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14. ABSTRACT The purpose of the CoE is to discover the molecular mechanisms and the modulation estrogen-induced apoptosis. The laboratory research project is focused on genomics and proteomics with a current focus on molecular interrogation to decipher mechanisms that may be applied to aid patient treatment. In parallel, but not supported by the CoE, is a pilot clinical study of estrogen-induced apoptosis in patients with metastatic breast cancer, that have had repeated cycles of successful antihormone therapy, but have subsequently failed and relapsed. A new low dose protocol is being completed. The molecular pharmacology laboratory of the Principle Investigator (PI) is advancing in all areas originally designated in the grant, e.g. a description of the time dependent changes in estrogen-responsive growth and apoptosis in model cell lines, an evaluation and description of the early proteomic pathways associated with estrogen-induced apoptosis dependent on the estrogen receptor (ER) co-activator AIB1 (SRC-3), the critical importance of the shape of the ER complex, the mechanism of c-Src activity that mediates apoptosis and the genomic spectrum of our endocrine resistant cell lines to define cell sensitivity to estrogen-induced apoptosis. We have discovered and described all the stages of the development of estrogen induced apoptosis through the intrinsic (mitochondrial) pathway that becomes reinforced by the extrinsic (death receptor) pathway. We have correlated rises in reactive oxygen species (ROS) with apoptosis and through RNAseq analysis, identified AP-1 (cFos and cJun) as the critical mediators for estrogen-induced apoptosis.

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INTRODUCTION

The Center of Excellence Grant is completing four independent, interconnected and synergistic tasks to achieve the goal and answer the overarching question: **to discover the mechanism of estrogen-induced breast cancer cell apoptosis and establish the clinical value of short-term low dose estrogen treatment to cause apoptosis in antihormone resistant breast cancer.** To achieve the goal, we had established an integrated organization (Fig. 1) with a first class advisory board that links clinical trials (**Task 1)** with laboratory models and mechanisms (**Task 2)** proteomics **(Task 3)** and genomics **(Task 4).**

Figure 1

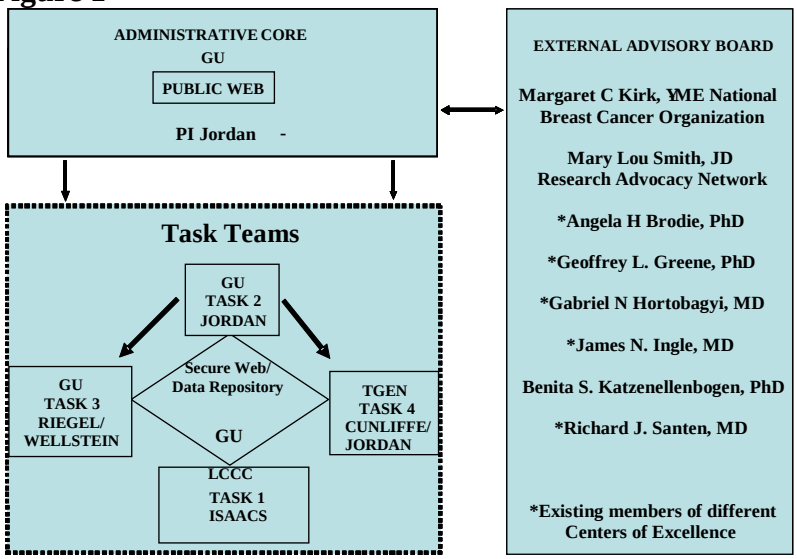


Figure 1. Organization of the COE.

Organization of the COE

Our work is having significant impact in the clinical trials community with the recent publication of the Womenôs Health Initiative study of estrogen replacement therapy alone in hysterectomized women that shows an ***actual*** decrease in the incidence of breast cancer [1,2]. This exciting new development in womenôs health finds its scientific foundation in our innovative grant and poised to define the mechanisms necessary to exploit estrogen therapy further in the clinic. The work that we are refining will form the basis of an invited series of reviews on the molecular mechanism of estrogen-induced apoptosis. Through the award of this Center of Excellence Grant from the DOD, we have demonstrated innovation in solving fundamental problems in womenôs health at the molecular level.

Body

TASK 1: (LCCC/Isaacs) - To conduct exploratory clinical trials to determine the efficacy and dose response of pro-apoptotic effects of estrogen [Estrace] in patients following the failure of two successful antihormonal therapies.

Here we report work completed on Task 1a at the LCCC and FCCC sites during this COE.

Task 1a: (Isaacs) - To confirm the efficacy of standard high dose estrogen (Estrace) therapy and then determine a minimal dose to induce tumor regression.

Clinical trial conducted by Claudine Isaacs, MD

Work Accomplished:

Our high dose estrogen protocol was approved both at Fox Chase Cancer Center and the Lombardi Cancer Center, Georgetown University, Washington, DC. The principal obstacle to progress clinically was the withdrawal of funding for clinical work by Astra Zenica. No funds were available in our Center of Excellence grant to conduct clinical trials. However, the fact that a clinical trial comparing high-dose (30mg daily Estrace) versus low dose Estrace (6mg Estrace daily) has now been published (Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A, Jamalabadi-Majidi S, Crowder R, Siegel BA. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 2009;302(7):774-80.) by others based on our laboratory work is a fundamental advance. The study demonstrates that low-dose estrogen has lower side effects than our current protocol using high-dose estrogen.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2a (Obiorah and Jordan) - Studies carried out by Dr. Ifeyinwa Obiorah in the Jordan laboratory at Georgetown University

Breast cancer cell apoptosis with phytoestrogens is dependent on an estrogen deprived state

Introduction

In this study we have evaluated the apoptotic and potential chemopreventive effects of phytoestrogens using a unique cell model that simulates a postmenopausal cellular environment. Genistein, coumestrol and equol, a gastrointestinal metabolite of diadzein are used in comparison to E_2 and equilin a constituent of conjugated equine estrogen (CEE) in hormone replacement therapy (HRT) to determine their proliferative and apoptotic potential using fully estrogenised and an estrogen deprived breast cancer cells respectively. Here, we test the hypothesis that the phytoestrogens have biologic effects similar to that of E_2 and CEE in breast cancer prevention and this may have clinical implications for the strategic use of phytoestrogens as alternatives to HRT in postmenopausal populations.

Work Accomplished:

Effect of phytoestrogens on breast cancer cells

Based on the controversy surrounding breast cancer risk and the use of phytoestrogens, we decided to determine the biological properties of the genistein, equol and coumestrol in comparison to E_2 and equilin in two different models of breast cancer cell models. Estrogens have been shown to regulate the growth of ER positive MCF-7 breast cancer cells. First, we tested the ability of test compound to induce proliferation in MCF7:WS8 cells which are estrogen responsive breast cancer cells grown in fully estrogenised medium. MCF-7:WS8 cells were grown in estrogen free media for 3 days and treated with various concentrations of genistein, coumestrol and equol and their effects were compared to E_2 and equilin (Fig. 2a-1A). The phytoestrogens, equol [EC_{50}: 1.72 x 10^{-9}], genistein [EC_{50}: 1.08 x 10^{-8}] and coumestrol[EC_{50}: 3.07 x 10^{-9}], all stimulated cell growth in a concentration related manner with maximum stimulation occurring at 0.1ÕM, whereas E_2 [EC_{50}: 3.11x 10^{-12}] and equilin [EC_{50}: 1.01 x 10^{-11}] maximally induced cell growth at 10 pM and 0.1 nM respectively.

Growth inhibition was observed with the phytoestrogens at 10ÕM with genistein (10^{-5}M vs 10^{-7}M; $P<0.05$). Next we investigated the growth properties of the genistein, equol and coumestrol in long term estrogen deprived MCF7:5C cells in comparison to E_2 and equilin (Fig. 2a-1B). Genistein[IC_{50}: 2.77 x 10^{-8}], equol[IC_{50}: 4.67 x 10^{-8}] and coumestrol[IC_{50}: 2.34 x 10^{-8}] drastically inhibited the growth of the MCF7:5C cells at higher concentrations compared to E_2. Maximum growth inhibition was observed with all phytoestrogens at 0.1ÕM. E_2 [IC_{50}: 2.06 x 10^{-11}] achieved maximum growth inhibition at 0.1nM, while equilin [IC_{50}: 2.32 x 10^{-10}] reached maximum growth inhibition at 1nM after 7 days of treatment.

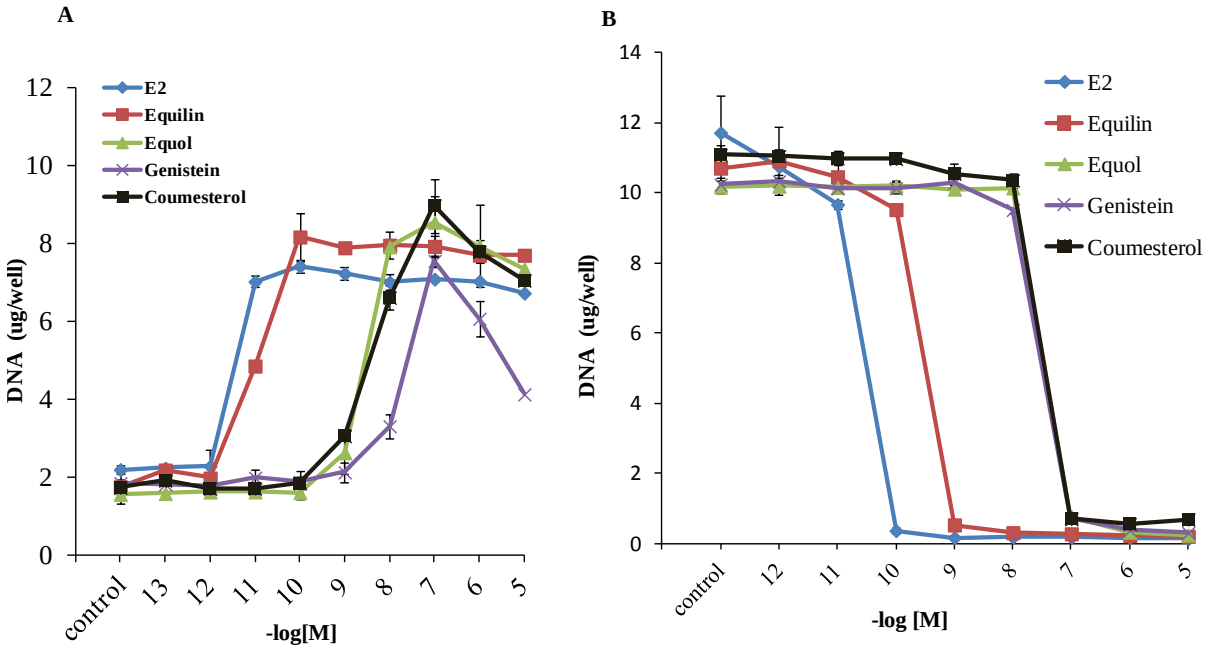


Figure 2a-1. Growth characteristics of 17β-estradiol, equilin and phytoestrogens in breast cancer cells *(A) MCF7:WS8 cells were seeded in 24-well plate and treated with steroidal and phytoestrogens over a range of doses for seven days. Cell growth was assessed as DNA content in each well. (B) Inhibition of cell growth in MCF7:5C cells by genistein, equol and coumestrol was assessed in comparison to E_2 and equilin. Each data point is average +/- SD of three replicates. [* P< 0.05].*

Phytoestrogens induce apoptosis in a long term estrogen deprived breast cancer cell line

Based on the fact that the decrease in cell growth observed with the steroidal estrogens is due to apoptosis[3] we investigated whether the anti-proliferative effects of the phytoestrogens was also due to an increase in apoptosis. MCF7:5C cells were treated with E_2 (1nM), equilin (1nM), genistein(1ÕM), equol(1ÕM) and coumestrol(1ÕM) for 72h and stained with annexinV-FITC and PI fluorescence and cells were analyzed using the flow cytometry. In the control treated group, only 6.8% of cells stained for apoptosis, whereas E_2 (24.56%), equilin (17.49%), genistein (14.79%), equol (14.89%) and coumestrol (17.83%) all show increased apoptotic staining compared to the control treated cells (Fig. 2a-2A). E_2, equilin and all phytoestrogens induced apoptotic genes; *BCL2L11/BIM*, *TNF*, *FAS* and *FADD* (Fig. 2a-2B-C) after 48h of treatment. Induction of these genes is consistent with the apoptotic status determined using the flow cytometry. In contrast to other reports which indicate that genistein causes a G2/M arrest, no checkpoint blockade was noted after treatment with all compounds, indicating that the initial response of the cells to estrogens is growth, then apoptosis in MCF7:5C cells.

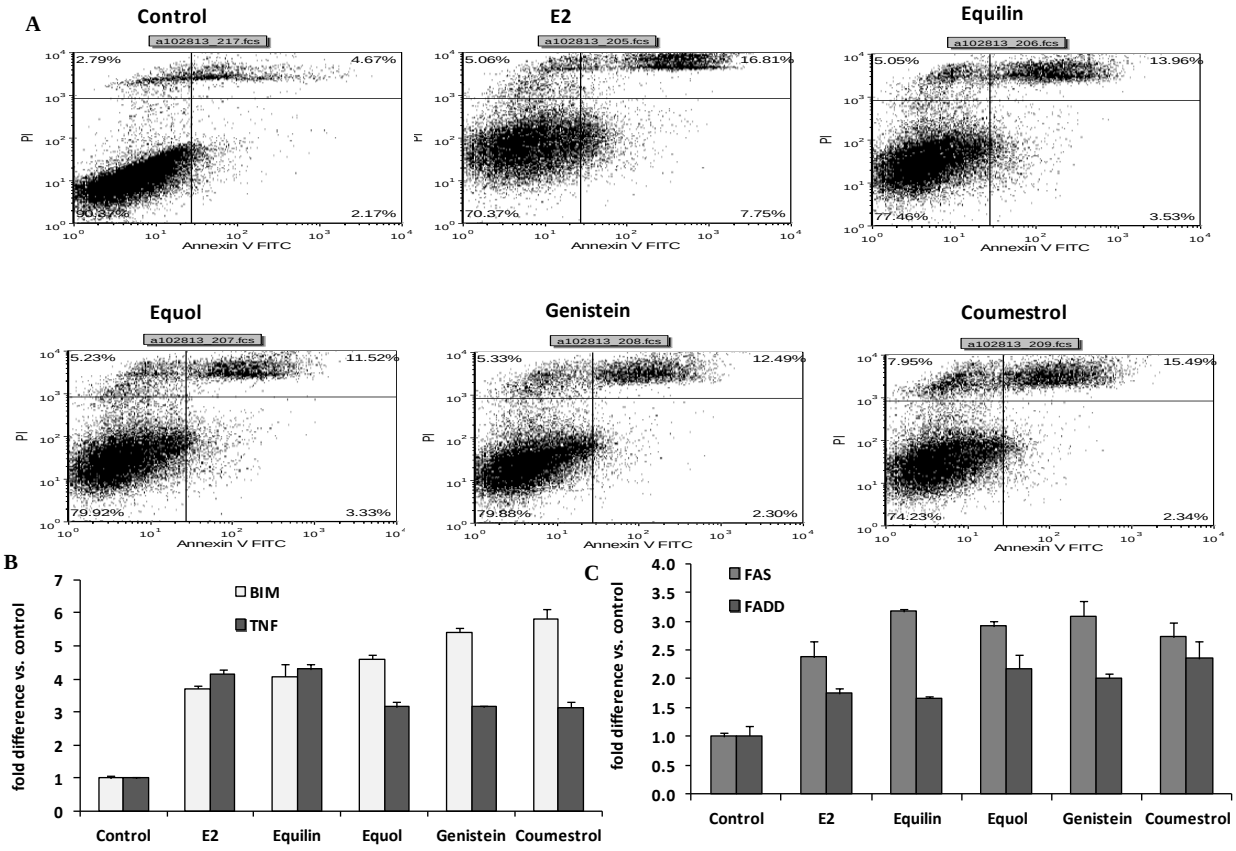


Figure 2a-2. Induction of apoptosis by phytoestrogens and steroidal estrogens *(A) MCF7:5C cells were treated with 0.1% ethanol vehicle (control), or 1nM E_2, 1nM equilin or phytoestrogens (1µM) for 72h and then stained with annexin v-FITC and propidium iodide and analysed by flow cytometry. Increased apoptotic effect is observed in the right upper and lower quadrant. E_2, equilin and phytoestrogens increase (B) BIM, TNF (C) FAS and FADD mRNA levels. PCR data values are presented as fold difference versus vehicle treated cells ± SEM. [* P< 0.05]*

Phytoestrogens possess estrogenic properties mediated through the estrogen receptor in the MCF7:5C cells

We explored the ability of phytoestrogens to regulate estrogen response genes in comparison to E_2 and equilin. Genistein, equol and coumestrol were all able to induce *TFF1/PS2* and *GREB1* (Fig. 2a-3A). Phytoestrogens have been shown to induce apoptosis through an estrogen receptor (ER) independent mechanism. To evaluate the involvement of ER in the effects of the phytoestrogens, we investigated their anti-proliferative effects in the presence of 4-hydroxytamoxifen (4OHT) (Fig. 2a-3B). The combination of various concentrations of 4OHT or ICI 182 780 with E_2, equilin and each phytoestrogens blocked estrogen induced apoptosis suggesting that the phytoestrogens mediate apoptosis via the ER. We sought to examine the effects of genistein, equol and coumestrol on the ER. Following treatment of MCF7:5C cells with E_2, equilin and the phytoestrogens for 24h, ERŭ levels were determined by western blotting. All phytoestrogens caused a decrease in the ERŭ protein levels in a comparable manner as E_2 and equilin (Fig. 2a-3C). Similarly the same effect was noted with all estrogens on the ERŭ mRNA levels (Fig. 2a-3D). Interestingly, E_2, equilin, genistein, equol and coumestrol, all have no effect on the ERɓ protein and mRNA levels suggesting different regulatory effects the phytoestrogens may have on the ERŭ and ERɓ.

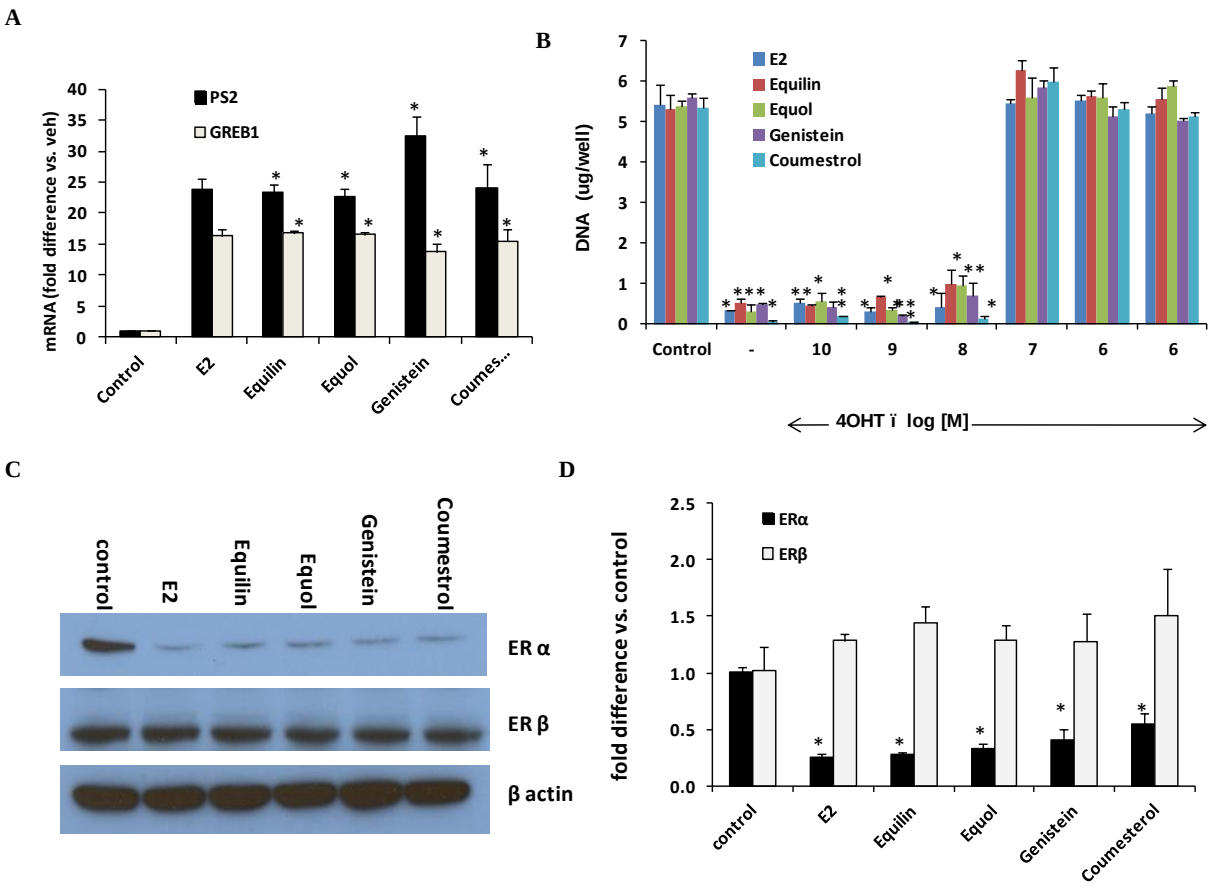


Figure 2a-3. Steroidal and phytoestrogens act as agonists via an estrogen receptor dependent mechanism *(A) MCF7:5C cells were treated with 0.1% ethanol vehicle (control), 1nM E_2, 1nM equilin or phytoestrogens (1µM). Total RNA was isolated after 24h and reverse transcribed and PS2, GREB1, progesterone receptor (PgR) mRNA levels was obtained using RT-PCR. (B) Various concentrations of 4-hydroxytamoxifen (4OHT) block steroidal estrogen- or phytoestrogen mediated growth inhibition (C) MCF-7:5C cells were treated with vehicle (control) and steroidal and phyto-estrogens for 24 hours. ERα and ERβ protein was detected by immunoblotting. (D) ERα and ERβ mRNA was quantified with real time PCR (RT-PCR). *,* P *< 0.05, compared with control.*

ERα is important for steroidal and phytoestrogen induced apoptosis and growth inhibition

To determine whether ER Ŭor ɓ is required for the antiproliferative and apoptotic effects of the estrogens, MCF7:5C cells were transfected with either ERŬ or ERɓ siRNA or nontarget siRNA (control) for 72h. Knockdown of ERŬ and ER ɓ protein level was determined by western blot (Fig. 2a-4A, D). RNA-interference mediated inhibition of ERŬ abolished both steroidal and phytoestrogen induced apoptosis (Fig. 2a-4B) and growth inhibition (Fig. 2a-4C) compared with cells transfected with the control siRNA. Interestingly, loss of ERɓ using siRNA did not prevent the ability of the steroidal or phytoestrogens to either induce apoptosis (Fig.2a-4E) or inhibit the growth (Fig.2a-4F) of the MCF7:5C cells. Taken together, this indicates that ERŬ is the initial site for the indicated estrogens to cause growth inhibition and apoptosis in the MCF7:5C cells.

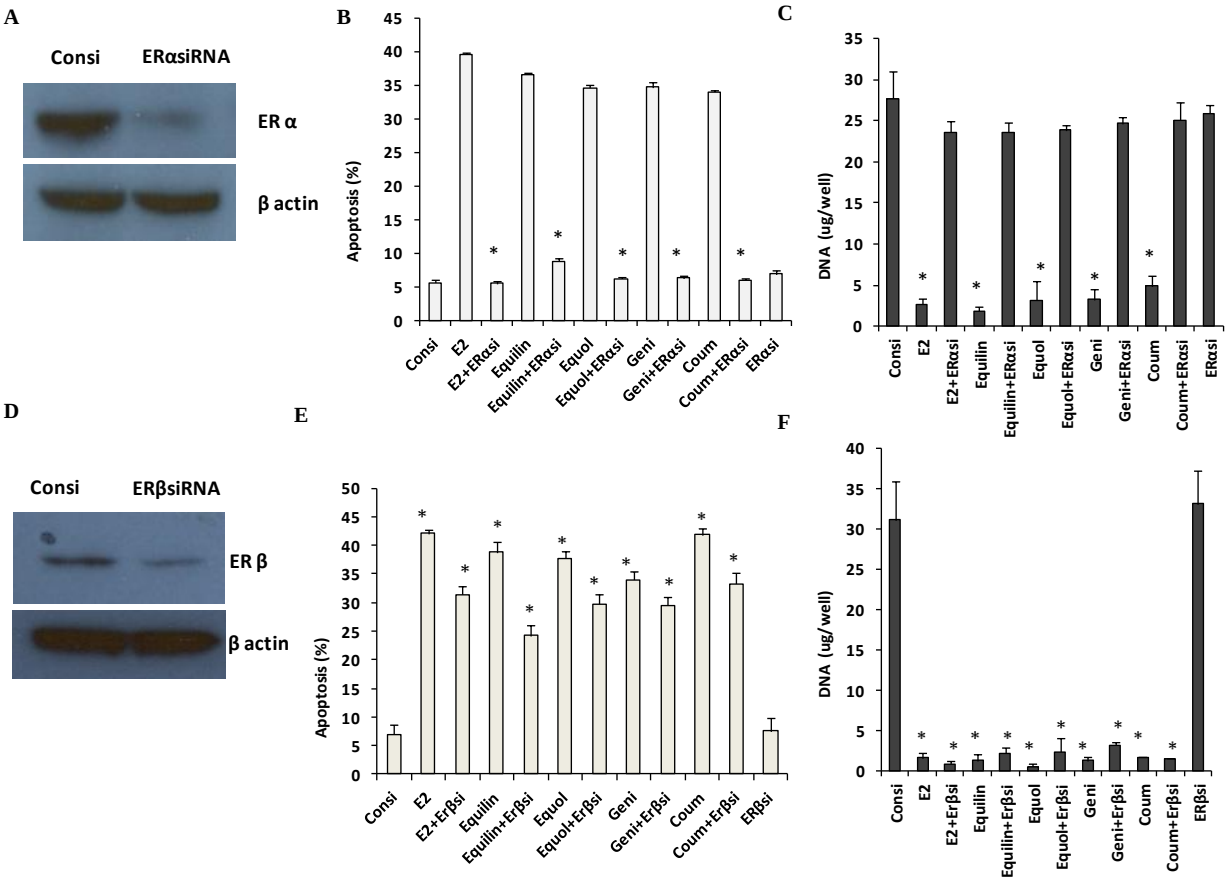


Figure 2a-4. ERα is required for estrogen induced growth inhibition and apoptosis *MCF7:5C cells were transfected with either non target RNA (consi) or siRNA of ERα for 72 h. (A) ERα was detected by immunoblotting. Then, cells were treated with either control (0.1% EtOH), 1nM steroidal estrogens or 1μM phytoestrogens for (B) 72h and apoptosis was determined using annexin V binding assay.(C) Growth inhibition in the transfected cells was assessed after 6 days of treatment with indicated compounds using DNA quantification assay. [* P< 0.05]*

Phytoestrogens induce endoplasmic reticulum stress and inflammatory stress response genes

Microarray analysis indicates that endoplasmic reticulum stress (ERS) and inflammatory response genes are top scoring pathways associated with E_2 induced apoptosis. To investigate whether phytoestrogens induce ERS genes, we used RT-PCR to quantitate mRNA levels. After 48h of treatment, genistein, equol, coumestrol and equilin and E2 all induce *DDIT3*(also known as CHOP), a marker of ERS associated with cell death, and inositol requiring protein 1 alpha (*IRE1α*) , an unfolded protein response sensor which is activated to relieve stress (Fig. 2a-5A). Significant induction of *IRE1α* and phospho-eukaryotic translation initiation factor-2Ŭ (p-eIF2Ŭ), another UPR sensor, protein levels occur by 24h (Fig. 2a-5B). Next we determined whether genistein, coumestrol and equol induce proinflammatory response genes using RT-PCR. At 48h, E_2, equilin and all phytoestrogens activate caspase 4, an inflammatory caspase; *CEBP β* which is known to bind to IL-1 response element in *IL6* and a downstream target of ERS; *IL6*, a proinflammatory cytokine; lymphotoxin beta (*LTB*),an inducer of inflammation response, (Fig. 2a-5C-D). This indicates that the phytoestrogens activate similar genes involved in the apoptotic pathway of E_2.

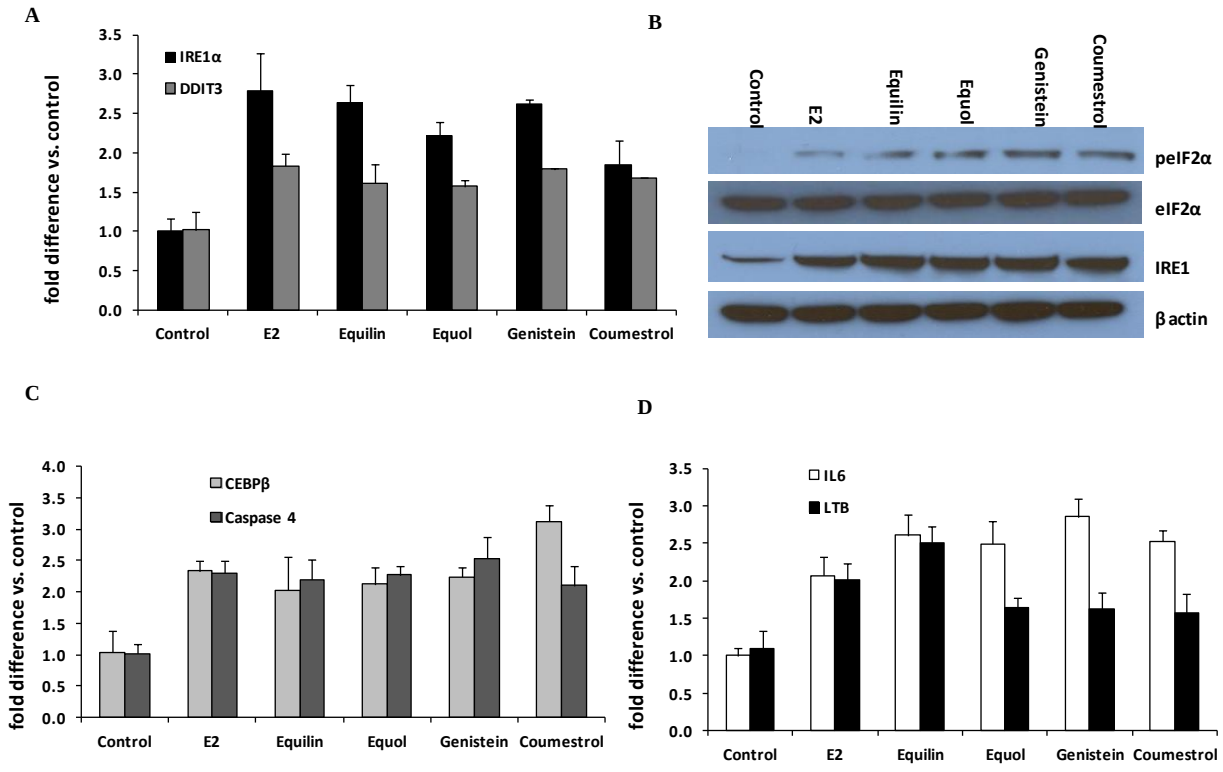


Figure 2a-5. Endoplasmic reticulum stress and inflammatory stress response are involved phytoestrogen induced apoptosis *(A) The indicated estrogens induce endoplasmic reticulum stress related genes, DDTT3 and IRE1α. (B) MCF-7:5C were treated with E_2 (1nM), equilin (1nM) or phytoestrogen (1μM) for 24h. IRE1α and phosphorylated eIF2α were used as indicators of UPR activation and their protein expression were examined by immunoblotting. Total eIF2α and β-actin were determined for loading controls. Indicators of inflammatory stress response (C) caspase4, CEBP β, (D) IL6 and LTB were activated by E_2, equilin and phytoestrogens. [* P< 0.05]*

Inflammation is required for phytoestrogen mediated apoptosis

Next we investigated the importance of inflammatory response in phytoestrogen mediated apoptosis. Dexamethasone, a synthetic glucocorticoid with potent anti-inflammatory properties was used to inhibit inflammation in the MCF7:5C cells. Cells were treated with 1nM E_2 or equilin or 1ÕM phytoestrogens and various concentrations of dexamethasone were added to block the biological effects of the compounds. Although dexamethasone has an inhibitory effect in the MCF7:5C cells, it was able to reverse the steroidal estrogen or phytoestrogen inhibited growth (Fig. 2a-6A). Similarly, flow cytometry studies revealed that 1ÕM dexamethasone reversed the apoptotic effects mediated by E_2, equilin, genistein, equol and coumestrol (Fig. 2a-6B). To determine that inflammatory stress response was inhibited by dexamethasone, MCF7:5C cells were treated with the indicated estrogens for 48h and total RNA was extracted and reverse transcribed. Dexamethasone inhibited the ability of all estrogens to induce caspase 4, *CEBPβ*, *BIM* and *TNF* (Fig. 2a-6C-D). Together, this suggests that inflammation is important for both steroidal and phytoestrogen mediated apoptosis.

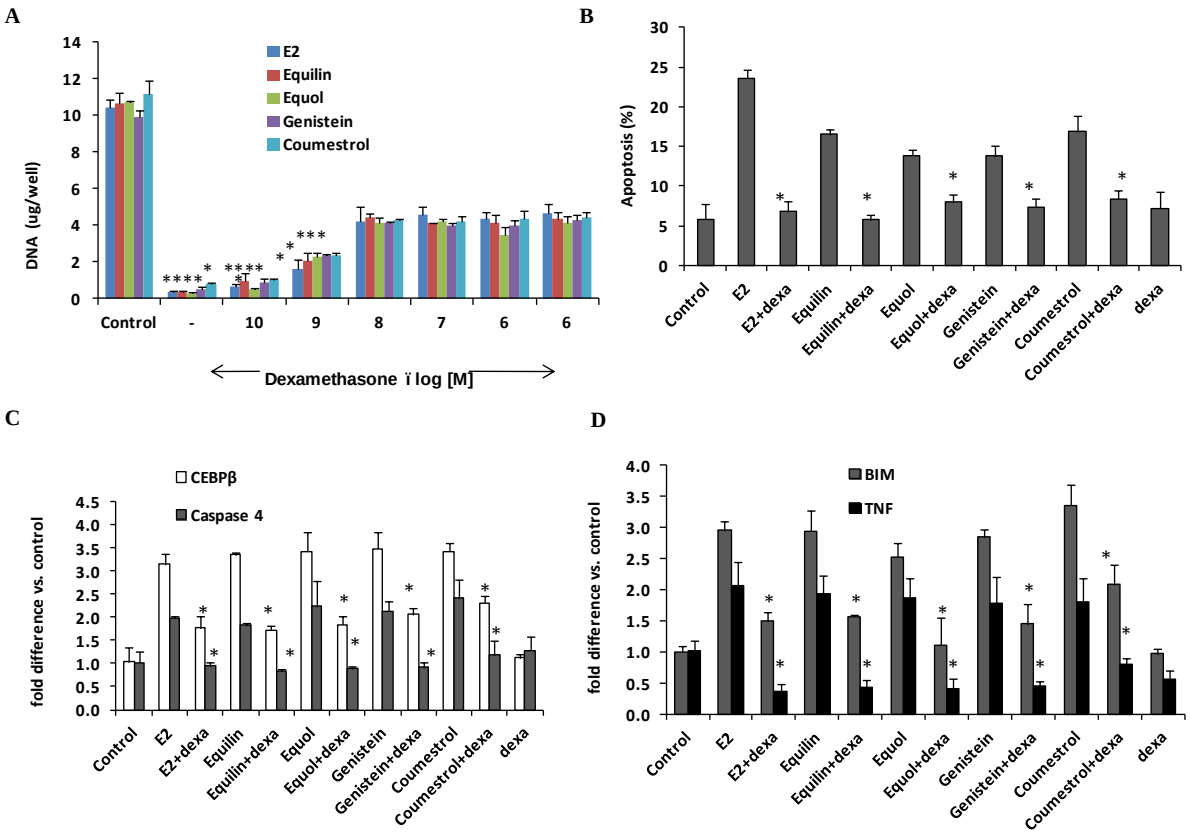


Figure 2a-6. Inflammation is important for phytoestrogen mediated apoptosis *(A) Cells were treated with the indicated estrogens in presence of increasing concentration of dexamethasone(dexa). (B) Dexamethasone completely reverses E_2, equilin and all phytoestrogen induced apoptosis. Apoptosis was assessed using the flow cytometry. Dexamethasone blocked the induction of (C) CEBP β,caspase 4 (D) BIM, and TNF by E_2, equilin and phytoestrogens. [* P< 0.05]*

DISCUSSION

Phytoestrogen consumption is associated with a decrease in the incidence of breast cancer in the Asian population probably due to early exposure to a high soy diet. This correlates to animal studies which suggest that it is due to mammary cell differentiation and a decrease in terminal end buds which are sites of early tumor proliferation[4]. Phytoestrogens increase cell growth of ER positive breast cancer cells but induce apoptosis at high concentrations in these cells. Although studies[5] may support use of phytoestrogens in postmenopausal women, their full chemopreventive properties is yet to be clearly defined. E_2 and CEE induce apoptosis in long term estrogen deprived breast cancer cells. Therefore we addressed the question of whether low concentrations of phytoestrogens will induce apoptosis in MCF7:5C cells which simulate a postmenopausal state that is dependent on the duration of estrogen deprivation following menopause. Genistein, equol and coumestrol all increase cell growth in MCF7:WS8, (which simulate the premenopausal or perimenopausal state) after 3 days of estrogen deprivation at low concentrations. These cells have adapted to an estrogen rich environment and will grow with a natural resupply of estrogens provided with exogenous phytoestrogens treatment. This correlates with the results of Andrade and colleagues [6]who show that long-term consumption of low GEN doses (Ò500 ppm) promotes MCF-7 tumor growth in vivo. However at low concentrations <1ÕM, all phytoestrogens inhibit cell growth. In contrast the phytoestrogens, although less potent than E_2 and equilin, induce apoptosis in MCF-7 cells that have undergone long term estrogen deprivation. Therefore a potential use of phytoestrogens at physiologic concentrations will be in an estrogen deprived environment

which is induced either by natural withdrawal of estrogens caused by menopause or by treatment with exhaustive anti-estrogen therapy for breast cancer with aromatase inhibitors or tamoxifen.

Studies [7,8] suggest that phytoestrogens possess anti-estrogenic properties which may be responsible for their chemopreventive effects. Here we show that the phytoestrogens do in fact induce estrogen responsive genes just like steroidal estrogens in the estrogen deprived MCF7:5C cells and that their growth inhibition and apoptosis are mediated through the ER. In contrast, it has been reported that genistein mediates apoptosis through an ER independent mechanism in the MCF-7 cells and the ability of phytoestrogens to induce apoptosis is observed maximally in the presence of E_2. It is important to note however that apoptosis was mediated by the phytoestrogens only at high concentrations in these studies. As another potential mechanism of apoptosis, phytoestrogens show increased binding affinity to ERб [9] which is thought to be responsible for its growth inhibitory properties. In our study, loss of ERб did not affect the anti-proliferative and apoptotic properties of the steroidal and phytoestrogens. However, we determined that knockdown of ERŬprevents both steroidal and phytoestrogen mediated growth inhibition and apoptosis suggesting that ER Ŭsignaling is required for their biological actions.

Genistein, equol and coumestrol induce ERS and inflammatory stress response, intrinsic and extrinsic apoptosis related genes which correlates with results of differential gene expression in response to E_2 interrogated using agilent based microarray analysis [10]. Activated ERS genes indicate that E_2 prevents protein folding leading to accumulation of unfolded proteins and widespread inhibition of protein translation and crosstalk with inflammatory response genes and subsequent induction of cell death. Inhibition of *PERK/EIF2AK3*, a key ERS sensor of UPR and inducer of pEIF2Ŭ prevents E_2 mediated apoptosis [11]. *PERK* is also known to induce apoptosis by sustaining levels of *DDIT3*, another major ERS gene involved in apoptosis, which is known to dimerize with *CEBPβ* under stress conditions. Ablation of *CEBPβ* using siRNA decreases expression of *DDIT3* suggesting a crosstalk between ERS and inflammatory stress response. Similarly, inhibition of caspase 4, an inflammatory response gene and a downstream target of ERS, using caspase 4 inhibitor-z-LEVD-fmk also blocks E_2 induced apoptosis. To show that inflammation is important in phytoestrogen induced apoptosis, dexamethasone was used to block inflammation globally, resulting in inhibition of all estrogen induced apoptosis and their ability to induce inflammatory response and apoptosis related genes. Therefore the clinical implication is that caution should be exercised in the use of steroidal anti-inflammatory agents in conjunction with these phytoestrogens, which could prevent the full chemopreventive benefits.

Successful use of estrogens to treat or prevent tumors is dependent on the timing of estrogen withdrawal. Estrogen therapy was the first chemical used in the treatment of advanced breast cancer in postmenopausal women and this therapy resulted in the regression of 30% of tumors in the first reported clinical trial [12]. It was noted that ñthe beneficial responses were three times more frequent in women over the age of 60 years than in those under that age; that estrogens may, on the contrary, accelerate the course of mammary cancer in younger women, and that their therapeutic use should be restricted to cases 5 years beyond the menopauseò [13]. Stoll and colleagues[14] noted that objective remission rate from estrogen treatment in 407 patients with advanced breast cancer was higher in women more than 5 years postmenopausal (35%) when compared to women who were less than 5 years postmenopausal (9%). In more recent clinical studies, about thirty percent of patients with advanced breast cancer who have been exposed to exhaustive antihormone therapy show an objective clinical response with estrogen therapy. CEEs reduced the incidence and mortality from breast cancer but this is probably because the majority of these women were over 65 years. Furthermore, 10 years adjuvant tamoxifen therapy produced a further reduction in recurrence and mortality from breast cancer when compared to 5 years of tamoxifen therapy suggesting that it was the womanôs own estrogen that destroys the appropriately sensitive tamoxifen resistant micrometastasis once long term tamoxifen is stopped.

In conclusion, it is important to note that in order to obtain the full breast cancer chemopreventive benefits of phytoestrogens, it is necessary to begin up to five years following menopause. Commencing soy consumption during perimenopause may cause growth of nascent ER positive breast tumors which

may increase breast cancer risk, whereas phytoestrogen therapy 5 years after menopause will most likely induce apoptotic cell death and enhanced patient survival.

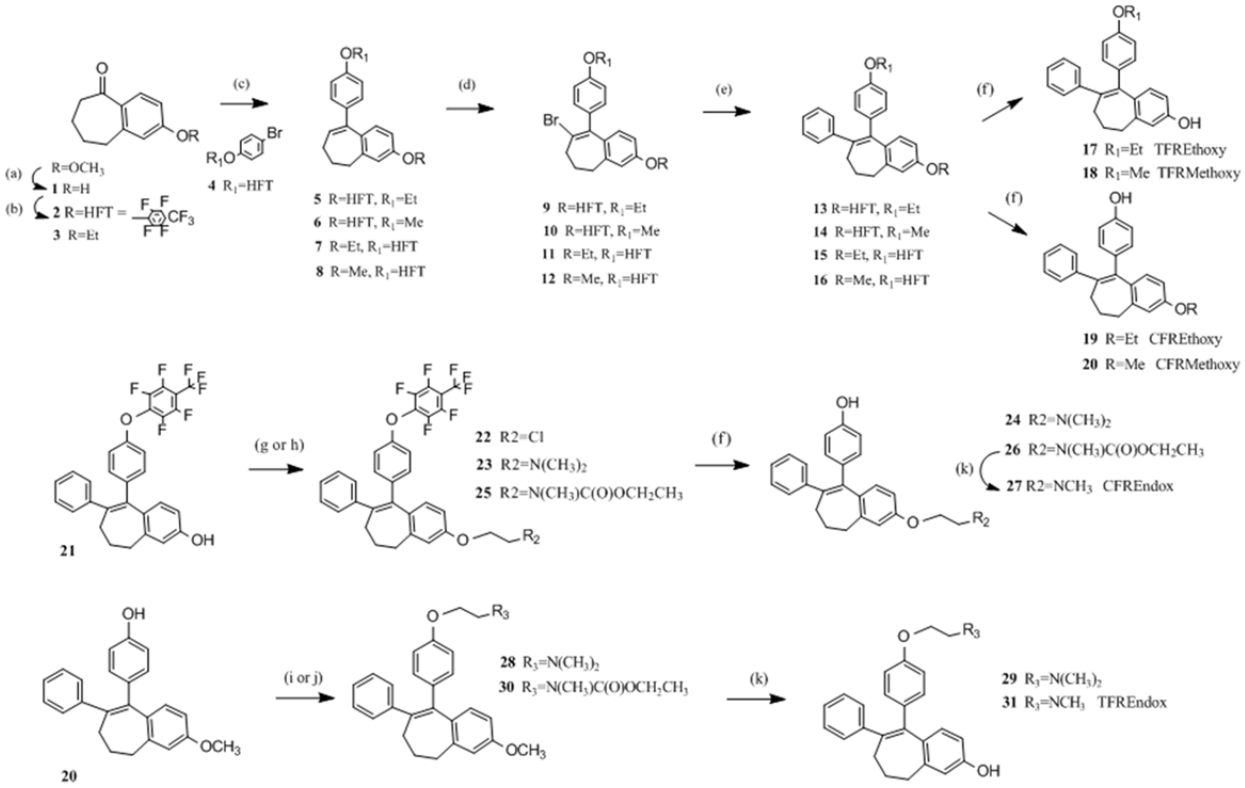
TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2b (Maximov and Jordan) - Studies carried out by Dr. Philipp Maximov in the Jordan laboratory at Georgetown University

Influence of the length and positioning of the antiestrogenic side chain of endoxifen and 4-hydroxytamoxifen on gene activation and growth of the estrogen receptor positive cancer cells.

Introduction

We synthesized a series of FR analogs of the alkylaminoethoxy side chain of 4OHT to link molecular modeling with cell replication in breast cancer (MCF-7:WS8) and prolactin synthesis in the rat pituitary gland cancer cell line GH3. We took these approaches to study structure-function relationships: reducing the antiestrogenic side chain of 4OHT and comparing results with bisphenol (BPTPE) and trihydroxytriphenylethylene (3OHTPE) [15], comparing E and Z FR isomers and finally the length and bulk of the antiestrogenic side chain of E-isomer of FR4OHT (EFR4OHT) (Scheme 2). Select ER-responsive genes (pS2, GREB1 and PgR) were measured following 48 hour incubation of all test compounds with MCF-7:WS8 cells as well as ER levels determined by Western blotting. Also we evaluated the impact of therapeutic concentrations of E-isomers of FR4OHT and FR endoxifen (FREndox) alone or in combination with therapeutic levels of Z-isomers on the growth of MCF-7:WS8 breast cancer cell line to estimate therapeutic relevance during breast cancer treatment with tamoxifen for tumor cell growth control by putative estrogenic metabolites.



Reagents and conditions: (a) $AlCl_3$, toluene, reflux, 21 hrs; (b) octylfuorotoluene, TBAHS, CH_2Cl_2, 1N NaOH, 16h; or EtI, K_2CO_3, acetone, rt; (c) (i) Mg, ether, reflux, 10h; (ii) HCl, EtOH, reflux, 2 hrs; (d) pyridine hydrobromide perbromide, CH_2Cl_2, rt; (e) ZnCl, phenyl lithium, $Pd(PPh_3)_4$, THF; (f) $NaOCH_3$ or NaOEt, DMF, 80° C, 4 hrs; (g) (i) $ClCH_2CH_2Cl$, TBAHS, 1N NaOH 16h; (ii) $(CH_3)_2NH$ in EtOH reflux 20h; (h) Br-R2, TBAHS, 1N NaOH, rt, 16h; (i) (i) NaH, DMF; (ii) Cl-R, 60° C, 3h; (j) HO-R, TPP, DIAD, THF, rt; (k) pyridine HCl, 140° C, 3h.

Fig. 2b-1 The scheme of synthesis of the isomerically stable fixed ring (FR) isomers of Methoxy, Ethoxy substituted triphenylethylene derivatives and fixed ring 4OHT and Endox.

Work Accomplished:

Chemistry.

Five novel FR4OHT analogs (ZFRMethoxy, ZFREthoxy, EFREthoxy, ZFREndox and EFREndox) were synthesized in a multistep sequence involving a Gringard reaction of a protected p-bromophenol with a substituted benzosuberone. Subsequent modifications provided two key intermediates that have a methoxy or heptafluorotolyl (HFT) protecting group on either of the phenolic groups. This versatile scaffold was important for the synthesis of the E and Z-isomers of FRMethoxy, FREthoxy, FR4OHT and FREndox compounds. Both isomers of FR4OHT (24 and 29 in Scheme 2) and CFRMethoxy (20) were synthesized according to McCague et al [16], while compounds 3OHTPE and BPTPE were synthesized according to Maximov et al [15].

Z fixed ring methoxy / E and Z fixed ring ethoxy analogs (ZFRMethoxy and ZFR/EFREthoxy). 2-Methoxyheptenone (benzosuberone) was demethylated to 1 (Scheme 2) by refluxing with aluminium chloride in toluene [17,18]. Phenol 1 was protected with octafluorotoluene using phase transfer reaction conditions to 2 or converted to the ethoxy analog 3 using ethyl iodide and potassium carbonate in acetone. Both compounds were treated with the Grignard reagent of a protected p-bromophenol that resulted in the formation of the ethoxy cycloheptene 5 and the methoxy analog 6 [16]. For compounds 7 and 8, 4-bromophenyl 2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenyl ether 4 was obtained by the method of Jarman and McCague [19]. This compound was converted to the Grignard reagent and reacted with suberone 3 which yielded 7 or reacted with 2-methoxybenzosuberone which led to 8. Bromine was introduced at the 8-position using pyridine hydrobromide perbromide (9-12) that was subsequently replaced with a phenyl moiety upon treatment with phenyl zinc chloride and a palladium catalyst yielding compounds 13-16. These key intermediates were selectively deprotected to provide either E or Z-isomers of FRMethoxy (18 and20) and FREthoxy (17 and 19) tamoxifen analogs.

E fixed ring Endoxifen (EFREndox). The synthesis of EFREndox 27 was first attempted by selective demethlyation of EFR4OHT 24 using 1-chloroethyl chloroformate both with and without magnesium oxide [20], as well as vinyl chloroformate [21] with no formation of product detected by LC-MS. In addition, demethylation using ruthenium chloride in methanol followed by treatment with hydrogen peroxide was also tried without success [22]. Also, the attempted direct methylamination of chloroethoxybenzocycloheptene 22 by heating with 33% methylamine in ethanol failed. Z-isomer of 4OHT (model compound) was converted to its N-oxide by stirring with 30% hydrogen peroxide in acetone but did not demethylate using selenium oxide [23]. Alternatively, we investigated several methods for attaching the protected ethylamine side chain directly onto phenol 21. Methods included reaction of 21 with ethyl (2-bromoethyl)(methyl)carbamate [20] by heating with cesium carbonate in DMF, heating with sodium hydride in DMF, and using phase transfer reaction conditions. All produced 25 in various yields with the last method giving the best overall yield. In the next step, the heptylfluorotolyl protecting group was removed using sodium methoxide in DMF to 26, followed by removal of the carbamate with pyridine HCl to 27. A faster route to EFREndox 27 could be achieved by starting with 14, where both protecting groups would be removed concurrently.

Z fixed ring Endoxifen (ZFREndox). The synthesis of TFREndox 31 was attempted with selective demethlyation of the Z tamoxifen 29 using vinyl chloroformate [21] with no product formation. The ZFREndox compound 30 was synthesized by heating compound 20 with ethyl (2-bromoethyl)(methyl)carbamate [21] and cesium carbonate in DMF but the reaction was not as efficient as using ethyl (2-hydroxyethyl)(methyl)carbamate [21], TPP and DIAD in THF. Both the

methoxide and carbamate protecting groups of 30 were removed simultaneously by heating with pyrdine HCl to give ZFREndox 31.

Pharmacology.

To assess estrogenic and antiestrogenic properties of the test compounds we employed a DNA quantification assay with the ER positive human breast cancer cell line MCF-7:WS8 as described in the Materials and Methods section, and have compared the results with the test compounds with previously described angular estrogens BPTPE and 3OHTPE [15]. Estradiol (E_2) induced growth of cells (Fig.2b-2A) in a concentration-dependent manner with maximal stimulation starting at a concentration of 10^{-11} M. All of the test compounds are partial agonists, and do not reach the same level of growth induction as with E_2. It is therefore not appropriate to calculate EC_{50} against E_2. However, they do cluster by their levels of growth induction. Compounds BPTPE, ZFRMethoxy, ZFREthoxy and EFR4OHT induce the same levels of growth of MCF-7:WS8 cells a concentration of 10^{-6} M with no statistical difference ($P<0.05$). Thus we estimated the potency of these compounds by comparing their EC_{50} concentrations (Fig.2b-2A). The results demonstrate that BPTPE is a much more potent partial agonist in MCF-7:WS8 cells (EC_{50} of $1.5x10^{-11}$M) than other test compounds in this group (Fig.2b-2A). The ZFRMethoxy and ZFREthoxy compounds with the shortest side chains have EC_{50} $3x10^{-10}$M, while EFR4OHT compound has the highest EC_{50} in this group of compounds of $1.5x10^{-8}$ M (Fig.2b-2A). The next group of compounds (EFRMethoxy, EFREthoxy and EFREndox) induce cell growth a little higher but statistically more significantly than the previous group ($P<0.05$), so their EC_{50} concentrations can be estimated between these compounds (Fig.2b-2A). EFRMethoxy compound has an EC_{50} of $4x10^{-9}$M, while EFREthoxy has EC_{50} of $2.7x10^{-9}$ M, and EFREndox has EC_{50} of $2x10^{-8}$ M. The ZFR4OHT and ZFREndoxifen, like the structurally similar Z-4OHT and endoxifen have no estrogenic properties over the whole concentration range of 10^{-12}-10^{-6} M (Fig.2b-2A) ($P>0.05$ for all concentration points when compared to each of their respective vehicle controls). Estrogenic properties on the growth of MCF-7:WS8 cells of 3OHTPE were previously described [15] and are not shown here. The EC_{50} $1.5x10^{-10}$M is similar to BPTPE.

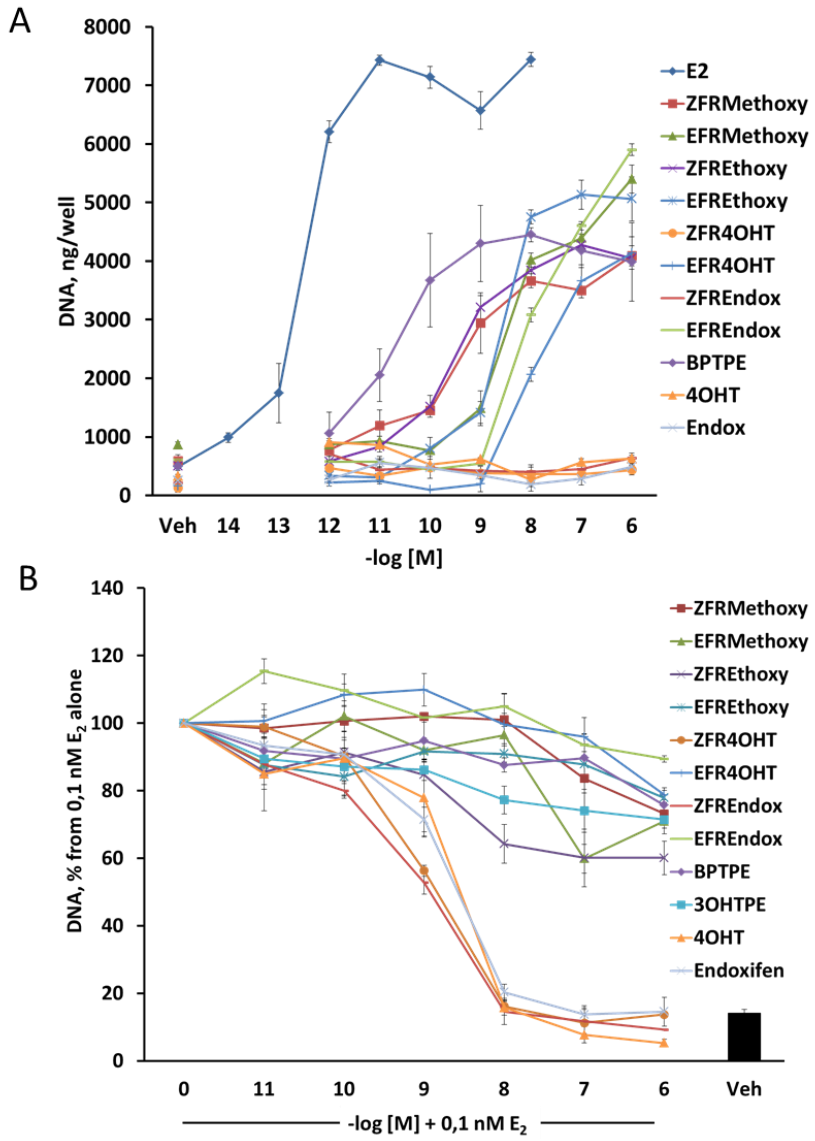


Figure 2b-2. *Assessment of estrogenic/antiestrogenic properties of the test compounds in MCF-7:WS8 ER-positive human breast cancer cell lines. A) treatments of the MCF-7:WS8 cells with compounds alone; B) treatments of MCF-7:WS8 cells with compounds in combination with 10^{-10} M E_2. All DNA content was normalized to a corresponding 10^{-10} M E_2 control of each of the experiments.*

To test the antiestrogenic properties of test compounds we employed the same DNA based growth assay with combination treatments with 10^{-10} M E_2. The Z-isomers of the FR4OHT and FREndox produce an equivalent antiestrogenic effect (average IC_{50} of $3x10^{-9}$ M in MCF7:WS8 cells) inhibiting 10^{-10} M E_2 completely ($P>0.05$ at 10^{-6} M points when compared to vehicle control) like 4OHT and endoxifen (Fig.2b-2B). ZFRMethoxy, ZFREthoxy, EFRMethoxy, EFREthoxy, EFR4OHT, ECFREndox, BPTPE and 3OHTPE compounds all have very weak antiestrogenic properties (Fig. 2b-2B), inhibiting E_2-stimulated cell growth by about 20% at top concentration ($P<0.05$ compared to control), however ZFREthoxy compound seems to have a little more antiestrogenic properties than the rest of the group by about 20% ($P<0.05$ at 10-6 M concentration), and EFREndox inhibits only by about 10% compared to vehicle control ($P<0.05$). All this is consistent with the intrinsic activity of test compounds alone (Fig. 2b-2A).

MCF-7:WS8 cells were treated with therapeutic concentrations of E and Z-isomers of FR4OHT and endoxifen found in postmenopausal breast cancer patients treated with tamoxifen [24]. Results show that pharmacological concentrations of tested E-isomers alone or in combination with Z-isomers were not able to induce significant cell growth ($P>0.05$ compared to control), compared to cell proliferation induced by postmenopausal levels of estrogens (E_1/E_2) found in postmenopausal women taking tamoxifen (Fig. 2b-3) ($P<0.05$ compared to control). The concentrations of estrogens corresponding to average levels of estrogens in postmenopausal women were $7.8x10^{-11}$ M for E_1 and $4.7x10^{-11}$ M for E_2 and were obtained from pervious publications [25,26]. The levels for the test compounds corresponding to mean therapeutic levels of tamoxifen metabolites in breast cancer patients taking tamoxifen were: ZFR4OHT- $5.81x10^{-9}$ M, ZFREndox- $29.1x10^{-9}$ M, EFR4OHT- $0.56x10^{-9}$ M, and EFREndox- $1.17x10^{-9}$ M [24].

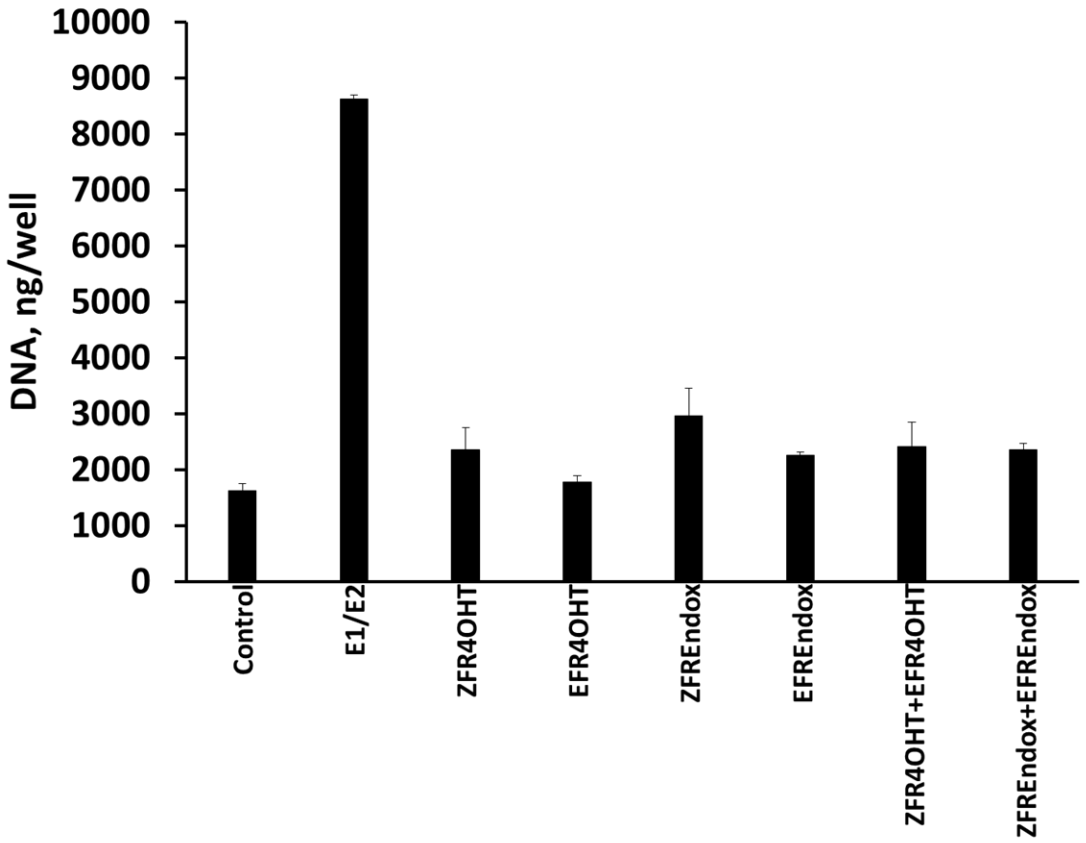


Figure 2b-3. *Assessment of estrogenic properties of different stable isomers of tamoxifen's metabolites 4OHT and endoxifen in MCF-7:WS8 at average therapeutic concentrations [24]. The levels for the tested compounds corresponding to mean therapeutic levels of tamoxifen metabolites were: ZFR4OHT- $5.81x10^{-9}$ M, ZFREndox- $29.1x10^{-9}$ M, EFR4OHT- $5,6x10^{-9}$ M, and EFREndox- $1.17x10^{-9}$ M.*

Real-Time PCR.

To assess the pharmacological properties the test compounds on estrogen responsive genes we used Real-Time Polymerase Chain Reaction (RT-PCR) in the ER positive rat pituitary tumor cell line GH3 to assess the modulation of the prolactin gene (Prl) and also in estrogen-responsive genes pS2, progesterone receptor (PgR) and GREB1 in MCF7:WS8 cells. All cells were first estrogen starved and then processed as described in the Methods section. Results of the Prl gene expression analysis show that, Prl gene in rat GH3 cells has elevated expression of mRNA in response to E_2 in a concentration-dependent manner (Fig.2b-4A) with maximal stimulation at 10^{-9} M ($P<0.05$ compared to control). All of the test compounds had shallow partial agonist dose

response curves (Fig.2b-4A). As a result of the inability of test compounds to induce maximal Prl gene actions higher than 40% of E_2 it is inappropriate to estimate EC_{50}. In combination with 1nM E_2 all test compounds exhibited antiestrogenic properties, however only ZFR4OHT, ZFREndox and 4OHT were able to completely inhibit 1nM E_2-induced Prl gene upregulation to control levels at their top concentration of 10^{-6} M (P>0.05) (Fig. 2b-4B). All other test compounds inhibited the effects of 1nM E_2 the levels of the intrinsic activity of compounds alone (Fig. 2b-4B).

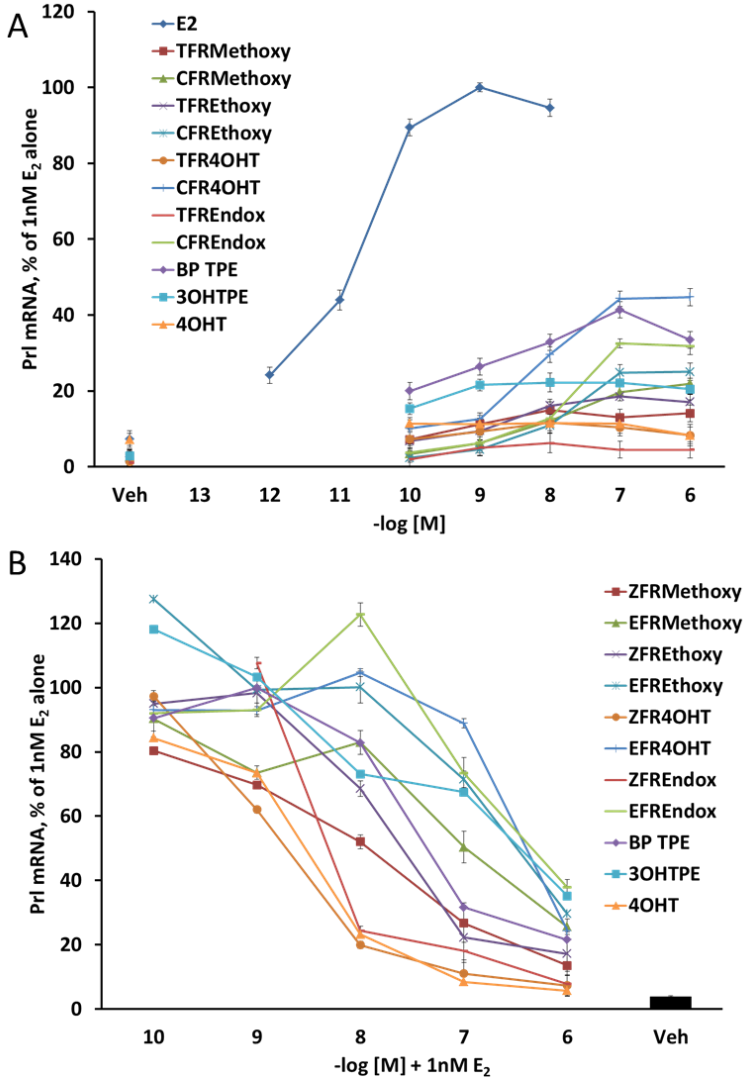


Figure 2b-4. *Assessment of estrogenic/antiestrogenic properties of the test compounds on inducing Prolactin (Prl) gene's mRNA expression in GH3 rat pituitary tumor cells. A) treatments of the GH3 cells with compounds alone. The fold change of the mRNA was first calculated using ΔΔCt method, corresponding 10^{-10} M E_2 control values were considered as 100% and all other treatments were calculated accordingly; B) treatments of the GH3 cells with compounds in combination with 10^{-9} M E_2. The fold change of the mRNA was first calculated using ΔΔCt method, corresponding 10^{-10} M E_2 control values were considered as 100% and all other treatments were calculated accordingly.*

RT-PCR of estrogen regulated genes pS2, GREB1 and PgR in MCF-7:WS8 cells treated with test compounds show a differential effect based on the structure of the ligands. Estradiol (10^{-10} M) induced expression of all test genes compared to vehicle control (Fig. 2b-5) after 48 hours of treatment (P<0.05 for all genes). Treatments with 3OHTPE and BPTPE produced a partial estrogenic effect on all genes (P<0.05 when comparing to E_2 treatment or vehicle control) and no

significant difference between each other (P>0.05) in any of the genes. Treatments with isomers of FRMethoxy and FREthoxy compounds produced partial estrogenic effects in all estrogen-responsive genes (P<0.05 when compared to Vehicle control), however E-isomers were able to produce a higher induction of expression in all studied genes than corresponding Z-isomers (P<0.05). ZFR4OHT, ZFREndox and 4OHT and Endox produced no significant effect on mRNA synthesis in pS2 and GREB1 genes (P>0.05 when compared to Vehicle control) and were similar to each other (P>0.05), however did induce 3-4 fold increase in PgR mRNA levels (Fig. 2b-5C) compared to vehicle control (P<0.05). EFR4OHT and EFREndox compounds were able to induce expression of all genes investigated (Fig. 2b-5), significantly higher than their Z-isomers (P<0.05). Higher than therapeutic concentrations of test compounds, in particular isomers of FR4OHT and FREndox, were chosen to demonstrate their ability to regulate estrogen responsive genes at concentrations consistent with their inhibitory effects on the estrogen-induced cell proliferation (Fig.2b-2B).

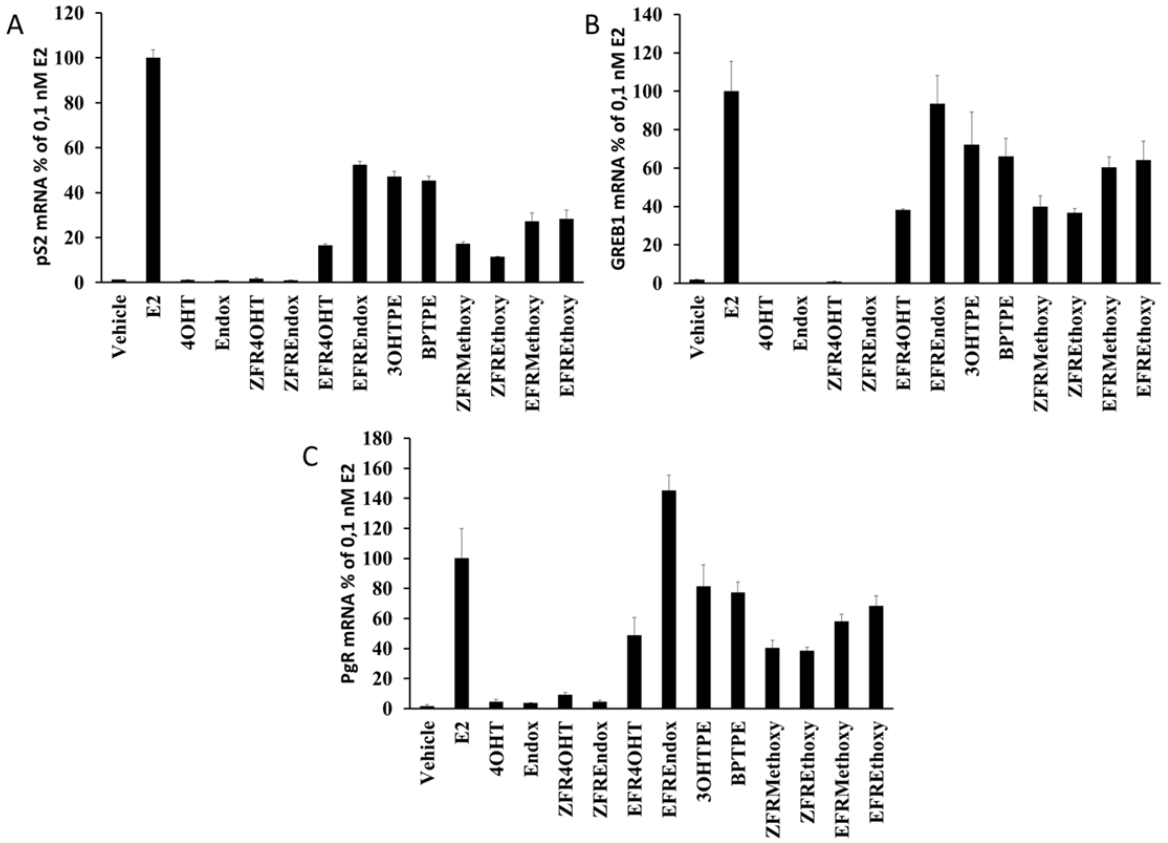


Figure 2b-5. *Assessment of estrogenic/antiestrogenic properties of the test compounds on inducing estrogen-responsive gene's mRNA expression in MCF-7:WS8 breast cancer cell line. A) pS2 gene; B) GREB1 gene and C) PgR gene. Treatment with E_2 was made at 10^{-10} M concentration, all of the other test compounds were treated at 10^{-6} M concentration. The fold change of the mRNA was first calculated using ΔΔCt method, corresponding 10^{-10} M E_2 control values were considered as 100% and all other treatments were calculated accordingly.*

Immunoblotting.

Immunoblotting was performed to assess the impact of the test compounds on the regulation of the ERŬ protein levels in MCF-7:WS8 cells. We starved the cells in the same way as estrogen starvation for cell proliferation assays. After 24 hour treatment with compounds, cells were harvested and processed as described in the Materials and Methods section. Results showed that 1nM E_2 reduces the level of ERŬ by about 60% as measured by densitometry. In contrast, 4OHT

and endoxifen and their ZFR analogs all caused an upregulation of the ERŬprotein. The estrogen-like E-isomers of FR4OHT and FREndox did not induce the down regulation of the protein. Fulvestrant (ICI) that degrades ERŬwas used as a positive control, and was able to downregulate the ERŬby more than 90%. Interestingly, compounds with shorter side chains like FRMethoxy and FREthoxy E and Z isomers and BPTPE and 3OHTPE were not able to induce any degradation of the ERŬlike E_2, despite their estrogenic properties in these cells, and actually upregulated the protein levels (Fig. 2b-6).

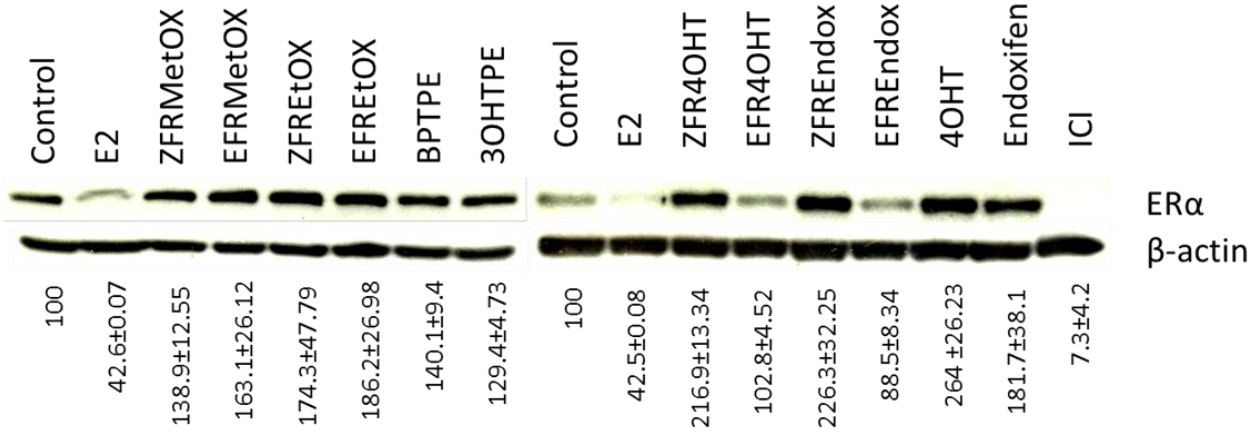


Figure 2b-6. *Immunoblotting results for test compounds after 24 hour treatment of MCF-7:WS8 breast cancer cells. Percent of control was calculated by comparison with the actin band. Immunoblotting experiments were repeated three times.*

Molecular Modelling

To study the binding mode of FR derivatives of endoxifen and 4OHT in the ER binding pocket, flexible docking simulations were carried out against both conformations of ER Ligand-Binding Domain (LBD), agonist (PDB codes 1GWR (ER LBD co-crystallized with E_2) [27], 3ERD (ER LBD co-crystallized with DES) [28], 3Q97 (ER LBD co-crystallized with ethoxytriphenylethylene isomers) [29] and antagonist (PDB codes 3ERT (ER LBD co-crystallized with 4OHT) [28], 1UOM (ER LBD co-crystallized with 2-phenyl-1-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-1,2,3,4-tetrahydro-isoquinolin-6-ol, PTI) [30], 2OUZ (ER LBD co-crystallized with lasofoxifene) [31]). The X-ray structures to be used for docking were selected based on the shape similarity between the investigated compounds and co-crystallized ligands of ER LBD complexes from PDB. In the following, the most relevant results obtained in docking simulations run against antagonist conformation 3ERT (Fig. 2b-7A) and two agonist conformations 1GWR (Fig. 2b-7B) and 3Q97 (Fig. 2b-7C) are discussed. We have selected this antagonist structure because the native ligand shows the highest structural similarity with the investigated compounds. The co-crystallized ligands were docked to their native experimental structures to evaluate the docking method efficiency. The best ranked docking poses of the native ligands recapitulate the binding mode of the ligand to the active site of the experimental structures and the same interactions with the aminoacids lining the binding pocket were found (Supplemental Figures S1, S2 and S3 in [32]).

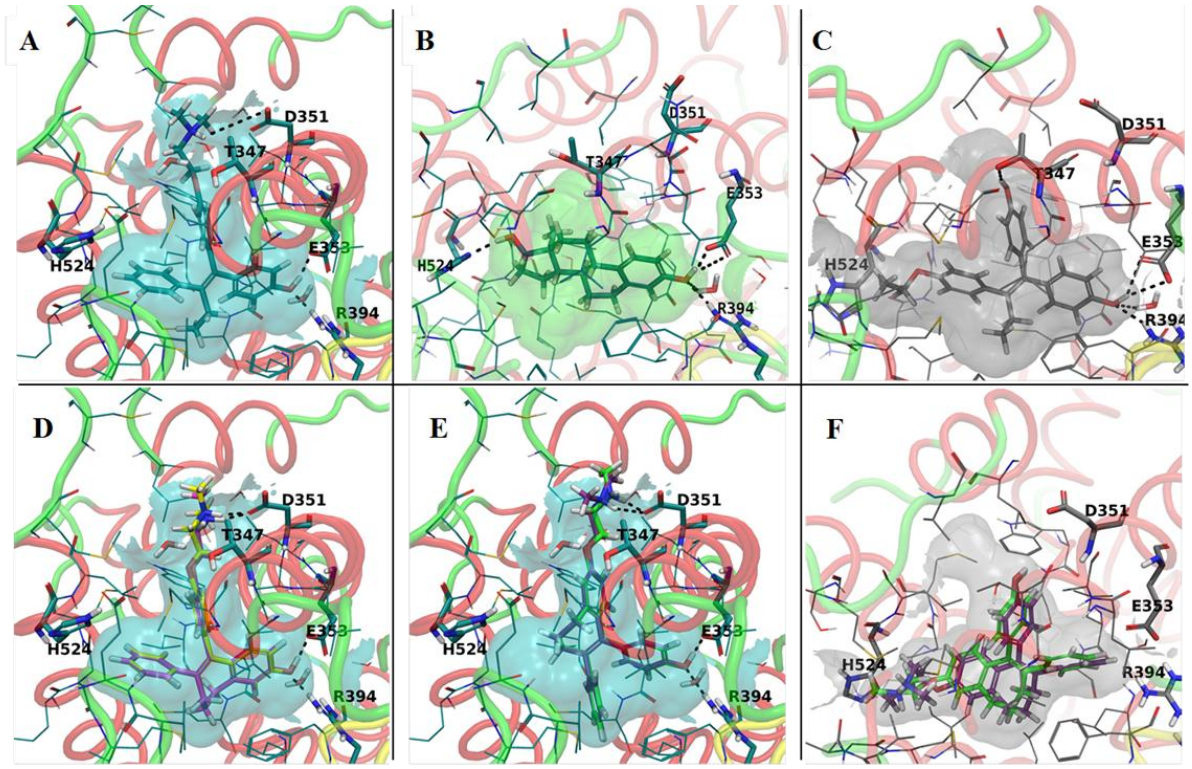


Figure 2b-7. *Representations of the experimental structures binding pockets used for modeling: A) the antagonist conformation- ER LBD co-crystallized with 4OHT (PDB code 3ERT); B) the agonist conformation of ER LBD co-crystallized with E_2 (PDB code 1GWR) C) the agonist conformation of ER LBD co-crystallized with a E-isomer of ethoxytriphenylethylene (PDB code 3Q97); the best docking poses of the Z-isomers to the LBD of ER (antagonist conformation; PDB code 3ERT) of: D) ZFREndox (yellow) and ZFR4OHT (magenta); E isomers of fixed ring 4OHT and Endoxifen do not fit very well into the antagonist conformation (3ERT): E) EFREndox (green) and EFR4OHT (purple); and the best docking poses of the Z-isomers to the LBD of ER (agonist conformation; PDB code 3Q97) of: F) EFREndox (green) and EFR4OHT (purple).*

The predicted binding mode of the ZFR4OHT and ZFREndox to the antagonist conformation of ER 3ERT is similar to that of 4OHT (Fig. 2b-7A). In these models the ligands are accommodated well in the binding pocket, the complex H-bond network involving aminoacids Asp351, Glu353 and Arg394 is recapitulated and similar hydrophobic interactions are encountered (Fig. 2b-7D). Conversely, the EFR4OHT and EFREndox are docked to the 3ERT binding site in a completely different alignment but forming the H-bonds with Asp351, Glu353 and Arg394 (Fig. 2b-7E). Although, the E-isomers form the H-bond network they do not fit the binding pocket of ER antagonist conformation as well as the Z-isomers, as can be seen from the docking scores (Table 1), especially the values for Emodel. E-isomers do not fill the binding pocket and are not involved in hydrophobic interactions with the important aminoacids of the binding site like the Z-isomers and 4OHT. These remarks are supported by the van der Waals (vdW) parameter which accounts for hydrophobic interactions and shows favorable values for Z-isomers (Table1). This binding alignment has been recapitulated in docking experiments performed for other experimental structures of ER LBD in antagonist conformation, 1UOZ and 2OUM (data not shown). These results show that it is highly probable for the E-isomers to be accommodated in a different conformation of ER LBD. Docking runs performed at the agonist conformations of ER (the receptor co-crystallized to E_2, PDB entry 1GWR (Fig. 2b-7B) and to DES, PDB entry 3ERD) has

led to conflicting results, thus, no valid docking pose could be found. For this reason other experimental structures of ER in the agonist conformation were selected from PDB, based on the 3D similarity between the co-crystallized ligands and E-isomers. The structure showing the highest shape similarity between the native ligand and E-isomers was selected, namely PDB entry 3Q97 (Fig. 2b-7C). Interestingly, this experimental structure contains two isomers, corresponding to E and Z-isomers of a triphenylethylene derivative, co-crystallized with ER LBD. The binding pocket of 3Q97 (Fig. 2b-7C) is wider and larger than the ones of 1GWR or 3ERD and it can accommodate the E-isomers. The top ranked docking poses of EFR4OHT and EFREndox are shown in Fig. 2b-8F and it can be seen they fit in the binding pocket. The Z- isomers were ranked with lower docking scores and were docked in an orientation similar with that from the antagonist conformation of ER. It can be concluded from these findings that the predicted binding mode of Z-isomers is similar with that of 4OHT and other antagonists of ER, showing higher values of the docking scores when compared with E-isomers docked to antagonist conformation of ER LBD. The former compounds do not fit into the encapsulated binding pocket of ER, corresponding to agonist conformation of the receptor, even if some degree of flexibility has been allowed to the receptor. It is highly probable for E-isomers to induce conformational changes to the active site of ER upon binding which would be reflected in the repositioning of helix 12 to a conformation related to that of the experimental structure 3Q97.

Compound	GScore	H bond	vdW	Coul	Emodel	CvdW
ZFREndox	-14.22	-1.5	-48.6	-15.4	-92.6	-64
ZFR4OHT	-13.22	-1.5	-50	-13.4	-92.6	-63.4
EFREndox	-10.65	-1.6	0.6	-7.9	38.6	-7.3
EFR4OHT	-10.59	-1.9	1.3	-9.4	37.8	-8.1

Table 2b-1. *Docking results for X-ray structure 3ERT. CvdW = Coul + vdW is the non-bonded interaction energy between the ligand and the receptor. Emodel is a specific combination of GScore. GlideScore (GScore in kcal/mol) is given by: GScore = a * vdW + b * Coul + Lipo + Hbond + Metal + Rewards + RotB + Site, where: vdW = van der Waals interaction energy; Coul = Coulomb interaction energy; Lipo = Lipophilic-contact plus phobic-attractive term; HBond = Hydrogen-bonding term; Metal = Metal-binding term (usually a reward); Rewards = Various reward or penalty terms; RotB = Penalty for freezing rotatable bonds; Site = Polar interactions in the active site. The coefficients of vdW and Coul are: a = 0.050, b = 0.150 for Glide 5.0 (the contribution from the Coulomb term is capped at -4 kcal/mol).*

The Z and E-isomers of FRMethoxy and FREthoxy compounds were also docked to the experimental structures of ER LBD in the agonist (PDB entries 1GWR and 3Q97) and antagonist (PDB entry 3ERT) conformations. Docking results analysis shows Z-isomers being better accommodated in the agonist conformation of ER than the E-isomers (Fig.2b-8B and 2b-8C). The Emodel and docking scores have higher values for Z-isomers. Few details indicate that it is possible for these isomers to bind to a conformation of ER similar to that of 3Q97. Thus, in the agonist structure 1GWR the alkoxy substituent is involved in clashes with the sidechains of Leu525 and Leu540 of helix12 while the fused rings system of the ZFREthoxy derivative is involved in clashes with Ile424 and Leu428 (Fig. 2b-8B). Thus, the best ranked docking poses of ZFRMethoxy and ZFREthoxy derivatives in the binding site of 3Q97 are free of these unfavorable contacts while a larger number of favorable interactions are formed with other hydrophobic aminoacids of the binding site (Fig. 2b-8C). The binding site of the antagonist conformation, 3ERT, is larger and exposed to the solvent and although the top ranked docking poses of Z-isomers form the H-bond network, the favorable hydrophobic contacts with Leu525 and Leu540 are missing (Fig. 2b-8A). As

a result, it can be concluded that it is highly probable for Z-isomers to bind to a conformation of ER similar to the experimental structure 3Q97. Regarding the E-isomers, the binding mode most frequently predicted by the docking poses is similar for the antagonist conformation, 3ERT (Fig. 2b-8D) and agonist conformation, 1GWR (Fig. 2b-8E) with the methoxy and ethoxy substituents pointing towards the region of the binding pocket lined by aminoacids Glu353 and Arg394. However, in this alignment clashes are encountered with these. Conversely, the top ranked docking poses at 3Q97 binding pocket show the alkoxy substituents oriented towards His524 in the opposite region of site and no H-bonds are formed (Fig. 2b-8H). Also, no clashes have been noticed with other aminoacids of the binding site.

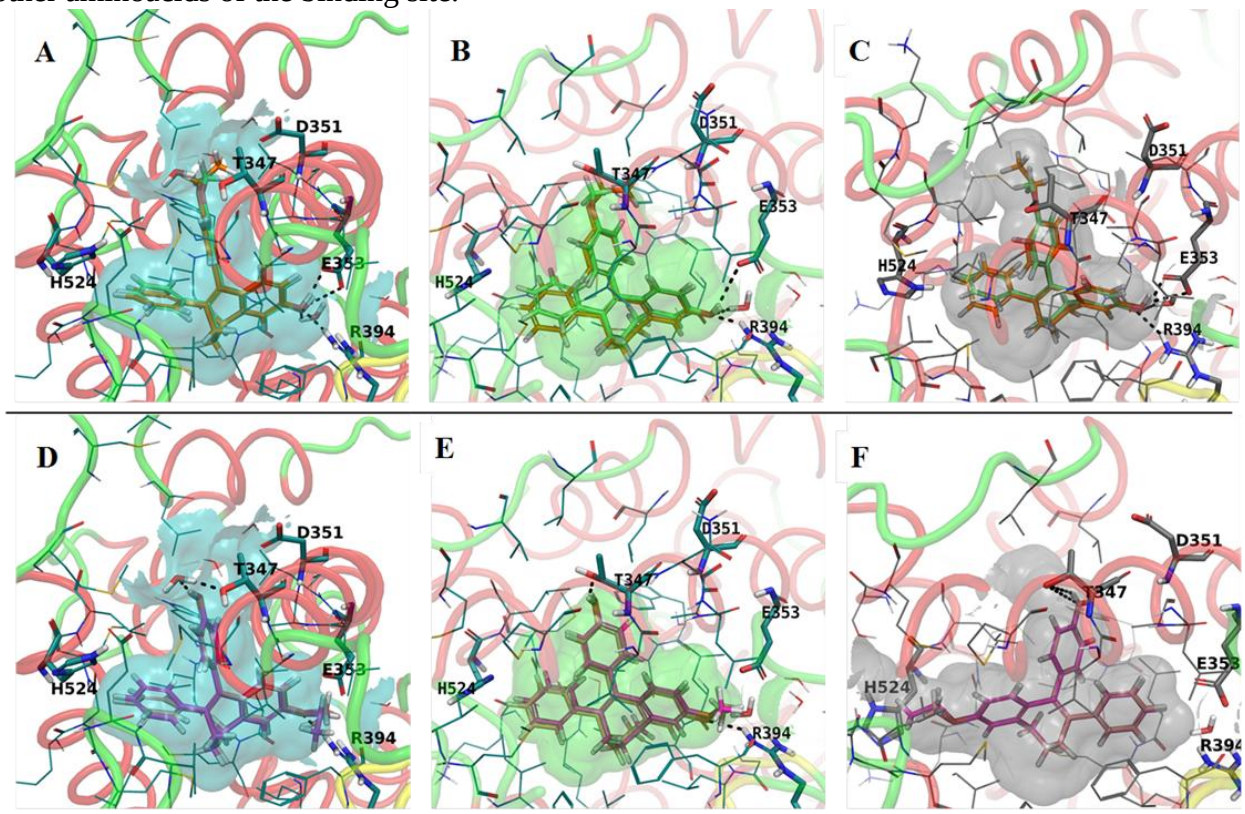


Figure 2b-8. *Representations of the experimental structures binding pockets used for modeling: A) the best docking poses of the Z-isomers to the LBD of ER (antagonist conformation; PDB code 3ERT) of ZFRMethoxy (green) and ZFREthoxy (orange); B) the agonist conformation of ER LBD co-crystallized with E_2 (PDB code 1GWR) of ZFRMethoxy (green) and ZFREthoxy (orange); C) the agonist conformation of ER LBD co-crystallized with a E-isomer of ethoxytriphenylethylene (PDB code 3Q97) of ZFRMethoxy (green) and ZFREthoxy (orange); D) the best docking poses of the Z-isomers to the LBD of ER (antagonist conformation; PDB code 3ERT) of EFRMethoxy (light pink) and EFREthoxy (magenta); E) the agonist conformation of ER LBD co-crystallized with E_2 (PDB code 1GWR) of EFRMethoxy (light pink) and EFREthoxy (magenta); F) the agonist conformation of ER LBD co-crystallized with a E-isomer of ethoxytriphenylethylene (PDB code 3Q97) of EFRMethoxy (light pink) and EFREthoxy (magenta).*

Discussion

The goal of this investigation is to link estrogenic/antiestrogenic ligand structures of tamoxifen metabolites with the well documented estradiol responses of cell replication or an estrogen target gene activation in cancer and apply biological endpoints to molecular modelling of the ER complex. This study has its origins with original published reports [16,33,34] of the

synthesis and evaluation of E and Z isomers of FR4OHT. We now extend earlier work with this study of E and Z ER endoxifen, investigate new Z and E FRMethoxy and FREthoxy derivatives of triphenylethylene (TPE) and compare our results with the angular estrogens BPTPE and 3OHTPE [15]. The biological endpoints used were cell replication in MCF-7:WS8 cells and the estrogen-regulated prolactin gene (Prl) in rat pituitary gland tumor GH3 cell line.

There are several important new findings with the structure-function relationship of new FR compounds. The length and positioning of the side chain of the new Z and E FR compounds govern estrogen-induced cell replication of MCF-7:WS8 cells (Fig. 2b-2A). The natural estrogen E_2 is extremely active as a full agonist over the range 10^{-14}-10^{-8} M, however each of the Z FR derivative are partial agonists, so comparative EC_{50} calculation are not appropriate. Nevertheless, BPTPE is a potent partial agonist (50% max of E_2 curve) over the range 10^{-12}-10^{-9} M. The Z FRMethoxy and Ethoxy partial agonist curve is displaced a log to the right and the EFRMethoxy and Ethoxy is displaced further. The E FR isomers of 4OHT and endoxifen are both low potency estrogens and this is consistent with their lower ligand-binding activity of the ER [35]. Only the nonsteroidal antiestrogens 4OHT and endoxifen and their ZFR derivatives were antiestrogenic on cell proliferation. By contrast, all compounds were antiestrogenic (fig. 2b-2B) at 1ÕM in the GH3 rat pituitary prolactin assay i.e.: down to the level of the partial agonist activity of each compound (Fig. 2b-2A). The inability of substituted angular estrogens to be unable to initiate prolactin gene synthesis fully but stimulate mouse vaginal cornification (which classifies them as estrogens) has been noted previously [36,37,38]. The partial gene regulation (pS2, GREB1 and PgR) is also noted with BPTPE and 3OHTPE as well as the E and Z FRMethoxy and Ethoxy TPEs. It is interesting to note that at 1 ÕM EFREndox is particularly active in triggering pS2, GREB1 and PgR (Fig. 2b-5) so the ability of the E isomers of FR4OHT and Endox were tested at therapeutic concentrations [24] to determine whether estrogen-induced cell replication could occur during therapy. None was noted (Fig. 2b-4).

Additionally, results from RT-PCR of the estrogen-responsive genes in MCF-7:WS8 cells show that the E-isomers are inducing higher expression of pS2, GREB1 and PgR genes mRNAs, and also Prl gene mRNA in rat GH3 cells. This contrasts with the Z-isomers. Considering all the results, it is possible to conclude that the E-isomers of the biologically active tamoxifen metabolites 4OHT and endoxifen have estrogenic properties in human breast cancer cells but this is not of biological significance during therapy with tamoxifen.

The most important general observation was the sensitivity of all the different TPE structures to trigger cell replication (Fig. 2b-2A). This supersensitivity is clearly required for cancers to survive through relentless cell replication. Antiestrogenic activity blocking replication requires a correctly positioned alkylaminoethoxy side chain [39]. By contrast, estrogen-regulated protein synthesis is much less successful with test compounds and the resulting complex is clearly less promiscuous, tending to create a biologically inert ñantiestrogenic complexò.

It is interesting to note that the accumulation of ER determined by Western blotting for all compounds is independent of estrogenic or antiestrogenic activity. The turnover of ER complexes is regulated by ubiquitinilation and proteosomal degradation [40] but it is clearly the shape of the ligand and the resulting conformation of the complex that determines accumulation or destruction. The shape of the ligand is critical; a planar class I (estradiol) ligand causes reduction of ER whereas non-steroidal antiestrogens such as 4OHT and endoxifen [41] cause the ER complex to accumulate. The same is true of angular TPEs [41] which are also all of the new FR compounds investigated here that bind to the ER. By contrast, fulvestrant (ICI 182,780) causes the rapid destruction of ER [42]. A previous study by Wu et al [43] demonstrated that endoxifen also caused rapid destruction of ER but this was not observed in this study. We used endoxifen obtained from the Mayo clinic and the Z FR endoxifen, both of which had the same accumulation of the ER.

Molecular modeling demonstrates that most likely the positioning of the E-isomers in the ligand-binding cavity of the ER is different due to repositioned side chains, potentially reducing the affinity to the receptor. However, this structural change also alters the pharmacological properties of the E-isomers, as they are more estrogenic rather than antiestrogenic. The molecular modeling shows that the E-isomers fit better into the ER conformation when the receptor is bound to a structurally similar E-isomer of ethoxytriphenylethylene where X-ray crystallography (PDB entry 3Q97) shows that the H12 of the LBD is actually closed, which resembles the conformation induced by estrogens [28]. This is also confirmed by the Western blotting results for the ER protein levels, which show that the Z-isomers of FR4OHT and endoxifen, being antiestrogens, are inducing upregulation of the ER protein levels, however the E-isomers are not inducing the same upregulation, indicating their different properties (Fig. 2b-6). However that is not the case with fixed-ring compounds with shorter side chains. In contrast, Z-isomers of FR 4OHT and endoxifen fit better into the antagonist conformation of the ER LBD [28]. Compounds with shorter side chain fit better into the conformation of the ER LDB that accommodates their E-isomers resulting in the H12 being closed. This results in estrogenic activity.

In summary, a well-defined series of compounds has been classified and characterized for cell growth and estrogen target protein synthesis. The important finding is that replication in the ER-positive breast cancer cell is extremely sensitive to stimulation by a broad range of synthetic estrogens. This supersensitivity to growth stimuli is the major survival mechanism of cancer. It is a simple principle based on growth to survive from any source through the ER signal transduction pathway. This promiscuous pathway is only stopped when the antiestrogenic side chain of antiestrogens interacts with Asp351 and Helix 12 is prevented from closing [44]. By contrast, the transcription of RNA for estrogen target genes such as prolactin is highly selective with these new compounds synthesized in this study. The compounds tend to become antiestrogenic (Fig. 2b-6) possibly because the conformation of the ER complex cannot recruit all necessary transcription factors. The conformation of the complex is critical. However, it is also important to appreciate that X-ray crystallography of complex 3Q97, that appears to be estrogen-like, only gives a glimpse at that one moment of time of low energy crystallization. We anticipate that progressive changes occur over time as the estrogen ER complex adapts to the changing environment within the cell. Biological end points are correlated with the receptor docking of a new intermediate form of the ER ligand-binding domain (PDB entry 3Q97). These data will be used in the future to decipher and to advance the understanding of the molecular mechanisms of estrogen-induced apoptosis [45].

These data and compounds will help us to further expand our knowledge about estrogen-induced apoptosis in estrogen-independent breast cancer cells through and the modulation of estrogen-induced apoptosis by the ER conformation. Currently we are finishing experiments with these compounds in MCF-7:5C cells and new data will be included in the appendix part of this report once published.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2c (Obiorah and Jordan) - Studies carried out by Dr. Ifeyinwa Obiorah in the Jordan laboratory at Georgetown University

Differences in the Rate of Oestrogen-induced Apoptosis in Breast Cancer by Estradiol and the Triphenylethylene Bisphenol

Introduction

We have addressed the paradox that an angular class II oestrogen, BP, can act as an inhibitor of oestrogen-induced apoptosis by adopting an ñantiestrogenic conformationò for the BP-ERŬ complex, but related triphenylethylenes are effective antitumor agens in patients. We have found that the trigger for oestrogen-induced apoptosis is dependent not only on the shape of the oestrogen-ERŬ complex, but also on the duration of oestrogen exposure.

Work Accomplished:

Differential expression of cell cycle genes induced by bisphenol and 17β oestradiol

To identify cell cycle genes associated with BP induced cell growth, MCF7 cells were treated with 1ÕM BP for 6h, 12h and 24h and compared to 1nM E_2 and 1ÕM 4OHT as positive and negative regulators of cell replication respectively. The antiestrogen, 4OHT was used to block the stimulatory effects of BP and E_2. We used RT-PCR array kits that contain 4 x 96 well plates to profile the expression of 84 genes key to cell cycle regulation. At 6h, E_2 induces several genes such as cyclin D1 (CCND1), CDK5R1, HERC5, CHEK2 and RBBP8 (Figure 2c-1A). Bisphenol and 4OHT only induced HERC5. Interestingly CCND1 was downregulated by BP at this time point. There was increased expression of cell cycle related genes by E_2 at 12h (Figure 2c-1B), which further increased by almost 2 fold at 24h (Figure 2c-1C). Similarly, BP induced 60% and 50% of the cell cycle related genes that were up-regulated by E_2 at 12h and 24h respectively. The rest of the cell cycle related genes induced by BP show an obvious trend of overexpression when compared to the control. Similarly, all cell cycle genes downregulated by BP are equally decreased by E_2 treatment. Unlike the estrogens, 4OHT did not activate the cell cycle related genes but rather blocked the effects of E_2 and BP. These results demonstrate that BP induces similar cell cycle related genes as E_2, although not as effectively.

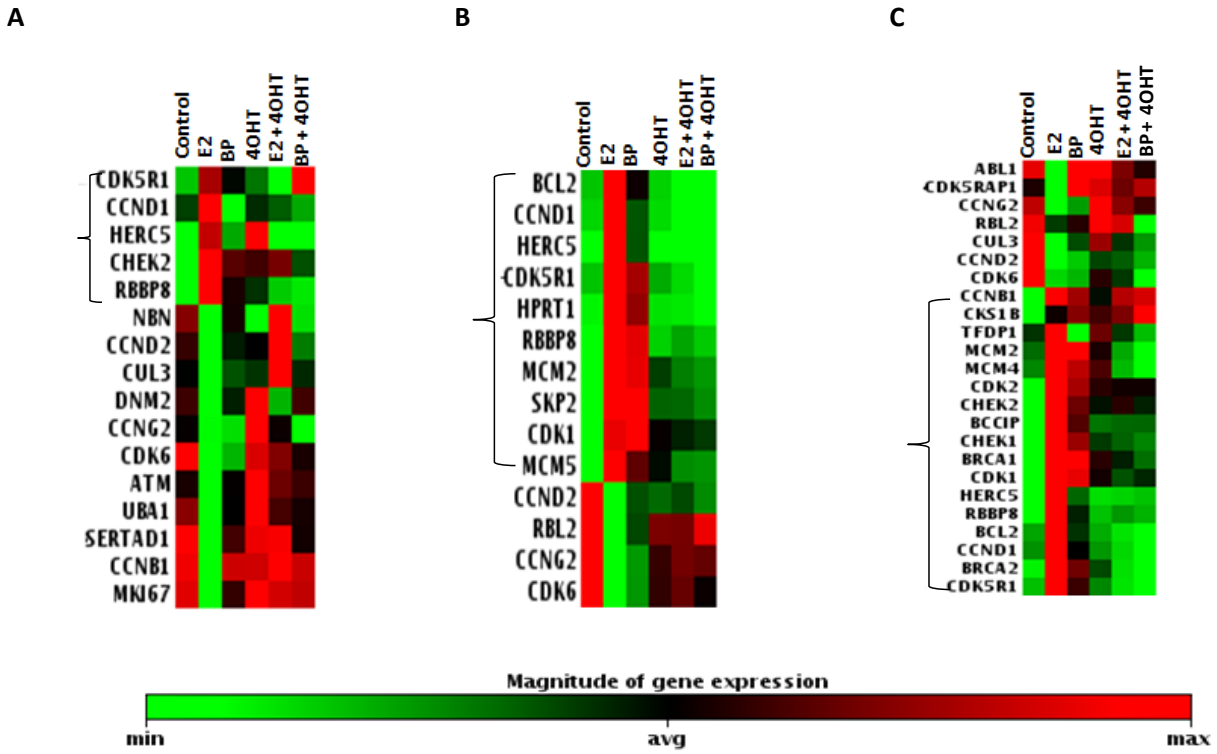


Figure 2c-1. *Heat map of the time course pattern of E_2 and BP-regulated expression of cell cycle genes. MCF-7 breast cancer cells were treated with either control, E_2 (1nM), BP (1µM) or 4OHT (1µM) over a period of 24h and 4OHT was used to block the effects of E_2 and BP. Genes which are at least 2.5 fold over-expressed (red) or under-expressed (green) as compared to control at p value of 0.05 at (A) 6h, (B) 12h and (C) 24h are presented. Cell cycle genes induced by E2 and BP are indicated in black.*

Effect of bisphenol on apoptosis in MCF7:5C cells

The planar type 1 oestrogen, E_2 induces apoptosis in long term oestrogen deprived MCF7 (MCF7:5C) cells. On the other hand, the angular oestrogen BP does not initially induce apoptosis in MCF7:5C cells and blocks E_2 induced apoptosis in a similar manner as does 4OHT. To evaluate the long term effects of BP, we treated MCF7:5C cells with 1 ÕM BP, 1nM E_2 and 0.1% ethanol vehicle (control). Growth of the cells was inhibited by E_2 after 3 days of treatment and the effect became maximal by 6 days of treatment (Figure 2c-2A). On the other hand, BP increases the growth of the cells up to 6 days of treatment (Figure 2c-2A) but causes 100% inhibition of growth by 9 days of treatment (Figure 2c-2B). The inhibition of growth observed with BP was further investigated for apoptosis using flow cytometry. Following 6 days of treatment, BP caused a 7 fold increase in the percent of cells (4.15% vs. 27.61%) undergoing apoptosis compared to the control (Figure 2c-2C) using Annexin V staining.

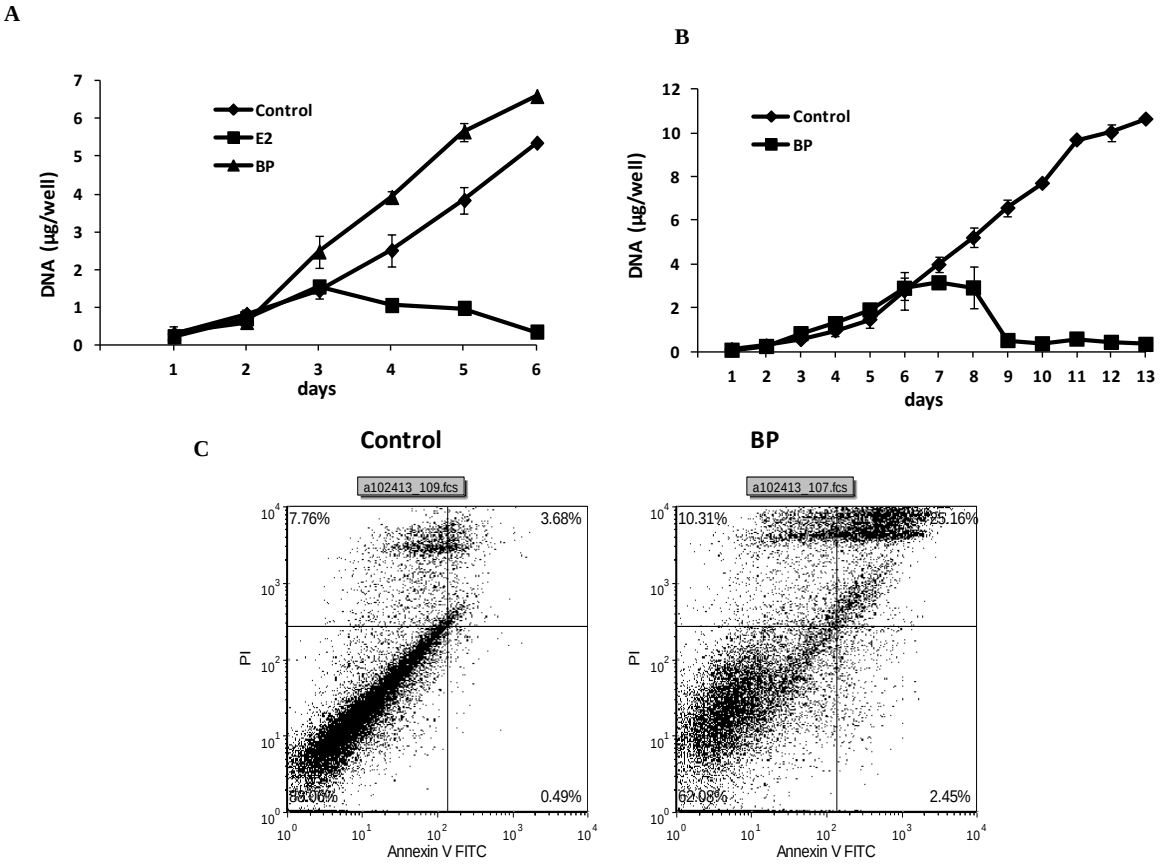


Figure 2c-2. ***Effect of BP in the growth and apoptosis of MCF7:5C breast cancer cells.*** *(A) Cells were seeded in triplicates and treated with either control, E_2 (1nM) or BP (1µM) and the cells were harvested daily for 6 days. (B) Treatment with BP versus the control was extended for 13 days and the DNA content of the remaining cells in each well was quantified. The data represent the mean of three independent experiments. (C) MCF7:5C cells were treated with control or BP (1µM) for 6 days and then stained with annexin v-FITC and propidium iodide and analysed by flow cytometry. Viable cells (left lower quadrant) are annexin v-FITC- and PI-, early apoptotic cells (right lower quadrant) are annexin v-FITC + and PI-, dead cells (left upper quadrant) are PI+ and late apoptotic cells (right upper quadrant) are annexin v-FITC + and PI +. Increased late apoptotic effect is observed in the right upper quadrant.*

Determination of the point of commitment for BP induced apoptosis

Next, we investigated the delayed response of BP, MCF7:5C cells were treated with BP [1ÕM] and 4OHT [1ÕM] was used to block the antiproliferative and apoptotic effects of BP at daily intervals over a range of 9 days. Cells were harvested after 13 days of treatment and total DNA was quantified using a fluorescent DNA quantification kit. Apoptosis induced by BP was blocked by daily additions of 4OHT for up to 3 days and afterwards the cells became committed to apoptosis mediated by BP (Figure 2c-3). 4OHT alone caused a small decrease in DNA similar to that observed at day 1,2,3. After day 3, an irreversible decline occurred with BP that was not rescued. The day 4 value was about 50% of the control or 4OHT alone values. Cells could not be rescued from BP induced apoptosis by 4OHT after 4 days of treatment suggesting that the cell commitment trigger for apoptosis has occurred. It is important to emphasize that each of the two ñrescueò experiments adds antiestrogens 4OHT or ICI 182,780 at specific days after BP and measures cellular DNA at 13 days. MCF7:5C cells are both committed to apoptosis after day 3 with either antiestrogen (Fig 2c-3).

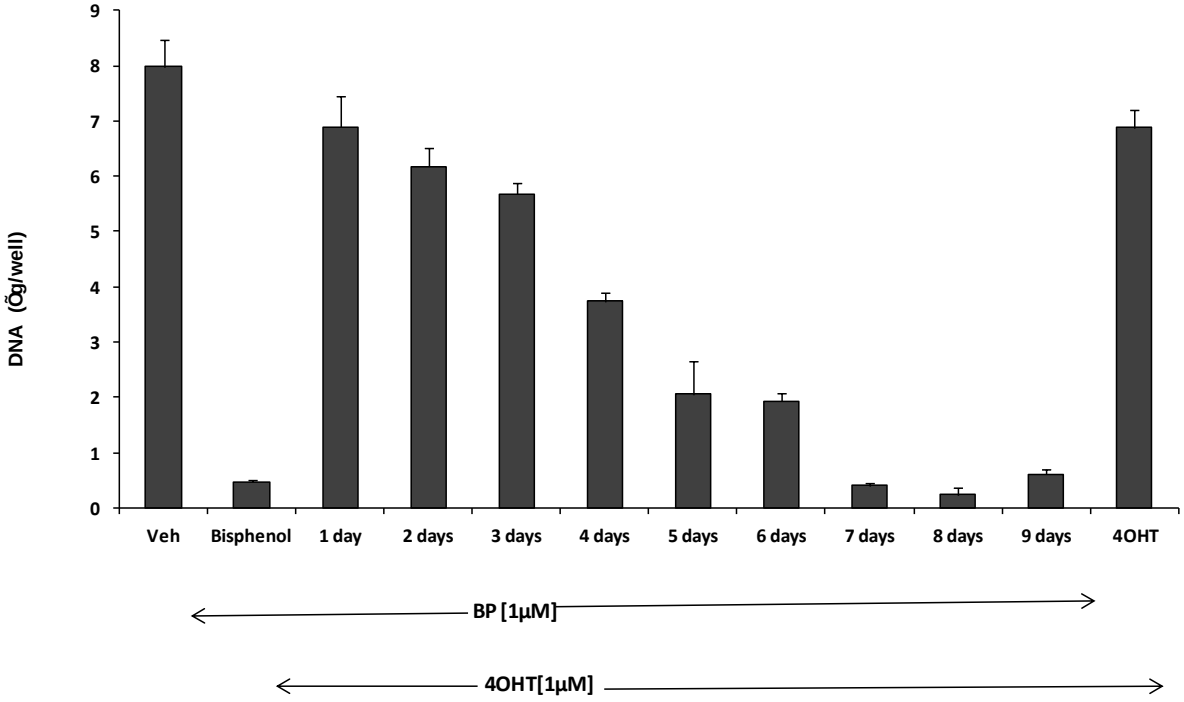


Figure 2c-3. ***Determination of the trigger point for BP induced apoptosis.*** *MCF7:5C cells were treated with BP (1µM) alone and 1µM 4OHT was added and used to block and reverse BP action daily over a period of 9 days. The cells were harvested after 14 days of treatment. The DNA content of the remaining cells was quantified using a fluorescent DNA quantification kit. The point of trigger for apoptosis induced by BP is determined by the time when the apoptotic effects of BP could not be blocked by 4OHT.*

Apoptosis related genes induced by bisphenol

To determine the early events preceding BP induced apoptosis, the induction of apoptosis related genes were investigated in MCF7:5C cells treated with BP [1ÕM], 0.1% ethanol vehicle (control), 1ÕM 4OHT and BP in combination with 4OHT (in triplicates) for 3, 4, 5 days. We used 384 well RT-PCR profiler plates to monitor expression of 370 apoptosis related human genes (see Methods). Comparative analysis showed that significant evidence of apoptotic gene induction did not occur until after 3 days of treatment. At 4 days (Figure 2c-4A) BP induces ERS related genes; DDIT3 and inflammatory stress (IS) response genes such as CEBPB, IFI6, IFI16 and DAPK1. At 5 days of treatment (Figure 2c-4B), there is continued increase in the up-regulation of ERS and IS-associated genes including LTA and caspase 4, an inflammatory caspase. Bim/BCL2L11 is important for E_2 induced apoptosis. Its acivation by E_2 occurs by 36h of treatment[46] and E_2 subsequently induces the TNF family of proapoptosis related genes. The induction of these genes by BP was investigated by extending the duration of treatment for 7, 8 and 9 days mRNA levels of BCL2L11 and TNF were quantified by RT-PCR. Upregulation of Bim/BCL2L11 (Figure 2c-5A), TNF (Figure 2c-5B), FAS (Figure 2c-5C) and FADD (Figure 2c-5D) was observed by 8 days of treatment with continued increase of all genes at 9 days of treatment with BP. These data indicate that there is a prolonged induction of ERS and IS-associated genes by 4 days of treatment with subsequent up-regulation of mitochondrial and TNF related apoptosis genes.

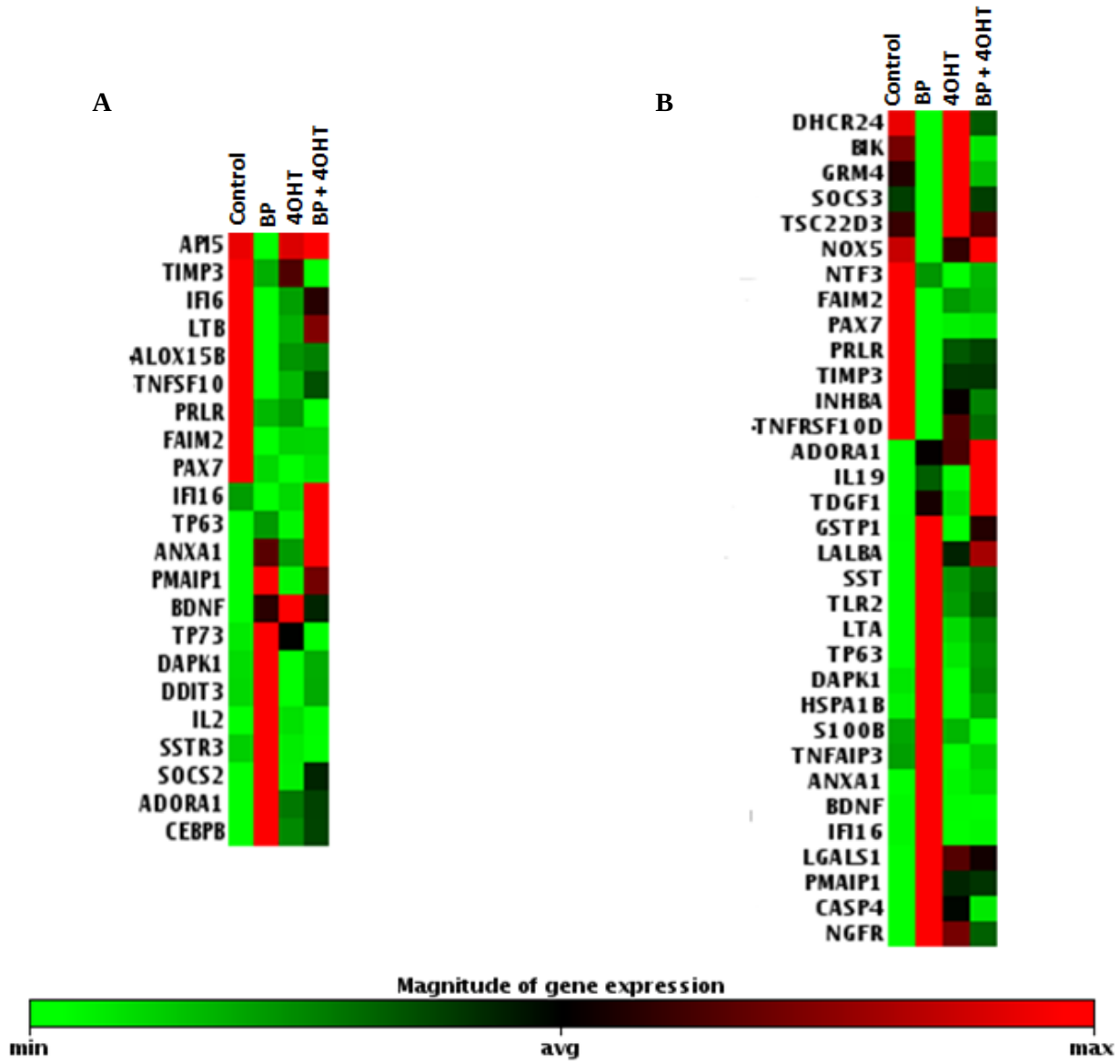


Figure 2c-4. ***Determination of apoptotic genes differentially expressed by BP treatment in MCF7:5C cells.*** *MCF7: 5C cells were treated with vehicle (control), 1µM BP, 1 µM 4OHT, in the presence or absence of BP over a period of 5days. Gene expression values were obtained and analyzed in comparison to the controls and heat maps were generated at (A) 96h and (B) 120h of treatment and the expressed genes listed. The selected genes were at least 2.5 fold over-expressed (red) or under-expressed (green) as compared to control at p value of 0.05.*

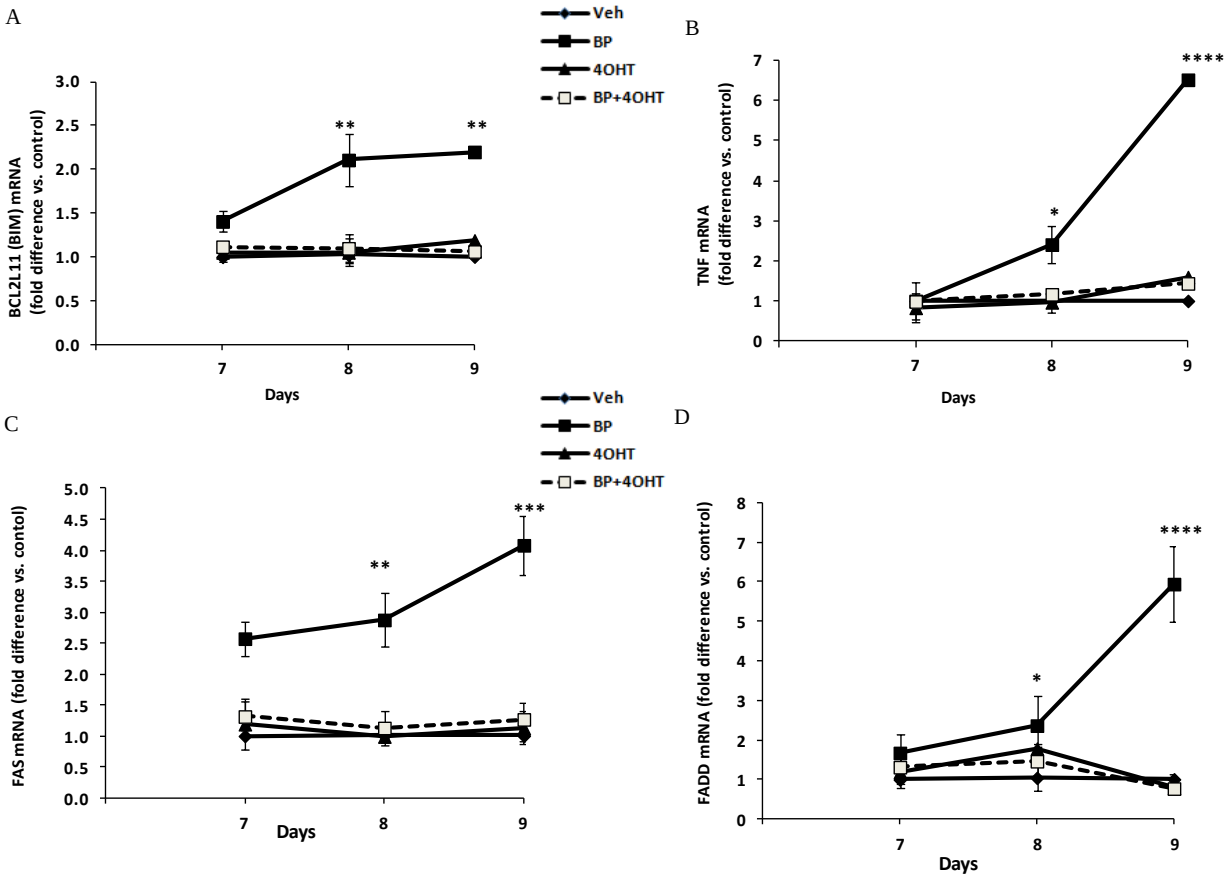


Figure 2c-5. ***Induction of apoptotic genes by BP. BP induces apoptotic genes after 7 days of treatment.*** *MCF7:5C cells were treated with Vehicle (Veh), BP (1 µM), 4OHT, 1µM or combination treatment of BP and 4OHT for 7-9 days. Total RNA was isolated and reverse transcribed and (A) BIM and (B) TNF (C) FAS and (D) FADD mRNA levels was determined using RT-PCR. PCR data values are presented as fold difference versus vehicle treated cells ± SEM. [* P< 0.05, ** p<0.005, *** p<0.0001, ****p<0.0005]*

Differential effect of bisphenol on cell cycle

Since the BP induced apoptosis is not apparent until the second week of treatment, we evaluated the effect of BP on the regulation of the cell cycle. MCF7:5C cells were treated with either vehicle control (0.1% ethanol), 1nM E_2 or 1ÕM BP for 24h, 48h and 96h and performed cell cycle analysis using flow cytometry (Figure 2c-6). As suspected, BP and E_2 cause a consistent increase in the S phase when compared to the control. Although, the trigger for apoptosis occurred for E_2 and BP at 36h and 96h (Figure 2c-3) respectively, no checkpoint blockade was noted after treatment with either compound and contrasts dramatically with early cell cycle arrest at G2/M with paclitaxel.

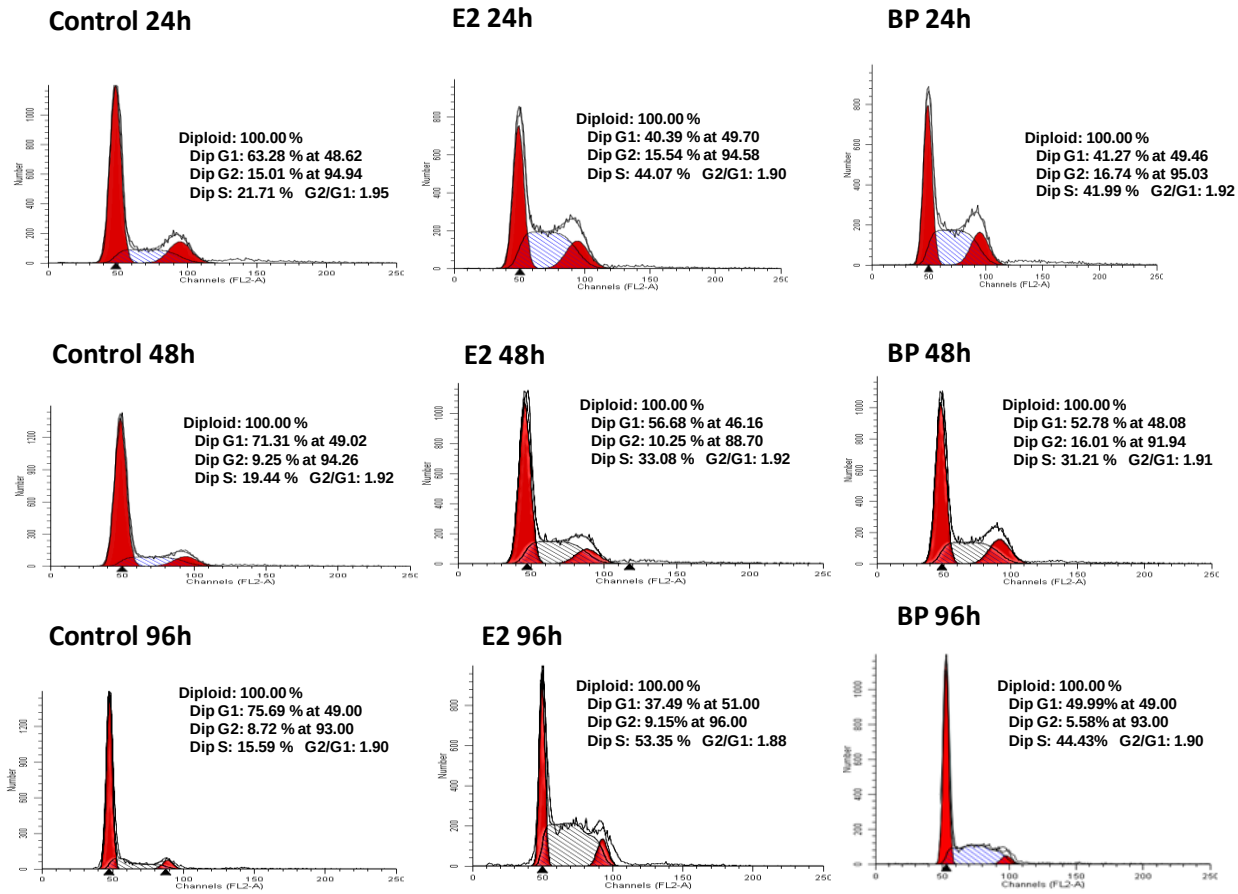


Figure 2c-6. ***Diverse effects of BP and E2 on cell cycle progression.*** *Distribution of the cells through the cell-cycle phases was analyzed by flow cytometry in cells treated with E_2 (1nM), BP (1 µM), or control for 24 h, 48h and 96h. The percentage of the cells in each fraction is calculated using the ModFit software. The y axis represents the number of cells and FL2-A represents the intensity of propidium iodide.*

Functional importance of caspase 4 in bisphenol induced apoptosis

Caspase 4, an inflammatory caspase, is upregulated in the MCF7:5C cells by 5 days of treatment with BP. To determine the role of caspase 4 in BP induced apoptosis, cells were treated with control or BP (1ÕM) and the effects of caspase 4 was blocked by caspase 4 inhibitor-z-LEVD-fmk (10ÕM). Growth inhibited by BP was reversed by z-LEVD-fmk (Figure 2c-7A). Proliferation was determined after 12 days of exposure to BP and quantified by DNA mass per well. Apoptosis induced after 6 days of exposure to BP was completely reversed by z-LEVD-fmk (Figure 2c-7B). Thus, the blockade of BP induced apoptosis by caspase 4 inhibitor-z-LEVD-fmk indicates that caspase 4 plays an important role for the induction of apoptosis by BP.

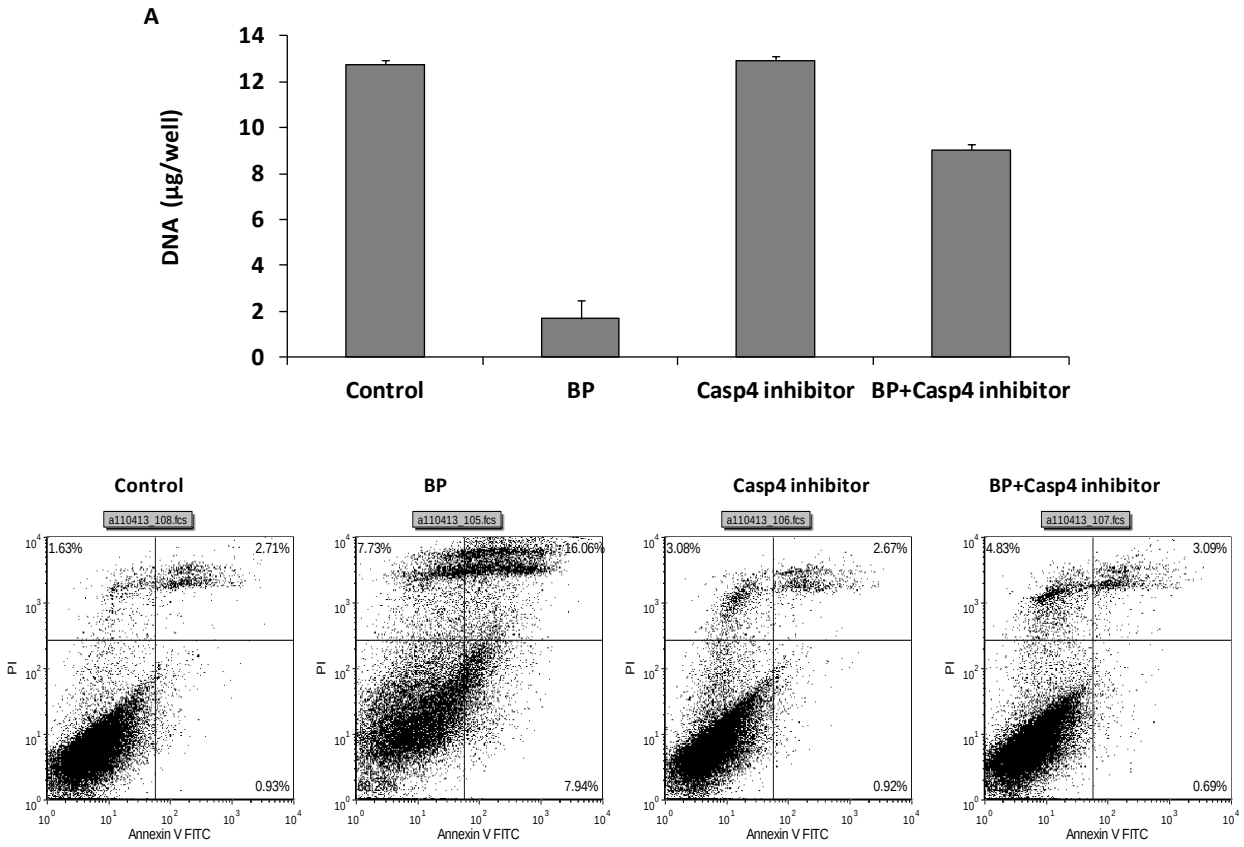


Figure 2c-7. Caspase 4 is important for BP induced apoptosis. MCF7:5C cells were treated with control(0.1% ethanol) or BP (1ÕM) or caspase 4 (casp4) inhibitor with or without BP for either (A) 12 days and assessed for cell proliferation or (B) for 6 days and evaluated for apoptosis. Apoptosis and inhibition of growth of cells were blocked by caspase 4 inhibitor z-LEVD-fmk (10 µM).

DISCUSSION

The aim of our study is to elucidate the growth and induction of apoptosis by BP in fully oestrogenised and long term oestrogen deprived breast cancer cells. The ERŬ in breast cancer cells can either initiate replication or trigger apoptosis based on the context of cell selection in estrogen replete or deprived environments[10,47] (Originally, oestrogens including, E_2 and TPE derivatives were discovered using a bioassay of the induction of vaginal cornification in ovarectomised mice. Replication and cornification of vaginal cells in the mouse was the early appropriate method of establishing the structure-function relationships of an oestrogenic TPE molecule. Initial structure-function studies *in vitro* established an ERŬ mediated mechanism for E_2 stimulate prolactin (an oestrogen responsive gene) synthesis in rat pituitary cells [35,48]. However, BP and other TPE derivatives were found to act as partial agonists with antioestrogenic properties at the prolactin gene *in vitro* [36] Structure-function relationship studies to modulate prolactin synthesis by extending the length of the ñantioestrogenic side chainò created an antioestrogen that blocked oestrogen stimulated prolactin synthesis [38]. These are the basic early facts of the pharmacological function of the oestrogen-ERŬ complex that now allows us to interpret our current findings on the modulation of apoptosis.

Our results show that BP induces cell cycle regulated genes that are similar to those activated by E_2 in MCF-7 cells. This correlates with the ability of BP to induce replication of MCF-7 cells in a comparable manner as E_2 [15]. On the other hand, 4OHT, which possesses a bulky alkylaminoethoxy side chain [28], failed to induce cell cycle regulated genes in a time dependent manner but rather blocks E_2 and BP mediated activation of cell cycle genes, therefore confirming its role as an antioestrogen. Although BP

possesses a bulky phenyl substituent, it does not have an akylaminoethoxy side chain. Molecular modeling studies suggest that the phenyl component of TPEs prevent the complete sealing of the ligand binding domain of the ERŬ by helix 12 [49,50]. The reduced number of gene changes noted with BP treatment compared with E_2 (Figure 2c-1) may be caused by differences in the structure of the ligand-ERŬ complex, thus resulting in a reduction in the full oestrogenic potential of BP induced replication. Additionally, BP unlike E_2 does not readily induce apoptosis in long term oestrogen deprived MCF-7 cells but rather appear to possess early antioestrogenic properties. Using cell proliferation assays, BP induces growth of MCF7:5C cells in the first week of treatment. In contrast, growth inhibition occurs after the third day of treatment with E_2. Inhibition of growth in oestrogen deprived MCF7:5C cells with BP is seen after 8 days of treatment. Similarly, apoptotic effects of BP are observed following 6 days of BP treatment using flow cytometry studies (Figure 2c-2). Previous studies have shown that MCF7:5C cells are resistant to the actions of 4OHT, which has the ability to reverse and block E_2 mediated apoptosis. Using 4OHT or ICI 182,780 to block and rescue the cells from BP induced apoptosis suggests that the trigger for apoptosis occurs with BP after 4 days of treatment. There was no evidence of cell cycle arrest with either E_2 or BP (Fig 2c-6) prior to apoptosis. This contrasts dramatically with our previous publication of E_2 and the rapid G2 blockade triggered by paclitaxel prior to apoptosis, no checkpoint blockade was noted after treatment with either compound and contrasts dramatically with early cell cycle arrest at G2/M with paclitaxel [46].

The apoptosis related genes clearly demonstrate that the majority of genes that are upregulated by BP at 4 days of treatment are ERS and IS response genes. DDIT3 also known as CHOP or GADD153 is a key ERS protein associated with cell death [51], whereas CEBPB, which is known to induce proinflammatory cytokines such as IL6 [52], is activated by ERS and is important for nuclear transport of DDIT3. There is a continued induction of similar proapoptotic genes at 5 days of treatment including caspase 4, an inflammatory caspase that predominantly localizes to the endoplasmic reticulum and undergoes cleavage and induces effector caspases in response to ERS. Upregulation of Bim, FAS, TNF and FADD mRNA are observed by 7 days of treatment with BP. Microarray analysis indicate ERS-mediated apoptosis as the top scoring pathway of apoptosis induced by E_2 in MCF7:5C cells [10]. Oestradiol induces ERS and IS response genes by 36h of treatment and apoptotic genes such as Bim and TNF are activated by 48h of treatment. A similar trend is observed with BP, however there is a prolonged ERS and IS with subsequent induction of caspase 4 at 5 days of treatment and mitochondrial and extra-mitochondrial apoptotic genes at 7 days of treatment. After 48h treatment with BP, there is no induction of apoptotic genes but an increase in growth (Figures 2c-2A, 2c-6) and the cells can be rescued from apoptosis with antioestrogens (Figure 2c-3).

The initial resistance to trigger apoptosis may also result from the antioestrogenic conformation BP creates with the ERŬ. Angular TPEs such as BP have a reduced tendency to promote recruitment of coactivators containing LxxLL motif [53]. We have previously shown that BP recruits the ERŬ and SRC3 to the PS2 promoter ERE less efficiently when compared to planar estrogens thus indicating that complete sealing of helix 12 of the LBD and interaction of coactivators with the TPE-ERŬ complex is necessary for the rapid activation of apoptosis observed with planar estrogens ([50] Depletion of SRC3 in the MCF7:5C cells and MCF-7 cells leads loss of E_2 induced apoptosis and growth respectively [54].

Since caspase 4 is specifically activated by ERS and it was induced by 2 fold with E_2 within 24h but by 2 fold by BP within 96h, a specific caspase 4 inhibitor [55] was used to block activation of caspase 4 in BP treated cells and this resulted in reversal of BP inhibited growth and apoptosis (Figure 2c-7). We previously reported that E_2 induced apoptosis can be blocked by a caspase 4 inhibitor [10]. Together, these results suggest that BP activates IS and ERS related genes which interact with resultant induction of caspase 4 between 4 and 5 days of treatment and subsequent activation of mitochondrial and extramitochodrial related apoptotic genes in the second week of treatment. This delayed sequence for BP contrast with early activation by E_2 [10].

In summary, we have used cell based assays and gene profiling studies to demonstrate the biological response of the TPE, BP on both growth and apoptosis. TPEs were among the first chemical therapy used in the treatment of advanced breast cancer in postmenopausal women. These data support the apoptotic mechanism of TPEs in early clinical practice.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2d (Obiorah and Jordan) - Studies carried out by Dr. Ifeyinwa Oborah in the Jordan laboratory at Georgetown University

Defining the Conformation of the Estrogen Receptor Complex That Controls Estrogen-Induced Apoptosis in Breast Cancer

Introduction:

To interrogate the relationship of structure of an estrogenic ligand to program the conformation of the ER complex, we synthesized a range of estrogenic TPEs , which are structurally similar to 4OHT. We and others hypothesize that the structure of the ligand governs the external surface of the ER complex with either planar estrogens or the TPEs [56,57]. As a result of the ligand shape, the estrogens can program the conformation of the estrogen-ER complex to modulate rapid or delayed apoptosis. The growth response of the ER positive breast cancer cells is very sensitive to a wide range of estrogenic ligands. This is to ensure cancer cell survival in austere estrogen environments. This may not be true for estrogen induced apoptosis and the ligand shape may be required to be more specific to trigger cell death. The estrogen deprived cancer cell is protected. We investigated the actions of clinically relevant planar estrogens (E2, diethylstilbestrol, equilin, estrone and equilenin), anti-estrogens (4OHT, endoxifen. raloxifene and bazedoxifene) and model TPEs (bisphenol, trihydroxytriphenylethylene and ethoxytriphenylethylene) on growth in MCF-7 cells and apoptosis in MCF-7:5C cells. In order to understand the biological activity of the TPE:ER , we employed a validated ER engineered assay using induction of the mRNA for the transforming growth factor (TGF-Ŭ) gene *in situ* in MDA-MB231 cell stably transfected with wild type ER or mutant D351G:ER [56] (Fig. 2d-1). We classified the structure of the ligands based on their ability to initiate TGFŬ mRNA synthesis through the ER complex. The biological assay predicts two extremes of the ligand ER complex based on known X-ray crystallography [58,59]: an ñestrogen-likeò shape and an ñantiestrogen-likeò shape. We find that the TPE:ER complex is antiestrogen-like which explains the delayed apoptosis in MCF7:5C cells compared to the estrogenic complex formed by the planar estrogens.

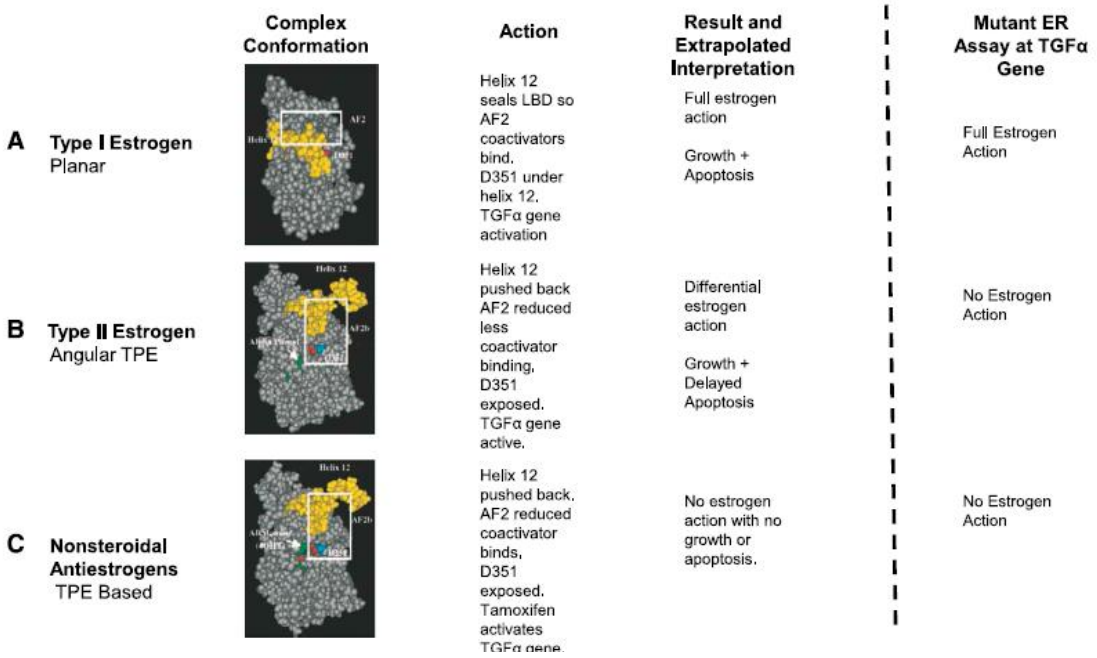


Fig.2d-1. ***Functional Test: Putative conformations of the complex with ligand in LBD for Type II estrogen to be "antiestrogenic" with regard to helix 12 positioning.*** *The assay discriminates between ligands (A) which allow helix 12 to seal the LBD or not (B) and (C). Sealing of helix 12 over the LBD is important for the ability of the ligands to trigger apoptosis.*

Work Accomplished:

Growth effects of estrogens and anti-estrogens in MCF-7 cells

To study the biological activity of the planar estrogens (Fig.2d-2A) which include E2, DES, equilin, estrone and equilenin), and triphenylethylenes (Fig.2d-2B) namely, EtOX (ethoxytriphenylethylene), 3OHTPE (trihydroxytriphenylethylene) and bisphenol, we tested their ability to induce cell proliferation in wild type MCF-7cells. As controls we used SERMs; 4OHT, endoxifen (endox), raloxifene (ral) and bazedoxifene (baze) (Fig.2d-2C) which are known anti-estrogens. MCF-7 cells were grown in estrogen free media for 3 days and treated with various concentrations of the indicated compounds and their effects were compared to E2. All planar estrogens (Fig. 2d-3A) were able to

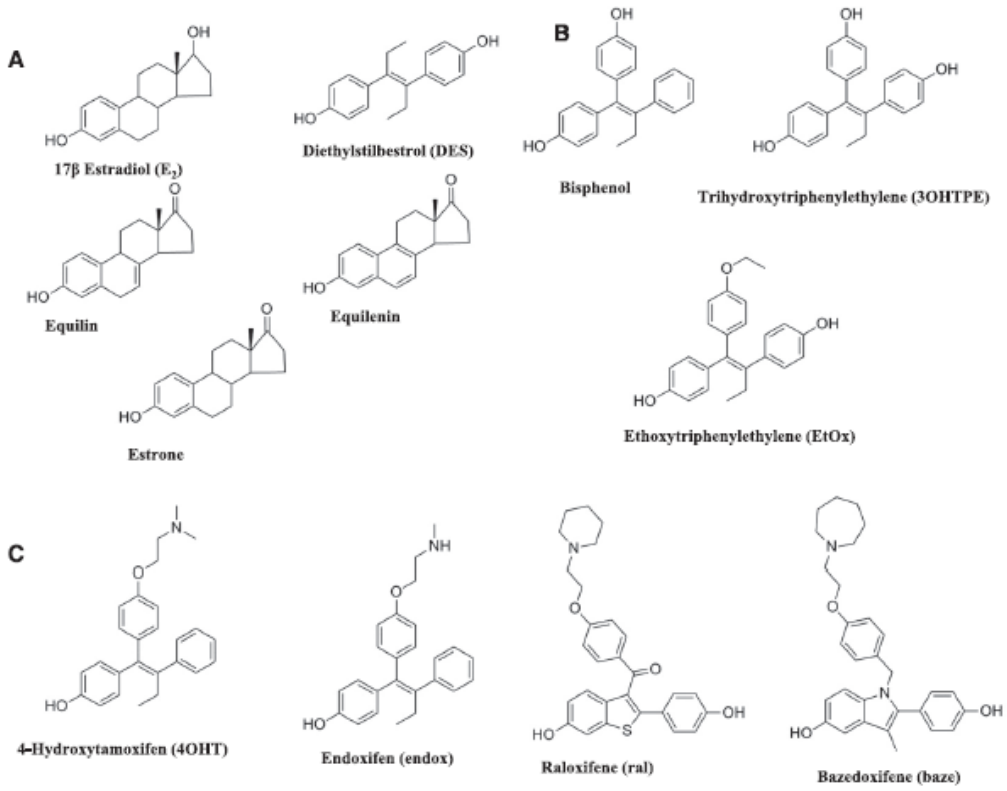


Fig.2d-2. ***Chemical structures of the compounds used in the experiments. (A)*** *Planar estrogens* ***(B)*** *Triphenylethylenes* ***(C)*** *Selective estrogen receptor modulators.*

induce cell proliferation in a concentration dependent manner to the maximum level as E2. DES, equilin and estrone induced cell proliferation with maximum stimulation occurring at 0.1nM, whereas equilenin reached maximal stimulation at 1nM as compared to 0.01nM for E2. Similarly, the triphenylethylenes tested were able to induce cell growth to the maximum level as E2, although their agonistic potency was less than E2 (Fig. 2d-3B). Bisphenol, EtOX and 3OHTPE all induced cell proliferation in a concentration dependent manner with maximum stimulation at 1-10 nM as compared to 0.01 nM for E2. Nonetheless, the TPEs all were potent estrogen-agonists in this assay. On the other hand, as expected, the SERMs,
4OHT, endox, ral and baze (Fig. 2d-3C) which are antiestrogens did not induce cell growth.

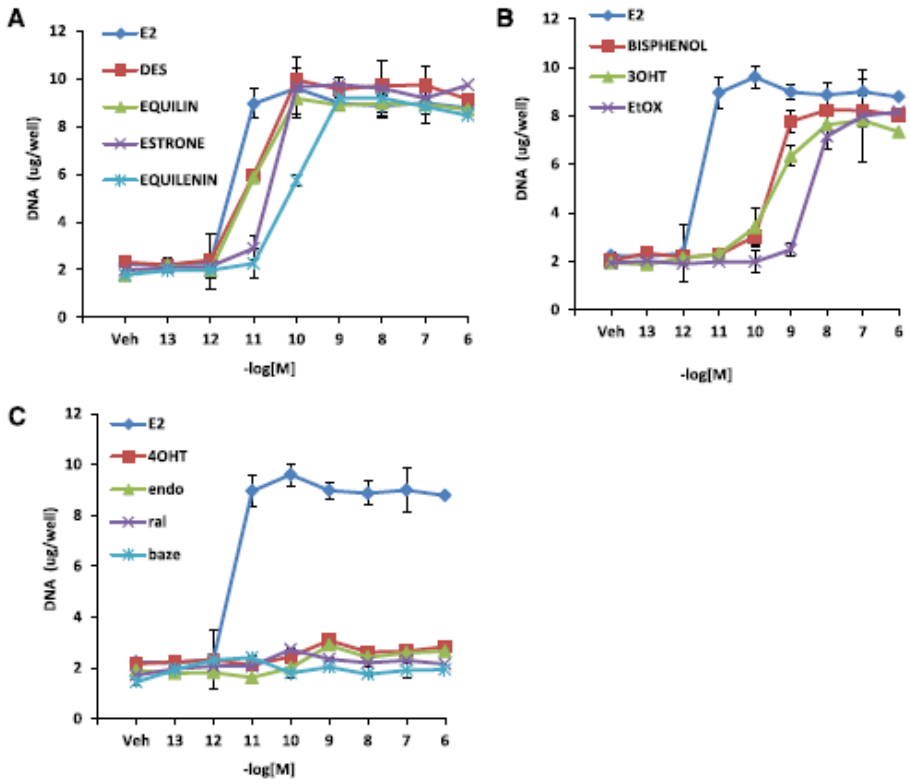


Fig.2d-3. ***Growth characteristics of planar estrogens and triphenylethylenes in MCF7:WS8 cells.****MCF7:WS8 cells were seeded in 24-well plate and treated with* ***(A)*** *planar estrogens over a rangeof doses for seven days. Cell growth was assessed as DNA content in each well. Induction of cellgrowth by* ***(B)*** *Triphenylethylenes and* ***(C)*** *SERMs was assessed in comparison to E2. Each datapoint is average +/- SD of three replicates.*

Effects of planar estrogens, TPEs and SERMS on apoptosis in MCF7:5C cells

We tested if TPEs and SERMS were able to induce apoptosis in long term estrogen deprived MCF7:5C breast cancer cells as effectively as E2. All planar estrogens were able to cause growth inhibition as effectively as E2 (Fig.2d-4A). All the planar estrogens achieved maximal growth inhibition in the range of 1 nM as compared to E2 which achieved maximal growth at 0.1 nM. To confirm that the decrease in cell proliferation was due to apoptosis, MCF-7:5C cells were treated with ethanol vehicle (control) E2 (1nM), or DES (1nM),equilin(1nM), estrone(1nM) and equilenin(1nM) for 72 hours, and annexin V ï FITC and PI fluorescence was determined by flow cytometry. In the control-treated group, only 5.9% stained positive for apoptosis, whereas, in the E2 treated group, cells that stained positive for apoptosis increased by 3 fold. Interestingly, the estrogenic triphenylethylenes did not inhibit the growth of MCF7:5C cells even at higher concentrations (Fig.2d-4B) at the end of a 7 day assay. Compared to E2, bisphenol, 3OHTPE and EtOX did not show any effective apoptosis even at micro-molar concentration and were comparable to that of the SERMs (Fig 2d-4C). Furthermore, the TPEs were able to block E2 mediated apoptosis in a similar manner to the SERMs (Fig. 2d-4D-E). However, the TPEs were able to induce apoptosis after 14 days of treatment (Fig.2d-4F), whereas the SERMs still did not induce apoptosis in the MCF7:5C cells.

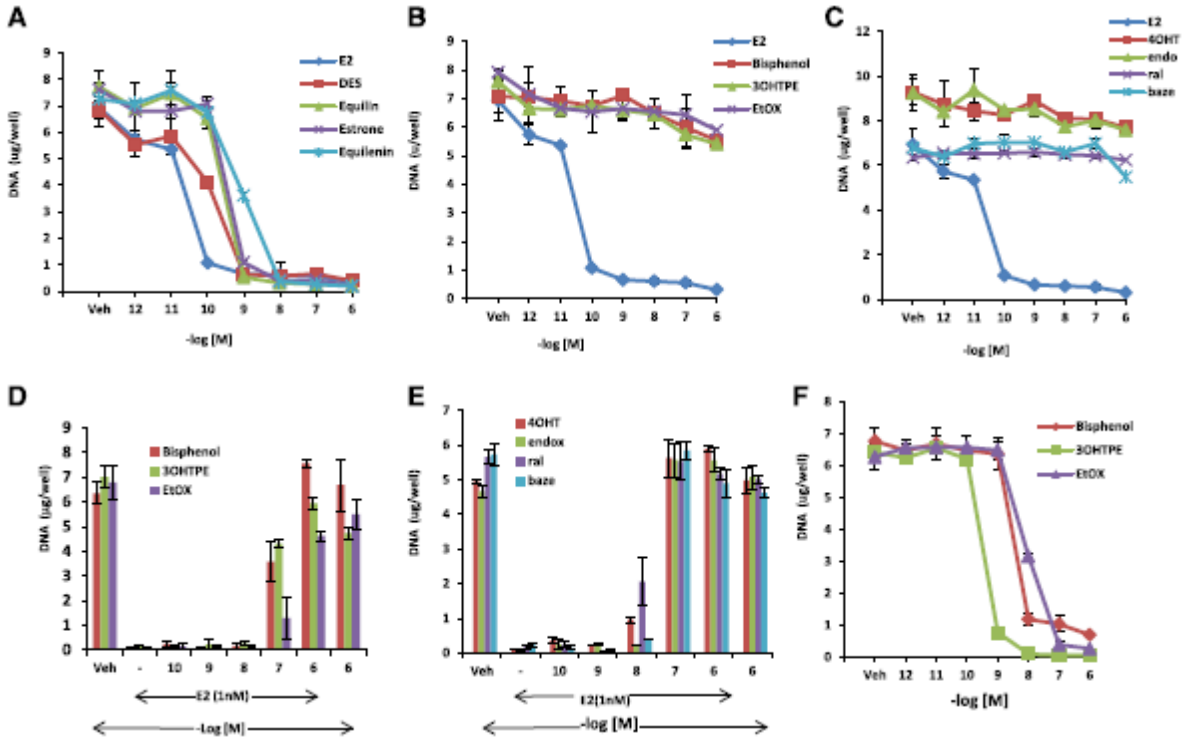


Fig.4. ***Differential effect of planar estrogens and triphenylethylenes in MCF7:5C cells.*** *Dose dependent effect of* ***(A)*** *planar estrogens* ***(B)*** *Triphenylethylenes and* ***(C)*** *SERMs on apoptosis of MCF7:5C cells treated for 7 days as indicated. Cells were treated with 1nM 17-β estradiol (E_2) in presence of increasing concentration of indicated TPEs* ***(D)*** *and SERMs* ***(E)***. ***(F)*** *Effect of TPE in MCF7:5C cells after 14 days of treatment. Each data point is average +/- SD of three replicates.*

Regulation of transforming growth factor alpha (TGFa) gene by planar and non-planar estrogens in MDA: MB-231 cells stably transfected with wild type ER alpha or D351G mutant ER alpha.

The TGFa gene is induced by 4OHT as effectively as E2 in MDA:MB-231 cells stably transfected with wild type ERa (MC2 cells). In contrast, in MDA:MB-231 cells stably transfected with a mutant D351ER (JM6 cells), 4OHT fails to induce expression of the TGFa gene but E2 retains its ability to induce the TGFa gene. We determined if the TPEs (3OHTPE, EtOX and bisphenol) and the planar estrogens (DES, equilin, estrone and equilenin) resembled E2 or 4OHT in inducing the TGFa gene expression by using the assay system summarized in Fig. 2d-1). As expected, all the planar estrogens were able to induce TGFa gene expression in a concentration dependent manner in both wild type Era (MC2) (Fig. 2d-5A) and D351G mutant ERa (JM6) cells (Fig. 2d-5D). On the other hand, the TPEs and tamoxifen metabolites; 4OHT and endox were able to induce TGFa gene expression in MC2 cells (Fig. 2d-5B) in a concentration dependent manner, whereas ral and baze do not activate the TGFa gene in this cell line (Fig. 2d-5C). By contrast, the TPEs, 4OHT and endox distinctly failed to induce TGFa gene expression (Fig. 2d-5E) in JM6 cells which expresses D351G mutant form of the ERa, rather they block E2 mediated TGFa induction(Fig. 2d-5F). Similarly, ral and baze are antiestrogenic in the mutant stable transfectant. These findings indicate that the TPEs possess antiestrogenic properties and binds with ERa in a manner which is distinctly different from the planar estrogens but strikingly resembles 4OHT and endox.

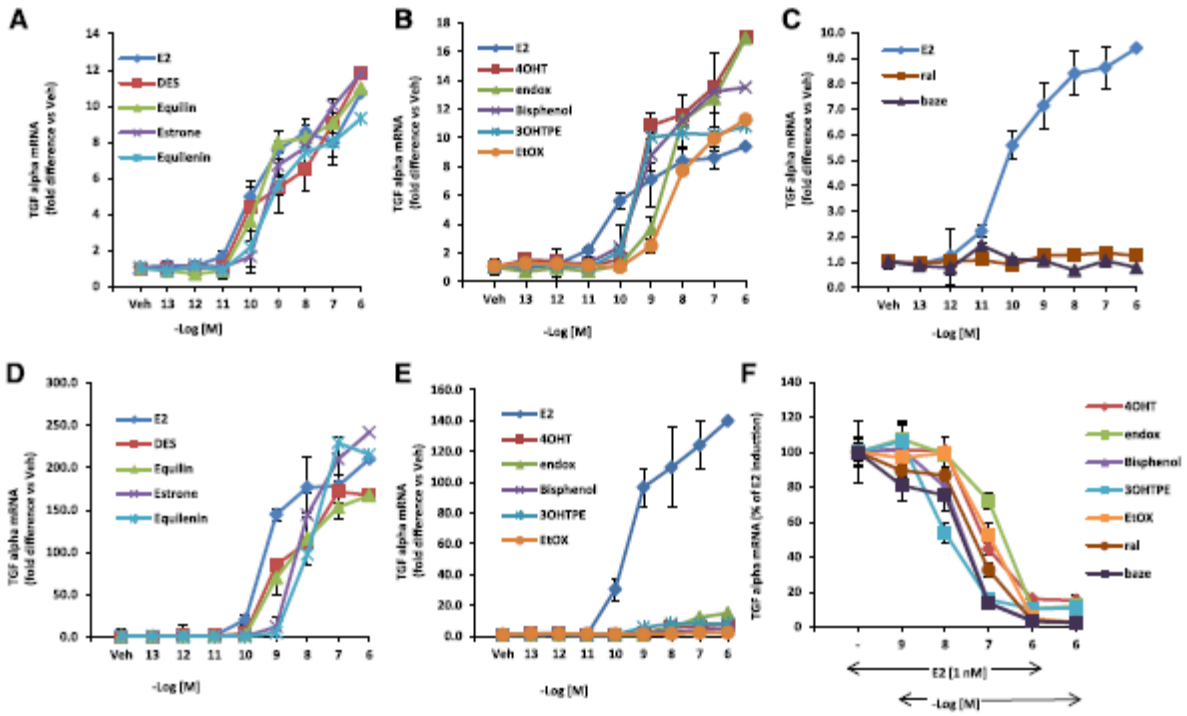


Fig.2d-5. ***The concentration-dependent action of test compounds using wild type(MC2) and mutant D351G ER (JM6) stable transfectants.*** *MC2 cells were treated with* ***(A)*** *planar estrogens* ***(B)*** *TPEs, 4OHT and endox for 24h atindicated concentrations and expression of TGFα RNA was measured using quantitative realtime PCR.* ***(C)*** *MC2 cells treated with ral and baze in a dose responsive manner. JM6 cells weretreated with* ***(D)*** *planar estrogens* ***(E)*** *E2, TPEs, active metabolites of tamoxifen (4OHT and endox) for 24 h with various concentrations and expression of TGFα RNA was measured usingquantitative real time PCR.* ***(F)*** *JM6 cells were treated with 1nM E_2 alone or in presence ofincreasing concentration of indicated TPEs and SERMs.* ***(A-E)*** *are represented as fold difference versus vehicle treated cells. Each data point is average +/- SD of three replicates.*

Recruitment of ER (estrogen receptor a) and SRC3 (steroid co-activator-3) at the proximal promoter of PS2 gene after treatment with triphenylethylenes

To further understand the ER mediated mechanism involved in the regulation of the model estrogen responsive gene PS2 by the TPEs in MCF7:WS8 and MCF7:5C cells we determined the recruitment of the ERa and SRC-3 protein at the proximal promoter of PS2 gene, which has a classical estrogen responsive element (Fig. 2d-6A), using ChIP (chromatin immunoprecipitation) assay after 45 minutes of treatment with TPEs (1 ÕM) and compared it with E2 (1nM) and 4OHT (1ÕM). The whole assay was repeated two further times with similar results occurring in each cell line . In MCF7:WS8 cells, E2 was able to recruit very high level of ERa at the PS2 promoter (Fig. 2d-6B) where more than 8% of input PS2 promoter region was occupied by ERa. On the other hand TPEs were ~50% as efficient as E2 treatment in terms of recruiting ERa whereas very low level (~20% of E2) of ERa recruitment was observed after 4OHT treatment. Recruitment of the co-activator SRC3, which is critical in inducing the estrogen responsive gene, was not observed at all after 4OHT treatment at the PS2 promoter. All the TPEs tested recruited only about 15-20% of SRC3 as compared to E2 treatment, which showed around 0.9 % of input PS2 promoter region was occupied by SRC3 protein. Interestingly, in MCF7:5C cells treated with E2, around 5% of input PS2 promoter region was occupied by ERa (Fig. 2d-6C). In MCF7:5C cells treated with TPEs had

50% less ERa occupancy and ~80% less SRC3 occupancy was observed as compared to E2 treatment in MCF7:5C cells, whereas no SRC3 recruitment was observed after 4OHT treatment. These ChIP data concurs with the PS2 mRNA induction level in MCF7:WS8 and MCF7:5C cells with their respective treatments.

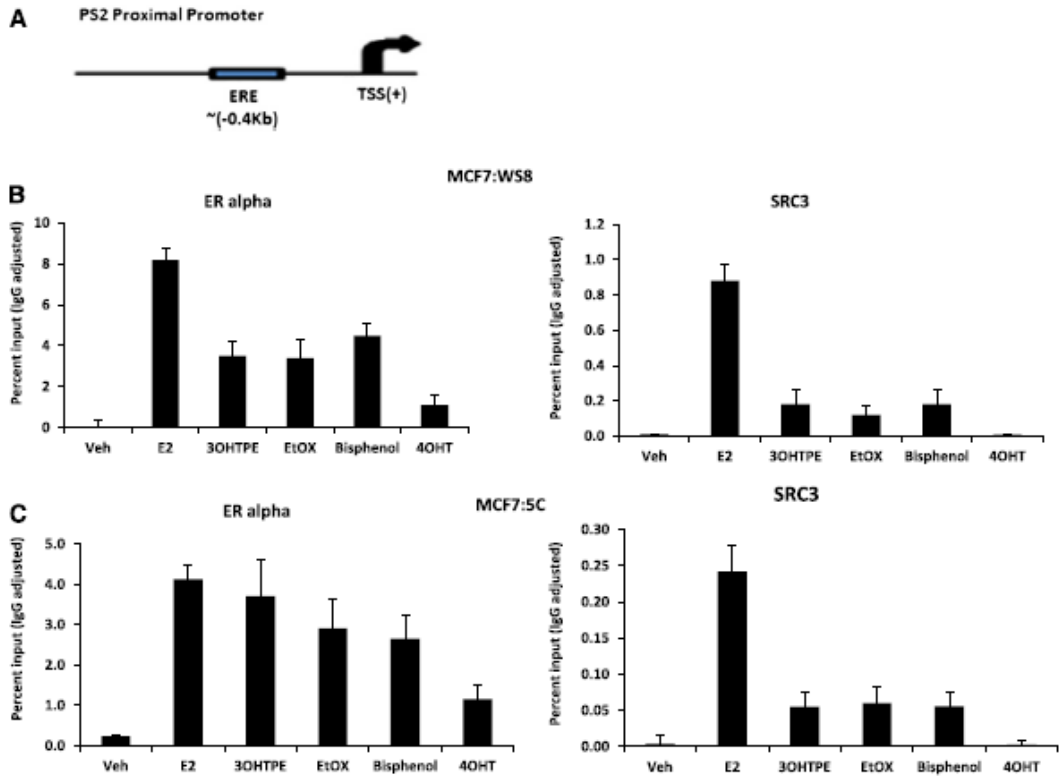


Fig.6. ***Recruitment of ER alpha and SRC3 (AIB1) at PS2 proximal promoter region containing ERE in MCF7:WS8 and MCF7:5C cells. (A)*** *Depiction of PS2 proximal promoter region and the ERE region relative to TSS (transcription start site).* ***(B)*** *MCF7:WS8 cells treated for 45 minutes with E_2 (1nM), 3OHTPE (1μM), EtOX (1μM), bisphenol (1μM) and 4OHT(1μM) and ChIP assay was performed as described in materials and methods.* ***(C)*** *MCF7:5C cells were treated identically as mentioned above and ChIP assay was performed under identical conditions. Data is represented as percent input of the starting chromatin used for the ChIP*

Induction of ERa expression by planar and non-planar estrogens

To test whether the structure the compounds create with the ER affects the ERa expression levels, 4 breast cancer cell lines, which include MCF-7:WS8, MCF7:5C, MC2 and JM6 cells, were treated with planar estrogens (1nM), TPEs (1Õ M) and SERMs (1Õ M)for 24h and ERa levels were determined by western blotting. ICI was included as a positive control. All planar estrogens and ICI caused decrease in the ERa protein levels in MDA-MB231 cells stably transfected with either wild type ER (MC2) (Fig. 2d-7A) or with the mutant receptor (JM6) (Fig. 2d-7B). On the other hand, the TPEs do not decrease the ERa protein levels in the MC2 cells, whereas 4OHT and endox cause accumulation of the receptor, while ral and baze cause moderate down regulation of the ER. In the JM6 cells, all TPEs and SERMs did not dramatically affect the ERa protein expression. As expected, all planar estrogens and ICI cause a decrease of ERa protein levels in MCF7:WS8 (Fig.

2d-7C) and MCF7:5C (Fig. 2d-7D) cells, whereas the tamoxifen metabolites caused increase in ERa protein expression. Interestingly the TPEs cause moderate decrease of ERa in MCF7:WS8 and MF7:5C cells compared to E2, and the reduction is more dramatic in the MCF7:5C cells. In contrast to the tamoxifen metabolites, ral and baze also cause a reduction in the protein levels of ERa in both MCF-7 derived breast cancer cell lines. ER a protein levels of all breast cancer cell lines used in the study are compared.

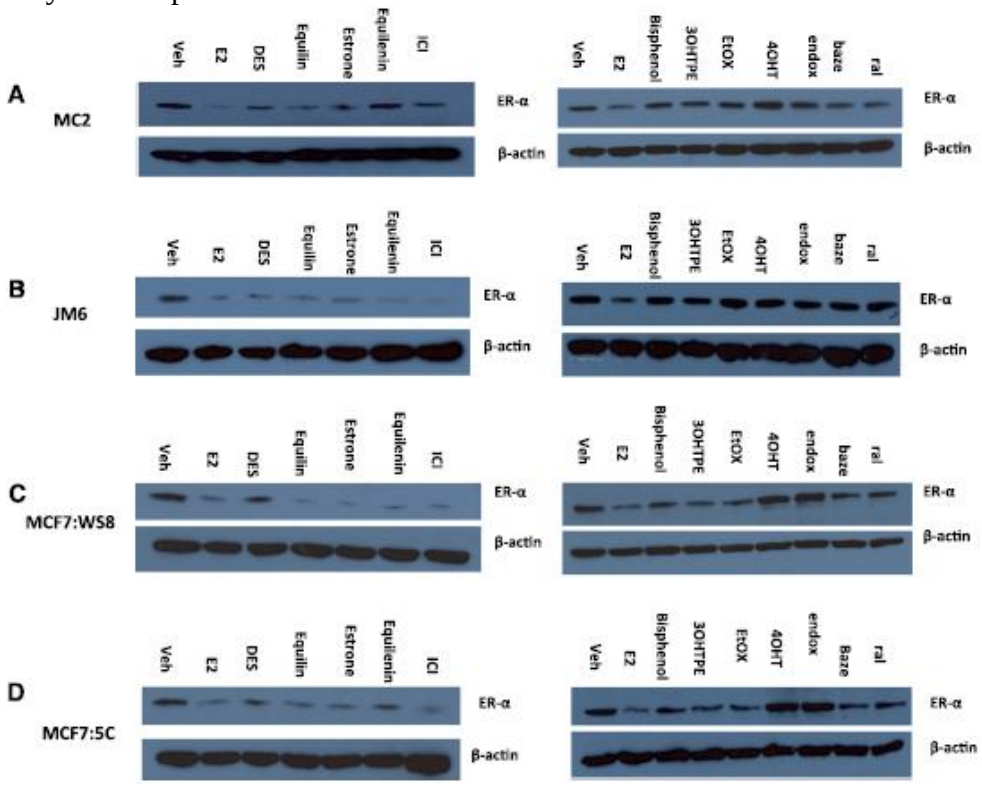


Fig.2d-7. ***Differential regulation of the ERα protein by planar and non-planar estrogens. (A)*** *MC2* ***(B)*** *JM6* ***(C)*** *MCF7:WS8* ***(D)*** *MCF7:5C cells were treated with* E_2 *(1nM), DES (1nM), equilin (1nM), estrone (1nM), equilenin (1nM), 3OHTPE (1μM), EtOX (1μM), bisphenol (1μM), 4OHT (1μM), endox (1μM), baze (1μM), ral (1μM) and cell lysates were analysed by western blotting by anti ERα antibody. Blot was reprobed by anti actin antibody.*

Binding of Bisphenol to the LBD of ER alpha

Next, the binding mode of the TPEs was investigated by the molecular docking of bisphenol to the ligand binding domain (LBD) of ERa. Thus the flexible docking of bisphenol into the LBD of the receptor co-crystallized with E2 and 4OHT (Fig. 2d-8A-B) were performed. The superimposition of the top ranked docking pose of the ligand onto the E2 co-crystallized with ERa, agonist conformation of the receptor, shows some incompatibility (Fig.2d-8C). Hence the resulting model revealed sterical clashes between bisphenol and ñLeu crownò, mostly with the side chains of Leu525 and Leu540. Due to this steric hindrance it is most unlikely for bisphenol to bind in a conformation of ERa that is similar to that of E2. On the other hand, when bisphenol is docked into the binding site of 4OHT co-crystallized with ERa (Fig. 2d-8D), the binding mode is similar to that of 4OHT.Namely the same alignment of the ligand in the binding pocket is noticed having the propensity to form the same hydrophobic contacts with the amino acids lining the binding cavity

and to recapitulate the complex H-bond network involving E353, R394 and highly order water molecule. Taken together, this data show that bisphenol and extrapolating TPEs would most likely bind to the ERa in the antagonist conformation of the receptor.

DISCUSSION

Estrogens are potent mitogens for the proliferation of breast cancer cells. In contrast, planar estrogens (class 1) can induce apoptosis of long term estrogen deprived MCF-7 cells (MCF7:5C) in a paradoxical manner. 4OHT has no effect in the MCF: 5C cells but rather blocks E2 mediated apoptosis [49]. TPEs which are structurally similar to 4OHT possess estrogenic properties in the MCF-7 cells at comparable concentrations to the planar estrogens. The TPEs (class II angular estrogens) do not rapidly trigger estrogen induced apoptosis in MCF7:5C cells, but block class 1

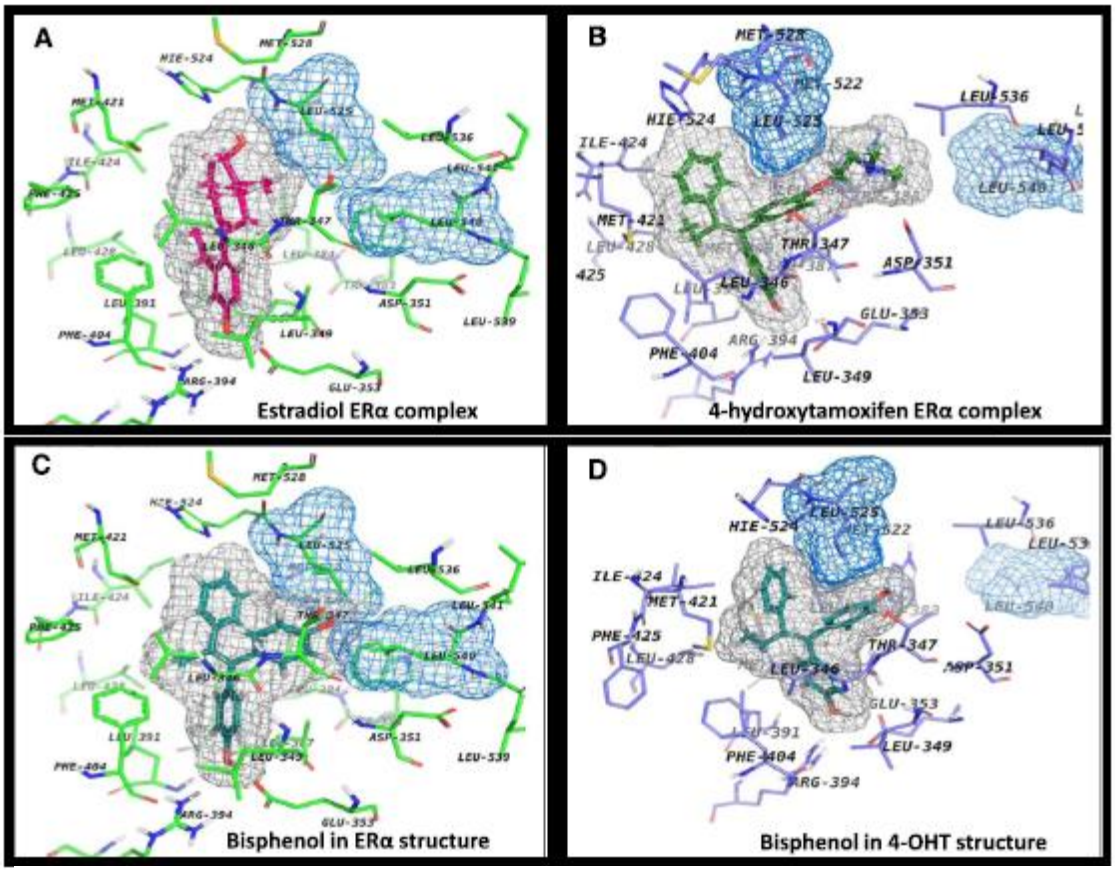


Fig.2d-8. ***The binding site of ERα with different ligands.*** *The ligands are depicted with their corresponding grid molecular surfaces colored in gray. Also, Leu525 and Leu540 are depicted as grid molecular surfaces colored in blue.* ***(A)*** *Agonist conformation of ERα with* E_2 *(depicted in magenta; PDB code: 1GWR).* ***(B)*** *Antagonist conformation of ERα with 4OHT (colored in green; PDB code: 3ERT).* ***(C)*** *Docking of bisphenol in agonist conformation (colored in cyan; PDB code: 1GWR).* ***(D)*** *Docking of bisphenol in antagonist conformation (colored in cyan: PDB code: 3ERT).*

planar estrogen induced apoptosis. However, prolonged treatment with the TPEs lead to an eventual induction of apoptosis in the MCF7:5C cells, whereas the cells continue to be resistant to the actions of the SERMs which are known antiestrogens. As a result of these aforementioned findings we initially proposed a hypothesis [49], that the TPE-ER complex mimics of an antiestrogen-ER complex and this may be responsible for the delay of apoptosis by the TPEs. We addressed the hypothesis in four ways: utilizing our validated functional assay to classify estrogens using the

induction of the TGFa gene [56] (Fig. 2d-1), binding of ER and recruitment of SRC3 to the promoter region of a model estrogen response gene (PS2) (Fig. 2d-6), ligand bound ER accumulation or reduction and putative ER docking experiments(Fig. 2d-8). We have previously demonstrated the critical importance of D351 in modulating the SERM:ER complex [60] for the estrogen-like actions of the 4OHT by removing the exposed surface charge by engineering a mutant ER D351G, which causes a conversion of the 4OHT:ER from being estrogenic to completely antiestrogenic at the TGFa gene [61,62]. The anchoring role of D351 in the activation of the helix 12 mutated ER has recently [63,64] been illustrated in tissue from metastatic breast cancer resistant to anti-hormones. Mutations of Y537 in helix 12 are shown to anchor to D351 to accomplish sealing of the unoccupied LBD by helix 12. This provides evidence of the clinical relevance of our assay system. To determine whether the conformation of the ER complex determines the triggering of apoptosis in long term estrogen deprived ER positive breast cancer cells, MCF-7:5C, we employed [56] an assay using induction of the mRNA for the TGF-a gene in situ in MDA-MB-231 cells stably transfected with cDNA wild-type(MC2) or D351G ER(JM6). As expected, all planar estrogens cause activation of the TGFa gene in the MC2 and JM6 cells. The planar estrogens are not affected by the mutation on D351 because upon binding to the ER, they are sealed within the LBD by helix 12 allowing for coactivator binding on the surface of helix 12 (AF-1) and gene activation. The TPEs induce TGFa gene at comparable concentrations as the tamoxifen metabolites, 4OHT and endox in the MC2 cells (Fig. 2d-5B), but lose this estrogen-like action in the JM6 cells (Fig. 2d-5E) and block E2 induction of TGFa (Fig. 2d-5F). The results of the TGF assay imply that TPEs adopt a 4OHT-like conformation with the ER with helix 12 pushed back and D351 exposed. By inference the ñantiestrogenic conformationò of the TPE: ER complex is responsible for the initial inhibition of E2 induced apoptosis. The short aminoethoxy side chain of the tamoxifen metabolites [62] and the absence of this side chain in the TPEs prevent adequate shielding of the charged D351, whereas the anti-estrogenic side chain of ral and baze provides effective interaction and neutralization of this charge [65] (Fig. 2d-5C). Thus, this prevents the induction of the TGFa gene by ral and baze. SRC3 has been shown to be extremely important in estradiol induced growth in breast cancer cells [66,67,68]. Additionally, SRC3 knockdown was found to reduce apoptosis induced by E2 in MCF7:5C cells [54]. Using ChIP assays we show that TPEs are able to recruit ERa but less efficiently when compared to E2 and this was further observed with SRC3 (Fig. 2d-6).The ER:TPE complex binds to the promoter with about 50% of E2 but SRC3 binding is <25% of E2. This suggests that treatment with TPEs influences the conformation of the liganded-ERa complex such that efficiency of ERa binding to ERE region is moderately inhibited whereas binding of SRC3 is severely inhibited as compared to E2 treatment which is a planar estrogen. This may also explain why bisphenol is a partial agonist at the prolactin gene and exhibits antiestrogen properties [36,37]. Of notable importance, the magnitude of SRC3 recruitment by the TPEs is far less in MCF7:5C cells (Fig. 2d-6C) when compared to MCF7:WS8 cells (Fig. 2d-6B) and may play a crucial role in manifesting the functional role of the TPEs in these cells. This observation may contribute to the robust cell replication in MCF-7 with TPEs but delayed apoptosis in MCF7:5C. Estradiol induces downregulation of the ER in breast cancer cells [69,70,71] and this process is inhibited by 4OHT, thereby causing accumulation of ER-a [72]. Similarly in all our cell lines, the planar estrogens all downregulate the ER while tamoxifen metabolites, 4OHT and endox, do not (Fig. 2d-7). The western blot analysis shows that the TPEs do not readily decrease ERa protein levels when compared to the planar estrogens. This illustrate the fact that the TPE:ER complex appears to be ñantiestrogen-likeò when compared to 4OHT and endox (Fig. 2d-7) However in the MCF7:5C cells, their ability to downregulate ERa protein levels is more apparent. The ER complex resembles the vehicle (control) rather than the extremes of E2 or 4OHT. Ral and baze also cause moderate decrease in ERa levels which concurs with previous studies done on these compounds [73]. Bourgon-Voillard and colleagues determined that class II ligands such as bisphenol had fewer

tendencies to promote recruitment of coactivators containing LxxLL motif and this appeared to be a requirement for the downregulation of the ER in MCF7 cells. Bourgoin-Voillard and colleagues [53] also illustrate the accumulation of the bisphenol:ER complex in MCF-7 cells using immunocytochemistry. The molecular modeling data (Fig. 2d-8) provide evidence that the TPEs bind to the ERa in a manner similar to that observed with 4OHT using x-ray crystallography. The bulky phenyl ring of the TPEs prevent helix 12 from sealing the LBD and will result in an initial steric hindrance when attempting to bind in the E2 -ER a conformation, resulting in their blockade of E2 induced apoptosis. However, continuous treatment of the MCF7:5C with the TPEs for 14 days result in induction of apoptosis similar to the planar estrogens. This suggests that the antiestrogenic conformation the TPEs create with the ER prevents immediate coactivator binding, causing a delay in the trigger for apoptosis but this delay disappears with prolonged treatment. This conclusion correlates with the Haddow clinical study [74], where postmenopausal women with advanced breast cancer were treated with TPE- like estrogens leading to about a 30% response rate during breast cancer therapy. The planar estrogens form compact estrogen-ER complex with excellent SRC3 binding and recruitment and it appears that this event is necessary to induce apoptosis in the MCF7:5C cells. On the other hand, angular TPEs form antiestrogen-like-ER complex with less SRC3 binding and recruitment, thereby leading to delayed apoptosis, whereas the SERMS do not recruit SRC3 so this results in no apoptosis.

In conclusion, we have advanced the hypothesis that TPE-ER conformation is initially similar to that of tamoxifen metabolites, 4OHT and endox and our molecular classification assay indicate that helix 12 is pushed back in the TPE-ER complex. The antiestrogenic conformation of the TPE-ER complex appears to be responsible for the initial blocking of apoptosis and reduction in coactivator recruitment observed with the TPEs in the MCF7:5C cells. It is important to stress that the evidence we present suggests that the TPE:ER complex conformation may in fact be in between the extreme structures of E2:ER and 4OHT:ER ligand binding domain [28,75]. Since prolonged treatment with TPEs causes triggering of ER mediated apoptosis similar to that of the planar estrogens but 4OHT does not, an intermediate conformation of the TPE:ER complex may be responsible for these observations.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2e (Sengupta, Obiorah and Jordan) - Studies carried out by Dr. Ifeyinwa Obiorah and Dr. Surojeet Sengupta in the Jordan laboratory at Georgetown University

Delayed triggering of estrogen induced apoptosis that contrasts with rapid paclitaxel-induced breast cancer cell death

Introduction:

The goal of this paper is to determine the critical trigger point for E_2-induced apoptosis. We have explored the differential gene expression as a prelude to determine the early molecular events in E_2-induced apoptosis in comparison with classic cytotoxic chemotherapy-induced apoptosis. Induction of mRNA levels of proapoptotic genes confirmed whether mitochondrial and tumour necrosis factor (TNF) apoptotic pathways were activated. We compared and contrasted the ability of E_2 and paclitaxel with arrest cell cycle to advance the molecular understanding of the new biology of E_2-induced apoptosis in therapy.

Work Accomplished:

Cell growth and apoptotic effects of E_2 and paclitaxel on MCF7:5C cells

We sought to compare the antiproliferative activity between paclitaxel and E_2 in the MCF7:5C cell line and explore their potential to induce apoptosis. Paclitaxel induced rapid inhibition of growth in a concentration-dependent manner with maximum inhibition at 0.1 µM. Fifty percent growth inhibition was achieved in 24 h (Figure 2e-1A), which increased to almost 100% after 48 h of treatment (Figure 2e-1B). In contrast, E_2 achieved maximal growth inhibition at 0.1 nM, and did not quantitatively prevent cell proliferation until after 72 h (Figure 2e-1C). Twenty-five percent of growth inhibition occurred at 96 h with E_2 treatment (Figure 2e-1D) and this increased to 80% at the 120-h time point (Figure 2e-1E). The decrease in cell number observed with E_2 and paclitaxel was further investigated to determine whether the growth inhibition was due to apoptosis. An increased apoptotic response (Figure 2e-2A) was detected by increasing the percentage of annexin V staining from control 3.92ï 21.49% by paclitaxel after 12 h treatment, whereas an apoptotic effect was observed at 72 h with E_2 (Figure 2e-2B). An apoptotic response was not detected after 24 h treatments with E_2 through annexin V staining. Experiments were repeated three times and a summary of results are represented. Similar results were observed with a DNA-binding dye, YO-PRO-1.

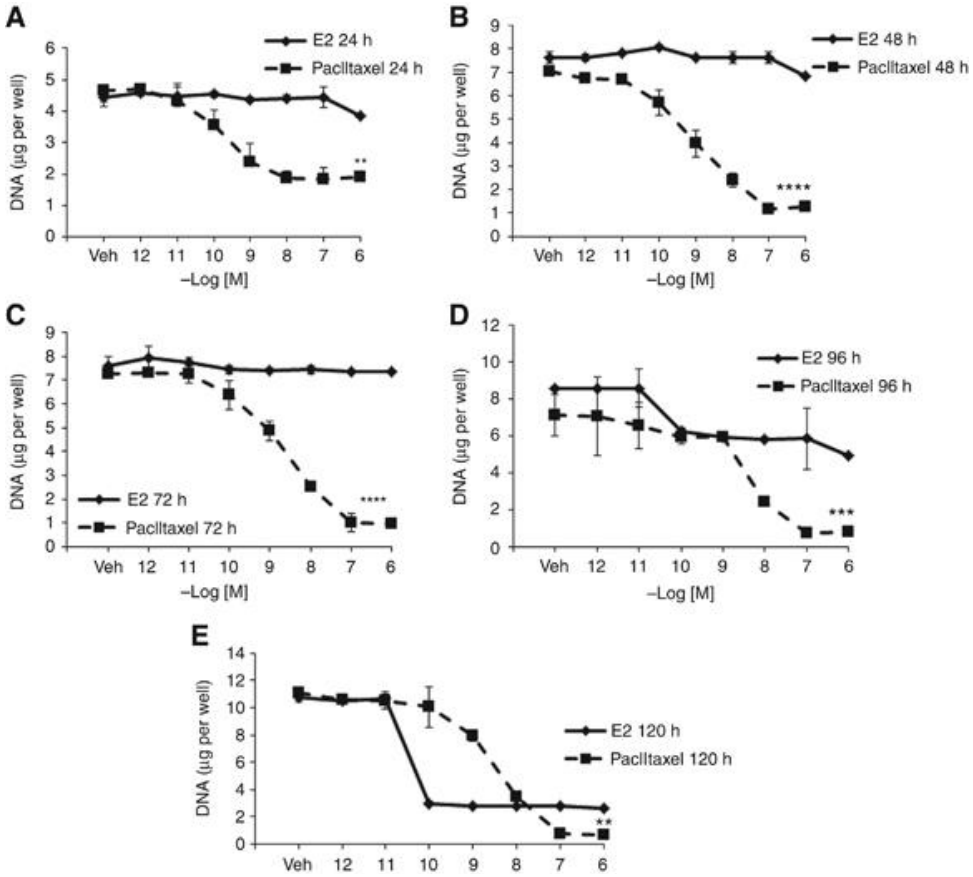


Fig.2e-1 ***Effect of E_2 and paclitxel on the growth characteristics in the MCF7:5C cells.*** *MCF5C cells were seeded in 24-well plate treated with the control vehicle (Veh) or E_2(♦) and paclitaxel (▪) over a range of doses and cells were harvested after (**A**) 24 h, (**B**) 48 h, (**C**) 72 h, (**D**) 96 h and (**E**) 120 h. Data points shown are the average of three replicates ±s.d. (**P<0.02,***P<0.0003,****P<0.0001).*

Determination of the critical trigger point of estradiol-induced apoptosis

Although E_2 treatment induces apoptosis of MCF7:5C cells in a concentration-dependent manner, the cells are unresponsive to the anti-oestrogen, 4OHT. Rather 4OHT blocks E_2-mediated apoptosis [49]. To further investigate the delayed response to E_2-mediated apoptosis and to determine the critical trigger point for E_2-induced apoptosis, we used 4OHT to block and rescue the cells from the apoptotic effect of E_2. In this way, we established when the cells are committed to cell death. MCF7:5C cells were treated with 1 nM of E_2, and subsequently 1 µM of 4OHT was used to block the apoptotic effects of E2 at the indicated time points over a range of 96 h after the addition of E_2. Cells were then all collected for DNA assay on day 7. Apoptosis triggered by E_2 was competitively inhibited and rescued for up to 24 h, and thereafter it lost the ability to rescue cells committed to E_2-induced apoptosis (Figure 2e-3). Between 24 and 36 h, the cells are committed to apoptosis despite the anti-oestrogenic action of 4OHT.These data suggest that the critical trigger for the commitment of the cell to the induction of apoptosis by E_2 lies between 24 and 36 h.

Differential gene expression of E_2-mediated apoptosis at the critical trigger point

To identify genes associated with E_2-induced apoptosis with a particular focus on the critical trigger time point, differential regulation of apoptotic gene expression in response to E_2 was interrogated in the MCF7:5C cells. At 24h, as expected, significant evidence of apoptotic gene induction is not apparent, rather proapoptotic genes such as *BAD* and *BCL2L10*, and Caspases 1, 9 and 10 are differentially downregulated by E_2. TNF-related genes, *TNFRSF8* and *TNFSF14*, are induced by both E_2 and 4OHT, and they do not have a definitive role in the TNF-mediated apoptosis but rather are involved in the T-cell response. Interestingly, at 36h (Figure 2e-4A), which represents the trigger point for apoptosis, E_2 induces proinflammatory genes such as *CEBPB*, *CEBPG* and *DAPK1*, and endoplasmic reticulum stress-related genes such as *DDIT3* and *ERN 1*. BCL2L11 (BIM), an important member of the mitochondrial pathway and an apoptosis activator, is also upregulated by E_2, suggesting an early involvement of the intrinsic pathway. Following 48h of E_2 treatment (Figure 2e-4B), the gene expression expands to involve the TNF-related genes such as *FAS*, *TNFRSF21* and *TNF*, and continued increased expression of endoplasmic reticulum stress and proinflammatory-related genes. In addition, p53 expression is increased at 48h. PMAIP 1 (also known as NOXA), a Bcl-2 homology (BH3) only family and a p53-regulated gene is also upregulated by E_2. 4OHT acted as an anti-oestrogen and was able to block most of the effects of E_2..

Paclitaxel induces TNF family of apoptosis-related genes in MCF7:5C cells

We further investigated expressed genes activated by paclitaxel that may define a molecular mechanism. Based on the biological experiments shown above (Figures 2e-1 and 2e-2B), paclitaxel-induced apoptotsis happened after 12h treatment and reached to a peak at 24h. We mainly focused on detecting gene regulation by paclitaxel at these two time points. Paclitaxel selectively activated the TNF family of apoptosis-related genes. After an

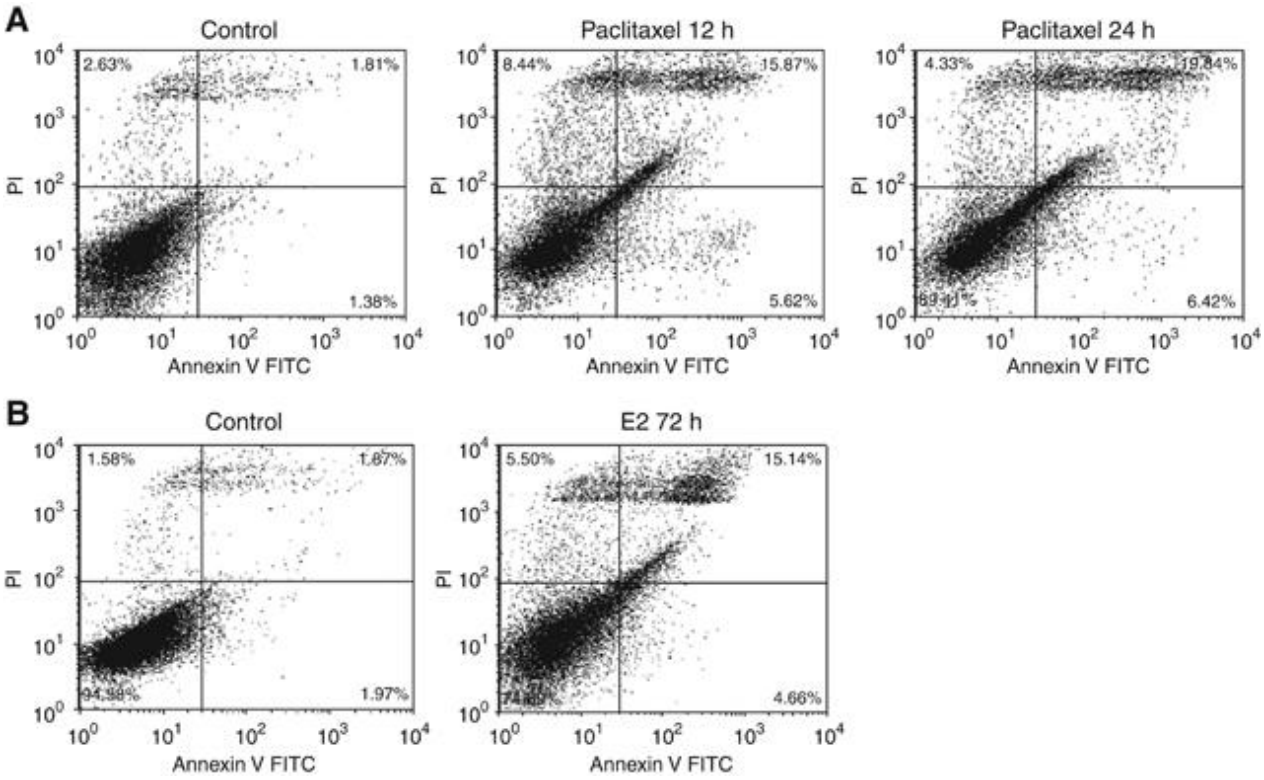
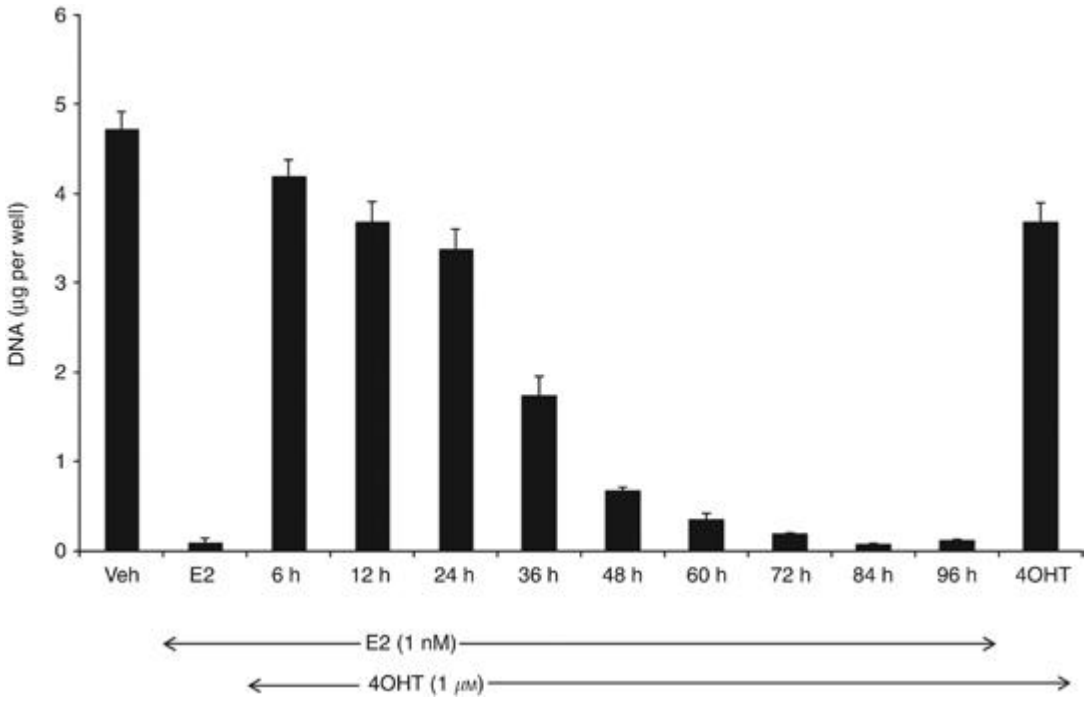


Fig 2e-2. ***Differential apoptotic effects of E_2 and paclitaxel.*** *MCF7:5C cells were treated with control or (**A**) paclitaxel (1 μM) for 12 and 24 h or (**B**) E_2 (1 nM) for 72 h, and then stained with annexin V-FITC and PI and analysed by flow cytometry. Viable cells (left lower quadrant) are annexin V-FITC− and PI−, early apoptotic cells (right lower quadrant) are annexin V-FITC+ and PI−, dead cells (left upper quadrant) are PI+ and late apoptotic cells (right upper quadrant) are annexin V-FITC+ and PI+. Increased staining for apoptosis is observed maximally in the right upper quadrant.*

Fig 2e- 3. ***Deciphering the trigger point for E_2-induced apoptosis.*** *Cells were treated with vehicle (Veh) or E_2 (1 nM) alone, and 1 μM 4OHT was added and used to block and reverse E_2 action at 6, 12, 24, 36, 48, 60, 72, 84 and 96 h. The cells were harvested after 7 days of treatment. The extent of apoptosis was determined by measuring the DNA content of the remaining cells in each well. The experiment was done in triplicates, and the data represent the mean of three independent experiments with 95% confidence intervals. The trigger point for E_2-mediated apoptosis was elucidated at the time when the apoptotic effects of E_2 could not be blocked by 4OHT.*


DNA (µg per well)
0
1
2
3
4
5
6
Veh
E2
6 h
12 h
24 h
36 h
48 h
60 h
72 h
84 h
96 h
4OHT
E2 (1 nM)
4OHT (1 µM)

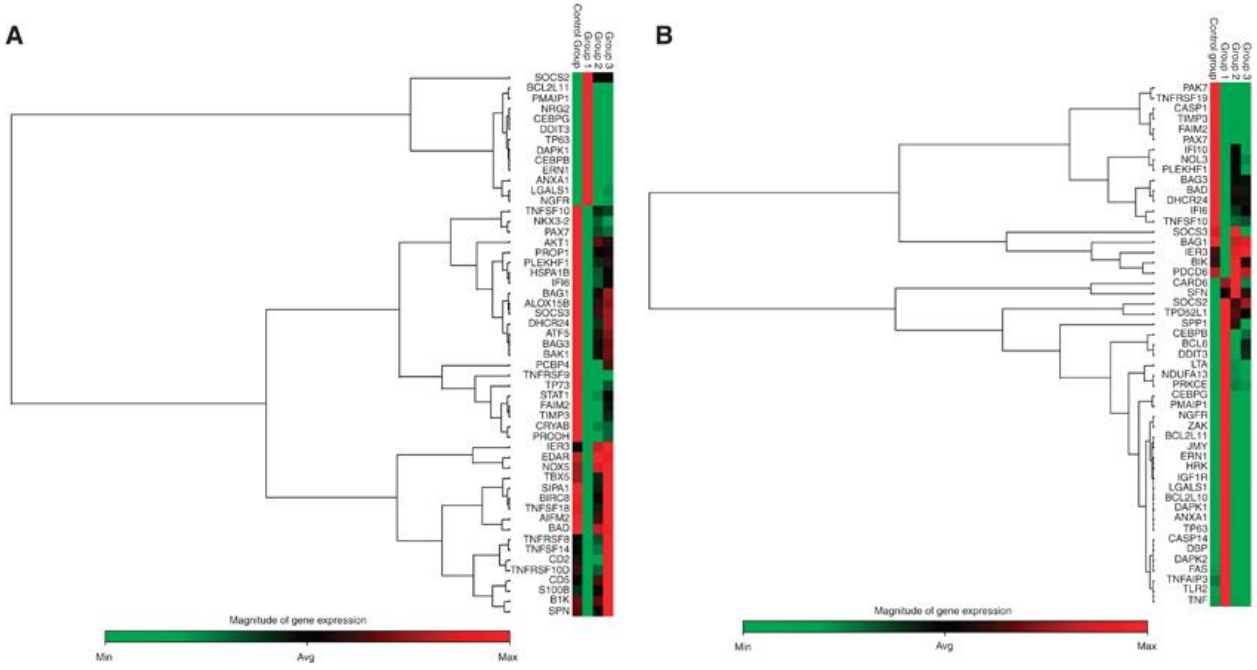
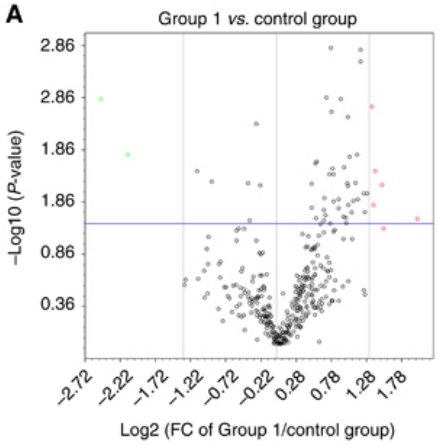

A
Control Group
Group 1
Group 2
Group 3
SOCS2
BCL2L11
PMAIP1
NRG2
CEBPG
DDIT3
TP63
DAPK1
CEBPB
ERN1
ANXA1
LGALS1
NGFR
TNFSF10
NKX3-2
PAX7
AKT1
PRDX1
PLEKHF1
HSPA1B
IFI6
BAG1
ALOX15B
SOCS3
DHCR24
ATF5
BAG3
BAK1
PCBP4
TNFRSF9
TP73
STAT1
FAIM2
TIMP3
CRYAB
PRODH
IER3
EDAR
NOX5
TBX5
SIPA1
ERRC2
TNFSF18
AIFM2
BAD
TNFRSF8
TNFSF14
CD2
TNFRSF10D
CD5
S100B
BIK
SPN
Magnitude of gene expression
Min
Avg
Max
B
Control group
Group 1
Group 2
Group 3
PAK7
TNFRSF19
CASP1
TIMP3
FAIM2
PAX7
IFI10
NOL3
PLEKHF1
BAG3
BAD
DHCR24
IFI6
TNFSF10
SOCS3
BAG1
IER3
BIK
PDCD6
CARD6
SFN
SOCS2
TPD52L1
SPP1
CEBPB
BCL6
DDIT3
LTA
NDUFA13
PRKCE
CEBPG
PMAIP1
NGFR
ZAK
BCL2L11
JMY
ERN1
HRK
IGF1R
LGALS1
BCL2L10
DAPK1
ANXA1
TP63
CASP14
DBP
DAPK2
FAS
TNFAIP3
TLR2
TNF
Magnitude of gene expression
Min
Avg
Max

Fig 2e-4. ***Heat map of E_2-mediated apoptotic genes that are differentially expressed by 36 and 48 h of treatment.*** *Cells were parsed into groups of three replicates per treatment per time point, and then treated with either 0.1% ethanol (control group), 1 nM E_2 (group 1), 1 μM 4OHT (group 2), in the presence (group 3) or in the absence of E_2 over a period of 48 h. Total RNA was extracted and reverse transcribed as described in Materials and Methods section. Samples were loaded onto customised PCR array plates with primers for indicated apoptotic genes. Gene expression values were obtained and analysed in comparison with the controls at (**A**) 36 h and (**B**) 48 h. The maximum expressed level of any given gene is represented by red colour and minimum levels are represented as green colour.*

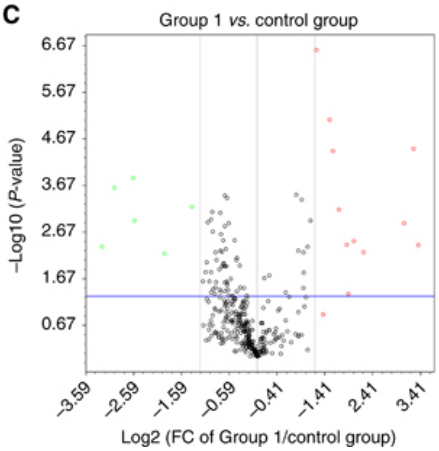
initial 12 h of treatment (Figure 2e-5A and B), paclitaxel stimulated TNFRSF10A (TNF receptor superfamily, member 10a) and TNFRSF10B (TNF receptor superfamily, member 10b), which are known to be activated by the ligand TNF-related apoptosis inducing ligand (TNFSF10/TRAIL), and causes death through the extramitochondrial pathway.TNFRSF19 (TNF receptor superfamily, member 19) induces apoptosis in a caspase-independent manner. In addition, TNF proapoptotic genes, including *FAS* and *TNF*, and other TNF proinflammatory genes such as *LTA, LTB* and *TNFAIP3*, are activated by 24 h of treatment with paclitaxel (Figure 2e-5C and D). Paclitaxel further induces NOXA and CDKN1A (p21) that are known to inhibit the activity of cyclin-CDK2 or -CDK4 complexes at the G1 phase. Although these two p53-regulated genes were upregulated by paclitaxel, p53 induction was not observed at 24 h. Unlike E_2, which increases BIM and TNF mRNA levels (Figure 2e-6A and B), paclitaxel was only able to induce TNF expression (Figure 2e-6C and D). These results highlight the differences in apoptosis-related genes induced by the two treatments.



B

Gene symbol	Fold upregulation
HSPA1B	2.8724
SEMA4D	2.687
TNFAIP3	2.9231
TNFRSF10A	2.6083
TNFRSF10B	2.654
TNFRSF19	3.2439

Gene symbol	Fold downregulation
GDNF	−3.9205
LTA	−2.6799



D

Gene symbol	Fold upregulation
ALOX15B	4.4146
ANXA1	2.7694
CDKN1A	2.5468
DAPK1	12.2063
LTA	3.0582
LTB	5.5024
NME5	9.8936
NTF3	3.5263
PMAIP1	3.3451
SNCA	11.3361
FAS	4.756
TNF	3.8363
TNFAIP3	4.3023

Gene symbol	Fold downregulation
CARD11	−5.0698
CRYAB	−8.1087
GDNF	−3.2651
NDUFA13	−6.7381
TBX5	−2.7497
TNFSF18	−5.1435

Fig 2e-5. ***Determination of apoptotic genes induced by a cytotoxic chemotherapy in MCF7:5C cells.*** *MCF7: 5C cells were treated with either 0.1% ethanol (control), or 1 μM paclitaxel (group 1) for 12 and 24 h. Gene expression values were obtained and analysed in comparison with the controls, and volcano plots were generated at 12 h of treatment (**A**) and the expressed genes listed (**B**). Similarly, gene expression levels are analysed after 24 h of paclitaxel treatment (**C**) and genes are listed in **D**. The genes selected were at least 2.5-fold overexpressed or under-expressed as compared with vehicle at P-value=0.05. Genes upregulated are represented in red and downregulated genes are represented in green.*

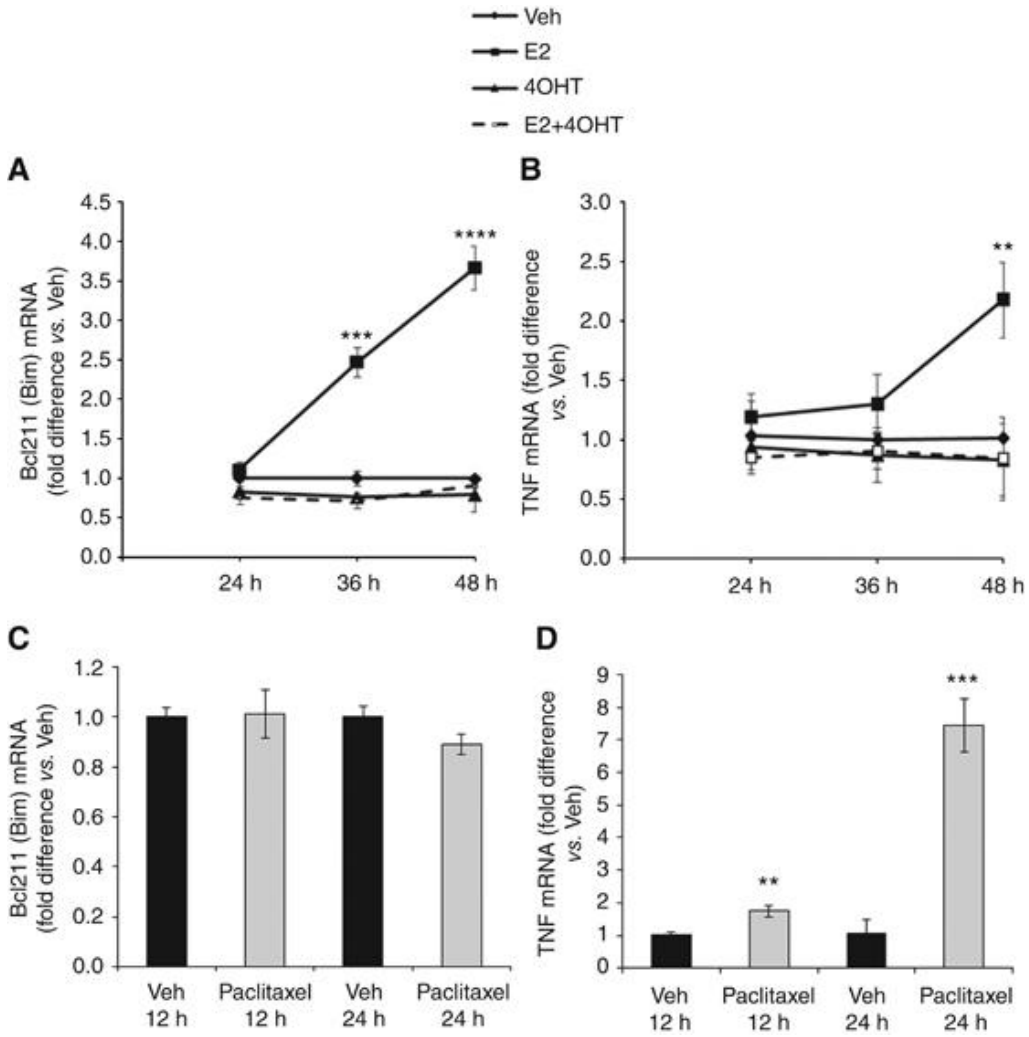


Fig 2e-6. ***E_2 activates both mitochondrial and extrinsic pathway of apoptosis, whereas paclitaxel activates only the extrinsic pathway.*** *MCF7:5C cells were treated with vehicle (Veh), 1 nM E2, 1 μM 4OHT or combination treatment of E_2 and 4OHT for 24, 36 and 48 h. Total RNA was reverse transcribed and assessed for (**A**) BIM and (**B**) TNF gene expression. Induction of (**C**) BIM and (**D**) TNF mRNA was determined in MCF7:5C cells treated with either Veh or 1 μM paclitaxel for 12 and 24 h using RT–PCR. PCR data values are presented as fold difference versus Veh-treated cells±s.e.m. (**P<0.02, ***P<0.0003, ****P<0.0001).*

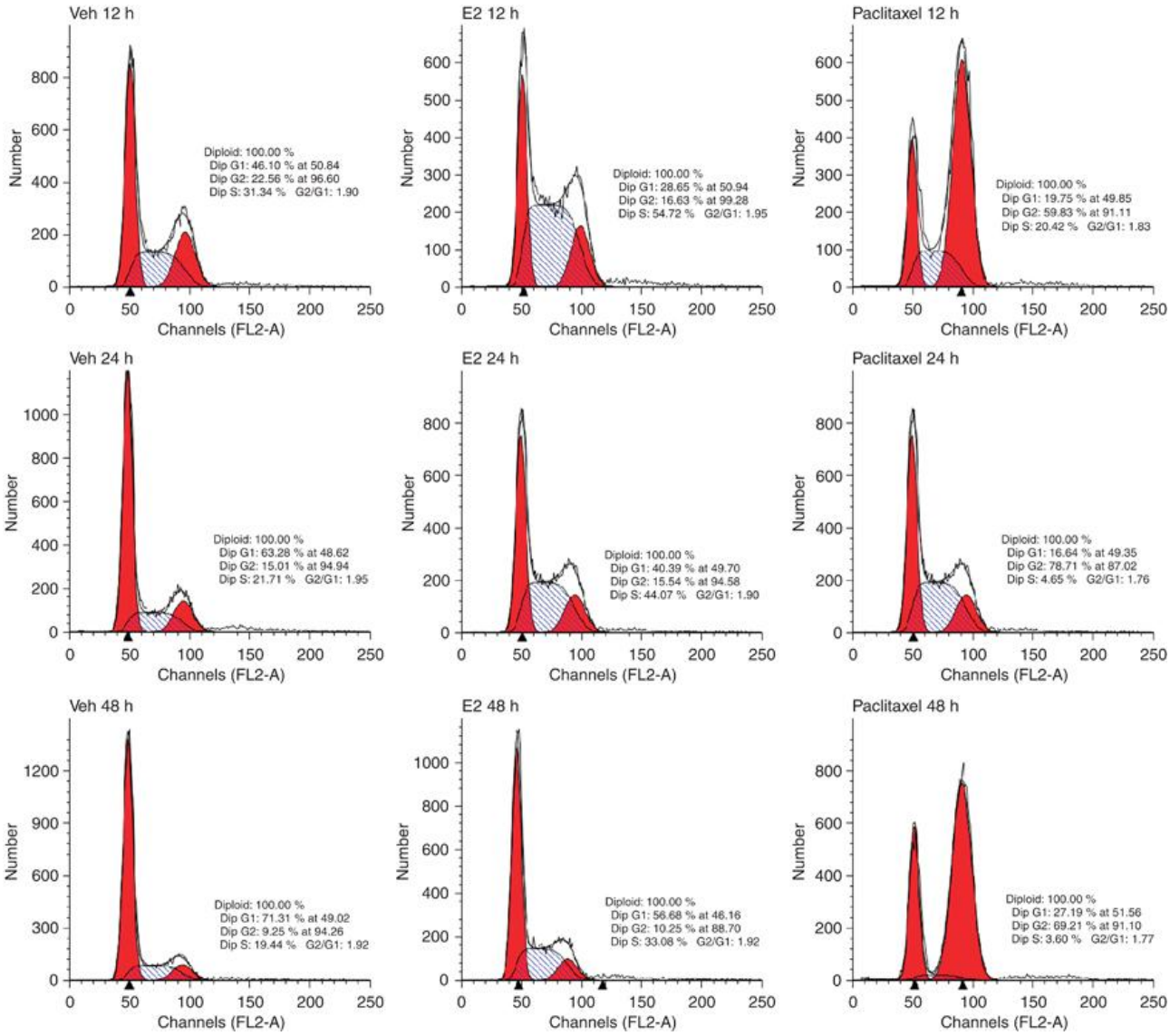


Fig 2e-7. ***Cell cycle analysis of the effects of E_2 and paclitaxel in the MCF7:5C cells.*** *Representative cell cycle profiles of MCF7:5C cells treated with either 0.1% ethanol (Veh), E_2 (1 nM) or paclitaxel (1 μM) for 12, 24 and 48 h. FL2-A represents the intensity of PI, and the y-axis represents the cell number.*

Differential effect of paclitaxel in induction of G2 blockade in comparison with E_2

Paclitaxel prevents progression of mitosis and activates the mitotic checkpoint, paving a path for apoptosis. To elucidate whether the apoptotic effects of paclitaxel in comparison with E_2 were mediated through cell cycle arrest, we performed cell cycle analysis in MCF7:5C cells using flow cytometry. Our results reveal that paclitaxel treatment causes accumulation of cells in G_2/M phase with a concomitant reduction in the number of cells in G_1 and S phase (Figure 2e-7) Cell cycle arrest in G_2/M phase was about threefold higher compared with control. In contrast, a G_1 or G_2 blockade was not observed with E_2 treatment. E_2 dramatically enhanced S phase at 12 h and rapidly increased to sevenfold by 48 h. This is consistent with our recent publication [11] that E2 increased S phase after 72 h treatment. Based on these observations, we hypothesise that the apoptotic effects

of paclitaxel in MCF7:5C cells results from a perturbation in the cell cycle check points, whereas E_2 induces cell proliferation finally resulting in apoptosis.

Discussion:

The molecular sequence of events resulting in either E_2-induced apoptosis or paclitaxel-induced apoptosis is completely different. E_2-induced apoptosis appears to be unique. Paclitaxel rapidly induces apoptosis of MCF7:5C cells, whereas E_2 shows a delayed process for the induction of apoptosis. Using 4OHT to block and rescue E_2-induced events necessary for an apoptotic response, we observed that the trigger for apoptosis occurs after 24 h and the cells become committed to apoptosis by and after 36 h. There is activation by E_2 of endoplasmic reticulum stress-related genes and proinflammatory genes at 36 h. Activation of the mitochondrial pathway was indicated by increased expression of BCL2L11, BIM, that continued to be upregulated at 48 h. Involvement of the extrinsic pathway was evidenced by induction of FAS, TNFRSF21 and TNF, and TNFAIP3 at 48 h. The TNF family genes are a group of cytokines that are involved in a number of processes including apoptosis [76,77] and inflammation [78]. The increased involvement of endoplasmic reticulum stress and inflammatory genes in E_2-induced apoptosis is not surprising because both pathways are known to intersect [79,80]. Multiple genes induced by E_2 are NF-κB responsive that is a major regulator of inflammatory response [81,82]. Upregulation of the observed genes provide a potential mechanism for E_2 to target a variety of inflammatory and apoptotic genes.

The importance of BIM and Bax have previously been noted and verified by selective increased expression of both proteins by E_2 [83]. Involvement of the extrinsic signalling pathway in E_2-induced apoptosis has been also observed. Osipo *et al* [84] showed that E_2-induced regression of tamoxifen-stimulated breast cancer tumours by activating the death receptor Fas and inhibiting the antiapoptotic/prosurvival factors NF-κB and HER2/neu. In addition, the growth of raloxifene-resistant MCF7 cells *in vitro* and *in vivo* was inhibited by E_2 by increasing Fas expression and reduced NF-κB activity [85]. However, unlike the present study, none of the previous studies investigated a time course of the intrinsic and extrinsic pathway in the MCF7:5C cells in E_2-induced apoptosis. Similar to our PCR array results, RNA sequencing of E_2-treated MCF7:5C cells revealed induction of multiple apoptosis-related genes [11]; therefore, deletion of a single gene is unlikely to significantly affect E_2-mediated apoptosis in the MCF7:5C cells. E_2 induces apoptosis in osteoclasts within 24 h [86] and is associated with upregulation of TGF-β and inhibition of E_2-treated cells with anti-TGF-β antibody inhibited E_2-induced apoptosis [87]. Therefore, this study show a unique sequential activation of endoplasmic reticulum stress, inflammatory-response genes as well as the intrinsic and extrinsic apoptosis-related genes in E_2-mediated apoptosis.

Paclitaxel, a cytotoxic chemotherapy extensively used in the treatment of breast cancer was used as a comparator to E_2 to demonstrate differences in the expression of apoptosis related genes. Paclitaxel selectively induces the TNF proapoptotic genes, but BIM expression was not noted. On the other hand, paclitaxel kills the MCF7 cells by displacement of BIM from the BIM/BCL2 complex [88]. Knockdown of BIM with siRNA significantly impairs the ability of paclitaxel to cause apoptosis in MCF7 cells [88,89]. In contrast, another study [90] showed that BIM was not required for paclitaxel-mediated apoptosis in MCF7 cells, and these apparent discrepancies could be because of differences that exist from MCF7 cell lines obtained from different sources. However, long-term deprivation of E_2 from the MCF7 cells may have induced changes in the microenvironment that may be responsible for the taxane to activate the TNF apoptosis-related genes. Flow cytometry studies show that E_2 causes both proliferation and apoptosis of the MCF7:5C cells, indicating that before the trigger for apoptosis occurs, the cells grow in response to

E_2. Because cells continue to divide with elevated S phase of cell cycles, the reduction of cell number by E_2 do not become evident until after 4 days of treatment. In contrast, paclitaxel causes an immediate G2 blockade by 12 h that may explain the rapid reduction of cell number.

In conclusion, the initial target site of E_2 is ER. E_2 induces endoplasmic reticulum stress and mitochondrial apoptotic genes and a later recruitment of the TNF family of apoptotic genes, whereas paclitaxel induces a G2/M blockade and rapidly induces TNF apoptosis-related genes. The unique delayed aspect of E_2-induced apoptosis in antihormone-resistant breast cancer creates a new dimension in our opportunities to apply the knowledge for this targeted therapy of clinical significance [1,91,92]. This natural process of E_2-induced apoptosis may have significant applications in the further understanding of the cellular biology of cancer.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2f (Sweeney and Jordan) - Studies carried out by Dr. Elizabeth Sweeney in the Jordan laboratory at Georgetown University

Mechanisms underlying differential response to estrogen-induced apoptosis in long-term estrogen-deprived breast cancer cells.

Introduction

Network enrichment analyses done using gene arrays in timecourse experiments show overexpression of apoptotic- and stress-related pathways in the MCF-7:5C cells after 24-96 hours of E_2 treatment; however, these analyses show the MCF-7:2A cells expressing more genes associated with glutathione metabolism during this time period of E_2 exposure (Fig. 2f-1). This suggests that the two cell lines respond to E_2 treatment using different signaling pathways. The MCF-7:5C cells respond by quickly inducing apoptosis, while the antioxidant pathway may be more relevant to the MCF-7:2A cells. Experiments were designed to interrogate the apoptotic, stress, and antioxidant pathways in both cell lines to distinguish signaling mechanisms in response to E_2.

The concept of estrogen-induced death [83,93] is important because of its clinical relevance. A clinical study published in 2009 [12] compared two doses of estrogen for second-line treatment after breast cancer patients had failed aromatase inhibitor therapy. The authors showed that after long-term anti-hormone therapy, no response is lost with the lower dose of estrogen; overall about 30% of women responded to estrogen treatment. The goal of this work is to uncover the mechanisms preventing the other 70% of patients from responding, and perhaps find ways to circumvent their resistance. To this end, MCF-7:2A cells were used as a model for estrogen-deprived breast tumors with the ability to evade estrogen-induced apoptosis in the clinic.

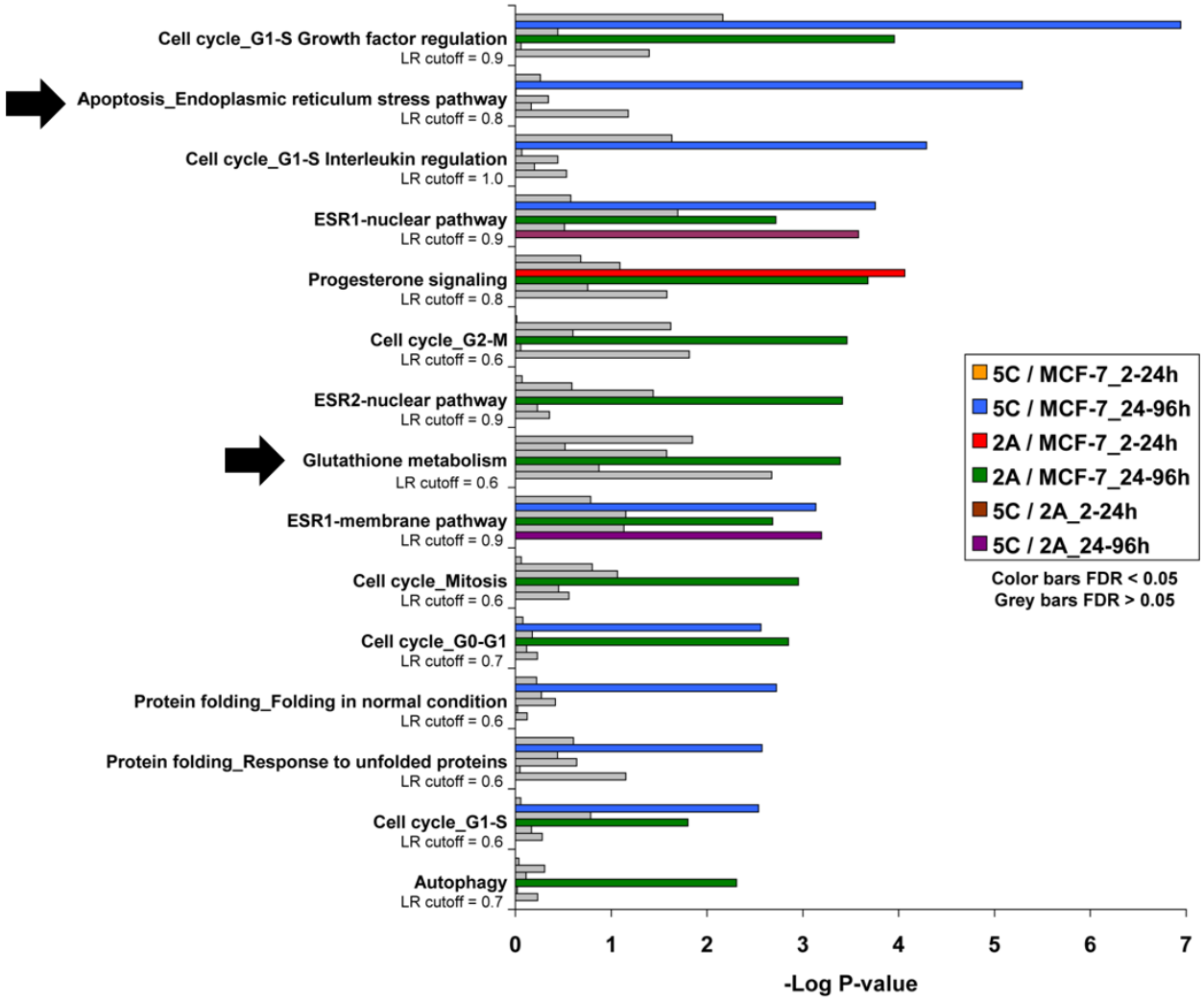


Figure 2f-1. ***Network enrichment analysis for MCF-7:WS8, MCF-7:5C and MCF-7:2A cells.*** *Global gene arrays were performed to compare activated gene networks associated with 1 nM E_2 treatment in the cell lines. Genes were analyzed after 2-24 h treatment and 24-96 h treatment.*

Work Accomplished:

MCF-7:2A initial response to E_2 The MCF-7:WS8, MCF-7:5C, and MCF-7:2A cell lines respond differently to 10^{-9} mol/liter (1 nM) E_2. In the presence of 1 nM E_2, MCF-7:WS8 cells are stimulated to proliferate over seven days, whereas MCF-7:5C cells are killed by this timepoint (Fig. 2f-2A). MCF-7:2A cell growth is unaffected by the presence of E_2 after one week, but their DNA is reduced by 50% after the second week of treatment (Fig. 2f-2A). Interestingly, MCF-7:2A cells are initially stimulated to proliferate in response to E_2. After 24 hours treatment with 1 nM E_2, both the mitogen-activated protein kinase (MAPK) and serine/threonine protein kinase Akt (AKT) pathways are activated, as shown by an increase in phosphorylated MAPK (p-MAPK) and phosphorylated AKT (p-AKT) proteins, respectively (Fig. 2f-2B). Further, MCF-7:2A cells treated with E_2 for 24 hours show an increase in the percentage of dividing cells compared with vehicle treatment (34.78% versus 20.17%), illustrated by S-phase in cell cycle analysis (Fig. 2f-2C).

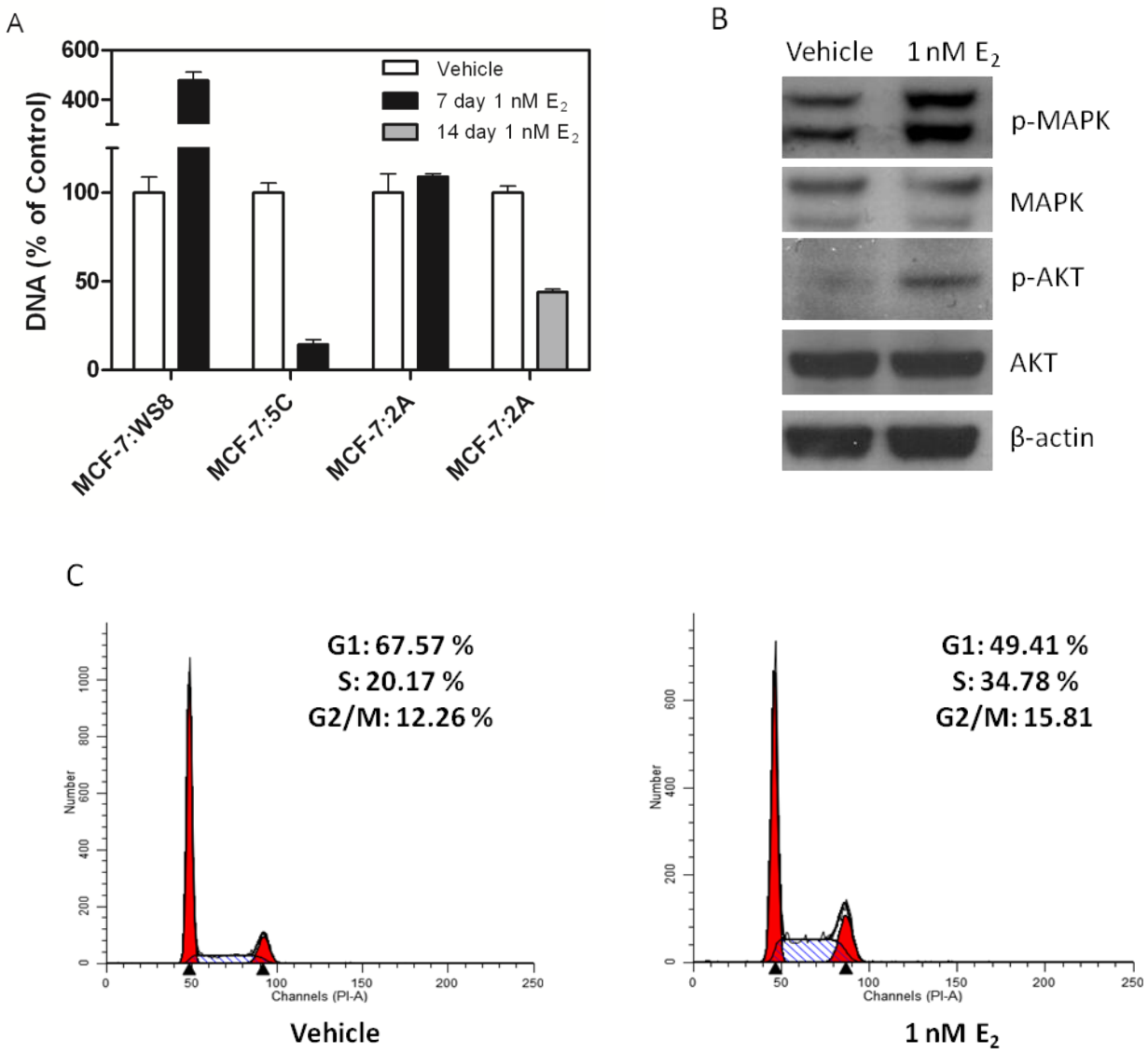


Figure 2f-2. ***MCF-7:2A growth response to E_2.*** *A. DNA was measured from MCF-7:WS8, MCF-7:5C, and MCF-7:2A cells after 7 or 14 days treatment with vehicle or 1 nM E_2. Values are normalized to vehicle-treated cells. Means represent samples in triplicate. B. MAPK and AKT growth pathway protein levels were measured by Western blot after 24 h vehicle or 1 nM E_2 treatment. β-actin was used as a loading control. C. Cell cycle analysis was performed after 24 h vehicle or 1 nM E_2 treatment.*

MCF-7:5C and MCF-7:2A UPR To determine whether the different biological effects observed in MCF-7:5C and MCF-7:2A cells is due to different patterns of the unfolded protein response (UPR), proteins associated with the UPR were measured over a 72 hour timecourse. Two markers of the UPR, phosphorylated eIF2Ŭ (p-eIF2Ŭ) and IRE1Ŭ, were visualized by Western blot in MCF-7:5C and MCF-7:2A cells in the presence of vehicle and 1 nM E_2 (Fig. 2f-3). p-eIF2Ŭ is directly downstream of protein kinase RNA-like endoplasmic reticulum kinase (PERK), a sensor which initiates UPR. Both cell lines show an increase in the protein expression of p-eIF2Ŭ and IRE1Ŭ by 72 hours of E_2 treatment, indicating activated UPR. Though MCF-7:2A cells show a slightly higher basal p-eIF2Ŭ level, no differences in UPR activation can be seen between the two cell lines.

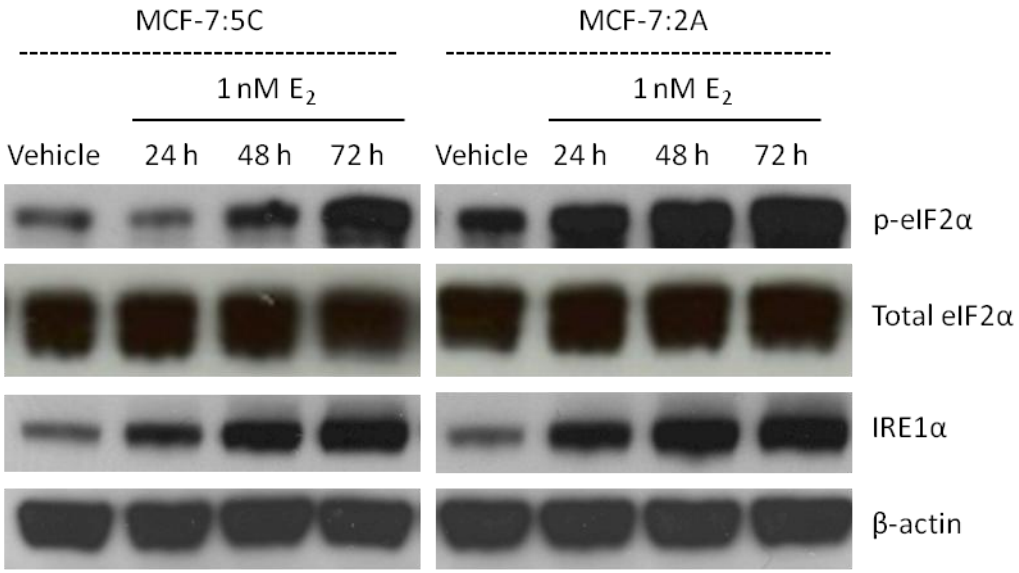


Figure 2f-3. ***MCF-7:5C and MCF-7:2A UPR.*** *Cell lines were probed for UPR-related proteins after treatment with vehicle or 1 nM E_2 for 24, 48, and 72 h. β-actin was used as a loading control.*

MCF-7:5C and MCF-7:2A estrogen-induced apoptosis *To determine whether MCF-7:2A cells experience apoptosis through the same mechanism as MCF-7:5C cells, RT-PCR was used to quantify mRNA levels of apoptosis-related genes. MCF-7:5C cells noticeably up-regulate LTA (4.19 ± 1.92 fold change), LTB (5.39 ± 1.82), TNFα (9.40 ± 3.86), and BCL2L11 (6.06 ± 0.87) after 72 hours of E_2 treatment, while MCF-7:2A cells show no major changes during this time period (Fig. 2f-4A). MCF-7:2A cells were then treated with E_2 for a longer time period to measure apoptosis-related genes during the time when they appear to die. MCF-7:2A cells increase both TNFα (33.55 ± 12.09 fold change) and BCL2L11 (3.71 ± 0.35 fold change) after 12 days of 1 nM E_2 treatment (Fig. 2f-4B). The up-regulated apoptosis-related genes correspond to the time when cell death is most apparent in both cell lines, during week one in MCF-7:5C cells, and during week two in MCF-7:2A cells.*

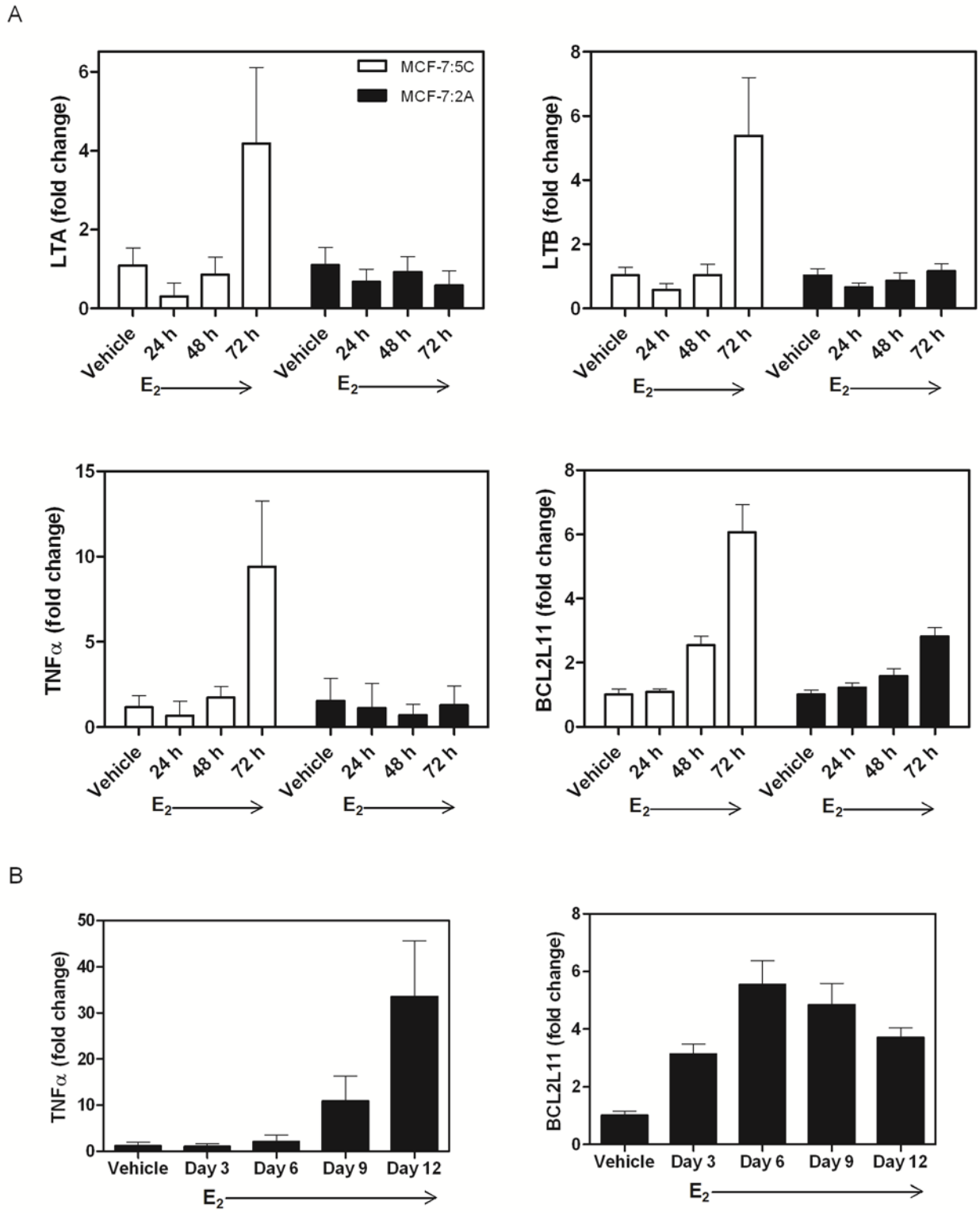


Figure 2f-4. ***Apoptosis-related genes in MCF-7:5C and MCF-7:2A cells.*** *A. MCF-7:5C and MCF-7:2A cells were treated with vehicle or 1 nM E_2 for 24, 48, and 72 h. LTA, LTB, TNFα, and BCL2L11 mRNA levels were measured using RT-PCR. 36B4 was used as an internal control. B. MCF-7:2A cells were treated with vehicle or 1 nM E_2 for 3, 6, 9, and 12 days. TNFα and BCL2L11 mRNA levels were then measured using RT-PCR. 36B4 was used as an internal control. Means represent at 7 to 18 replicates.*

MCF-7:5C and MCF-7:2A oxidative stress Heme oxygenase 1 (HMOX1) was used as an indicator to illustrate when MCF-7:5C and MCF-7:2A cells experience oxidative stress. After 72 hours of 1 nM E_2 treatment, HMOX1 mRNA was increased 4.61-fold in MCF-7:5C cells (Fig. 2f-5A), suggesting this cell line undergoes oxidative stress at this time point. MCF-7:2A cells did not generate an up-regulation of HMOX1 mRNA until 12 days of 1 nM E_2 treatment when it increased 10.03-fold (Fig. 2f-5B), suggesting an earlier protective mechanism inherent in these cells to prevent oxidative stress longer than MCF-7:5C cells.

Glutathione is a potent antioxidant and was quantified in MCF-7:5C and MCF-7:2A cells to illustrate a potential protective mechanism in MCF-7:2A cells against oxidative stress (Fig. 2f-6A). In fact, MCF-7:2A cells have significantly more basal glutathione than do MCF-7:WS8 and MCF-7:5C cells (Fig.2f-6A). Buthionine sulfoximine (BSO) is a synthetic amino acid that blocks glutathione synthesis by inhibiting gamma-glutamylcysteine synthetase. One hundred ÕM BSO dramatically decreases glutathione levels in both MCF-7:5C and MCF-7:2A cells (Fig. 2f-6B). To ask the question of whether glutathione is protecting MCF-7:2A cells from oxidative stress and E_2-induced apoptosis, HMOX1 was measured following treatment with vehicle, 1 nM E_2 alone, 100 ÕM BSO alone, and 1 nM E_2 + 100 ÕM BSO after 24, 48, and 72 hours (Fig. 2f-6C). MCF-7:2A cells show increased HMOX1 mRNA at 72 hours after treatment with 100 ÕM BSO and 1 nM E_2 + 100 ÕM BSO (3.57 Ñ 0.36 and 2.60 Ñ 0.70 fold changes, respectively), suggesting a protective role of glutathione in these cells. Reactive oxygen species (ROS) increased 634% over vehicle in MCF-7:2A cells after 12 days of the combination treatment (Fig. 2f-6D). Furthermore, 1 nM E_2 + 100 ÕM BSO treatment caused a significant decrease in DNA after 14 days treatment (Fig. 2f-6E), suggesting that oxidative stress is a key factor in determining E_2-induced MCF-7:2A cell death.

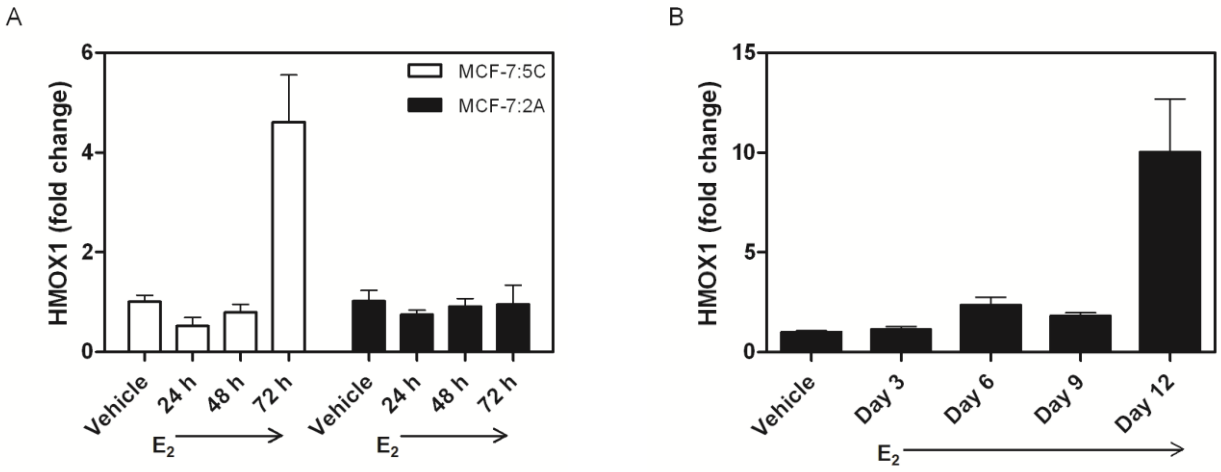


Figure 2f-5. ***MCF-7:5C and MCF-7:2A HMOX1 regulation.*** *A. MCF-7:5C and MCF-7:2A cells were treated with vehicle or 1 nM E_2 for 24, 48, and 72 h; HMOX1 mRNA was measured using RT-PCR. 36B4 was used as an internal control. Mean represents 18 replicates. B. MCF-7:2A cells were treated with vehicle or 1 nM E_2 for 3, 6, 9, and 12 days; HMOX1 mRNA was measured using RT-PCR. 36B4 was used as an internal control. Means represent at least 8 replicates.*

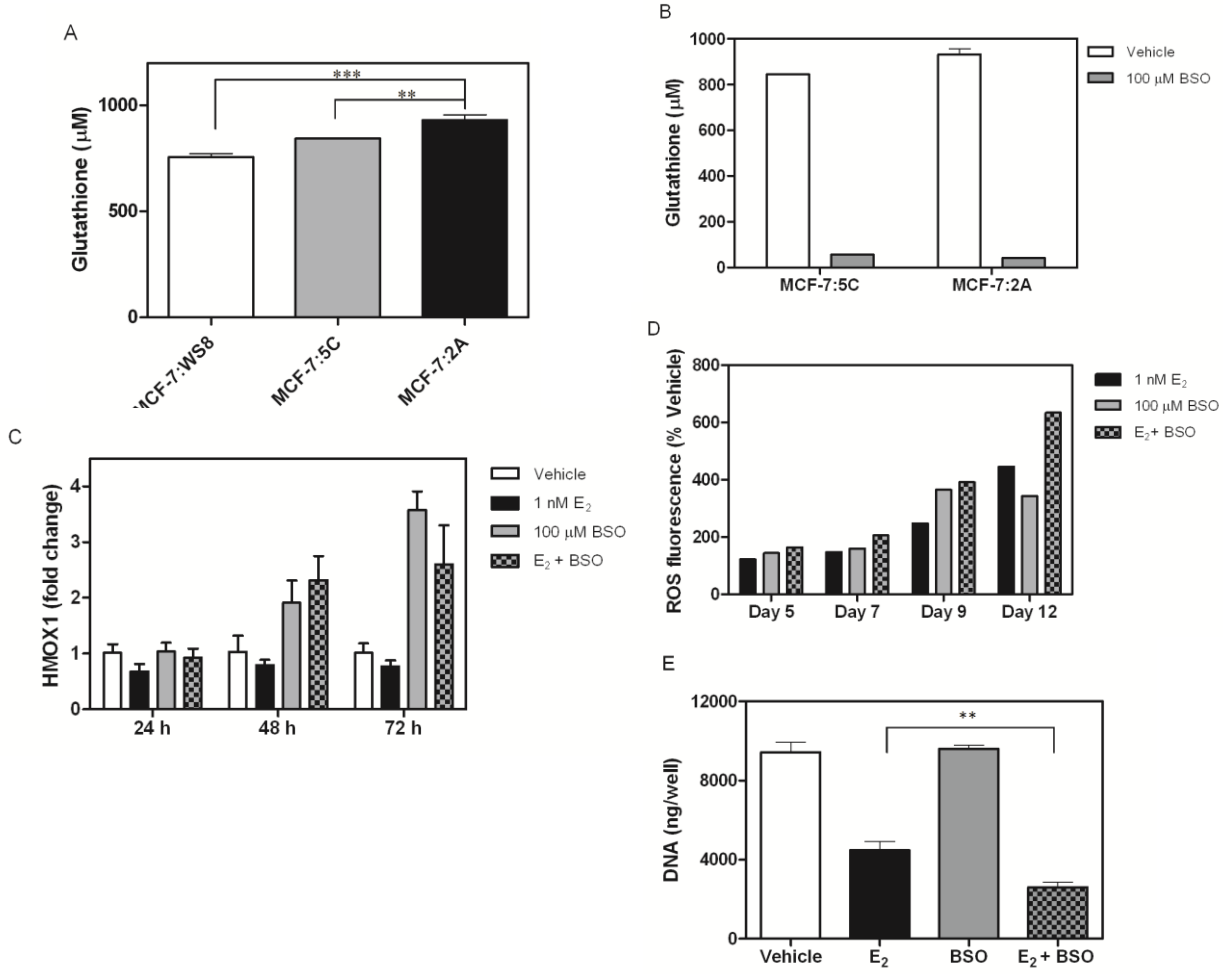


Figure 2f-6. MCF-7:2A *oxidative stress and glutathione.* *A. Total basal glutathione (GSSG+GSH) levels were measured in MCF-7:WS8, MCF-7:5C, and MCF-7:2A cells. Means represent samples in triplicate. B. Total glutathione in MCF-7:5C and MCF-7:2A cells were quantified after 72 h treatment of vehicle or 100 µM BSO. Means represent samples in triplicate. C. MCF-7:2A cells were treated for 24, 48, and 72 h with either vehicle, 1 nM E_2, 100 µM BSO, or 1 nM E_2 + 100 µM BSO; HMOX1 mRNA was measured using RT-PCR. 36B4 was used as an internal control. Means represent at least 8 replicates. D. MCF-7:2A were subjected to the aforementioned treatments for 5, 7, 9, and 12 days, and ROS levels were measured. Data is normalized to vehicle treatment. E. MCF-7:2A cells were treated likewise, and DNA was harvested and quantified after two weeks. Means represent samples in triplicate. **p<0.01, ***p<0.001*

MCF-7:5C and MCF-7:2A IGFR Insulin-like growth factor receptor beta (IGF-1Rß) up-regulation is another mechanism through which MCF-7:2A cells could receive anti-apoptotic advantage over MCF-7:5C cells. MCF-7:2A cells exhibit 2.71-fold greater basal IGF-1Rß mRNA than MCF-7:5C cells (Fig. 2f-7A). This is consistent at the protein level as shown by Western blot, where MCF-7:2A cells exhibit more IGF-1Rß protein expression than MCF-7:5C cells (Fig. 2f-7B). When treated with an IGF-1Rß inhibitor (10 ÕM AG1024) for 7 days, MCF-7:2A cells show significantly decreased DNA content when compared to vehicle and 1 nM E_2 treatments (Fig. 2f-7C). Combination treatment of 1 nM E_2 + 10 ÕM AG1024 decreased DNA content significantly more than either treatment alone (Fig. 2f-7C), suggesting an integral role of IGF-1Rß in MCF-7:2A cells evading E_2-induced apoptosis. To interrogate this further, growth pathway proteins were measured in response to 10 ÕM AG1024 treatment. MAPK and AKT

pathways are both blocked by the IGF-1Rδ inhibitor after 72 hours as shown by decreased p-MAPK and p-AKT levels when compared to vehicle-treated MCF-7:2A cells (Fig. 2f-7D).

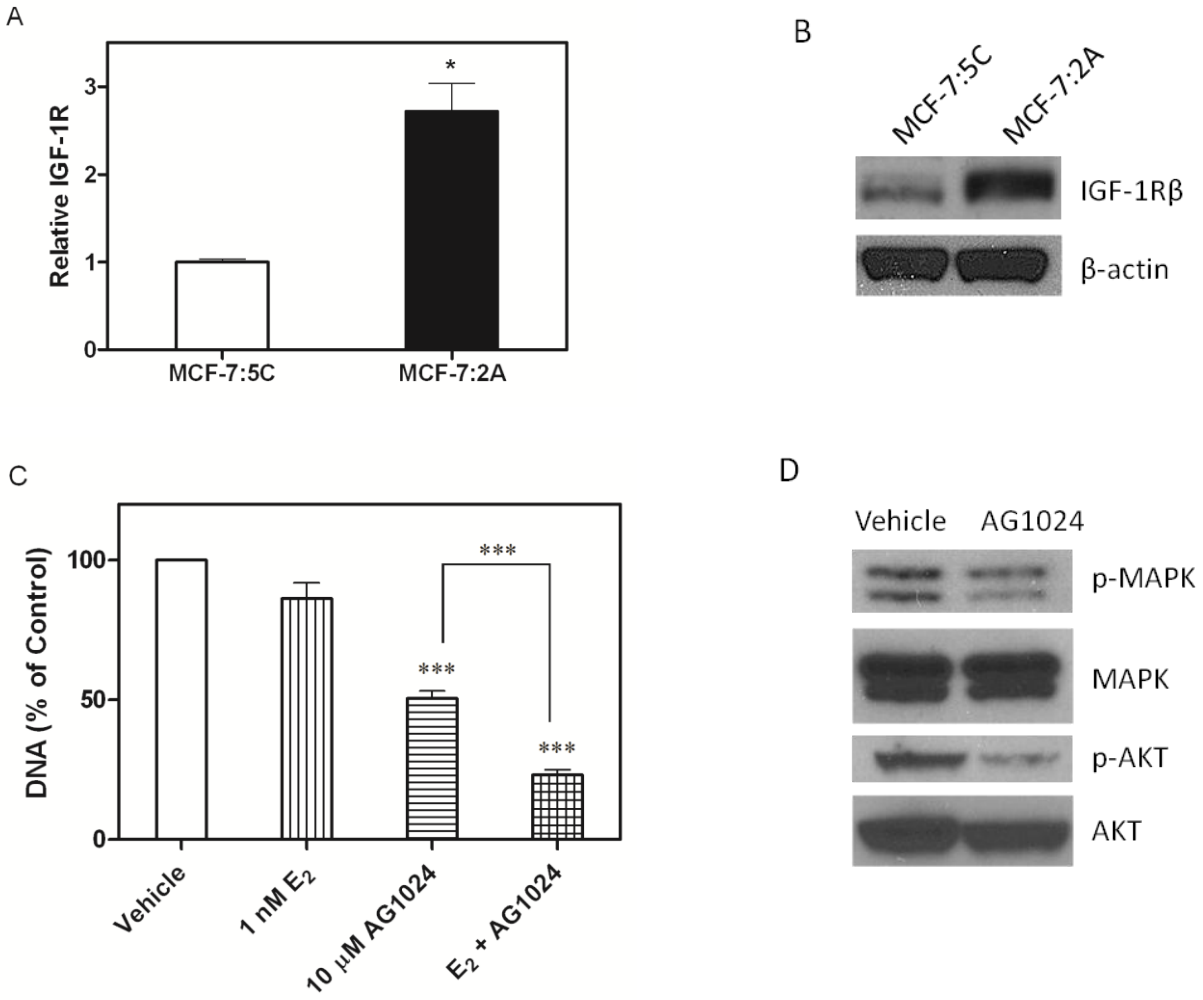


Figure 2f-7. ***MCF-7:2A IGF-1Rβ.*** *A. Basal IGF-1Rβ mRNA was measured in MCF-7:5C cells and MCF-7:2A cells via RT-PCR. MCF-7:2A values are normalized to MCF-7:5C. 36B4 was used as an internal control. Means represent samples in triplicate. B. Basal IGF-1Rβ protein levels were measured in MCF-7:5C and MCF-7:2A cells by Western blot. β-actin was used as a loading control. C. MCF-7:2A cells were treated with vehicle, 1 nM E_2, 10 μM AG1024, or 1 nM E_2 + 10 μM AG1024. DNA was harvested and quantified after seven days. Means represent samples in triplicate. D. MCF-7:2A cells were treated for 72 h with vehicle or 10 μM AG1024. Growth pathway protein levels were visualized via Western blot. Total MAPK and total AKT were used as loading controls. *p<0.05, ***p<0.001*

Discussion

This study investigated the mechanisms through which MCF-7:2A cells evade E_2-induced apoptosis in vitro as a means to understand resistant breast cancer cells after long-term anti-hormone therapy in the clinic. After failure on an aromatase inhibitor, approximately 30 percent of breast cancer patients will respond to treatment with estrogen [12]; their nascent or remaining breast tumors will become cytostatic or disappear with physiological levels of estrogen. Further, estrogen replacement therapy (ERT) has been shown to reduce the risk of breast cancer in hysterectomized post-menopausal women [1], perhaps due to estrogen-deprived breast cancer cells undergoing estrogen-induced apoptosis before resulting in clinically apparent disease. This study sought to discriminate between estrogen-deprived breast tumors that will

quickly respond to treatment with E_2 versus those that will respond more slowly and less dramatically. We modeled these different scenarios with MCF-7:5C and MCF-7:2A cell lines, respectively.

We have found that the UPR, associated with endoplasmic reticulum stress (ERS), is a fundamental element in E_2-induced MCF-7:5C cell apoptosis [10]. In this setting, E_2 triggers UPR and rapidly causes apoptosis within one week of treatment. Two main sensors of the UPR, IRE1Ŭand PERK, are activated in both cell lines similarly. PERK activation is confirmed by p-eIF2Ŭ expression, since eIF2Ŭ is phosphorylated by activated PERK. In MCF-7:2A cells, the same sensors are activated as in MCF-7:5C cells (Fig. 2f-3), but significant cell death is not apparent at the same timepoint (Fig. 2f-2A). Despite similar signaling patterns, the biological responses between the two cell lines differ. Our data suggested that another mechanism was preventing cell death after E_2-induced UPR in MCF-7:2A cells.

Oxidative stress is a critical pathway for MCF-7:5C and MCF-7:2A cells to undergo E_2-induced apoptosis [11]. MCF-7:2A cells inherently exhibit stronger survival and antioxidant mechanisms than MCF-7:5C cells (Figs. 2f-4-6). This relationship is consistent with previously published data showing that MCF-7 cells with higher levels of glutathione peroxidase 1 (GSHPx-1) can survive better under oxidative stress conditions[94], such as hydrogen peroxide treatment, and that MCF-7 cells can increase antioxidant enzymes (i.e. manganese superoxide dismutase, MnSOD) to prevent TNF-mediated apoptosis [95]. Activation of E_2-induced apoptosis in MCF-7:2A cells also seems to require TNF family member up-regulation (Figs. 2f-4A and 4B). Oxidative stress occurs concurrently with up-regulation of apoptosis-related genes in the TNF family. Whether increased TNFŬ causes oxidative stress or oxidative stress causes increased TNFŬis not yet documented in this setting.

Increased IGFR promotes anti-hormone resistance in breast cancer, likely through growth factor receptor crosstalk and aberrant ER, MAPK, and AKT signal transduction pathway activation [96]. Our data correlate with these findings in that higher IGF-1Rɓ mRNA and protein expression confer a growth advantage and apoptotic resistance in MCF-7:2A cells despite treatment with E_2 (Fig. 2f-7). This suggests an IGF-1Rɓ signaling pathway that can circumvent normal ER signaling in long-term estrogen-deprived breast cancer cells. Studies using hepatocellular carcinoma cells (HCC) have demonstrated that IGF-1R overexpression can potentially cause increased glutathione transferase (GST) and protection from oxidative stress [97]. Although this mechanism is shown in liver cancer cells, it may apply to our models of breast cancer as well. Perhaps the higher level of IGF-1Rɓ in MCF-7:2A cells generates the increased glutathione levels necessary to escape cell death in the presence of E_2.

The evidence thus far shows that TNF family member gene expression, protection against oxidative stress, and growth factor signaling are major mechanisms underlying the different biological responses to E_2 seen in MCF-7:2A cells versus MCF-7:5C cells. Despite similar UPR signaling patterns, MCF-7:2A cells resist ERS-induced death longer and stronger than MCF-7:5C cells. Additional studies may provide further insight into the connection between IGF-1Rɓ and glutathione in MCF-7:2A cells, and how this relationship functions in the presence and absence of a stressor such as E_2. In order to effectively treat breast cancer patients who have undergone exhaustive anti-hormone treatment, and to explain why ERT can prevent breast cancer in some post-menopausal women, the examination of breast cancer cell models of estrogen deprivation is proving invaluable. By understanding mechanisms that prevent apoptosis in these breast cancer cells, we can translate key findings into clinical practice.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2g (Sengupta and Jordan) - Studies carried out by Dr. Surojeet Sengupta in the Jordan laboratory at Georgetown University

Cyclin Dependent Kinase-9 Mediated Transcriptional De-regulation of cMYC as a Critical Determinant of Endocrine-Therapy Resistance in Breast Cancers

Introduction:

To determine the relevance and mechanism of cMYC over-expression in imparting estrogen-independence to the endocrine-resistant breast cancer cells we used a panel of MCF7 ERŬ+ breast cancer cells which are known to proliferate in the absence of estrogen and exhibit different sensitivities to the anti-hormone therapies. The different MCF7 cell line derivatives used were MCF7:5C [98], MCF7:2A [99], MCF7/LCC1 [100], MCF7/LCC2 [101] and MCF7/LCC9 [102,103]. All these cells mimic aromatase inhibitor resistance as they can grow in an estrogen-deprived condition. In addition, MCF7:5C and LCC2 cells are also resistant to anti-estrogens, 4-hydroxy -tamoxifen (4OHT) whereas LCC9 cells demonstrate resistance to 4OHT and fulvestrant. All these cell lines cells showed high expression of cMYC protein as compared to parent MCF7 cells and estrogen-independent growth of all the resistant cells was drastically inhibited by a cMYC inhibitor, 10058-F4 (F4). For focused studies we chose MCF7:5C cells as we have extensive experience with this cell line and the LCC1, LCC2 and LCC9 cells showed modest estrogen stimulation of growth [100,101,102] despite being estrogen-independent. On the other hand MCF7:5C cells undergo apoptosis after estrogen treatment [10,83]. This is a documented response clinically, following the development of anti-hormone resistance [91].
This study dissects the upstream molecular mechanism involved in the transcriptional over-expression of cMYC oncogene in the endocrine-therapy resistant cells, which imparts estrogen-independence. In addition, we present CDK9 as a potential target for therapeutic intervention which can suppress the deregulated transcriptional over-expression of cMYC leading to complete inhibition of estrogen-independent proliferation of the endocrine-resistant breast cancer cells.

Work Accomplished:

Levels of cMYC and estrogen-independent growth of ERα+ endocrine resistant breast cancer cells

We found that all the endocrine-therapy resistant breast cancer cells used in this study, namely, MCF7:5C, MCF7:2A, MCF7/LCC1, MCF7/LCC2 and MCF7/LCC9 cells overexpress cMYC mRNA (Figure 2g-1A) and protein (Figure 2g-1B) as compared to parental MCF7 cells. All the resistant cells showed ~3-4 fold higher growth as compared to the parental MCF7 cells (Figure 2g-1C) over a 4 day period. Cell cycle analysis of MCF7:5C cells revealed more than 2 fold higher ñSò phase cells than in MCF7 cells and 5 fold higher proliferation over a six day period
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To determine if the high levels of cMYC mRNA was due to the elevated transcriptional activity or stability of the transcripts we performed a pulse chase assay and found that the cMYC mRNA had a similar rate of degradation in MCF7 and MCF7:5C cells.

Inhibition or depletion of cMYC blocks estrogen-independent proliferation of ERα+ endocrine resistant cells cells

We determined the functional role of cMYC over-expression in estrogen-independent growth of the endocrine-therapy resistant breast cancer cells by blocking the cMYC action using a pharmacological inhibitor 10058-F4 which has been shown to specifically inhibit actions of cMYC by blocking its interaction with MAX [104] and stabilizing the MYC monomer [105]. cMYC the resistant cells (Figure 2g-2A) whereas only 18% growth inhibition was observed in MCF7 cells inhibition. Furgher experiments with MCF7:5C cells showed that 10058-F4 was selectively able to inhibit its growth in a dose-dependent manner as compared to MCF7 cells over a four day period (Figure 2g-2B). Cell cycle analysis confirmed that the decrease in proliferation resulted from a 57% reduction in the óSô phase cells of the MCF7:5C cells (Figure 2g-2C). In comparison, there was only 6% decrease in the óSô phase cells of the parental MCF7 cells. We also used the targeted approach to confirm the role of cMYC in MCF7:5C cells, by depleting cMYC levels using short interfering RNA (siRNA). Two different siRNA against cMYC depleted the levels of its protein in MCF7:5C cells which led to 50-75% reduction in the number of óSô phase cells (Figure 2g-2D) with a concurrent inhibition of cell growth over a period of four days. Reduced phosphorylation of retinoblastoma protein was also evident in the cells depleted of cMYC protein.

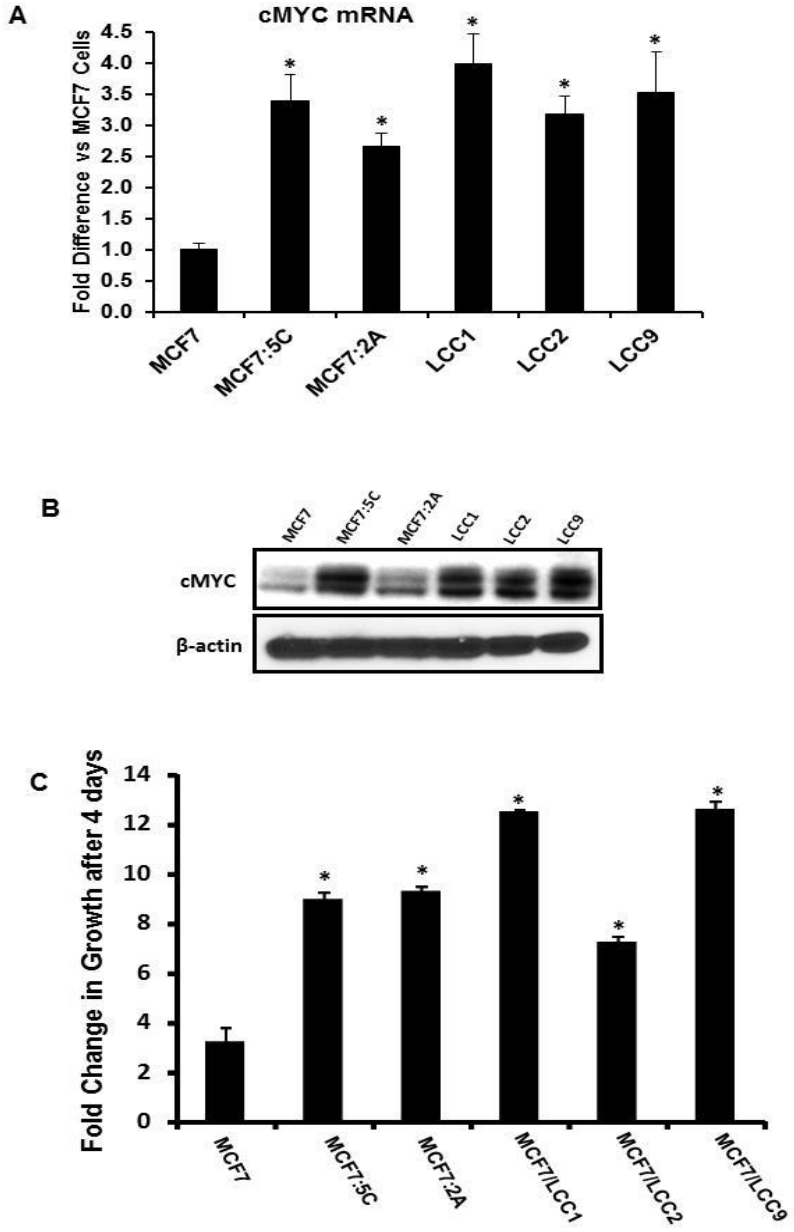
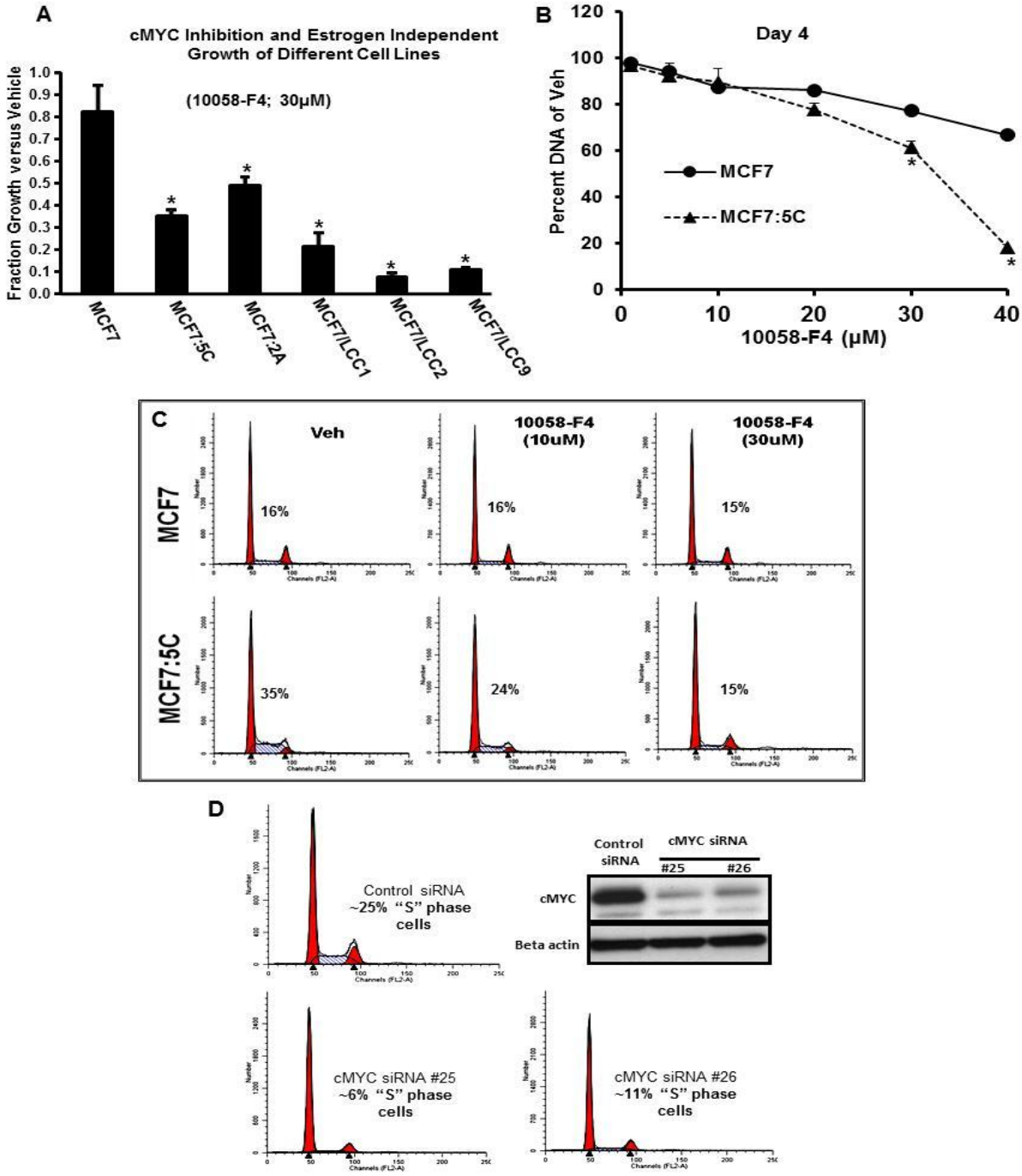


Figure 2g-1. *Levels of cMYC and estrogen independent growth of endocrine therapy resistant breast cancer cells. (A) cMYC mRNA levels were measured in different MCF7 derivative endocrine therapy resistant cells using RT-PCR. Data is represented as fold difference in cMYC mRNA versus MCF7 cells. (B) Western blot of cMYC protein in MCF7 and. Beta actin was used as a loading control. (C) Estrogen independent growth of MCF7 and other endocrine therapy resistant breast cancer cells over a 4 day period. Un-treated cells were grown and total DNA was measured on day 4 after seeding. The data is represented as fold change in growth versus day '0'. (* p<.05 versus MCF7 cells)*


A
cMYC Inhibition and Estrogen Independent Growth of Different Cell Lines
(10058-F4; 30μM)
Fraction Growth versus Vehicle
MCF7
MCF7:5C
MCF7:2A
MCF7/LCC1
MCF7/LCC2
MCF7/LCC9
B
Day 4
Percent DNA of Veh
MCF7
MCF7:5C
10058-F4 (μM)
C
Veh
10058-F4 (10uM)
10058-F4 (30uM)
MCF7
MCF7:5C
16%
16%
15%
35%
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D
Control siRNA
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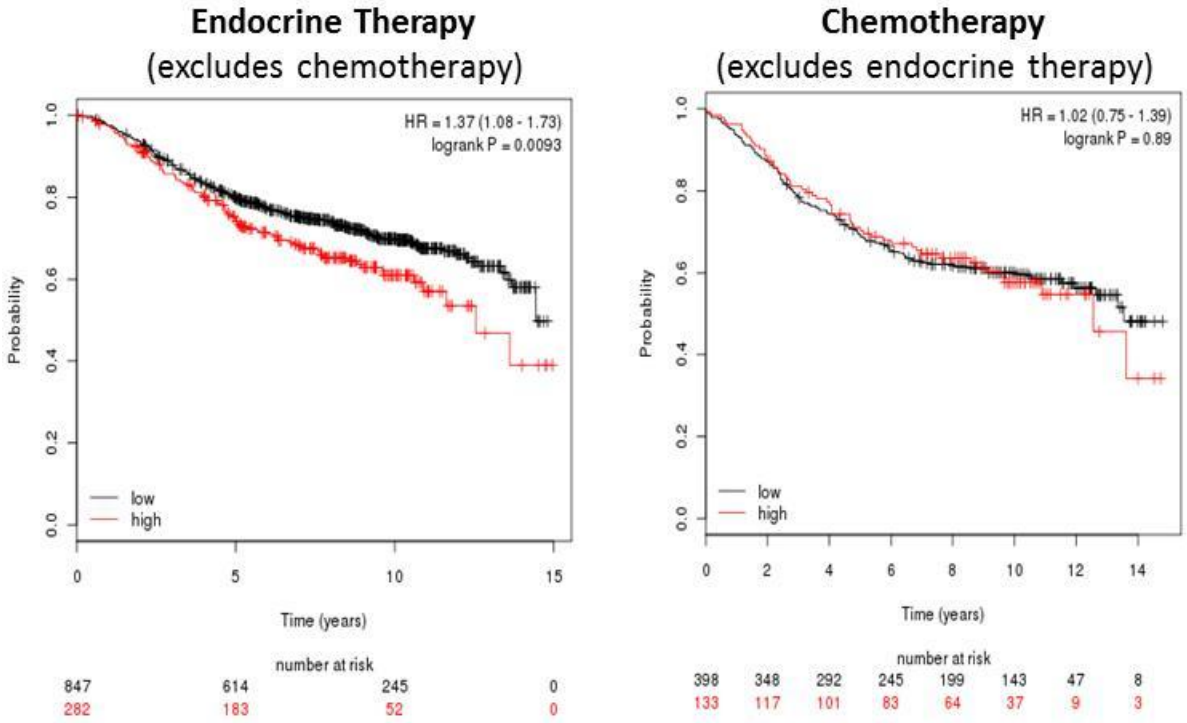
Control siRNA
cMYC siRNA
#25
#26
cMYC
Beta actin
cMYC siRNA #25
~6% "S" phase cells
cMYC siRNA #26
~11% "S" phase cells

Figure 2g-2. ***Inhibition or depletion of cMYC blocks estrogen independent growth of endocrine therapy resistant breast cancer cells. (A)*** *Total DNA was measured from the MCF7 and the resistant breast cancer cells after four days of treatment with 30μM, cMYC inhibitor (10058-F4). (* p<.05 versus MCF7 cells)* ***(B)*** *Total DNA was measured from the MCF7 and MCF7:5C cells after four days of treatment with cMYC inhibitor (10058-F4) with indicated concentration. (* p<.05 versus MCF7 cells)* ***(C)*** *"S" phase cells were assessed using cell cycle analysis of MCF7 and MCF7:5C cells treated with indicated concentration of cMYC inhibitor for 24 hrs. The numbers on each graph represents the percentage of "S" phase cells.* ***(D)*** *Assessment of "S" phase cells using cell cycle analysis 48 hours after siRNA mediated depletion of cMYC using two different siRNA (#25 and #26). The inset shows the western blot of cMYC protein levels after depletion of cMYC.*

Figure 2g-3. ***cMYC gene expression correlates with relapse free survival (RFS) in endocrine therapy but not chemotherapy treated patients.*** *The Kaplan-Meier plots show the association of cMYC gene expression and RFS in endocrine therapy or chemotherapy treated ERα+ breast cancer patients. The top 25% percent highest expressing cMYC patients (top quartile; in red) were compared with the rest of the 75% patient population (in black).*



cMYC gene expression correlates with RFS in endocrine therapy but not chemotherapy treated patients

The Kaplan-meier plots were generated for cMYC gene association with RFS of early breast cancer patients who received endocrine-therapy or chemotherapy only as an adjuvant treatment. We used the on-line tool (www.kmplot.com) which has a combined data set from various annotated breast cancer studies and can be used to study the association of a single gene with patients outcome using various user defined parameters [106]. The top 25% percent highest cMYC expressing patients (top quartile) were compared with the rest of the 75%. Kaplan-Meier plots (Figure 2g-3) reveal that high levels of cMYC expression is associated with poor RFS (P value; 0.0093) in 1129 patients treated with endocrine therapy only (Tamoxifen or AIs) whereas this association was not observed in the 531 patients (P value; 0.89) treated with chemotherapy only.

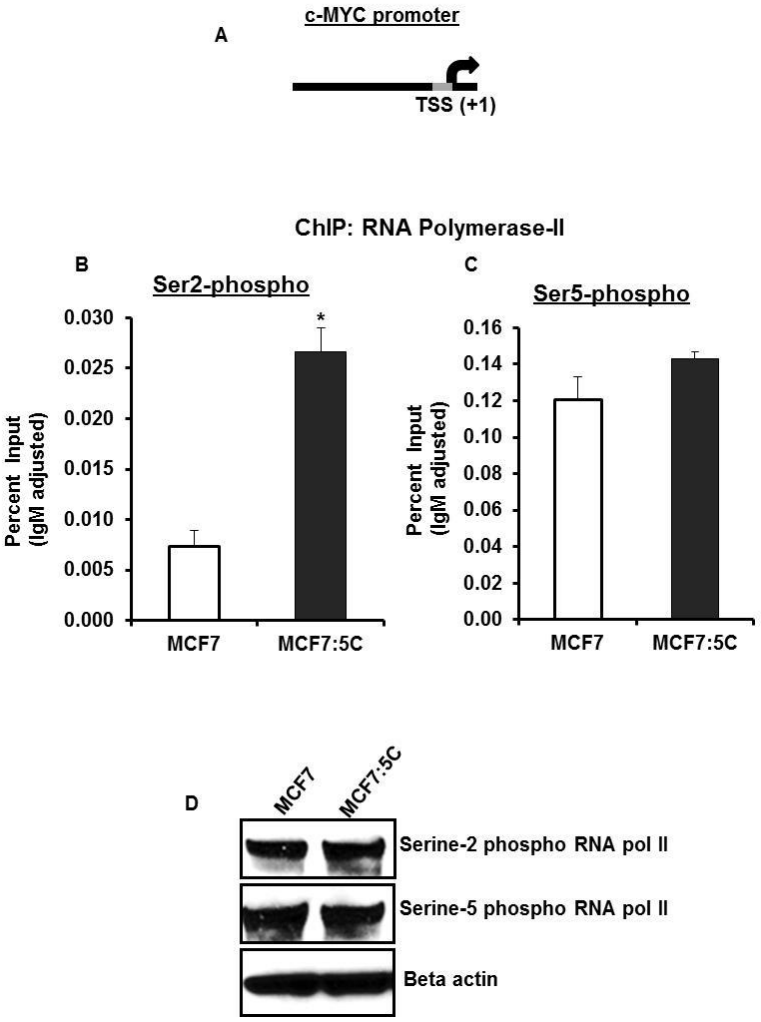


Figure 2g-4. ***Recruitment of serine-5 and serine-2 - phosphorylated RNA polymerase II at the cMYC promoter. (A)*** *Schematic presentation of cMYC promoter showing the transcription start site (TSS). The grey box represents the region (~150bp upstream of TSS) probed using real-time PCR following ChIP assay.* ***(B)*** *Recruitment of serine-2 phosphorylated RNA polymerase II and* ***(C)*** *serine-5 phosphorylated RNA polymerase II was assessed by ChIP assay followed by real-time PCR in MCF7: and MCF7:5C cells. Values are represented as percent input of the starting chromatin, adjusted for control IgM recruitment for each sample. (* p<.05 versus MCF7 cells)* ***(D)*** *Total protein levels of serine-2 and serine-5 phosphorylated RNA polymerase II in MCF7 and MCF7:5C cells.*

Recruitment of phospho-serine-2 and phospho-serine-5 RNA polymerase II at the cMYC promoter in MCF7:5C and MCF7 Cells

To further determine the mechanism of steady-state transcriptional over-expression of the cMYC mRNA in MCF7:5C cells we probed the proximal promoter of the cMYC gene (Figure 2g-4A) in terms of recruitment of phosphorylated serine-5 and phosphorylated serine-2 RNA polymerase II, which is responsible for the initiation and the elongation of the transcription of RNA, respectively. ChIP assay using phospho-specific RNA polymerase II antibodies revealed that in MCF7:5C cells the recruitment of serine-2 phosphorylated RNA polymerase II was more than 3 fold higher than parental MCF7 cells (Figure 2g-4B). However, no difference was observed in the recruitment of serine-5 phosphorylated RNA polymerase II at the cMYC promoter in MCF7:5C and MCF7 cells (Figure 2g-4C). We further confirmed that the total levels of phosphorylated serine-2 or serine-5 RNA polymerase was not different in MCF7:5C cells as compared to MCF7 cells (Figure 2g-4D).

Levels of cyclin dependent kinase 9 (CDK9) and its role in estrogen-independent growth of endocrine-therapy resistant cells

CDK9 is a major kinase which is responsible for the phosphorylation of serine-2 RNA polymerase II [107,108] and the elongation of RNA transcripts [109]. We therefore examined the total CDK9 levels in the endocrine-therapy resistant cells and observed an over-expression in all the cells as compared to the MCF7 cells. In MCF7:5C cells, the total as well as the phosphorylated CDK9 levels were elevated by 2.5 and 3.1 fold respectively (Figure 2g-5A). We also observed a slight increase in the levels of CTDP/ FCP1 protein in MCF7:5C cells, which is known to dephosphorylate CDK9 [107] (Figure 2g-5A). Interestingly, FCP1 has also been reported to stimulate transcription elongation [110]. Next, we used a specific, potent, competitive inhibitor of CDK9, known as CAN 508 [111] to study the role of CDK9 in estrogen-independent growth of MCF7:5C cells and compared it with the parental MCF7 cells. A dose dependent effect was observed in MCF7:5C cells where 30ÕM of CAN 508 compound completely inhibited its growth over a six day period (Figure 2g-5B). Furthermore, 30 ÕM of CAN 508 drastically blocked the growth of all endocrine-therapy resistant breast cancer cells used in this study whereas it had

minimal growth inhibitory effect on the parental MCF7 cells.

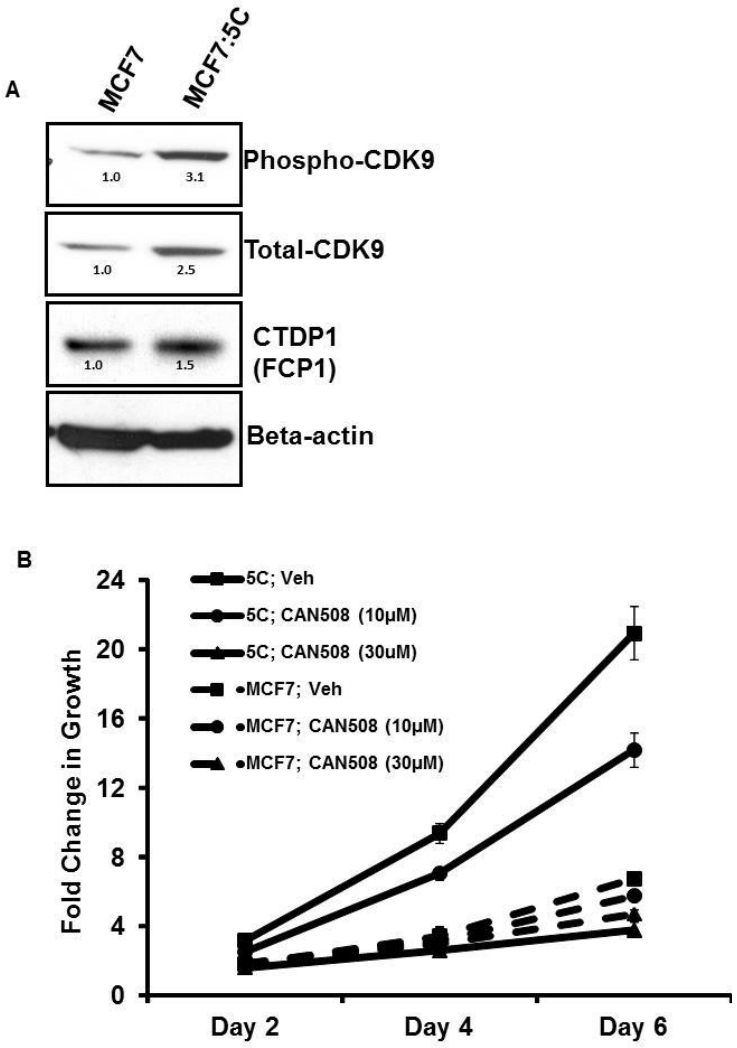
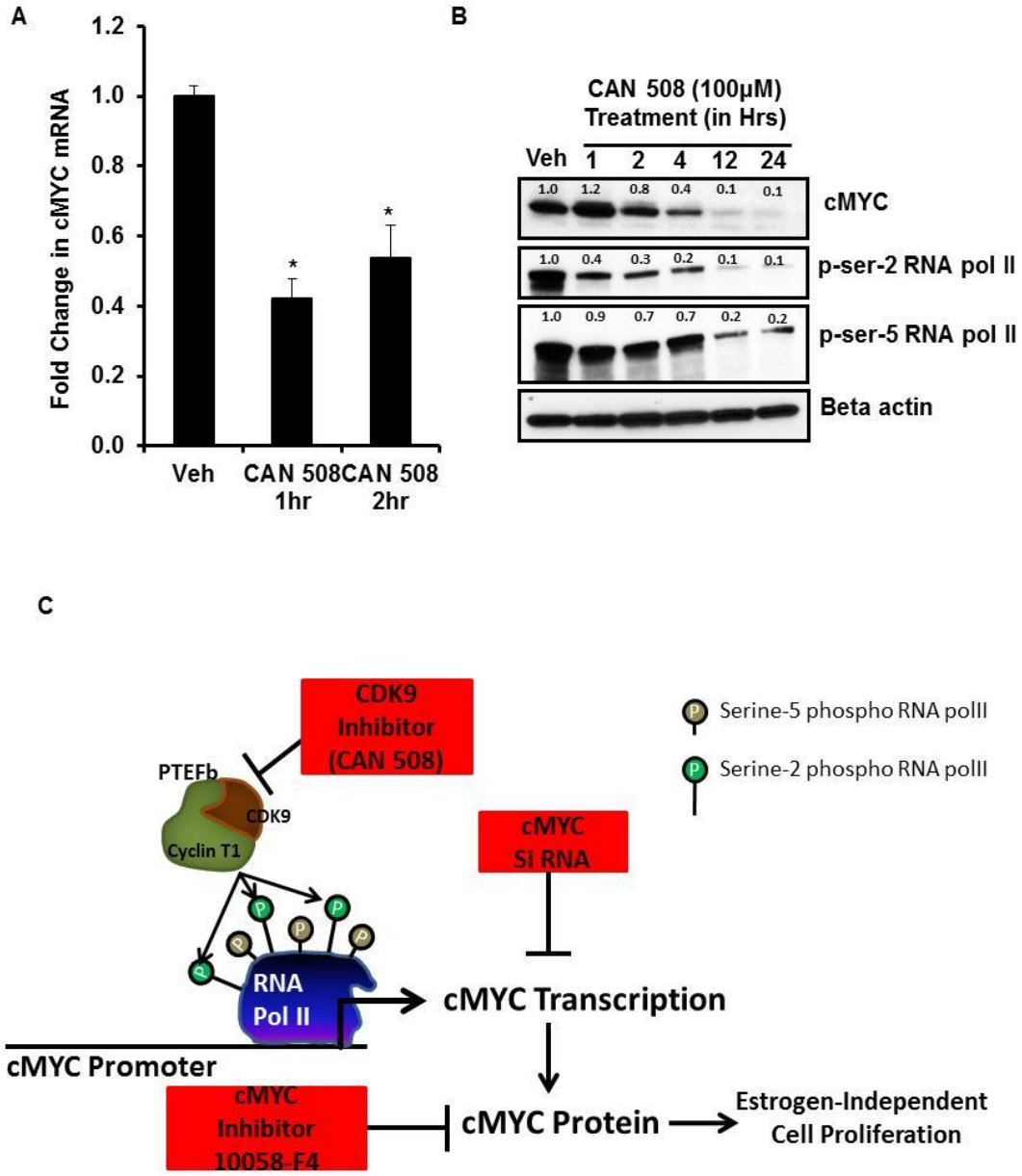


Figure 2g- 5. ***Total CDK9 levels and effect of its inhibition on estrogen-independent growth.*** ***(A)*** *Protein levels of phospho and total CDK9 and CTDP1 was assessed using western blotting in MCF7 and MCF7:5C cells. The numbers above each band correspond to the fold change in protein levels versus MCF7 cells adjusted for beta actin levels for each sample.* ***(B)*** *Total DNA was measured to assess the growth of MCF7 and MCF7:5C cells after 2, 4 and 6 days of treatment with indicated doses of the CDK9 inhibitor, CAN508.*

Figure 2g-6. ***Reduction of cMYC mRNA and protein by CDK9 inhibition and the proposed model of cMYC transcriptional regulation in MCF7:5C cells. (A)*** *Levels of cMYC mRNA was measured by quantitative RT-PCR in MCF7:5C cells after one and two hrs of CDK9 inhibition by 100μM of CAN508. (* p<.05 versus vehicle (Veh) treatment).* ***(B)*** *Protein levels of cMYC, phospho-serine-2 and serine-5 RNA polymerase II after inhibition of CDK9 by 100μM of CAN508 for indicated time points. The numbers above each band correspond to the fold change in protein levels versus vehicle (Veh) treatment adjusted for beta actin levels for each sample. (**C**) The cartoon depicts our findings on the CDK9 mediated cMYC transcriptional regulation and its role in estrogen-independent growth of the MCF7:5C cells.*



CDK9 inhibition blocks transcription of cMYC RNA and levels of cMYC protein in MCF7:5C cells

Inhibition of CDK9 in MCF7:5C cells by using CAN 508, resulted in approximately 60% decrease in cMYC mRNA within one hour of treatment (Figure 2g-6A). This was followed by time dependent decline in cMYC protein levels (Figure 2g-6B). Concomitant inhibition of serine-2 phosphorylated RNA polymerase II CTD was also observed within an hour of treatment (Figure 2g-6B) indicating its role in cMYC transcription. As evident, serine-5 phosphorylation of RNA polymerase II CTD was not much altered within 4 hours of CDK9 inhibition. Although later time points showed marked reduction in serine-5 phosphorylation, along with serine-2 phosphorylation which was most likely due to secondary effects of CDK9 inhibition. Inhibition of CDK9 also completely blocked the phosphorylation of retinoblastoma (Rb) protein within twelve hours of treatment in the MCF7:5C cells.

Discussion:

Accumulative evidence indicates that cMYC overexpression and subsequent genes up-regulated in breast cancers are associated with resistance to AIs [112] and antiestrogens [113,114]. This study establishes the role and mechanism of cMYC regulation in the estrogen-independent growth of ERŬ+, endocrine-resistant breast cancer cells. All the resistant cell models used in this study are MCF7-derived cell lines. Importantly, MCF7 cells retain the ERŬ protein after acquiring endocrine therapy resistance which mimics the clinical scenario as 80% of the endocrine-therapy resistant breast cancer patients are ERŬ positive [115]. Interestingly, despite the limited availability of cell lines, significant translational advances have occurred [103]. Based on our results, we decipher a novel mechanism of transcriptional over-expression of cMYC in resistant breast cancer cells (Figure 2g-6C) which involves CDK9 mediated hyper-phosphorylation of serine-2 RNA polymerase-II CTD at the promoter of cMYC gene. This, in turn, is responsible for the transcriptional elongation and overexpression of cMYC. Our analysis of the annotated breast cancer patientôs database (Figure 2g-3) suggested that over-expression of cMYC correlates with the failure of endocrine therapy (but not chemotherapy) and eventual relapse of the disease.

Ectopic overexpression of cMYC in MCF7 cells is reported to be sufficient to confer resistance to endocrine therapy [114,116]. We observed elevated cMYC levels in the ERŬ+, endocrine therapy ï resistant breast cancer cells (Figure 2g-1A and B) which proliferated in the absence of estrogen. A previous study has also reported high cMYC levels in long-term estrogen deprived cells [117]. Inhibition of cMYC or its depletion blocked the proliferation of the cells (Figure 2g-2A demonstrating the critical role of cMYC overexpression in estrogen-independent growth of these resistant breast cancer cells. The reduction in óSô phase cells (Figure 2g-2C and D) was achieved by de- phosphorylation of tumor suppressor retinoblastoma (Rb) protein which is known to arrest the cells in G1 phase of the cell-cycle [118].

Further, using a pulse chase assay, we ascertained that the high basal level of cMYC mRNA in the MCF7:5C cells was due to the high rate of transcription and not enhanced stability of the transcripts. Since therapeutic targeting of cMYC is not feasible, we studied the upstream factors responsible for cMYC transcriptional over-expression from its natural proximal promoter in the MCF7:5C cells. Transcription of cMYC gene is regulated at the elongation step by promoter-proximal pausing of RNA polymerase II in eukaryotes [119,120]. Importantly, cMYC is a well-defined estrogen-regulated gene [121] and the estrogen-induced growth of the hormone responsive breast cancer cells is contingent upon the expression of cMYC gene in these cells as majority of growth related genes which are estrogen regulated are cMYC target [122]. In MCF7 cells, studies have demonstrated [123] that the proximal promoter of the cMYC gene is pre-loaded with RNA polymerase II which is phosphorylated at serine 5 of its CTD, in the absence of estrogen. However, phosphorylation of serine-2 of CTD of RNA polymerase II is needed to overcome the elongation

block of the transcripts which is achieved after estrogen stimulation. Our findings are consistent. In MCF7 cells we observed high levels of serine-5 phosphorylation, and low serine-2 phosphorylation of RNA polymerase II CTD at the cMYC promoter under basal conditions (Figure 2g-4B and C). In contrast, under identical condition, the phosphorylation of serine-2 of CTD of RNA polymerase II is markedly elevated in MCF7:5C cells (Figure 2g-4A and B) which drives the higher transcriptional elongation of cMYC.

The kinase complex responsible for the phosphorylation of serine-2 of RNA polymerase II CTD and inducing transcriptional elongation is known as positive transcriptional elongation factor-b (PTEF-b) which is composed of CDK9 and cyclin T1 [124,125,126]. Our observation of higher levels of CDK9 in MCF7:5C cells (Figure 2g-5A) and in other resistant breast cancer cells strongly suggested that it is responsible for elevated serine-2 phosphorylation of RNA polymerase II CTD at the cMYC promoter of MCF7:5C cells. Indeed, using a pharmacological agent, CAN 508, which specifically inhibits CDK9 activity [111,127] the growth of MCF7:5C cells (Figure 2g-5B) as well as other endocrine therapy resistant MCF7 derived ERŬ+ breast cancer cells were selectively inhibited. This demonstrated that the estrogen-independent growth of the endocrine therapy resistant breast cancer cells was driven by CDK9. We further confirmed that inhibition of CDK9 led to the reduction of cMYC mRNA levels within one hour of treatment in MCF7:5C cells followed by the protein level (Figures 2g-6A and B). The concurrent decrease in global serine-2 (but not serine-5) phosphorylation of RNA polymerase II CTD (Figures 2g-6B) suggested that CDK9 was responsible for cMYC transcriptional over-expression in the resistant cells. In addition, we confirmed that CDK9 inhibition reduced the level of phospho-Rb protein in a similar manner as cMYC depletion in the MCF7:5C cells. This supports our hypothesis that the growth suppressive effect of CDK9 inhibition reduces cMYC levels in the endocrine-therapy resistant breast cancer cells. Furthermore, we found that CDK9 or cMYC inhibition was not deleterious to the immortalized human epithelial cells (MCF10A) indicating that CDK9 can be a potential novel therapeutic target.

Since we did not detect any difference in the global level of serine-2 phosphorylated RNA polymerase II CTD between MCF7:5C and its parental MCF7 cells (Figure 2g-4D); further studies are required to establish the chromatin modifications at the cMYC promoter which ensue in the process of acquiring resistance. These changes are crucial as it allows the RNA polymerase II CTD to be hyper-permissive for serine-2 phosphorylation, thus ensuring elongation of the cMYC transcripts in the MCF7:5C cells. Intriguingly, recent reports have indicated that in hematological malignancies bromo-domain containing protein 4 (BRD4), which has been known to recruit CDK9 and regulate serine-2 phosphorylation of RNA polymerase II [128,129], is involved in cMYC overexpression [130,131].

In this study, we have delineated the transcriptional mechanism of cMYC over-expression, endocrine-therapy resistant, ERŬ+ breast cancer cells, and propose that recruitment of hyper-phosphorylated serine-2 RNA polymerase II at the cMYC promoter which is mediated by CDK9, is responsible for the estrogen independent proliferation of these cells. We therefore suggest that there will be a potential clinical benefit by using CDK9 inhibitors in the treatment of endocrine therapy resistant breast cancers.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2h (Fan and Jordan) - Studies carried out by Dr. Ping Fan in the Jordan laboratory at Georgetown University

Inhibition of c-Src Blocks Estrogen-induced Apoptosis and Restores Estrogen Stimulated Growth in Long-term Estrogen Deprived Breast Cancer Cells

Introduction

The description of the evolution of tamoxifen resistance *in vivo* to trigger rapid tumor regression with physiological concentrations of E_2 [84,132,133] was rapidly followed by similar reports *in vitro* with populations or selected clones of MCF-7 cells triggering apoptosis with physiological E_2 after long-term E_2 deprivation [83,93]. Thus E_2 deprivation produces the same selective pressure on MCF-7 cells as selective ER modulators (SERMs) to create selective cellular populations vulnerable to E_2-induced apoptosis [85,132]. All of these laboratory data with MCF-7 cells provide the scientific rationale for the subsequent finding that high dose (30mg daily) or low dose (6mg daily) E_2 produces a 30% clinical benefit rate in patients failing aromatase inhibitor therapy [12].

Overall, the new biology of E_2 action to trigger apoptosis translates appropriately to the responsiveness of human breast cancer in the clinical setting. As a result, we have used our cellular models to elucidate the molecular mechanisms that modulate E_2-induced apoptosis through inducing endoplasmic reticulum stress and oxidative stress [11]. Recently, we have found that the oncogene c-Src is activated in two long-term E_2-deprived breast cancer cell models and is involved in the process of stress induced by E_2 [134]. Preclinical data in endocrine resistant models demonstrate that the crosstalk between ER and c-Src is an important resistance mechanism [135,136]. Blockade of c-Src signaling pathways is an attractive strategy to circumvent the resistance to antihormone therapy in breast cancer [137,138]. Here, we ask the question of what are the consequences of long-term physiological concentrations of E_2 in combination with the c-Src inhibitor on the shift of adaptive populations in E_2-deprived breast cancer cells?

To mimic the clinical administration of a c-Src inhibitor, we treated MCF-7:5C cells with different combinations in a long-term (8 weeks) study to further investigate the therapeutic potential of the combination of the c-Src inhibitor and E_2 on the growth of MCF-7:5C cells compared with either E_2 alone or PP2 alone. Contrary to our original hypothesis that the c-Src inhibitor would enhance the apoptotic effects of E_2, the c-Src inhibitor prevented E_2-induced apoptosis and allowed E_2 to stimulate growth. One major mechanistic change that reversed the E_2 response was that the c-Src inhibitor cooperated with E_2 to increase IGF-1Rϐ growth pathways, which was an important determinant for the signaling pathways of phosphatidylinositol-3 kinases/Akt and mitogen-activated protein kinase (MAPK). Furthermore, long-term combination treatment transcriptionally up-regulated EMT inducers, Twist1 and Snail, and disrupted E-cadherin mediated cell-cell adhesion. These data not only demonstrate the important role of c-Src in modulating E_2-induced apoptosis but also have implications for the poor performance with c-Src inhibitors in ER positive antihormone resistant patients in clinical trials.

Work Accomplished:

The c-Src inhibitor completely blocked E_2-induced apoptosis in MCF-7:5C cells.

We have found that non-receptor tyrosine kinase, c-Src, is activated in long-term E_2 deprived breast cancer cell lines MCF-7:5C and MCF-7:2A and functions as an important transducer to mediate E_2-induced stresses. Here, to mimic the clinical administration of the c-Src inhibitor, we long-term (8 weeks) treated MCF-7:5C cells with a specific c-Src inhibitor, PP2, alone or in combination with E_2 to investigate the therapeutic potential in long-term E_2 deprived MCF-7:5C cells. MCF-7:5C cells exhibited a cobblestone-like epithelial phenotype (Fig. 2h-1A). PP2 treated cells appeared smaller and more contracted, with decreased cell spreading (Fig. 2h-1A). And apoptotic impairment could be observed under microscope in E_2 alone treated cells (Fig. 2h-1A). In contrast, combination E_2 and PP2 abolished the growth inhibitory actions by E_2 alone or PP2 alone and the resulting cell line (MCF-7:PF) grew vigorously displaying a spindle-like morphology (Fig. 2h-9A). Further analysis of cell cycles showed that both the c-Src inhibitor and E_2 could clearly arrest cell cycles in G1 phase which was a marker of growth inhibition. However, combination PP2 and E_2 was unable to arrest cell cycles in G1 (Fig. 2h-1B). These data confirmed that E_2-initiated apoptosis requires c-Src tyrosine kinase pathway.

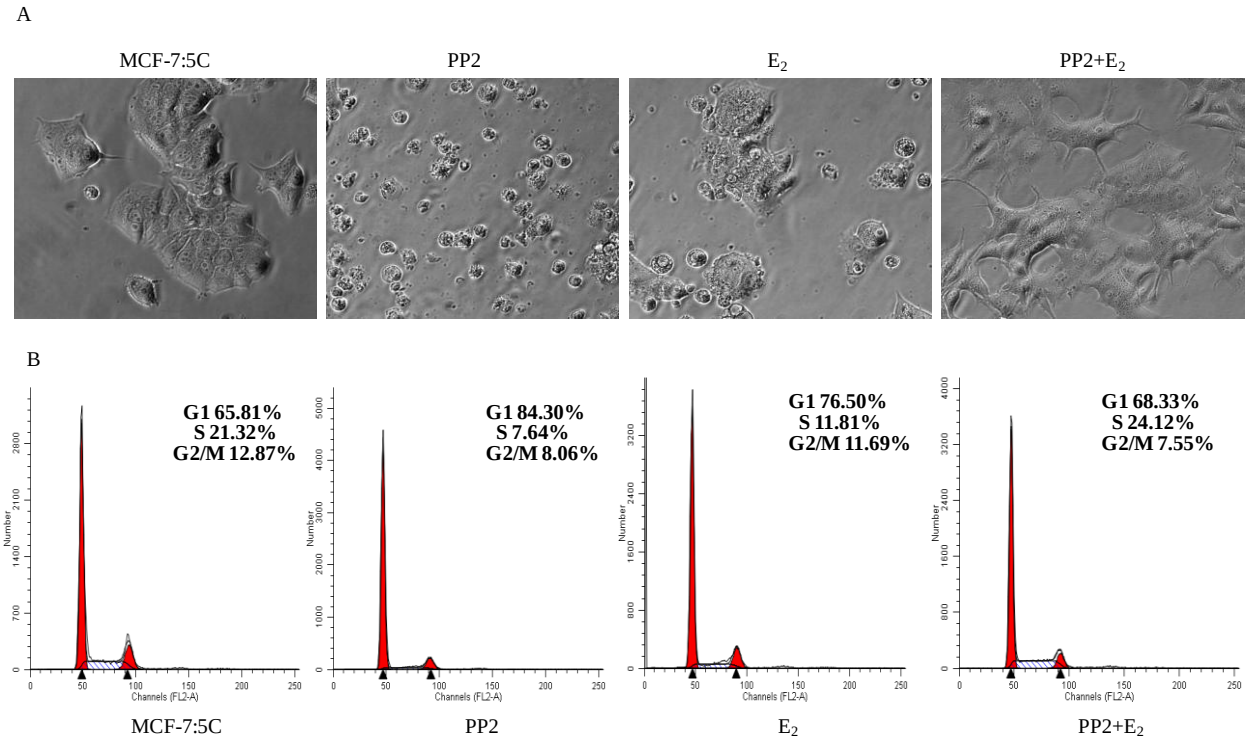


Figure 2h-11. ***A. The c-Src inhibitor completely blocked E_2-induced apoptosis after long-term treatment.*** *The morphological changes after 8 weeks treatment with different combination. MCF-7:5C cells were long-term treated with vehicle (0.1% EtOH), PP2 (5×10^{-6} mol/L), E_2 (10^{-9} mol/L), and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) in the T_{25} flasks, respectively. Cells were photographed under bright field illumination at (×20) magnification (Zeiss).* ***B. The c-Src inhibitor blocked G1 arrest of cell cycles induced by E_2 after long-term treatment.*** *Cell cycles changes after different treatment. MCF-7:5C and differently long-term treated cells were harvested and gradually fixed with 75% EtOH on ice. After staining with propidium iodide (PI), cells were analyzed through flow cytometry. All the data shown were representative of at least three separate experiments with similar results.*

Inhibition of c-Src converted E_2 from inducing apoptosis to stimulating growth in MCF-7:PF cells.

To further investigate the mechanisms underlying the c-Src inhibitor blocking the apoptosis induced by E_2, the response to E_2 by differently long-term treated cells was first evaluated. Physiological levels of E_2 still caused growth inhibition in MCF-7:5C cells and long-term PP2 treated cells (Fig. 2h-2A). Long-term treatment with E_2 initially caused massive apoptosis, but small fraction of surviving cells subsequently re-grew. Although apoptotic morphology could be observed under microscope at this time point (Fig. 2h-2A), E_2 did not decrease cell number compared with control cells (Fig. 2h-2A). It implied an imbalance between apoptosis and cell growth caused by E_2. In contrast, the resulting cell line (MCF-7:PF) by a combination of PP2 and E_2 grew vigorously in culture with physiological levels of E_2 (Fig. 2h-2A). This stimulation by E_2 could be completely blocked by pure antiestrogen ICI182,780 which demonstrated that proliferation was mediated by ERŬ (Fig. 2h-2B). Very similarly, the c-Src inhibitor also converted E_2 response from apoptosis to proliferation in another long-term E_2-deprived MCF-7:2A cells, a late-apoptosis cell line (Fig. 2h-2C). It is known that blocking c-Src tyrosine kinase increases ERŬ expression after 24h treatment (6). This elevated level of ERŬ was stably expressed after long-term treatment (Fig. 2h-2D). As expected, E_2 alone and MCF-7:PF cells expressed lower levels of ERŬ due to E_2 down-regulation (Fig. 2h-2D). The ERŬ mRNA levels were consistent with protein expression (data not shown). There was no change in ERɓ expression after long-term treatment (Fig. 2h-2E). This suggested that MCF-7:PF cells have functional ERŬ in response to E_2.

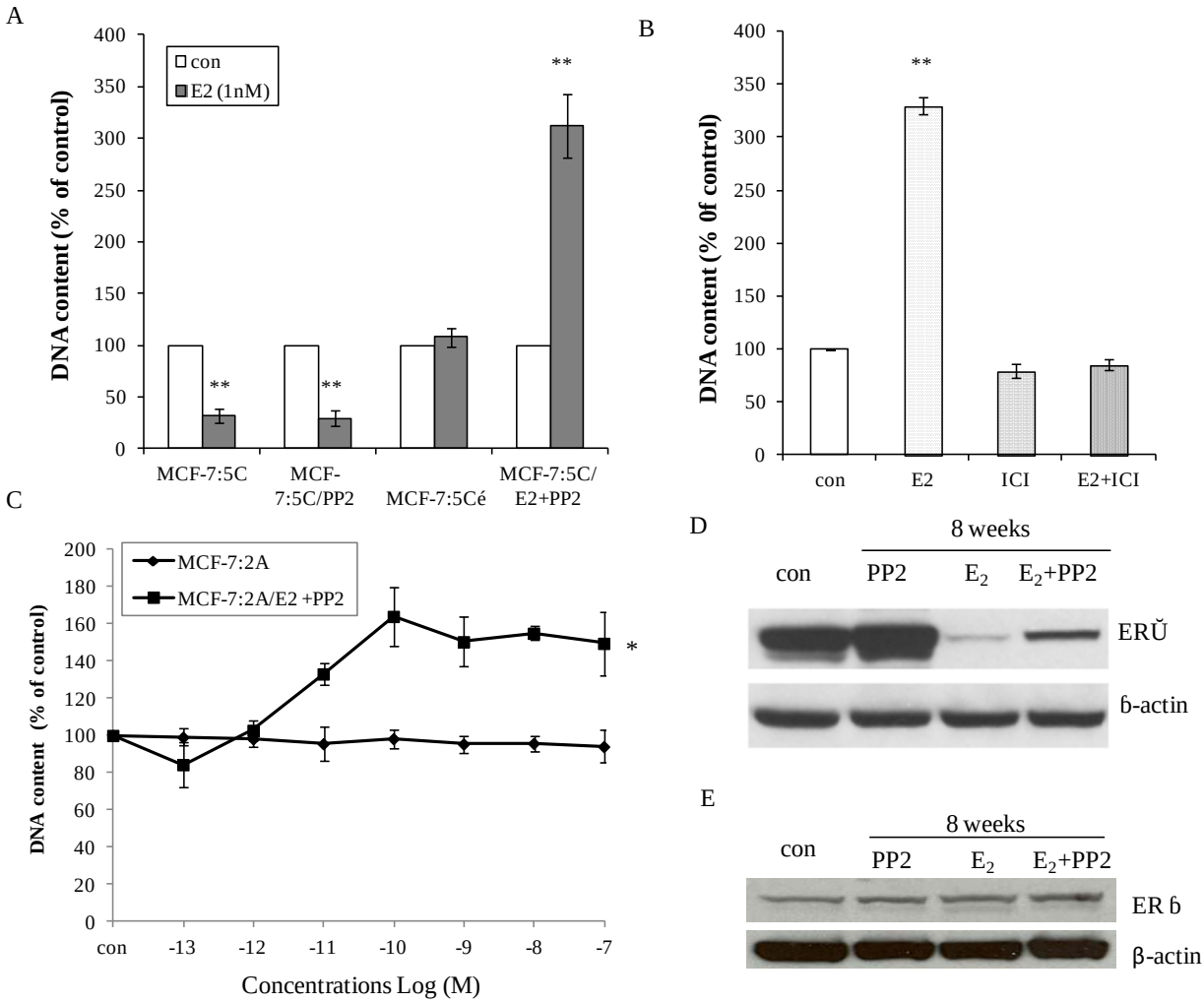


Figure 2h-2. ***The c-Src inhibitor converted E_2 responses from inducing apoptosis to stimulating growth.*** *Differently treated cells were seeded in 24-well plates in triplicate, respectively. After one day, cells were treated with vehicle (0.1% EtOH) and E_2 (10^{-9} mol/L) respectively. The cells were harvested*

*after 7 days treatment and total DNA was determined using a DNA fluorescence quantitation kit. P<0.001, ** compared with control.* ***2B. E_2 proliferative effect was blocked by ICI 182,780.*** *MCF-7:PF cells were seeded in 24-well plates in triplicate. After one day, the cells were treated with vehicle (0.1% EtOH), E_2 (10^{-9} mol/L), ICI 182,780 (10^{-6} mol/L), and E_2 (10^{-9} mol/L) plus ICI 182,780 (10^{-6} mol/L) respectively. The cells were harvested as above and total DNA was determined using a DNA fluorescence quantitation kit. P<0.001, ** compared with control.* ***2C. Inhibition of c-Src converted E_2 from inducing apoptosis to stimulating growth in MCF-7:2A cells.*** *MCF-7:2A cells were long-term (8 weeks) treated with PP2 (5×10^{-6} mol/L) and E_2 (10^{-9} mol/L) as in MCF-7:5C cells. Long-term combination treated cells and MCF-7:2A cells were plated in 24-well plates in triplicate. After one day, cells were treated with different doses of E_2 as indicated. The cells were harvested after 7 days treatment and total DNA was determined using a DNA fluorescence quantitation kit. P<0.05, * compared with MCF-7:2A cells.* ***2D. Changes of ER alpha after long-term treatment*** *Cell lysates of differently long-term treated cells were harvested. ER alpha was examined by immunoblotting with primary antibody. Immunoblotting for ß-actin was detected for loading control.* ***2E. Changes of ER beta after long-term treatment*** *Cell lysates of differently long-term treated cells were harvested. ER beta was examined by immunoblotting with primary antibody. Immunoblotting for ß-actin was detected for loading control.*

The c-Src inhibitor was additive with E_2 to elevate endogenous ER target genes in MCF-7:PF cells.

Although ER expression levels were quite different (Fig. 2h-2D), the estrogen response element (ERE) activity was similar among differently treated cells (Fig. 2h-3A). It was interesting to find that c-Src inhibitor dramatically elevated E_2 inducible gene pS2 mRNA (Fig. 2h-3B) although the mechanisms were unclear. Moreover, the c-Src inhibitor was additive with E_2 to increase pS2 mRNA in MCF-7:PF cells (Fig. 2h-3B). Another ER target gene, progesterone receptor (PR), was undetectable in MCF-7:5C cells compared with wild type MCF-7 cells (Fig. 2h-3D). However, adding back E_2 in the medium could recover PR expression in E_2 alone treated cells and MCF-7:PF cells (Fig. 2h-3C and 2h-3E). The c-Src inhibitor PP2 alone did not regulate PR expression (Fig. 2h-3E). Nevertheless, it synergized with E_2 to up-regulate PR mRNA although without consistent highest protein expression (Fig. 2h-3C and 2h-3E), which implied existence of a post-translational modification of PR in MCF-7:PF cells.

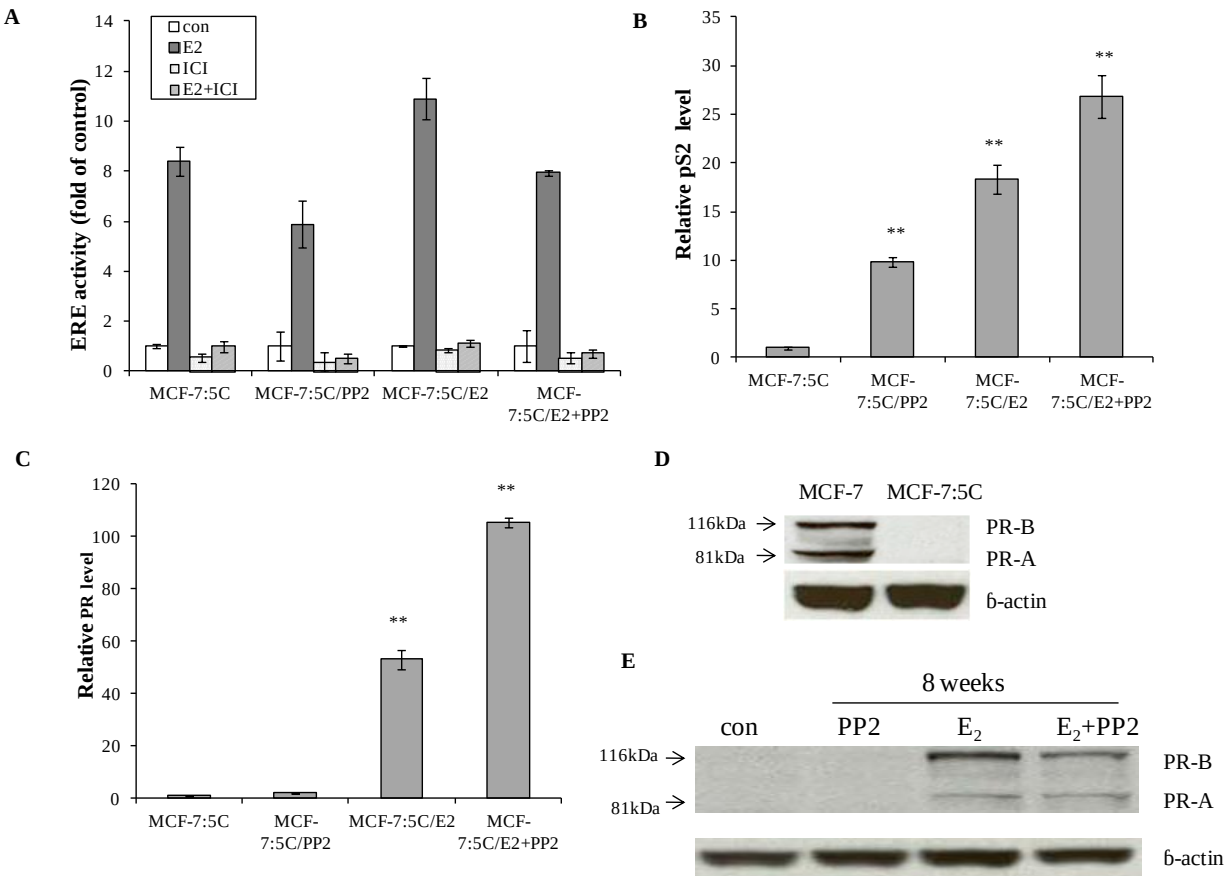


Figure 2h-3. ***A. ERE activity in different cells*** *MCF-7:5C and differently long-term treated cells were seeded in 24-well plates in triplicate and transfected with ERE firefly luciferase plasmid plus renilla luciferase plasmid as in Materials and Methods, respectively.* ***3B. The c-Src inhibitor collaborated with E_2 to up-regulate pS2 mRNA****. MCF-7:5C and differently long-term treated cells were grown in 6-well plates in triplicate, respectively. The RNA was harvested in TRIzol for real-time PCR analysis. P<0.001, ** compared with control.* ***3C. The c-Src inhibitor collaborated with E_2 to up-regulate PR mRNA****. The RNA of different cells was harvested in TRIzol for real-time PCR analysis. P<0.001, ** compared with control.* ***3D. PR expression levels were different between wild-type MCF-7 cells and MCF-7:5C cells.*** *Cell lysates were harvested from MCF-7 cells and MCF-7:5C cells. PR was examined by immunoblotting with primary antibody against it. Immunoblotting for ß-actin was detected for loading control.* ***3E. PR changes after long-term treatment.*** *Cell lysates were harvested from differently treated cells. PR was examined by immunoblotting with primary antibody. Immunoblotting for ß-actin was detected for loading control. All the data shown were representative of at least three separate experiments with similar results.*

The c-Src inhibitor synergized with E_2 to elevate transcriptional activity of PR in MCF-7:PF cells.

As shown in Fig. 2h-3E, E_2 alone treated cells and MCF-7:PF cells had similar levels of PR protein. To further investigate the function of PR, progestin (R5020) was used to examine the activity of progesterone response element (PRE). Interestingly, the progestin uniquely activated PRE activity in MCF-7:PF cells which could be blocked by anti-progestin RU486 (Fig. 2h-4A). They also had different cell growth responses to progestin with stimulating growth in MCF-7:PF cells but not in E_2 alone treated cells (Fig. 2h-4B). It has been described that activation of MAPK results in phosphorylation of PR on Ser294, affecting transcriptional function of PR [139]. In agreement with this report, we observed that phosphorylated MAPK and PR (Ser294) levels in MCF-7:PF cells were higher than that in E_2 alone treated cells (Fig. 2h-4C). Inhibition of MAPK with U0126 effectively blocked the phosphorylation of PR

on Ser294 in MCF-7:PF (Fig. 2h-4D). Although anti-progesterone RU486 blocked PRE activity induced by progestin in MCF-7:PF cells (Fig. 2h-4A), it could not inhibit cell growth activated by progestin whereas itself significantly promoted MCF-7:PF cell growth (data not shown). This estrogenic effects of RU486 [140] on MCF-7:PF cells were very similar as on wild-type MCF-7 cells (data not shown). Then, specific siRNA was used to knockdown of PR that effectively inhibited MCF-7:PF cell growth (Fig. 2h-4E). All of these results demonstrated that extracellular signal MAPK may modify PR and affect the transcriptional activity of PR.

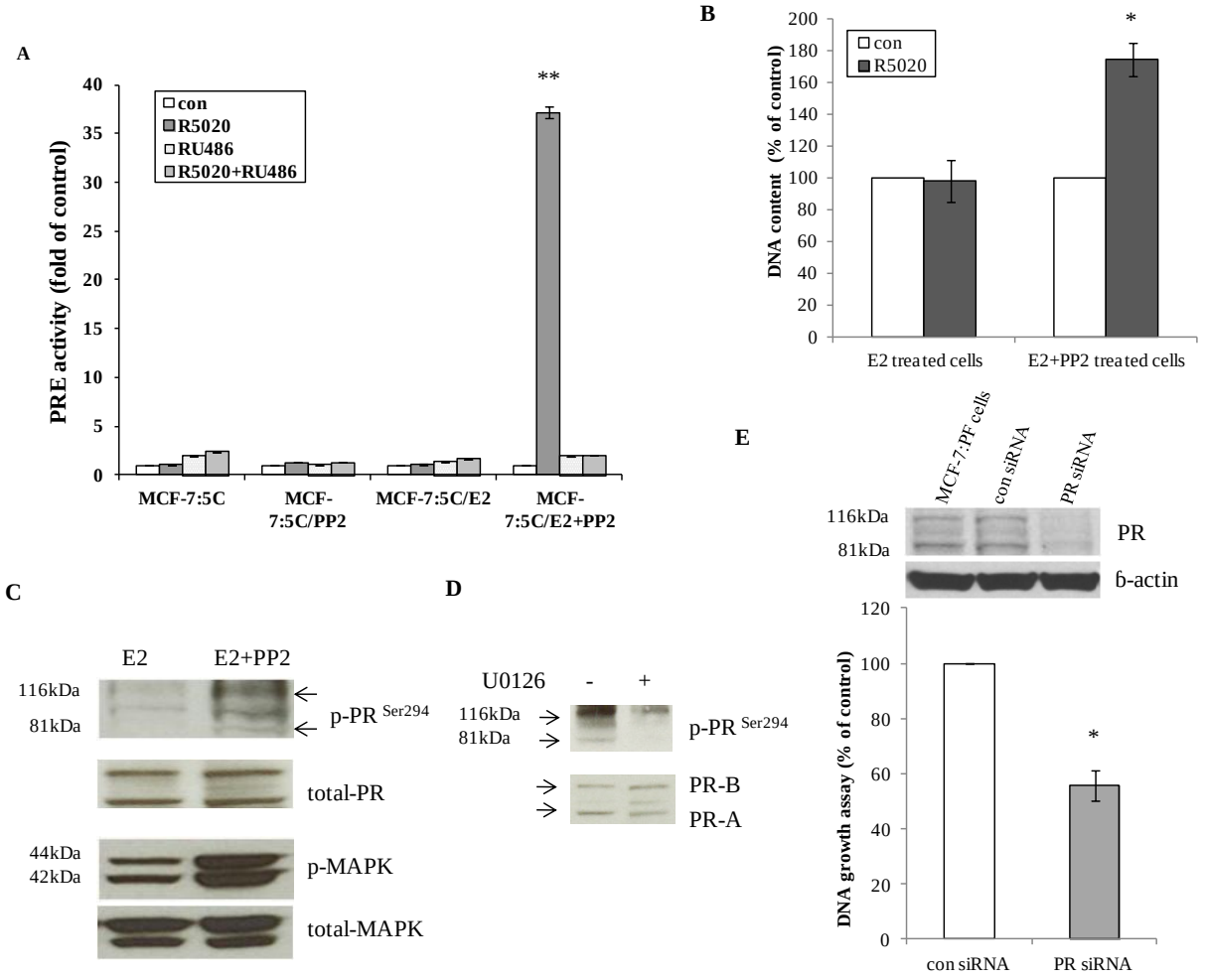


Figure 2h-4. ***4A. The c-Src inhibitor synergized with E_2 to activate PR transcriptional activity.*** *Progestin significantly activated PRE activity in MCF-7:PF cells. Differently long-term treated cells were transfected with PRE firefly luciferase plasmid plus renilla luciferase plasmid as in Materials and Methods. The cells were treated with vehicle (0.1% EtOH), progestin (10^{-8}mol/L), RU486 (10^{-6}mol/L), and RU486 (10^{-6}mol/L) plus progestin (10^{-8}mol/L) in triplicate for 24 hours. P<0.001, ** compared with control.* ***4B. Different responses to progestin between E_2 alone treated cells and MCF-7:PF cells.*** *E_2 alone treated cells and MCF-7:PF cells were plated in 24-well plates in triplicate. After one day, cells were treated with vehicle (0.1% EtOH) and progestin (10^{-8} mol/L) respectively. Cells were harvested after 7 days treatment and total DNA was determined as above. P<0.05, * compared with E_2 alone treated cells.* ***4C. MCF-7:PF cells had higher phosphorylated PR than E_2 alone treated cells.*** *Cell lysates of MCF-7:PF cells and E_2 alone treated cells were harvested. Phosphorylated PR and MAPK were examined by immunoblotting with primary antibodies. Total PR and MAPK were used as loading controls.* ***4D. The PR was phosphorylated by MAPK in MCF-7:PF cells.*** *MCF-7:PF cells were treated with vehicle (0.1% DMSO) and MAPK inhibitor U0126 (10^{-5}mol/L) for 48 hours. Phosphorylated PR*

was examined by immunoblotting with primary antibody. Total PR was used as loading control. ***4E. Knockdown of PR by siRNA blocked cell growth.*** *MCF-7:PF cells were transfected with control siRNA and specific PR target siRNA as manufacture's instruction. Cell lysates were harvested after 72 hours to detect PR levels by immunoblotting with primary antibody. Immunoblotting for ß-actin was detected for loading control. As a parallel experiment, cells were harvested after 5 days transfection for DNA growth assay as above. P<0.05, * compared with control siRNA.*

The c-Src inhibitor collaborated with E_2 to enhance insulin like growth factor-1 receptor beta (IGF-1Rβ) which drove growth pathways in MCF-7:PF cells.

c-Src mediates the interaction between growth factor receptors and ER in breast cancer [135]). These two growth regulatory pathways are tightly linked in ER positive breast cancer [135]. Our observations showed that both the c-Src inhibitor and E_2 could increase IGF-1Rϐ expression after long-term treatment. Moreover, PP2 and E_2 were additive to elevate IGF-1Rϐ in MCF-7:PF cells (Fig. 5A and 5B). To investigate the potential role of IGF-1Rϐ in MCF-7:PF cells, a specific inhibitor of IGF-1Rϐ, AG1024, was utilized to block receptor tyrosine kinase activity, which effectively abolished MAPK and Akt pathways (Fig. 5C) and inhibited cell growth (Fig. 5D) in MCF-7:PF cells. Importantly, the AG1024 completely abolished E_2 stimulation in a dose dependent manner in MCF-7:PF cells (Fig. 5D). These data indicated that IGF-1Rϐ is linked tightly with the ER function in MCF-7:PF cells.

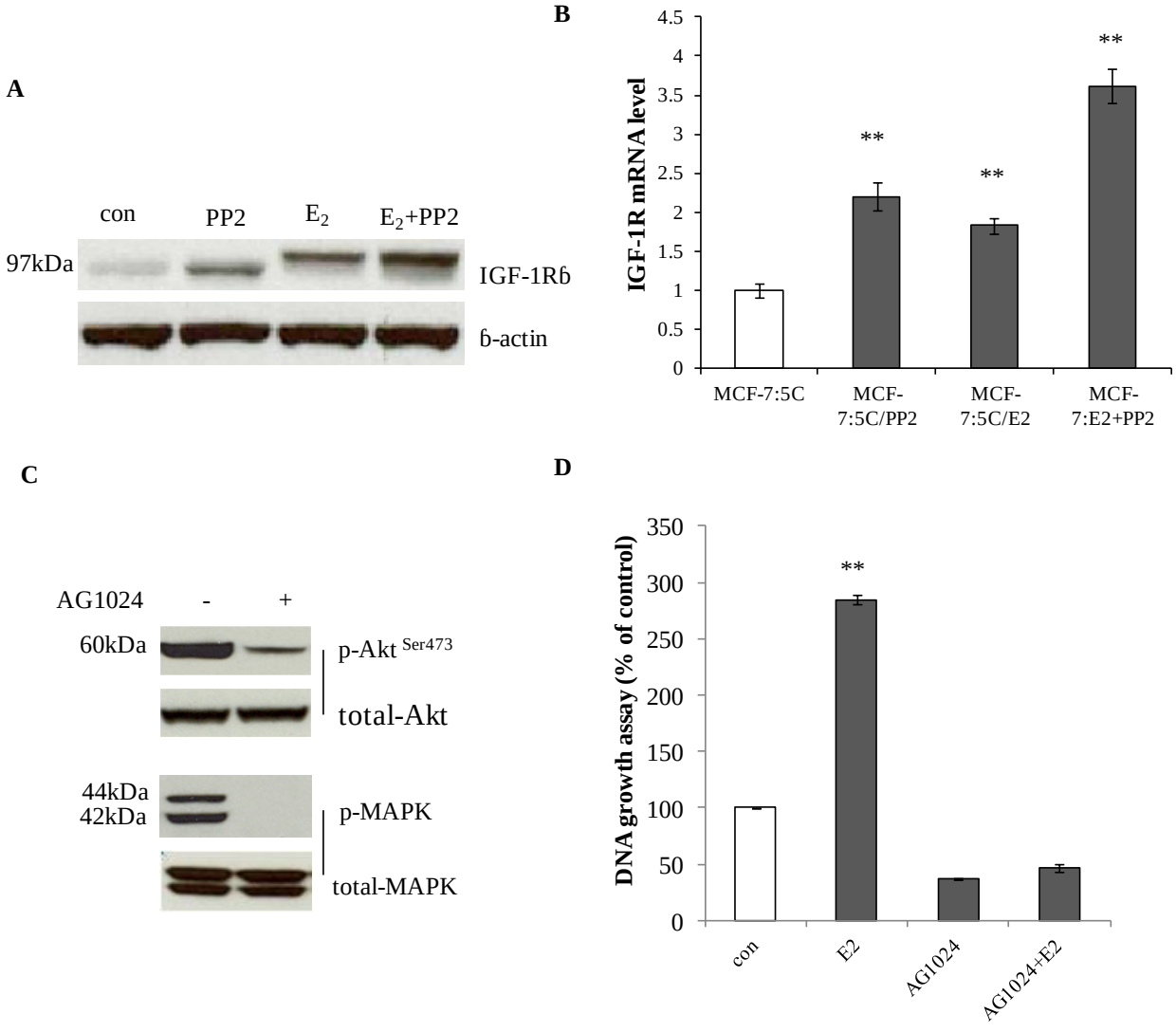


Figure 2h-5. ***5A. The c-Src inhibitor collaborated with E_2 to elevate IGF-1Rβ.*** *Cell lysates of differently long-term treated cells were harvested. IGF-1Rβ was examined by immunoblotting with primary antibody. ß-actin was detected for loading control.* ***5B. IGF-1Rβ mRNA changes were consistent with protein levels.*** *The RNA of differently long-term treated cells was harvested as above. $P<0.001$, ** compared with control.* ***5C. Activation of Akt and MAPK pathways by IGF-1Rβ in MCF-7:PF cells.*** *MCF-7:PF cells were treated with vehicle (0.1% DMSO) and AG1024 (10^{-5}mol/L) for 48 hours. Cell lysates were harvested. Phosphorylated Akt and MAPK were determined by immunoblotting with primary antibodies. Total Akt and MAPK were examined for loading controls.* ***5D. IGF-1R inhibitor completely blocked E_2 stimulation in MCF-7:PF cells.*** *MCF-7:PF cells were treated with vehicle (0.1% EtOH), E_2 (10^{-9}mol/L), AG1024 (10^{-5}mol/L), and E_2 (10^{-9}mol/L) plus AG1024 (10^{-5}mol/L) for 7 days respectively. The cells were harvested and DNA content was determined as above. $P<0.001$, ** compared with control. All the data shown were representative of at least three separate experiments with similar results.*

Inhibition of c-Src disrupted E-cadherin-mediated cell-cell adhesion and resulted in epithelial-mesenchymal transition (EMT) in MCF-7:PF cells.

Activation of c-Src kinase has been documented in E-cadherin mediated cell-cell adhesion, which is thought to play an important role in cancer invasion and metastasis [141]. Therefore, we sought to examine changes of E-cadherin associated signals after long-term treatment with the c-Src inhibitor in MCF-7:5C cells. Contrary to the effects on wild-type MCF-7 cells [141], PP2 reduced E-cadherin but increased N-cadherin and fibrinogen in MCF-7:5C cells (Fig. 2h-6A), which are characterized features of EMT [141]. EMT is regulated by various signal transduction pathways including extracellular signal-regulated kinase (ERK) and Wnt. The c-Src inhibitor effectively blocked c-Src phosphorylation in both PP2 alone treated cells and MCF-7:PF cells (Fig. 2h-6B) whereas long-term E_2 treated MCF-7:5C cells still maintained the higher level of phosphorylated c-Src (Fig. 2h-6B). Although the c-Src inhibitor blocks phosphorylated MAPK in the early stage[11], PP2 clearly increased MAPK but continuously blocked Akt after long-term treatment (Fig. 2h-6B). Additionally, inducers of the EMT include several transcription factors such as Snail, Twist, as well as the secreted transforming growth factor beta (TGFb). In our cell model, the c-Src inhibitor collaborated with E_2 to increase Snail and Twist1 in MCF-7:PF cells (Fig. 2h-6C). Nevertheless, MCF-7:PF cells had higher migratory capacities than MCF-7:5C cells, evaluated using a Boyden chamber migration assay (Fig. 2h-6D and 2h-6E). All of these results suggested that multiple EMT regulators are significantly modified after long-term combination treatment.

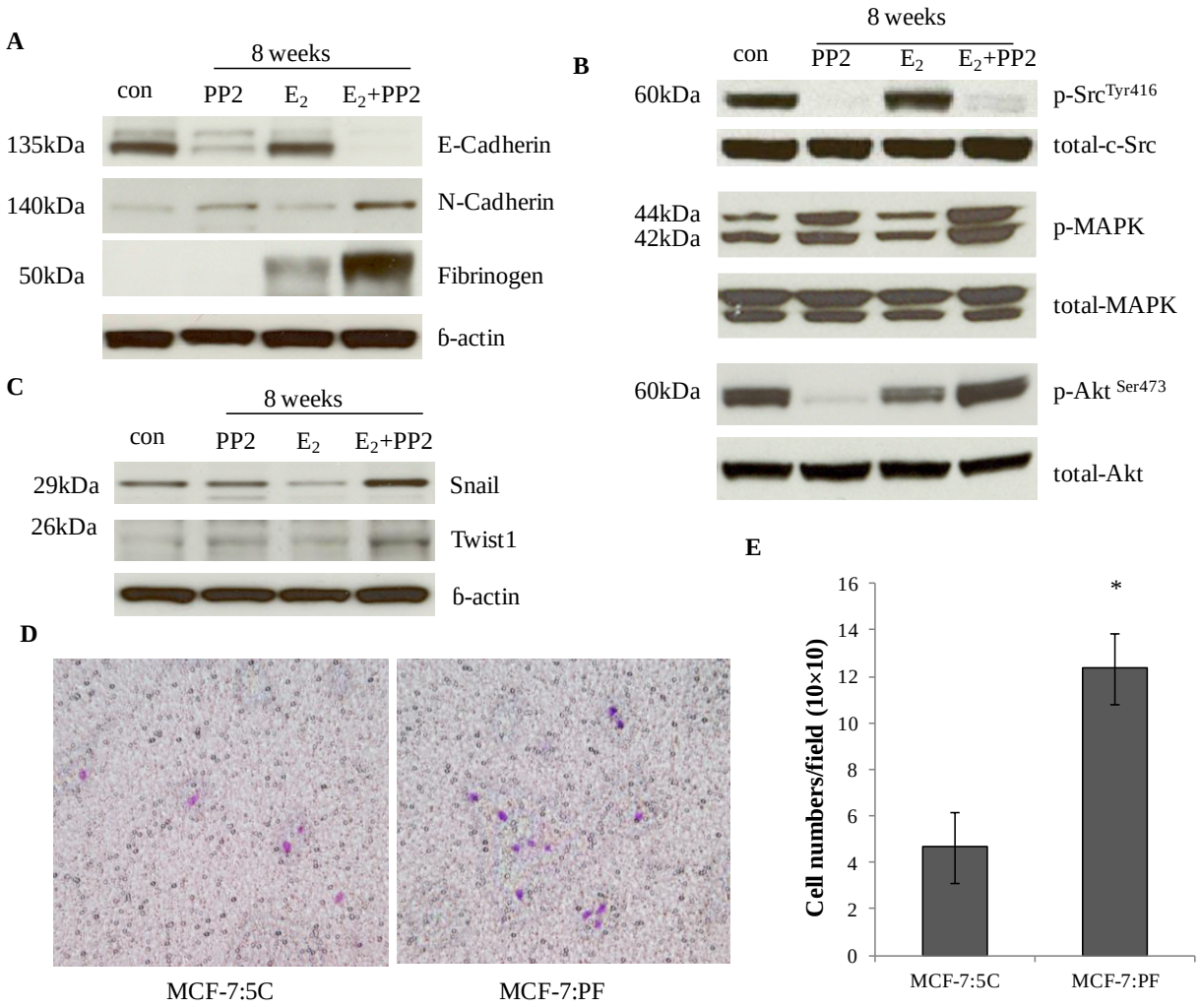


Figure 2h-6. ***6A. The c-Src inhibitor promoted EMT after combination with E_2 in MCF-7:5C cells.*** *Cell lysates of differently treated cells were harvested. E-cadherin, N-cadherin, and fibrinogen were examined by immunoblotting with primary antibodies. ß-actin was detected for loading control.* ***6B. Signaling pathways changes after long-term combination treatment.*** *Cell lysates of differently treated cells were harvested. Phosphorylated c-Src, MAPK, Akt were examined by immunoblotting with primary antibodies. Total c-Src, MAPK, and Akt were detected for loading controls.* ***6C. Transcription factors Twist1 and Snail were up-regulated.*** *Cell lysates of differently treated cells were harvested. Twist1 and Snail were examined by immunoblotting with primary antibodies. ß-actin was detected for loading control.* ***6D.*** *Migratory capacities of MCF-7:PF cells, compared with MCF-7:5C cells. MCF-7:5C cells and MCF-7:PF cells were loaded in Boyden chambers as in Materials and Methods. Images were taken under bright field illumination at (×10) magnification (Olympus).* ***6E. MCF-7:PF cells had higher migratory capacities than MCF-7:5C cells.*** *Migrated cells were stained as in Materials and Methods. Cell numbers were counted in at least four microscopic fields at (10×10) magnification. $P<0.05$, * compared with MCF-7:5C cells. All the data shown were representative of at least three separate experiments with similar results.*

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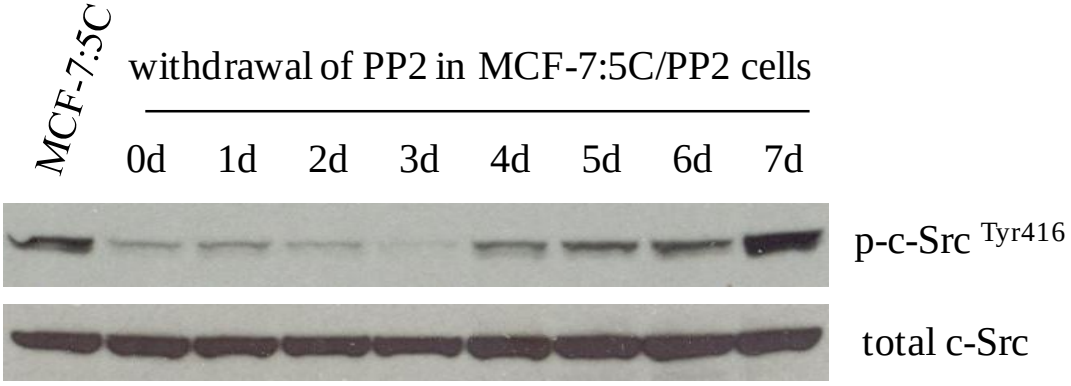

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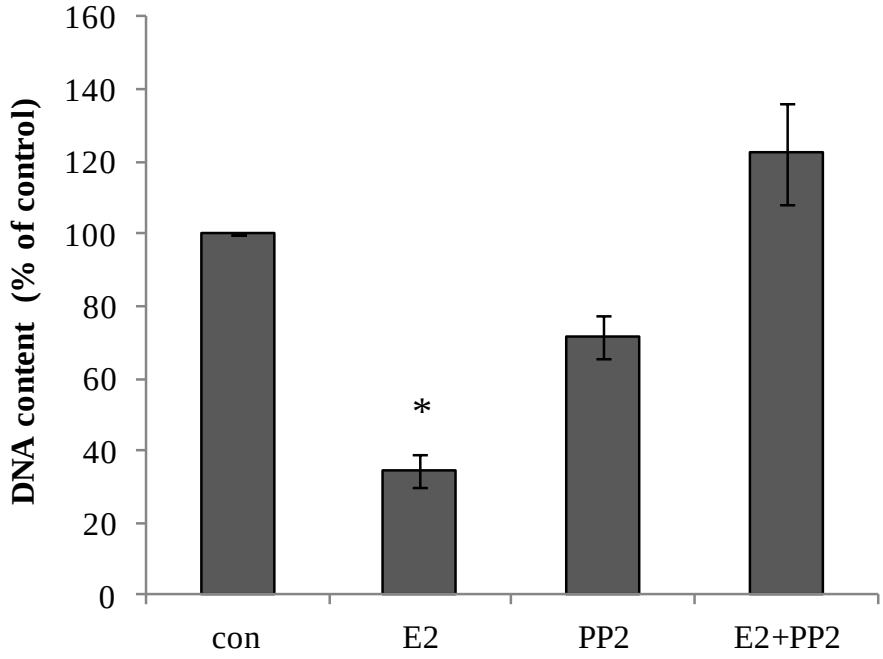

Figure 2h-7. ***Phosphorylation of c-Src affected the cell response to E_2 in long-term PP2 treated cells.*** *A, Changes of c-Src phosphorylation after withdrawal of PP2 in long-term PP2 treated cells. Long-term PP2 treated cells were washed with PP2 free medium for different time points as indicated, compared with MCF-7:5C cells. Cell lysates were harvested. c-Src phosphorylation was examined by immunoblotting with primary antibody. Immunoblotting for total c-Src was detected for loading control. B, Inhibition of c-Src blocked E_2-induced apoptosis in long-term PP2 treated cells. MCF-7:5C/PP2 cells were plated in 24-well plates in triplicate. After one day, cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9} mol/L), PP2 (5×10^{-6} mol/L), and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L), respectively. Cells were harvested after 7 days treatment. DNA content was examined as above. $P<0.05$, * compared with control. All the data shown were representative of at least three separate experiments with similar results.*

Discussion

Resistance to aromatase inhibitors is an important clinical problem. We have demonstrated in the laboratory that two long-term E_2-deprived MCF-7 breast cancer cell lines respond to physiological concentrations of E_2 by triggering apoptosis [10]. This laboratory observation has clinical relevance for the prevention or treatment of E_2-deprived diseases; however, only 30% of patients receive clinical benefit [12]. This prompted us to investigate strategies to increase the therapeutic responsiveness in aromatase inhibitor resistant breast cancer. The oncogene c-Src is activated in E_2-deprived breast cancer cell lines [134]. Many observations highlight c-Src as an important therapeutic target to overcome endocrine resistance in breast cancer. We chose an eight-week treatment period in the laboratory to mimic the clinical criteria to evaluate the efficacy of endocrine therapy. Unexpectedly, the c-Src inhibitor converted the E_2 response from inducing apoptosis to stimulating growth in two long-term E_2 deprived breast cancer cell lines (Fig. 2h-2A). Most importantly, we found that the c-Src inhibitor enhanced the action of E_2 to up-regulate IGF-1Rϐ which, in turn, promoted the MCF-7:PF cells to grow (Fig. 2h-5A and 2h-5D). Furthermore, the combination treatment enhanced embryonic transcription factors and repressed E-cadherin expression (Fig. 2h-6A and 2h-6C), a characteristic feature of EMT in the generation of invasive tumor cells.

We sought to find the mechanisms by which the c-Src inhibitor blocked the apoptosis induced by E_2 after long-term combination treatment. Our recent observations show that the c-Src inhibitor effectively blocks oxidative stress and extrinsic apoptotic pathways induced by E_2 within 72 hours [11] since these pathways are mediated by the c-Src tyrosine kinase. Paradoxically, physiological levels of E_2 still were able to induce apoptosis in long-term PP2 treated cells as in the original MCF-7:5C cells when the drug was washed out (Fig. 2h-2A). We further found that c-Src phosphorylation was gradually recovered after withdrawal of PP2 from the medium (Fig. 2h-7A). Including PP2 in the medium could completely abolish E_2-induced apoptosis in MCF-7:5C/PP2 treated cells (Fig. 2h-7B). These data confirmed that c-Src phosphorylation is required for E_2-induced apoptosis (Fan P, et al. Eur J Cancer 2012, Fan P, et al. Cancer Res 2013) which is a critical initial protective response for cell survival. However, unlike 4-hydroxytamoxifen, the c-Src inhibitor cannot completely block apoptosis-induced by E_2 during 72 hours of exposure[11]. This implies that other adaptation responses can potentially occur in cell populations after long-term combination treatment. It is well known that growth factor receptors crosstalk with c-Src and the ER pathways in breast cancer cells[135]. E_2 started to increase IGF-1Rϐ levels after 4 hours treatment which could be blocked by 4-hydroxytamoxifen in MCF-7:5C cells, demonstrating an ER-dependent mechanism. Blockade of c-Src further enhanced levels of IGF-1Rϐ (Fig. 2h-5A). Importantly, inhibition of IGF-1R effectively blocked cell growth and completely abolished proliferation induced by E_2 (Fig. 5D). These results highlighted the importance of phosphorylated IGF-1R in the mediation of E_2-stimulated growth. To determine whether autophosphorylation of IGF-1R by ligand IGF-1 plays an important role in the activation of receptor, we observed that IGF-1 levels were almost undetectable and neither E_2 nor PP2 up-regulated the IGF-1 expression in our cell models through real-time PCR (data not shown). It is also necessary to note that IGF-1R is required for the activation of Akt (Fig. 5C) which is also an important pathway to suppress apoptosis.

In addition to up-regulation of IGF-1Rϐ, expression of PR was strictly regulated by E_2 (Fig. 2h-3C and 2h-3D). Interestingly, the function of PR was quite different between cells treated with E_2 alone and MCF-7:PF cells (Fig. 2h-4A), even though they had similar PR protein levels (Fig. 2h-3C). Our preliminary data suggested that this may be related to PR phosphorylation on Ser294 by extracellular signaling ERK/MAPK (Fig. 2h-4B and 2h-4C). Consistently, other groups have reported that PR transcriptional activity is regulated by a balance between the degree of PR phosphorylation and sumoylation which can dramatically alter genetic expression. In this study, the c-Src inhibitor activated MAPK signal (Fig. 2h-6B) which increased transcriptional activity of PR induced by E_2 and finally activated the response to progestin in MCF-7:PF cells (Fig. 2h-4A and 2h-4B).

We addressed the question of why the c-Src inhibitor increased the extracellular signaling ERK/MAPK in MCF-7:5C cells. Our recent publication [134] shows that the c-Src inhibitor exerts different effects on two basic growth pathways, Akt and MAPK, in different breast cancer cell lines. The c-Src inhibitor continuously inhibited Akt pathway (Fig. 2h-6B) but transiently blocked MAPK in MCF-7:5C cells (Fig. 2h-6B). The association of c-Src with the membrane cytoskeleton has been well documented. Evidence implicates [142] a role for c-Src in the regulation of the formation of focal adhesions and the extracellular matrix to affect subsequent signaling pathways. In our study, the c-Src inhibitor disrupted E-cadherin-mediated cell-cell adhesion and made the cell gain mesenchymal cell markers such as N-cadherin and fibrinogen (Fig. 2h-6A), a characterized feature of EMT. Deposition of fibrinogen into the extracellular matrix serves as a scaffold to support binding of growth factors to activate extracelluar signaling ERK/MAPK (Fig. 2h-6A and 2h-6B). EMT, a complex reprogramming process of epithelial cells, plays an important role in tumor invasion and metastasis. Current studies show that EMT is controlled by a group of embryonic transcriptional factors, such as Zeb-1/2, Twist1, and Snail, and each of these factors is capable of directly repressing E-cadherin expression (Fig. 2h-6C) [143]). These results suggested that the antiestrogen resistant breast cancer cell is clearly reprogrammed with regard to the variations of those signaling pathways. Therefore, further studies are required to uncover the precise interaction among these EMT inducers that may hold promise for developing novel strategies to inhibit EMT and cancer metastasis.

In summary, this study suggested that physiological levels of E_2 (probably the patientôs own E_2) is able to induce apoptosis in long-term E_2-deprived breast cancer. However, administration with a c-Src inhibitor will cause the tumor to grow after aromatase inhibitor resistance, with a variety of signaling networks regulated by the c-Src inhibitor to promote an aggressive phenotype. These data raise a concern regarding the ubiquitous use of c-Src inhibitors in advanced aromatase inhibitor-resistant breast cancer especially when combined with E_2.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2i (Fan and Jordan) - Studies carried out by Dr. Ping Fan in the Jordan laboratory at Georgetown University

Introduction

Mitochondria are important intracellular organelles involved in apoptosis via an intrinsic pathway [144]). Although the molecular mechanisms of E_2-induced apoptosis are not fully understood, evidence indicates that mitochondrial related caspase pathways are involved[83,93]. Similarly, a variety of events in apoptosis focus on mitochondria, including the loss of mitochondrial transmembrane potential, release of cytochrome *c*, and participation of pro- and antiapoptotic Bcl-2 family proteins [144,145]. However, accumulating evidence suggests that the endoplasmic reticulum where members of the Bcl-2 family of proteins localize, is also a major point of integration of pro-apoptotic signaling or damage sensing. The endoplasmic reticulum senses local stress such as unfolded protein through a set of pathways known as the unfolded protein response (UPR), which activates three transmembrane sensors PRK-like endoplasmic reticulum kinase (PERK), inositol-requiring 1 alpha (IRE-1Ŭ), and activating transcription factor 6 (ATF-6) in endoplasmic reticulum. Depending on the duration and degree of stress, the UPR can provide either survival signals by activating adaptive and antiapoptotic signals, or death signals by inducing cell death programs [146].

We have found that E_2 changes the cell number according to the treatment period in long-term E_2 deprived breast cancer cell lines MCF-7:5C and MCF-7:2A [10]. E_2 has the capacity to decrease around 80 percentage of cell number in MCF-7:5C cells after 7 days treatment whereas in MCF-7:2A cells after two weeks treatment. Unexpectedly, the c-Src inhibitor effectively rescues the decreasing of cell number by E_2 in two long-term E_2 deprived cell lines [134]. The goal of this study is to identify the mechanisms underlying the early stage of E_2-induced apoptosis and the function of c-Src in the process of E_2-initiated apoptosis. To that end, we demonstrate that E_2 triggers endoplasmic reticulum stress and oxidative stress which activate two main apoptotic pathways, the mitochondrial ('intrinsic') and death receptor ('extrinsic') pathways, whereas c-Src plays an essential role in mediating stress responses induced by E_2 in MCF-7:5C cells. These findings have important clinical implications for the appropriate application of combination therapies in advanced aromatase inhibitor resistant breast cancer.

Work Accomplished:

c-Src mediated estrogen-activated growth pathways in long-term estrogen deprived breast cancer cells MCF-7:5C.

It is well documented that E_2 stimulates growth and prevents apoptosis in wild-type breast cancer cells and estrogen-responsive osteoblast cells. In contrast, physiological concentrations of E_2 induce apoptosis in long-term E_2 deprived breast cancer cells [83,93]. c-Src plays a critical role in relaying ER signaling pathways in breast cancer cells. To investigate the function of E_2 and c-Src in long-term E_2-deprived breast cancer cells MCF-7:5C, a specific c-Src tyrosine kinase inhibitor, PP2, was utilized to block phosphorylation of c-Src (Fig. 2i-1A). It also effectively abolished the growth pathways including the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT pathways in MCF-7:5C cells (Fig. 2i-1A). E_2 activated c-Src through ER

since 4-hydroxytamoxifen (4-OHT) completely suppressed phosphorylation of c-Src (Fig.2i- 1B). Although our previous finding showed that E_2 initiates apoptosis in MCF-7:5C cells, E_2 was able to activate growth pathways in MCF-7:5C cells (Fig. 2i-1C). These actions were blocked by the c-Src inhibitor, PP2 (Fig. 2i-1C). Even though the characteristic E_2-induced apoptosis occurs after 72 hours treatment, cell numbers were initially increased by E_2 with a high percentage in S phase (Fig.2i-1D). All of these results suggested that E_2 caused an imbalance between growth and apoptosis in MCF-7:5C cells.

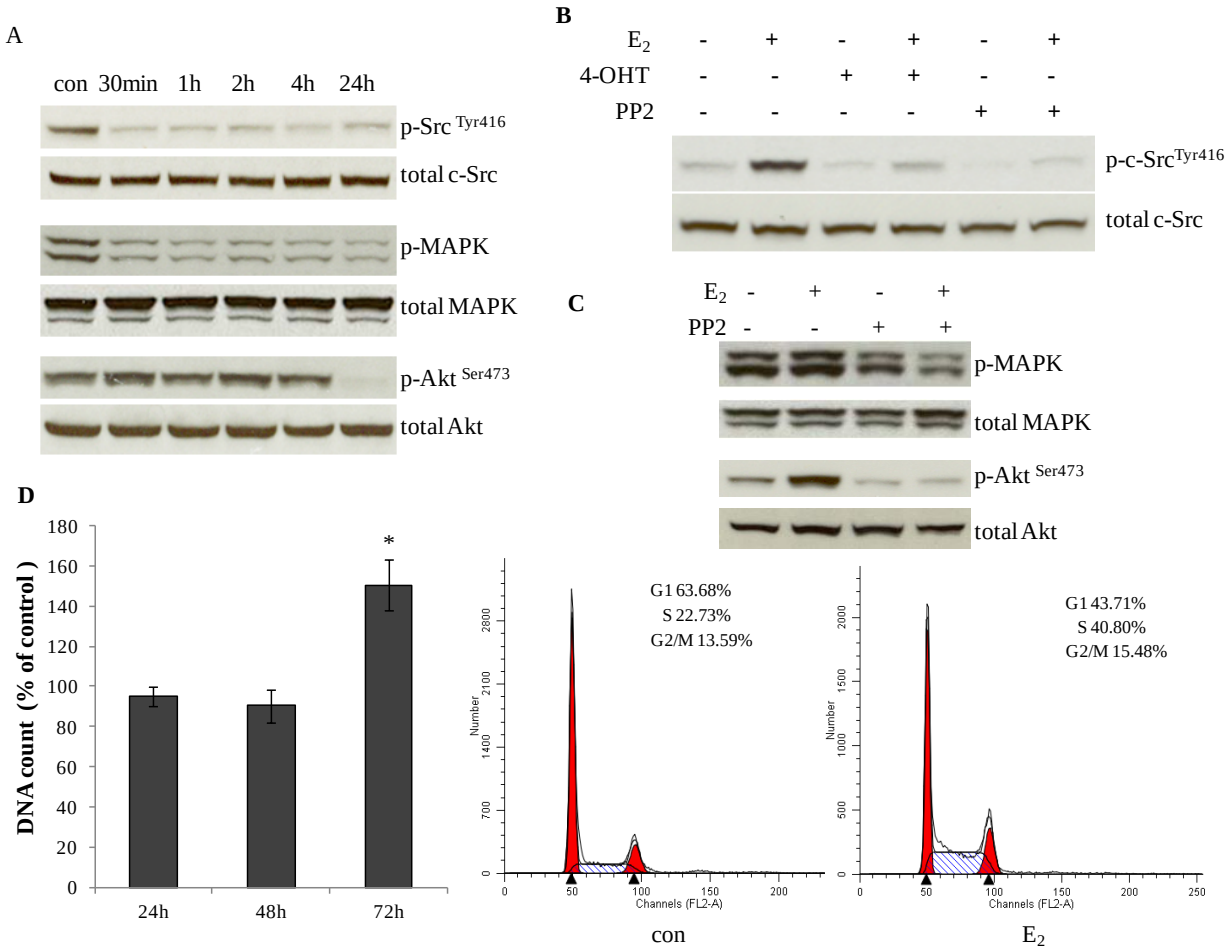

Figure 2i-1. ***1A. The c-Src inhibitor blocked growth pathways in MCF-7:5C cells.*** *MCF-7:5C cells were treated with vehicle (0.1% DMSO) and PP2 (5×10^{-6} mol/L) for different times as indicated. Cell lysates were harvested. Phosphorylated c-Src, MAPK, and Akt were examined by immunoblotting with*

primary antibodies. Immunoblotting for total c-Src, MAPK, and Akt were determined for loading controls. ***1B. Estrogen activated c-Src phosphorylation in MCF-7:5C cells.*** *MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9} mol/L), 4-OHT (10^{-6} mol/L), E_2 (10^{-9} mol/L) plus 4-OHT (10^{-6} mol/L), PP2 (5×10^{-6} mol/L), and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) for 48 hours. Cell lysates were harvested. Phosphorylated c-Src was examined by immunoblotting with primary antibody. Immunoblotting for total c-Src was determined for loading control.* ***1C. The c-Src inhibitor blocked genomic pathway induced by E_2 in MCF-7:5C cells.*** *MCF-7:5C cells were treated with vehicle (0.1%DMSO), E_2 (10^{-9} mol/L), PP2 (5×10^{-6} mol/L), and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) for 24 hours. Cell lysates were harvested. Phosphorylated MAPK and Akt were examined by immunoblotting with primary antibodies. Immunoblotting for total MAPK and Akt were determined for loading controls.* ***1D, E_2 increased S phase of cell cycles in MCF-7:5C cells.*** *MCF-7:5C cells were treated with vehicle and E_2 for different durations. Total DNA was determined using a DNA fluorescence quantitation kit. As a parallel experiment, MCF-7:5C cells were treated with vehicle and E_2 for 72 hours. Cells were fixed for cell cycles analysis. $P<0.05$, * compared with respective control.*

c-Src mediated the non-genomic pathway activated by E_2 in long-term estrogen deprived breast cancer cells MCF-7:5C which was not involved in the process of apoptosis.

E_2 was able to activate the non-genomic pathway within minutes (Fig. 2i-2A) which was blocked by the c-Src inhibitor (Fig. 2i-2A). To further investigate the function of the non-genomic pathway in the E_2-induced apoptosis, a synthetic ligand, estrogen dendrimer conjugate (EDC), was used to treat MCF-7:5C cells which is very ineffective in stimulating transcription of endogenous E_2 target genes. ER target gene pS2 was selected as a biomarker to measure the dose responsive manner to activate transcriptional activity by EDC (Fig. 2i-2B). In agreement with the result in reference 5, only higher dose of EDC (10^{-6} mol/L) activated pS2 but not in EDC (10^{-8} mol/L) (Fig. 2i-2B). Very similar as E_2, the EDC (10^{-8} mol/L) activated the non-genomic pathway which was blocked by the c-Src inhibitor (Fig. 2i-2C). However, the EDC (10^{-8} mol/L) had no capacity to induce apoptosis (Fig. 2i-2D and 2i-2E). These results indicated that EDC activated the non-genomic pathway but without capacity to activate genomic pathway and did not induce apoptosis in MCF-7:5C cells in certain concentration. All of these data suggest that the non-genomic pathway does not play a critical role in the E_2-induced apoptosis.

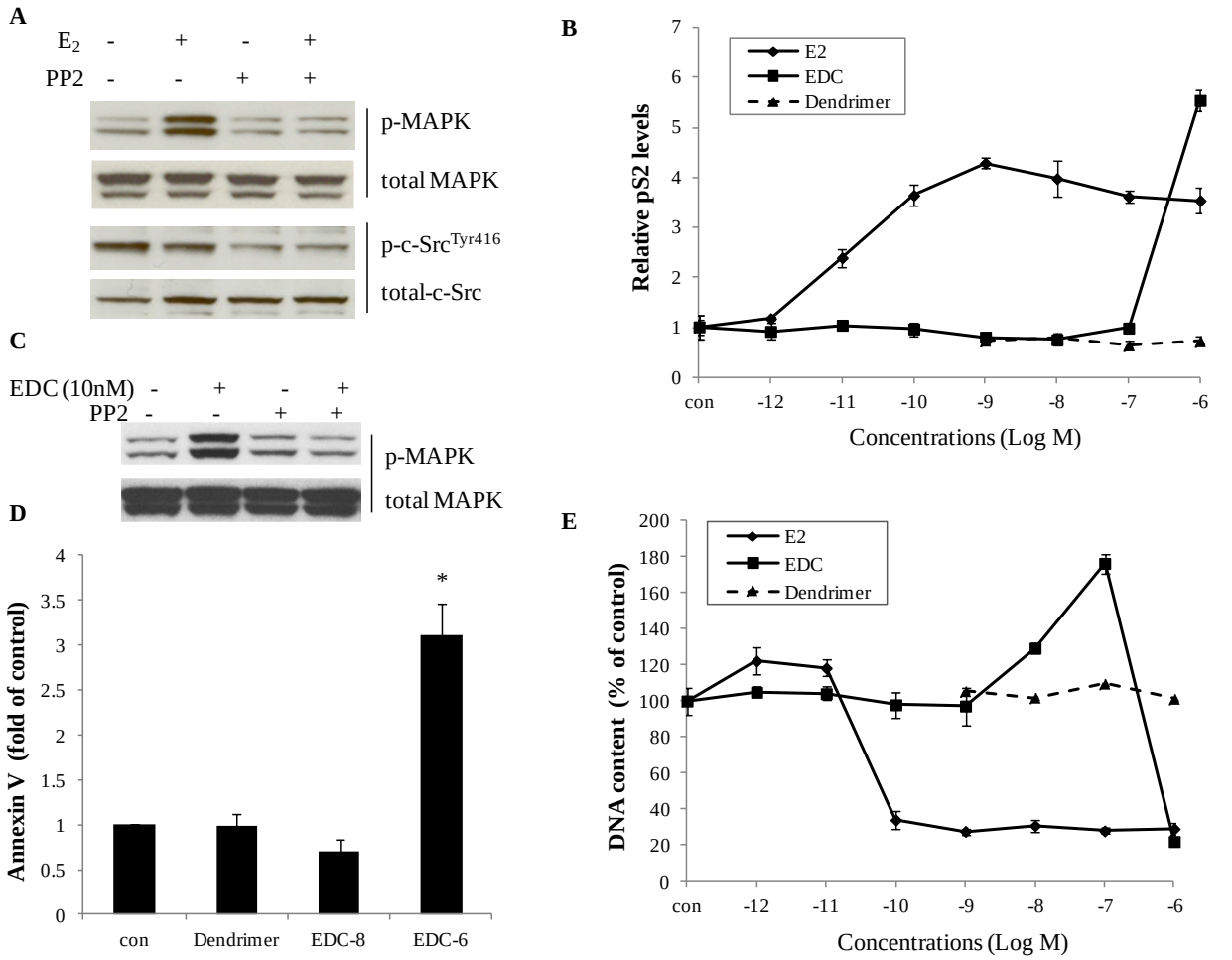

Figure 2i-2. ***2A.The c-Src inhibitor blocked non-genomic pathway induced by E_2 in MCF-7:5C cells.*** *MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9} mol/L), PP2 (5×10^{-6} mol/L), and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) for 10 mins. Cell lysates were harvested. Phosphorylated MAPK was examined by immunoblotting with primary antibody. Immunoblotting for total MAPK was determined for loading control.* ***2B. Activation of pS2 by different concentrations of estrogen dendrimer conjugate (EDC).*** *MCF-7:5C cells were treated with vehicle (0.1% MeOH), different concentrations of EDC, E_2, and empty dendrimer as indicated for 8 hours in triplicate. Cells were harvested in TRIzol for real-time PCR.* ***2C. The c-Src inhibitor blocked the non-genomic pathway activated by EDC****. MCF-7:5C cells were treated with vehicle (0.1% MeOH), EDC (10^{-8} mol/L), PP2 (5×10^{-6} mol/L), EDC (10^{-8} mol/L) plus PP2 (5×10^{-6} mol/L) respectively for 15 minutes and the cell lysates were harvested. Phosphorylated MAPK was examined by immunoblotting with primary antibody. Immunoblotting for total MAPK was used for loading control.* ***2D. Detection of apoptosis by different doses of EDC*** *MCF-7:5C cells were treated with vehicle (0.1% MeOH), EDC (10^{-8} mol/L), and EDC (10^{-6} mol/L) for 72 hours. Cells were harvested for the analysis of apoptosis through Annexin V binding assay.* ***2E. Cell growth curves after EDC treatment*** *MCF-7:5C cells were treated with vehicle (0.1% MeOH), different concentrations of EDC, E_2, and empty dendrimer as indicated for 7 days in triplicate. Cells were harvested and total DNA was determined using a DNA fluorescence quantitation kit.*

Inhibition of c-Src suppressed estrogen-induced apoptosis in MCF-7:5C cells.

We have shown that long-term E_2 deprivation increases c-Src activity [134]. Therefore, we addressed the question of whether the c-Src inhibitor, PP2, in combination with E_2 would enhance apoptosis in MCF-7:5C cells. Unexpectedly, the c-Src inhibitor blocked apoptosis initiated by E_2 (Fig. 2i-3A). To confirm

that inhibition of c-Src could block E_2-induced apoptosis, a specific siRNA was utilized to knock down c-Src in MCF-7:5C cells (Fig. 2i-3B), which reduced the percentage of Annexin V binding induced by E_2 (Fig. 2i-3C). Further experiments showed that E_2 disrupted mitochondrial membrane potential ($\Delta\psi_m$) after 48 hours treatment which was measured by flow cytometry using rhodamine 123 (Rh123) (Fig. 2i-3D). The c-Src inhibitor PP2 and 4-OHT both prevented reduction of Rh123 retention induced by E_2 (Fig. 2i- 3D). These data demonstrated that E_2-triggered apoptosis utilize the c-Src tyrosine kinase pathway. All of these findings suggested that the c-Src plays a critical role to mediate E_2-induced apoptosis.

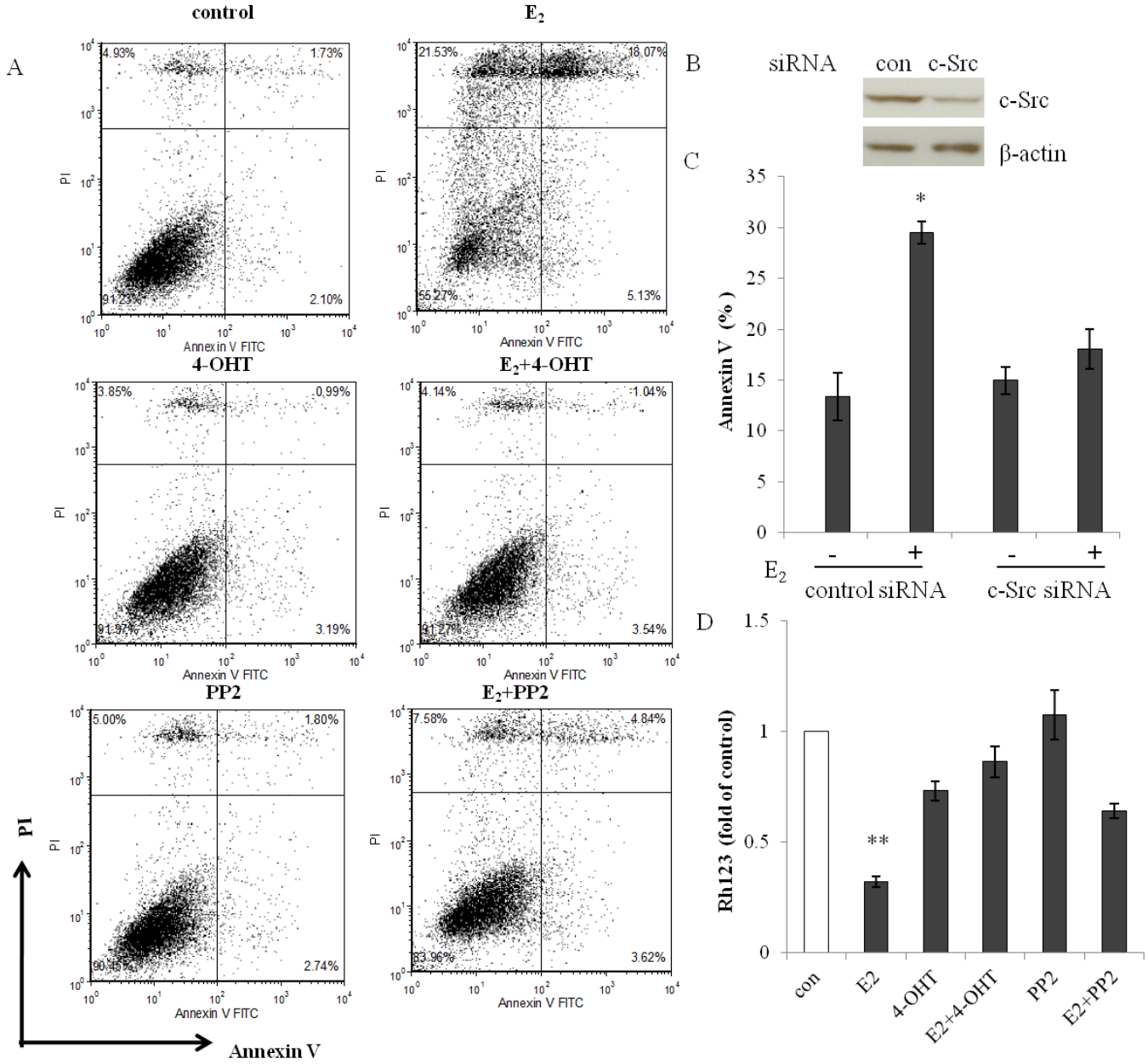

Figure 2i-3. ***3A. Inhibition of c-Src blocked E_2-induced apoptosis.*** *MCF-7:5C cells were treated with different compounds respectively as above for 72 hours and Annexin V binding assay was used to detect apoptosis.* ***3B. Knockdown of c-Src through interfering RNA*** *MCF-7:5C cells were transfected with siRNA of c-Src for 72 hours using non-target siRNA as control. c-Src was detected by immunoblotting. The β-actin was used for loading control.* ***3C. Knockdown of c-Src blocked E_2-induced apoptosis.*** *MCF-7:5C cells were transfected with c-Src siRNA and non-target siRNA as above. Then, they were treated with vehicle (0.1% EtOH) and E_2 (10^{-9}mol/L) respectively for 72 hours. Apoptosis was detected through*

*Annexin V binding assay. P<0.05, * compared with control.* ***3D. The c-Src inhibitor blocked the reduction of mitochondrial potential induced by E_2.*** *MCF-7:5C cells were treated with different compounds respectively as above for 48 hours and cells were harvested to detect mitochondrial potential through Rh123. P<0.001, ** compared with control.*

Suppression of E_2-induced apoptosis by the c-Src inhibitor was independent of the classical estrogen response element (ERE) regulated transcriptional genes in MCF-7:5C cells.

The ER is the initial site for E_2 to induce apoptosis since anti-estrogens ICI 182,780 and 4-OHT completely block apoptosis triggered by E_2 [83]. In addition to the mediation of ER growth pathways, c-Src is involved in the process of ligand-activated ER ubiquitylation [147]. Therefore, blockade of c-Src tyrosine kinase with PP2 further increased ERŬprotein and mRNA expression levels in MCF-7:5C cells (Fig. 2i-4A). E_2 activated estrogen response element (ERE) activity which could be blocked by 4-OHT but not by PP2 (Fig. 2i-4B). It was interesting to find that the c-Src inhibitor alone could up-regulate E_2 inducible gene pS2 and was additive with E_2 to elevate pS2 mRNA level (Fig. 2i-4C). Another important ER target gene progesterone receptor (PR) has been regarded as an indicator of a functional ER pathway, since expression of PR is regulated by E_2. Although the c-Src inhibitor alone did not elevate PR expression, it dramatically synergized with E_2 to up-regulate PR mRNA (Fig. 2i-4D). All of these results demonstrated that blockade of c-Src increased expression of classical ER target genes. It also implied that classical ER pathway might not directly involve in the E_2-induced apoptosis.

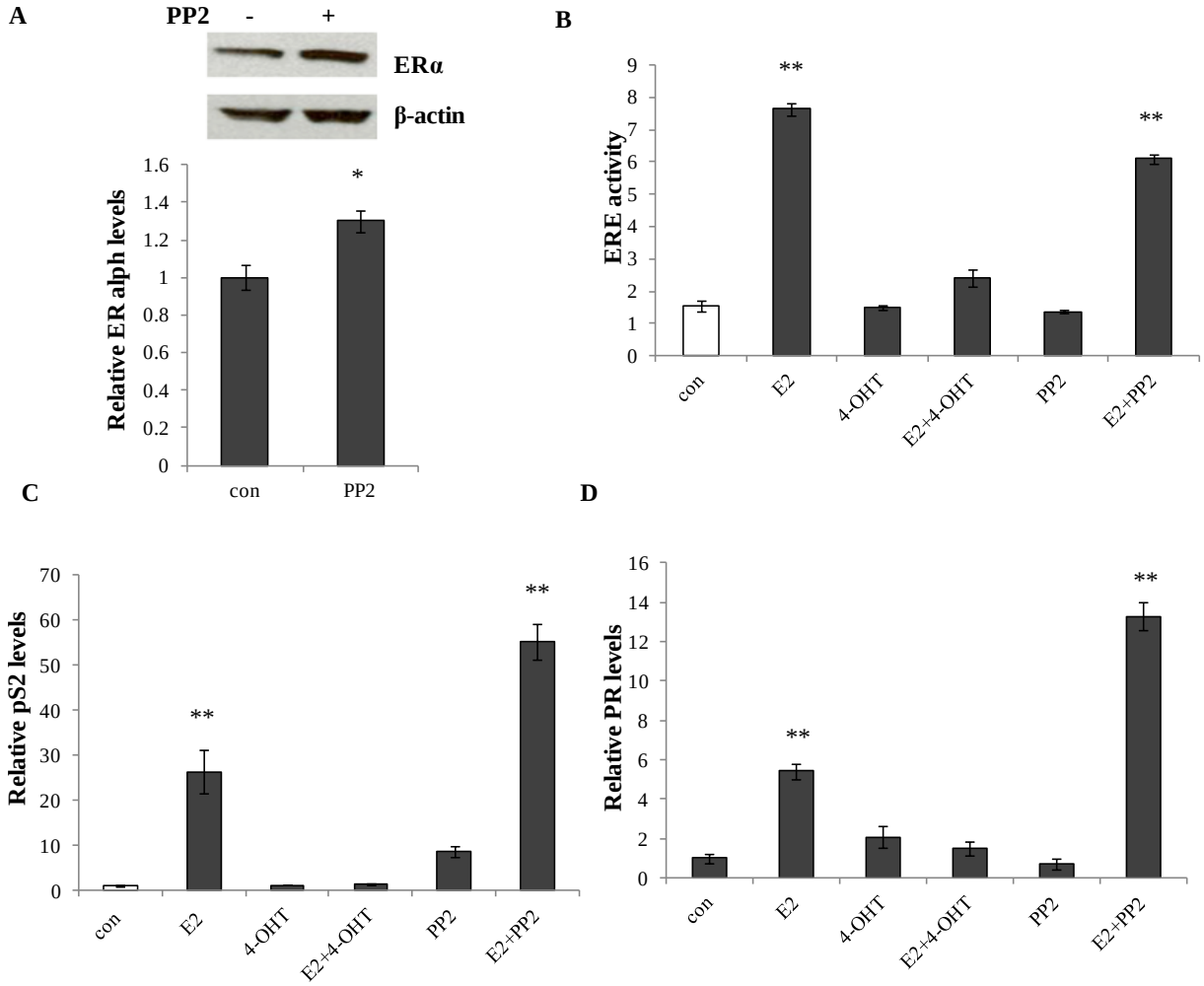

Figure 2i-4. ***4A. The c-Src inhibitor increased levels of ER alpha.*** *MCF-7:5C cells were treated with vehicle (0.1% DMSO) and PP2 (5×10^{-6}mol/L) respectively for 24 hours. ERα protein was detected by immunoblotting. ERα mRNA was quantified with qPCR. P<0.05, * compared with control.* ***4B. The c-Src inhibitor did not block ERE activity induced by E_2.*** *MCF-7:5C cells were transfected with ERE firefly luciferase plasmid plus renilla luciferase plasmid. Then, cells were treated with different compounds respectively for 24 hours to detect ERE activity. P<0.001, ** compared with control.* ***4C. The c-Src***

inhibitor further activated pS2. *MCF-7:5C cells were treated with different compounds respectively for 24 hours. The pS2 mRNA was quantified with qPCR. P<0.001, ** compared with control.* ***4D. The c-Src inhibitor further increased PR levels.*** *MCF-7:5C cells were treated with different compounds respectively for 72 hours. The PR mRNA was quantified with qPCR. P<0.001, ** compared with control.*

c-Src was involved in the process of triggering apoptosis-related genes by E_2 in MCF-7:5C cells.

To further investigate the mechanisms of the suppression of E_2-induced apoptosis by PP2, RNA-seq analysis was performed to examine the genes regulated by E_2 to trigger apoptosis in MCF-7:5C cells. A wide range of apoptosis-related genes were activated by E_2 (Fig. 2i-5A), which were functionally classified into three groups: TP53-related genes (such as TP63, PMAIP1, and CYFIP2), stress-related genes (such as HMOX1, PPP1R15A, ZAK, NUAK2 *etc.*), and inflammatory response-related genes (such as LTB, FAS, TNFRSF21, and CXCR4 *etc.*). Most were stress-related genes (Fig. 2i-5B). Consistent with the biological experiments, 4-OHT and PP2 both blocked apoptosis-related genes induced by E_2 but to a different extent in MCF-7:5C cells (Fig.2i-5A). The majority of these apoptosis-related genes were confirmed by real-time PCR with similar changes noted as in RNA-seq analysis (Fig. 2i-5C). E_2 dramatically increased p63 mRNA levels (Fig. 2i-5C) but did not arrest cells in the G1 phase. In fact, S phase was markedly elevated in MCF-7:5C cells (Fig. 2i-1D). Heme oxygenase 1 (HMOX1) which is active at high concentrations of heme, catalyzes the degradation of heme and is thought to function as an oxidative stress indicator. In breast cancer cells, cytochrome *c* is a major source of heme protein found in the inner membrane of the mitochondrion. E_2 markedly increased HMOX1 in MCF-7:5C cells (Fig. 2i-5C) thereby confirming that E_2 may damage the mitochondria and caused cytochrome *c* release. All of these data suggested that E_2 widely activated intrinsic and extrinsic apoptosis pathways and c-Src was directly involved in mediating apoptosis.

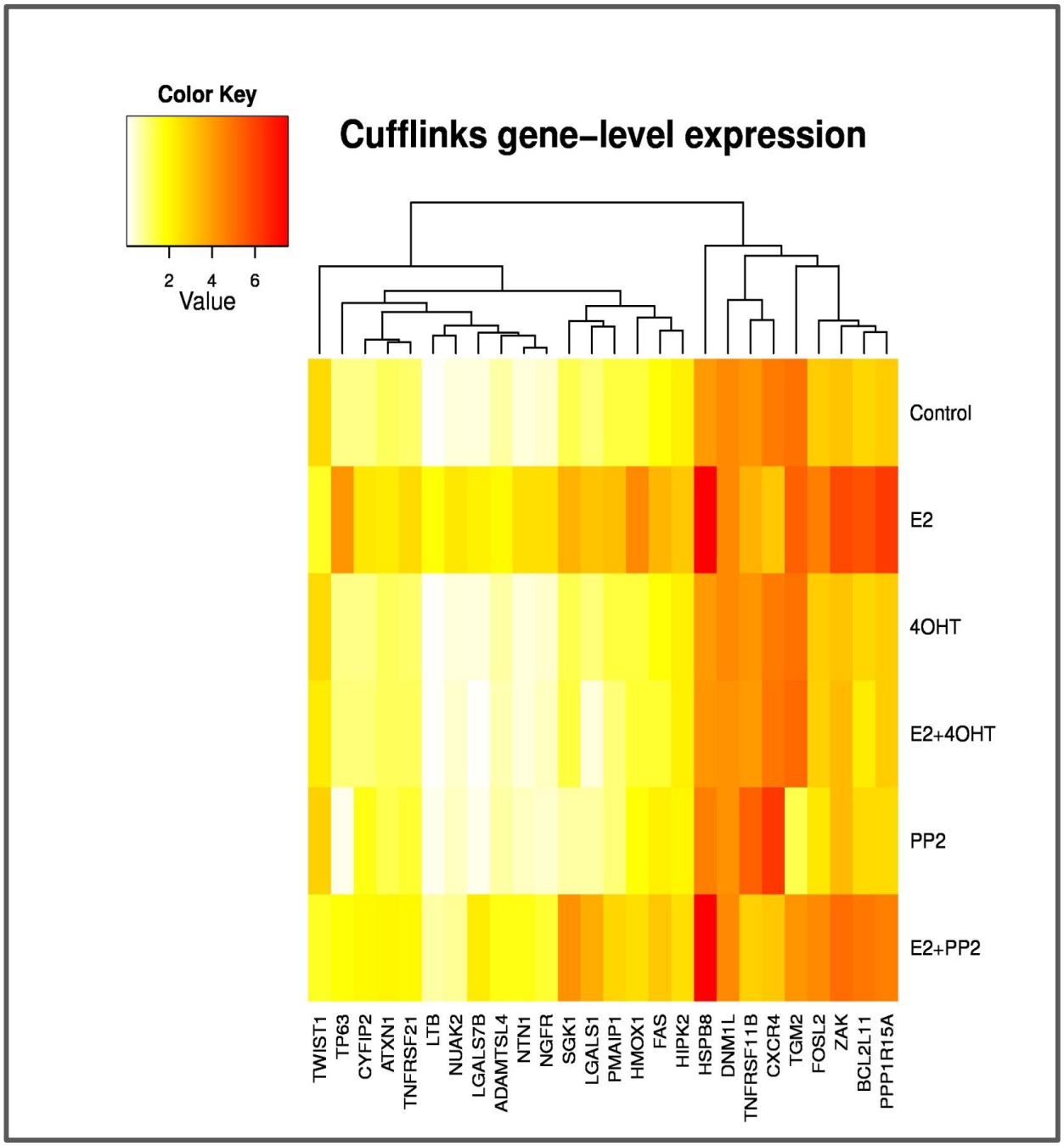

Figure 2i-*5A. Estrogen activated apoptosis-related genes in MCF-7:5C cells analyzed through RNA-seq.* *MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9} mol/L), 4-OHT (10^{-6} mol/L), E_2 (10^{-9} mol/L) plus 4-OHT (10^{-6} mol/L), PP2 (5×10^{-6} mol/L), and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) for 72 hours. Cells were harvested and RNA was isolated with kit (Qiagen) for RNA-seq analysis.*

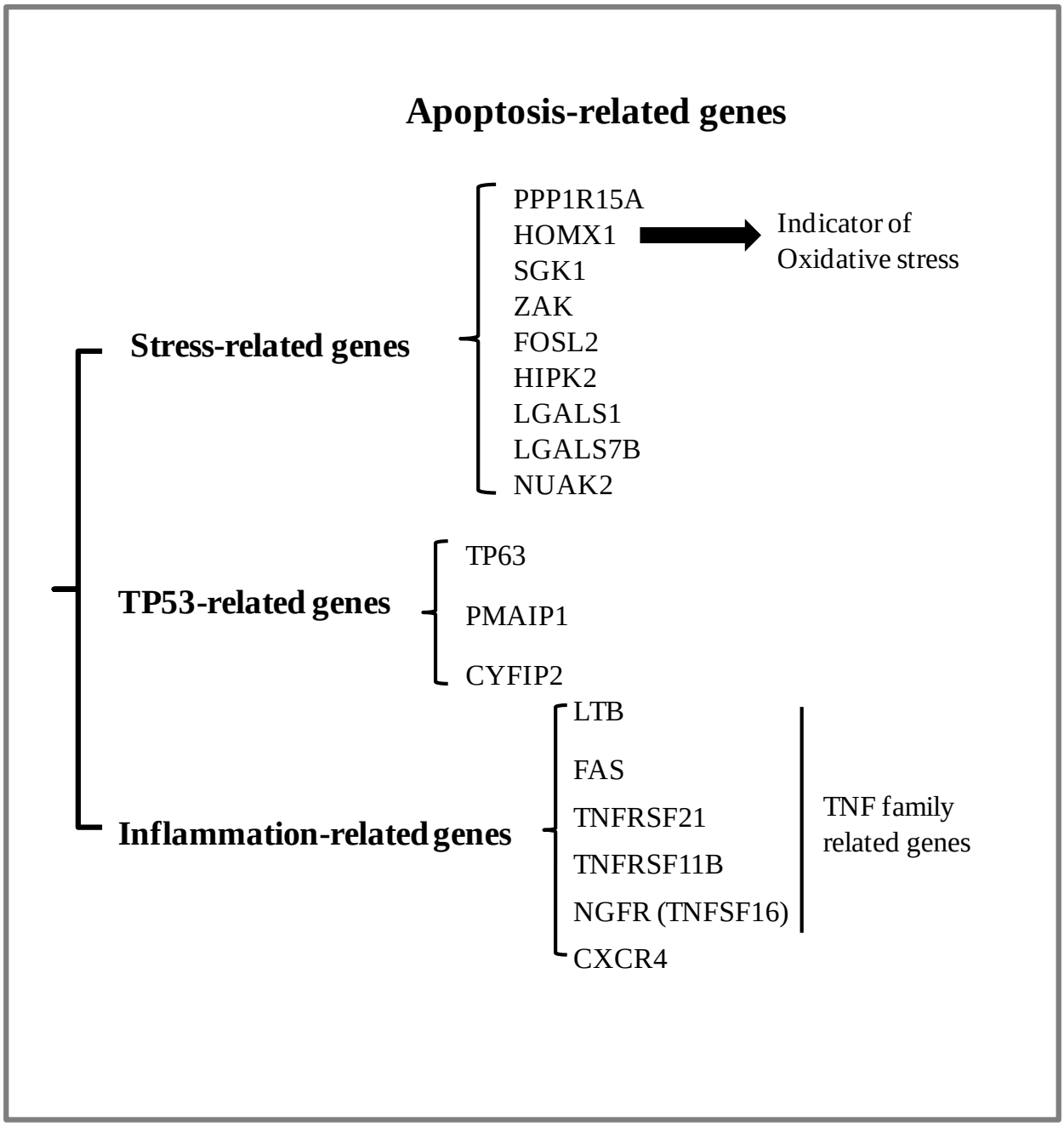

Figure 2i-5B. ***The apoptosis-related genes selected by RNA-seq were functionally divided into three groups as shown above.*** *Estrogen widely activated apoptosis-related genes to trigger apoptotic cascades in MCF-7:5C cells.*

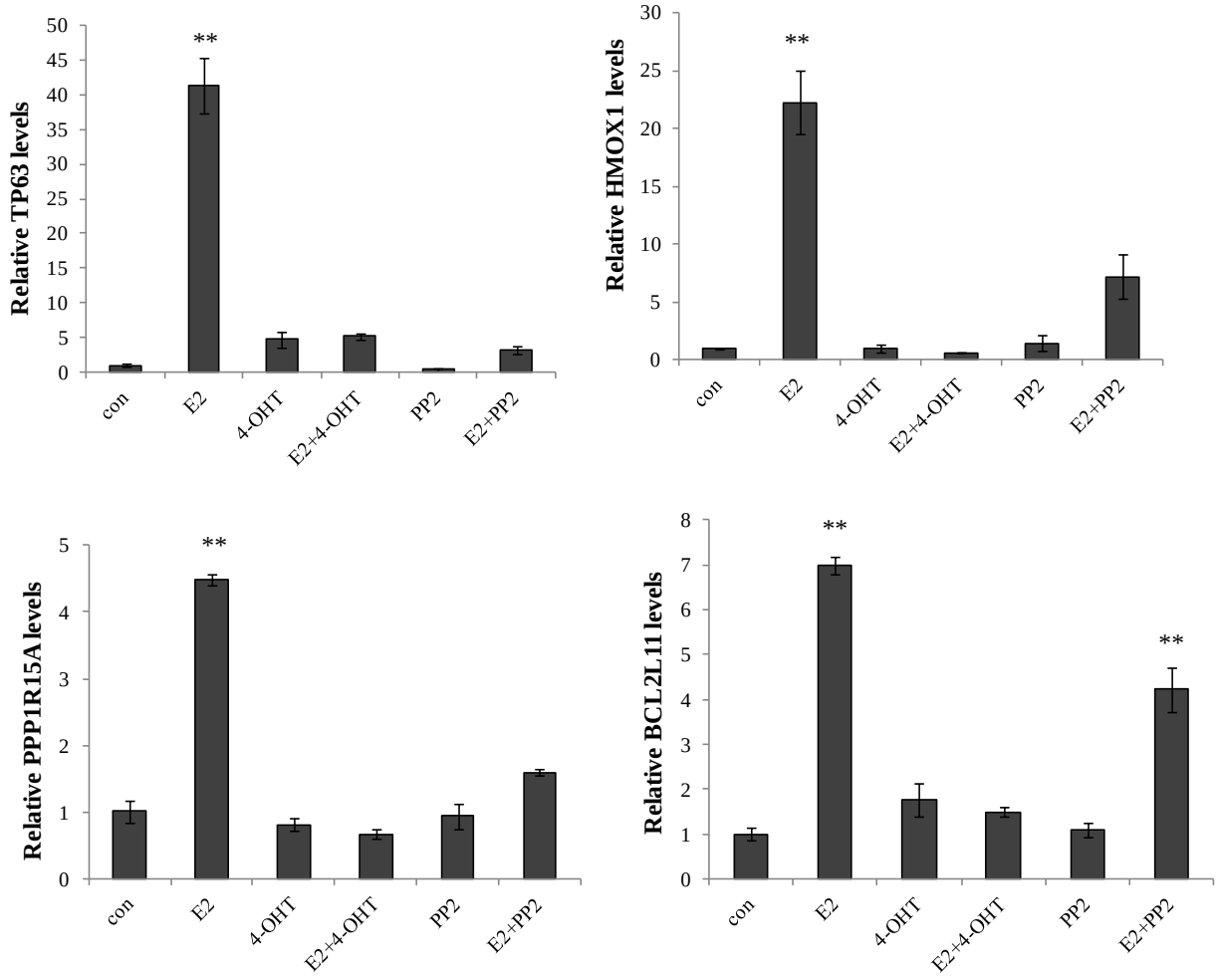

Figure 2i-5C. ***RNA-seq data were confirmed by real-time PCR.*** *MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9} mol/L), 4-OHT (10^{-6} mol/L), E_2 (10^{-9} mol/L) plus 4-OHT (10^{-6} mol/L), PP2 (5×10^{-6} mol/L), and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) for 72 hours. Cells were harvested and RNA was isolated with kit (Qiagen) for real-time PCR analysis.*

The c-Src inhibitor blocked estrogen-induced oxidative stress in MCF-7:5C cells.

Reactive oxygen species (ROS) are the product of oxidative stress by mitochondria, whereas an increase in ROS contributes to degenerative changes in mitochondrial function. Under physiological conditions, cellular ROS levels are tightly controlled by low-molecular-weight radical scavengers and by a complex intracellular network of enzymes such as catalases (CAT) and superoxide dismutases (SOD). Under conditions of lethal stress, ROS are considered as key effectors of cell death. Intracellular ROS were detected by CM-H_2DCFDA through flow cytometry (Fig.2i-6A). Detectable ROS appeared after 48 hours of treatment with E_2. The production of ROS reached a peak after 72 hours treatment (Fig.2i-6A and 6B). Blocking ER (by 4-OHT) and c-Src (by PP2) abolished ROS generation induced by E_2 (Fig.2i-6C), indicating that both ER and c-Src were upstream signals of ROS. Free radical scavengers Mn-TBAP, catalase, and sodium formate (SF) which respectively act on superoxide radical (O_2^-), H_2O_2, and hydroxyl radical (OH^-), were utilized to suppress the production of ROS. Our results suggested that H_2O_2 and OH^- were the major sources of ROS induced by E_2. This conclusion was based on the observation that catalase and sodium formate inhibited E_2-induced apoptosis, whereas Mn-TBAP was less effective (Fig. 2i-6D). The RNA-seq analysis demonstrated that E_2 did not significantly regulate antioxidant enzymes such as

catalases (CAT) and superoxide dismutases (SOD) in MCF-7:5C cells (data not shown). Our results suggest that E_2 has the potential to damage mitochondria to cause oxidative stress.

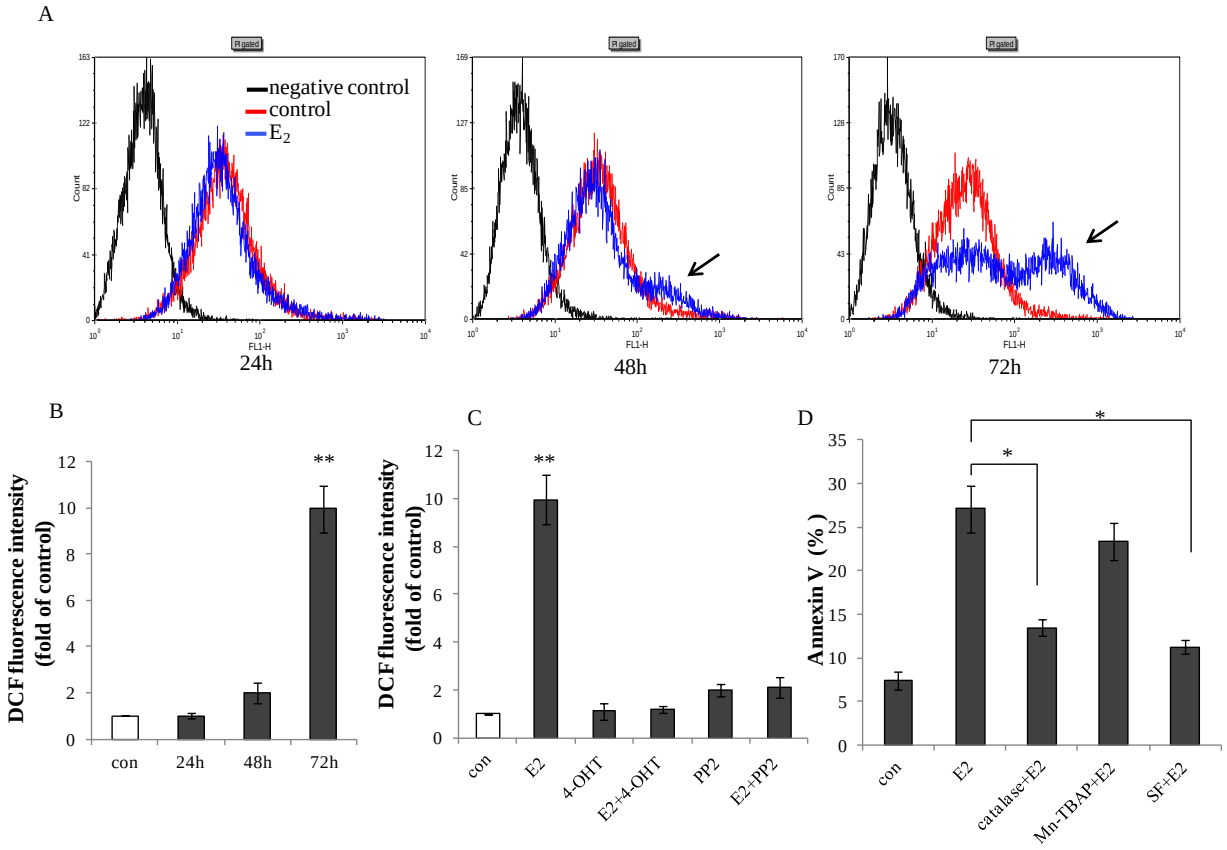

Figure 2i-6. ***The c-Src inhibitor blocked estrogen-induced oxidative stress in MCF-7:5C cells. 6A. E_2 increased ROS production in MCF-7:5C cells.*** *MCF-7:5C were treated with vehicle and E_2 for different durations. ROS was detected through flow cytometry with CM-H2DCFDA staining.* ***6B. Quantification of ROS production induced by E_2*** *ROS production induced by E_2 was compared with control. $P<0.001$, ** compared with control.* ***6C. The c-Src inhibitor reduced ROS production induced by E_2.*** *MCF-7:5C cells were treated with different compounds as above. ROS production was detected through flow cytometry. $P<0.001$, ** compared with control.* ***6D. Free radical scavengers prevented E_2-induced apoptosis.*** *MCF-7:5C cells were treated with vehicle (0.1% EtOH), E_2 (10^{-9}mol/L), catalase (5000U/mL) plus E_2 (10^{-9}mol/L), Mn-TBAP(5×10^{-5}mol/L) plus E_2 (10^{-9}mol/L), sodium formate (2×10^{-3}mol/L) plus E_2 (10^{-9}mol/L) for 72 hours. Apoptosis was detected through Annexin V binding assay. $P<0.05$, * compared with E_2 treated group.*

The c-Src inhibitor blocked estrogen-induced tumor necrosis factor (TNF) family signaling in MCF-7:5C cells.

As shown above in Fig. 2i-5A and 2i-5B, E_2 activated TNF family-related genes (such as LTB, FAS, TNFRSF21, and CXCR4 etc.). We confirmed through real-time PCR that members in TNF family members (TNF alpha, LTA, and LTB) were clearly up-regulated by E_2 but blocked by 4-OHT and the c-Src inhibitor in MCF-7:5C cells (Fig. 2i-7A, 2i-7B, and 2i-7C). Of all TNF family members, TNF is the most potent inducer of apoptosis through extrinsic pathway which is mediated by death receptors. We found that low-dose TNF alpha (5ng/ml) could increase the cleavages of caspase 9 and PARP in MCF-7:5C cells (Fig.2i-7D). TNF alpha could significantly inhibit cell growth (Fig.2i-7E). These data demonstrated that E_2-induced apoptosis in MCF-7:5C cells utilized both intrinsic (mitochondria) and extrinsic (TNF family) pathways.

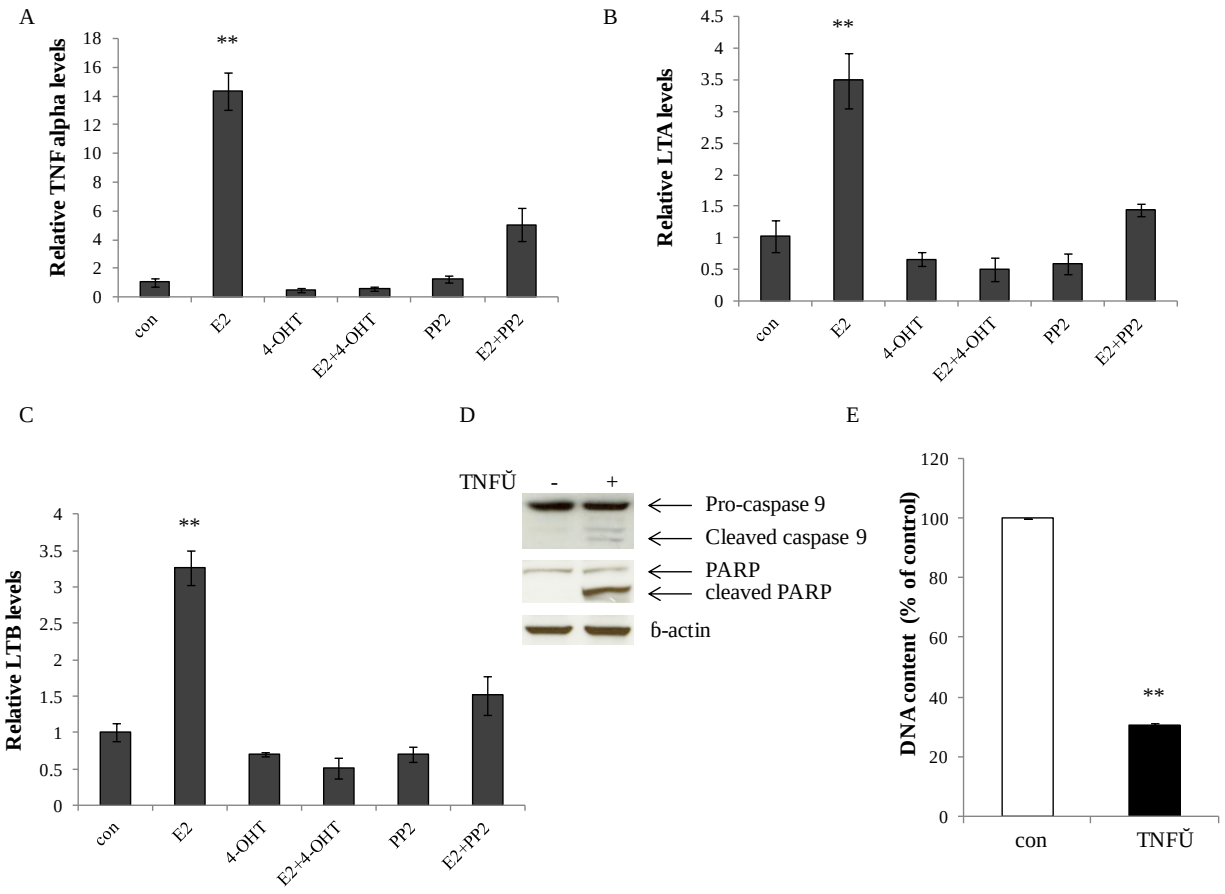

Figure 2i-7. ***7A. The c-Src inhibitor and 4-OHT blocked TNF alpha up-regulated by E_2.*** *MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9}mol/L), 4-OHT (10^{-6}mol/L), E_2 (10^{-9}mol/L) plus 4-OHT (10^{-6}mol/L), PP2 (5×10^{-6} mol/L), and E_2 (10^{-9}mol/L) plus PP2 (5×10^{-6} mol/L) for 72 hours. RNA was isolated with kit (Qiagen) for real-time analysis with specific primers for TNF alpha. P<0.001, ** compared with control.* ***7B. The c-Src inhibitor and 4-OHT blocked LTA up-regulated by E_2.*** *MCF-7:5C cells were treated with different compounds as above. RNA was isolated with kit (Qiagen) for real-time analysis with specific primers for LTA. P<0.001, ** compared with control.* ***7C. The c-Src inhibitor and 4-OHT blocked LTB up-regulated by E_2.*** *MCF-7:5C cells were treated with different compounds as above. RNA was isolated with kit (Qiagen) for real-time analysis with specific primers for LTB. P<0.001, ** compared with control.* ***7D, TNF alpha increased the cleavages of caspase 9 and PARP.*** *MCF-7:5C cells were treated with vehicle (0.1% DMSO) and TNF alpha (5ng/ml) for 24h. Cell lysates were harvested. Cleavages of caspase 9 and PARP were examined by immunoblotting with primary antibodies. Immunoblotting for β-actin was determined for loading controls.* ***7E, TNF alpha inhibited cell growth in MCF-7:5C cells.*** *MCF-7:5C cells were treated with vehicle (H2O) and TNF alpha (5ng/ml) for 7 days. Cells were harvested and DNA content was determined as above. P<0.001, ** compared with control.*

c-Src was involved in estrogen-induced endoplasmic reticulum stress in MCF-7:5C cells.

Our previous global gene array data show that E_2 activates genes related to endoplasmic reticulum stress in MCF-7:5C cells [10]. To relieve stress, sensors of unfolded protein responses (UPR) are activated as initial responses. In this study, a significant induction of UPR sensors, inositol-requiring protein 1 alpha (IRE1Ŭ) and PERK/eukaryotic translation initiation factor-2Ŭ (eIF2Ŭ), by E_2 occurred after 24 hours of treatment and was further increased by prolonging treatment times in MCF-7:5C cells (Fig. 2i-8A). The antiestrogen 4-OHT completely abolished the response (Fig. 2i-8A). The PERK inhibitor blocked

phosphorylation of eIF2Ŭ and prevented E_2-induced apoptosis (Fig. 2i-8B and 2i-8C), confirming that endoplasmic reticulum stress was important in the apoptosis initiated by E_2. Phosphorylated eIF2Ŭ closely associates with an important cellular energy sensor, adenosine monophosphate (AMP)-activated protein kinase (AMPK), to regulate protein translation and apoptosis. AMPK, which phosphorylates many metabolic enzymes to stimulate catabolic pathways and increases the capacity of cells to produce ATP , was significantly activated after 48 hours treatment with E_2 (Fig. 2i-8D). The c-Src inhibitor, PP2, blocked the phosphorylation of eIF2Ŭ but not IRE1Ŭ induced by E_2 (Fig. 2i-8E). PP2 also prevented the activation of AMPK after E_2 treatment (Fig. 2i-8F). All of these data indicate that c-Src acts as an important transducer in the protein kinase pathways (eIF2Ŭ and AMPK) of stress response (Fig. 2i-8E and 2i-8F) that result in apoptosis.

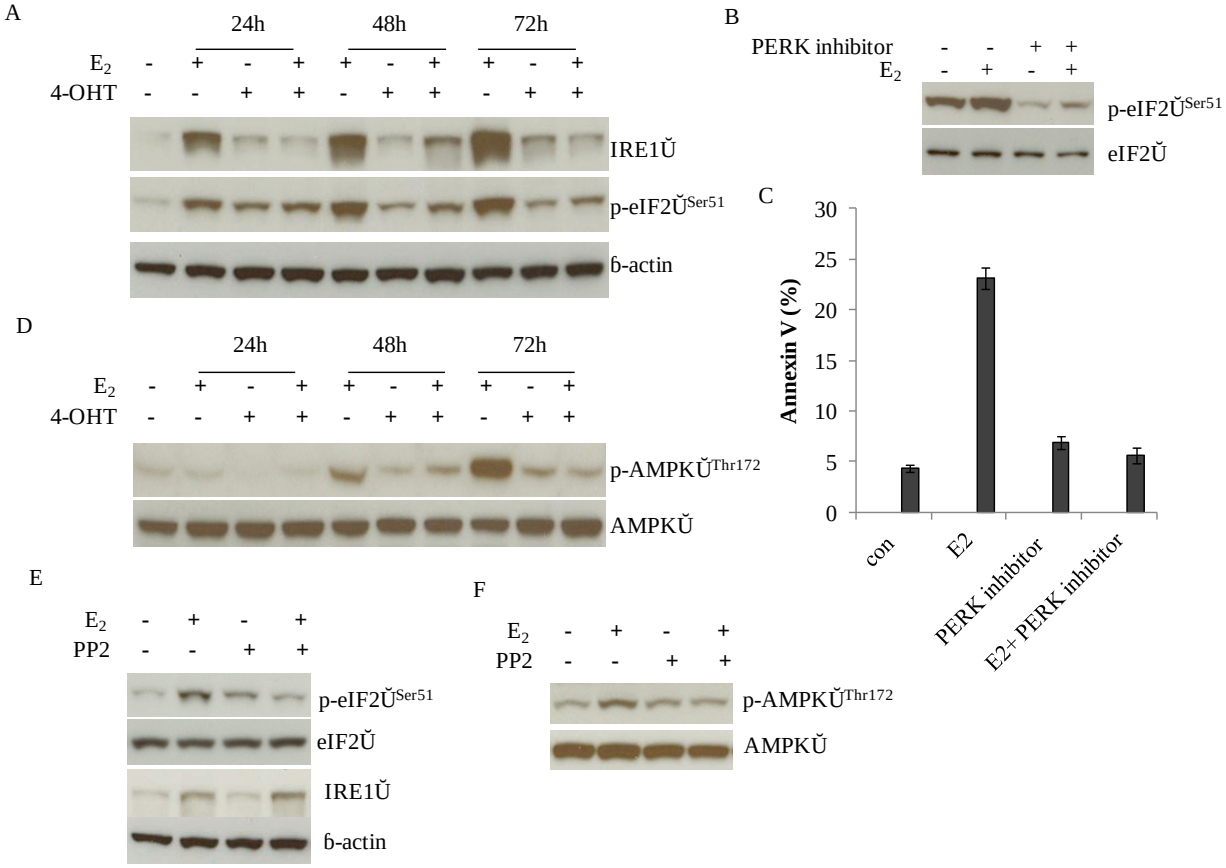

Figure 2i-8. ***8A. E_2 induced endoplasmic reticulum stress in MCF-7:5C cells.*** *MCF-7:5C were treated with E_2 (10^{-9}mol/L) or combined with 4-OHT (10^{-6}mol/L) for different durations. IRE1α and phosphorylated eIF2α were used as indicators of UPR activation.* ***8B. The PERK inhibitor blocked phosphorylation of eIF2α.*** *MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9}mol/L), PERK inhibitor (1×10^{-5}mol/L), E_2 (10^{-9}mol/L) plus PERK inhibitor (1×10^{-5}mol/L) respectively for 24 hours. Phosphorylated eIF2α was examined as the downstream of PERK. Total eIF2α was determined for loading control.* ***8C. The PERK inhibitor blocked E_2-induced apoptosis in MCF-7:5C cells.*** *MCF-7:5C cells were treated with E_2 or combined with PERK inhibitor respectively for 72 hours. Apoptosis was detected through Annexin V binding assay.* ***8D. E_2 activated energy stress sensor AMPK in MCF-7:5C cells.*** *MCF-7:5C cells were treated with E_2 or combined with 4-OHT as above. Phosphorylated AMPK was examined by immunoblotting. Total AMPK was determined for loading control.* ***8E. The c-Src inhibitor blocked phosphorylation of eIF2α but not IRE-1 α.*** *MCF-7:5C cells were treated with E_2 or combined with PP2 for 24 hours. IRE1α and phosphorylated eIF2α were examined by immunoblotting. Total eIF2α and β-actin were determined for loading controls.* ***8F. The c-Src inhibitor blocked***

phosphorylation of AMPK. *MCF-7:5C cells were treated with E_2 or combined with PP2 for 48 hours. Phosphorylated AMPK and total AMPK were examined by immunoblotting.*

Discussion

We have previously investigated the inhibitory effects of E_2 on long-term endocrine resistant breast cancer tumor growth *in vivo* [84,85,132]. And we have confirmed this therapeutic effect is related with the apoptosis induced by E_2 [83]. This scientific discovery has been used in the clinical trials to treat aromatase inhibitor resistant breast cancer patients and 30% of patients receive benefit [12]. The potential limitation on translational research in the treatment of hormone responsive breast cancer is that only four ER positive breast cancer cell lines are available to use routinely [103]. Only MCF-7 of the four produces the phenotype of E_2-induced apoptosis observed clinically [83,93]. The purpose of establishing long-term E_2 deprivation *in vitro* models is to mimic administration of an aromatase inhibitor that reduces levels of circulating estrogen in clinical studies ([98]. After a period of proliferative quiescence lasting a few months, the return of proliferation is similar to the relapses observed 12-18 months after primary hormonal therapy in patients. Multiple pathways are involved in the adaptive response to the pressure of E_2 deprivation ([148]. Although MCF-7 cells grown long term have been shown to differ substantially in various properties depending upon the number of passages and geographic source of the cell lines, induction of apoptosis by physiological concentrations of E_2 is the common characteristic of these *in vitro* model systems[83,93]. Nevertheless, how E_2 induces apoptosis is at present unclear. Our new observation [134] that a c-Src inhibitor paradoxically can block E_2-induced apoptosis naturally demands further study. We examined this aspect of c-Src pharmacology to describe fully this phenomenon and gain an insight into the convergence of ER and c-Src pathways for the modulations of an apoptotic trigger in breast cancer. Here, for the first time we document that c-Src participates in the mediation of stress responses induced by E_2 to widely activate apoptosis-related genes involved in the intrinsic and the extrinsic apoptosis pathways.

The ER is the initial point for E_2 to induce apoptosis since anti-estrogens ICI 182,780 and 4-OHT completely block apoptosis triggered by E_2 [83]. Contradictory to the traditional apoptosis mechanism caused by cytotoxic chemotherapy with cell cycles arrest, E_2-induced apoptotic cells simultaneously undergo proliferation with an increased S phase of cell cycle resulting in increased cell number despite p53 family members being up-regulated. E_2 exerts a dual function on MCF-7:5C cells, with both initial proliferation and the apoptosis. In other words, the initial response of E_2 to stimulate growth is the up-regulating of classical transcriptional activity by ER (Fig. 2i-4B) without any detected apoptotic changes in the first 24 hours. Activation of apoptotic genes appeared after 48 hours treatment with E_2 (data not shown), and reached a peak by 72 hours (Fig. 2i-5C). Consistently, characteristic apoptosis occurred at 72 hours (Fig. 2i-2A). These data suggest that the higher rate of proliferation by E_2 might activate other pathways to trigger apoptosis. Our data demonstrate that E_2 caused endoplasmic reticulum stress which activated the unfolded protein response (UPR) within 24 hours (Fig. 2i-8A). The initial aim of UPR is to restore normal function of the cell, however, if the damage is too severe to repair, the UPR ultimately initiates cell death through activation of the apoptotic pathway [149].

c-Src functioned as an important downstream signal of ER in MCF-7:5C cells, which was activated by E_2 (Fig.2i-1B) and demonstrated multiple levels of association with ER. An important finding in this study is that c-Src tyrosine kinase is critical for E_2-induced apoptosis (Fig. 2i-3). This, therefore, raised the question of the actual role played by c-Src in the process of apoptosis induced by E_2. c-Src mediated PI3K/AKT and MAPK growth pathways by E_2 (Fig. 2i-1C). However, specific inhibitors of PI3K/Akt (LY294002) and MAPK (U0126) could inhibit cell growth but not prevented E_2-induced apoptosis in MCF-7:5C cells, which imply that MAPK/Akt growth pathways are not directly involved in the apoptosis-induced by E_2. In MCF-7:5C cells, E_2 activated the non-genomic pathway after 10 minutes treatment and the c-Src inhibitor blocked the non-genomic pathway (Fig. 2i-2). Detectable elevation of c-

Src phosphorylation appeared after 30 mins treatment with E_2 (Fig. 2i-2). Consistent stimulation of c-Src appeared after 24 hours treatment and gradually increased when extending to 48 hours (Fig. 2i-1B). All of these data suggest that c-Src activation is a direct effect resulting from E_2. To further explore the function of the non-genomic pathway in the process of E_2-induced apoptosis, EDC was used to treat MCF-7:5C cell which is very ineffective in stimulating transcription of endogenous E_2 target genes [150]. The EDC (10^{-8} mol/L) activated the non-genomic pathway but without capacity to activate genomic pathway and did not induce apoptosis in MCF-7:5C cells (Fig. 2i-2). All of these results suggest that the non-genomic pathway does not play a critical role in the E_2-induced apoptosis.

Additionally, E_2 activated classical ERE activity but the c-Src inhibitor could not block the response (Fig. 2i-4B). Furthermore, the c-Src inhibitor collaborated with E_2 to up-regulate endogenous ER target genes pS2 and PR (Fig. 2i-4C and 2i-4D). All of these results imply that classical ER transcriptional pathways are not directly involved in E_2-induced apoptosis. Similarly, Zhang *et al* [151] reported that the inhibitory effects of E_2 on cell growth are independent of the classical ERE regulated transcriptional genes**.** Our global gene array data suggest that E_2 signaling can occur through a non-classical transcriptional pathway involving the interaction of ER with other transcription factors such as activator protein-1 (AP-1) and Sp1, which may regulate stress responses [10]. In the present study, E_2 initiated UPR (Fig. 2i-8A), increased ROS production (Fig. 2i-6A), and widely activated apoptosis related genes (Fig. 2i-5A). The c-Src was involved in the stress responses and inhibition of c-Src decreased the expression of apoptosis related genes induced by E_2, which are critical mechanisms for the blockade of c-Src to prevent E_2-induced apoptosis.

Overall, E_2 induces endoplasmic reticulum and mitochondrial stresses in MCF-7:5C cells, which subsequently up-regulates apoptosis-related genes to activate intrinsic and extrinsic apoptotic pathways. Unexpectedly, c-Src tyrosine kinase plays a critical role in the stress response induced by E_2. These data clearly raise a concern regarding the ubiquitous use of c-Src inhibitors to treat patients with advanced aromatase inhibitor-resistant breast cancer, thereby undermining the beneficial effects of E_2-induced apoptosis.

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TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2j (Sengupta and Jordan) - Studies carried out by Dr. Surojeet Sengupta in the Jordan laboratory at Georgetown University

Molecular Mechanism of Action of Bisphenol and Bisphenol-A Mediated by Estrogen Receptor alpha in Growth and Apoptosis of Breast Cancer Cells

Work Accomplished:

Earlier studies from our laboratory have identified that the amino acid aspartate at 351 (which is in the helix 3 (H3)) of the ERŭLBD is critically important for maintaining the integrity of antiestrogenic activity of keoxifene (RAL) and 4OHT [61,152]. Earlier, the mutation of ERŭ encoding amino acid 351which substituted the aspartate to tyrosine amino acid was detected in one of the xenograft tumors stimulated by tamoxifen in the athymic mice [153]. Further investigations have revealed that changing the amino acid aspartate 351of the ERŭto glycine (D351G) abolishes the estrogenic effect of 4OHT but does not affect estradiol action on TGFŭgene activation in the ER negative breast cancer cells stably transfected with either wild type ERŭ or D351G mutated ERŭ [62]. Using these models, estrogens were classified as either type I, which have the planar structures or type II, which have the angular or non-planar structures [56,154]. A recent confirmatory study evaluated the ability of several type-I and II liganded ERŭto associate with the specific peptide motif ñLXXLLò which co-activators use to interact with the ERŭ[53].
A previous study [49] from our laboratory indicated that the conformation of the ERŭcomplex can govern the estrogen-induced apoptosis in the MCF7:5C breast cancer cells. The present study dissects the ERŭmediated effect of two structurally similar estrogenic ligands, namely, bisphenol (BP) and bisphenol-A (BPA) (Fig. 2j-1), on two critical physiological responses, *i.e.* growth and apoptosis in the breast cancer cells. BP is structurally related to 4OHT with E2-like agonistic properties, whereas BPA has been characterized as an endocrine disruptor with weak estrogenic properties. Using various investigative tools, this study underscore the fact that minor difference in the shape of the ERŭ-liganded complex has profound modulation on estrogen-induced apoptosis but not on estrogen-induced replication of breast cancer cells.

Differential effect of Bisphenol and Bisphenol-A in inducing apoptosis in MCF7:5C cells but not growth in MCF7 cells

BP (Fig. 2j-1) a tri-phenylethylene (TPE) is a known partial estrogenic ligand which can induce growth of the ERŭpositive breast cancer cells [15] and can also partially initiate prolactin synthesis from primary culture of cells from immature rat pituitary glands [36]. Another compound with similar chemical structure, bisphenolï A (BPA) (Fig. 2j-1) is also a well characterized but weak estrogenic ligand [155]. Here we evaluated the ability of these two estrogenic compounds to induce growth and apoptosis in MCF7 and MCF7:5C cells respectively, as both these responses are dependent on estrogen agonistic action. As expected, BP as well as BPA was able to induce the concentration dependent growth in the MCF7 cells (Fig. 2j-2A). BPA was less potent compared to

BP as maximal growth was achieved by BP at 10^{-9}M concentration as compared to 10^{-6}M for BPA. By comparison, E2 induced maximal growth at 10^{-11}M concentration in the MCF7 cells. In the case of MCF7:5C cells, which undergo apoptosis with E2 treatment [10,83], a marked contrast was observed between BP and BPA in the induction of apoptosis. BPA was able to induce apoptosis to the same extent as E2 in these cells at a higher (10^{-6}M) concentration (Fig. 2j-2B) as compared to E2 which achieved maximal effect at 10^{-10}M. However, BP failed to induce apoptosis even at 10^{-5}M concentration (Fig. 2j-2B). We further investigated that if BP was actually binding to the ERŬin the MCF7:5C cells by treating these cells with BP in combination with 10^{-9}M of E2. BP was able to block the effect of E2 in the MCF7:5C cells (Fig. 2j-2C) in a concentration dependent manner indicating that the effect of BP was through the ERŬ, thus inhibiting the E2 action. On the other hand, BPA was not able to block the effect E2 action (Fig. 2j-2C). In addition, we also show that the estrogenic effect of BPA (10^{-6}M) in inducing apoptosis in MCFF7:5C cells was completely blocked by BP (10^{-6}M) as well as 10^{-6}M of 4OHT (Fig. 2j-2D).

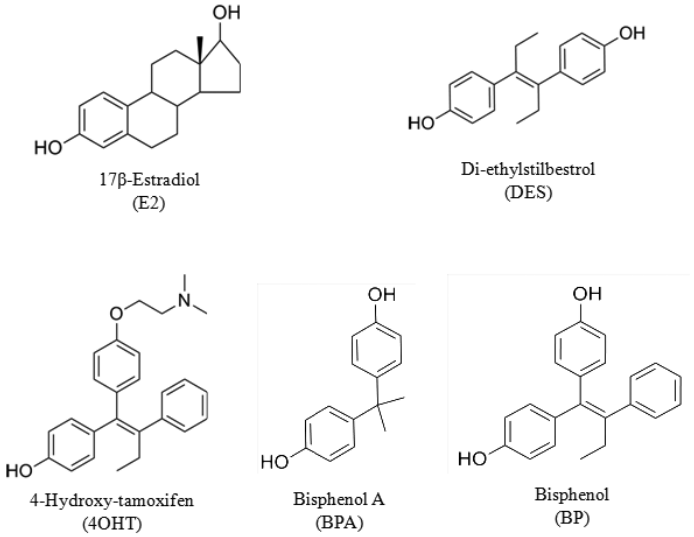


Figure 2j-1. *Chemical structures of 17β-estradiol (E2), Di-ethylstilbestrol (DES), 4-Hydroxy tamoxifen (4OHT), Bisphenol (BP) and Bisphenol-A (BPA).*

Regulation of estrogen responsive gene trefoil factor 1(TFF1 or PS2) by bisphenol and bisphenol-A

We next investigated the transcriptional regulation of a well characterized estrogen-regulated gene, TFF1 (PS2) [156] by BP and BPA and compared it with E2 and 4OHT. MCF7 cells were treated for 4 hours with the 0.1% ethanol (veh), E2 (10^{-9}M), 4OHT (10^{-6}M), BP (10^{-6}M and 10^{-5}M) or BPA (10^{-6}M and 10^{-5}M) and the transcripts levels of PS2 gene were measured using real-time PCR. Two different concentrations (10^{-6}M and 10^{-5}M) were used for BP and BPA because BPA is a weak estrogen and we wanted to evaluate the concentration dependent regulation of these compounds. As expected, PS2 mRNA was up-regulated around five fold by E2 (10^{-9}M) compared to vehicle treatment and 4OHT (10^{-6}M) which completely failed to induce the levels of PS2 mRNA (Fig. 2j-3A). On the other hand, BP treatment at 10^{-6}M concentration moderately (~2 fold) up-regulated the PS2 mRNA levels and higher concentration (10^{-5}M) of BP failed to further increase

Figure 2j-2. ***Differential effect of bisphenol (BP) and bisphenol-A (BPA) on growth and apoptosis of ERα positive breast cancer cells. A.*** *Dose dependent effects of BP, BPA and (estradiol) E2 on growth of MCF7 cells treated for six days as indicated. The black bar denotes the level of DNA in vehicle treated cells over a six day period. The growth is measured as amount of DNA present in each well. (*p<.05 versus vehicle treatment)* ***B.*** *Dose dependent effect of BP, BPA and E2 on apoptosis of MCF7:5C cells treated for six days as indicated. The black bar denotes the level of DNA in vehicle treated cells over a six day period. The growth is measured as amount of DNA present in each well. (*p<.05 versus vehicle treatment)****C.*** *Dose dependent effect of BP and BPA on E2 (1nM)-induced apoptosis in MCF7:5C cells, treated over a six day period. The growth is measured as amount of DNA present in each well. (*p<.05 versus 1nM E2 treatment)* ***D.*** *Effect of BP (10^{-6}M) and 4OHT (10^{-6}M) on BPA (10^{-6}M) induced apoptosis in MCF7:5C cells over six day period. (*p<.05 versus vehicle treatment; # p<.05 versus BPA treatment) The data is presented as percent of growth considering the vehicle treated cells as 100 percent. Each value is average of at least three replicates +/- S.D.*

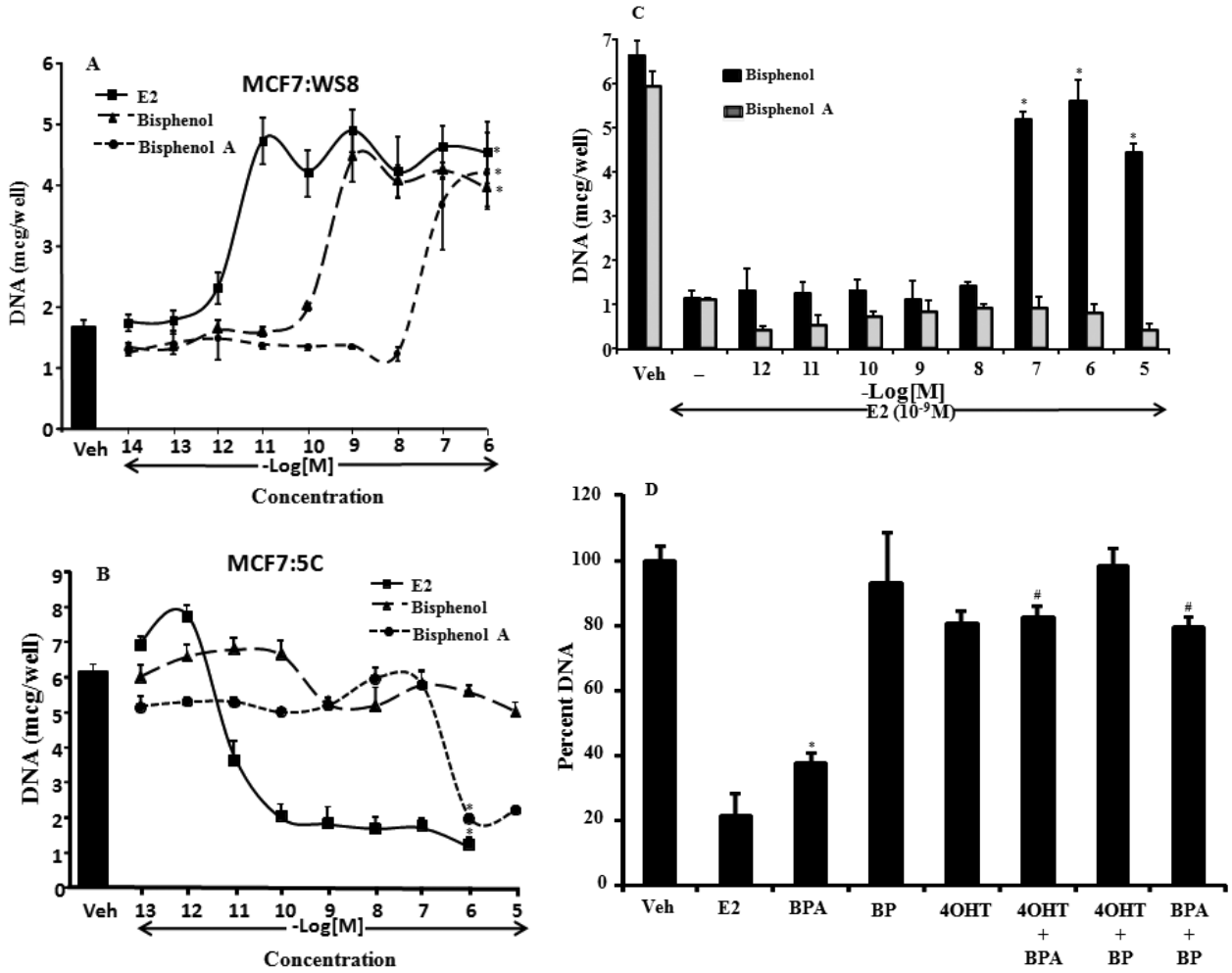
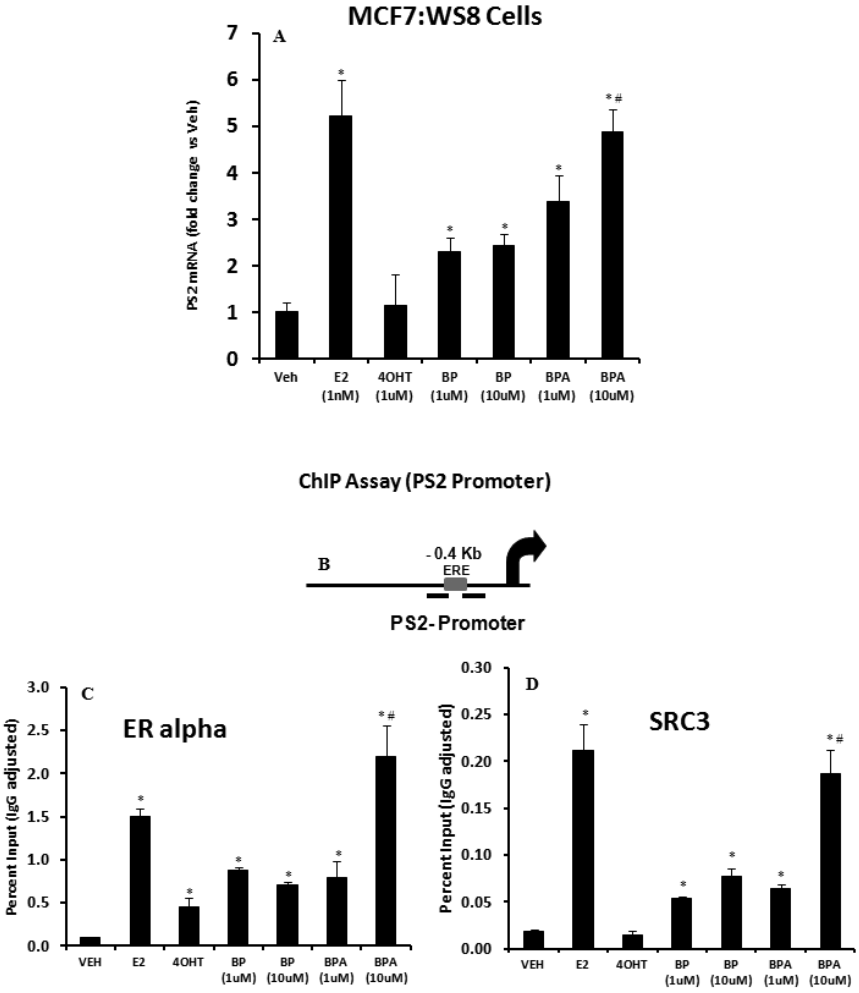


Figure 2j-3. ***Regulation of PS2 (TFF1) gene by bisphenol (BP), bisphenol-A (BPA) compared with 17-beta estradiol (E2) and 4-hydroxy-tamoxifen (4OHT) and recruitment of estrogen receptor alpha (ER alpha) and steroid receptor co-activator-3 (SRC3) at the estrogen responsive element (ERE) of proximal promoter of PS2 gene followed by 45 minutes treatments of bisphenol (BP), bisphenol-A (BPA) compared with 17-beta estradiol (E2) and 4-hydroxy-tamoxifen (4OHT) in MCF7 cells. A.*** *MCF7 cells were treated with indicated treatments for 4hrs and harvested for total RNA. Total RNA was reverse transcribed and assessed for PS2 gene expression levels using real time PCR. 36B4 gene was used as an internal control. All values are represented in terms of fold difference versus vehicle treatment. (*p<.05 versus vehicle treatment; #p<.05 versus 1µM BPA and 10µM BP treatment)* ***B.*** *Schematic representation of the PS2 proximal promoter containing an ERE (grey box) and the black bars represent the primers used for RT-PCR.* ***C.*** *Recruitment of ERα at the PS2 proximal promoter, by ChIP assay after 45 minutes of indicated treatment. (*p<.05 versus vehicle treatment; #p<.05 versus 1µM BPA and 10µM BP treatment)* ***D.*** *Recruitment of SRC3 at the PS2 proximal promoter, by ChIP assay after 45 minutes of indicated treatment. All the values are representated as percent input of the starting chromatin material and after subtracting the IgG control for each sample. (*p<.05 versus vehicle treatment; #p<.05 versus 1µM BPA and 10µM BP treatment)*



the levels of PS2 (Fig. 2j-3A). Conversely, cells treated with BPA exhibited concentration dependent increase in up-regulation of the PS2 mRNA and the magnitude of up-regulation with high concentration (10^{-5}M) of BP was equivalent to the E2-mediated up-regulation of PS2 mRNA (Fig. 2j-3A).

Recruitment of ERα and SRC3 at the promoter of TFF1 gene after treatment with BP and BPA

To understand the differences in the molecular mechanism of the transcriptional activation of PS2 gene in vivo by BP and BPA in comparison to E2 and 4OHT treatment we performed chromatin immuno-precipitation (ChIP) assay to evaluate the recruitment of ERŬ and SRC3 at the promoter region of TFF1 (PS2) gene (Fig. 2j-3B) which has a well characterized functional estrogen responsive element (ERE) [157]. MCF7 cells were treated with either 0.1% ethanol (veh), E2 (10^{-9}M), 4OHT (10^{-6}M)**,** BP (10^{-6}M or 10^{-5}M) or BPA (10^{-6}M or 10^{-5}M) for 45 minutes and thereafter harvested for ChIP assay. The results (Fig. 2j-3C) reveal that both concentrations of BPA (10^{-6}M and 10^{-5}M) recruited ERŬ to the PS2 promoter with ERE in a concentration dependent manner which was equivalent to results obtained with E2 treatment. In contrast BP did not show a concentration related effect and the levels of ERŬ plateaued at 50% of either E2 or BPA (Fig. 2j-3C). Recruitment of the co-activator, SRC3 (AIB1), which plays a key role in transcriptional activation of several estrogen regulated genes including PS2 gene [158,159], followed the similar pattern as the ERŬ (Fig. 2j-3D). BPA treatment at both the concentrations (10^{-6}M or 10^{-5}M) recruited SRC3 in a concentration dependent manner to become equivalent to levels observed with E2 treatment whereas BP treatment (both concentration) plateaued at 50% of E2 or BPA recruitment levels (Fig 2j-3D). As expected, 4OHT treatment did not recruit SRC3 and was comparable to vehicle treatment. The ChIP data correlates very well with the observed pattern of transcriptional activation of PS2 gene (Fig. 2j-3A) under same treatment conditions.

Differential induction of transforming growth factor alpha (TGFα) gene by BP and BPA in MDA: MB-231 cells stably transfected with wild type ERα or D351G mutant ERα.

Previous studies from our laboratory have established an in vitro system to evaluate and differentiate the conformation of liganded ERŬ induced by planar and non-planar ligands [56]. Activation of TGFŬ gene in MDA: MB 231 cells stably transfected with wild type (wt) ERŬ (MC2 cells) or mutant ERŬ (JM6 cells, D351G; which has the aspartate substituted with glycine at amino acid 351), is used as a marker to distinguish the ERŬ interactions between planar and non-planar estrogen ligands [56]. We treated the MC2 and JM6 cells with increasing concentrations of BP and BPA and measured the TGFŬ induction in these cells. E2 was used as a positive control. In MC2 cells, (wt ERŬ), all the tested ligands induced TGFŬ transcripts level to similar levels (Fig. 2j-4A). Induction of TGFŬ by BPA was observed at higher concentrations whereas BP and E2 had similar effects (Fig. 2j-4A). On the other hand, in JM6 cells (mutant; D351G ERŬ), BP failed to induce TGFŬ transcription even at higher concentrations (Fig. 2j-4B), whereas E2 and BPA treatment induced TGFŬ (Fig. 2j-4B), although the maximal induction with BPA was observed at higher concentration (10^{-5}M) which was less than 50% of E2 treatment. We further confirmed that E2-induced TGFŬ stimulation in JM6 cells was completely blocked by BP and 4OHT in a dose dependent manner; whereas co-treatment of BPA in presence of E2 failed to inhibit it (Fig. 2j-4C).

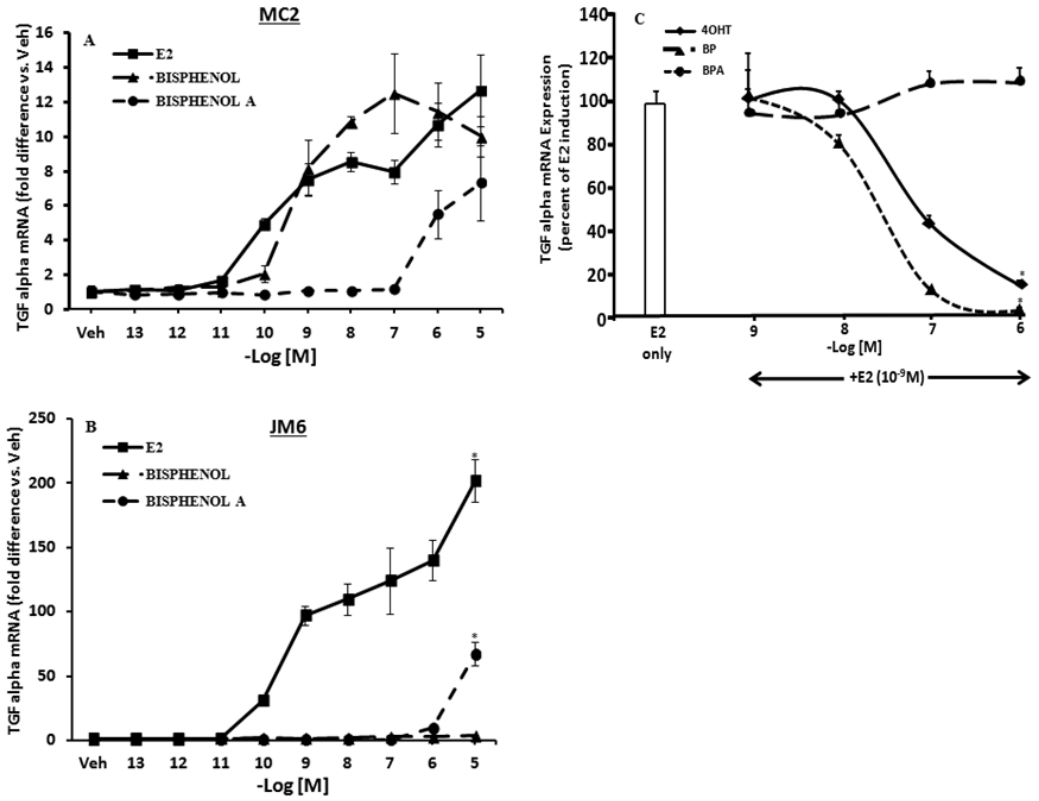


Figure 2j-4. ***Induction of TGFα mRNA by E_2, BP, and BPA in MDA:MB 231 cells stably transfected with wild type ERα (MC2 cells) or D351G mutant ERα (JM6 cells). A.*** *MC2 cells were treated with 17β estradiol (E2), bisphenol (BP) or bisphenol-A (BPA) at indicated concentration for 48 hrs and cells were harvested for total RNA. Total RNA was reverse transcribed and real time PCR (RTPCR) was performed to assess the expression of TGFα using 36B4 as an internal control. The values are presented as fold difference versus vehicle treated cells.* ***B.*** *JM6 cells were treated with 17β estradiol (E2), bisphenol (BP) or bisphenol-A (BPA) at indicated concentrations for 48 hrs and cells were harvested for total RNA. Total RNA was reverse transcribed and real time PCR (RTPCR) was performed to assess the expression of TGFα using 36B4 as an internal control. The values are presented as fold difference versus vehicle treated cells. (*p<.05 versus10^{-5}M BP treatment)* ***C.*** *JM6 cells were treated with E2 alone or in combination with different concentration of bisphenol (BP), bisphenol-A (BPA) or 4-hydroxy tamoxifen (4OHT) as indicated for 48 hrs. The values are presented as percentage of expression of TGFα mRNA considering the E_2-induced levels as 100 percent. (*p<.05 versus 1nM E2 and 1nM E2 +10^{-6}M BPA treatment)*

Molecular docking of BP and BPA to the LBD of ER alpha

To determine the binding mode of BPA and BP to ERŭ, the ligands were docked to the agonist and antagonist conformations of the receptor. The experimental structure, 3ERT, was selected from PDB for the antagonist conformation of ERŭ (Fig. 2j-5A) containing 4OHT, while for the agonist conformation two experimental structures were selected, namely the receptor co-crystallized with E2, 1GWR (Fig. 2j-5B) and DES, 3ERD (Fig. 2j-5C), respectively.

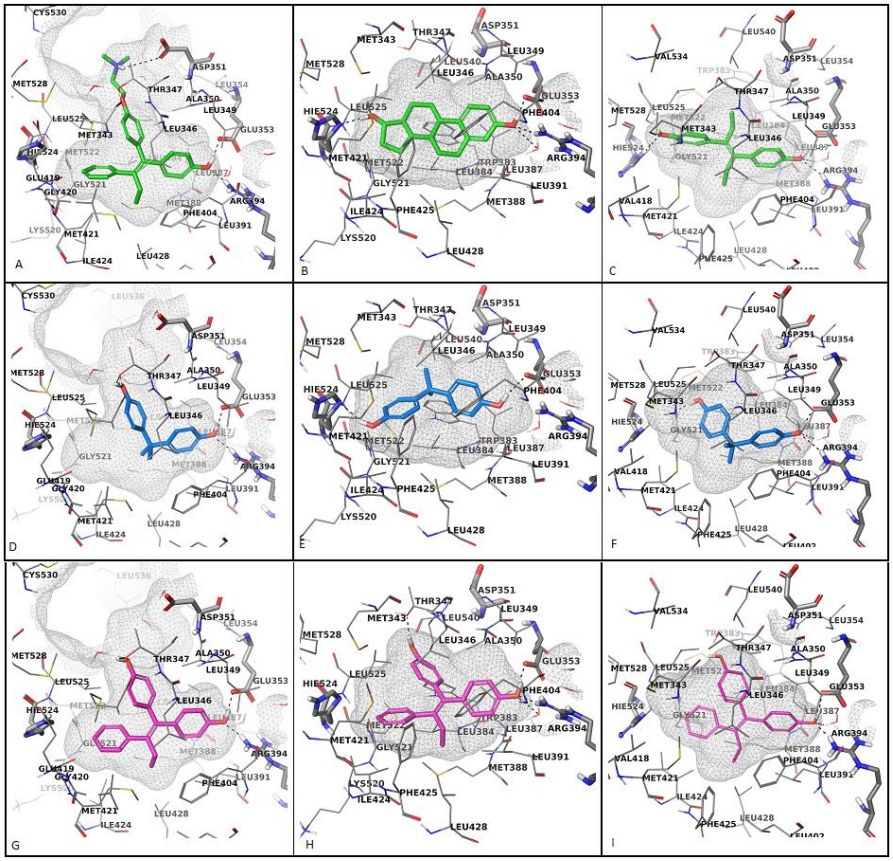


Figure 2j-5. ***Molecular docking of BP and BPA with ERα ligand binding domain.*** *Cross-sectional representations of ERα binding sites in the antagonist (**A**) with 4OHT and agonist (**B, C**) with 17β estradiol and DES conformations. The top ranked docking poses of BPA into the binding site of 3ERT (**D**), 1GWR (**E**), 3ERD (**F**) are displayed with C atoms colored in magenta while the best docking solutions of BP computed for 3ERT (G), 1GWR (**H**), 3ERD (**I**) are represented with C atoms colored in blue. The amino acids involved in H-bond contacts are depicted as sticks and the rest of the amino acids lining the binding site are shown as lines having the C atoms colored in gray. Only polar hydrogen atoms are shown, for simplicity.*

When BPA is docked to the antagonist conformation, 3ERT, it is oriented perpendicular with the binding pocket and in this alignment it has the propensity to form the H-bond network involving E353, R394 and a water molecule (Fig. 2j-5D). Additionally, a hydrogen bond with the hydroxyl group of T347 is formed. In this alignment the binding site is poorly occupied and the hydrophobic contacts with the amino acids lining the bottom of the binding site are missing.

In the case of BPA two highly probable binding modes have been identified. The first one has been mostly predicted when the ligand has been docked into the binding sites of ERŭ co-crystallized with E2 and DES, the structure 3ERD_Ůusing the SP mode. The ligand is placed across the binding site in a similar orientation with the native ligands, having the two methyl groups involved in hydrophobic contacts with the side chains of amino acids W383, L384, L525, and L540. Also, BPA forms H-bonds with H524 and E353 (Fig. 2j-5E). When docking calculations have been run in the XP mode of Glide a second alignment of the top tanked poses in the binding site of 3ERD_Ůand 3ERD_ŭ has been noticed. This orientation involves the formation of H-bonds between the hydroxyl groups of BPA and amino acids G521, E353 and R394 (Fig. 2j-5F). Apart

from the H-bonds formation, the methyl groups are involved in hydrophobic contacts with amino acids L346, F404, and L428. Also, this binding mode has been encountered for 6 out of 10 poses resulted from the docking of BPA into the experimental structure 1GWR.

The predicted binding modes of BP to the open and closed conformation of ER are similar, forming the H-bond network between E353, R394 and the highly ordered water molecule and an additional H-bond with the hydroxyl group of T347 (Fig. 2j-5G, 2j-5H, 2j-5I). The composite score, Emodel, shows that BP is better accommodated in the binding site of the open or antagonist conformation of ERŬand it is more likely for the ligand to bind at this conformation of ER. Similar results have been obtained using the Induced Fit docking method, which accounts for both the ligand and protein flexibility [15].

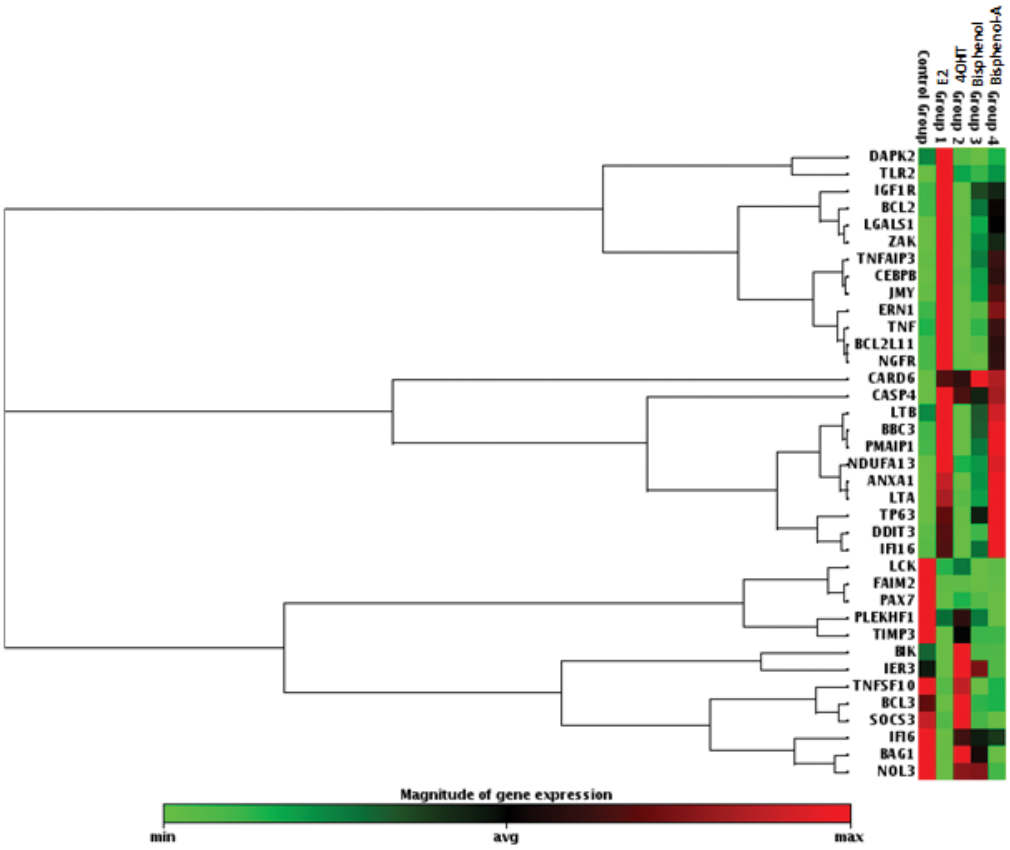
The comparative analysis of the composite score Emodel for the agonist and antagonist top ranked docking poses of BPA has shown that the binding mode predicted for the antagonist conformation is highly improbable and it is more likely for BPA to bind to a conformation of ERŬ closely related with the agonist one. Two distinct binding modes of BPA to the agonist conformations of ERŬ have been predicted with tight Emodel scores and cannot be clearly discriminated which alignment is correct or at least with the highest probability of being right. The docking scores calculated for E2, DES and BPA shows the binding affinity of BPA to ERŬis much lower when compared with the binding affinities of E2 or DES to ERŬ.

Comparative analysis of regulation of apoptotic genes by BP, BPA, 4OHT and E2 in MCF7:5C cells using apoptotic gene RT-PCR profiler

We thereafter determined the effect of BP and BPA treatment in regulating the apoptosis related genes in MCF7:5C cells and compared it with E2 and 4OHT as a positive and negative inducer of apoptosis respectively. We used the RT-PCR profiler assay kits for apoptosis from a commercial vendor which uses 384 well plates to profile the expression of 370 apoptosis related human genes (Qiagen; SABiosciences Corp, Fredrick, MD; Cat#330231 PAHS-3012E). To select a single time point of treatment with the ligands we first treated the MCF7:5C cells with E2 (10^{-9}M) for 24, 48 and 72 hrs (in triplicates) and created an apoptotic gene signature throughout these time points after comparing them with vehicle treatment. This gene signature was generated by comparing the expression level of all the genes with vehicle treatment and selecting the genes which were at least 2.5 fold over-expressed or under-expressed as compared to vehicle treated cells. The fold change was calculated by delta-delta Ct method using the web based tool, RT^2 profile PCR array data analysis version 3.5 (Qiagen; SABiosciences Corp, Fredrick, MD).

After carefully analyzing the gene list generated by E2 treatments over the above said time period we selected 48 hrs as the time point to treat MCF7:5C cells with BP, BPA and 4OHT and compare the expression of the apoptosis related genes with the gene signature of the E2 treatment at 48 hrs. This particular time point was selected because the MCF7:5C cells undergo apoptotic changes after E2 treatment during this time period [83] and also because after 48 hrs of E2 treatment the cells are committed to apoptosis, as 4OHT treatment cannot rescue these cells after this time point (unpublished observations).

Figure 2j-6. ***Heat map of apoptotic genes which are at least 2.5 fold up- or down-regulated by 48 hrs of treatment of 17-β estradiol 10^{-9} M (E2), versus vehicle and its relative comparison of their expression with 4-hydroxy tamoxifen, 10^{-6} M (4OHT), bisphenol, 10^{-6} M (BP) and bisphenol A, 10^{-6} M (BPA) treatment after 48 hrs in MCF7:5C cells.*** *The maximum expressed level of any given gene is represented by red color and minimum levels are presented as green color. Control group and group 1, 2, 3, 4 are the re-presentation of the vehicle, E2, 4OHT, BP and BPA treatments respectively. The gene expression levels in each treatment group are the average of three independent biological replicates.*



Next, we analyzed the changes in the overall expression profiles of apoptotic genes by E2, 4OHT, BP, and BPA versus vehicle (Veh) treatment at 48 hrs using the same apoptosis RT profiler. For any gene to be considered as differentially expressed we set the cut-off as 2.5 fold up- or down-regulation versus the vehicle treatment. Using this criterion we created a gene-list for up-regulated and down-regulated genes for each treatment group. We thereafter generated a heat map (Fig. 2j-6) in which we selected all the genes which were at least 2.5 fold up- or down-regulated by E2 treatment and compared it with other ligand treatments. This heat map clearly demonstrates that the genes which are up-regulated at least 2.5 fold after 48 hrs of E2 treatment are not up-regulated in 4OHT or BP treatment. In contrast, the majority of the genes up-regulated by BPA treatment were shown to be the same genes up-regulated by the E2 treatment. Many of these genes are up-regulated by BPA to the similar extent as E2 and others show a distinct trend of over-expression as compared to vehicle (Fig. 2j-6). Nevertheless, down-regulated genes follow a different pattern. The pattern of

genes down-regulated by BP treatment resembles the pattern observed with E2 and BPA treatment and not with the pattern of 4OHT treatment (Fig. 2j-6). Approximately 53% and 61% of down-regulated genes are in common with E2 treatment and with the treatment of BP and BPA respectively (Supplementary Table, ST 2).

Discussion:

The chemical structures of the ligands which bind to ERŬ are critical in determining the biological effects in the estrogen responsive cells and tissues. Minor changes in the ligand structures can alter the way these ligands interact with the ERŬ protein and transform the conformation of the liganded ï ERŬ complex in the cells. Structure-function relationships have been studied extensively using various biological endpoints, such as modulation of prolactin gene expression in primary cell cultures of rat pituitary glands [36,37,38], or TGFŬ activation in stably transfected wt and mutant ERŬ in MDA:MB 231 cells [56]. The current study dissects, compares and contrasts the mechanism of action of BP and BPA, two structurally similar ligands of ERŬ, which have opposing effects on apoptosis but not on the growth of estrogen responsive breast cancer cells.
The results of this study established that unlike BPA and E2, BP was not functioning as an estrogen-agonist in inducing apoptosis in MCF7:5C cells while both compounds (BPA and BP) were estrogenic in inducing growth in MCF7 cells. This clearly indicated differential requirement of ERŬ mediated molecular action to achieve two distinct physiological responses in the breast cancer cells. Activation of estrogen responsive gene PS2 by these compounds in MCF7 cells suggested that higher concentrations of BPA was as effective as E2 but BP treatment failed to achieve E2-like stimulation, even with higher concentration. This phenomenon was observed because BP has a high ERŬ binding affinity and can maximally induce PS2 gene at lower concentration and raising the concentration did not enhance the induction because it failed to recruit sufficient co-activator (SRC3) at the PS2 gene promoter. This was most likely due to insufficient ERŬ recruitment at the promoter and inaccessibility of the co-activator interacting surface of BP liganded ERŬ. A recent study [53] however suggested that BP-liganded ERŬ cannot bind to a peptide containing the co-activator interacting domain. This discrepancy can be attributed to the fact that our studies were performed in live cells chromatin as opposed to using an *in vitro* ELISA based system. This indicates that binding of liganded ERŬ and its interaction with other co-regulators can be modulated by other factors involved in transcriptional complex.

On the other hand BPA at higher concentration engaged SRC3 to a similar level as E2 treatment. The fact that higher concentration of BPA was required to recruit ERŬ and SRC3 to the similar levels as E2 treatment is because itôs binding affinity with ERŬ is very low (RBA, 0.073) [155] and therefore higher concentrations of the ligand is required to drive the kinetics towards the activated state. In the case of BP, it has a strong binding affinity to the ERŬ (RBA, 96.0) [37] and therefore maximal activation is achieved at lower concentration and increasing concentration do not enhance the activation. Overall, these results indicate that binding mode of BPA and E2 are similar whereas BP might bind differently to ERŬ. Indeed, our molecular docking studies determined that BPA binds to the ERŬ in two possible ways, both similar to agonistic mode of binding. Also docking scores calculated in this study predicted very low binding affinity of BPA to ERŬ, which is in excellent agreement with previous reports [160,161,162]. In contrast, modeling studies suggested antagonistic mode of binding (as in 4OHT) for BP to the ERŬ. To confirm the molecular modeling we used a biological model system which can distinguish between planar and angular estrogen ligands [56,154] by measuring the transcriptional activation of TGFŬ in MDA:MB 231 cells stably transfected with wt ERŬ (MC2 cells) or mtERŬ (D351G) (JM6 cells). Results (Fig 2j-4B) show that BP treatment failed to activate TGFŬ transcription similar to 4OHT [56] in JM6 cells

whereas BPA treatment was similar to E2 action, albeit with lower potency. This consolidated our finding that the mode of action of BP is more like 4OHT rather than E2. Importantly, the structure of BP is identical to 4OHT except for the basic di-methylamine-ethoxy side chain. The absence of the side chain contributes towards the enhanced estrogenic properties of BP with AF-1 fully engaged in ER responses to stimulate growth, as H12 of the ERŬprotein liganded with BP may not be properly restrained. This contrasts with 4OHT or RAL, where the restricted structure of the co-activator-interacting interface for binding of SRC3 or other co-activators now has limited AF-1 and AF-2 activity for growth. Of note, 4OHT and BP liganded ERŬwas less efficiently recruited to the PS2 promoter ERE which may also contribute towards lesser recruitment of SCR3 for BP as recruitment of ERŬprecedes the co-activator binding [156].

The fact that SRC3 is essential for E2- induced apoptosis in the MCF7:5C cells [54] as well as E2-mediated growth of MCF7 cells [68] coupled with the findings of this study, leads to the hypothesis that the estrogen-mediated growth of MCF7 cells is more sensitive and can be induced even if the conformation of the liganded-ERŬ complex allows only partial interaction of co-activators as in case of BP binding. In contrast, complete and robust interaction of co-activator with the liganded-ERŬcomplex must be needed for rapid induction of apoptosis in MCF7:5C cells.

Indeed, using an ñapoptosisò pathway focused real-time PCR based profiler consisting of 370 genes, this study further illustrated that apoptosis related genes were similarly up-regulated by E2 and BPA treatments after 48 hrs of treatment whereas BP and 4OHT showed very few up-regulated genes and the TPE based compounds did not have a similar profile of up-regulated genes during this time frame. By comparing the gene list (Supplementary table, ST2), which includes all the genes up- or down-regulated at least 2.5 fold by the treatments, it is evident that 66% of up-regulated genes are common between E2 and BPA treatment, whereas only 8% genes are commonly up-regulated by BP or 4OHT treatment.

Interestingly, a different pattern was observed for the down-regulated genes as both BP and BPA treatment exhibited common down-regulated genes as E2 and distinctly different from 4OHT. This suggests that the conformational requirement of liganded- ERŬmay be different for up-regulation and down-regulation of genes. Furthermore, it indicates that the up-regulated apoptotic genes are responsible for triggering and executing apoptosis since up-regulated genes are differentially regulated by BP and BPA but not the down-regulated genes. These observations merits further investigations.

By employing structurally related ligands and using MCF7:5C and parental MCF7 cells we have demonstrated that depending upon the biological response the same molecule can function as an E2-antagonist or ï agonist respectively. Based on these data it is reasonable to speculate that genistein and related phytoestrogens may also induce apoptosis in MCF7:5C cells as their binding to ERŬLBD is similar as E2 and DES [163] and function as type I estrogens [154]. In conclusion, this study provides evidence that binding of ERŬ with different ligands that program conformational changes of the liganded-ERŬ, determines the transcriptional profile of the responsive genes by virtue of interaction with co-regulators.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2k (Fan and Jordan) - Studies carried out by Dr. Ping Fan in the Jordan laboratory at Georgetown University

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Modulating Therapeutic Effects of c-SRC Inhibitor via Estrogen Receptor and HER2 in Breast Cancer Cell Lines

Introduction

Estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) are two successful therapeutic targets in breast cancer. c-Src functions as an important adapter protein with ER and HER2, which validates it as an attractive target for the treatment of breast cancer. However, a very recent phase II clinical trial shows that a c-Src inhibitor, dasatinib, had limited single-agent activity in patients with HER2+ and/or hormone receptor (HR)+ advanced breast cancer*[164]* Therefore, a specific c-Src inhibitor, PP2, was utilized to block c-Src activity to identify targeted vulnerabilities affected by ER and HER2 in a panel of breast cancer cell lines (*i.e.* MCF-7, T47D, ZR-75-1, BT474, MCF-7:5C, MCF-7:2A, MDA-MB-231, Sk-Br-3, MCF-7/F, and T47D:C42). The antiproliferative effect of PP2 correlated with blocking c-Src mediated ERK/MAPK and/or PI3K/Akt growth pathways. The signaling pathways of c-Src were modulated by ER and HER2 in breast cancer cells. Inhibition of c-Src tyrosine kinase predominantly blocked ER negative breast cancer cell growth, particularly the triple (*i.e.* ER, PR, and HER2) negative cells. In contrast, ER negative Sk-Br-3 cells with highest HER2 phosphorylation were resistant to PP2, in which over-activated HER2 directly regulated growth pathways but not through c-Src. However, blocking c-Src recovered ER expression which made Sk-Br-3 cells regain responses to 4-hydroxytamoxifen. The majority of ER positive breast cancer cells were not sensitive to PP2 regardless of wild-type or endocrine resistant cell lines. Overall, c-Src mediates the essential role of growth pathways in ER negative breast cancer cells without HER2 over-activation. ER expression and HER2 over-activation are two important predictive biomarkers for the resistance to a c-Src inhibitor. These data provided an important therapeutic rationale for patient selection in clinical trials with c-Src inhibitors in triple negative breast cancer.

Work Accomplished:

Baseline levels of ER, HER2, and c-Src activation in a panel of breast cancer cell lines

We addressed the question whether expression of ER and growth factor receptors would affect the therapeutic effects of the c-Src inhibitors in breast cancer cells. To answer this question, a panel of wild-type (MCF-7, T47D, ZR-75-1, BT474, MDA-MB-231, and Sk-Br-3) and endocrine resistant (MCF-7:5C, MCF-7:2A, MCF-7/F, and T47D:C42) breast cancer cell lines were investigated. Baseline levels of ER, HER2, EGFR, and c-Src were measured by immunoblot analysis. They all keep their biological characteristics with differential levels of ER, PR, HER2, and EGFR (Fig. 2k-1A and 2k-1B). All cell lines expressed detectable levels of total c-Src, whereas they manifested different levels of phosphorylated c-Src (Fig. 2k-1C). Although there is no clear relationship between c-Src phosphorylation and HR expression (Fig. 2k-1D) after normalized by total c-Src among tested cell lines, interestingly, we observe that c-Src is activated in resistant cell lines compared with respective parental cell lines (MCF-7:5C, MCF-7:2A, and MCF-7/F versus MCF-7, T47D:C42 versus T47D).

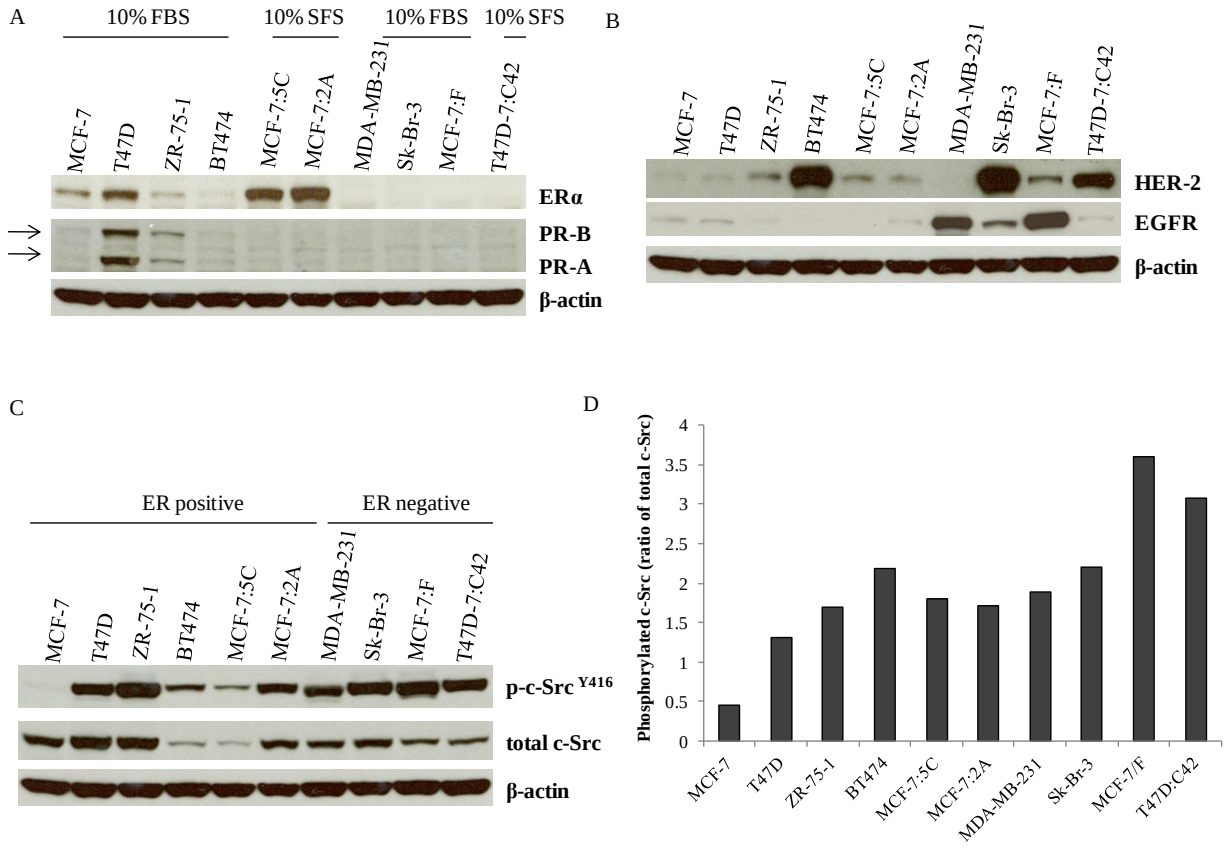


Figure 2k-1. ***Baseline expression of hormone receptors and growth factor receptors in different cell lines. 1A. Baseline ERα and PR expression levels in different cell lines.*** *MCF-7, T47D, ZR-75-1, BT474, MDA-MB-231, Sk-Br-3, and MCF-7/F cells were cultured in estrogenized medium (10% FBS). MCF-7:5C, MCF-7:2A, and T47D:C42 cells were cultured in phenol red free medium containing charcoal-stripped serum (10% SFS). Cell lysates were harvested. ERα and PR expression levels were examined by immunoblotting with primary antibodies. Immunoblotting for β-actin was determined for loading control.* ***1B. Baseline HER2 and EGFR expression levels in different cell lines.*** *Cell lysates were harvested as above. HER2 and EGFR expression levels were examined by immunoblotting with primary antibodies. Immunoblotting for β-actin was determined for loading control.* ***1C. Baseline c-Src phosphorylation in different cell lines.*** *Cell lysates were harvested as above. Phosphorylated c-Src and total c-Src were detected by immunoblotting with primary antibodies. Immunoblotting for β-actin was used for loading control.* ***1D. Quantification of phosphorylated c-Src by total c-Src.*** *Phosphorylated c-Src in different cell lines was quantified by the total c-Src using Quantity One software from Bio-Rad.*

Inhibitory effects of the c-Src inhibitor on ER positive wild-type breast cancer cells

All ER positive wild-type breast cancer cells were cultured in estrogenized medium. The specific c-Src inhibitor, PP2, effectively blocked phosphorylation of c-Src in all cell lines (Fig. 2k-2A). However, PP2 could not inhibit all cell growth (Fig. 2k-2B). T47D and BT474 cells were responsive to PP2 with 50% and 40% inhibition after 7 days treatment, respectively (Fig.2k-2B), whereas MCF-7 and ZR-75-1 cells were resistant to PP2 treatment (Fig.2k-2B). Further investigation showed that antiproliferative effects of PP2 were correlated with inhibition of ERK/MAPK and/or PI3K/Akt pathways. PP2 could not continuously block growth pathways in resistant cells such as MCF-7 and ZR-75-1 (Fig. 2k-2C). In contrast, PP2 effectively inhibited both signaling pathways in T47D and BT474 cells (Fig. 2k-2C).

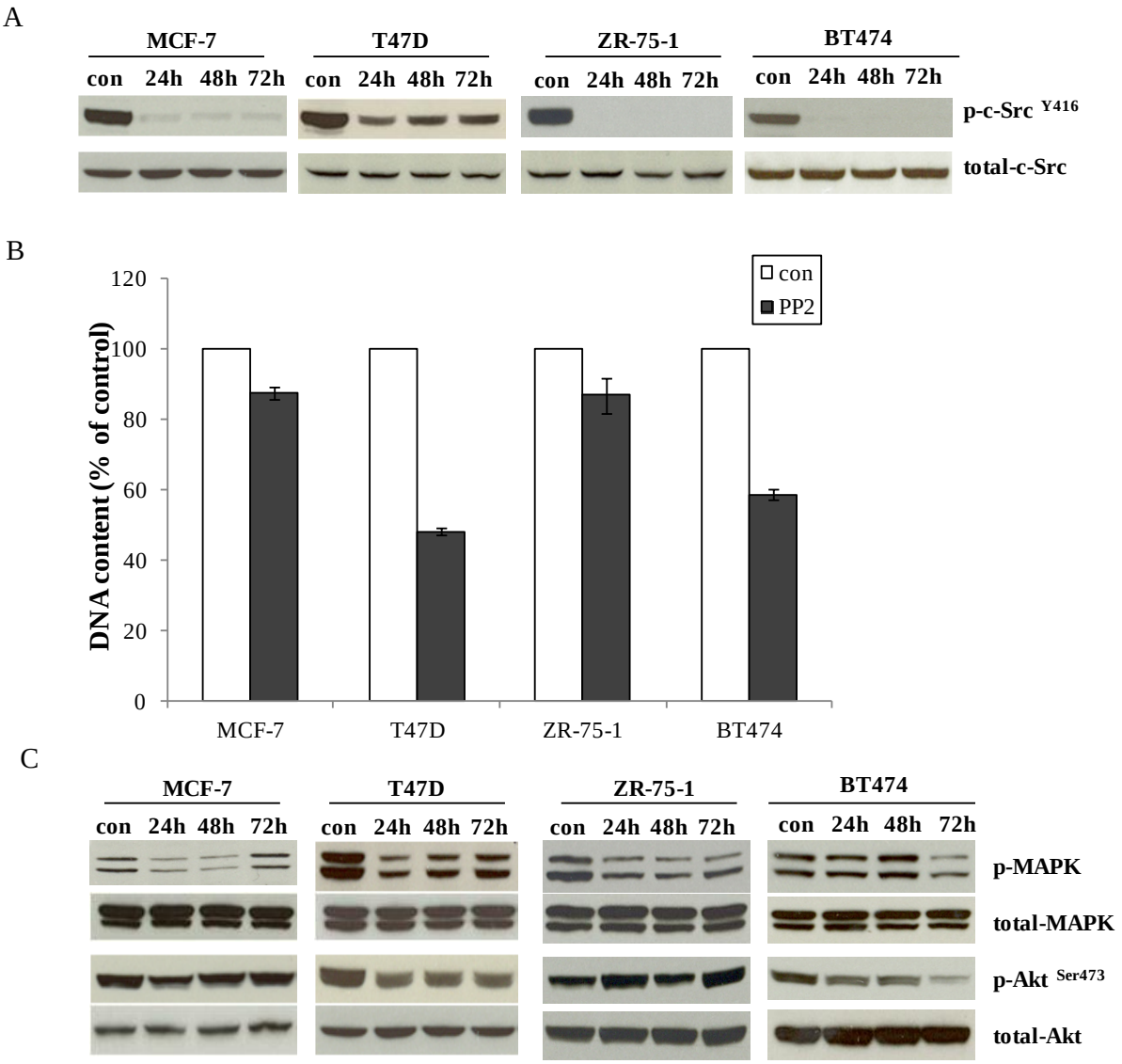


Figure 2k-2. ***Effects of the c-Src inhibitor on ER positive wild-type cell lines. 2A. Blocking c-Src phosphorylation in ER positive wild-type cell lines by PP2.*** *ER positive wild-type cells were treated with PP2 (5μM) in estrogenized medium at time points as indicated and cell lysates were harvested. Phosphorylated c-Src was detected by immunoblotting with primary antibody. Immunoblotting for total c-Src was used for loading control.* ***2B. Inhibitory effects of PP2 on wild-type ER positive cells.*** *Wild-type ER positive cells were seeded in 24-well plates in triplicate in estrogenized medium. After one day, the cells were treated with vehicle (0.1%DMSO) and PP2 (5μM) respectively. The cells were harvested after 7 days treatment and total DNA was determined using a DNA fluorescence quantitation kit.* ***2C. Signaling pathways changes in ER positive wild-type cells after PP2 treatment.*** *Cell lysates were harvested as above. Phosphorylated MAPK and Akt were examined by immunoblotting with primary antibodies. Immunoblotting for total MAPK and Akt were used for loading controls.*

Inhibitory effects of the c-Src inhibitor varied under conditions with or without basal E_2 in ER positive wild-type breast cancer cells.

Since basal estrogen levels in the culture medium affect the biological function of the ER positive wild-type breast cancer cells [165], we investigated inhibitory effects of the c-Src inhibitor on ER positive wild-type cells under conditions with (10% FBS) or without (10% SFS) basal estrogen. Two distinct modulations of c-Src phosphorylation existed in ER positive wild-type cells after short-term absence of

E_2. MCF-7 and ZR-75-1 cells had the same pattern with enhanced c-Src phosphorylation, conversely, c-Src phosphorylation was down-regulated in T47D and BT474 cells (Fig. 2k-3A). The PP2 effectively blocked c-Src phosphorylation in four wild-type breast cancer cells under conditions with 10% SFS (Fig. 2k-3B). However, inhibition by PP2 varied in ER positive wild-type cells under these two conditions (Fig. 2k-3C). MCF-7 cells were effectively responsive to PP2 under conditions without basal E_2 (10% SFS), conversely, T47D cells were completely resistant to PP2 in phenol red free medium (Fig. 2k-3C). Four ER positive wild-type breast cancer cells were stimulated by E_2 to grow with different sensitivity (Fig.2k-3D). Notably, PP2 could not block the proliferation induced by E_2 in MCF-7 and ZR-75-1 cells but partially abolished E_2 stimulation in T47D and BT474 cells (Fig. 2k-3D). These results indicated that c-Src might play a distinct role in mediating E_2 signaling in wild-type cells.

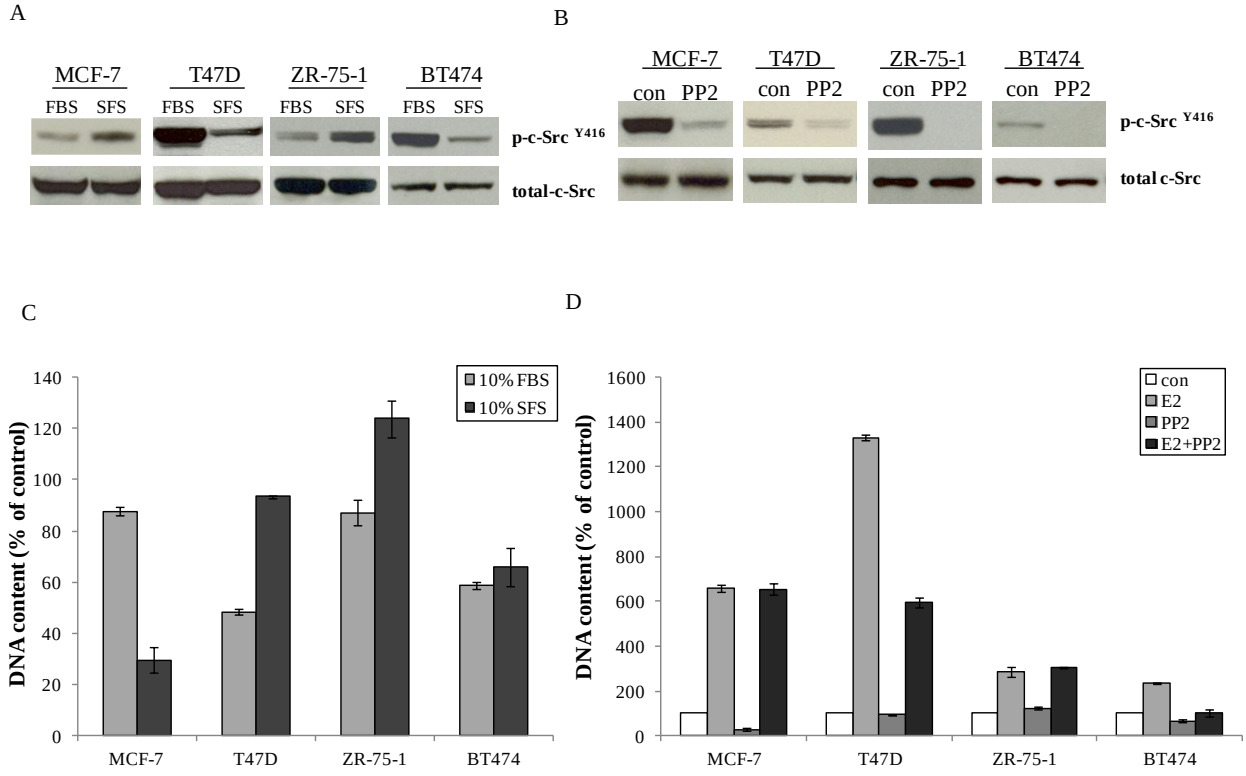


Figure 2k-3. ***Effects of the c-Src inhibitor on ER positive wild-type cell lines under conditions with or without basal E_2. 3A. c-Src phosphorylation changed after short-term absence of E_2 in ER positive wild-type cells.*** *Wild-type ER positive cells were cultured under conditions with basal estrogen (10% FBS) or without basal estrogen (10% SFS) for 3 days, respectively. Cell lysates were harvested. Phosphorylated c-Src was examined by immunoblotting with primary antibody. Immunoblotting for total c-Src was determined as loading control.* ***3B. Blocking c-Src phosphorylation in ER positive wild-type cell lines by PP2 under the conditions without basal estrogen.*** *Wild-type ER positive cells were cultured under the conditions without basal estrogen (10% SFS) for 3 days. Then cells were treated with PP2 (5μM) in 10% SFS medium for 24h and cell lysates were harvested. Phosphorylated c-Src was detected by immunoblotting with primary antibody. Immunoblotting for total c-Src was used for loading control.* ***3C.Growth inhibitory effects of PP2 on ER positive wild-type cells under conditions with or without basal E_2.*** *Wild-type ER positive cells were cultured under conditions with basal estrogen (10%FBS) or without basal estrogen (10% SFS) for 3 days, respectively. Then, they were seeded in 24-well plates in triplicate. After one day, the cells were treated with vehicle (0.1%DMSO) and PP2 (5μM) in estrogenized medium (10%FBS) or E_2 free medium (10%SFS), respectively. The cells were harvested after 7 days treatment and total DNA was determined as above.* ***3D. The PP2 had different effects on E_2 stimulation in ER positive wild-type cells.*** *Wild-type ER positive cells were changed to E_2 free medium for 3 days.*

Then, they were seeded in 24-well plates. After one day, the cells were treated with vehicle (0.1% EtOH), E_2 (10^{-9}mol/L), PP2 (5μM), and E_2 (10^{-9}mol/L) plus PP2 (5μM) respectively in E_2 free culture medium. The cells were harvested after 7 days treatment and total DNA was determined as above.

Effects of the c-Src inhibitor on ER positive long-term estrogen deprived breast cancer cells

In two long-term estrogen deprived breast cancer cells (MCF-7:5C and MCF-7:2A), that overexpress ER, PP2 could block c-Src activation (Fig. 2k-4A) and abolished about 25% of proliferation in MCF-7:5C cells but without any inhibition in MCF-7:2A cells (Fig.2k-4B). The inhibitory effects of PP2 were consistent with blocking growth pathways in different cells. Phosphorylated Akt was abolished in MCF-7:5C cells but without continuous inhibition of MAPK. PP2 could not continuously block both growth pathways in MCF-7:2A cells (Fig. 2k-4C). Our previous data showed that E_2 has therapeutic function to induce apoptosis in long-term E_2 deprived breast cancer cells [83]. We reasoned that a combination of PP2 with E_2 would enhance E_2-induced apoptosis. Surprisingly, PP2 did not enhance the growth inhibitory effects of E_2 on these two cell lines but blocked the growth inhibition induced by E_2 (Fig. 2k-4D). These data implied that E_2-triggered apoptosis might be utilizing c-Src tyrosine kinase as an important signaling pathway. We are currently investigating the mechanisms of how the c-Src inhibitor blocks E_2-triggered apoptosis.

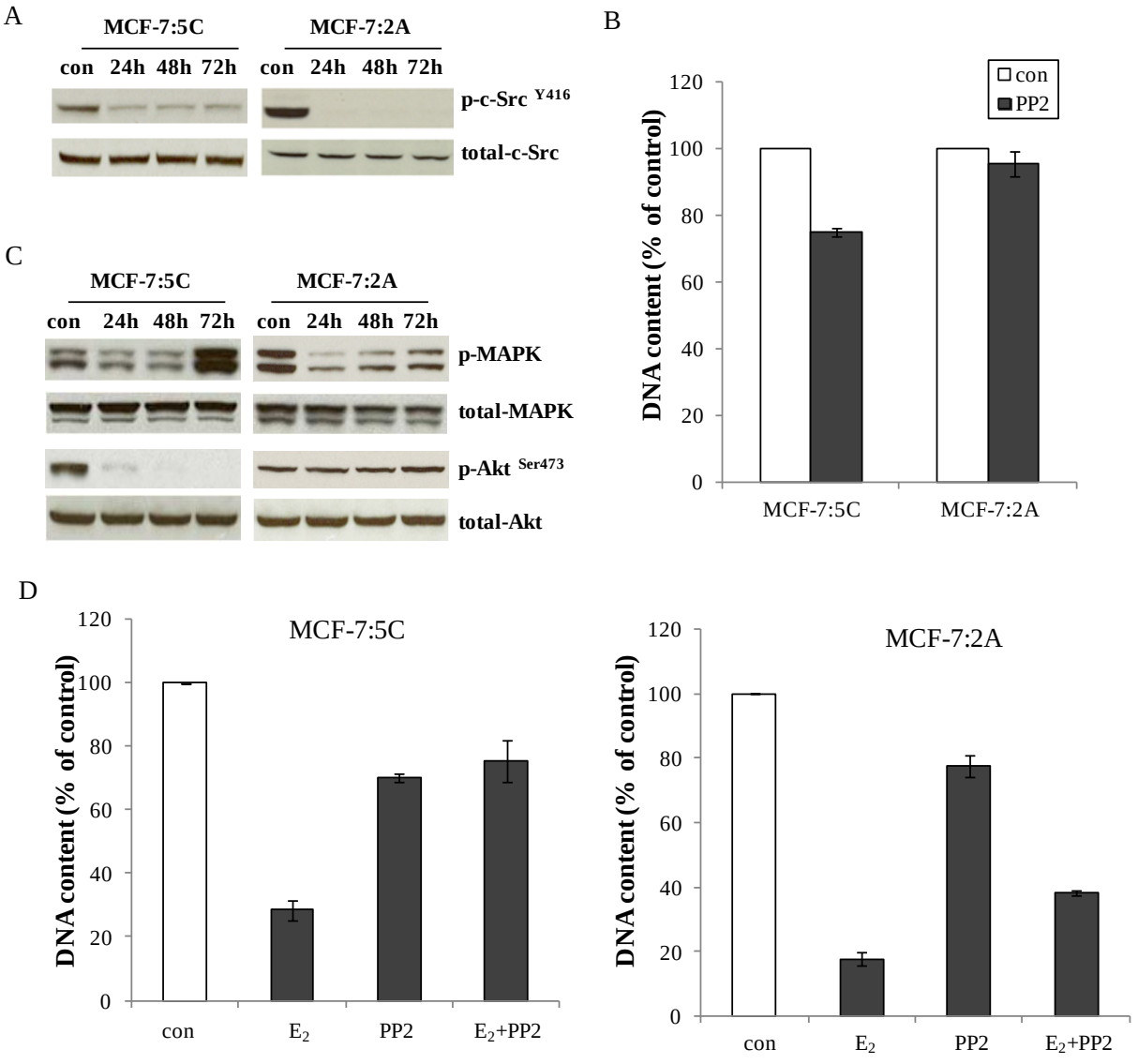


Figure 2k-4. ***Effects of the c-Src inhibitor on ER positive endocrine resistant cell lines. 4A. Blocking c-Src phosphorylation in endocrine resistant ER positive cells.*** *MCF-7:5C and MCF-7:2A cells were treated with PP2 (5μM) at time points as indicated and cell lysates were harvested. Phosphorylated c-Src was detected by immunoblotting with primary antibody. Immunoblotting for total c-Src was used for loading control.* ***4B. Growth inhibitory effects of PP2 on endocrine resistant ER positive cells.*** *MCF-7:5C and MCF-7:2A cells were seeded in 24-well plates in triplicate. After one day, the cells were treated with vehicle (0.1% DMSO) and PP2 (5μM) respectively in culture medium. The cells were harvested after 7 days treatment and total DNA was determined as above.* ***4C. Signaling pathways changes in endocrine resistant ER positive cells after PP2 treatment.*** *Cell lysates were harvested as above. Phosphorylated MAPK and Akt were examined by immunoblotting with primary antibodies. Immunoblotting for total MAPK and Akt were used for loading controls.* ***4D. The PP2 blocked E_2-induced inhibition in MCF-7:5C and MCF-7:2A cells.*** *MCF-7:5C cells were seeded in 24-well plates as above. After one day, the cells were treated with vehicle (0.1% EtOH), E_2 (10^{-9}mol/L), PP2 (5μM), and E_2 (10^{-9}mol/L) plus PP2 (5μM) respectively. The cells were harvested after 7 days treatment and total DNA was determined as above. MCF-7:2A cells were seeded in 6-well plates. After one day, the cells were similarly treated as in MCF-7:5C cells. The cells were harvested after 14 days treatment and total DNA was determined as above.*

The c-Src inhibitor effectively blocked ER negative breast cancer cell growth.

The inhibitory effects of the c-Src inhibitor, PP2, on ER negative breast cancer cell lines were examined in two wild-type MDA-MB-231 and Sk-Br-3 and two resistant cell lines MCF-7/F (ICI 182,780 resistance) and T47D:C42 (long-term estrogen deprived). PP2 blocked the phosphorylation of c-Src in all ER negative cells (Fig. 2k-5A). However, the growth inhibitory effects of the c-Src inhibitor were different. PP2 could inhibit 80% of cell growth in MDA-MB-231 cells. In contrast, PP2 exerted no inhibitory effects on Sk-Br-3 cells with HER2 overexpression (Fig. 2k-5B). Inhibition of c-Src could efficiently suppress around 60% of cell growth in both resistant cells, MCF-7/F and T47D:C42 (Fig. 2k-5B). The triple negative MDA-MB-231 cell line was the most sensitive to PP2. These results demonstrated that HER2 amplification might be an indicator for resistance to the c-Src inhibitors in clinical trials. Further investigation indicated that PP2 effectively blocked the MAPK and Akt pathways in the c-Src inhibitor sensitive cells, whereas MAPK and Akt phosphorylation were increased in Sk-Br-3 cells (Fig.2k-5C). The data implied that HER2 might drive the growth pathways in Sk-Br-3 cells.

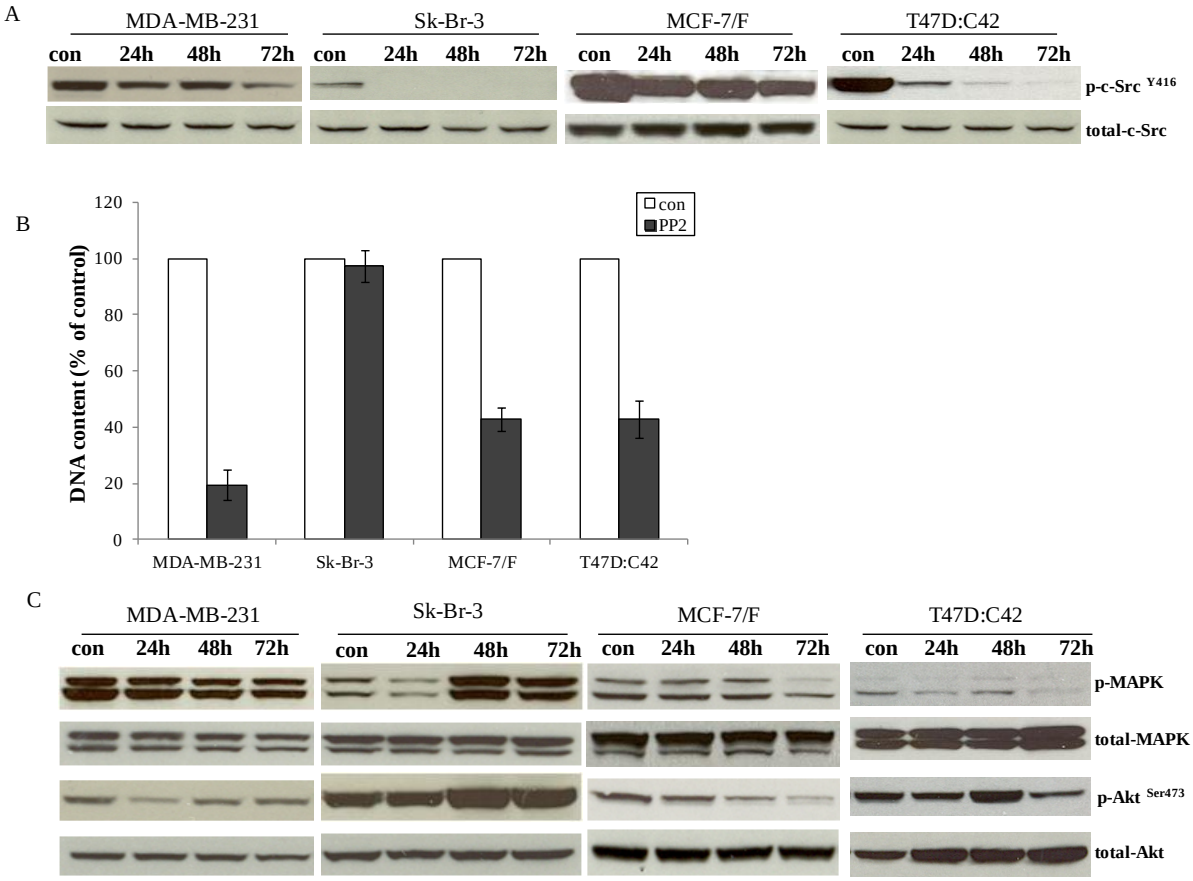


Figure 2k-5. ***Effects of the c-Src inhibitor on ER negative cell lines. 5A. Blocking c-Src phosphorylation in ER negative cell lines by PP2.*** *ER negative cells were treated with PP2 (5μM) for different times as indicated and cell lysates were harvested. Phosphorylated c-Src was detected by immunoblotting with primary antibody. Immunoblotting for total c-Src was used for loading control.* ***5B. Inhibitory effects of PP2 on ER negative cells.*** *ER negative cells were seeded in 24-well plates in triplicate. After one day, the cells were treated with vehicle (0.1%DMSO) and PP2 (5μM) in 10% SFS medium. The cells were harvested after 7 days treatment and total DNA was determined as above.* ***5C. Signaling pathways were changed in ER negative cells after PP2 treatment.*** *ER negative cells were treated with PP2 (5μM) for different times as indicated and cell lysates were harvested. Phosphorylated MAPK and Akt were examined by immunoblotting with primary antibodies. Immunoblotting for total MAPK and Akt were determined for loading controls.*

Activation status of HER2 determined the inhibitory effects of the c-Src inhibitor.

c-Src is known to bind to HER2 and is thus activated in HER2-overexpressing cancer cells. BT474 and Sk-Br-3 cells overexpress endogenous HER2 (Fig. 2k-1B), however, they had different responses to PP2 (Fig.2k-2B and 2k-5B). To examine whether HER2 activation affects the inhibitory rate of PP2, phosphorylation of HER2 was evaluated. Among tested cell lines, Sk-Br-3, BT474, and T47D:C42 cells had elevated though different levels of HER2 activation. As a control, HER2 was undetectable in MDA-MB-231 cells (Fig2k-.6A). HER2 was highly activated in Sk-Br-3 cells compared with BT474 cells which made it hypersensitive to lapatinib, a dual tyrosine kinase inhibitor of HER2 and EGFR (Fig.2k-6B). The growth inhibitory effects by lapatinib corresponded to the levels of phosphorylated HER2 (Fig. 2k-6B). We observed that HER2 hyper-activation rendered breast cancer cell completely resistant to PP2, the higher HER phosphorylation, the lower responsive rate to PP2 (Fig. 2k-6B). This was further confirmed by S phase changes through flow cytometric analysis (Fig. 2k-6C). Lapatinib reduced S phase in cells with higher HER2 phosphorylation, conversely, PP2 was effective in

cells with lower HER2 phosphorylation (Fig. 2k-6C). Lapatinibôs antitumor activity was associated with blocking phosphorylation of HER2 and the subsequent inhibition of its downstream signaling pathways (Fig. 2k-6D). Lapatinib blocked MAPK and Akt pathways in Sk-Br-3 and BT474 cells, but it exerted no inhihition in MDA-MB-231 cells, which demonstrated that antiproliferative effects of lapatinib also correlated with inhibitory ability of growth pathways.

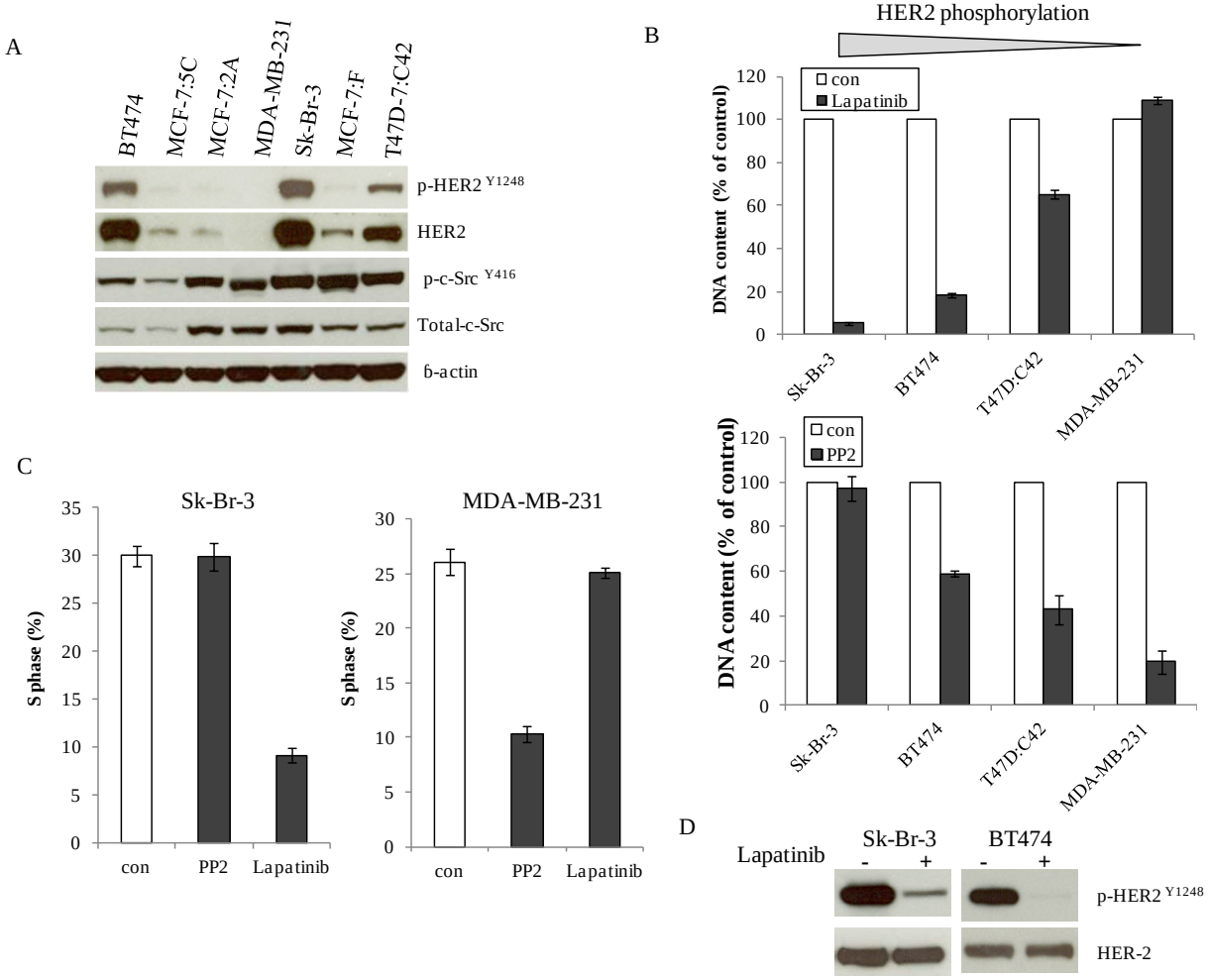


Figure 2k-6. ***Activation status of HER2 determined the inhibitory effects of the c-Src inhibitor. 6A. Baseline HER2 phosphorylation in different cell lines.*** *Cell lysates were harvested from different cells. Phosphorylated HER2 and total HER2 were examined by immunoblotting with primary antibodies. Immunoblotting for β-actin was determined for loading control.* ***6B. Inhibitory effects of the HER2 inhibitor and the c-Src inhibitor on cells with elevated HER2 phosphorylation.*** *Sk-Br-3, BT474, T47D:C42, and MDA-MB-231 cells were seeded in 24-well plates in triplicate. After one day, the cells were treated with vehicle (0.1%DMSO), lapatinib (1μM), and PP2 (5μM) in 10% SFS medium. The cells were harvested after 7 days treatment and total DNA was determined as above.* ***6C. S phase changes after lapatinib and PP2 treatment.*** *Sk-Br-3 and MDA-MB-231 cells were treated with vehicle (0.1% DMSO), lapatinib (1μM), and PP2 (5μM) for 24h. Cells were harvested and fixed with 75% EtOH. Cell cycles were analyzed through flow cytometery.* ***6D. Blocking HER2 phosphorylation after lapatinib treatment.*** *Sk-Br-3 and BT474 cells were treated with vehicle (0.1%DMSO) and lapatinib (1μM) for 24h. HER2 phosphorylation was examined by immunoblotting with primary antibody. Immunoblotting for total HER2 was determined for loading control.*

Blocking c-Src tyrosine kinase recovered ERα expression and reduced HER2 levels in ER negative Sk-Br-3 cells.

c-Src may drive estrogen-dependent ERŭ proteolysis in a subset of ER negative breast cancer ([147]. c-Src did not play a critical role in mediating growth pathways in Sk-Br-3 cells (Fig. 2k-5B). To study whether the c-Src inhibitor can regulate ER turn-over in breast cancer cells with HER2 amplification, we found that PP2 could recover ERŭ expression in Sk-Br-3 cells (Fig. 2k-7A). Real-time PCR analysis showed that mRNA levels of ERŭ was increased after PP2 treatment in Sk-Br-3 cells (Fig. 2k-7B) which implied that c-Src was involved in the regulation of ERŭ not only in the protein level but also at the transcription level. We further demonstrated that PP2 decreased HER2 levels in Sk-Br-3 cells after extending treatment time (Fig. 2k-7C). This result also implied a complicated feedback loop existed between c-Src and HER2 in Sk-Br-3 cells. Importantly, Sk-Br-3 cells acquired responses to 4-hydroxytamoxifen and ICI 182,780 after short-term treatment with PP2 (Fig. 2k-7D). Therefore, it is plausible that the simultaneous interruption of c-Src tyrosine kinase and targeting ER might be an effective treatment for breast cancer cells with HER2 amplification.

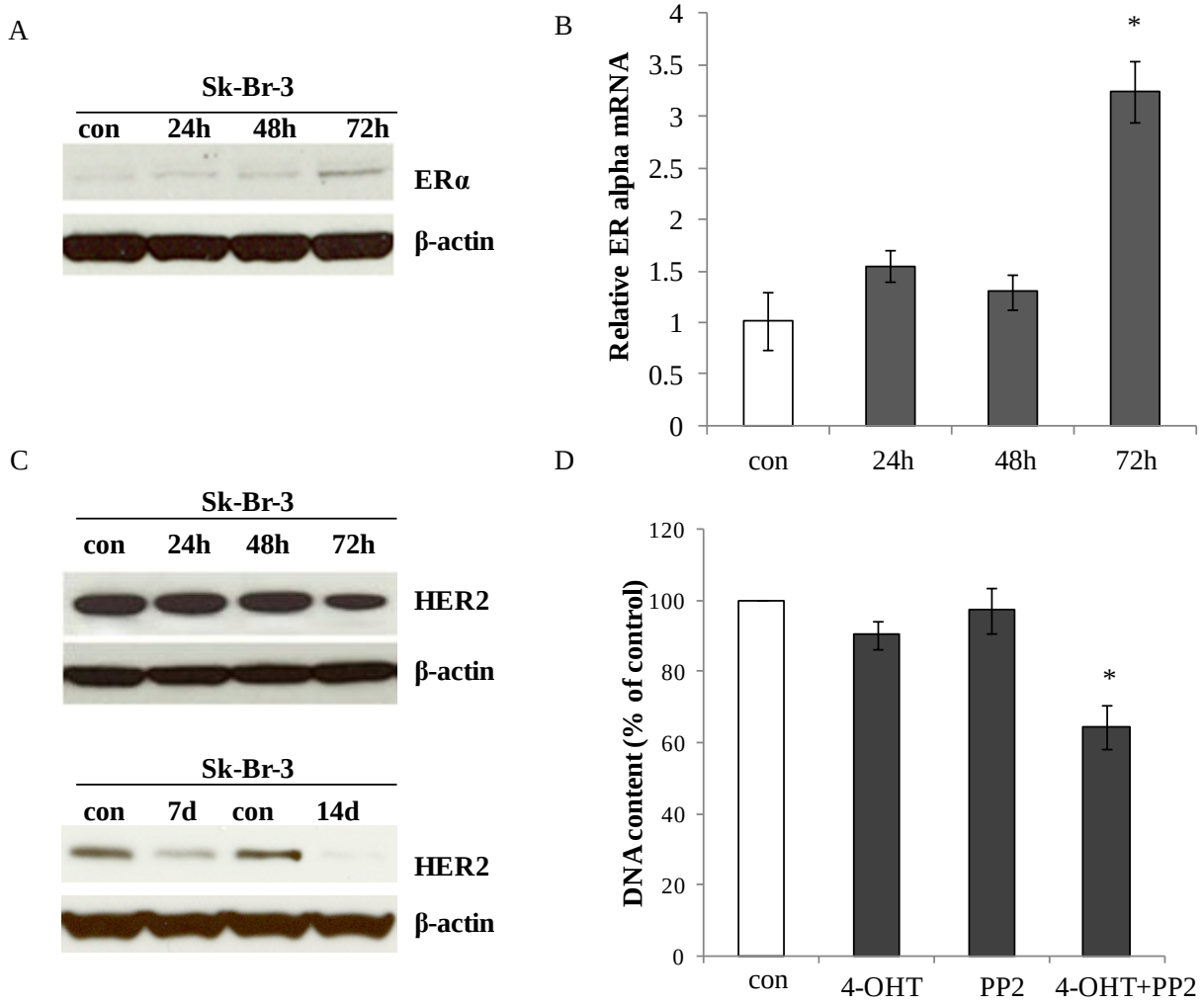


Figure 2k-7. Blocking c-Src sensitized cell to antiestrogen in Sk-Br-3 cells. *7A*. ERα expression was elevated in Sk-Br-3 cells after PP2 treatment. Sk-Br-3 cells were treated with PP2 as indicated. ERŭ expression was examined by immunoblotting with primary antibody. Immunoblotting for ϐ-actin was determined for loading control. ***7B*. ERα mRNA was increased in Sk-Br-3 cells after PP2 treatment.** Sk-Br-3 cells were treated with PP2 (5ε M) for the times as indicated. The RNA was harvested in TRIzol for real-time PCR analysis. $P<0.05$, * compared with control. ***7C*. HER2 expression was downregualted in Sk-Br-3 cells after PP2 treatment.** Sk-Br-3 cells were treated with PP2 for the times as indicated. HER2 was examined by immunoblotting with primary antibody. Immunoblotting for ϐ-actin was

determined for loading control. ***7D*. The PP2 sensitized Sk-Br-3 cells to 4-hydroxytamoxifen.** Sk-Br-3 cells were treated with vehicle, 4-OHT (1εϚ), PP2 (5εM), and 4-OHT (1εϚ) plus PP2 (5εM) in 10% FBS medium. The cells were harvested after 7 days treatment and total DNA was determined as above. $P<0.05$, * compared with control.

Discussion

We employed a panel of well characterized breast cancer cell lines (MCF-7, T47D, ZR-75-1, BT474, MDA-MB-231, and Sk-Br-3) and resistant cell lines (MCF-7:5C, MCF-7:2A, MCF-7/F, and T47D:C42) to identify biomarkers associated with the inhibitory actions of a specific c-Src inhibitor, PP2. PP2 blocked c-Src tyrosine kinase activity in all cell lines tested. However, the antiproliferative effects of PP2 were associated with the inhibition of ERK/MAPK and/or PI3K/Akt growth pathways. ER positive and HER2 hyperactivation were two important clinically related markers that were associated with the inability of PP2 to inhibit both wild-type and different resistant breast cancer cells. Triple-negative breast cancer cells, defined by a lack of expression of estrogen, progesterone and HER2 receptors, were the most sensitive to the c-Src inhibitor.

The therapeutic mechanisms of the c-Src inhibitor are to block its phosphorylation and subsequent growth pathways [166]. It has been reported that cancer cells which do not manifest detectable c-Src phosphorylation are resistant to the c-Src inhibitor [167]. Generally, cells with higher c-Src activity were more sensitive to PP2 (Fig. 2k-5B), but not all cells with elevated c-Src tyrosine kinase activity were able to be effectively inhibited by the c-Src inhibitor such as ZR-75-1, MCF-7:2A, and Sk-Br-3 cells (Fig. 2k-2B and 5B). Thus, the level of c-Src phosphorylation is not sufficient to distinguish responsive cells from cells resistant to the c-Src inhibitor. Growth inhibition also depends on whether c-Src directly mediates growth pathways in a special type of cell. We consistently found that the levels of MAPK phosphorylation and/or Akt phosphorylation were reduced by PP2 in responsive cell lines but not in resistant cell lines (Fig.2k- 2C, 2k-4C, and 2k-5C).

The non-receptor tyrosine kinase c-Src acts as a critical molecule in relaying ER signaling, including nongenomic and genomic actions [168]. Its activity is modulated by E_2 through multiple mechanisms, leading to breast cancer cell proliferation, invasion, and metastasis. Consistently, the growth inhibitory effects by the c-Src inhibitor on ER positive cells appear to be more complex than on ER negative cells in present work. Most ER negative breast cancer cells were sensitive to the inhibition by PP2 (Fig. 2k-5B). However, the majority of ER positive cells were not sensitive to PP2 regardless of whether they were wild-type or long-term estrogen deprived cells (Fig. 2k-2B and 2k-4B). Although PP2 had moderate ability to inhibit some ER positive wild-type cell growth (Fig. 2k-2B), inhibitory effects by it varied under conditions with or without basal E_2 (Fig. 2k-3C). Our results also demonstrated that c-Src mainly mediated E_2 responses which included E_2-stimualted growth and E_2-induced apoptosis in ER positive cells (Fig. 2k-3D and 2k-4D). These functions might disturb the therapeutic effects of the c-Src inhibitor on ER positive cells. Although the c-Src inhibitor shows limited activity in ER positive cells as a single-agent, c-Src is consistently activated after ER targeting treatment with tamoxifen[135,136,169] which plays a critical role in mediating migration and invasion in tamoxifen resistant cells. Therefore, combined the c-Src inhibitor and ER blockade may delay endocrine resistance and increase the therapeutic effects.

The function of c-Src has been linked to its association with the HER2/Neu epidermal growth factor receptor family members [170]. In this study, increased expression of EGFR (MDA-MB-231 and MCF-7/F) did not affect the inhibitory effects of PP2, but HER2 overexpression was an indicator for the resistance to PP2 (Fig. 2k-5B). Finn *et al* [171] also reported HER2 amplification was a predictive marker for resistance to a c-Src inhibitor, dasatinib, in breast cancer cells. However, both BT474 and Sk-Br-3 cells overexpress endogenous HER2, they had differential responses to PP2 (Fig.2k-2B and 2k-5B). Further investigation demonstrated that status of HER2 activation determined the inhibitory rate of PP2, the higher HER2 phosphorylation, the lower inhibitory rate of PP2 (Fig. 2k-6B and 2k-6C). HER2 was highly activated in Sk-Br-3 cells compared with BT474 cells which made it hypersensitive to the HER2 inhibitor but not the c-Src inhibitor (Fig.2k-6A and 2k-6B). Therefore, status of HER2 activation may be

a better predictive biomarker for resistance to the c-Src inhibitor than currently available total HER2 determined by immunohistochemistry (IHC) or fluorescent *in situ* hybridization (FISH).

The triple negative MDA-MB-231 cells are characterized by a point mutation at codon 13 in the *K-RAS* gene [172]. This mutation is responsible for the constitutive phosphorylation of ERK1/2 which leads to a very aggressive cancer phenotype. Among tested cell lines, we observed that PP2 could not completely block c-Src phosphorylation in MDA-MB-231 cells within the first 24h (Fig. 2k-2A, 2k-3B, 2k-4A, and 2k-5A). But the level of c-Src phosphorylation was gradually decreased (Fig. 2k-5A). We prolonged treatment time to 4 days, the level of c-Src phosphorylation was clearly decreased. It is unclear how EGFR and *K-RAS* regulate the function of c-Src in MDA-MB-231 cells. The c-Src inhibitor, PP2, effectively suppressed growth in MDA-MB-231cells, which demonstrated that triple negative breast cancer cells depend on c-Src to proliferate (Fig. 2k-5B). Two independent studies support our observation by showing that the majority of dasatinib sensitive breast cancer cell lines were ñbasalò type or ñtriple-negativeò [171,173]). The hyper-sensitivity to the c-Src inhibitors provides a good therapeutic option for the clinical triple negative breast cancer (TNBC) patient. However, the TNBC is actually a highly diverse group of cancer, so that the determination of ER, PR and HER2 is not a precise classification to subtype this aggressive disease. MDA-MB-231 cells can not represent clinical TNBC model. Recent Phase II clinical trial shows that single-agent dasatinib has limited activity in unselected patients with TNBC, which suggests that a strategy of better patient selection with gene signatures is required to further evaluate the potential of the c-Src inhibitors in TNBC patient.

In summary, this study demonstrated a complex association exists among ER, HER2, and c-Src in different breast cancer cell lines. Moreover, our results underscored that ER expression and HER2 overexpression (especially over-activation) might be causes of resistance to a c-Src inhibitor in breast cancer. Our findings may be of value for future clinical investigation to determine the therapeutic efficacy of c-Src inhibitors in ER negative breast cancer with or without HER2 over-activation.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2l (Lewis-Wambi and Jordan) - Studies carried out by Dr. Joan Lewis-Wambi in the Jordan laboratory at Fox Chase Cancer Center

Introduction

In this study, we investigated the effects of Bazadeoxifene (BZA), 4-hydroxytamoxifen (4OHT), endoxifen, raloxifene, and the pure antiestrogen fulvestrant (ICI 182,780) on the growth of MCF-7:5C and MCF-7:2A breast cancer cells and determined the mechanism of action of BZA in these cells. We found that all of the SERMs inhibited E2-stimulated MCF-7 and T47D breast cancer cell growth, however, only BZA significantly inhibited the hormone-independent growth of MCF-7:5C and MCF-7:2A cells. The inhibitory effect of BZA was associated with cell cycle arrest and ERŬ and cyclin D1 downregulation which was completely reversed by ERŬ or cyclin D1 suppression. Together, these data show that BZA is distinct from the other members of the SERM family in its ability to inhibit the growth of breast cancer cells that are resistant to long-term estrogen deprivation.

Work Accomplished:

BZA Inhibits the Growth of Hormone-Independent MCF-7:5C and MCF-7:2A Breast Cancer Cells.

We first compared the growth characteristics of hormone-dependent MCF-7 and T47D breast cancer cells to those of long-term estrogen deprived MCF-7:5C and MCF-7:2A cells in the presence of E2. Cells were grown in estrogen-free media and then treated with 10^{-14} M to 10^{-8} M E2 for 7 days and cellular DNA was measure as an index of growth. In parallel, cells were also treated with 10^{-9} M E2 for 2 to 12 days and then harvested and counted using a hemocytometer. Fig. 2l-1A shows that E2 treatment stimulated the growth of MCF-7 and T47D cells in a concentration-dependent (top panel) and time-dependent manner (bottom panel) with maximum stimulation at 10^{-9} M, however, in MCF-7:5C and MCF-7:2A cells, E2 treatment at similar concentrations had the opposite effect causing either complete growth inhibition in MCF-7:5C cells or partial growth inhibition in MCF-7:2A cells. This current finding is consistent with our previously published work [83,174] which showed that physiologic concentrations of E2 induced programmed cell death (apoptosis) in MCF-7:5C and MCF-7:2A cells throught activation of the mitochondrial death pathway.

Next, we determined the inhibitory effects of BZA and other SERMs on MCF-7, T47D, MCF-7:5C, and MCF-7:2A cells. For experiments, MCF-7 and T47D cells were grown in fully estrogenized media and MCF-7:5C and MCF-7:2A cells were grown in estrogen-free media and then treated with 10^{-12} M to 10^{-6} M BZA, RAL, FUL, 4OHT, or ENDOX for 7 days and cellular DNA was measured as an index of growth. Fig. 2l-1B shows that all of the tested SERMs along with the pure antiestrogen FUL inhibited E2-stimulated growth in MCF-7 and T47D cells and hormone-independent growth in MCF-7:2A cells in a concentration-dependent manner, however, in MCF-7:5C cells, only BZA and FUL inhibited the growth of these cells with no effects observed with RAL, 4OHT, and ENDOX. BZA reduced the growth of MCF-7:5C cells in a concentration dependent manner causing a 80% reduction at 10^{-8} M whereas FUL reduced the growth by 55% at a similar concentration.

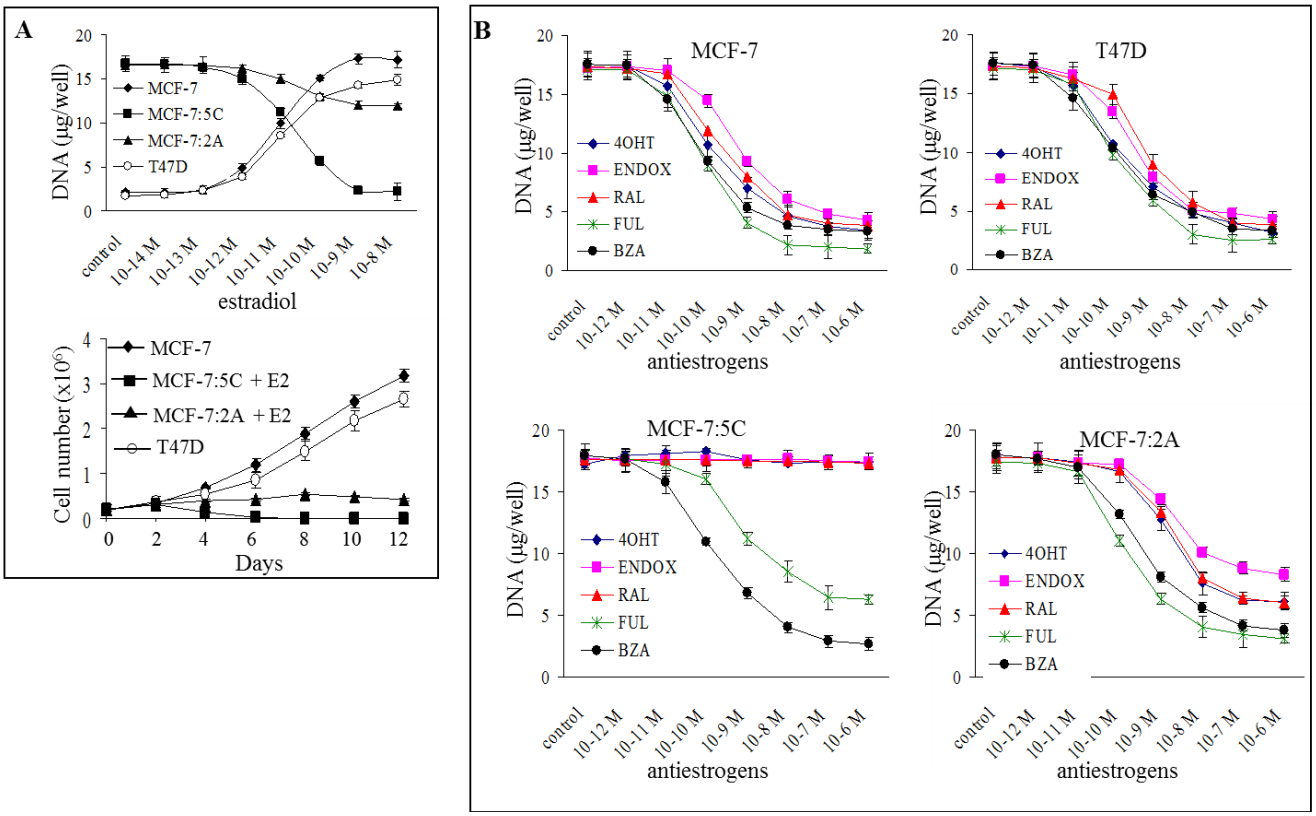


Figure 2l-1. *Effects of E2 and SERMs on the growth of hormone-dependent MCF-7 and T47D cells versus hormone-independent MCF-7:5C and MCF-7:2A cells. A, MCF-7 and T47D cells were grown in phenol red-free RPMI medium supplemented with 10% charcoal stripped FBS for 3 days prior to the start of the experiment. On the day of the experiment, all cell lines were seeded in phenol red-free RPMI medium supplemented with 10% charcoal stripped FBS at 30,000 per well in 24-well dishes and after 24 h were treated with 10-14 to 10-8 M E2 for 7 days, with retreatment every other day. At the conclusion of the experiment, cells were harvested and proliferation was assessed as cellular DNA mass (□g/well) using a DNA quantitation kit. The effect of E2 on proliferation of the different cell lines over a 12-day period was also determined by cell counting. B, the effects of antihormones at inhibiting E2-stimulated growth in MCF-7 and T47D cells and hormone-independent growth in MCF-7:5C and MCF-7:2A cells. Cells were seeded as described above except MCF-7 and T47D cells were grown in fully estrogenized media and then treated with 10-12 M to 10-6 M fulvestrant (FUL), bazedoxifene (BZA), raloxifene (RAL), 4-hydroxytamoxifen (4OHT), or endoxifen (ENDOX) for 7 days with retreatment on alternate days. Proliferation was assessed as cellular DNA mass (□g/well) as described in the methods section. C, effects of SERMs on reversing E2-induced growth inhibition of hormone-independent MCF-7:5C cells. Cells were seeded as described above and then treated with 10-9 M E2 alone or E2 combined with increasing concentrations (10-12 M to 10-6 M) of BZA, RAL, 4OHT, or ENDOX for 7 days and processed as described above. All data are presented as the mean from three different experiments in tripicate.*

BZA Downregulates ERα Protein in MCF-7:5C and MCF-7:2A Cells.

Since BZA dramatically reduced the growth of MCF-7:5C cells, we next determined whether BZA had actions similar to that of 4OHT or FUL at the level of ERα stability/degradation. We treated MCF-7:5C, MCF-7:2A, MCF-7, and T47D cells with 10^{-9} M E2 or 10^{-7} M FUL, 4OHT, RAL, or BZA for 24 hours and monitored ERα protein level. As shown in Fig. 2l-2A, ERα protein was highly expressed in MCF-7:5C and MCF-7:2A cells compared to MCF-7 and T47D cells and treatment with BZA dramatically downregulated ERα protein in MCF-7:5C and MCF-7:2A cells however it did not significantly reduce ERα levels in MCF-7 and T47D cells. The ability of BZA to downregulate ERα protein in MCF-7:5C and MCF-7:2A cells was greater than that of RAL and almost comparable to that of the pure antiestrogen ICI 182780 (FUL) which strongly downregulated ERα in all of the cell lines. E2 treatment also markedly downregulated ERα protein in MCF-7:5C cells and the other cell lines (Fig. 2l-2A), however, 4OHT stabilized ERα against degradation in MCF-7 and T47D cells (Fig. 2l-2A), which is consistent with previous studies [165]. We also examined the effect of the tamoxifen metabolite, endoxifen, on ERα expression in the different cell lines and found that endoxifen failed to down-regulate ERα in all of the tested cell lines. Our finding is in contrast to that of Wu and coworkers (2009) who reported that endoxifen downregulates ERα in breast cancer cells.

We also performed dose response and time response studies in MCF-7:5C and MCF-7 cells to determine the concentration and time of BZA treatment that optimally downregulated ERα protein. Fig. 2l-2B showed that BZA reduced ERα protein levels in MCF-7:5C cells in a concentration dependent manner with maximum inhibition at 10^{-6} M and this down-regulation was observed as early as 4 hours after treatment (Fig. 2l-2C, top). BZA also downregulated ERα mRNA levels in MCF-7:5C cells compared to control and this level of inhibition was comparable to that of fulvestrant (Fig. 2l-2C, bottom). To show that the decreased ERα protein by BZA was due to protein degradation, we used MG132 to inhibit the proteosome in MCF-7:5C and MCF-7 cells. We found that inhibition of proteosome activity completely blocked ERα degradation in response to BZA and E2 with partial reversal with fulvestrant (Fig. 2l-2D). We further determined whether BZA might affect ERα protein expression by inhibiting its synthesis. We treated MCF-7:5C cells with 0.5 to 5.0 εM cycloheximide (CHX) for 4 h to address this question. The impact of CHX on ERα protein expression was much less dramatic than that of BZA (data not shown), which suggest that BZA-induced down-regulation of ERα protein is not likely to involve protein synthesis inhibition. Together, these data show that BZA downregulates ERα and that it differs from other SERMs in its ability to regulate cell growth and ERα protein expression.

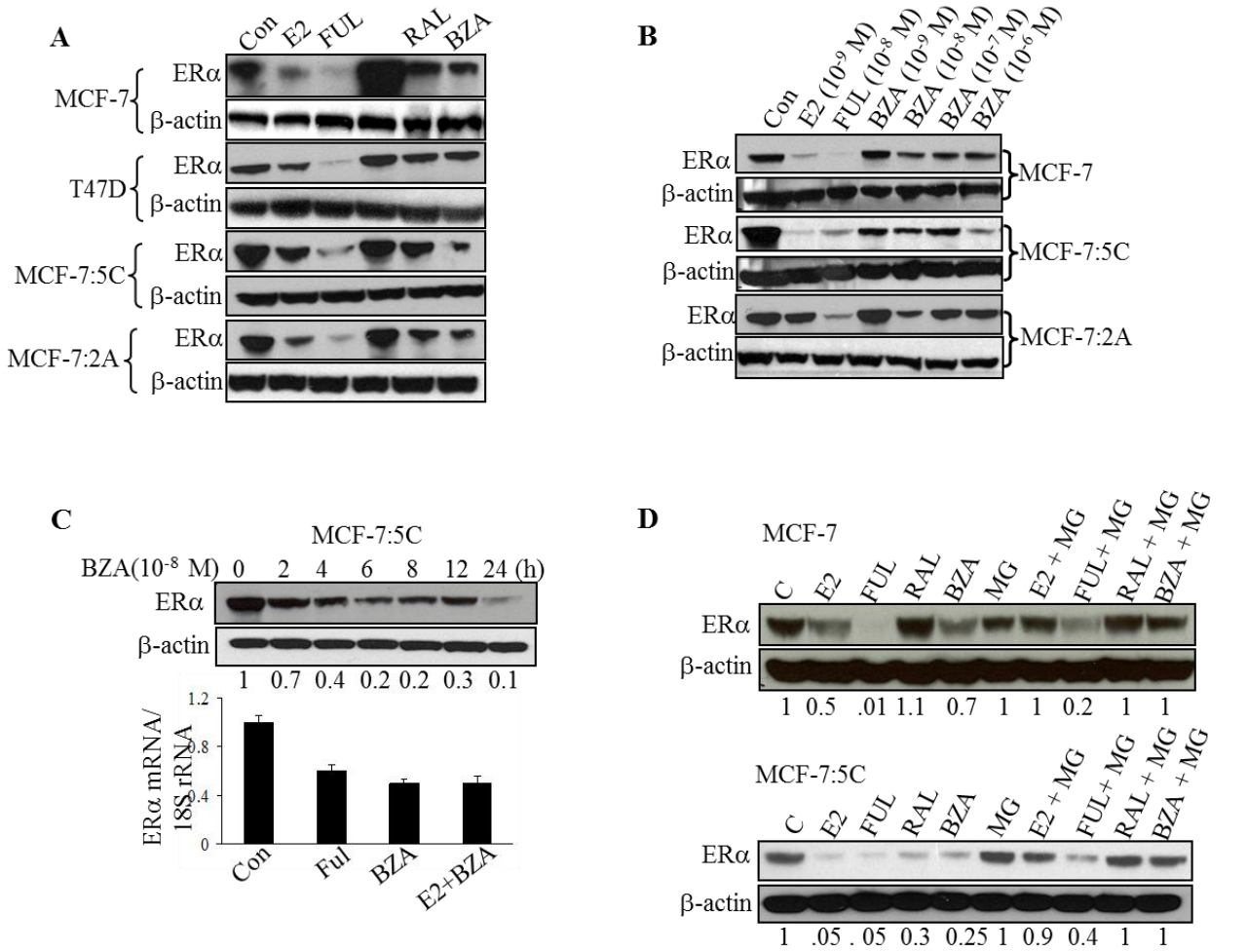


Figure 2l-2. *Effects of SERMs on ERα expression and stability in hormone-dependent MCF-7 and T47D cells and hormone-independent MCF-7:5C and MCF-7:2A cells. A, Western blot analysis of ERα protein levels in MCF-7, T47D, MCF-7:5C, and MCF-7:2A cells in response to 24-h treatment with 10^{-9} M E2 or 10^{-7} M FUL, 4OHT, RAL or BZA. β-actin was used as a loading control. B, Western blot analysis of ER□ protein levels in MCF-7:5C and MCF-7 cells following treatment with 10^{-9} M to 10^{-6} M for 24 h. For comparison, cells were also treated with 10^{-9} M E2 or 10^{-8} M FUL. C, Western blot analysis of ERα protein levels in MCF-7:5C cells in response to 10^{-8} M BZA treatment over a 24h time period. Quantitated protein levels were normalized to □-actin. Densitometric quantitation relative to the control is shown on the bottom of the immunoreactive bands. Also shown is ERα mRNA levels in MCF-7:5C cells treated with 10^{-9} M E2, 10^{-8} M BZA, or the combination of E2 plus BZA for 24 hours. The amount of ERα mRNA was determined by real-time RT-PCR and normalized to the internal control 18S rRNA. Each data point represents the average of four biological replicates from three independent experiments. D, Western blot analysis of ERα protein levels in MCF-7 and MCF-7:5C cells pretreated with the proteosome inhibitor MG132 (4 nmol/L) for 4 hours and then treated as indicated for 8 h. β-actin levels are shown as protein loading controls. All experiments were repeated three times and the same results were obtained.*

BZA Inhibits ER Transcriptional Activity in MCF-7:5C Cells.

To determine whether BZA blocks ER function, we next examined the transcriptional activation of an estrogen response element (ERE) in MCF-7, T47D, MCF-7:5C, and MCF-7:2A cells. For experiment, cells were transiently transfected with an ERE-luciferase reporter plasmid and treated for 24 h with 10^{-10} M E2 or 10^{-8} M BZA either alone or in combination with E2. The results of these studies showed that the ERE was activated maximally by E2 in all of the cell lines with T47D showing the most robust E2 response (Fig. 2l-3A). BZA, as an individual treatment, reduced basal ERE luciferase activity in MCF-7:5C and MCF-7:2A cells and in combination with E2 was able to completely block E2-induced ERE activity in these cells (Fig. 2l-3A).

To further test whether BZA is able to block E2 activation of endogenous genes, we analyzed the expression level of pS2 mRNA in MCF-7:5C cells using qRT-PCR. The pS2 gene is often used as a prognostic marker in breast cancer cells and is frequently used in studies of ER action. Furthermore, it is suggested that estrogen regulates the expression of pS2 through an imperfect ERE in the pS2 promoter [175]. Our results showed that basal pS2 mRNA level was ~3.5-fold higher in MCF-7:5C cells compared to wild-type MCF-7 cells and E2 treatment further increased basal pS2 mRNA level in MCF-7 and MCF-7:5C cells which was completely blocked by BZA (Fig. 2l-3B). Notably, we also found that siRNA knockdown of ERŬ(Fig. 2l-3C) significantly reduced the basal proliferation of MCF-7:5C cells and markedly reduced the responsiveness of these cells to BZA (Fig. 2l-3C, bottom). Suppression of ERŬ also significantly reduced cyclin D1 protein levels in MCF-7:5C cells. Overall, these results show that BZA is a potent antiestrogen capable of blocking ER-mediated transcriptional activation in hormone-independent breast cancer cells and that the inhibitory effect of BZA is dependent on ERŬexpression.

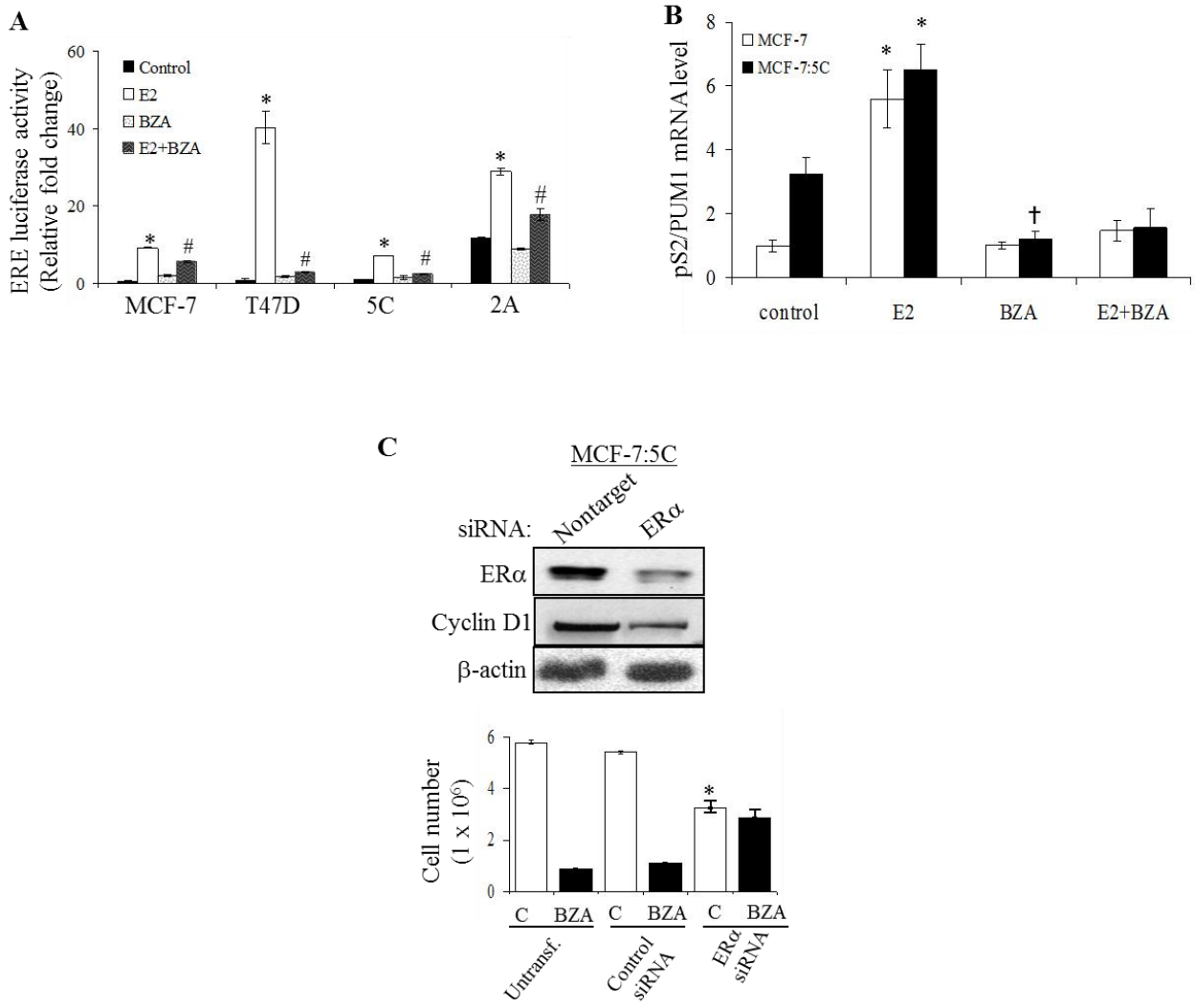


Figure 2l-3. *BZA inhibits constitutive ERα transcriptional activity and expression of endogenous ER-regulated genes in hormone-independent and hormone-dependent breast cancer cells. A, ERE luciferase activity in hormone-dependent MCF-7 and T47D cells and hormone-independent MCF-7:5C and MCF-7:2A cells. For experiment, cells were transiently transfected with an ERE-luciferase reporter construct and treated with 10^{-9} M E2, 10^{-7} M BZA, or E2+BZA for 24 h. Luciferase values are reported as relative fold change compared to control (untreated cells). *, significance at the p < 0.001 level (ANOVA) compared with control. #, significance at the p< 0.01 compared with E2 treatment. B, real time RT-PCR analysis of pS2 mRNA gene expression in MCF-7 and MCF-7:5C cells after indicated treatments for 24 h. Each data point represents the average of four biological replicates. *, significance at the p < 0.001 level compared with control. #, significance at the p < 0.05 level compared with MCF-7:5C control. C, MCF-7:5C cells were transfected with nontarget (control) or ERα siRNAs for 48 h. Transfected cells were then harvested for Western blot analysis (top panel) or treated with 10^{-7} M BZA for an additional 4 days followed by cell counting using a hemocytometer (bottom panel). Data shown are representative of three independent experiments with duplicate (*, p < 0.01 versus nontarget transfected cells).*

BZA blocks cell cycle progression in MCF-7:5C cells and downregulates cyclin D1.

Since BZA significantly reduced the growth of MCF-7:5C cells, we next examined its effect on cell cycle progression. For experiment, MCF-7 and MCF-7:5C cells were treated with 10^{-9} M E2, 10^{-8} M BZA, or E2 plus BZA for 48 h followed by propidium iodide staining and flow cytometric analysis. The results showed that in MCF-7:5C cells, BZA treatment increased the proportion of cells in G0/G1 phase from 60% (control) to 81% at 48 h and it reduced the proportion of S phase cells from 33% to 9%, however, in MCF-7 cells, E2 treatment increased the proportion of S-phase cells from 19% to 42% at 48 h with no effect observed with BZA alone (Fig. 2l-3A). Notably, the inhibitory effect of BZA on cell cycle in MCF-7:5C cells was almost comparable to that of fulvestrant; however, none of the other SERMs induced G1 arrest in these cells (data not shown).

The G-1ï induced cell cycle block in MCF-7:5C cells was further investigated by measuring protein expression of G_1-phaseï specific cyclin D1. MCF-7 and MCF-7:5C cells were treated with E2, BZA, RAL, 4OHT or FUL and then collected at 24 h for immunoblot analysis. Fig. 2l-4B shows that in parental MCF-7 cells, treatment with E2 increased cyclin D1 protein ~5-fold above control with significant induction with 4OHT and BZA, however, in MCF-7:5C cells, cyclin D1 protein was constitutively overexpressed and treatment with BZA dramatically reduced basal cyclin D1 protein to an almost undetectable level. Notably, none of the other tested compounds including the pure antiestrogen fulvestrant had any significant effect on cyclin D1 protein in MCF-7:5C cells (Fig. 2l-4B, bottom). Further analysis showed that the inhibitory effect of BZA on cyclin D1 occurred as early as 2 h and persisted up to 24 hours (Fig. 2l-4C, top left). BZA also completely reduced constitutive cyclin D1 mRNA level (Fig. 2l-4C, bottom) and cyclin D1 promoter activity (Fig. 2l-4C, top right) in MCF-7:5C cells compared to parental MCF-7 cells and it completely blocked cyclin D1 induction by E2 in these cells. Lastly, we found that siRNA knockdown of cyclin D1 significantly reduced the basal growth of MCF-7:5C cells and it significantly reduced the inhibitory effect of BZA in these cells (Fig. 2l-4D). It should be noted that cyclin D1 protein was also significantly reduced in MCF-7:5C cells due to ER knockdown (Fig. 2l-2D), thus suggesting an important collaboration between cyclin D1 and ER in mediating the growth inhibitory effect of BZA in MCF-7:5C cells.

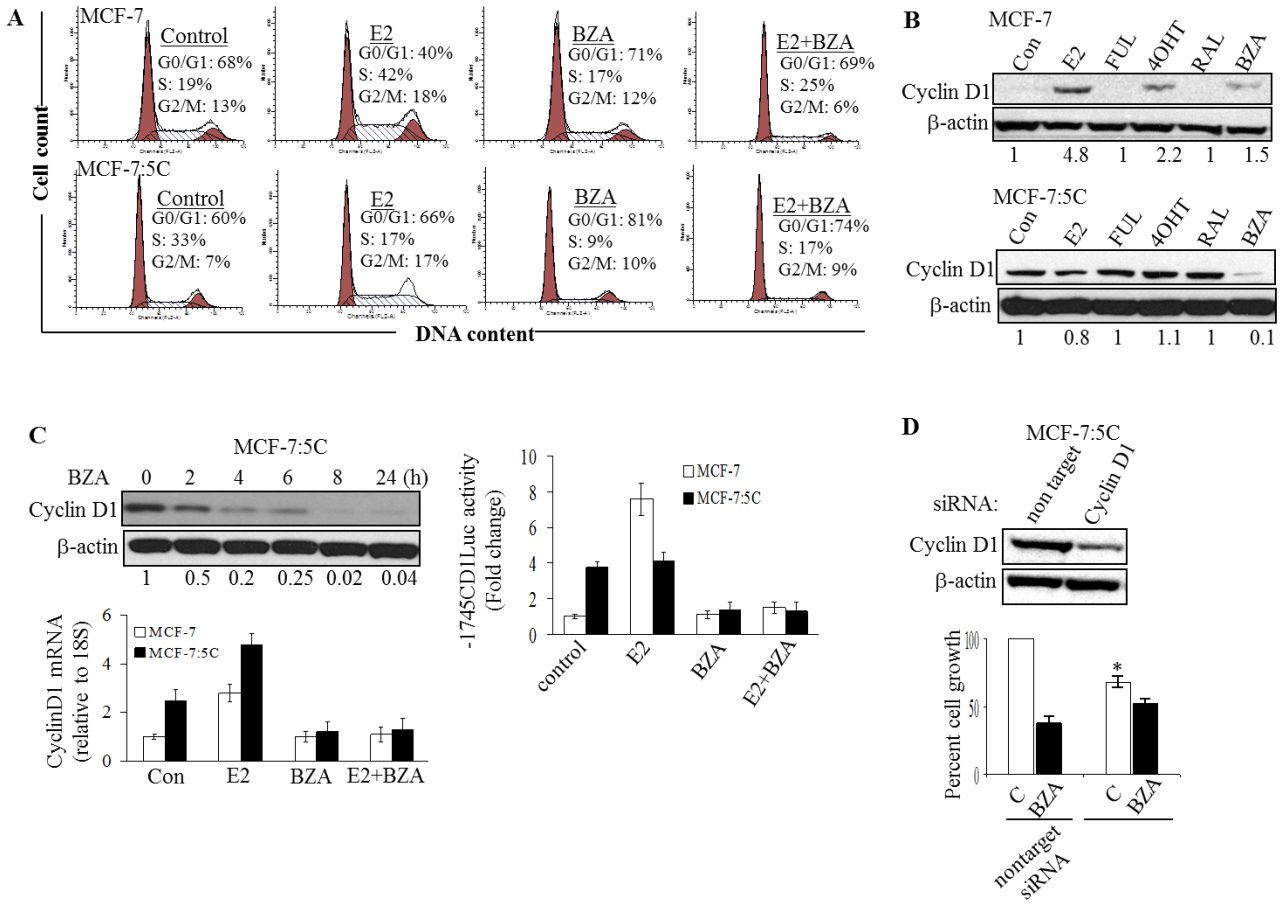


Figure2l-4. *Effects of BZA on cell cycle progression and cyclin D1 regulation in MCF-7 and MCF-7:5C cells. A, cell cycle distribution was determined by propidium iodide staining of DNA content and flow cytometry. Cells were treated with 10-9 M E2, 10-7 M BZA, or E2 plus BZA for 24 and 48h. Thirty-thousand cells per sample and three replicates per group were collected. Representative histograms are shown. B, Western blot analysis of cyclin D1 expression level in MCF-7 and MCF-7:5C cells following treatment with BZA and other SERMs. Prior to experiment, MCF-7 cells were switched from fully estrogenized media to estrogen-free media for 3 days and then treated with ethanol vehicle (control), 10-9 M E2 alone, or 10-9 M E2 plus FUL (10-7 M), RAL (10-7 M), 4OHT (10-7 M), or BZA (10-7 M) for 24 h. MCF-7:5C cells, however, did not require a media switch since they are hormone-independent and are routinely grown in estrogen-free media. MCF-7:5C cells were treated as described above for MCF-7 cells. Quantitated protein levels normalized to β-actin are indicated. C, BZA regulation of cyclin D expression and promoter activity in MCF-7:5C cells. Cells were treated with 10-7 M BZA for the indicated time points. Cyclin D1 protein and mRNA levels were determined by Western blot and quantitative RT-PCR, respectively with □-actin and 18S rRNA as internal controls. For cyclin D1 promoter activity experiment, MCF-7 and MCF-7:5C cells were cotransfected with a full-length cyclin D1 promoter plasmid (-1745CDLUC) and Renilla luciferase control plasmid overnight and then treated with 10-9 M E2, 10-8 M BZA, or E2 + BZA for 24 h. Luciferase activity was measured as described in materials and methods. D, proliferation of MCF-7:5C cells transfected with the nontarget or cyclin D1 siRNA. Cells were transfected and then seeded at 15,000 per well in 24-well dishes. Medium was replenished the day after seeding on day 0 and every other day thereafter. Cells were collected on day 5 and counted using a hemocytometer. All data are presented as the mean from three independent experiments with duplicate (*, $p < 0.01$ versus nontarget transfected cells).*

Molecular modeling and docking of BZA into the ligand binding site of ER□□□

Molecular modeling and docking studies were carried out to understand the bioactively bound conformation of BZA and the molecular basis for its interaction with ER□. Using the available X-ray crystallographic data, the flexible docking of BZA into the ligand binding domain (LBD) of ERŭ co-crystallized with RAL was performed and for comparison reasons, RAL was also docked in its native protein structure. The superimposition of the docked solution and experimental structure of RAL showed that the docking model recapitulated the orientation of the native ligand in the active site and the same interactions with the key aminoacids of the binding cavity were formed with RMSD of 0.362 when compared with the crystal structure. The experimental structure of ERŭ co-crystallized with the agonist E2 (PDB code 1gwr) is displayed in Fig. 2l-5A, while the antagonist conformations of ERŭ bound to 4OHT (Fig. 2l-5B), RAL (Fig. 2l-5C), BZA (Fig.2l-5D), and FUL (Fig.2l-5E) are also presented in the figure. The analysis of the results demonstrates that BZA (Fig. 2l-5D) binds to ERŭ in an orientation similar with RAL (Fig. 2l-5C), i.e. an antagonist conformation and has the tendency to form the same hydrophobic contacts with the aminoacids lining the binding cavity. Also, the same complex H-bond network is formed with D351, E353, R394, H524 and a highly ordered water molecule, located in the vicinity of residues E353 and R394. Additionally, the docked structure of BZA was compared to 4OHT bound to ERŭ (Fig. 2l-5B) as well as BZA superimposed in the binding site of 4OHT-ERŭ complex. It is interesting to note that for the 4OHT bound receptor (Fig. 2l-5B), the H-bond interaction between BZA and H524 is missing due to the different orientation of this aminoacid in the binding site compared with the RAL-ERŭ complex. It can be concluded that BZA binds at the antagonist conformation of ER□ LBD, in a similar alignment with RAL not 4OHT or FUL, and forms, basically, the same favorable hydrophobic and hydrophilic interactions with the key aminoacids of ER□ binding site.

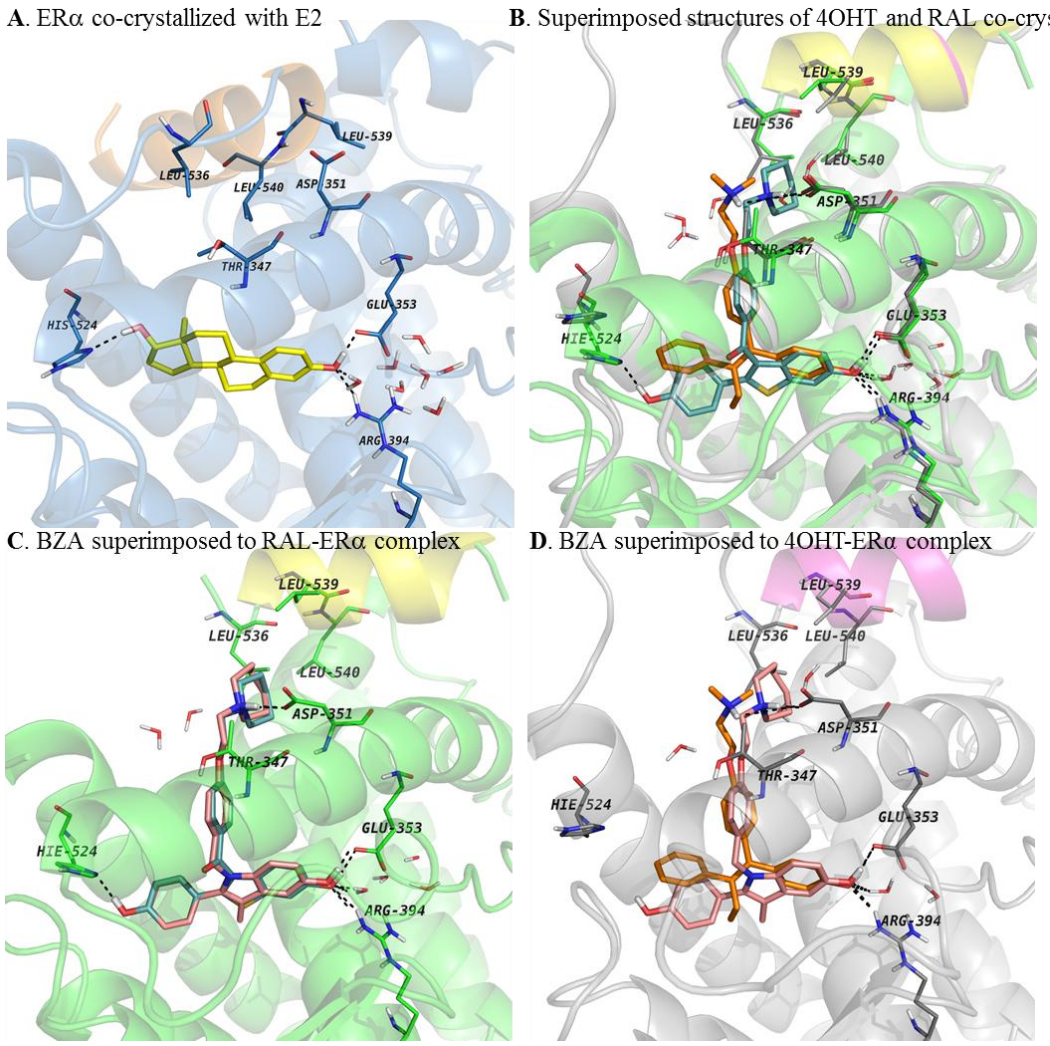


Fig. 2l-5. *Molecular modelling of ERα binding site with various ligands. A, agonist conformation of ERα co-crystallized with E2; helix 12 is depicted in orange and lays over the binding site sealing the ligand inside it. The antagonist conformations of the receptor are shown in panels B, C, D, and E. X-ray structures of ERα co-crystallized with 4OHT (B), raloxifene (C), bazedoxifene (D), or fulvestrant (E) docked into the ERα-raloxifene crystal structure. Helix 12 is depicted in magenta for 4OHT bound conformation and yellow for raloxifene and bazedoxifene. Also the key aminoacids lining the binding site are displayed and the network of hydrogen bonds in which they are involved with the ligands is shown in black dashed lines. Carbon atoms are colored in yellow for E2, orange for 4OHT, cyan for raloxifene and pink for bazedoxifene. These images show the differences between the agonist (A) and antagonist conformation (B, C, E) of ERα and present the alignment of bazedoxifene in the binding site of ERα which is very similar with raloxifene's orientation and the same interactions with the key aminoacids of the binding cavity are encountered*

Discussion

While TAM [176,177] and RAL [178] are the best known of the SERMs, promising results have been observed with the targeted development of newer and more tissue-specific SERMs, many of which are under investigation for postmenopausal osteoporosis. Of the newer SERMs in development, Phase 3 clinical data have shown that BZA is effective in preventing and treating

postmenopausal osteoporosis, without adverse effects on the endometrium or breast [179]. BZA in combination with conjugated estrogens has also been shown to relieve hot flashes and improve vulvovaginal atrophy and its symptoms [180,181]. In the present study, we report for the first time that BZA, in addition to inhibiting the growth of hormone-dependent MCF-7 and T47D breast cancer cells, also inhibits the growth of breast cancer cells that have acquired resistance to long-term estrogen deprivation (i.e. hormone-independent/aromatase inhibitor resistant). Specifically, we found that BZA at 10^{-8} M inhibited the growth of hormone-independent MCF-7:5C breast cancer cells by ~75-85% and this inhibitory effect was associated with G1 arrest and cyclin D1 and ERŬ down-regulation. BZA also completely blocked E2-induced ERE luciferase activity and reduced basal pS2 mRNA levels in MCF-7:5C and MCF-7:2A cells. BZA was the only SERM capable of inhibiting hormone-independent growth of MCF-7:5C cells with no effects observed with 4OHT, endoxifen, or RAL. Notably, our molecular modeling studies indicated that BZA bound the ligand binding domain of ERŬ in an antagonist orientation similar to RAL, but distinct from 4OHT and fulvestrant (Fig.2l-5), and it formed the same hydrophobic and hydrophilic contacts as RAL with the aminoacids lining the binding cavity of the receptor. It should be noted that BZA and RAL are similar in structure, however, despite their similarities, there are reported structural differences between them [182,183]. In particular, BZA possesses a core binding domain that consists of a 2-phenyl-3-methyl indole whereas RAL has a benzothiophene core and TAM has a trans-stilbene core. Additionally, the side chain affecter region of BZA is connected to the core binding region via a methylene hinge, whereas RAL has its side chain connected via a carbonyl hinge and TAM has its side chain directly connected to its core binding region and. Finally, BZA contains a hexamethylenamine ring at the side chain terminus whereas RAL and TAM contain a piperidine ring and dimethylamine, respectively [183]. These structural differences are thought to explain the 10-fold lower binding affinity of BZA for ER□ compared to RAL (IC_{50} of 26 nM versus 2.4 nM), however, these differences do not appear to affect the potency of BZA as an antagonist in breast cancer cells, as demonstrated in this study. Indeed, these findings help to further distinguish BZA from the other SERMs such as TAM and RAL and they support the concept that subtle but moderate structural differentiation can dramatically impact the ability of a ligand to regulate cell proliferation.

Previous research has indicated that deregulation of ERŬ expression is a driving force in the initiation and progression of estrogen-sensitive breast tumors [184,185]. Indeed, it has been suggested that alterations in pathways leading to ERŬ synthesis and/or degradation underlie the deregulation of ERŬ and its consequent manifestations, including enhanced proliferation in breast tumors [186]. ERŬ is the predominant receptor isoform expressed in breast cancer cells, and increased numbers of ERŬ-expressing cells as well as increased individual cell ER□ content can be observed at the earliest stages of breast tumorigenesis. Previously, we have shown that ER□ mRNA and protein levels are significantly elevated in breast cancer cells that have been adapted to grow in an estrogen-depleted environment [83,187,188]. This particular type of regulation in which ERŬ levels are increased following estrogen deprivation has been described as a Model I response [165]. A Model I response is characterized by an ERŬ that is expressed at high levels in the absence of estrogen and is subsequently down-regulated following estrogen binding, primarily through repression of the steady-state level of the mRNA. In the present study, we found that basal ERŬ protein levels were upregulated greater than 2-fold in hormone-independent MCF-7:5C and MCF-7:2A breast cancer cells compared to MCF-7 cells and treatment with BZA (10^{-8} M) induced proteasome-mediated degradation of ERŬ in these cells which was reversed by the broad spectrum proteasome inhibitor MG132. The ability of BZA to degrade ERŬ in MCF-7:5C cells was rapid and robust occurring as early as 4 h after treatment with maximum degradation at 24 h and it was comparable to the pure antiestrogen fulvestrant which also completely degraded ERŬ. Notably, BZA and fulvestrant were the only compounds that markedly reduced the growth of both MCF-

7:5C and MCF-7:2A breast cancer cells and blocking BZA-induced ER□ degradation with MG132 dramatically reduced its growth inhibitory effects on these cells (data not shown). The importance of ER□ in mediating the antagonist effects of BZA in hormone-independent MCF-7:5C cells was further confirmed by siRNA knockdown experiments which showed a 60% reduction in the ability of BZA to inhibit the growth of these cells. Suppression of ER□ also significantly reduced the basal growth of MCF-7:5C cells and E2-induced growth in wild-type MCF-7 cells, which is consistent with recent findings by Ariazi and coworkers [189]. It should be noted; however, that degradation or suppression of ER□ is not the only mechanism by which an antagonist can inhibit cell proliferation. For example, TAM has been shown to stabilize ER□ protein against degradation in breast cancer cells [99,165,187,188], however, it is a potent antagonist in the breast with the ability to block E2-stimulated proliferation and E2-induced ERE activity in these cells. Based on these findings, we speculate that, in the absence of estrogen, the unliganded ER□ drives the proliferation of hormone-independent breast cancer cells; however, in the presence of BZA, the ability to inhibit cell proliferation is dependent on receptor degradation.

Apart from ERŭ, BZA also significantly reduced cyclin D1 expression in MCF-7:5C breast cancer cells. Specifically, we found that cyclin D1 protein was constitutively overexpressed by 3-to 5-fold in hormone-independent MCF-7:5C cells compared to wild-type MCF-7 cells and treatment with BZA completely reduced it to an undetectable level. In addition, we found that suppression of cyclin D1 reduced the basal growth of MCF-7:5C cells and it significantly reduced the inhibitory effect of BZA in these cells. Suppression of cyclin D1 also significantly reduced ER□ protein levels in MCF-7:5C cells with similar effects observed following ER□ suppression, thus suggesting a link between cyclin D1 and ER□ in these cells. Cyclin D1 is a breast cancer oncogene whose overexpression has been linked to poor prognosis in ER□ and PgR-positive breast cancers [190]. It is a multifunctional G_1-phase cyclin whose regulatory effects are particularly important in breast development and cancer [191]. Cyclin D1 is regulated by estrogen and progesterone [192] and it contributes to poor treatment response of ER-positive tumors by acting downstream to promote hormone agonist- and antagonist-independent proliferation [193]. There is also evidence that cyclin D1 can interact with ER coactivators to activate estrogen response element (ERE) in a ligand-independent manner [194] and this interaction is not inhibited by antiestrogens [194]. Notably, we found that BZA not only reduced basal cyclin D1 protein and mRNA levels but also completely reduced its promoter activity in hormone-independent MCF-7:5C cells and these changes were associated with decreased cell proliferation. In contrast, RAL, 4OHT, endoxifen, and FUL failed to inhibit cyclin D1 expression in MCF-7:5C cells, and these compounds, with the exception of fulvestrant, did not have any growth inhibitory effect in MCF-7:5C cells.

In conclusion, it is clear from clinical data that BZA in combination with conjugated estrogens represents a new form of therapeutic agents for the treatment of postmenopausal symptoms and prevention of postmenopausal osteoporosis. The fact that it does not stimulate the breast or endometrium and is very effective at inhibiting the proliferation of endocrine-resistant breast cancer cells highlights its widespread therapeutic potential and demonstrates that not all SERMs are alike. Our data also suggest that the overexpression of ERŭ and cyclin D1 in MCF-7:5C cells might be driving the hormone-independent growth of these cells and that the ability of BZA to downregulate ERŭ and cyclin D1 is critical to treat and possibly reverse antihormone resistance in breast cancer.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2m (Maximov and Jordan) - Studies carried out by Dr. Philipp Maximov in the Jordan laboratory at Fox Chase Cancer Center

Introduction

Here we offer the hypothesis that, the shape of the ER complex with either planar estrogens (Class I) or angular estrogens (Class II), can modulate the apoptotic actions of estrogen through the shape of the resulting complex. We have previously synthesized a range of estrogenic TPEs, and all of these compounds will stimulate estrogen-stimulated growth of MCF-7 cells [15]. Here we investigate the actions of 4-OHT and our model TPEs (Fig. 2m-1) on estradiol-induced apoptosis in MCF-7:5C cells [195]. We have discovered that the angular TPE estrogens do not cause rapid estrogen-induced apoptosis, even though they are potent stimulators of breast cancer cell growth. They do, in fact, block estradiol-induced apoptosis as effectively as 4-OHT, a known antiestrogen. We offer the hypothesis that the shape of the ER complex and its ability to bind co-activators and transport them to the correct part of the cell, is fundamentally important for the initiation of estrogen-induced apoptosis.

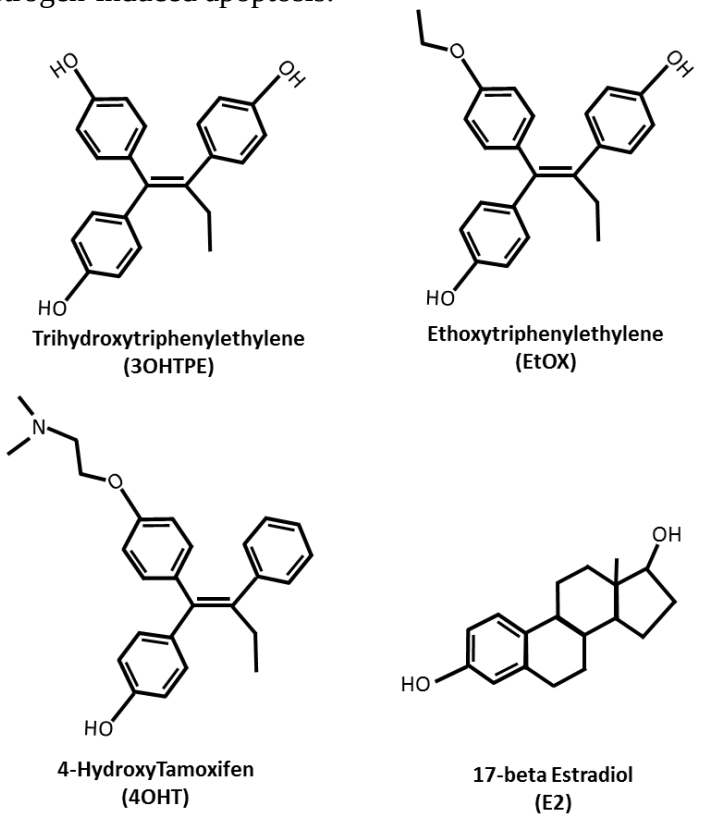


Figure 2m-1. *Structure of the compounds used in the study. Trihydoxytriphenylethylene (3OHTPE), Ethoxytriphenylethylene (EtOX), 4-Hydroxytamoxifen (4OHT) and 17-beta Estradiol (E2).*

Work Accomplished:

Reversal of E2-induced apoptosis in MCF7:5C cells by 4OHT, 3OHTPE and EtOX

17-beta Estradiol induces apoptosis in ER+ MCF7:5C cells [195] which are long-term E2 deprived MCF7 breast cancer cells. Our aim was to evaluate the 4-OHT and the TPEs, 3OHTPE and EtOX (Fig. 2m-1), for their ability to reverse the apoptosis induced by E2 in MCF7:5C cells in a concentration dependent manner. Interestingly, the triphenylethylenes 3OHTPE and EtOX has been previously reported to be complete estrogenic as they can induce proliferation of MCF7 cells, unlike 4OHT [15]. We found that 3OHTPE and EtOX were able to block the E2-induced apoptosis of MCF7:5C cells similar to the 4OHT in a concentration dependent manner (Figure 2m-2) as evident by DNA growth assay. The compounds alone at 10^{-6} M concentration were not able to induce significant apoptosis of MCF7:5C cells (Figure 2m-2) whereas, as expected, drastic apoptosis was induced by E2 (1nM) alone.

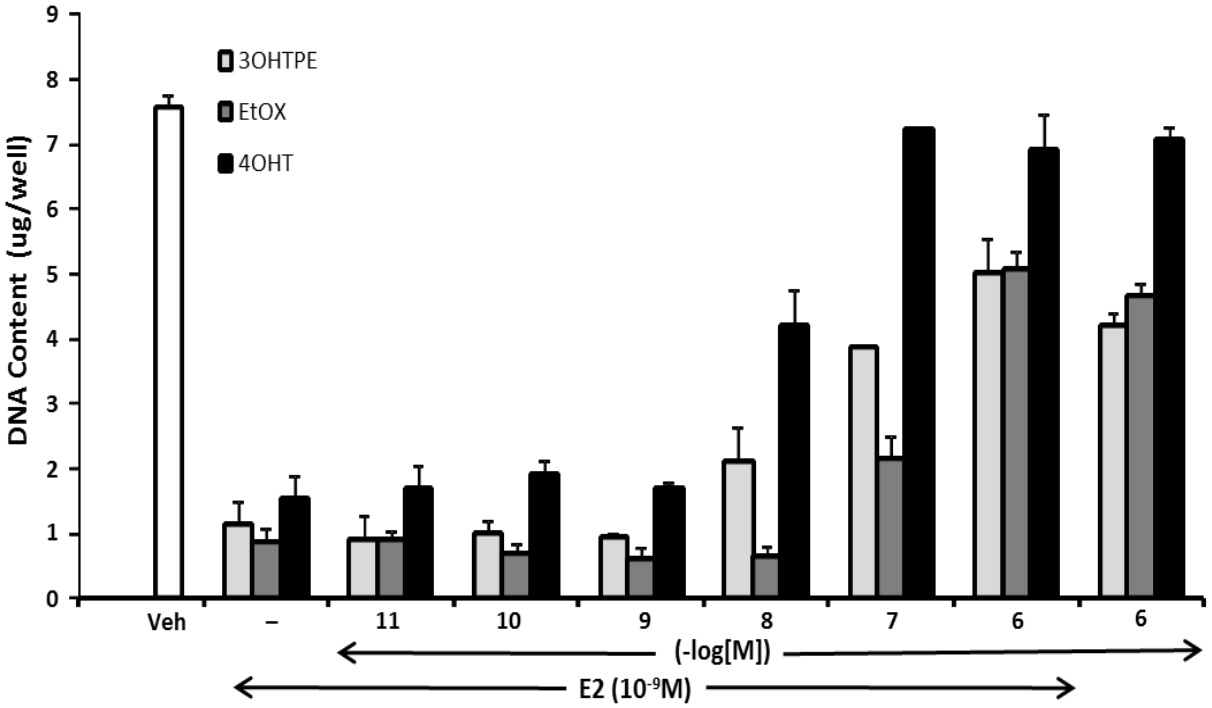


Figure 2m-2. *Reversal of E2-induced apoptosis of MCF7:5C cells by 4OHT, 3OHTPE and EtOX. MCF7:5C cells were treated with either vehicle (Veh), E2 (10−9 M) alone or E2 in combination with increasing concentration of the indicated compounds. Cells were also treated with compounds alone at 10−6 M concentration. After 7 days of treatment the total DNA in the wells were estimated as a measure of cell survival.*

Estrogen receptor alpha levels are not decreased by 4OHT, 3OHTPE and EtOX

Treatment with estrogen in MCF7 cells causes a rapid destruction of ER alpha protein levels whereas 4OHT retards the destruction of ER alpha levels [196]. Interestingly, despite being acting as an estrogen agonist in MCF7 cells [15] the triphenylethylenes, 3OHTPE and EtOX did not reduce the protein levels of ER alpha after 24 hrs of treatment at 10^{-6} M concentration, as evident by western blot analysis of ER alpha protein levels which is similar to 4OHT treatment (Figure 2m-3). As expected, ER alpha protein levels were drastically reduced after treatment with E2 (1nM) for 24 hrs in MCF7 cells (Figure 2m-3).

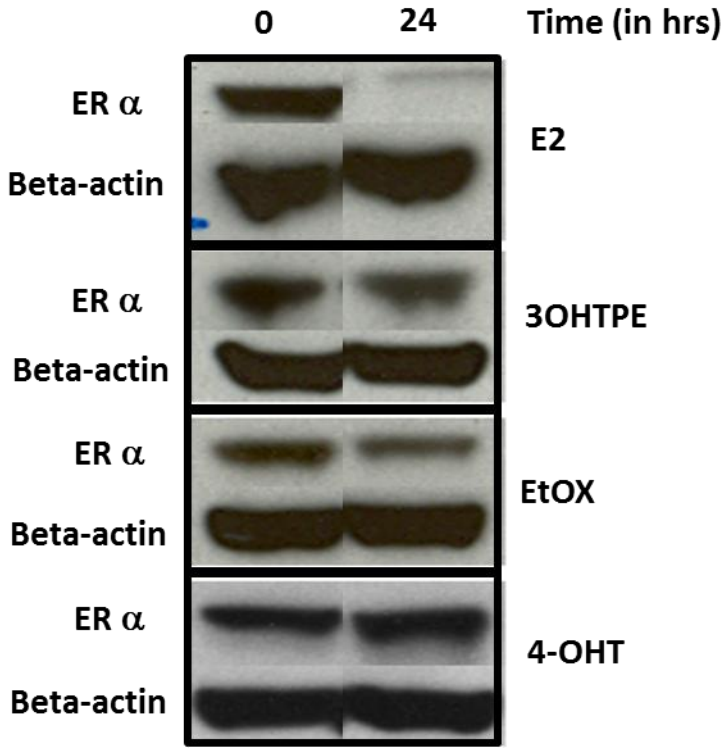


Figure 2m-3. *Levels of ER alpha protein after treatment with E2, 3OHTPE, EtOX or 4OHT. MCF7 cells were treated with E2 (10−9M), 3OHTPE (10−6M), EtOX (10−6M) or 4OHT (10−6M) for 24 hrs and total protein was isolated to estimate the ER alpha levels by western blotting. Levels of beta-actin were measured to ensure equal loading.*

Induction of apoptosis by E2, 4OHT, 3OHTPE and EtOX

We further evaluated the apoptotic induction by 4OHT, 3OHTPE and EtOX and compared with E2 in MCF7:5C cells using a dye-based kit which can measure the cells undergoing apoptosis as detailed in materials and methods. We found that E2 (1nM) produced drastic increase in apoptotic cells after 5 days of treatment, whereas 4OHT (10^{-6}M) was completely ineffective (Figure 2m-4). 3OHTPE (10^{-6}M) induced a modest level of apoptosis and a very slight apoptotic induction was observed after EtOX (10^{-6}M) treatment for 5 days (Figure 2m-4).

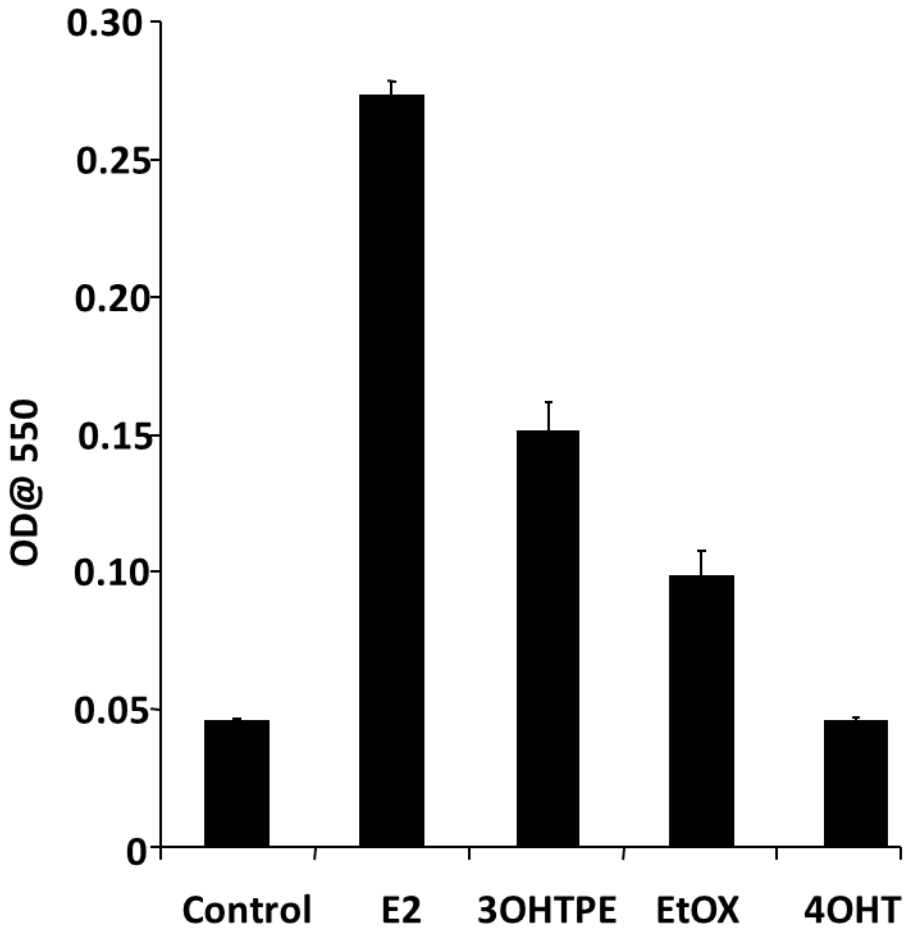
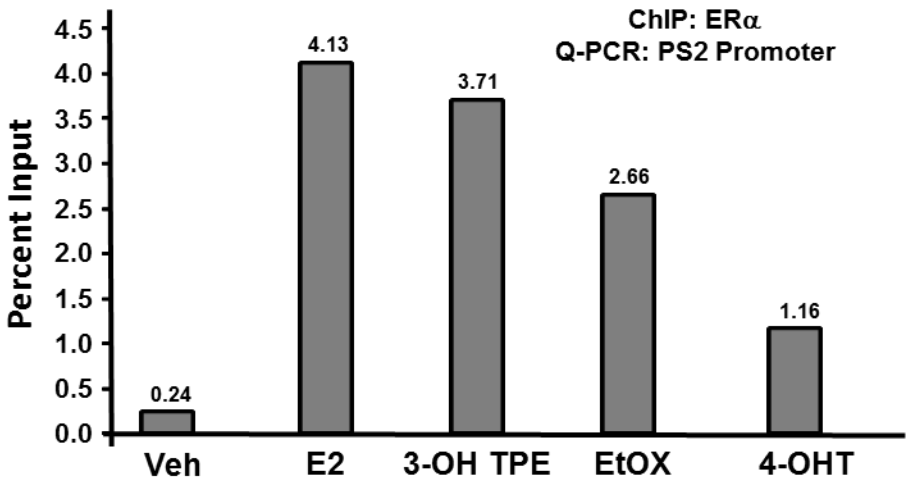


Figure 2m-4. *Induction of apoptosis by E2, 3OHTPE, EtOX or 4OHT. MCF7:5C cells were treated with E2 (10−9M), 3OHTPE (10−6M), EtOX (10−6M) or 4OHT (10−6M) and the induction of apoptosis was measured using a dye-based kit as detailed in materials and methods.*

Recruitment of ER alpha and SRC3/AIB1 at the Promoter of PS2 (TFF1) gene by E2, 4OHT, 3OHTPE and EtOX

PS2 (TFF1) transcription is induced by E2 through a classical estrogen responsive element (ERE) at the promoter of the gene and its mechanism has been extensively studied [156,197]. We therefore evaluated the binding of the ER alpha and SRC3/ AIB1 to the PS2 promoter after 45 minutes of treatment with 4OHT (10^{-6}M), 3OHTPE (10^{-6}M) or EtOX (10^{-6}M) in comparison with E2 (1nM) in the MCF7:5C cells. Around 17 fold increase in ER alpha recruitment was recorded with E2 treatment as compared to vehicle treatment at the PS2 promoter (Figure 2m-5A). In comparison, ~15 and ~11 fold increase in ER alpha recruitment was observed after treatment with 3OHTPE and EtOX respectively, whereas only ~5 fold increase in ER alpha recruitment was observed with 4OHT treatment (Figure 2m-5A). Interestingly, in case of SRC3/AIB1, very low levels of recruitment was observed after treatment with 3OHTPE and EtOX as compared with E2 treatment (Figure 2m-5B), whereas SRC3/ AIB1 was not recruited at all after treatment with 4OHT (Figure 2m-5B).



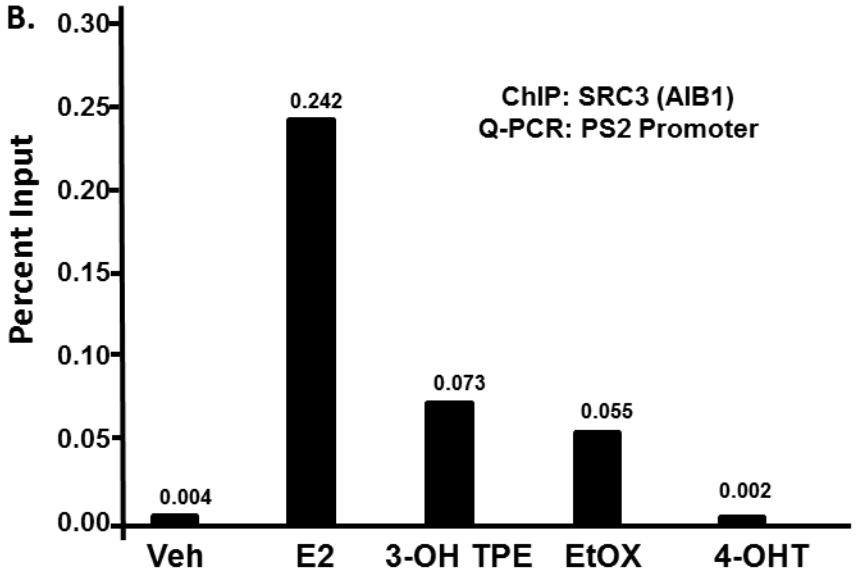


Figure 2m-5. *Recruitment of ER alpha (A) or SRC3 (AIB1) (B) at the promoter of PS2 (TFF1) gene. MCF7:5C cells were treated with E2 (10−9M), 3OHTPE (10−6M), EtOX (10−6M) or 4OHT (10−6M) for 45 minutes and cells were fixed with 1.25% formaldehyde before isolating the chromatin. ChIP was performed using ER alpha or SRC3 antibody and the immunoprecipitated DNA was quantified using specific primers for PS2 promoter by quantitative real time PCR. The values at the top of each bar represents the percent input after subtracting the negative control (raggit IgG).*

Binding of EtOX to the LBD of ER alpha

In an attempt to clarify the binding mode of EtOX to ERŭ, the flexible docking of this compound into the LBD (ligand binding domain) of the receptor co-crystallized with 4OHT (Figure 2m-6B) was performed and also, the best ranked ligand-receptor complex was superimposed onto the agonist conformation of ERŭ (Figure 2m-6B). The results show that when EtOX is fitted in the binding site of the agonist conformation of ERŭ (1gwr) the ethoxy side chain of the ligand is bumping the side chains of L525 and L540 (Figure 2m-6C) and it is unlikely for the ligand to bind in this conformation of the receptor. In contrast, when EtOX is docked into the binding site of ERŭ

antagonist conformation (Figure 2m-6D) the top ranked pose is fitted well in the binding cavity and probably, it binds to an antagonist-related conformation of the receptor.

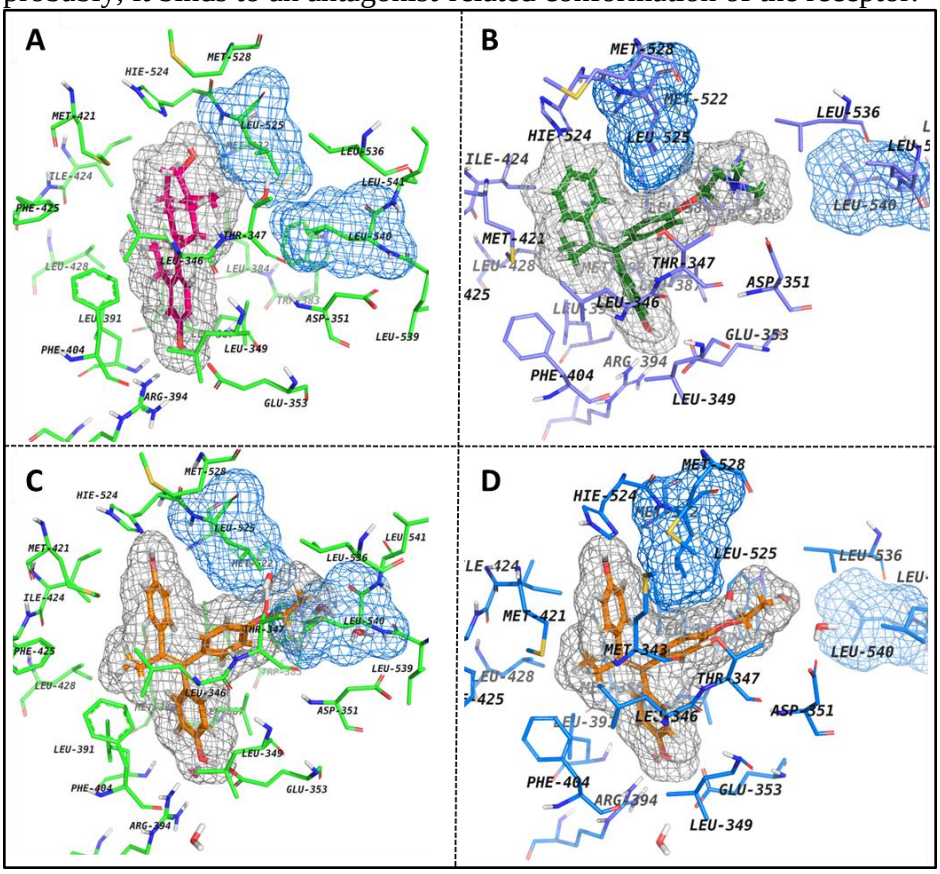


Figure 2m-6. *ERalpha binding site presented with different ligands. All ligands are shown with their corresponding molecular surfaces depicted as gray grids. Also, Leu525 and Leu540 are shown with their molecular surface depicted as blue grids. A) The agonist conformation of ERalpha co-crystallized with E2 (colored in magenta) (PDB code: 1GWR); B) 4OHT (depicted in green) co-crystallized with ERalpha - the antagonist conformation of the receptor (PDB code: 3ERT); C) EtOX (colored in orange) is superimposed in the agonist conformation of the receptor (PDB code: 1GWR) and D) same ligand is docked in the antagonist conformation of ERalpha (3ERT).*

Discussion

Estrogen-induced apoptosis can be reversed in a concentration related manner by the nonsteroidal antiestrogen 4-OHT. It is important to point out that in the ER+ MCF-7:5C cells used in this study, 4-OHT, though it binds to the ER, blocking apoptosis, does not produce any effect on cell growth when administered alone. These cells are completely resistant to the actions of nonsteroidal antiestrogens. The major finding in this study is that the test TPEs that are all fully estrogenic on cell replication in MCF-7 cells [15], also inhibit estrogen-induced apoptosis. Based upon our previous report on the molecular classifications of estrogens [198], this leads to the suggestion that the angular TPEs are creating a shaped ER complex that is analogous to that observed in x-ray crystallography with 4-OHT [28]. Indeed, molecular modeling (Figure 2m-6) demonstrates that the angular TPE would be unlikely to fit in the estradiol ER complex because steric hindrance would prevent helix 12 from sealing the LBD.

It seems that the TPEs can affect the ER complex in ways similar to 4-OHT. 4-OHT is known to retard the destruction of the 4-OHT ER complex [165,196]. Similarly, the TPEs do not

facilitate the rapid destruction of the TPE ER complex (Figure 2m-3). Thus, western blot analysis shows that the TPE ER levels are analogous to 4-OHT ER levels rather than estradiol ER-like i.e.: rapidly destroyed. Indeed, LeClercqôs group [53] have recently confirmed and extended our molecular classifications of estrogens, with a larger series of compounds and have also shown that an angular TPE does not cause the destruction of the ER complex in a manner analogous to estradiol when MCF-7 cells are examined by immunohistochemistry for the ER.

In a preliminary study, we have examined, using the ChIP assay the binding of the ER alpha in the promoter region of the TFF1 (PS2) gene. The E2-ER complex has robust binding in the promoter region (Figure 2m-5A) and SRC-3 is detected presumably bound to the ER complex (Figure 2m-5B). In contrast, 4-OHT ER complexes only have modest binding of ER alpha and virtually no SRC-3 in the promoter region. The TPEs permit some binding of the TPE ER complex in the promoter region but there are lower levels of SRC-3 and a reduced ability to stimulate PS2 synthesis (data not shown). A major conclusion of LeClerqôs paper [53] is that the putative Class II estrogens (angular estrogens) that do not permit the appropriate sealing of the LBD with helix 12 do not efficiently bind co-activators. Our respective studies are therefore in agreement.

In summary, the proposed hypothesis that the TPE-ER complex significantly changes the shape of the ER to adopt a conformation that mimics that adopted by 4-OHT when it binds to the ER. A co-activator now has difficulty in binding to the TPE-ER complex appropriately, but whereas this does affect cell replication, it dramatically impairs the events that must be triggered to cause apoptosis. Future studies will confirm or refute our hypothesis based upon the known intrinsic activity of mutant ERs and their capacity to investigate estrogen-target genes. Naturally, the absolute proof of our hypothesis would be the solution of the x-ray crystallography of a TPE-ER complex.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2n (Balaburski and Jordan) - Studies carried out by Dr. Gregor Balaburski in the Jordan laboratory at Fox Chase Cancer Center

Introduction:

Our goal was to revisit this question by utilizing wildtype MCF-7 cells to recreate a raloxifene-resistant variant of MCF7 cells *in vitro*. The failure of wild-type MCF-7 cells to create acquired resistance *in vivo* would expose an inadequacy of laboratory models or imply that acquired raloxifene resistance would not occur in the clinic. This was not the case as the answer is yes to the first question and the answer to the second question requires clinical investigation. We subsequently used the new model *in vivo* to evaluate the actions of physiological estrogen and raloxifene on the growth responses of raloxifene-stimulated tumors passaged over a decade in ovariectomized athymic mice. This laboratory strategy mimics the clinical duration of raloxifene exposure.

Work Accomplished:

Verification of cell line identity.
DNA profiling of the cell lines was conducted using the Power Plex 1.2 System resulting in the generation of allelic scores for 8 polymorphic STR loci and the amelogenin locus which are presented in Fig. 2n-1A along with the scores for MCF-7 and ECC-1 cells reported in the ATCC STR database. Data from the amelogeningene amplification were consistent with all samples being of female origin as expected. Allelic score data from the 8 polymorphic STR loci reveal a pattern almost identical among the 5 MCF7 lines that is very closely related to the scores reported for MCF-7 by the ATCC, and consistent with their presumptive identity. Scores for 5 of the 8 loci (D13S317, D7S820, D16S539, TH01 and TPOX) were identical among the study and ATCC MCF-7 cells (Fig. 2n-1A, areas of identity highlighted in pink), but there was some evidence of genetic drift in some of the study lines. ATCC MCF-7 cells have D5S818 allelic scores of 11 and 12, whereas 4 of the study lines (WS8, 5C, 2A and ICI) only have one allele (12) (allelic loss highlighted in green), whereas the MCF7-RAL cells have two alleles at this locus: 12 and 13 (variant allele highlighted in blue). Similarly, for the vMA locus, the ATCC cells have alleles 14 and 15, as do the 5C, 2A and RAL cells, whereas the WS8 and ICI cells only have one allele; 15. Scores for the CSF1PO locus were identical among the lines showing a single allele (10), with the exception of the 5C cells that have an additional allele at this locus (11). The minor variations in the DNA profile exhibited by the MCF-7 cells are similar to the sort of genetic drift that has been seen previously among sub-lines of cells cultured independently [199], and overall these fingerprinting data confirm the presumptive identity of the lines as being of MCF-7 origin. Furthermore, the profiles from the study cell lines derived from MCF-7 (WS8) show that they are more closely related to each other than to the ATCC MCF-7 cells, again consistent with their having been derived from a common ancestor subline.

A.

cell line:	D5S818		D13S317		D7S820		D16S539		vWA		TH01		Amelogenin		TPOX		CSF1PO	
	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2
MCF-7 ATCC	11	12	11	11	8	9	11	12	14	15	6	6	X	X	9	12	10	10
MCF7-WS8 p24		12	11	11	8	9	11	12		15	6	6	X	X	9	12	10	10
MCF7/5C p217		12	11	11	8	9	11	12	14	15	6	6	X	X	9	12	10	11
MCF7/2A p549		12	11	11	8	9	11	12	14	15	6	6	X	X	9	12	10	10
MCF7/ICI p42		12	11	11	8	9	11	12		15	6	6	X	X	9	12	10	10
MCF7/RAL p83	12	13	11	11	8	9	11	12	14	15	6	6	X	X	9	12	10	10

B.

cell line	D5S818		D13S317		D7S820		D16S539		vWA		TH01		Amelogenin		TPOX		CSF1PO	
	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2
MCF-7 ATCC	11	12	11	11	8	9	11	12	14	15	6	6	X	X	9	12	10	10
MCF7 (GMB) p184	11	12	11	11	8	9	11	12	14	15	6	6	X	X	9	12	10	10
MCF7-RAL (GMB) p74	11	12	11	11	8	9	11	12	14	15	6	6	X	X	9	12	10	10
ECC1 ATCC	10	11	9	12	9	10	9	9	14	15	9	10	X	X	8	8	11	12

Figure 2n-1. *Verification of cell line identity by DNA fingerprinting. See Materials and methods.*

Development of a novel raloxifene-resistant tumor cell line, MCF7-RAL.

To examine the effects of long-term raloxifene treatments on breast cancer cell growth we derived a novel breast raloxifene-resistant cell line, MCF7-RAL (GMB). The MCF7-RAL (GMB) cells were developed by continuously passaging cells in estrogen-free media supplemented with 1 εM raloxifene for at least 1 year. The fingerprinting data from the independently obtained MCF7 cells p184 and MCF7-RAL p74 (GMB) cells reveal a pattern of allelic scores that is identical to the scores reported for the ATCC MCF-7 cells, and highly divergent from the pattern reported for non-related cells such as the ATCC ECC-1 cells (Fig. 2n-1B). These data suggest that the cell lines used in this study are in fact of ATCCMCF-7 origin and not a variant of the MCF-7WS8 clone. For clarity the MCF-7RAL (GMB) are referred to as MCF-7RAL throughout this paper. Currently, the MCF7-RAL cells have been propagated in RAL containing medium for approximately 10 years. The growth characteristics *in vitro* were compared and contrasted with wild-type MCF-7. Within 3 days of treatment the MCF7 cells are significantly (p=0.02) stimulated by 1 nM E2, 2.2-fold increase as compared to vehicle-treated controls (Fig. 2n-2A). Maximum induction, 4.8-fold increase as compared to control was observed at day 15. The E2-induced growth of the MCF7 cells was blocked by 1 εM FUL treatments. In contrast to E2, 1 εM RAL did not stimulate the growth of the MCF7 cells. Similarly to the MCF7 cells, within 3 days of treatments, E2 significantly (p=0.02) induced the growth of the MCF7-RAL cells (Fig. 2n-2B). Maximum E2 induction was observed at day 9, 2.67-fold increase as compared to control. At day 3 of treatment RAL also significantly (p=0.02) induced the growth of the MCF7-RAL cells. Maximum RAL induction was observed at

day 6, 2.1-fold increase as compared to the controls. The E2 and RAL-induced growth of the MCF7-RAL cells was significantly inhibited by 1 εM FUL treatments within 3 (p=0.04) and 6 days (p=0.02) of treatment, respectively. In addition, the MCF7-RAL cells were spontaneously growing. To further characterize the RAL-resistant phenotype of the MCF7-RAL cells we determined the protein expression levels of ERL To determine the protein levels of ERLin MCF7 and MCF7-RAL cells we treated the cells with EtOH, 1 εM RAL, 1 nM E2 and 1 εM FUL for 48 h. The ERLprotein levels in the MCF7-RAL cells are regulated in an identical manner as in the parental MCF7 cells (data not shown). Treatments with 1 nM E2 and 1 εM FUL decreased the protein levels of ERL, while treatments with 1 εM RAL maintained the protein expression of ERL The levels of total MAPK and total AKT in the MCF7-RAL cells appeared to remain unchanged, regardless of treatment, when compared to the parental, MCF7 cells. However, the levels of phosphorylated MAPK, increased in the EtOH-treated MCF7-RAL cells (data not shown). Luciferase reporter assays indicated that 1 nM E2

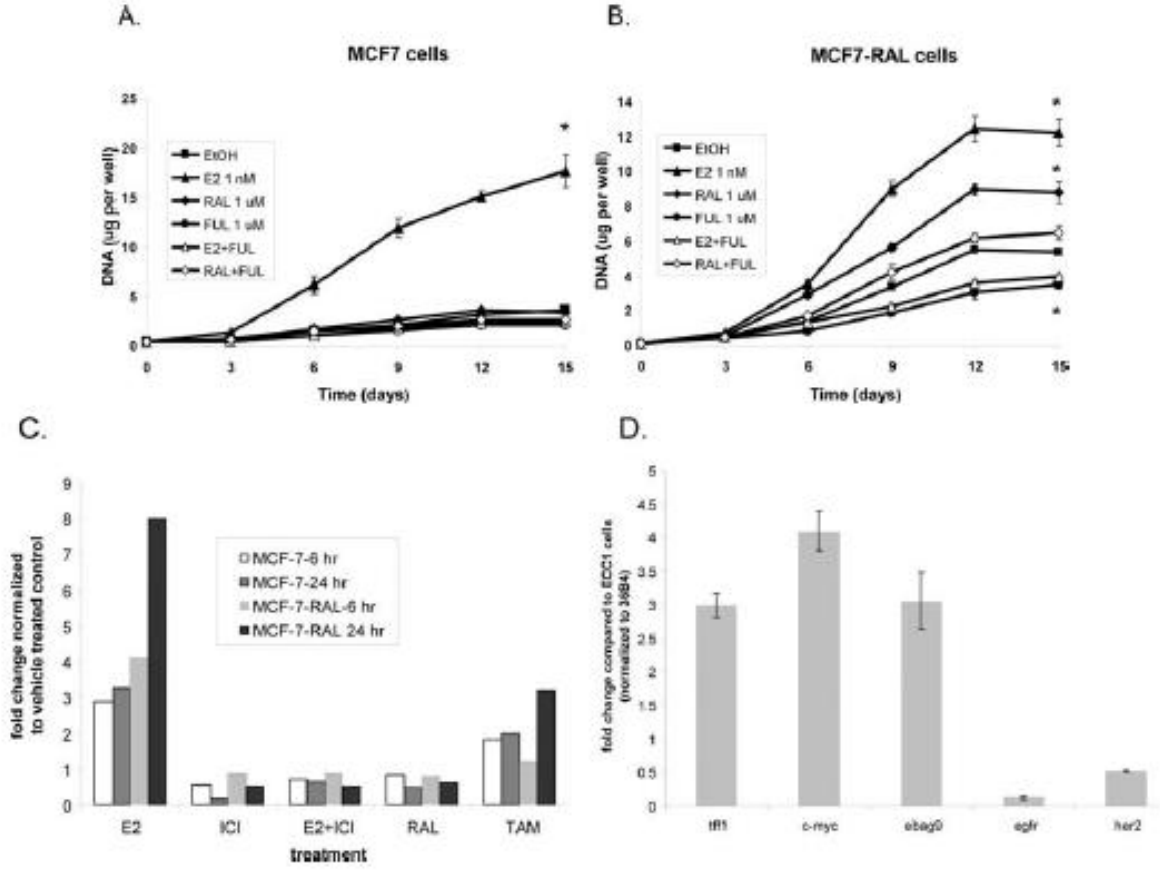


Figure 2n-2. *The MCF7-RAL cells are spontaneously growing cells that are stimulated by raloxifene (RAL) and 17ß-estradiol (E2). (A) Three days before seeding the MCF7 cells were cultured in E2-free conditions, RPMI-yellow media with charcoal stripped FBS. The MCF-7 cells were than seeded in a 24-well plate and 24-h post seeding the cells were treated with vehicle, 1 nM E2, 1 μM RAL, 1 μM fulvestrant (FUL) and combination of drugs as described in Materials and methods. (B) MCF7-RAL cells were seeded and treated in an identical manner as in (A). (C) MCF-7 and MCF7-RAL cells were either E2 or RAL starved for 3 days before transfection with the appropriate reporters. Twenty-four h post transfection the cells were treated with vehicle control (EtOH), 1 nM E2, 1 μM RAL, 1 μM TAM, 1 μM FUL and combination of 1 nM E2 and1 μM FUL. Luciferase activity was measured 6 and 24 h after post treatment. (D) Expression of ER·-regulated genes in MCF7-RAL cells in steady state. Error bars = standard error of the mean (SEM); *p<0.05, statistically significant finding as compared to EtOH-treated cells*

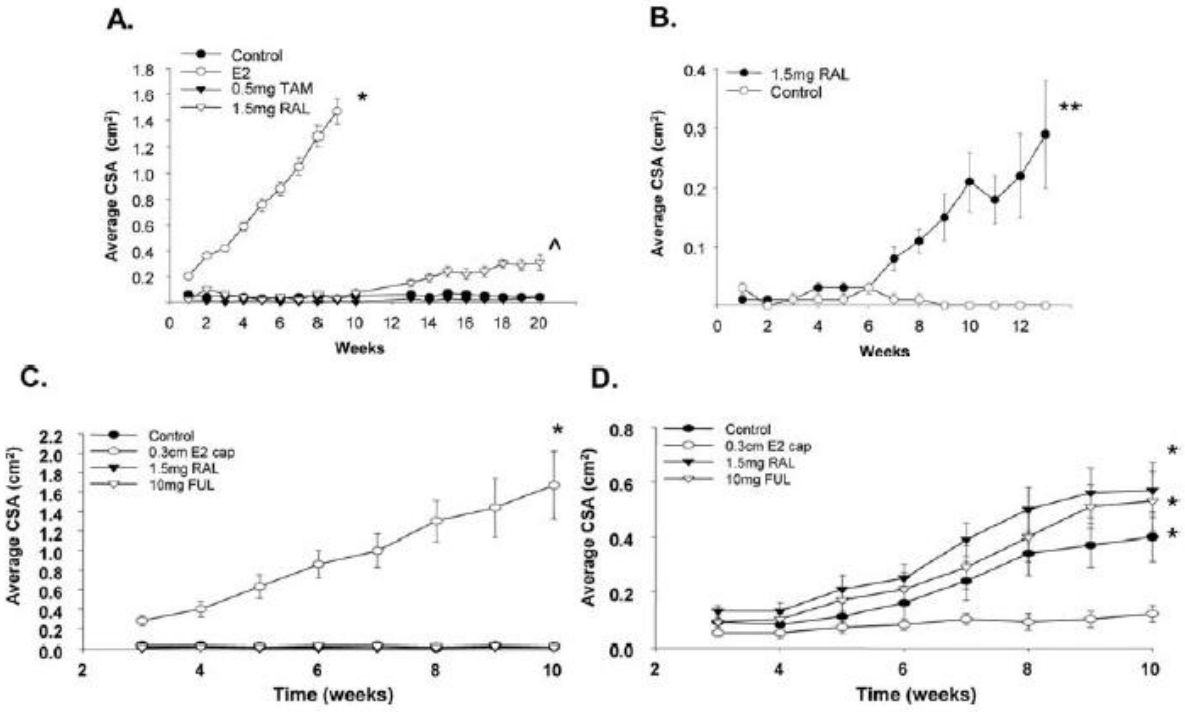


Figure 2n-3. *Establishment of MCF7-RAL tumor xenograft model. (A) MCF7-RAL-resistant cells (1x107) were injected into the axillary mammary fat pads of ovariectomized athymic mice. The mice were then divided into 4 groups and treated as follows: placebo, implanted with silastic 0.3-cm E2 capsule, orally gavaged with RAL (1.5 mg daily) and TAM (1.5 mg daily). (B) A single tumor from the RAL-treated group was transplanted (passage 1) into 20 naïve ovariectomized athymic mice and divided into 2 groups: placebo and RAL treated. Error bars = SEM; *p<0.0001, E2 vs. all other treatment groups; ^p=0.048 RAL vs. control. **p=0.05, RAL vs. control (C) MCF7-E2 and MCF7-RAL tumor xenogratfs were bi-transplanted into each ovariectomized athymic mouse (total of 40). The MCF7-E2 tumor was implanted in the left and the MCF7-RAL tumor was implanted in the right axillary mammary fat pad. The mice were randomized into groups of 10 and implanted with 0.3-cm E2 capsule or treated with RAL (1.5 mg daily), FUL (5 mg s.c., twice a week) or no treatment (control). (C) MCF7-E2 tumors; (D) MCF7-RAL tumors; error bars = SEM; *p<0.05, E2 vs. all other treatment groups.*

treatments significantly induced transcriptional activation of the reporter in MCF7 and MCF7-RAL cells (Fig. 2n-2C) consistent indicating similar activity of ERL in the parental and resistant cell line. Fulvestrant (FUL) and RAL treatments did not induce activation of the reporter. Furthermore, FUL treatments abolished the E2-dependent reporter activity. TAM treatments significantly induced reporter activity in both MCF7 and MCF7-RAL cells at the 24-h time point. The MCF7-RAL cells grew spontaneously and were inhibited by FUL treatment (Fig. 2n-2B). To further characterize the RAL-resistant phenotype of the MCF7-RAL cells at steady state, we determined by quantitative real-time PCR, the basal mRNA expression of ERL-regulated genes in MCF7 and MCF7-RAL cells (Fig. 2n-2D). In the basal state, the MCF7- RAL cells exhibited 3-fold up-regulation of *tff-1*, 4.1-fold upregulation of the *c-myc* and 3.1-fold up-regulation of *ebag9*. In contrast, the levels of *egfr* and *her2* were down-regulated by 7.7- and 1.99-fold, respectively.

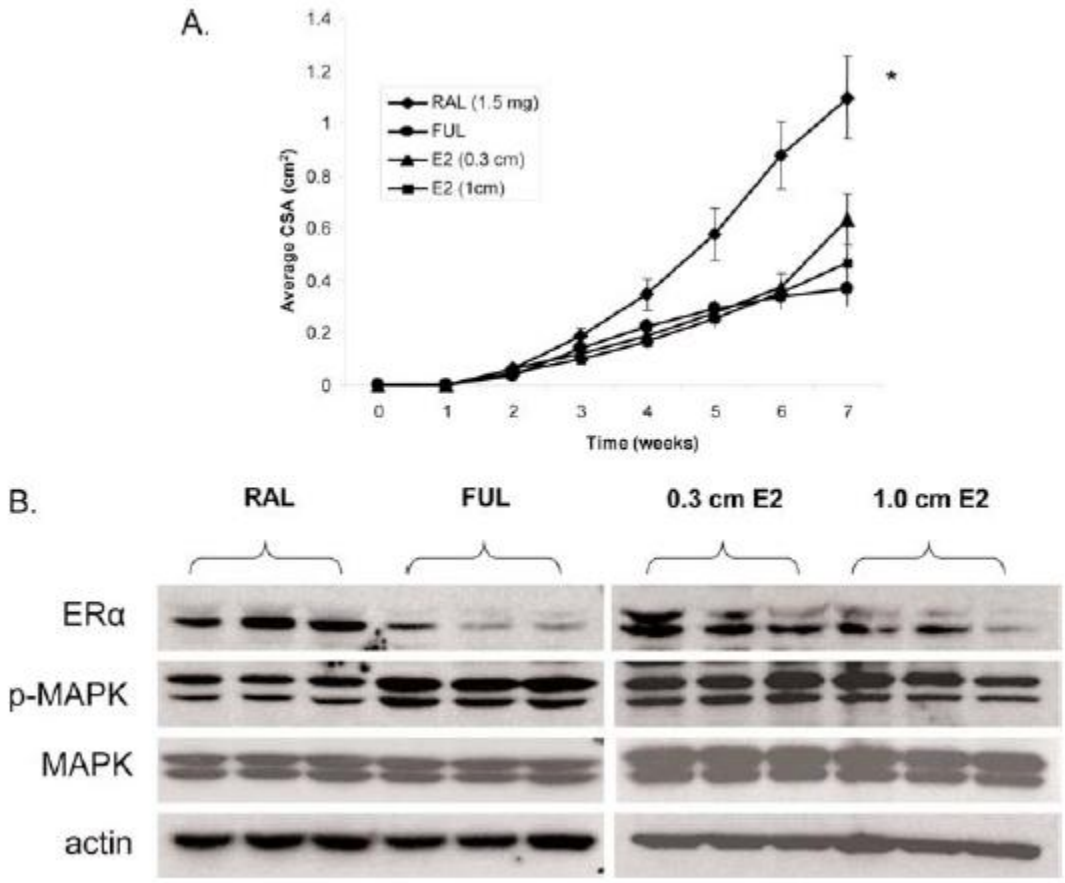


Figure 4. *Pre- and post-menopausal concentrations of E2 significantly impair the growth of long-term RAL-treated MCF7-RAL xenografts. (A) MCF7-RAL tumor xenografts serially transplanted for at least 8 years were implanted into 45 ovariectomized athymic mice. The animals were treated with RAL (1.5 mg daily), FUL (5 mg s.c twice weekly) or implanted with either 0.3-cm or 1.0-cm silastic E2 capsules. (B) Western blot analysis of protein extracts collected from (A). *p=0.001 RAL vs. all other treatment groups.*

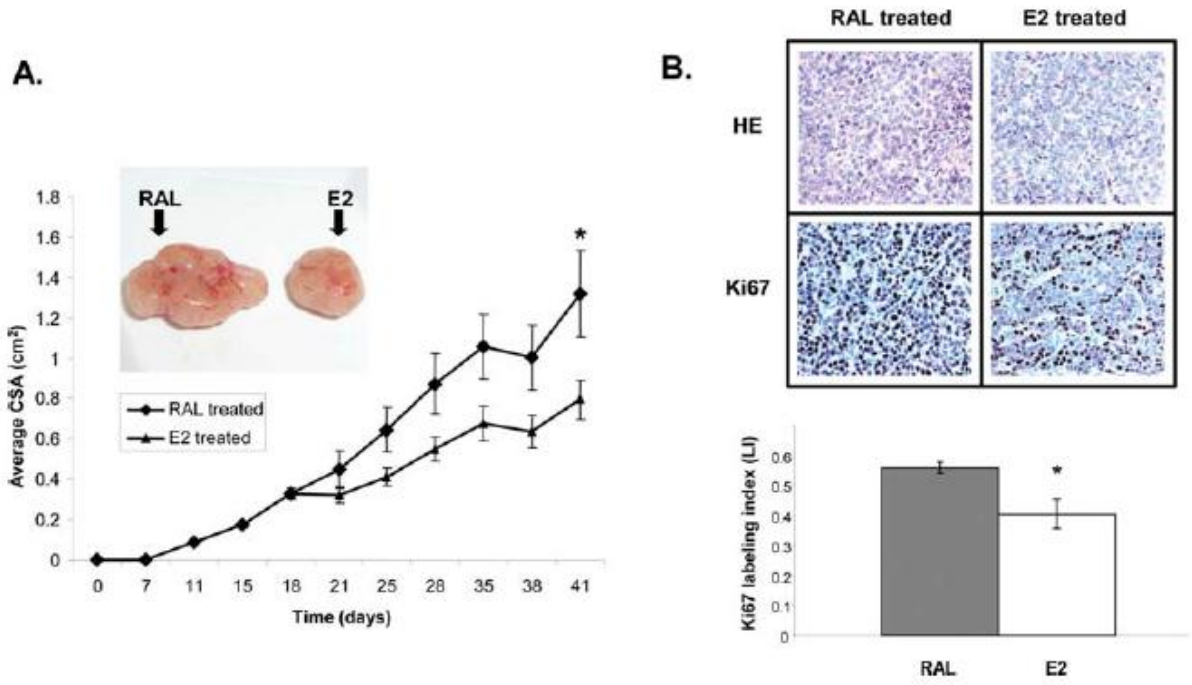


Figure 2n-5. *17ß-estradiol treatments impair the growth of established MCF7-RAL xenografts. (A) Long-term RAL-treated MCF7-RAL xenografts were implanted into 30 ovariectomized athymic mice and the animals were treated with RAL until the cross sectional area (CSA) of the tumors reached 0.3 cm2. The animals were than randomized into 2 groups: continued RAL treatments or implanted with 0.3-cm E2 capsules. Estradiol treatments significantly impaired*
*the growth of the MCF7-RAL xenografts by day 38 (20 days post-introduction of E2). Insert: representative images of E2- and RAL-treated tumors. (B) Histological analysis of tumors from (A). *p=0.02 RAL vs. E2.*

Development of an MCF7-RAL xenograft tumor model.

To develop MCF7-RAL xenograft tumor model *in vivo*, 1x107 MCF7-RAL cells were injected into the mammary fat pads of nude athymic mice as described in Materials and methods. The mice were treated with vehicle, implanted with 0.3-cm silastic E2 capsule or orally gavaged with 1.5 mg daily RAL or 0.5 mg daily TAM. At week 9, average cross sectional area (CSA) of the estradiol-treated group was 1.47 cm2, significantly greater ($p<0.0001$) than the control and the other treatment groups (Fig. 2n-3A). The E2-treated mice grew large tumors and were sacrificed at week 10 because of ethical considerations. By week 15, palpable tumors were observed in the RAL-treated group (average CSA = 0.24 cm2) which were significantly larger than the control group ($p=0.048$) (Fig.2n-- 3A). At week 20, a single tumor from the raloxifene treated group was excised, resected and transplanted into 20 ovariectomized athymic mice (Fig. 2n-3B). The mice were divided into control (no treatment) and a RAL- (1.5 mg daily) treated group. Starting at week 7, RAL promoted tumor growth which by the conclusion of the experiment at week 13 was statistically significant as compared to the control group ($p<0.05$) (Fig. 2n-3B). To further characterize the MCF7-RAL tumor xenograft model and to determine the effects of E2 and RAL on estrogen and raloxifene-dependent breast tumor growth, we bitransplanted MCF7-E2 and MCF7-RAL tumors on opposite sides in the

axillary mammary fat pads of the same animal. MCF7-E2 xenografts were implanted into the left and the MCF7-RAL xenografts were implanted into the right mammary fat pad of 40 ovariectomized athymic mice. As anticipated the E2-treated MCF7-E2 tumors displayed robust tumor growth and at week 10 the mean tumor size was 1.67 cm2 (Fig. 2n-3C). No tumor growth was observed in the control, RAL- and FUL-treated group (Fig. 2n-3C). In contrast, at week 10, RAL and

FUL stimulated MCF7-RAL tumor growth while the E2-treated tumors exhibited minimal growth (Fig. 2n-3D). At week 10, the mean size of the RAL- and FUL-treated tumors was 0.57 and 0.53 cm2, respectively. Interestingly, spontaneous tumor growth was observed in the control MCF7-RAL (at this point considered passage 3) (mean tumor size = 0.4 cm2, $p<0.05$ as compared to the E2 group) (Fig. 2n-3D).

Long-term RAL treatments of the MCF7-RAL tumor xenogratfs.

To determine the effects of E2 on long-term RALtreated MCF7-RAL xenografts, we evaluated the effects of pre- and post-menopausal levels of E2 [200] on the growth of MCF7-RAL tumors that were serially transplanted and continuously treated with RAL for at least 8 years. The MCF7-RAL tumor xenografts were transplanted into 45 ovariectomized athymic mice that were treated with RAL, FUL and 0.3- or 1.0-cm silastic E2 capsules (Fig. 2n-4A)

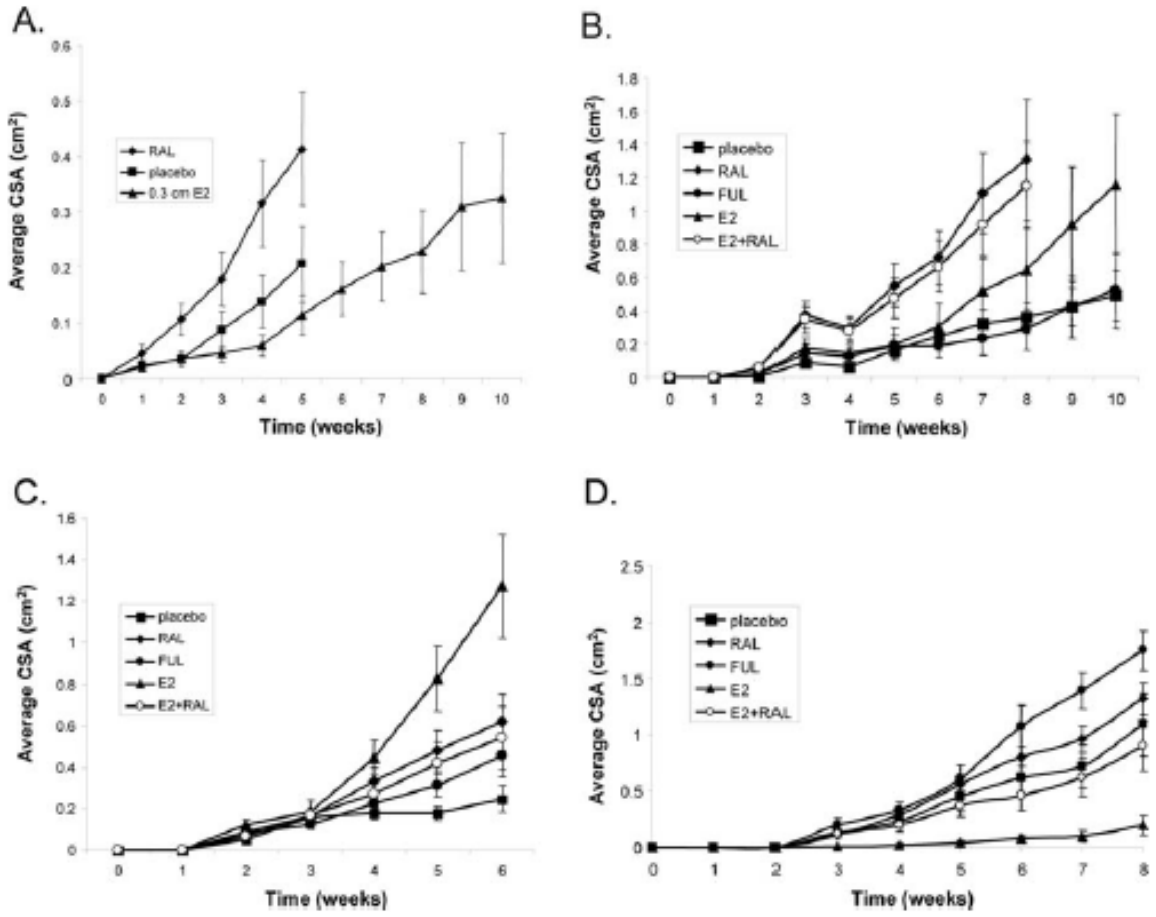


Figure 2n-6. *Long-term estrogen and raloxifene treatments result in changes in the phases of SERM resistance. (A) MCF7-RAL tumor xenografts were implanted into 45 ovariectomized athymic mice, the mice were divided into 3 groups and were either left untreated, treated with RAL (1.5 mg/daily) or implanted with 0.3-cm E2 capsules. (B) E2-treated tumors from (A) were resected and re-transplanted into 25 ovariectomized athymic mice that were either left untreated or treated with RAL (1.5 mg/daily), FUL (5 mg subcutaneously, twice weekly), implanted with 0.3-cm E2 capsules and combination of RAL and E2. (C) E2-treated tumors from (B) were serially retransplanted into 25 ovariectomized athymic mice that were either left untreated or treated with RAL, FUL, implanted with 0.3-cm E2 capsules and combination of RAL and E2. (D) RAL-treated tumors from (C) were implanted into naive animals and continuously treated with RAL for 28 weeks before being implanted into 25 naive animals that were either left untreated or treated with raloxifene, FUL, implanted with 0.3-cm E2 capsules and combination of RAL and E2. See Results for a precise description of the evolution of raloxifene resistance and statistical significance of the findings in the individual experiments.*

At week 7, the RAL-treated xenografts exhibited a statistically significant ($p<0.001$) RAL-stimulated growth (mean CSA = 1.1 cm2) as compared to the FUL, 0.3 and 1.0 cm E2-treated tumors (mean CSA = 0.37, 0.63, 0.46 cm2, respectively). There were no statistical differences between the FUL, 0.3 and 1.0 cm E2-treated tumors. To further characterize the effects of E2 on the long-term RAL-treated MCF7-RAL tumor xenografts we analyzed the ERŁexpression of the xenografts (Fig. 2n-4B). The long-term RAL-treated MCF7-RAL xenografts continue to express ERŁand RAL treatments increased the expression of ERŁwhile FUL treatments down-regulated the expression of ERŁ No differences in ERŁprotein expression was observed between the two different concentrations of E2- treated tumors.

Estrogen treatments inhibit the growth of established MCF7- RAL tumors.
To determine the effects of E2 on established MCF7-RAL tumors, MCF7-RAL tumor xenografts were implanted into ovariectomized athymic nude mice and the animals were treated with RAL until the average CSA of the tumors reached 0.3 cm2. At this point the animals were randomized into 2 groups: 1) continued RAL treatments and 2) implanted with 0.3-cm E2 capsules (Fig. 2n-5A). Within 3 days post-E2 implantation, there were visible morphological and size differences between the RAL- and E2-treated tumors
(Fig. 2n-5A insert). At day 7, the mean CSA was 0.64 cm2 for the RAL-treated and 0.41 cm2 for the E2-treated tumors. At day 17 the CSA of the RAL-treated tumors was 1.00 cm2 and the CSA of the E2-treated tumors was 0.64 cm2 (p=0.03). At the end-point of the experiment statistically significant differences (p=0.02) were observed between the RAL-treated tumors (average CSA = 1.32 cm2) and the E2-treated tumors (average CSA = 0.79 cm2) (Fig. 2n-5A). Histological analysis of the RAL- and E2-treated tumors at the conclusion of the experiment (Fig. 2n-5B) by hematoxylin and eosin staining indicated that there are no significant morphological changes between the two treatment groups. However, significant differences in the expression of Ki-67, a known marker of proliferation, were observed between the two groups. There were significant statistical differences (p=0.02) between the average labeling index (LI) of the
RAL-treated group and the E2-treated group which were 0.56Ñ0.04 and 0.40Ñ0.09, respectively.

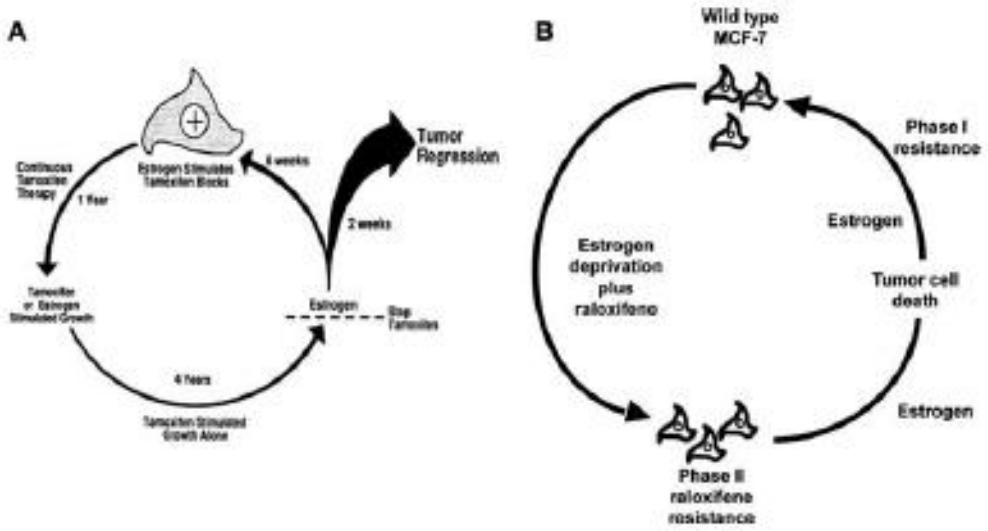


Figure 7. *Proposed model of the evolution of acquired raloxifene resistance in ER·-positive MCF-7 breast cancer. On the left (Fig. 7A) is our original proposal from cyclical evolution of acquired resistance to tamoxifen in a clonal derivative (MCF-7 WS8) of wild-type MCF-7 cells originally acquired from Dr Dean Edwards (University of Texas, San Antonio, TX) in 1985. All steps in the cycle (17,18) were documented with experimental data in the peer reviewed literature. On the right (Fig. 7B) is a summary of our current results that illustrate the cyclical evolution of acquired resistance to raloxifene in wild-type MCF-7 cells (MCF-7 GMB) acquired from Dr Myles Brown (Dana Farber Cancer Center, Harvard University, Boston, MA) in 1995. The technique of employing an estrogen-deprived environment with raloxifene accelerates the evolution to phase II-acquired resistance where estradiol causes tumor regression. This process can be reversed through phase I-acquired resistance in a continuous estrogenic environment so tumor growth is again controlled by raloxifene treatment. Continuous raloxifene does again cause phase II-acquired resistance and exposes estrogen-induced tumor regression.*

Effects of long-term estrogen treatments on the growth of MCF7-RAL tumors.

To determine the effects of long-term E2 treatments on the growth of MCF7-RAL tumor xenografts we transplanted long-term RAL-treated MCF7-RAL tumors into 45 ovariectomized athymic mice. The mice were divided into 3 groups: no treatment, RAL and 0.3 cm E2 (Fig. 2n-6A). Three weeks post-implantation the average CSA of the tumors were 0.17, 0.08 and 0.09 cm2 for the RAL, placebo and the 0.3-cm E2-treated tumors. At week 5, differences could be observed between the treatment groups; the average CSA of the RAL-treated tumors was 0.41 cm2 and the average CSA of the 0.3-cm E2-treated tumors was 0.11 cm2. The average CSA of the untreated tumors was 0.2 cm2 indicating spontaneously growing tumors. The 0.3-cm E2 treatment was continued for additional 5 weeks and at week 10 the average CSA was 0.32 cm2. At that point the E2-treated tumors were excised, resected and bitransplanted into 25 ovariectomized athymic mice. The animals were divided into 5 groups: RAL, placebo, 0.3 cm E2, FUL and combination of E2- and RAL-treated (Fig. 2n-6B). Treatment with RAL continued to induce the growth of the MCF7-RAL tumor and at week 8, the average CSA was 1.3 cm2. At week 8, the average CSA of the placebo and the FUL-treated tumors was 0.36 and 0.29 cm2. Unexpectedly, E2 treatments either individually or in combination with RAL induced the growth of the MCF7-RAL xenografts. At week 8 the average CSA of the E2-treated tumors was 0.64 cm2 and combination of E2 and RAL treatments resulted in tumor growth (average CSA = 1.15 cm2). The placebo, E2 and FUL treatments were continued and at week 10 the average CSA of the tumors was: 0.49, 1.16 and 0.52 cm2. The growth rates of the RAL-treated tumors were significantly different compared to the placebo ($p=0.003$) and FUL-treated, tumors ($p=0.005$). However, the growth rate of the E2- and E2 + RAL-treated tumors was indistinguishable from the RAL treated tumors. At week 10 the E2-treated tumors were excised, resected and implanted into 25 naive animals. The treatments were identical to the previous experiment and consisted of placebo, RAL, FUL, E2 and combination of E2 and RAL (Fig. 2n-6C). At week 3 post-implantation there were no significant differences in the average CSA between the various treatments, and the average CSA was 0.05, 0.08, 0.08, 0.12 and 0.06 cm2 for the placebo, RAL-, FUL-, E2- and the E2 + RAL-treated tumors. However, dramatic changes were observed at week 4 as E2 treatments started to induce significant tumor growth (average CSA = 0.44 cm2). In contrast RAL inhibited the estrogen-induced tumor growth as the combination of E2 + RAL treatments average CSA was 0.27 cm2. The average CSA of the RAL-treated tumors was 0.33 cm2 and the placebo- and FUL-treated tumors were 0.18 and 0.22 cm2, respectively. At conclusion of the experiment at week 6, the E2-treated tumors reached average CSA of 1.27 cm2. The average CSA of the RAL-treated tumors was 0.62 cm2 and the E2 + RAL group was 0.54 cm2. The growth rate of the E2- treated group was significantly different ($p<0.01$) from all other groups with the exception of the RAL-treated tumors, but approached significance ($p=0.06$). Upon conclusion of the experiment at week 6 the RAL-treated tumors were excised, resected and implanted into ovariectomized athymic animals that were continuously treated with RAL. Following 28 weeks of continuous RAL treatments the long-term treated MCF7-RAL tumor xenografts were implanted into 25 animals that were divided into 5 groups and treated as follows: placebo, RAL, E2, FUL and E2 + RAL (Fig. 2n-6D). Within 3 weeks of treatments highly statistically significant differences ($p<0.01$) emerged between the E2-treated tumors and all other treatment groups. At week 3 tumor growth was observed in the placebo, FUL, RAL and E2 + RAL while negligible tumor growth was observed in the E2-treated group (average CSA = 0.008 cm2). These differences persisted throughout the duration of the experiment and at its conclusion at week 7, the average CSA of the E2-treated tumors was 0.1 cm2. In contrast, significant tumor growth was observed in all other treatment groups. Paradoxically,

maximum tumor growth was observed in the FUL treatment groups (average CSA = 1.4 cm2). Significant tumor growth was also observed in the RAL group (CSA = 0.96 cm2) and in the placebo group (CSA = 0.72 cm2). The average CSA of the E2 + RAL group was 0.62 cm2, indicating that E2 treatments significantly inhibited the RAL-stimulated tumor growth (p=0.03).

Discussion

In a previous study, we used a select clone of MCF7 cells (MCF7-WS8) [201] that is extremely sensitive to estrogen stimulation, to create an MCF7 raloxifene-resistant cell line *in vitro* (MCF7-RAL) [85]. In a short-term growth experiment *in vivo* MCF7-RAL cells grew into tumors in response to raloxifene and tamoxifen but estradiol inhibited tumor growth [85]. This biological response to SERMs and estradiol is classified as phase II SERM resistance (1). We have now addressed the question of the predictable creation and evolution of SERM resistance with raloxifene *in vivo* using a wild-type MCF7 cell line from a source that is external to our laboratory. The origins of the line (MCF7 GMB) were confirmed by genotyping (Fig. 2n-1) and unlike the MCF7-WS8 cells were similar to the wild-type MCF7 from ATCC and the original MCF7 cells derived by Soule [202]. We created a new MCF7-RAL cell line that is able not only to grow in response to raloxifene *in vitro* but eventually grow in response to raloxifene *in vivo* with phase II resistance, i.e. estradiol inhibited tumor growth (Fig. 2n-3). However, in this 10-year retransplantation study *in vivo* we demonstrate the reversal of the biological characteristics of phase II anti-hormoneresistant tumor growth with long-term estradiol therapy to phase I resistance; i.e., estradiol- or raloxifene-stimulated growth, and then predominately estradiol-stimulated growth. Raloxifene now acts as an anti-estrogen, inhibiting estradiol stimulated growth (Fig. 2n-6C). Thus raloxifene has the potential to cause the classic evolution of SERM resistance in the clinical setting and reverse the process during long-term physiologic estrogen therapy. Nevertheless clinical studies need to be considered to evaluate the efficacy of estrogen on patients whose breast tumors develop during long-term raloxifene treatment to prevent osteoporosis [203]. Current antihormonal therapies used for the treatment of breast cancer (tamoxifen or aromatase inhibitors) can develop acquired resistance in the clinical cells. The best clinical responses to estrogen are observed with high-dose (15 mg) DES therapy following exhaustive anti-hormonal therapy [204,205]. Indeed, one patient had a complete response during the 5-year to DES therapy administered continuously and a further 5-year disease-free response following the cessation of therapy [204]. In contrast, no complete responses were observed in the study of Ellis *et al* [91] probably because the patient population was not selected based on exhaustive anti-hormonal therapy but only failure of therapy following aromatase inhibitors.

Experience in the laboratory demonstrates that long-term (>5 years) tamoxifen treatment is necessary to cause the evolution of tamoxifen resistance *in vivo* to expose the apoptotic actions of physiologic estrogen [132]. Consistent with these observations, a profound antitumor effect was noted with physiologic estrogen after 10 years of alternating treatments with raloxifene and physiologic estrogen (Fig. 2n-6D).

With regard to treatment strategies for SERM-resistant disease, it is important to note that the response to the injectable steroidal pure anti-estrogen fulvestrant is unpredictable (Fig. 2n-6). At some stages of acquired resistance, fulvestrant acts as an antitumor agent but at other stimulates tumor growth (Fig. 2n-6). This may in part explain the low reported efficacy of fulvestrant in clinical trials treating patients who already have acquired resistance to tamoxifen or aromatase inhibitors. However, it also appears that the recommended monthly doses of fulvestrant used clinically may be suboptimal and in fact actually enhance tumor growth in tumors with phase II resistance with physiologic estrogen present [84]. A recent clinical study on metastatic breast cancer demonstrates that doubling the monthly dose of fulvestrant enhances antitumor activity [206]. In a laboratory

study, an antitumor dose of fulvestrant in athymic animals implanted of estrogen [84]. In the present study, despite using repeated subcutaneous injections of fulvestrant weekly, tumor growth was enhanced in some tumor passages with long-term acquired resistance to raloxifene (Fig. 2n-6D). It appears that the efficacy of fulvestrant may depend both upon bioavailability, pharmacokinetics and, as yet, unresolved pharmacodynamic factors of the steroidal antiestrogens at unknown targets within the tumor with acquired raloxifene resistance. Two further conclusions emerged from the present study. The variant of MCF7 cells that is closely related to wild-type MCF-7 from ATCC could develop acquired resistance to raloxifene *in vitro* and the resulting cell line MCF7-RAL grew in response to either estradiol or raloxifene (Fig. 2n-2).

MCF7-RAL cells exhibited gene activation consistent with autonomous growth (Fig. 2n-2C). The cells responded to estradiol both *in vitro* and *in vivo* as a growth stimulus but only developed raloxifene-stimulated tumors *in vivo* after 5 months of continuous treatment. This was confirmed by retransplantation into raloxifene-treated ovariectomized athymic mice (Fig. 2n-3B). In contrast to MCF7-RAL cells *in vitro*, estradiol is no longer a growth stimulus *in vivo* and completely inhibits tumor development (Fig. 2n-3D). This new biology of estrogen action classifies the MCF7-RAL cells as phase II-resistant *in vivo*. Secondly, the observation that treatment with tamoxifen *in vivo* (Fig. 2n-3A) did not result in tumor growth and that this MCF7 variant could not be used to develop acquired tamoxifen resistance *in vitro* (H. Liu, unpublished), was unusual and unanticipated based on previous studies over two decades. All cells died during incubation with 4-hydroxy-tamoxifen. This observation is currently under investigation as it may provide insight into the cytocidal actions of tamoxifen. Based on this long-term study, and studies using prostate cancer cells, a general principle is emerging in cancer endocrinology. An androgen-independent cell line, LNCaP 104 R2 was derived from the androgen-dependent cell line, LNCaP 104 S [207]. The LNCaP 104 R2 cells are androgenindependent, continue to express the androgen receptor (AR) and low concentrations of androgen in the media inhibited their growth. Implantation of the LNCap104-R2 cells in male athymic-castrated nude mice resulted in tumor growth, that was inhibited by implantation of testosterone capsules [208].

In a subsequent study utilizing the LNCaP 104-R2 tumor model, Chuu *et al* [209] significantly impaired established tumor growth with androgen treatments; approximately 2 months post-cell injections. However, within 40 days of initiation of androgen treatments tumor growth resumed, which was a clear indication that the tumors adapted to the presence of the androgen and utilized it for growth. Subsequent androgen withdrawal inhibited tumor growth. These data are consistent with the assumption that androgendependent and androgen-independent tumor cells coexist in prostate cancer patients resulting in positive selection of androgen-independent tumor cells during androgen ablation therapies, resulting in androgen-independent growth. Therefore, intermittent androgen replacement therapy has been tested in recent years [210]. Nearly 20 years ago, we first described the antitumor potential of physiologic estrogen to destroy what is now known as phase II-acquired tamoxifen resistance [133]. We noted that the interplay of apoptotic estrogen and tamoxifen would create a cyclical method for controlling the growth of ER-postitive breast cancer by purging with estrogen at the appropriate time and then continuing anti-hormone therapy [133]. The cycles could be repeated. This original work is summarized in Fig. 2n-7. Our current 10-year *in vitro* and *in vivo* study of the evolution of acquired raloxifene resistance was initiated to explore the potential of raloxifene to exhibit acquired resistance in breast cancer during the long-term treatment and prevention of osteoporosis [203]. We conclude that the predictable evolution of acquired resistance to the SERM tamoxifen and estrogen deprivation (aromatase inhibitors) also occurs with raloxifene. The current conclusions are summarized in Fig. 2n-7, following the creation of MCF7- RAL cells *in vitro* is a raloxifene/estrogen-free environment which was then transplanted into athymic mice. The

development of phase II-acquired resistance i.e.: estrogen-induced apoptosis or estrogen-inhibited tumor growth [45] occurs with raloxifene and the principle is also true for the evolution of acquired androgen withdrawal in prostate cancer in the laboratory [207,208,209]. Preliminary studies to translate these laboratory findings to aid patients have shown merit [91,204,205]. Further understanding of the mechanism of sex steroid induced apoptosis [211] and the definition of vulnerable tumors following exhaustive anti-hormonal therapy have the potential to identify appropriate patient populations to amplify the effectiveness of a sex steroid apoptotic trigger in metastatic breast cancer and possibly prostate cancer [212].

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2o (Sengupta and Jordan) - Studies carried out by Dr. Surojeet Sengupta in the Jordan laboratory at Fox Chase Cancer Center

Estrogen Regulation of X-Box Binding Protein-1 and its Role in Estrogen Induced Growth of Breast and Endometrial Cancer Cells

Introduction:

We report the estrogen regulation of endogenous XBP1 and show that co-activators SRC-1 and SRC-3 along with ERŭ are recruited at the promoter and/or enhancer elements of XBP1 gene. By depleting XBP1 levels using siRNA we also show that XBP1 is required to mediate the estrogen-induced growth of MCF7 breast and ECC1 endometrial cancer cells.

Work Accomplished:

Estrogen upregulates XBP1 in MCF7 and ECC1 cells and is a primary responsive gene

We first studied the dose-response of (E2) on XBP1 mRNA regulation in MCF7 breast cancer cells after eight hours of E2 treatment using quantitative real-time PCR. Our data shows (Fig. 1A) that XBP1 mRNA was induced by E2 in a dose dependent manner. Low dose of E2 (10^{-14} M) was not able to induce any upregulation of XBP1 levels, whereas 10^{-12} M of E2 achieved the peak induction. Higher doses of E2 treatment induced XBP1 levels similar to10^{-12} M of E2. We then studied the regulation of the XBP1 at various time points after 1nM (10^{-9}M) E2 treatment and found that it is upregulated as early as two hours after estrogen treatment and maintains an elevated level even after 48 hrs of estrogen treatment in breast cancer (MCF7) as well as in endometrial cancer (ECC1) cells (Fig. 1B and 2A). This upregulation was completely abrogated in the presence of fulvestrant, a complete anti-estrogen, indicating an ERŭmediated mechanism (Fig. 1C and 2B). Pre-treatment with CHX, an inhibitor of protein synthesis, in the presence of E2 did not alter the E2-mediated upregulation suggesting de novo protein synthesis is not required for the estrogen mediated

upregulation of XBP1(Fig. 1C and 2B)

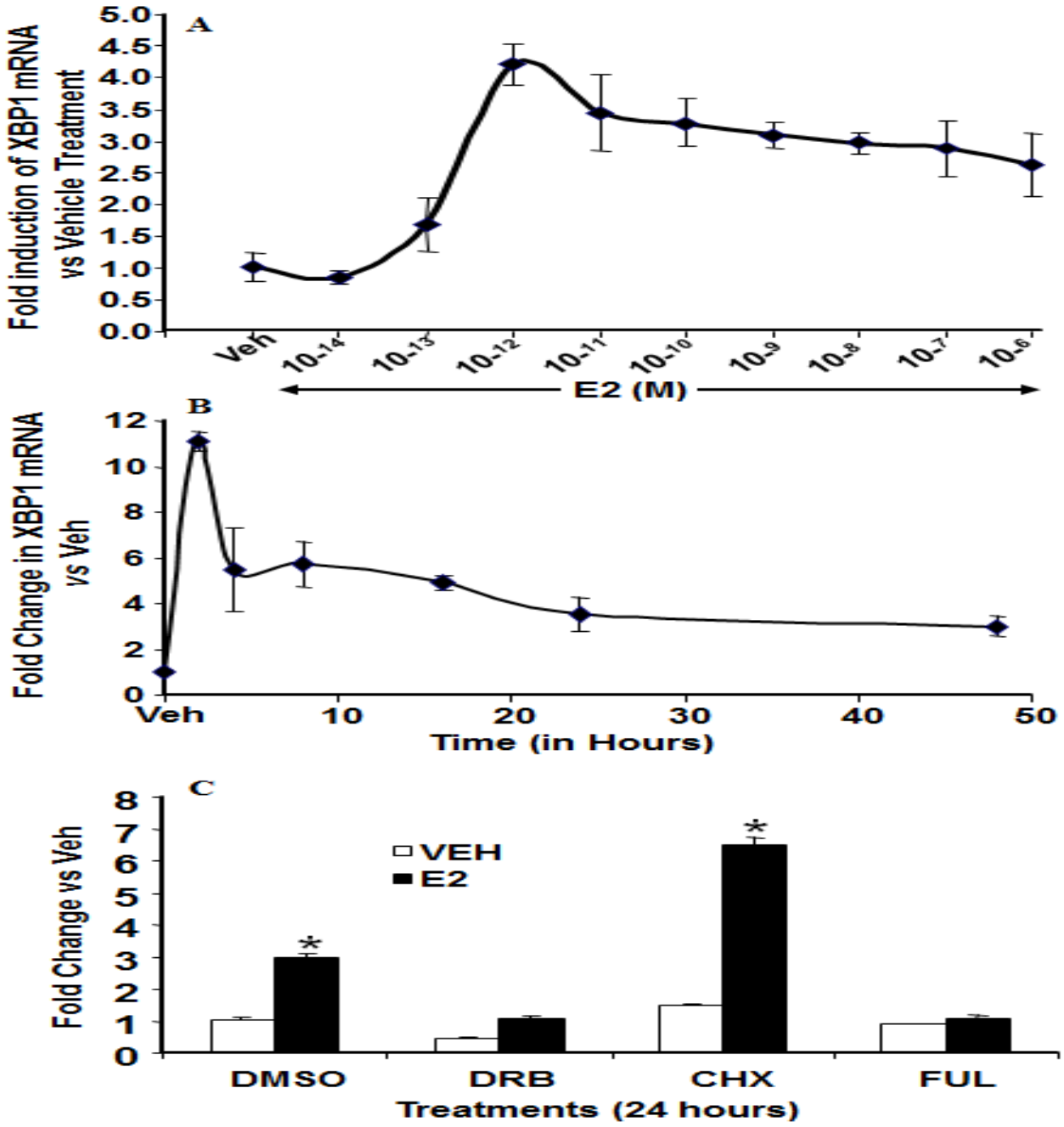
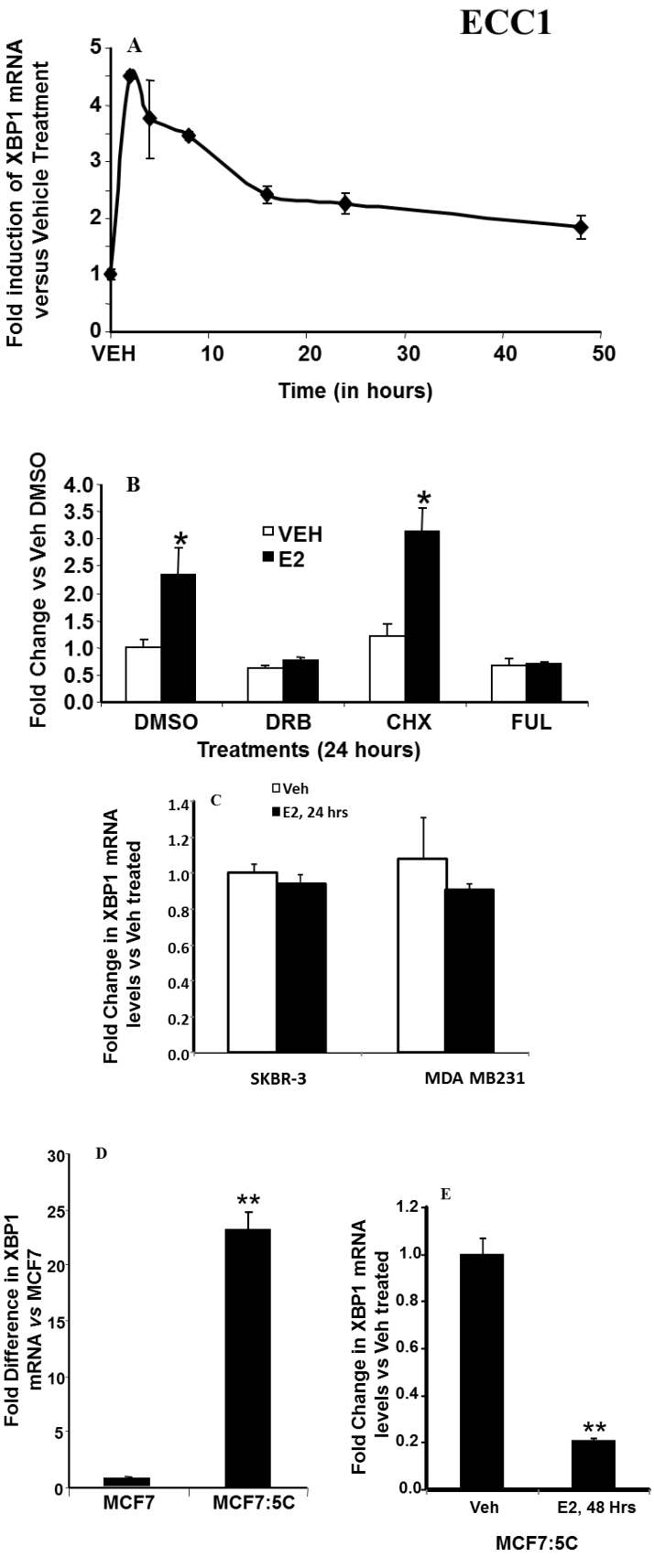


Fig 2o-1. *E2-mediated up-regulation of XBP1 in MCF7 cells. MCF7 cells were treated with different concentration of E2 for eight hours and expression of XBP1 was measured using quantitative real time PCR and compared with vehicle treated cells (**A**). MCF7 cells treated with E2 (1nM) for 2, 4, 8, 16, 24 or 48 hrs and expression of XBP1 was measured using quantitative real time PCR and compared with vehicle treated cells (**B**). MCF7 cells were treated with CHX (10µg/ml), DRB (75µM), or FUL (1mM) in absence or presence of E2 (1nM) for 24 hrs and expression of XBP1 was assessed using real-time PCR (**C**). * Denotes P<.05 compared to respective vehicle treated group.*

Conversely, pretreatment with DRB, a transcriptional inhibitor, completely blocked the up-regulation of XBP1 demonstrating involvement of transcriptional machinery in upregulation of XBP1 by estrogen (Fig. 1C and 2B).
We also studied the regulation of XBP1 in ER negative breast cancer cells SKBR3 and MDA MB 231 cells. As expected, XBP1 was not regulated by E2 in these cells (Fig. 2C). Furthermore we investigated the levels of XBP1 in long term estrogen-deprived MCF7 cells, known as MCF7:5C cells [195] which are estrogen-deprived resistant cells. Paradoxically low levels of E2 induce apoptosis in these cells [83]. Basal levels of XBP1 mRNA were found to be around twenty-three fold higher in MCF7:5C cells as compared to MCF7 cells (Fig 2D). Interestingly, E2 treatment for 48 hrs drastically down-regulated the XBP1 levels in MCF7:5C cells (Fig 2E) which coincides with estrogen-induced apoptosis in these cells.

Recruitment of ERα and other factors at the promoter and enhancer regions of XBP1 gene
To further confirm the direct involvement of ERŬ in transcriptional induction of XBP1 gene we performed ChIP assay to assess the recruitment of ERŬ, SRC1, SRC3 and serine-2-phosphorylated RNA polymerase II at ~ 0.3 Kb (promoter) and ~ 13 Kb (enhancer) upstream of the transcription start site of XBP1 gene (Fig 3A) in the MCF7 cells treated with vehicle or 1nM E2 for 45 minutes. We found higher occupancy of ERŬ and SRC3 at the enhancer region, but not at the promoter region. The occupancy of these factors at the enhancer region was further stimulated after 45 minutes of E2 treatment as compared to vehicle treatment (Fig 3B and 3C). In contrast, serine-5-phosphorylated RNA polymerase

Fig 2o-2. *E2-mediated up-regulation of XBP1 in ECC1 cells. ECC1 cells treated with E2 (1nM) for 2, 4, 8, 16, 24 or 48 hrs and expression of XBP1 was measured using quantitative real time PCR and compared with vehicle treated cells (**A**). ECC1 cells were treated with CHX (10μg/ml), DRB (75μM), or FUL (1mM) in absence or presence of E2 (1nM) for 24 hrs and expression of XBP1 was assessed using real-time PCR (**B**). SKBR-3 and MDA-MB-231 cells were treated with E2 (1nM) or vehicle (0.1% ethanol) for 24 hrs and expression of XBP1 was measured using quantitative real time PCR and compared with vehicle treated cells (**C**). Total RNA from MCF7 and MCF:5C were isolated and expression of XBP1 was measured using quantitative real time PCR relative to MCF7 cells (**D**). MCF7:5C cells were treated with vehicle (0.1% ethanol) or E2 (1nM) for 48 hrs and expression of XBP1 was measured using quantitative real time PCR and compared with vehicle treated cells (**E**).*
** Denotes p<.05 compared to vehicle treated group (**B**). ** Denotes P<.05 compared to MCF7 cells (**D**) or vehicle treated group (**E**).*


ECC1
A
Fold induction of XBP1 mRNA versus Vehicle Treatment
Time (in hours)
VEH
B
Fold Change vs Veh DMSO
VEH
E2
DMSO
DRB
CHX
FUL
Treatments (24 hours)
C
Veh
E2, 24 hrs
Fold Change in XBP1 mRNA levels vs Veh treated
SKBR-3
MDA MB231
D
Fold Difference in XBP1 mRNA vs MCF7
MCF7
MCF7:5C
E
Fold Change in XBP1 mRNA levels vs Veh treated
Veh
E2, 48 Hrs
MCF7:5C

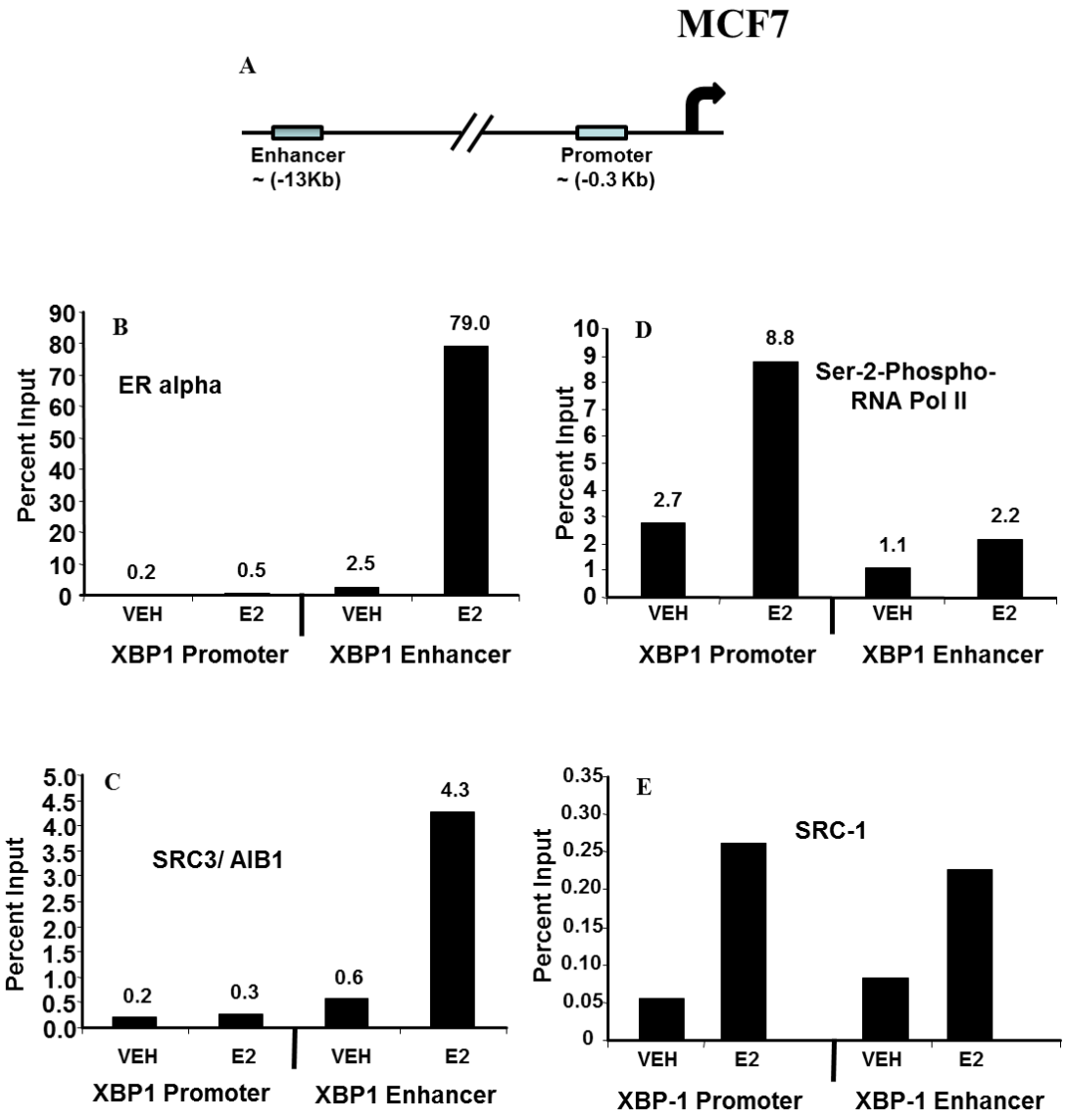


Fig 2o-3. *Recruitment of ERα, phospho-serine-2-RNA polII, SRC1 and SRC3, at the proximal promoter and distal enhancer region of XBP1 gene assessed by chromatin immuno-precpitation assay. MCF7 cells were treated with vehicle or E2 (1nM) for 45 minutes and ChIP assay was performed as mentioned in materials and methods. Schematic representation of the promoter and enhancer regions of XBP1 gene (**A**). The extent of recruitment of the factors indicated is shown for promoter and enhancer region of XBP1 gene. The data is expressed as percent input of 1/20th part of starting chromatin material in each case after subtracting the non-specific binding. The data shown is representative of three separate experiments with similar results.*

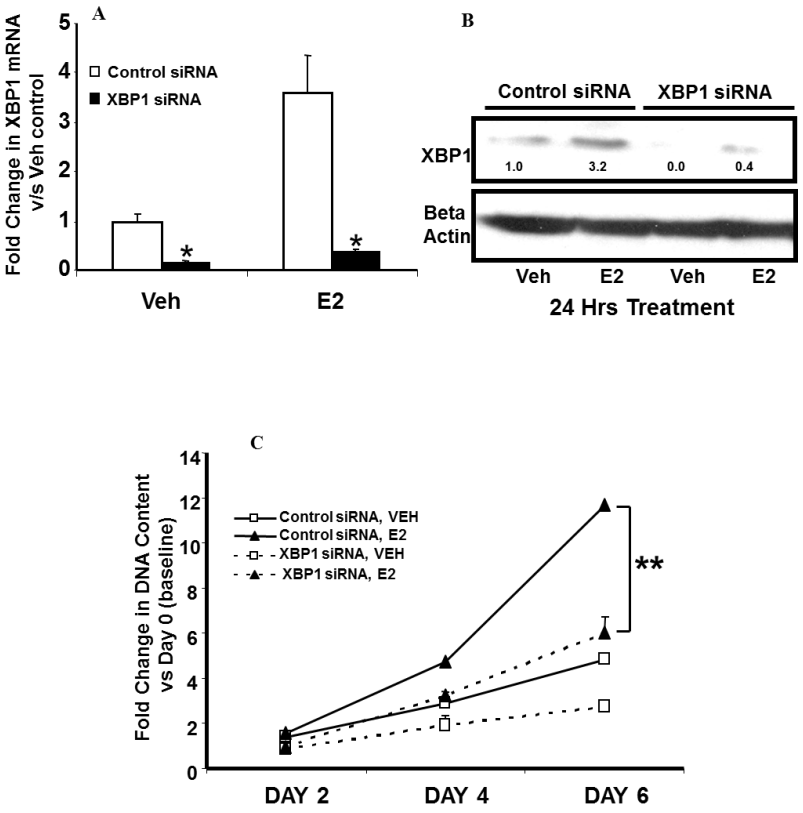
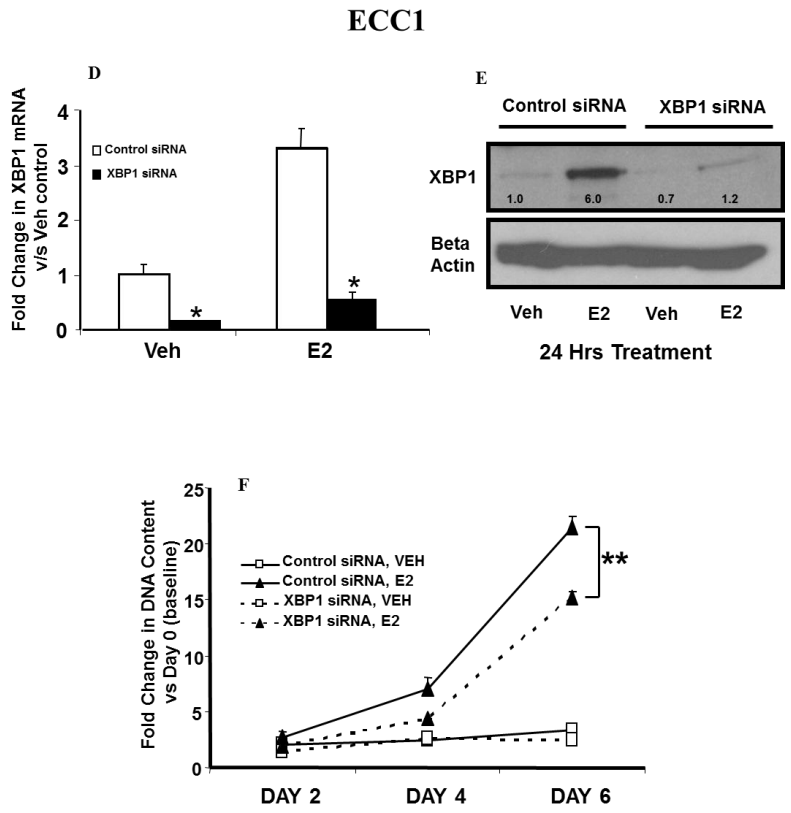


Fig 4. *Short interfering RNA (siRNA) mediated knockdown of XBP1 inhibits growth of MCF7 and ECC1 cells and its effect on estrogen mediated growth. MCF7 and ECC1 cells, transfected with XBP1 siRNA or control siRNA, were treated with E2 (1nM) or vehicle for 24 hrs and the extent of knock-down was assessed using quantitative real time PCR compared with control siRNA, vehicle treated cells (**A & D**) and western blotting (**B & E**). Subsequently cells were re-seeded and the growth of the cells was monitored over six day period. Total DNA content was measured as a marker of growth and the fold change in DNA content was calculated compared to the number of cells at the time of start of the treatment (baseline) (**C & F**). * Denotes P<.05 compared to control siRNA group and ** denotes p<.005, using unpaired Student's 't' test. The western blots were scanned and quantified. Levels of XBP1 normalized for beta-actin, relative to control siRNA-vehicle treated cells, are indicated below each band.*



II was found to be recruited fourfold more at the promoter region than at the enhancer region after 45 minutes of E2 treatment (Fig 3D). Occupancy of SRC1 was stimulated after E2 treatment in both promoter and enhancer region of XBP1 gene (Fig 3E). These results indicate that the enhancer region of XBP1 is involved in the regulation of estrogen-induced transcriptional stimulation of XBP1 gene.

Fig 2o-5. *Estrogen receptor α levels in MCF7 cells treated with control or XBP1 siRNA. MCF7 cells were transfected with control or XBP1 siRNA and subsequently treated with vehicle or E2 for 24 hrs. Estrogen receptor α levels were assessed by western blotting. Levels of beta actin are shown as loading control. The western blots were scanned and quantified. Levels of ER alpha protein normalized for beta-actin, relative to control siRNA-vehicle treated cells, are indicated below each band.*

XBP1 depletion inhibits estrogen mediated growth

To investigate the functional importance of XBP1, we evaluated the effect through loss of function using a pool of siRNA against XBP1 in MCF7 and ECC1 cells. The extent of XBP1 knockdown was confirmed by real time PCR and western blotting (Fig. 4A and 4B; 4D and 4E). A growth assay was performed after XBP1 knockdown by siRNA and total DNA content was used as a measure to determine the cell growth over a six day period. A parallel identical growth assay was performed using pool of non-targeting control siRNA for comparison. XBP1 knockdown attenuated the E2-induced growth of MCF7 and ECC1 cells by 49 and 30 percent respectively as compared to cells treated with control siRNA (Fig. 4C and 4F). These data indicated that the level of XBP1 expression is critical for inducing estradiol mediated growth of breast and endometrial cancer cells.

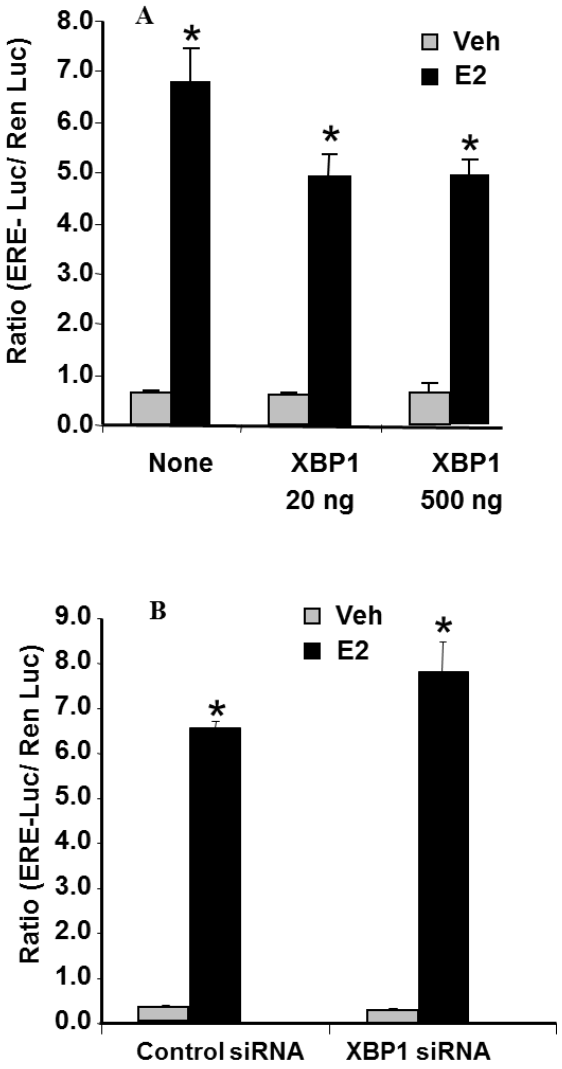


Fig 2o-6. *ERE mediated luciferase activity in XBP1 overexpressing or XBP1 depleted cells. MCF7 cells were transfected with empty vector (none), 20ng or 500ng of XBP1 expressing plasmid and ERE mediated luciferase activity was assessed in absence or presence of 1nM E2 (**A**). MCF7 cells transfected with control or XBP1 siRNA were used to assess ERE mediated luciferase activity in presence or absence of 1nM E2 (**B**). Renilla luciferase activity was used as internal control and all values are represented as a ratio of ERE-luciferase and renilla luciferase activity.*

We further investigated if the levels of ERŬ were altered in the XBP1 depleted MCF7 cells as compared to control siRNA treated MCF7 cells. No differences were detected in levels of ERŬ in XBP1 depleted cells as compared to control siRNA treated cells in presence of vehicle or E2 for 24 hrs (Fig 2o-5). This rule out the possibility that growth inhibition of XBP1 depleted cells was due to altered ERŬ levels.

XBP1 over-expression or XBP1 depletion does not affect ERE mediated transcriptional activity

To understand the underlying mechanism by which XBP1 may influence estrogen mediated growth we examined the effect of XBP1 over-expression or depletion on the transcriptional activity of ER from a classical ERE. We performed ERE-luciferase reporter assay in the MCF7 cells transiently transfected with XBP1 expression plasmid or XBP1 siRNA. No differences were observed (Fig 2o-6A and 6B) in transcriptional activity (as measured by luciferase activity) of the ERE-luciferase reporter in the cells either over-expressing XBP1 or the cells depleted of XBP1 as compared to their respective controls. This result suggests that levels of XBP1 in the cell may not affect the classical transcriptional activity of ERŬmediated through the direct binding of ERE.

Discussion:

Estrogen is the prime growth regulator of ER positive breast and endometrial cancer cells. To better understand the induction of estrogen mediated growth in breast cancer, several studies [213,214] have explored the estrogen-induced transcriptional network using DNA microarrays to identify downstream pathways. In these studies, many of the estrogen-regulated genes are identified as transcription factors which may be collectively responsible for the phenotypic manifestations of estrogen-induced growth of ER positive cancer cells. However, in the majority of cases, the precise role of the downstream events in growth is not understood. One solution which can be used to dissect the complexities of clinical tissues is to interrogate estrogen responsive cell lines as a recent study [215] has noted that the genes are similarly regulated by estrogen in breast cancer cells in vitro and human breast tumors. This provides a unique opportunity to study the underlying mechanism, by manipulating specific genes in the cells, which may influence the progression of ER positive cancers and also provide potential targets for therapeutic intervention. In this context, some recent reports have identified the important roles played by the estrogen-regulated genes such as FOXA1 [197,216,217], GREB1 [218] and GATA-3 [216,219] in regulating estrogen-induced growth in breast cancers.

In the present study we evaluated the phenotypic effects of the E2-regulated gene XBP1, which has been consistently shown to be highly co-expressed with ER alpha in breast cancer patients and is also known to be up-regulated by estrogen in the ER positive breast cancer cells in vitro [213,215,220,221,222,223,224] . Although it is well established that XBP1 plays a key role in UPR and endoplasmic reticulum stress by acting as a transcription factor for the genes involved in UPR, its role in E2- dependent ER positive cancers is not fully understood.

Our results confirm that XBP1 is an E2-regulated gene which is in agreement with previous studies [197,213]. The E2-induction of XBP1 is mediated by ERŬ and does not need de-novo protein synthesis for the up-regulation, as CHX did not alter the regulation. Treatment with DRB, an inhibitor of transcription completely blocked the upregulation of XBP1 by estrogen, indicating transcriptional regulation. The ChIP data further confirmed direct binding of ERŬ, SRC1, SRC3 and serine-2-phosphorylated RNA polymerase II at the promoter and/ or enhancer region of the XBP1 gene. Interestingly, recruitment of ERŬand SRC3 (AIB1) was higher at the enhancer region of the XBP1 gene and very minimal at the promoter region. ERŬrecruitment was induced dramatically at the enhancer region after 45 minutes of E2 treatment. Recruitment of ERŬwas also accompanied by SRC1 and SRC3 at the enhancer region. However, as expected, recruitment of serine-2-phosphorylated RNA polymerase II was higher at the promoter region than the enhancer region. These data strongly suggest that the enhancer region of XBP1 gene, which is approximately 13 Kb upstream of transcription start site, is involved in the transcriptional regulation of XBP1 by ER. Indeed, recent studies [225,226,227] have indicated that distal enhancers of E2 induced genes GREB1 and carbonic anhydrase12 are involved in the transcriptional regulation by ER. It has been

shown that the distal enhancer can interact with the proximal promoter region of these estrogen regulated genes by intra-chromosomal looping.

This study reports for the first time that the XBP1 level is critical for E2-induced growth of ER-positive breast and endometrial cancer cells as evidenced by marked inhibition of E2-induced growth of XBP1 deficient MCF7 and ECC1 cells. This specifically demonstrates that the endogenous level of XBP1 and its up-regulation by estrogen is intimately involved in the growth regulation of estrogen responsive breast and endometrial cancer cells. A recent study demonstrated that overexpression of XBP1 in ER positive breast cancer cells can lead to anti-estrogen resistance, by regulating genes associated with apoptosis and cell cycle progression [228].

To further understand the mechanism by which XBP1 may influence the E2-induces growth, we hypothesized that levels of XBP1 may affect the ERE- mediated transcriptional activity of ER. To test this we depleted or over-expressed XBP1 and performed ERE-luciferase reporter assay. Our data shows that the level of XBP1 in the MCF7 cells does not affect the transcriptional activity of ER mediated through classical ERE binding. This indicates that XBP1 may influence the growth of the cells by either regulating a sub set of genes directly under the control of XBP1 or may also modulate the E2 regulation of the genes which are not exclusively regulated by classical ERE mediated transcription. These data are, however, in contrast to a previous study [229] where XBP1 over-expression activated ER transcriptional activity in a ligand independent manner. The differences in the results may be attributed to the exogenous over-expression of ERŬin the previous study whereas in the present study we relied on the intrinsic activity of ER in the MCF7 cells. Further investigations are required to address the associated mechanism of action.

In summary, our results demonstrate that XBP1 expression is estrogen regulated at the transcriptional level and the enhancer region of XBP1 gene may play a critical role in regulating E2 mediated transcriptional activation. Our findings show that expression level of XBP1 is critical in achieving optimal E2-induced growth in breast and endometrial cancer cells without influencing the classical ERE mediated transcriptional activity. Our findings also provide an explanation for the strong correlation observed between ERŬand XBP1 expression in the breast cancer patients. Taken together, we suggest that this novel mechanism for the regulation of cancer cell growth via XBP1 may be exploited as a novel drug target in future studies of anti-hormonal resistance in ER positive cancer cells.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

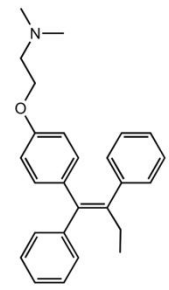
Task 2p (Maximov and Jordan) - Studies carried out by Dr. Philipp Maximov in the Jordan laboratory at Fox Chase Cancer Center

Structure Function Relationships of Estrogenic Triphenylethylenes Related to the Anti-estrogens Endoxifen and 4- Hydroxytamoxifen.

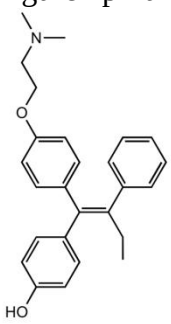
Introduction

Planar (E2) or non-planar (TPEs) compounds are both classified as estrogens based on their actions to cause growth of the immature rodent uterus or provoke vaginal cornification in castrate animals. However, knowledge of the structure of the 4OHTAM: ER LBD complex [28] led to the idea that all estrogens may not be the same in their interactions with ER [56]. Previous studies suggest that non-planar TPEs with a bulky phenyl substituent prevents helix-12 from completely sealing the LBD pocket [56]. This physical event creates a putative óanti-estrogen likeô configuration within the complex. However, the complex is not anti-estrogenic because Asp351 is exposed to communicate with AF-1 thereby causing estrogen-like action. Thus, there are putative Class I (planar) and Class II (non-planar) estrogens [56]. A similar classification and conclusion has been proposed [230], but the biological consequences of this classification are unknown.

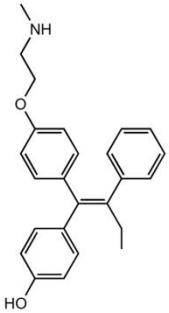
In this report we further addressed the hypothesis that the shape of the ER complex can be controlled by the shape of an estrogen. We have synthesized a range of hydroxylated TPEs to establish new tools to investigate the relationship of shape with estrogenic activity through the exposure of asp351. For convenience, the structures of nonsteroidal antiestrogens described in the text are illustrated in Figure 2p-1and the test compounds in Scheme 1.



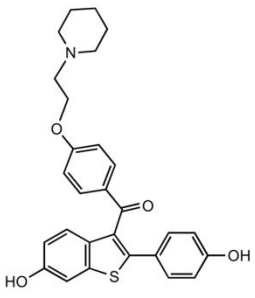
tamoxifen



4-OH tamoxifen



endoxifen

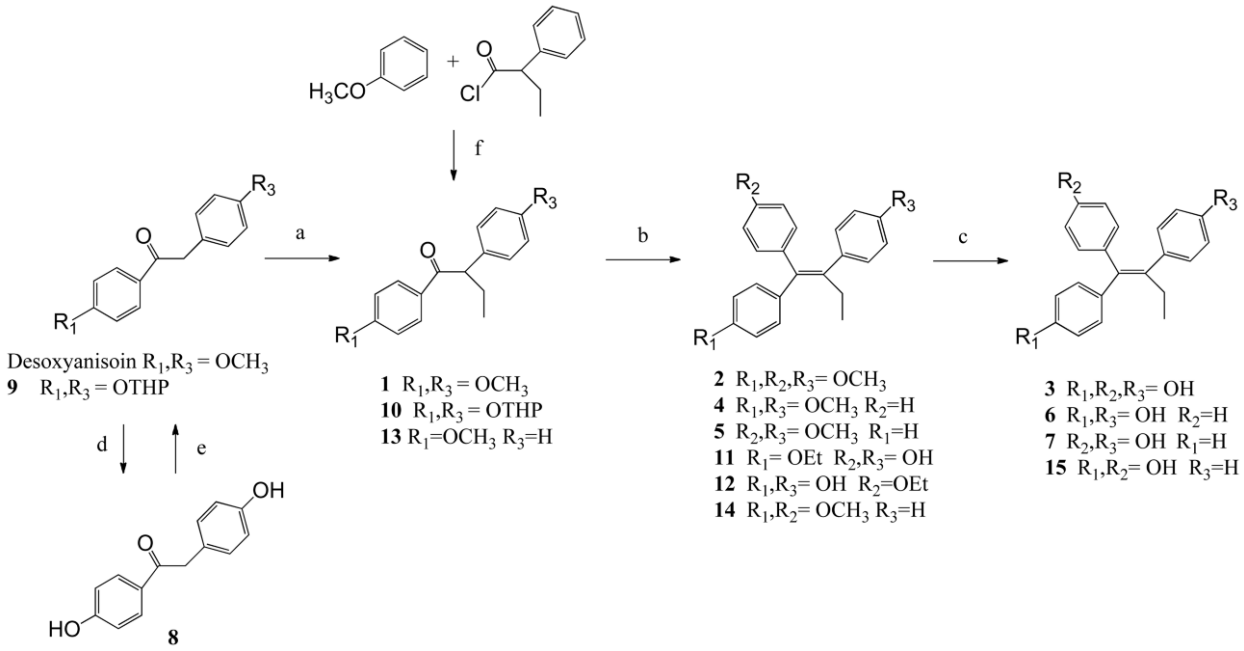


raloxifene

Figure 2p-1.*The formula of tamoxifen and its hydroxylated metabolites endoxifen, and 4OHTAM. The related SERM raloxifene is shown for comparison.*

Work Accomplished:

Chemistry. The general synthetic routes used to prepare substituted 1,1,2-tribenzyl-but-1-ene compounds are outlined in Scheme 1.



Scheme 1. Synthesis of substituted 1,1,2-tribenzyl-but-1-ene compounds Reaction conditions: (a) KOtBu, ether, 1 hr, then, EtI, reflux 12 hrs; (b) 4-BrMgC6H4R2, THF,refluxed 12 hrs, then, H_3PO_4, refluxed 2 hrs; (c) BBr_3, CH_2Cl_2, 4 days; (d) HI, AcOH, 130–140° C, 4 hrs; (e) C_5H_8O, p-$CH_3C_6H_4SO_3H$-H_2O, 0° C 4.5 hrs; (f) AlC_{l3}, CS_2, 20 ° C, 22 hrs.

Desoxyanisoin was treated with potassium t-butoxide followed by reflux with ethyl iodide to give 1 in 74% yield. Intermediate 1 was refluxed with the formed Grignard reagent of 4-bromoanisole, then treated with phosphoric acid to yield 2. Removal of the methoxide groups was accomplished with boron tribromide to give 3. Isomers 6-7 were synthesized from 1 by treatment with the formed Grignard reagent of bromobenzene followed reflux in phosphoric acid to yield isomers 4-5. Removal of the two methoxides was accomplished with boron tribromide resulting in isomers 6-7. Compounds 11-12 were obtained by reaction of desoxyanisoin with glacial acetic acid and hydroiodic acid to give 8 in 90% yield. Dihydroxy 8 was protected using 3,4-dihydro-2H-pyran and p-toluene sulfonic acid to form 9. Compound 9 was treated with potassium t-butoxide followed by reflux with ethyl iodide to yield 10 in 87%. Compound 10 was refluxed with the formed Grignard reagent of 4-bromophenetole, followed by acid hydrolysis using phosphoric acid to yield isomers 11-12. Synthesis of 15 proceeded from reaction of anisole with 2-phenylbutyryl chloride to form monomethoxide 13 in 94% yield. Compound 13 was coupled with 4-methoxyphenyl magnesium bromide, followed by phosphoric acid to produce 14. The methoxides of 14 were treated with boron tribromide to give dihydroxy 15.

Pharmacology. We compared and contrasted the estrogen-like properties of the hydroxylated TPEs to promote proliferation in the ERŭ-positive human breast cancer cell line MCF-7:WS8.

Compounds were compared with the tamoxifen metabolites 4-OHT and endoxifen which have a high affinity for the ER (because of the appropriately positioned phenolic hydroxyl) but are antiestrogenic because of the alkylaminoethoxy-side chain. To compare the biological activities of the tested TPEs we employed DNA proliferation assays which are described in the materials and methods.

Figure 2p-2 shows that our MCF-7:WS8 human breast cancer cells were exquisitely sensitive to E2 which produced a concentration-dependent increase in growth with maximal stimulation at $1x10^{-11}$ M. All of the TPEôs were potent agonists with the ability to stimulate MCF-7:WS8 breast cancer cell growth, however, their agonist potency was less compared to E2, which had a EC_{50} of $1x10^{-12}$ M. The most potent of the phenolic TPEs was bisphenol (15) with an EC_{50} of approximately $5x10^{-11}$ M. The second potent were the E and Z-isomers of the diphenolic TPEs, compounds 6 and 7, which both had an EC_{50} of approximately $1x10^{-10}$ M. The triphenolic TPE (3) was slightly less active with an EC_{50} of approximately $1.5x10^{-10}$ M whereas the ethoxy TPE (12) was the least potent with an EC_{50} of approximately $4x10^{-9}$ M. The EC_{50} values for all the tested compounds are outlined in Table 1. The compound 12 was prepared to replicate a molecule without the alkyl nitrogen group of 4-OHT or endoxifen and this derivative had reduced estrogenic potency comparison to the other TPEs, however, the molecule remained a full estrogen agonist in our proliferation assays. The metabolites, 4-OHT and endoxifen, had no significant agonist effect in MCF-7:WS8 cells, however, these compounds at 1 ÕM were able to completely inhibit estradiol-stimulated MCF-7:WS8 breast cancer cell growth (Figure 2p-3), thus confirming their role as antagonists/antiestrogens. Similar experiments performed with compounds 3 and 12 showed an inability to block estradiol-stimulated growth in MCF-7:WS8 cells at concentrations up to 1 ÕM (Figure 2p-3). Based on these findings, compounds 3 and 12 were classified as estrogens with a pharmacology, in this assay, indistinguishable from the natural planar estrogen E_2.

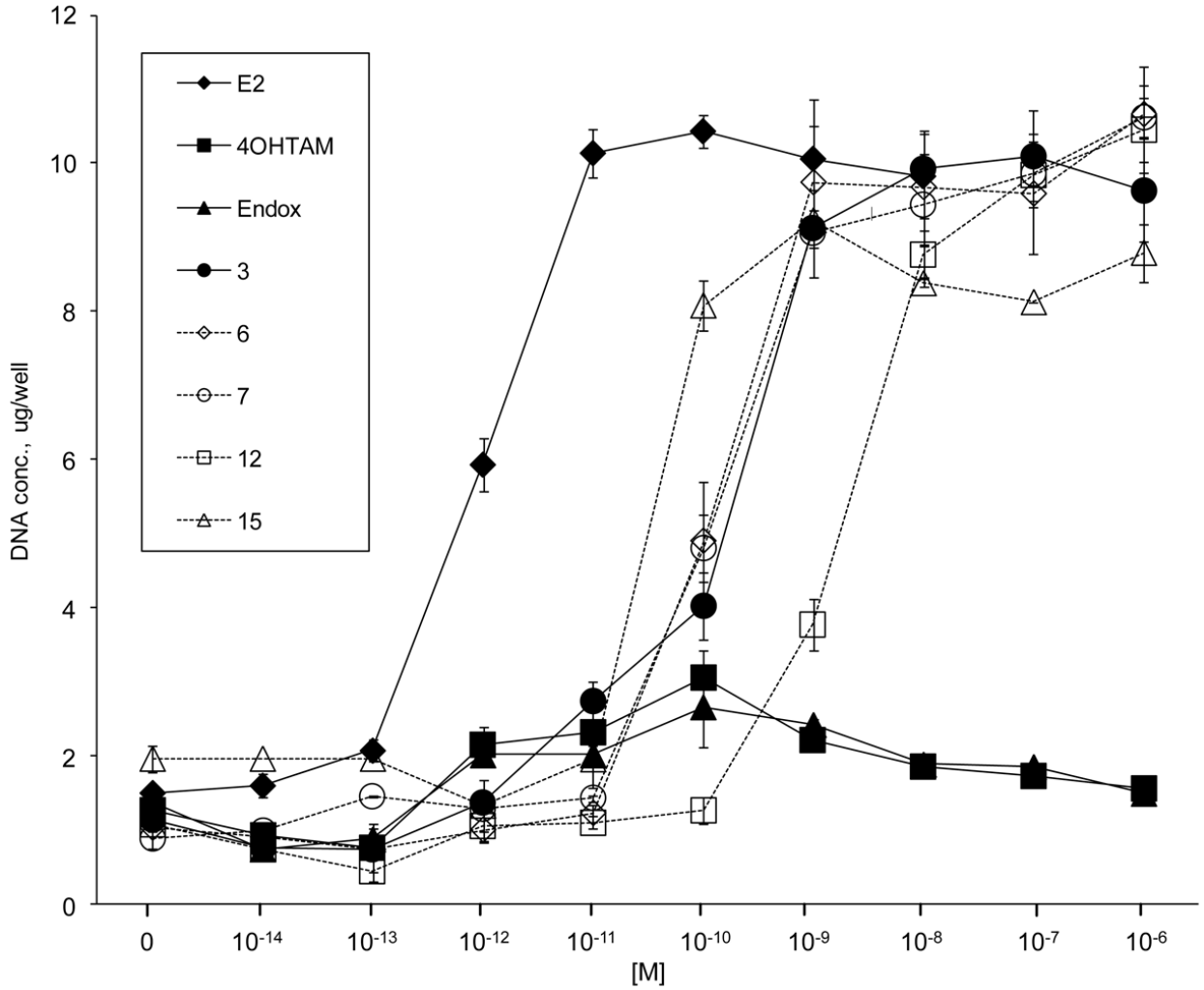


Figure 2p-2. *Effects of E2, test TPEs 3, 6, 7, 12 and 15, and antiestrogens 4OHTAM and endoxifen on the proliferation of MCF-7:WS8 breast cancer cells. Cells were treated with the indicated compounds for 7 days***.**

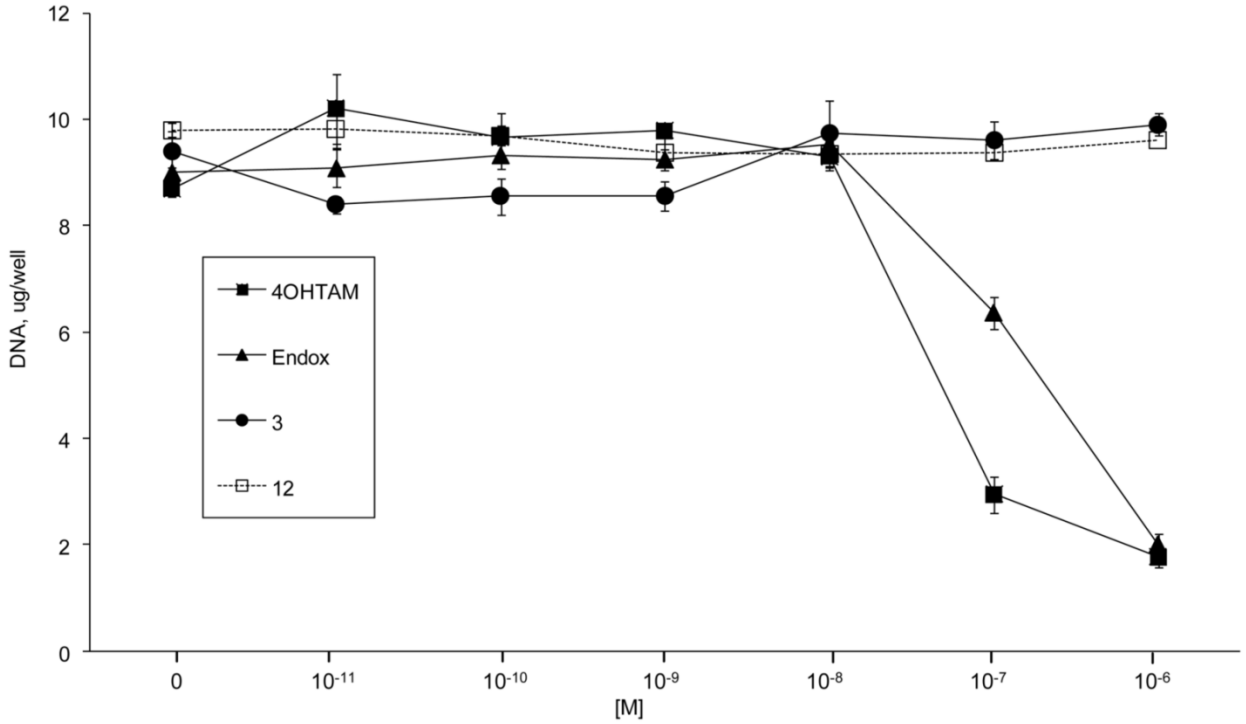


Figure 2p-3. *The ability of the tested TPEs 3 and 12 and 4OHTAM and endoxifen to inhibit estradiol stimulated MCF-7:WS8 breast cancer cell growth. Cells were treated with indicated compounds for 7 days.*

It is interesting to note that compounds 6 and 7, which are the E and Z-isomers of the diphenolic TPEs, were equivalent in their agonistic potency, thus suggesting that isomerization occurs *in vitro* given an equilibrium mixture. This phenomenon has been noted previously with the E isomer of 4-OHT [33] but the true pharmacology of the separate isomers was eventually resolved by the synthesis of fixed ring analogs [33,34]. Both the E and Z isomers of 4-OHT are antiestrogenic because they block the proliferation of estradiol-stimulated growth in MCF-7 breast cancer cells and they inhibit estradiol-stimulated prolactin gene activation. The E isomer is however approximately 1/100 the potency of the Z isomer.

To determine the ability of the test TPEs to activate the ER, MCF-7:WS8 cells were transiently transfected with an ERE-luciferase reporter gene encoding the firefly reporter gene with 5 consecutive EREs under the control of a TATA promoter. The binding of ligand-activated ER complex at the EREs in the promoter of the luciferase gene activates transcription. The measurement of the luciferase expression levels permits a determination of agonist activity of the TPE:ER complex. Figure 2p-4 shows that all the phenolic TPEs were estrogenic, but E_2 was 100 times more potent than the most potent TPE bisphenol (15). The order of potency was as follows: E2 > 15 > 3 > 7 > 6 > 12 > 4-OHT. None of the tested TPEs were antiestrogenic in this assay.

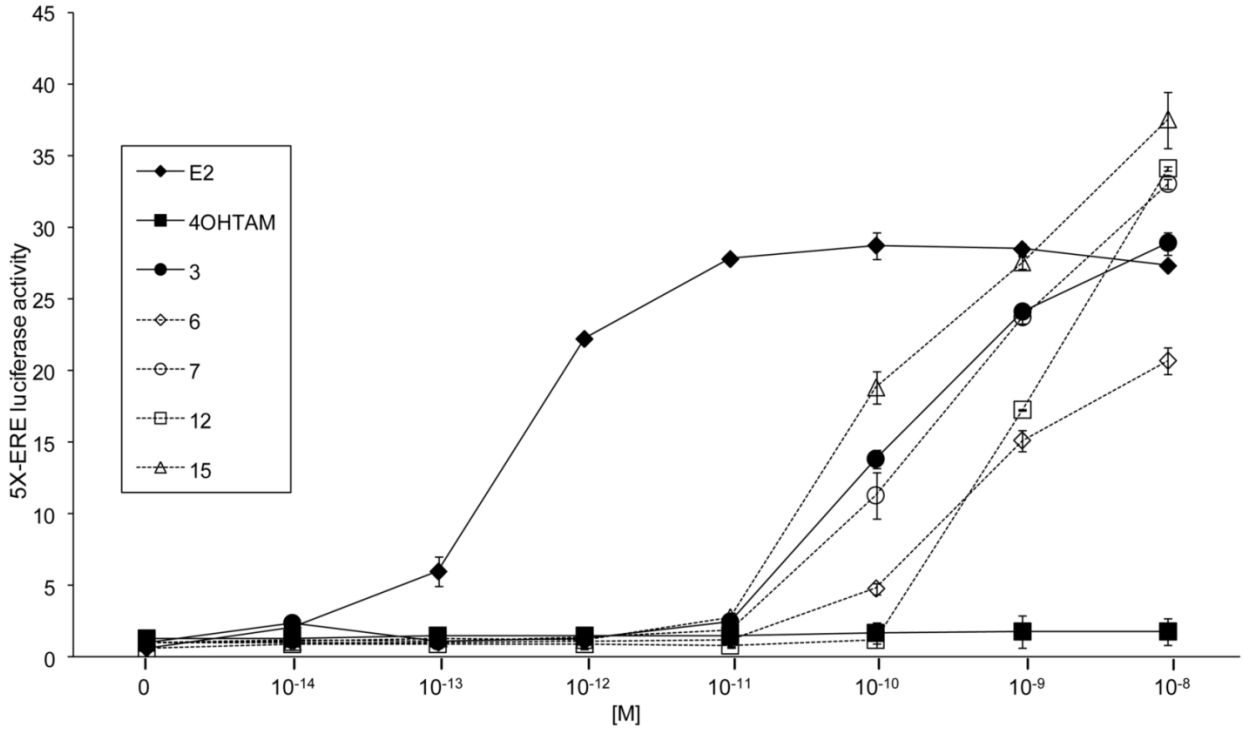


Figure 2p-4. ERE luciferase assay in MCF-7:WS8 cells transiently transfected with an ERE luciferase construct, and treated with E2, test TPEs 3, 6, 7, 12 and 15, and 4OHTAM.

Our goal was to confirm and advance the hypothesis that the shape of the estrogen ER complex was different for planar and nonplanar (TPE ï like) estrogens. This hypothesis has been advanced independently by ourselves [56,154] and Gustôs group [230]. Through a series of studies using mutant ER expression in an ER negative breast cancer cell line, we found that the mutant D351G ER completely suppressed estrogen-like properties of 4-OHT at an endogenous TGFŬtarget gene[62]. Use of this assay led us to classify planar estrogens (DES or E_2) as Class I and non-planar estrogens (TPE-type) as Class II. A broad group of compound structures were used in this study to establish whether a Class II compound could become non-estrogenic with the D351G ER mutant.

Our series of phenolic TPEs were evaluated in the ER-negative breast cancer cell line T47D:C42 [188] which was transiently transfected with an ERE luciferase plasmid and either the wild-type ER or the D351G mutant ER. Figure 2p-5A shows that in the presence of the wild-type ER all of the tested TPE compounds were potent agonists with the ability to significantly enhance ERE luciferase activity. In contrast, when the D351G mutant ER gene was transfected with the ERE luciferase reporter only the planar E_2 was estrogenic whereas the TPEs did not activate the ERE reporter gene (Figure 2p-5B). Overall, these results confirm the importance of Asp351 in ER activation by TPE ligands to trigger estrogen action.

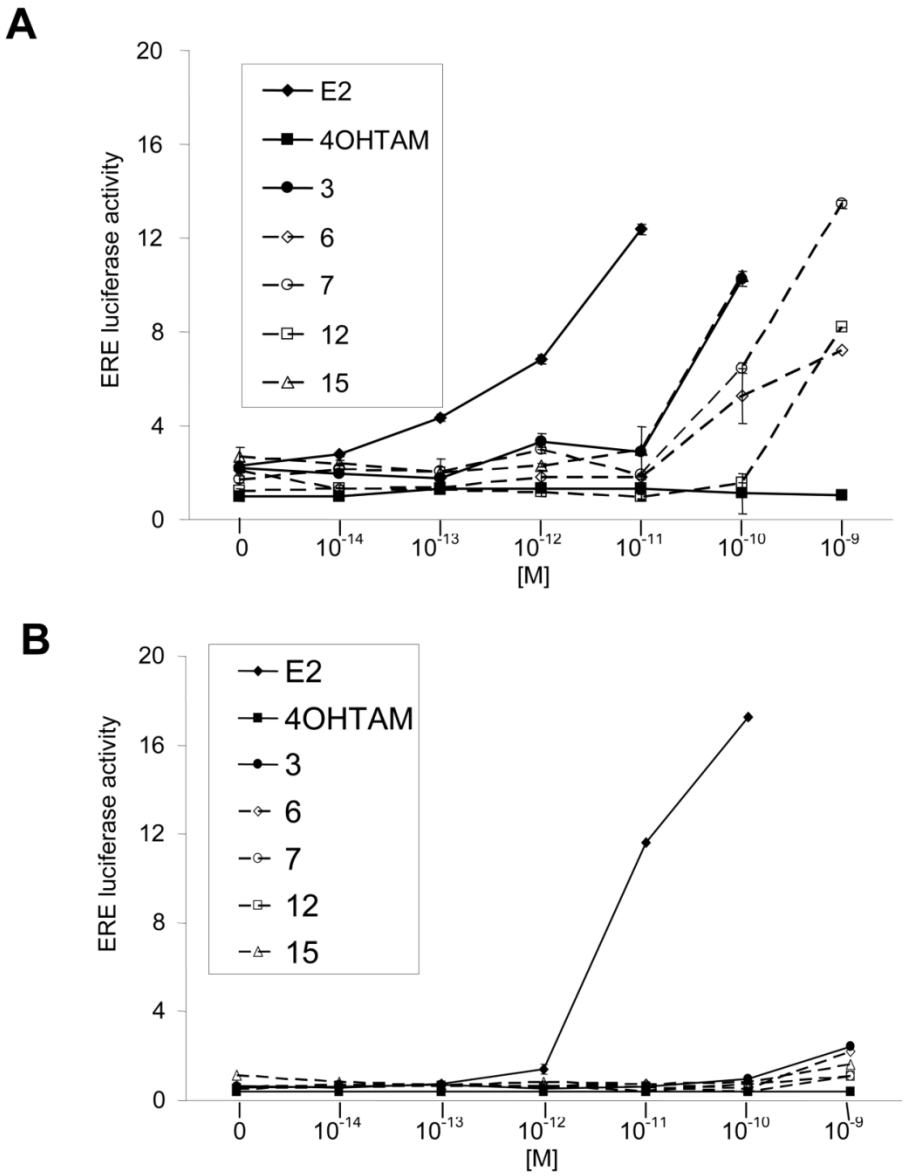


Figure 2p-5. *Luciferase assay in ER-negative T47D:C4:2 cells, transiently transfected with ERE luciferase and wild type (A) and D351G (B) mutant ER constructs respectively, and treated with E2, tested TPEs 3, 6, 7, 12 and 15, and 4OHTAM. Results demonstrate that substitution of Asp351 to Gly in ER, abrogates the agonistic activity on all tested TPEs (class II estrogens), except planar E2 (class I estrogen).*

Analysis of the induced fit models for tested TPEs.

Data analysis was performed on top ranked poses for each of the tested TPEs and for comparison reasons on 4OHTAM (Figure 2p-6A). The top ranked structure from induced fit for 4OHTAM has a ligand RMSD of 0.55 ¡ compared with the experimental structure. In addition to the low ligand RMSD there is a good similarity between the 3ert crystal structure and the top ranked structures from docking (Figure 2p-6B), the conformations of D351, E353, R394, T347, H524 and the rest of aminoacids which line the binding site are nearly superimposable in both structures. Also, the well-known network of H-bonds is formed between 4OHTAM and E353, R394 and water molecules. The most significant difference is that in the top docked pose of 4OHTAM the

antiestrogenic chain is moved closer to D351 to form the interaction between the amino group of 4OHTAM and carboxylate of D351. Induced fit docking of the TPE derivatives: 3, 6, 7, 12, 15 and endoxifen in the ligand binding domain of ERalpha (3ert) has yielded ligand poses which display a binding mode (Figure 2p-6B) very similar with that of 4OHTAM in the ER binding site (Figure 2p-6A). Thus, the superimposition of the top ranked poses of each ligand onto the 4OHTAM co-crystallized with ERalpha (binding cavity filled with water) shows the ligands binding to the receptor in a similar mode with 4OHTAM, having the propensity to form the same hydrophobic contacts with the aminoacids lining the binding cavity. Furthermore, the complex H-bond network is formed with E353, R394, H524 and a highly ordered water molecule positioned between E353 and R394 (Figure 2p-6B). Interestingly, a H-bond has been noticed between the hydroxyl group of 15, 3, 7 and the side chain of T347 is stabilized by an additional interaction with a water molecule from close proximity and precludes the interaction of the ligands with D351. The situation is different when water is removed from the binding site. In this case the OH is shifted so that the H interacts with the carboxylate group of D351 and the HO group of T347 is shifted to form a H-bond with the oxygen. (data not shown). The molecular docking results have shown that most of the compounds form the H-bond network encountered in the case of agonists (E353, R394, H524, water) and display hydrophobic interactions with the aminoacids lining the binding site. An interesting interaction is the hydrogen bond with T347 which seems to be stabilized by a water molecule and it was observed in different docking simulations (flexible and rigid). However, analysis of other ER crystal structures has not revealed additional data to confirm this interaction. Additional work has to be done to verify the hypothesis (docking, binding energy calculations through semiempircal and/or *ab-initio* methods, etc). The interaction with D351 is weak (3.8 ï 4 ¡) and it was mostly noticed when the simulations were run with the receptor without water in the binding site. This would mean that D351 is exposed and not shielded so it could communicate intrinsic estrogenic properties of the complex to AF-1.

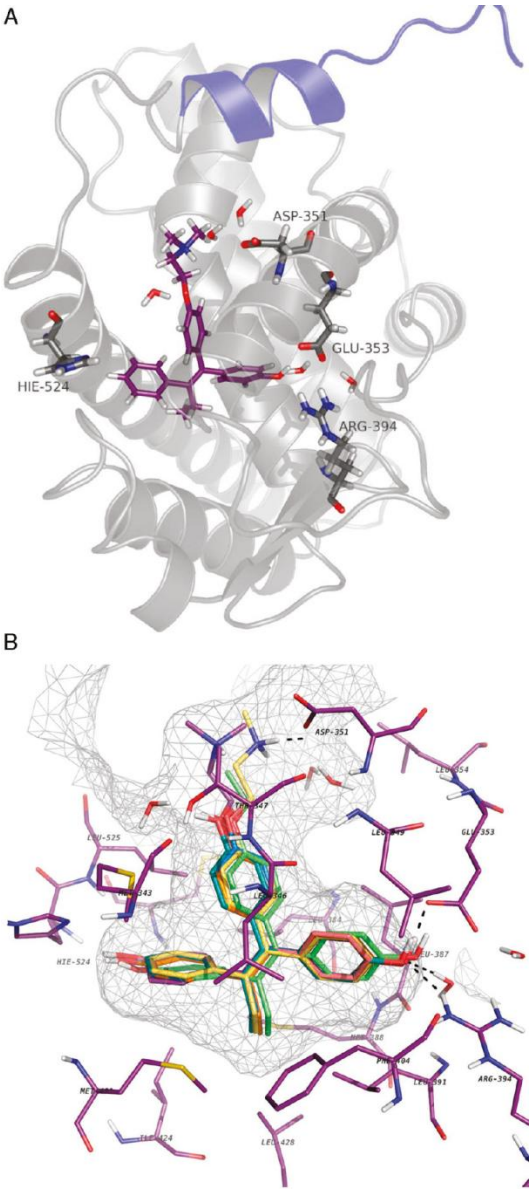


Figure 2p-6. *(A) Cartoon representation of the human ERR ligand binding domain complexed with 4-hydroxy tamoxifen, the antagonist conformation of the receptor. Helix 12 is depicted in blue, the amino acids involved in theH-bond networkwith the ligand are displayed as sticks. and the ligand is colored in purple. (B) Molecular docking of TPE derivatives into the binding site of ERR. For comparison reasons, the top ranked ligand-protein complex is superimposed on the crystal structure of the receptor cocrystallized with 4-OHT; the amino acids lining the biding sites of both complexes are shown and the complex H-bond network between ligand and the binding site is displayed. The induced fit docking poses of the ligands are colored as follow: 15 in cyan, 3 in blue, 6 in orange, 7 in pink, 12 in green, Endoxifen in yellow, while the crystal structure is depicted in purple.*

The best poses of the tested TPEs 3, 6, 7, 12, 15 and endoxifen, obtained from docking simulations ran against the antagonist conformation of the ER, were superimposed on the experimental agonist conformation of the ER (ER co-crystallized with estradiol, PDB code 1GWR) (Figure 2p-7A). This has shown that these ligands are unlikely to be accommodated in the agonist conformation of the ER due to the sterical clashes between ñLeu crownò, mostly Leu525 and

Leu540, helix 12 and ligands as depicted in figure 2p-7B, indicating, that these ligands most likely bind to ERôs conformation more closely related with the antagonist form.

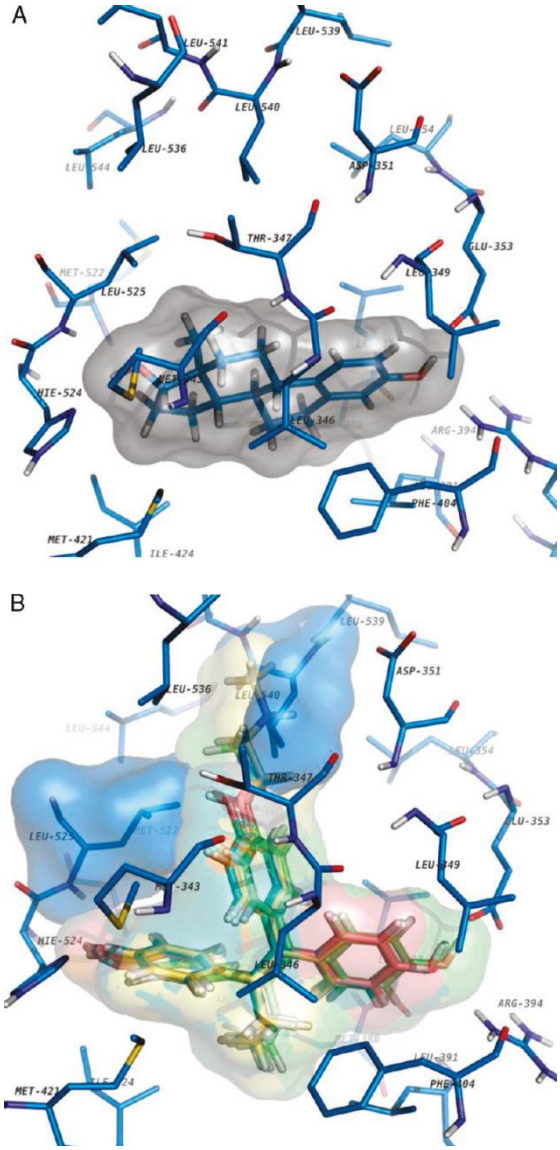


Figure 2p-7. *(A) View of ERR binding cavity. The X-ray crystal structure of ERR complexed with estradiol (PDB code 1GWR), the agonist conformation of the receptor. The amino acids lining the binding site are depicted as sticks colored by element. The color code is blue for carbon, red for oxygen, gray for nitrogen, and yellow for sulfur. The ligand is represented as sticks having the same colored code like the receptor and the ligand's surface is colored in gray. (B) View of ERR binding cavity. The best poses of BisPhen, TriOHTPE, EDiOHTPE, ZDiOHTPE, Z4EthoxDiOHTPE, Endox obtained from docking simulations ran against the antagonist conformation of the receptor are superimposed on the agonist conformation of the receptor, ERR cocrystallized with estradiol (PDB code 1GWR). The amino acids involved in steric clashes with the ligands, Leu525 and Leu540, are depicted as molecular surfaces colored in blue while the rest of amino acids lining the binding site are depicted as sticks colored by element, the color code is blue for carbon, red for oxygen, gray for nitrogen, and yellow for sulfur. The ligands are represented in sticks with the associated molecular surfaces. They respect the same coloring code with the exception of carbons which are colored as*

follow: BisPhen in cyan, TriOHTPE in blue, EDiOHTPE in orange, ZDiOHTPE in pink, ZEthoxDiOHTPE in green, Endoxifen in yellow. For clarity, waters and hydrogen atoms were omitted from the binding site.

Discussion

The aim of this structure function relationship study was to evaluate the pharmacological properties of synthetic TPEs as estrogens in MCF-7 human breast cancer cells using the DNA proliferation assay and ERE luciferase assays. Our results showed that all of the synthesized TPEs possessed potent estrogen-like properties in our MCF-7 human breast cancer cells. These TPEs markedly increased cell growth and enhanced ERE luciferase activity. In contrast, the tamoxifen metabolites 4OHTAM and endoxifen, which possess an alkylaminoethoxy sidechain in their structure, failed to induce growth or increase ERE luciferase activity thus confirming their role as antiestrogens.

X-ray crystallography of ER-4OHTAM and ER-Raloxifene complexes demonstrate that the presence of the alkyaminoethoxy sidechain of 4OHTAM is crucial for the ER to gain an antagonistic conformation by displacing the H12 of the receptor by 4OHTAMôs bulky sidechain, thus preventing the binding of the coactivators [28]. Based on the results of our proliferation assays and the luciferase assays, it is clear that repositioning of the hydroxyl groups changed the biological potencies of the tested TPE compounds which lowered their estrogenic potency compared to that of E2. However, the fact that these TPEs were able to significantly induce growth and ERE activation in MCF-7:WS8 cells demonstrated that they are still full agonists. The absence of the alkyaminoethoxy sidechain on the tested TPEs does not allow these compounds to act as antiestrogens, like 4-OHT or endoxifen, which posseses the alkyaminoethoxy sidechain [28]. However, despite the changes in biological potencies of the tested TPEs, due to repositioning of the hydroxyl groups and addition of the ethoxy group, these compounds also maintained their ability to activate the ERE as was demonstrated in our ERE luciferase assays.

Another interesting aspect in our study is the importance of Asp351 in activation of the ER thereby acting as a molecular test for the presumed structure of the TPE:ER complex. Based on the X-ray crystallography of the ER in complex with 4OHTAM [28] and raloxifene[75], it was determined that the basic side chains of these antiestrogens are in proximity of Asp351 in the ER. It was hypothesized that this interaction with raloxifene actually neutralizes and shields Asp351 preventing it from interacting with ligand-independent activating function 1 (AF-1). In contrast, 4OHTAM possesses some estrogenic activity, because the side chain is too short [28]. Substitution of Asp351 leads to loss of estrogenic activity of the ER bound with 4OHTAM [60,62]. Results from ERE luciferase assays in T47:C4:2 cells transiently transfed with wild type and D351G mutant ER expression plasmids demonstrated that wild type ER was activated by all of the tested TPEs, however substitution of Asp351 by Gly prevented the increase of ERE luciferase activity by all TPEs and only planar E2, which does not interact with Asp351 at all, or exposes it on the surface of the complex, was able to activate ERE in D351G ER transfected cells. This confirms and expands the classification of estrogens, where planar estrogens such as E2 are classified as class I and all TPE-related estrogens are classified as class II estrogens based on the mechanism of activation of the ER [56].

It is important to note that all of the tested TPEs were agonists in our wild type ER assay systems, however, extensive studies of the structure function relationship of phenolic TPEs by Gust and co-workers [231,232], demonstrated that some of these compounds were potent antagonists in their MCF-7:2a cells stably transfected with an ERE luciferase plasmid. Specifically, these

investigators found that compounds 1,1,2-Tris(4-hydroxyphenyl)but-1-ene and 1,1-Bis(4,4ô-hydroxyphenyl)-2-phenylbut-1-ene, which correspond to compounds 3 and 15 in this study, were able to completely inhibit estradiol-stimulated ERE luciferase activity at 100 nM. The antagonistic potency of these compounds, however, did not correlate with results from the cytotoxicity assays performed in their wild-type MCF-7 cells [231,232]. Both compounds 1,1,2-Tris(4-hydroxyphenyl)but-1-ene (designated as 3 in this study) and 1,1-Bis(4,4ô-hydroxyphenyl)-2-phenylbut-1-ene (designated as 15 in this study) produced weak cytotoxic effects only at concentrations above 5 uM which were well beyond the concentration range used in our study. Thus it is possible that the variation in findings between our laboratory and that of Gust and coworkers [231,232] might be due to differences in our in vitro model systems and our experimental design.

We have confirmed and advanced the hypothesis [56,154,230] that estrogens can be classified into planar Class I compounds (E2) and non-planar Class II compounds (TPEs). Armed with these new tools, we are now poised to examine the biological consequences of estrogen classification based on the shape of the resulting ER complex.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2q (Sengupta and Jordan) - Studies carried out by Dr. Sengupta in the Jordan laboratory at Fox Chase Cancer Center

Experimental treatment of oestrogen receptor (ER) positive breast cancer with tamoxifen and brivanib alaninate, a VEGFR-2/FGFR-1 kinase inhibitor: A potential clinical application of angiogenesis inhibitors

Introduction:
We have addressed the hypothesis that combining tamoxifen, a SERM with a sub-therapeutic dose of brivanib alaninate would be a beneficial strategy for long-term therapy in the treatment of breast cancer. We report the first studies testing the efficacy of brivanib alaninate to control tumour growth of ER regulated SERM sensitive (MCF-7 E2) and SERM stimulated (MCF-7 Ral, MCF-7 Tam), and endometrial (EnCa 101) tumours. We find that the combination of tamoxifen and brivanib alaninate in a laboratory model provided a therapeutic advantage for the control of breast tumour growth over tamoxifen or brivanib alaninate alone.

Work Accomplished:

Immunohistochemistry

Immunohistochemistry was performed on representative MCF-7 E2 tumours to determine whether the VEGFR-2 receptor was expressed in response to estradiol and 2 weeks of tamoxifen. We also determined VEGFR-2 receptor expression in response to estradiol, estradiol withdrawal and the treatment with the pure anti-oestrogen, fulvestrant. This analysis demonstrated the presence of VEGFR-2 on both tumour cells and endothelial cells (Fig. 2q-1A). In addition, VEGFR-2 and VEGFA expressions were increased on tumour cells in the presence of estradiol. It is interesting to note that the combination of estradiol and 2 weeks of 125 ε g tamoxifen did not apparently change VEGFR-2 or VEGFA expression in comparison to estradiol treatment alone. However, as noted in Fig.2q- 6A, tamoxifen was not effective at controlling established estradiol stimulated tumour growth during the two-week treatment period. With estradiol withdrawal alone, and the subsequent destruction of the ER with fulvestrant, there was very little expression of VEGFR-2 or VEGFA on the tumour cells (Fig. 2q-1A and B).

Effects of different doses of brivanib alaninate in SERM sensitive MCF-7 E2 tumours

We evaluated the effects of a low dose (0.05 mg/g) and high dose (0.1 mg/g) of brivanib alaninate on estradiol stimulated tumour growth. The high dose was based on data demonstrating the highest effective dose with minimal toxicity and the low dose that was chosen was half of the high dose and the minimally effective dose as determined by Bristol Myers Squibb (Princeton, NJ) [233]. Statistical comparisons were done to determine whether there was a difference in the average CSA of tumours treated with estradiol versus those that received the

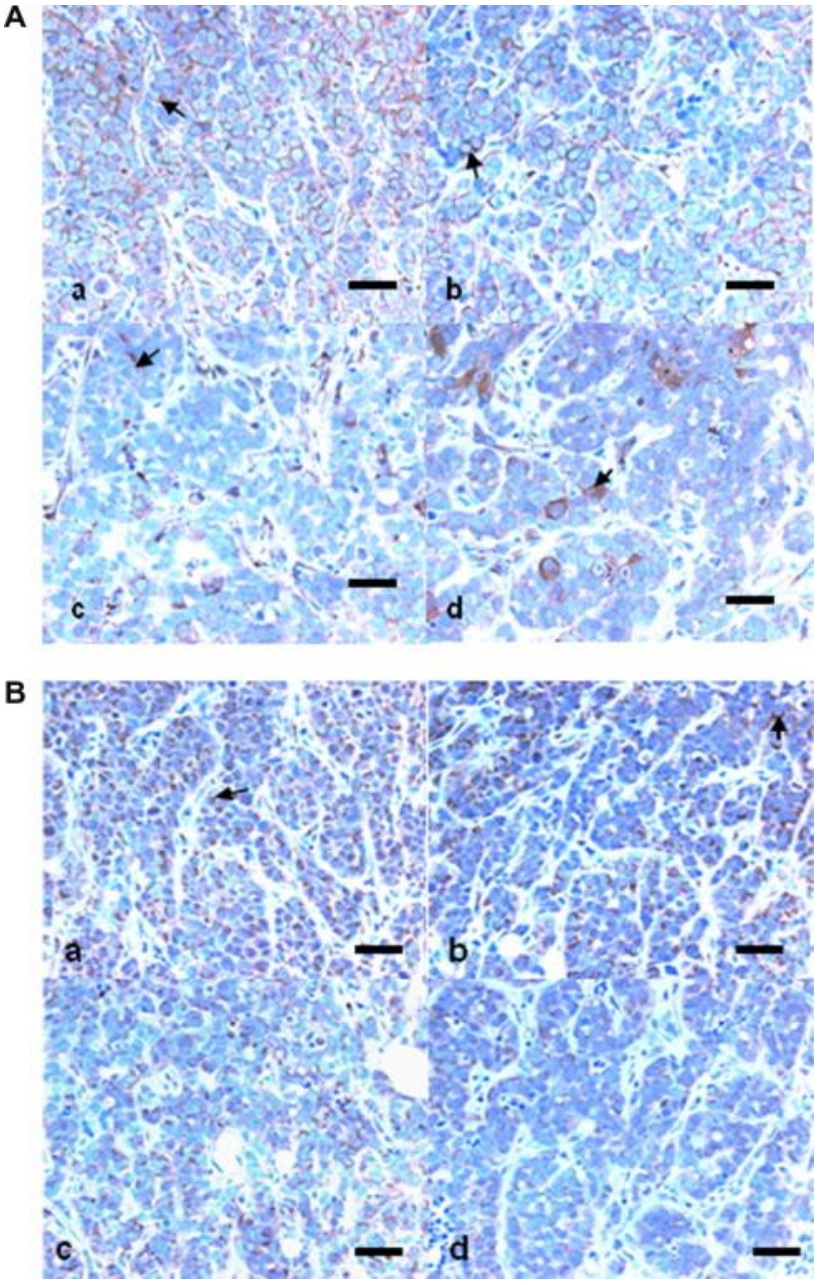


Fig. 2q-1. *The distribution of the VEGFR-2 receptor (A) and VEGFA (B) in the MCF-7 E2 tumour model. Tumour bearing animals were treated with estradiol (a), estradiol and 2 weeks of 125 μg tamoxifen (b), estradiol and then 2 weeks of estradiol withdrawal (c), and estradiol followed by 2 weeks of estradiol withdrawal and fulvestrant (d). VEGFA and VEGFR-2 expressions decreased with estradiol withdrawal. The bars represent 50 μm.*

high dose (0.1 mg/g) or low dose (0.05 mg/g) of brivanib alaninate in the presence of estradiol. Estradiol caused tumour growth, while the high dose of brivanib alaninate produced a dramatic decrease in estradiol-stimulated growth (Fig. 2q-2A). The average difference in tumour CSA at 6 weeks in the mice that received the high dose of the brivanib alaninate and estradiol versus estradiol was ï 0.37 cm^2 (p = 0.001, α = 0.025). There was no significant difference (0.13 cm^2) in the average CSA of tumours treated with estradiol only and those treated with estradiol and the low dose of brivanib alaninate (p = 0.202, α = 0.025). The tumour tissue was further evaluated with H and E staining (Fig. 2q-2B). The purpose of this analysis was to detect differences in the amount of necrotic tissue. In tumours in which angiogenesis and thus, oxygen and nutrient delivery is blocked, there would be a decrease in tumour cell viability and hence an increase in necrosis. In tumours that received brivanib alaninate, there was an increase in tissue necrosis as exemplified by the areas that

stain pink only. The necrosis was most prominent in the tumours treated with the high dose of the brivanib alaninate. There was mild necrosis in the tumours that were treated with the low dose of the brivanib alaninate.

Western immunoblotting of tumour extracts did not reveal a difference in total VEGFR-2, but there was less phosphorylation at the tyrosine 951 residue of VEGFR-2 in brivanib alaninate treated animals (Fig. 2q-2C). The presence of ER and phospho-ER demonstrated active tumour tissue and an activated ER. There was very little VEGFR-1, VEGFR-3 or FGFR-1 (data not shown) detected by immunoblotting. The use of RTPCR analysis confirmed a significant increase in VEGFA ($p = 0.02$) and a non-significant increase in human VEGFR-2 in tumours that were treated with the high dose of brivanib alaninate (Fig. 2q-2D). There was a significant decrease in mouse VEGFR-1 and mouse VEGFR-2 in tumours that were treated with the higher ($p = 0.02$, $p = 0.04$) dose of brivanib alaninate. ER mRNA decreased slightly, but significantly (Fig. 2q-2C) in those tumours that were treated with the higher dose of brivanib alaninate ($p = 0.04$), but there was no increase in

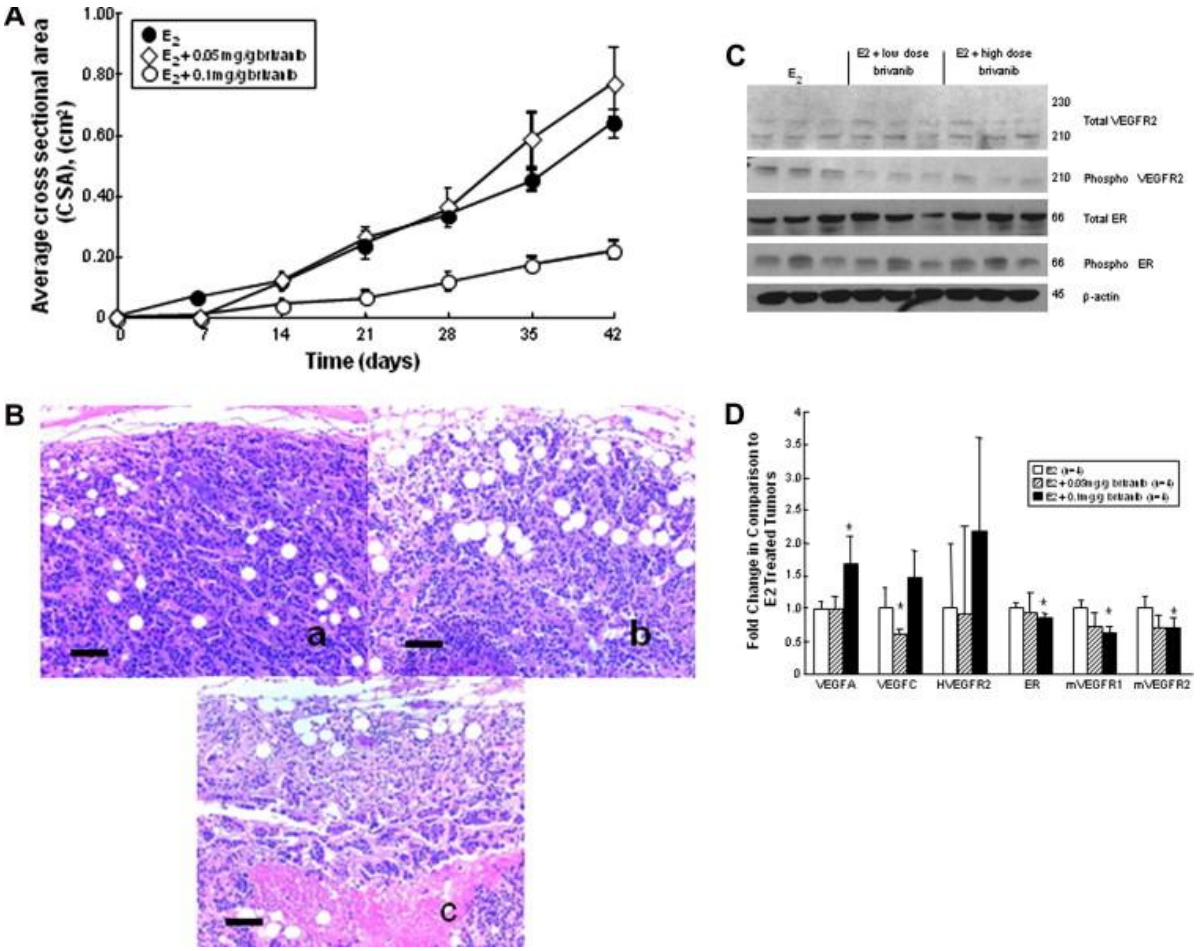


Fig. 2q-2. *The growth characteristics of MCF-7 E2 tumours treated with estradiol alone or with estradiol and the lower (0.05 mg/g) and higher doses (0.1 mg/g) of brivanib alaninate. There were five ovariectomised, athymic mice and 10 tumours per group. The drug treatment resulted in a decreased average CSA of the tumours at the higher dose (0.1 mg/g) ($p = .001$, $\alpha = 0.025$), but there was no difference between the group treated with the low dose (0.05 mg/g) of brivanib alaninate and the oestrogen only group ($p = 0.2$, $\alpha = 0.025$). There were no significant differences in animal body weights between groups. H and E staining is shown in panel B and reveals that with increases in the dosing of the drug, there was an increase in the amount of necrotic tissue (*). The bar represents 100 μm. Panel C demonstrates that there was no significant change in the total amount of VEGFR-2 expressed by the tumours, but there was a decrease in the phosphorylation pattern of the tumours treated with brivanib alaninate, regardless of the dose given. The presence of ER and its phosphorylated form was indicative of active tumour tissue in all the samples. Panel D demonstrates analysis by RTPCR. There was a significant increase in VEGFA in the high dose group in comparison with tumours treated with oestrogen only ($p = 0.02$). There was a small, but significant decrease in ER mRNA in the high dose (0.1 mg/g) group ($p = 0.04$). VEGFC transcription decreased significantly in tumours treated with the low dose (0.05 mg/g) of brivanib alaninate. Mouse VEGFR-1 and mouse VEGFR-2 mRNA, which represented the endothelial component of the tumour, significantly decreased in the high dose (0.1 mg/g) group ($p = 0.02$, $p = .04$).*

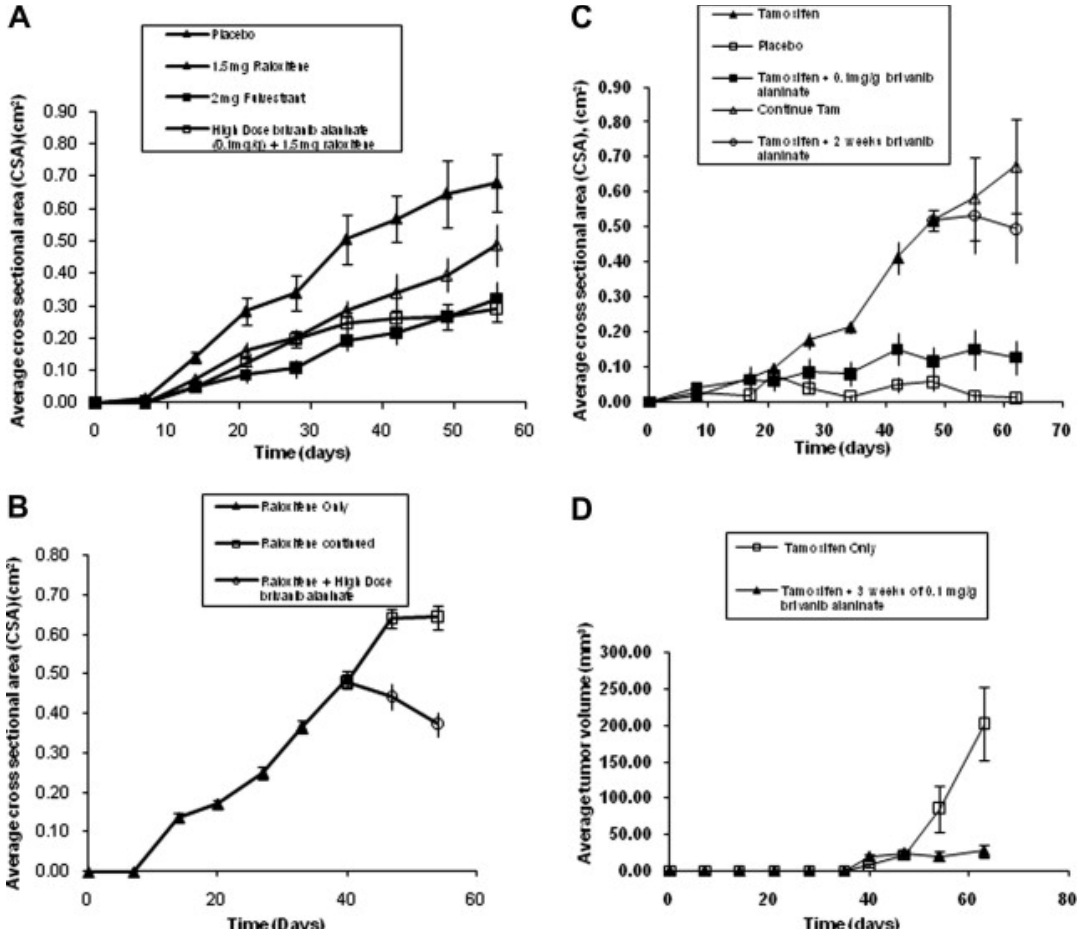


Fig. 2q-3. *The anti-tumour effects of high dose (0.1 mg/g) brivanib alaninate on the growth of human tumours with acquired resistance to the SERMs raloxifene or tamoxifen. There were no significant differences in animal body weights between groups. Unless stated otherwise, all groups had 5 ovariectomised athymic mice with 10 tumours. (A) Raloxifene stimulated MCF-7 Ral. Groups were treated with raloxifene (1.5 mg daily by gavage), vehicle, fulvestrant (2 mg SQ 5 d per week), or raloxifene plus brivanib alaninate (0.1 mg/g by gavage). Brivanib alaninate (0.1 mg/g) significantly prevented the growth of raloxifene treated tumours ($p < 0.001$, $\alpha = 0.016$). (B) Raloxifene (1.5 mg daily by gavage) stimulated MCF-7 RAL. Twenty ovariectomised athymic mice were randomised into two groups of 10 mice each with continued raloxifene treatment (total of 17 tumours in the group) or raloxifene plus high dose brivanib alaninate (0.1 mg/g by gavage) (total of 19 tumours in the group). There was a significant decrease in tumour size with brivanib alaninate ($p < 0.001$, $\alpha = 0.025$). (C) Tamoxifen (1.5 mg daily by gavage) stimulated MCF-7 TAM tumours. Athymic, ovariectomised mice were initially placed into three groups to receive 1.5 mg tamoxifen (8 mice, 16 tumours), 1.5 mg tamoxifen plus 0.1 mg/g brivanib alaninate (4 mice, 6 tumours) or control vehicle (5 mice, 10 tumours). The group that received tamoxifen was randomised to continue tamoxifen (4 mice, 8 tumours) or receive tamoxifen with 0.1 mg/g brivanib alaninate (4 mice, 8 tumours) once the tumours reached an average CSA of 0.5 cm^2. The VEGFR inhibitor produced significant decreases in tamoxifen-stimulated growth rate in early implanted ($p < 0.001$, $\alpha = 0.025$) or established ($p < 0.001$, $\alpha = 0.025$) tumours. (D) Treatment of tamoxifen-stimulated (500 μg tamoxifen by gavage daily) EnCa 101 endometrial tumours was continued in two groups of 10 ovariectomised, athymic mice (20 tumours per group for 40 d). One group then received concomitant high dose brivanib alaninate (0.1 mg/g by gavage) for 3 weeks. Tumour volume was significantly decreased in animals treated with brivanib alaninate and tamoxifen compared to tamoxifen alone ($p < 0.001$, $\alpha = 0.025$).*

ER protein by Western blotting analysis (Fig. 2q-2C). There was a significant decrease in transcription of VEGFC mRNA ($p = 0.04$) in tumours treated with the lower dose of brivanib alaninate (Fig. 2q-2D). There was very little or no VEGFB, VEGFD, mouse VEGFR-3 or human VEGFR-1 present in the tumours as evidenced by high CT values (>35) detected by RTPCR analysis (data not shown).

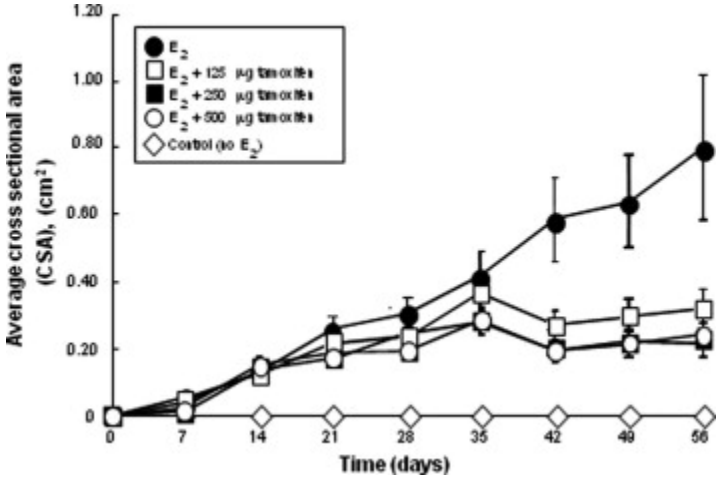


Fig. 2q-4. *The effect of daily oral tamoxifen dosing on the estradiol-stimulated growth of MCF-7 E2 tumours delivered by an implanted 0.3 cm sustained release silastic capsule. There were five ovariectomised, athymic mice and 10 tumours per group. There was a dose-dependent decrease in estradiol stimulated tumour growth. The tumours did not grow without estradiol. The lowest dose of tamoxifen, 125 µg, suppressed tumour growth by 63%, whereas the higher doses (250 µg and 500 µg) suppressed tumour growth by 75%. There were no significant differences in animal body weights between groups.*

Effect of brivanib alaninate on SERM stimulated tumour growth

To establish that an inhibitor of VEGFR-2 would block the growth of SERM stimulated tumours and as a consequence, would have the potential to retard the development of acquired SERM resistance in ER positive cancers, a series of models and designs was explored. The MCF-7 Ral tumour model[234] grows without raloxifene, and to a greater extent in the presence of raloxifene. Fulvestrant retards tumour growth [234]. This is illustrated in Fig. 2q-3A. Statistical comparisons were done to determine whether there was a difference in the average CSA of tumours treated with raloxifene versus those treated with placebo, fulvestrant or high dose brivanib alaninate (0.1 mg/g). Raloxifene stimulated tumour growth was significantly decreased in the presence of high dose brivanib alaninate (0.1 mg/g) administered with raloxifene and the difference in average CSA was 0.391 cm^2 after 8 weeks ($p < 0.001$, $\alpha = 0.016$). A similar difference in average CSA (0.366 cm^2) was also observed with tumours treated with raloxifene versus tumours treated with fulvestrant ($p < 0.001$, $\alpha = 0.016$). There was no significant difference between the average CSAs of tumours

(0.212 cm^2) in the presence or absence of raloxifene ($p = 0.024$, $\alpha = 0.016$). The addition of high dose brivanib alaninate (0.1 mg/g) to a daily regimen of 1.5 mg of raloxifene (0.1 mg/g) caused a rapid decrease in tumour growth (decrease in average CSA = ï 0.294 cm^2) ($p < 0.001$, $\alpha = 0.025$) in established raloxifene stimulated tumours (Fig. 2q-3B) over a two-week period. At the time of randomisation, the group that was treated with raloxifene (1.5 mg) and brivanib alaninate (0.1 mg/g) demonstrated no difference in average CSA (ï .0146 cm^2) than those that received raloxifene (1.5 mg) only ($p = 0.73$, $\alpha = 0.025$). Our MCF-7 Tam SERM stimulated model showed similar effects with brivanib alaninate. Statistical comparisons were done to determine whether there was a difference in the average CSA of tumours treated with 1.5 mg tamoxifen daily versus vehicle or 1.5 mg tamoxifen + 0.1 mg/g brivanib alaninate. The difference in CSA between those tumours that received 1.5 mg tamoxifen daily versus 1.5 mg tamoxifen and the high dose brivanib alaninate (0.1 mg/g) daily ($p < 0.001$, $\alpha = 0.025$) was 0.395 cm^2. A similar difference in CSA (0.484 cm^2) was observed between tumours treated with tamoxifen alone versus control treated with vehicle only ($p < 0.001$, $\alpha = 0.025$). The tamoxifen (1.5 mg/daily) treated group was then randomised to continue 1.5 mg/d tamoxifen or 1.5 mg/d tamoxifen + high dose brivanib alaninate for 2 weeks. At the time of randomisation, the group that was treated with tamoxifen (1.5 mg) and brivanib alaninate (0.1 mg/g) demonstrated no difference in average CSA than those that received tamoxifen (1.5 mg/g) only ($p = 0.76$, $\alpha = 0.25$). The addition of brivanib alaninate (0.1 mg/g) caused a rapid tumour regression (difference in average CSA = ï 0.261 cm^2) of established tumours after 2 weeks of treatment ($p < 0.001$, $\alpha = 0.025$) (Fig. 2q-3C). There was a significant decrease in blood vessel density (CD31 counts) in the group that received 0.1 mg/g brivanib alaninate and 1.5 mg tamoxifen for 2 weeks (average MVD/sq. mm = 76) in comparison with the group that continued receiving 1.5 mg tamoxifen (average MVD sq./mm = 156) ($p = 0.003$).

Finally, the tamoxifen-stimulated EnCa 101 endometrial tumour model [235] was also used to evaluate the efficacy of brivanib alaninate (0.1 mg/g). Animals with bi-transplanted tumours were treated with 500 ε g of tamoxifen daily by oral gavage for 40 d and then randomised. One group received 500 ε g of tamoxifen and 0.1 mg/g brivanib alaninate daily. The other group continued to receive 500 ε g of tamoxifen. At the time of randomisation, the group that was treated with tamoxifen (500 ε g) and brivanib alaninate (0.1 mg/g) had a larger average volume (difference = 40 mm^3) than those that received tamoxifen (500 ε g) only ($p = 0.002$, $\alpha = 0.025$). Despite this initial difference, over a three-week period, animals treated with tamoxifen alone subsequently had an average tumour volume (difference = 0.168 mm^3) that was much greater than those animals treated with brivanib alaninate in combination with tamoxifen ($p < 0.001$, $\alpha = 0.025$) (Fig. 2q-3D). All models demonstrated that a VEGFR-2 inhibitor, brivanib alaninate would prevent the growth of SERM stimulated tumours.

Determination of tamoxifen dosing in SERM sensitive MCF-7 E2 tumours

We determined an anti-oestrogenical dose of tamoxifen that would be approximately 50% effective in blocking estradiol stimulated tumour growth. Previously, 1.5 mg/d of tamoxifen has been used to almost completely block oestrogen stimulated tumour growth [236]. The differences in the CSAs of tumours treated with estradiol and 125 ε g tamoxifen (ï 0.368 cm^2, $p = 0.01$, $\alpha = 0.016$), estradiol and 250 ε g tamoxifen (ï 0.479 cm^2, $p = 0.001$, $\alpha = 0.016$) or estradiol and 500 ε g tamoxifen (ï 0.479 cm^2, $p < 0.001$, $\alpha = 0.016$) versus estradiol alone were significant (Fig. 2q-4). A dose of 125 ε g was chosen for further testing in combination with brivanib alaninate to determine whether there would be an improvement in therapeutic efficacy.

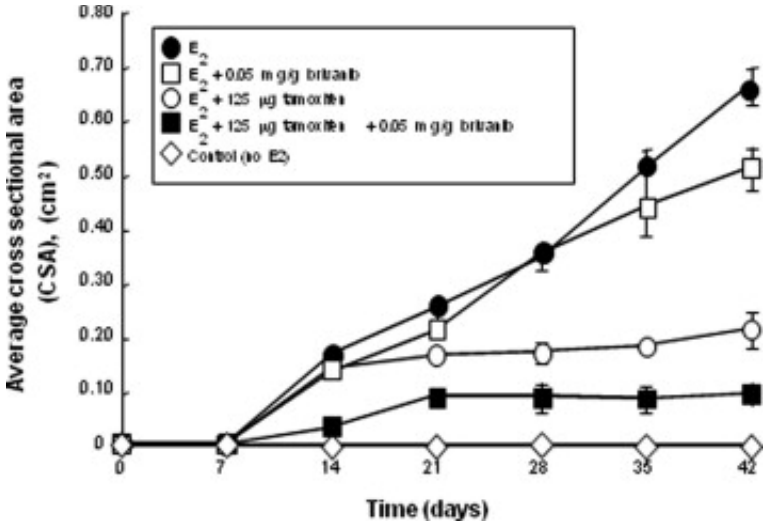


Fig. 2q-5. *The effect of a combination of tamoxifen (125 µg daily oral dose) and 0.05 mg/g brivanib alaninate on the growth of established estradiol stimulated MCF-7 E2 tumours. There were five ovariectomised, athymic mice and 10 tumours per group. The combination of 125 µg tamoxifen with 0.05 mg/g brivanib alaninate improved the effects of 125 µg tamoxifen (*p < *0.01,* Ŭ = *0.025) or 0.05 mg/g brivanib alaninate (*p < *0.001,* Ŭ = *0.025). There were no significant differences in animal body weights between groups.*

The combined effect of a lower dose of tamoxifen and brivanib alaninate in SERM sensitive MCF-7 E2 tumours

We hypothesise that a sub-therapeutic dose of brivanib alaninate may enhance a sub-optimal effective daily dose of tamoxifen (125 ε g) and thus improve tumour growth control. The strategy of limiting angiogenesis would optimise long-term anti-oestrogen therapy. Statistical comparisons were done to determine whether there was a difference in the average CSA of tumours treated with 125 ε g tamoxifen + 0.05 mg/g brivanib alaninate versus 125 ε g tamoxifen or 0.05 mg/g brivanib alaninate. The results illustrated in Fig. 2q-5 demonstrated that the combination of 125 ε g of tamoxifen and 0.05 mg/g of brivanib alaninate significantly improved the anti-tumour action tamoxifen or brivanib alaninate alone after 6 weeks. The difference in average CSAs of tumours treated with 125 ε g tamoxifen and 0.05 mg/g brivanib alaninate versus those treated 125 ε g tamoxifen (ï 0.128 cm^2, $p = 0.01$, $\alpha = 0.025$) was significant. Similarly, there was a significant difference in the CSA of those tumours treated with the combination therapy and those treated with brivanib alaninate (ï 0.449 cm^2, $p < 0.001$, $\alpha = 0.025$).

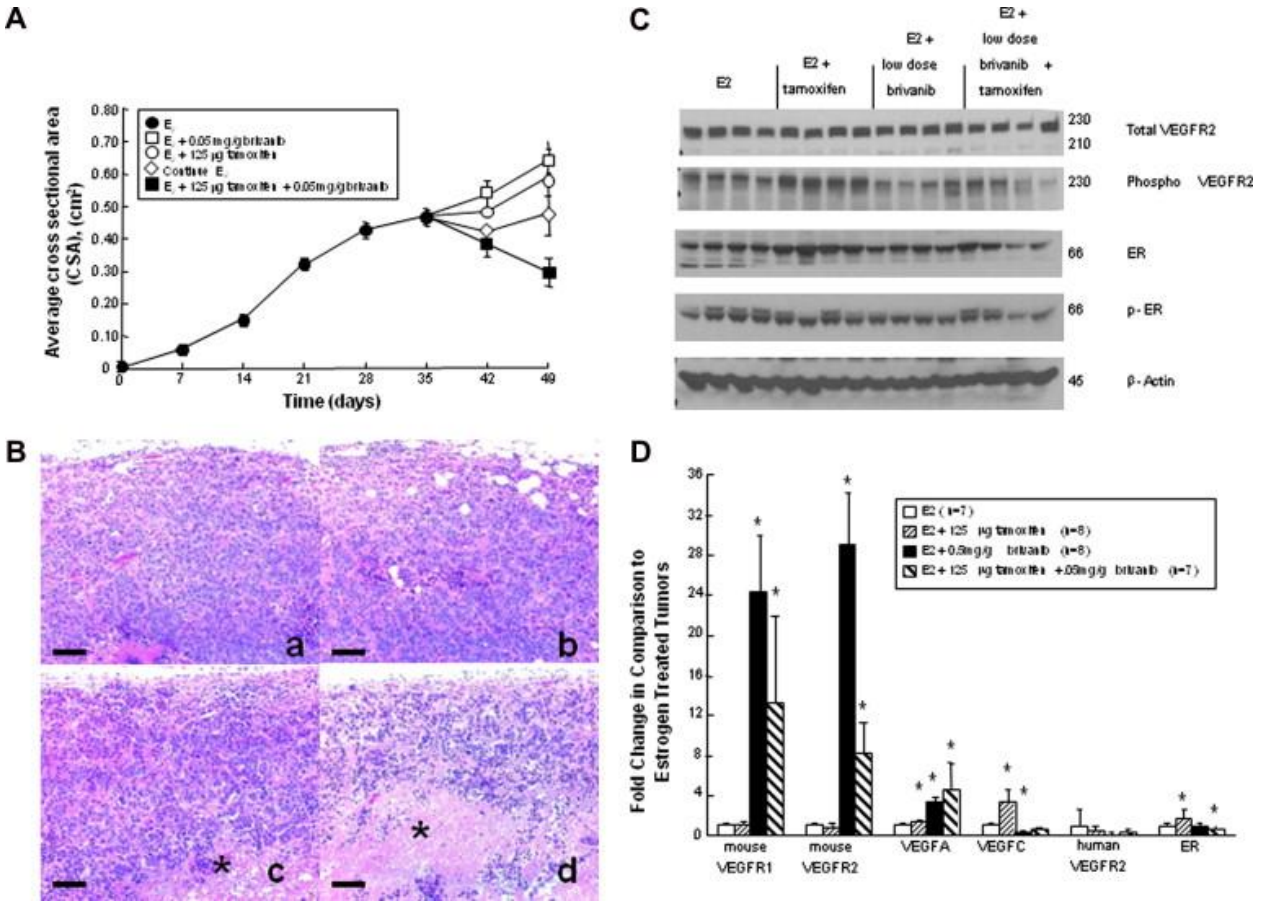


Fig.2q-6. Panel A: *the efficacy of a combination of 125 μg tamoxifen and 0.05 mg/g brivanib alaninate on the growth of established estradiol stimulated MCF-7 E2 tumours. There were five ovariectomised, athymic mice and 10 tumours per group. Tumours were grown to approximately 0.46 cm^2 and treated with the treatment regimens as indicated. There were no significant differences in animal body weights between groups. However, the decrease in average CSA was significant when comparing the combination treatment to tamoxifen (125 μg) treated tumours (*p *= 0.01, Ŭ= 0.025) or those treated with 0.05 mg/g brivanib alaninate (*p *= 0.007, Ŭ= 0.025).* Panel B: *H and E staining demonstrated an increase in necrotic tissue when brivanib alaninate was given alone or with tamoxifen. Once again, the bar represented a 100 μm distance.* Panel C: *Western blot analysis of tumour tissue did not illustrate a decrease in total VEGFR-2, regardless of the treatment group. The addition of brivanib alaninate, decreased the phosphorylation of VEGFR-2. Expression of ER and phosphorylated ER in all tumours, demonstrated the presence of active tumour tissue.* Panel D: *relative fold change in the mRNA levels of angiogenic factors in tumours relative to estradiol treatment alone. Mouse VEGFR-1 and mouse VEGFR-2 mRNA increased dramatically in tumours that received the inhibitor (*p *= 0.001,* p *= 0.001) or the inhibitor plus tamoxifen (*p *= 0.002,* p *= 0.002). VEGFA mRNA increased in tumours in response to tamoxifen treatment (*p *= 0.01) brivanib alaninate treatment (*p *= 0.001) and the combination of brivanib alaninate plus tamoxifen (*p *= 0.002). VEGFC increased in tamoxifen treated tumours (*p *= 0.001) and decreased in tumours treated with brivanib alaninate (*p *< 0.004). There was a significant, but small decrease in ER mRNA (*p *= 0.04) in tumours treated with the combination of tamoxifen plus brivanib alaninate and an increase in ER mRNA in tamoxifen treated tumours (*p *= 0.04).*

The combined effect of a lower dose of tamoxifen and brivanib alaninate in established SERM sensitive MCF-7 E2 tumours

The goal of this experiment was to obtain sufficient tumour tissue for molecular analysis to evaluate the actions of tamoxifen and brivanib alaninate. The results are summarised in Fig. 2q-6 and Fig. 2q-7. The short-term combination of brivanib alaninate and tamoxifen decreased tumour size during the two-week period, whereas neither tamoxifen alone nor the brivanib alaninate alone prevented an increase in established tumour size (Fig. 2q-6A). Statistical comparisons were done to determine whether there was a difference in the average CSA of tumours treated with 125 ε g tamoxifen + 0.05 mg/g brivanib alaninate versus 125 ε g tamoxifen or 0.05 mg/g brivanib alaninate. There was no difference in size at the time of randomisation (tamoxifen versus combination therapy (p = 0.87) and brivanib versus

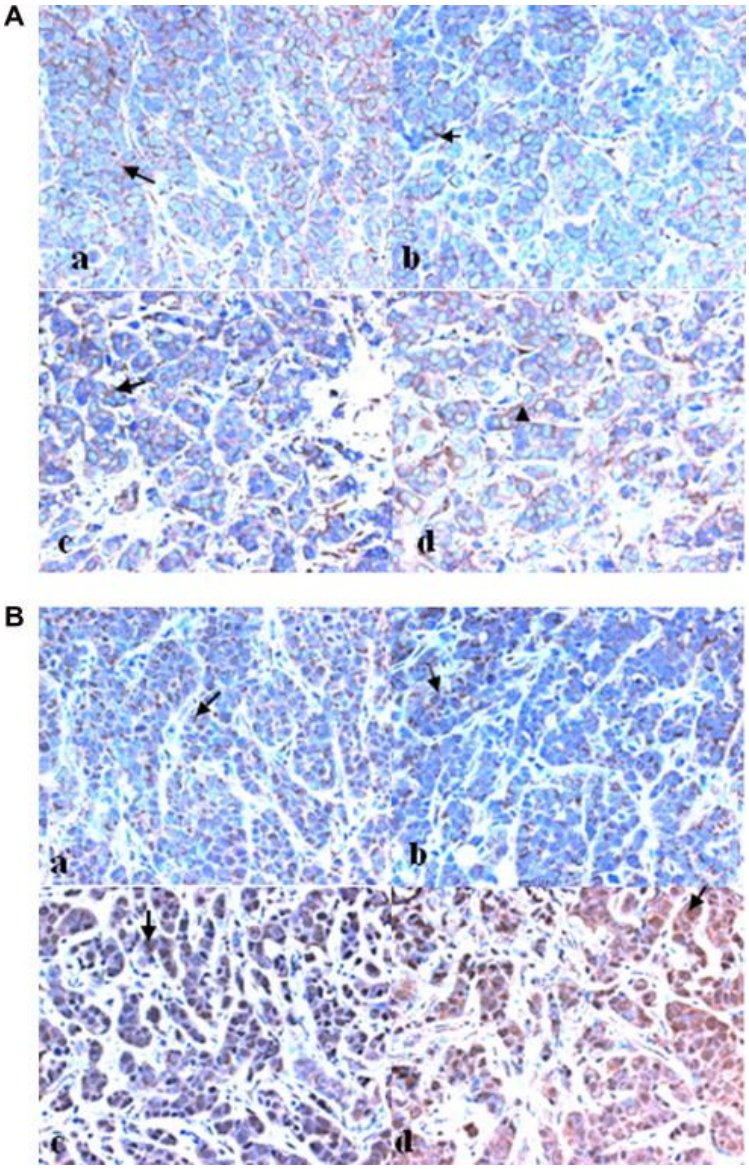


Fig.2q-7. *Panel A: there is no change in total VEGFR-2 expression by IHC in MCF-7 E2 tumours treated with estradiol (a), estradiol and 2 weeks of 125 μg tamoxifen (b), estradiol and 2 weeks of 0.05 mg/g brivanib alaninate (c), or estradiol and 2 weeks of the combination of 125 μg tamoxifen and 0.05 mg/g brivanib alaninate (d). Panel B: by IHC, the VEGFA staining intensity is greatest with 2 weeks of the combination of 125 μg tamoxifen and 0.05 mg/g brivanib alaninate (d). Staining intensity is the same with estradiol (a), estradiol and 2 weeks of 125 μg tamoxifen (b), estradiol, and 2 weeks of 0.05 mg/g brivanib alaninate (c). The bars represent 50 μm.*

combination therapy (p = 0.29)). The average CSA was significantly different between tumours treated with 125 ε g tamoxifen versus those treated with 125 ε g tamoxifen and .05 mg/g brivanib

alaninate (ï 0.292 cm^2, $p = 0.01$, $\alpha = 0.025$). The same observation was noted for those tumours treated with 0.05 mg/g brivanib alaninate versus those treated with 0.05 mg/g brivanib alaninate and 125 ε g tamoxifen (ï 0.341 cm^2, $p = 0.007$, $\alpha = 0.025$). Consistent with our findings, illustrated in Fig.2q- 2B, representative histological analysis in this experiment confirmed (Fig. 2q-6B) increased necrosis in tumours that received only brivanib alaninate or brivanib alaninate plus tamoxifen.

Western immunoblotting (Fig. 2q-6C) demonstrated a decrease in phosphorylation of the VEGFR-2, but not total VEGFR-2 in the two groups that received brivanib alaninate. Total ER expression was reduced in the group receiving tamoxifen and the brivanib alaninate compared to tamoxifen alone.

RTPCR analysis (Fig. 2q-6D) demonstrated an increase in mRNA for mouse VEGFR-1 and mouse VEGFR-2 in tumours that receive brivanib alaninate with ($p = 0.002$, $p = 0.002$) or without ($p = 0.001$, $p = 0.001$) tamoxifen. VEGFA mRNA is increased with tamoxifen ($p = 0.01$), brivanib alaninate ($p = 0.001$) or both drugs ($p = 0.002$) in combination. VEGFC increased with the tamoxifen treated group ($p = 0.001$), but decreased in the groups treated with the brivanib alaninate ($p = 0.004$). ER mRNA levels increased ($p = 0.04$) with the tamoxifen treated group, but decreased with the group that received both the VEGFR inhibitor and tamoxifen ($p = 0.04$).

We further validated our molecular studies with immunohistochemistry. There was little change in total VEGFR-2 (Fig. 2q-7A), which was consistent with the findings in Western immunoblotting. VEGFA staining intensity increased in the tumours treated with tamoxifen and brivanib alaninate, which is consistent with the increased VEGFA mRNA seen in RTPCR analysis (Fig. 2q-7B). The nuclear staining of the VEGF in the presence of brivanib (Fig. 2q-7C) could be consistent with the report by Rosenbaum-Dekel et al [237]. with the nuclear localisation of L-VEGF, but no specific antibody was available to test the hypothesis.

Discussion

We report the first study to explore the potential of combining tamoxifen with low dose brivanib alaninate to block the growth of ER positive breast cancer. Previous studies have demonstrated the efficacy of brivanib in mouse models of human hepatocellular carcinoma [238] and to inhibit growth in ER negative H3396 xenografts in athymic mice [239]. Our strategy is to employ an anti-oestrogen (tamoxifen) to block oestrogen stimulated VEGF production and to use a combination with blockers of VEGFR-2 to reduce angiogenic survival mechanisms in both the tumour and endothelial cells to enhance tumour cell death. Our results demonstrate that the strategy is feasible. We have advanced the idea with the demonstration that a VEGFR-2 inhibitor, brivanib alaninate can not only inhibit the growth of small SERM stimulated implants derived from MCF-7 cells with acquired resistance to tamoxifen and raloxifene, but also can inhibit SERM stimulated growth of established tumours in athymic mice (Fig. 2q-3Aï C). Additionally, brivanib alaninate inhibits tamoxifen-stimulated endometrial cancer (EnCa 101) growth (Fig. 2q-3D). Thus, the ability of a VEGFR-2 inhibitor to block the growth of tumours with acquired SERM resistance supports the idea that this strategy might improve adjuvant therapies.

Angiogenesis is important for tumour growth and metastasis. Stable transfection of MCF-7 cells with the VEGF gene results in hormone independent growth *in vivo* and tamoxifen resistance [240]. This is supported by the recent work by Aesoy and coworkers [241] using an anti-oestrogen resistant cell line (LCC2) *in vitro* that has constitutive VEGF secretion relative to wildtype MCF-7

cells. MCF-7 cells respond to 4-hydroxytamoxifen with a reduction in VEGF, but the anti-oestrogen resistant variant LCC2 does not. Oestrogen has been shown to increase the synthesis of VEGFA [242] and anti-oestrogens inhibit the process [241,242]. This observation was validated in our tumour models as the expression of VEGFA and VEGFR-2 is increased in the presence of oestrogen and decreased with oestrogen withdrawal (Fig. 2q-1A and B). As there is strong evidence for the oestrogen mediated regulation of angiogenesis, combining an anti-oestrogen with an antiangiogenic inhibitor to diminish tumour growth is a reasonable therapeutic approach.

There are fewer side-effects such as malignant hypertension with angiogenesis inhibitors when used lower doses [243]. At higher doses, therapeutic efficacy may be diminished when drug dosing is reduced or abbreviated. Therefore, we advanced the concept of dual inhibition of angiogenesis further and tested a combination of sub-effective tamoxifen (125 εg) daily and the sub-therapeutic VEGFR-2 inhibitor brivanib alaninate (0.05 mg/g daily). The combination significantly decreased tumour growth compared with estradiol and either drug alone. This was true for the prevention of early tumour development following initial implantation (Fig. 2q-5) or during the short-term treatment of established tumours (Fig. 2q-6 and Fig. 2q-7). Thus, we have shown that using a combination of lower, more tolerable doses of two drugs that are as efficacious as higher, less tolerable doses of either drug used alone, is a viable alternative for adjuvant therapy.

Drug treatments were evaluated in established tumours to provide tissue to investigate molecular mechanisms. Total VEGFR-2 levels did not change in the tumours with treatment (Fig. 2q-6 and Fig. 2q-7A), but the phosphorylation patterns were different (Fig. 2q-6C). Brivanib alaninate inhibits phosphorylation of the VEGFR-2 receptor. This confirmed the reported mechanism of action [238] of brivanib alaninate as an inhibitor of the VEGFR-2 tyrosine kinase. Treatment of established tumours with tamoxifen alone increased phosphorylation of VEGFR-2 and this increase in phosphorylation was inhibited when brivanib alaninate was combined with tamoxifen. Thus, it is possible to explain why a significant decrease in tumour size resulted from the use of a two-drug combination rather than a single drug that was individually ineffective in established tumours.

Similarly, transcription of VEGFC mRNA increased during tamoxifen treatment, but this was abrogated with brivanib alaninate. This is an important finding because VEGFC also activates VEGFR-2 [244]. There was a compensatory rise in VEGFA with tamoxifen, brivanib alaninate, or the combination of the two drugs. However, with the combination of tamoxifen and brivanib alaninate, the compensatory mechanisms of the tumour to overcome blockade of the ER and VEGFR-2 failed as evidenced by increased tumour necrosis. The compensatory rise in VEGFA was validated by IHC in tumours treated with the combination of tamoxifen and brivanib alaninate. Overall, our findings confirm and extend the recent findings of Aesoy and co-workers [241] who demonstrate a breast cancer cell survival of VEGF/VEGFR-2/p38 feedback loop in cells resistant to anti-oestrogens.

Classically, the VEGF pathway in tumours has been thought to result from VEGF secretion from tumour cell activation of VEGF receptors on endothelial cells. However, accumulating evidence suggest that VEGFR-2 is most likely found on both cancer cells and endothelial cells [241,245,246,247]. By using IHC to localise VEGFR-2 in the MCF-7 tumour model, there is demonstrable expression of VEGFR-2 on the breast cancer cells (Fig. 2q-1A). Moreover, there is evidence of oestrogen mediated regulation of VEGFR-2 expression on tumour cells as VEGFR-2 expression decreases with the withdrawal of 17ϐ-estradiol. Ryden [248] also demonstrated that

VEGFR-2 is expressed on tumour material from patients. These findings strengthen the argument to target VEGFR-2 in breast cancer.

By using RTPCR to differentiate between mouse and human VEGFR-2, we were able to evaluate the response to therapy in the endothelial (mouse) and the tumour cell (human) components. Interestingly, when the brivanib alaninate is started at the time of implantation there is a significant decrease in mouse VEGFR-1 and VEGFR-2. There was a trend towards an increase in human VEGFR-2 in mice treated with the higher dose of brivanib alaninate, with a significant decrease in mouse VEGFR-2 mRNA. When the angiogenesis inhibitor was given to mice with established tumours, there was a trend towards a decrease in human VEGFR-2 mRNA with a significant increase in mouse VEGFR-1 and VEGFR-2 mRNA. Thus, when the human VEGFR-2 is blocked, this then affects the endothelial component and the cells attempt to manufacture more receptor.

The ER is central to oestrogen-regulated events. As reported in previous studies, tamoxifen blocks the E2-mediated down-regulation of ER mRNA (Fig.2q- 6D) and there is an increase in total ER expression (Fig.2q- 6C). Interestingly, the co-administration of brivanib alaninate prevented the tamoxifen induced increase in ER mRNA (Fig. 6C) and there was a decrease in total ER expression (Fig. 6D). It appears that the administration of an inhibitor of VEGFR-2 can modulate the ER during the anti-tumour process and this is an area worthy of further investigation. Conversely, the expression of VEGFR-2 on the cancer cells in response to oestrogen is clearly important to maintain control of tumour growth. These observations further validate the use of a combination of an anti-oestrogen and an angiogenesis inhibitor.

In addition to inhibiting VEGFR-2, the inhibitor has also shown activity against FGFR-1 in other tumour models, and is thus useful as a dual inhibitor for angiogenesis [238]. In the present study, however, we were unable to detect FGFR-1 in our specific model.

Despite the encouraging results obtained in the present study, several recent reports [249,250,251] of either the development of resistance to antiangiogenic drugs[250] or enhanced metastatic spread with low dose antiangiogenic drugs [249,251] deserve consideration. Clinical trials have shown that the majority of human tumour types do not respond to inhibitors of integrin as an antiangiogenic strategy. Laboratory models now show [251] that low concentrations of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ inhibitors increase tumour growth via VEGFR-2 trafficking. This promotes endothelial cell migration to VEGF. In related studies, inhibitors of VEGFR can either enhance tumour cell seeding in ómetastatic assaysô[249] or cause adaptiveï evasive responses by tumours with greater malignancy and increased invasiveness [250]. Clearly, the complexity of the angiogenic survival signalling pathways present a challenge to seek the clinical relevance of pre-clinical pharmacology. Nevertheless, in a recent review, Ebos and co-workers[252] contend that it remains unclear whether antiangiogenic therapy will lead to increased invasion or metastases after long- or short-term treatments. There are more than 40+ adjuvant clinical trials in progress, so the question of the premature tumour resistance caused by low dose antiangiogenesis inhibitors will probably be answered first in the clinical setting [252]. With this concern in mind, we are currently considering an initial short-term testing platform in ER positive metastatic breast cancer that has failed exhaustive endocrine therapy [253]. It is known that apoptosis and tumour regression can be induced by both high or low dose oestrogen clinically [91,205], but we propose to use low dose oestrogen to reduce thromboembolic events. The therapeutic application of low dose oestrogen treatment is a direct translation of laboratory studies over the past 15 years [132,133]. By combining a dose escalation schedule of brivanib alaninate, we

will be able to monitor tumour response precisely for the 12-week treatment schedule. These preliminary clinical data will guide our future adjuvant applications.

In summary, antiangiogenic agents have been utilised clinically in patients who have breast cancer that is refractory to other agents [243]. In these instances, to see a partial clinical benefit, higher doses that are potentially toxic have to be used. The observations that elevations of VEGFA and VEGFR-2 are associated with poor prognosis and response to tamoxifen therapy [248,254] suggests that a strategy to combine anti-hormone treatment with an antiangiogenic strategy may have merit to test in clinical trials. Based on an increasing laboratory database that implicates an elevation in angiogenic factors in endocrine resistant breast cancer in the presence of tamoxifen [241], we have provided evidence that a combination of tamoxifen plus a low dose dual inhibitor of VEGFR-2 and FGFR-1, brivanib alaninate, effectively controlled tumour growth. The strategy of combining a tyrosine kinase inhibitor of VEGFR-2 has the advantage of reducing toxicity, permitting long-term therapy and therefore compliance to enhance efficacy for adjuvant tamoxifen therapy. Indeed, the strategy of inhibiting angiogenesis, might in fact, improve responsiveness of those ER positive tumours that are refractory to tamoxifen alone. We believe this issue should be addressed in clinical trial.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2r (Ariazi and Jordan) - Studies carried out by Dr. Eric Ariazi in the Jordan laboratory at Fox Chase Cancer Center

The G Protein–Coupled Receptor GPR30 Inhibits Proliferation of Estrogen Receptor–Positive Breast Cancer Cells

Introducion:
We investigated GPR30 largely in ER-positive MCF-7 with some comparisons to ER-negative SKBr3 breast cancer cells. First, a statistical association was sought between GPR30 and ERŬ-positive status in publicly available breast carcinoma microarray data sets. Next, the contribution of ERŬ and GPR30 in several E2-responsive activities, including regulation of GPR30 expression, intracellular Ca2+ mobilization, cellular growth, and cell cycle progression, was studied using receptor-specific ligands and small interfering RNA (siRNA) methodology.

Work Accomplished:

Increased GPR30 mRNA expression associates with ERα- positive status in 1,250 breast carcinomas.
Evidence of a relationship between GPR30 and ERŬexpression was sought by mining publicly available and well-annotated gene expression microarray data sets across five independent cohorts comprising 1,250 breast carcinomas. In the NKI cohort (n =
254), data were collected using two-color oligonucleotide microarrays (Fig. 2r-1A). According to the nonparametric Mann-Whitney rank sum test, GPR30 mRNA levels were significantly higher in ERŬ-positive versus ERŬ-negative tumors ($P < 0.0001$). The upper range of GPR30 expression was 7.7-fold higher on a linear scale in the ERŬ-positive compared with ERŬ-negative carcinomas. In addition, GPR30 and ERŬmRNA levels correlated as continuous variables (Pearson's coefficient $\rho = 0.30$, adjusted $P < 0.0001$). The other four cohorts used one color Affymetrix oligonucleotide microarrays (Fig. 2r-1B). In each of these four cohorts, GPR30 mRNA levels were significantly higher in the ERŬ-positive compared with the ERŬ-negative breast cancers (Uppsala cohort, n = 244, $P = 0.040$; Stockholm cohort, n = 159, $P = 0.0091$; EMC cohort, n = 344, $P = 0.0050$; TRANSBIG cohort, n = 198, $P = 0.0024$).

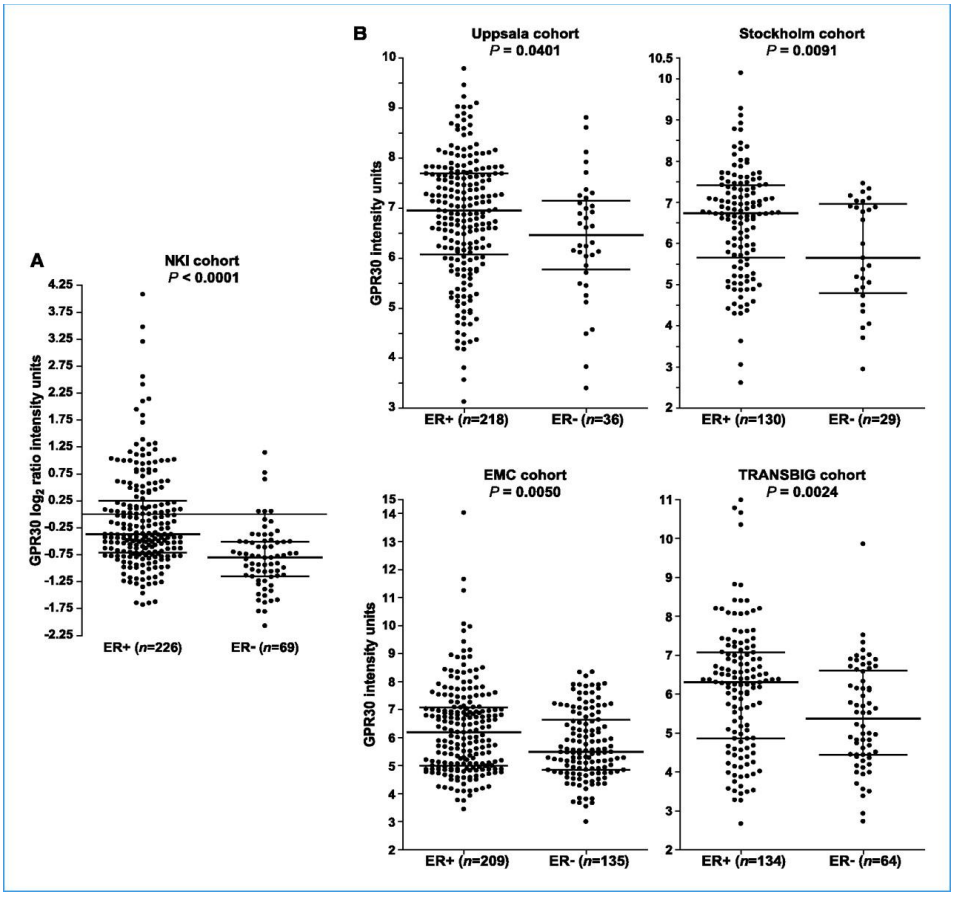


Figure 2r-1. *GPR30 mRNA expression shows an association with ERα-positive status in human breast carcinomas. A, GPR30 mRNA levels in the NKI cohort derived from two-color arrays. Expression values are normalized log_2 ratio intensity units corresponding to a single tumor cRNA hybridized against a pooled reference cRNA from all tumors. B, GPR30 mRNA levels in the Uppsala, Stockholm, EMC, and TRANSBIG cohorts all derived from one-color arrays. Expression values are MAS5.0 normalized intensity units. A and B, sample sizes of ERα-positive (ER+) and ER-negative (ER−) cancers are shown, and bars indicate the 75th, 50th (median), and 25th percentiles. Significance was assessed using the nonparametric Mann-Whitney rank test.*

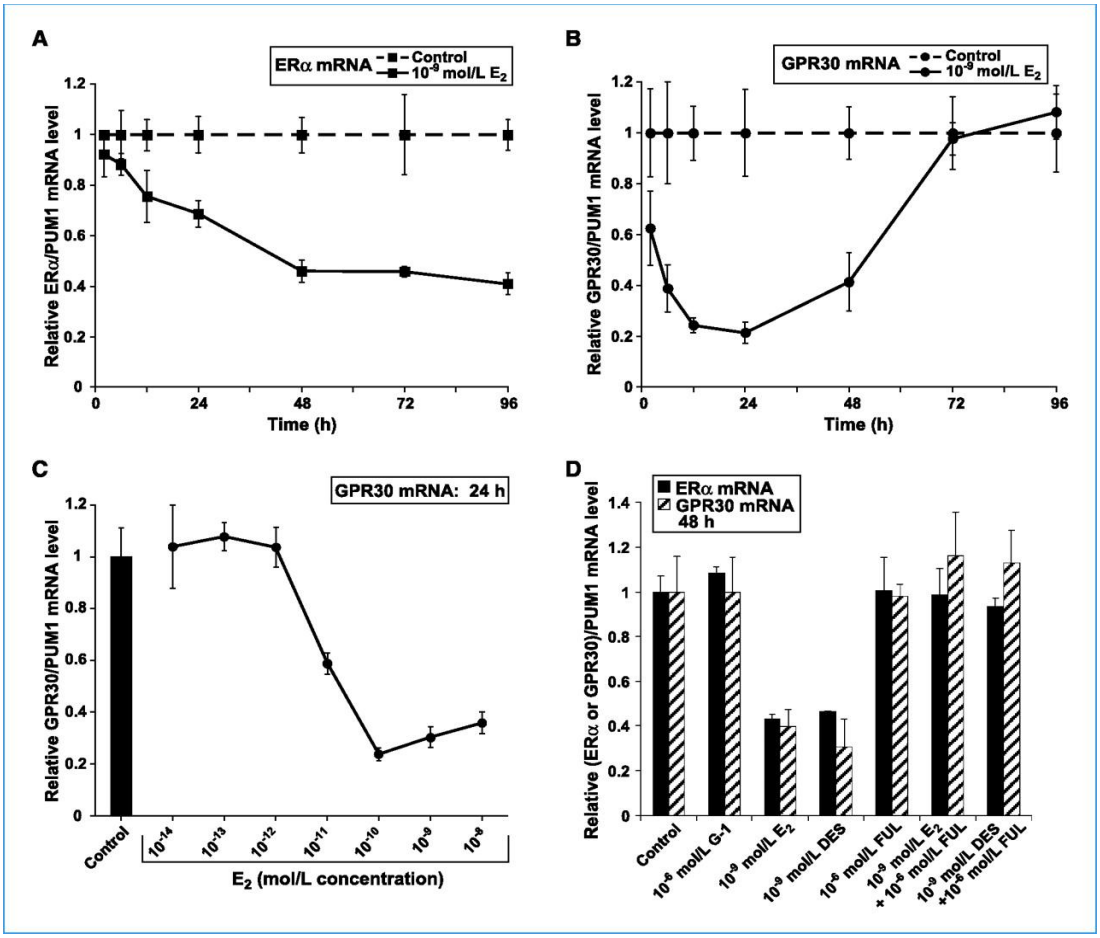


Figure 2r-2. *E_2 represses ERα and GPR30 mRNA levels via ER and not GPR30 in MCF-7 cells. E_2 regulation of ERα (A) and GPR30 mRNA (B) levels across a time course. MCF-7 cells were treated with 10^{-9} mol/L E_2 or with the vehicle ethanol alone for 2, 6, 12, 24, 48, 72, and 96 h. C, GPR30 mRNA levels in response to 24-h treatment with a serial dilution series of E_2. D, ERα and GPR30 mRNA levels in response to 48-h treatment with ER and GPR30 ligands as determined by qPCR. Each data point represents the average of six (A and B) or four (C and D) biological replicates. FUL, fulvestrant.*

E2 downregulates GPR30 mRNA expression via ER and not GPR30.
GPR30 regulation in response to E2 was investigated. MCF-7 cells were treated with E2 or without E2 (control, vehicle only) over a 96-hour time course followed by determination of ERŭand GPR30 mRNA levels by qPCR. As expected, E2 steadily downregulated ERŭmRNA levels by 59% over 96 hours (Fig. 2r-2A). E2 also downregulated GPR30 but with faster kinetics than with ERŭ(Fig. 2r-2B); GPR30 mRNA levels were decreased by 37% at 2 hours (P = 0.0013) and by 79% at 24 hours (P < 0.0001). Afterwards, GPR30 mRNA levels rebounded. Additionally, GPR30 mRNA expression decreased in a concentration-dependent manner from 10ī 12 mol/L E2 to 10ī 10 mol/L E2 (Fig. 2r-2C). The GPR30- specific agonist G-1 did not alter GPR30 mRNA expression, but the ER-specific agonist DES did repress GPR30 expression relative to control treatment by 54% P = 0.0009), which was very similar to the effect of E2 (Fig. 2r-2D). Fulvestrant completely blocked E2 and DES

effects. Therefore, E2 likely acted via ER and not GPR30 to transiently downregulate GPR30 mRNA expression.

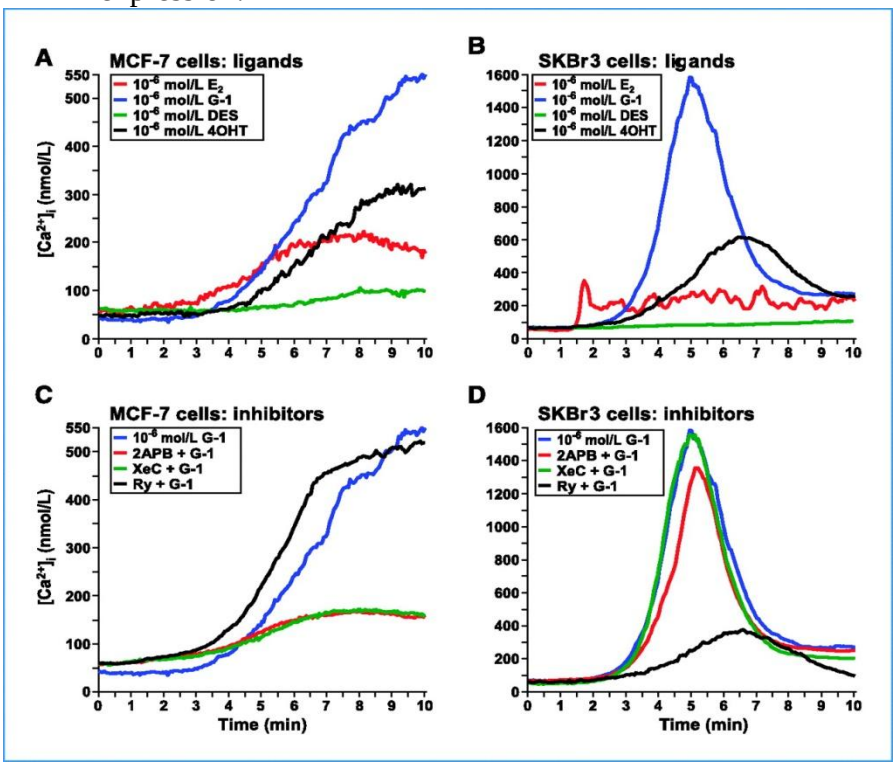


Figure 2r-3. *ER ligands that also activate GPR30 induce Ca^{2+} mobilization responses in both ER-positive MCF-7 and ER-negative SKBr3 cells. Ligand-induced Ca^{2+} responses (A and B) and blockade of G-1–induced responses using Ca^{2+} channel inhibitors (C and D) in MCF-7 and SKBr3 cells. Cells were loaded with Fura-2 AM, and intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ were determined in individual cells versus time using fluorescence microscopy. Cells were perfused with all ligands at 10^{-6} mol/L starting at 1 min. 2APB was used at 10^{-4} mol/L, XeC at 10^{-5} mol/L, and Ry at 10^{-5} mol/L. SKBr3 cells with flat, not rounded, morphology were imaged. G-1–induced Ca^{2+} traces in A and B were redrawn in C and D, respectively.*

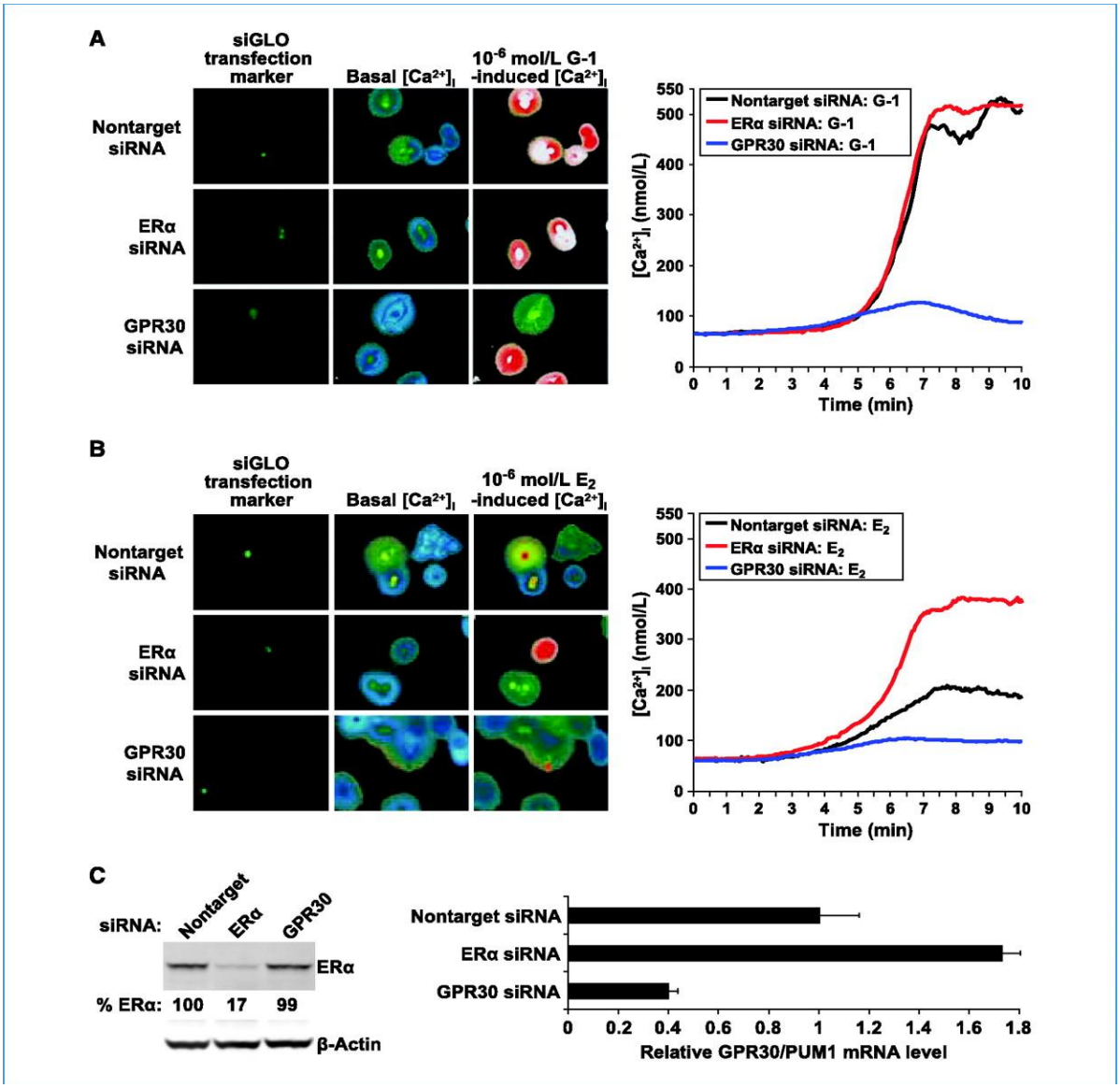


Figure 2r-4. *GPR30 and not ERα mediates E2-induced Ca2+ mobilization in MCF-7 cells. G-1–induced (A) and E2-induced (B) Ca2+ responses. Cells were transfected with nontargeting pool, ERα, and GPR30 siRNAs. Transfected cells were labeled using siGLO Green and appear green. Ca2+ imaging was performed 48 h following the transfection as in Fig. 3. Low levels of basal [Ca2+]i are visualized as blue and then green, whereas higher levels of [Ca2+]i are seen as red and then white. C, ERα protein levels were measured by immunoblotting and GPR30 mRNA levels by qPCR in siRNA-transfected cells 48 h following transfection*

GPR30 and not ERα mediates E2-induced Ca2+ mobilization responses.

To begin to delineate whether endogenous ERŬand/or GPR30 mediates E2-induced Ca2+ responses in breast cancer cells, changes in intracellular Ca2+ concentrations [Ca2+]i were measured in ER-positive MCF-7 and ER negative SKBr3 breast cancer cells at the single-cell level using Fura-2 AM (Fig. 2r-3). In ER-positive MCF-7 cells (Fig. 2r-3A), E2 induced [Ca2+]i by 112 Ñ1.6 nmol/L (n = 47 cells, P = 0.0063), G-1 by 511 Ñ3.4 nmol/L (n = 58 cells, P = 0.0007), and 4OHT by 234 Ñ3.4 nmol/L (n = 31 cells, P = 0.0017), whereas DES did not significantly increase the [Ca2+]I (change =

41 Ñ0.8 nmol/L, n = 23 cells, P = 0.66). In ER-negative SKBr3 cells (Fig. 2r-3B), the rank order of ligand-induced cytosolic Ca2+ increases was the same as in MCF-7 cells, but the magnitude of the increases was much greater and the responses were transitory instead of sustained. In ER-negative SKBr3 cells, E2 induced oscillating increases in [Ca2+]i with an average maximum of 294 Ñ1.6 nmol/L (n = 36 cells, P = 0.0037). G-1 and 4OHT induced transitory increases in [Ca2+]I of 1,517 Ñ 10.3 nmol/L (n = 79 cells, P = 0.0001) and of 558 Ñ2.7 nmol/L (n = 37 cells, P = 0.0013), respectively, whereas DES did not ([Ca2+]i change = 51 Ñ1.3 nmol/L, n = 21 cells, P = 0.26). Therefore, because G-1 and two ER ligands that also bind GPR30, E2 and 4OHT, but not ER-selective DES, elicited Ca2+ responses in both ER-positive MCF-7 cells and ER negative SKBr3 cells, they likely did so via GPR30. Two of the major Ca2+ channels, inositol triphosphate receptors (IP3R) and Ry receptors (RyR; ref. 28), were tested for whether they mediated G-1ï induced Ca2+ mobilization. The pharmacologic probes 2APB and XeC, both of which inhibit IP3Rs, and Ry, which at high concentrations blocks RyRs, were used. Cells were pretreated for 30 min before inducing

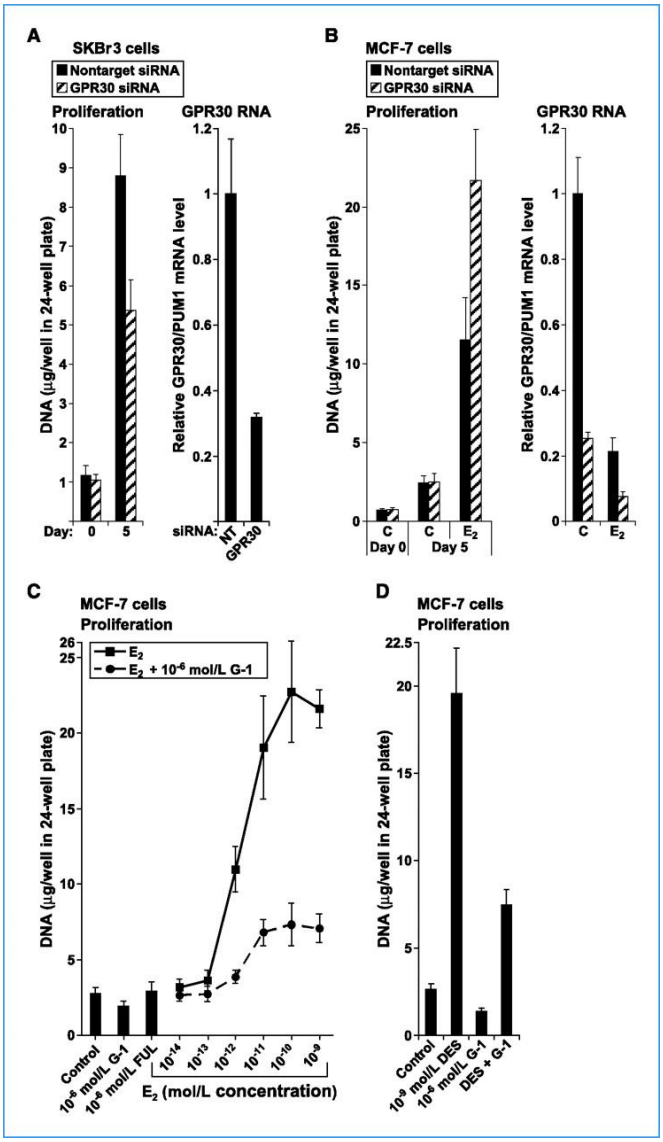
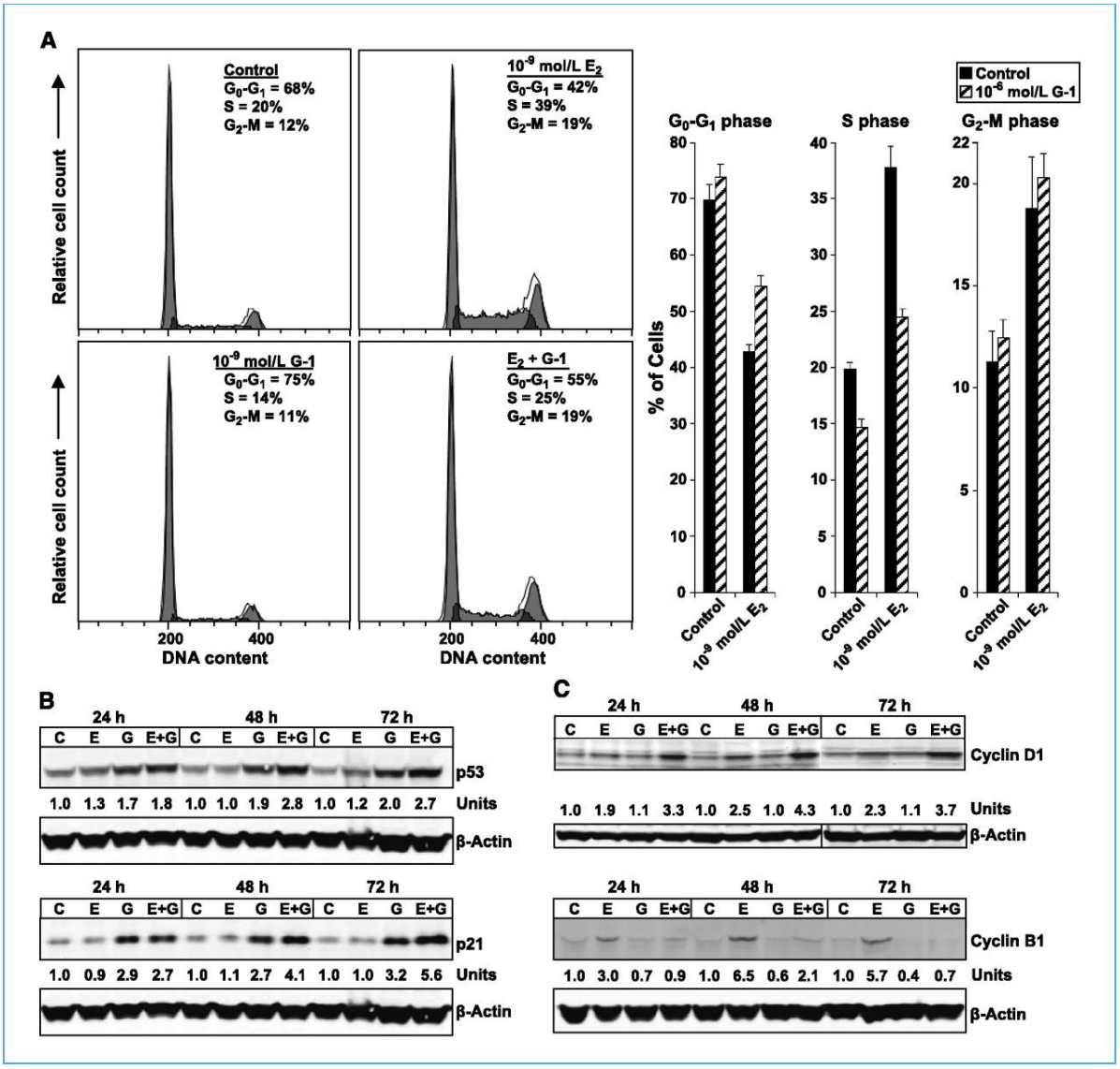


Figure 2r-5. *GPR30 promotes growth of ER-negative SKBr3 but inhibits growth of ER-positive MCF-7 cells. Proliferation of SKBr3 (A) and MCF-7 (B) cells transfected with the nontargeting pool and GPR30 siRNAs. Cells were transfected and then seeded at 15,000 per well in 24-well dishes. Medium was replenished the day after seeding on day 0 and every other day thereafter. Cells were collected on days 0 and 5. SKBr3 cells were cultivated in their passage medium, and MCF-7 cells in estrogen-free medium supplemented with 10−9 mol/L E2 or without E2 [control (C)]. Proliferation was assessed as cellular DNA mass (µg/well) using 24 replicate wells. GPR30 mRNA levels were determined by qPCR 48 h following the transfection in both cell lines, and in MCF-7 cells, after 24 h of 10−9 mol/L E2 or control treatment. C and D, proliferation of MCF-7 cells over 6 d treated with a serial dilution series of E2 (C) or with 10−9 mol/L DES (D) in the presence and absence of 10−6 mol/L G-1. Twelve replicate wells were used per group.*

with G-1. In MCF-7 cells, both 2APB and XeC blocked G-1ï induced [Ca2+]i increases by 78% (both 2APB + G-1 versus G-1 alone and XeC + G-1 versus G-1 alone, P = 0.0018) but Ry did not. In contrast, in SKBr3 cells, Ry blocked G-1ï induced [Ca2+]i increases by 80% (Ry + G-1 versus G-1 alone, P = 0.0006), whereas XeC did not. In addition, 2APB allowed G-1 to almost fully induce [Ca2+]i increases, although the response was significantly lower by 17% versus G-1 alone (P = 0.0094), but this was likely due to blockade of storeoperated Ca2+ entry, another activity of 2APB. Therefore, GPR30 was coupled to IP3Rs in ER-positive MCF-7 cells but to RyRs in ER-negative SKBr3 cells. To confirm that GPR30 and not ERŬmediates Ca2+ mobilization in response to E2 in MCF-7 cells, cells were transfected with siRNAs targeting these receptors (characterization of siRNAs in Supplementary Materials and Methods) and a nontargeting siRNA pool as a control. First, the GPR30 siRNA was validated by showing that it led to an almost complete blockade of G-1ï induced Ca2+ responses ([Ca2+]I increase = 58 Ñ1.3 nmol/L, n = 19 cells, P = 0.62; Fig. 2r-4A). Next, E2-induced Ca2+ mobilization responses were investigated (Fig. 2r-4B). In nontargeting siRNAï transfected cells, E2 induced an [Ca2+]i increase of 159 Ñ1.6 nmol/L (n = 14 cells, P = 0.0075). However, in ERŬsiRNAï transfected cells, E2 caused almost a 2-fold further rise in [Ca2+]i (314 Ñ3.2 nmol/L, n = 17 cells; P = 0.0006). In GPR30 siRNA transfected cells, the E2-induced Ca2+ response was blocked ([Ca2+]I increase = 52 Ñ0.8 nmol/L, n = 16 cells, P = 0.35). ERŬand GPR30 expression were depleted in the appropriate siRNA-transfected cells (Fig. 2r-4C). However, GPR30 mRNA levels were increased by 73% in ERŬ-depleted cells, a finding consistent with the prior observation that E2 and DES repressed GPR30 expression (Fig. 2r-2Bï D). Hence, the 2-fold potentiation of E2-induced [Ca2+]i in ERŬ-depleted cells likely reflected, at least in part, the increased GPR30 expression.

GPR30 functions to promote growth of ER-negative SKBr3 cells but to inhibit growth of ER-positive MCF-7 cells.

Figure 2r-6. *G-1 inhibits cell cycle progression in E2-stimulated MCF-7 cells by producing a block at G1 phase. A, cell cycle distribution as determined by propidium iodide staining of DNA content and flow cytometry. Cells were synchronized by 3-d cultivation in estrogen-free medium and then treated as indicated for 24 h. Thirty-thousand cells per sample and three replicates per group were collected. Representative histograms are shown. Immunoblot analyses of p53 and p21 (B) and of cyclin D1 and cyclin B1 (C) protein levels. MCF-7 cells were control (C)-, 10−9 mol/L E2 (E)-, and 10−6 mol/L G-1 (G)–treated as indicated. Quantitated protein levels normalized to β-actin are indicated.*



The role of GPR30 in cellular proliferation was examined by transfecting cells with nontargeting and GPR30 siRNAs and then measuring cellular DNA mass after 5 days of growth. First, SKBr3 cells were evaluated (Fig. 2r-5A). The number of cells seeded in each group was similar as indicated by a lack of difference in DNA masses at day 0. After 5 days of growth, DNA mass was 45% lower in GPR30 siRNA compared with nontargeting siRNAï transfected cells ($P < 0.0001$). Thus, the function of GPR30 was to promote growth of SKBr3 cells, in accordance with other reports (9, 10). Next, MCF-7 cells were similarly evaluated (Fig. 2r-5B). Again, equivalent numbers of nontargeting and GPR30 siRNAï transfected cells were seeded as indicated by DNA masses at day 0. After 5 days, GPR30 depletion did not affect basal growth (control treatment). However, GPR30 depletion did potentiate E2-stimulated growth by 2.1-fold (nontargeting versus GPR30 siRNAï transfected cells, $P < 0.0001$). Analysis of progesterone receptor (PgR) and TFF1 (pS2) mRNA levels by qPCR indicated no significant differences in their induction by E2 between nontargeting and GPR30 siRNAï transfected cells (data not shown). Therefore, in contrast to SKBr3 cells, the function of GPR30 in MCF-7 cells was to inhibit growth. The role of GPR30 in MCF-7 cell proliferation was further evaluated by examining effects of G-1 on E2-stimulated (Fig. 2r-5C) and DES-stimulated (Fig. 2r-5D) growth over 6 days. G-1 blocked the concentration-dependent growth stimulatory

response of E2 (all E2 treatment groups versus paired E2 + G-1 treatment groups, P = 0.0001, one-way ANOVA); in particular, G-1 inhibited 10ī 10 mol/L E2-stimulated growth by 77% relative to E2 alone (P < 0.0001, t test). G-1 also blocked DES stimulated growth by 72% (DES versus DES + G-1, P < 0.0001). Additionally, in both the E2 and DES experiments, G-1 inhibited basal (control treatment) growth by 32% (P < 0.0001) and 47% (P < 0.0001), respectively. Therefore, G-1ï activated GPR30 blocked growth of ER-positive breast cancer cells but did so independently of ligand-activated ER.

G-1–activated GPR30 blocks cell cycle progression at G_1 phase

The effect of G-1 on cell cycle progression was investigated. MCF-7 cells were synchronized by estrogen withdrawal and then treated with E_2 and G-1 for 24 hours followed by propidium iodide staining and flow cytometric analysis (Fig. 2r-6A). Treatment with G-1 alone significantly decreased the proportion of S-phase cells from 19.8% (control) to 14.7% (G-1; $P < 0.0001$). Importantly, the addition of G-1 to E_2 led to retention of an additional 11.6% of the cells in G_1 phase (42.7% in E_2-treated cells versus 54.4% in E_2 + G-1ï treated cells, $P < 0.0001$) and prevented 13.2% of cells from entering S phase (37.7% in E_2-treated cells versus 24.5% in E_2 + G-1ï treated cells, $P < 0.0001$). Therefore, G-1 blocked E_2-stimulated cells from cell cycle progression at the G_1 phase. The G-1ï induced cell cycle block was further investigated by measuring protein expression of the tumor suppressor p53, the cyclin-dependent kinase inhibitor (CDK-I) p21 (Fig. 2r-6B), the G_1-phaseï specific cyclin D1, and the G_2/M-phaseï specific cyclin B1 (Fig. 2r-6C). MCF-7 cells were treated with E_2 and G-1 and then collected at 24, 48, and 72 hours for immunoblot analysis. Both p53 and p21 proteins were upregulated in G-1 and E_2 + G-1ï treated cells across all time points compared with control-treated cells (Fig. 2r-6B). As expected, E_2 upregulated both cyclins D1 and B1 across the time course compared with control treatment, whereas G-1 alone did not (Fig. 2r-6C). However, the addition of G-1 to E_2 potentiated the upregulation of cyclin D1 while nearly completely preventing cyclin B1 accumulation compared with E_2 alone across the time points. Because cyclin D1 is induced during G_1 phase and degraded in S phase [255,256], whereas cyclin B1 accumulates during G_2-phase and degrades on M-phase entry [257], these data are consistent with G-1 blocking cell cycle progression in G_1 phase before cyclin D1 degradation occurred and before cyclin B1 accumulated.

Discussion

Filardo and colleagues[258] and Kuo and colleagues [259] have previously shown in breast carcinomas a positive association between GPR30 and ERŬexpression by immunohistochemistry and qPCR, respectively. We confirmed and extended this finding by examining gene expression microarray data sets of five independent patient cohorts comprising 1,250 breast cancers. In all cohorts, high GPR30 mRNA levels showed an association with ERŬpositivity (Fig. 2r-1). It is unknown why high GPR30 levels would be selected for in ERŬ-positive breast carcinomas given GPR30 attenuates growth of ER-positive breast cancer, but some GPR30-dependent functions may be necessary for tumorigenesis and cell survival, such as activation of adenylyl cyclase, PI3K, and MAPK [260,261]. Additional roles of GPR30 may be needed for disease progression, such as in cell migration [262,263], which may then promote metastasis [258].

The interplay between GPR30 and ERŬwas further investigated using MCF-7 breast cancer cells. E_2 repressed GPR30 expression in a time- and concentration-dependent manner (Fig. 2r-2B and C). In

addition, DES but not G-1 downregulated GPR30; therefore, ER mediated this effect (Fig. 2r-2D). The inverse functional relationship between ERŬand GPR30 was also shown by depleting ERŬ, which led to derepression of GPR30 mRNA expression and consequently potentiated E_2-induced Ca^{2+} mobilization responses (Fig. 2r-4). E_2 is known to downregulate ERŬexpression in MCF-7 cells as a negative feedback regulatory loop to prevent over responsiveness [165]. Likewise, GPR30 may also be negatively regulated by E_2 via ER to prevent excessive GPR30-dependent activity, such as aberrantly high $[Ca^{2+}]_i$. Interestingly, the maximum increases in $[Ca^{2+}]_i$ were much larger in SKBr3 cells than in MCF-7 cells (Fig. 2r-3B versus Fig. 2r-3A). It is possible that this was due to the lack of ERs in SKBr3 cells, which translated into a lack of negative feedback regulation.

GPR30 depletion decreased growth of SKBr3 cells (Fig. 2r-5A) but potentiated E_2-stimulated growth in MCF-7 cells (Fig. 2r-5B), indicating that GPR30 functions to promote SKBr3 but to inhibit MCF-7 cellular proliferation. Also in MCF-7 cells, G-1 profoundly inhibited E_2-stimulated (Fig. 2r-5C) and DES-stimulated growth (Fig. 2r-5D) as well as decreased the percentage of cells entering S phase (Fig. 2r-6A). However, these G-1 effects occurred in both the presence and the absence of E_2. These findings in MCF-7 cells complement those of Ahola and colleagues [264] who reported that transient GPR30 overexpression decreased the percentage of proliferating MCF-7 cells independent of E_2. Indeed, GPR30 likely does not directly regulate ER transcriptional activity because there were no significant differences in E_2-induced mRNA expression of well-established ER target genes *PgR* and *TFF1* between GPR30 siRNAï transfected and nontargeting siRNAï transfected cells (data not shown).

Rather, we propose that GPR30 antagonizes growth of MCF-7 cells by inducing sustained increases in cytosolic Ca^{2+} concentrations (Figs. 2r-3A and 4), in contrast to transitory increases in SKBr3 cells where GPR30 promotes growth. Aberrant sustained increases in intracellular Ca^{2+} levels can lead to inhibition of proliferation and induce apoptosis [265]. For example, the plasma membrane Ca^{2+}-ATPase (PMCA) pumps Ca^{2+} across the plasma membrane out of the cell to lower cytosolic Ca^{2+} levels after Ca^{2+} increases. Partial inhibition of PMCA in MCF-7 cells causes a moderate increase in intracellular Ca^{2+} levels, which leads to inhibition of proliferation by altering cell cycle kinetics [266]. Additionally, the mechanism of action of numerous antitumor agents involves increases in $[Ca^{2+}]_I$ [267].

It is possible that differences in GPR30-coupled Ca^{2+} signaling, which mediate sustained versus transitory responses, associate with ER status. In support of this hypothesis, GPR30 was coupled to differing Ca^{2+} channels: to IP_3Rs in ER-positive MCF-7 cells but to RyRs in ER-negative SKBr3 cells. Alternatively, sustained versus transitory Ca^{2+} responses could have been due to potential alterations in factors that participate in lowering cytosolic Ca^{2+}, such as plasma membrane or sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase pumps. We intend to explore these possibilities involving differing Ca^{2+} responses in future studies.

As shown by propidium iodide staining and flow cytometry, G-1 induced a cell cycle block at the G_1 phase (Fig. 2r-6A). Consistent with a G_1-phase arrest, G-1 increased accumulation of the tumor suppressor p53, the CDK-I p21, and the G_1-phaseï specific cyclin D1 but prevented E_2-induced accumulation of the G_2/M-phaseï specific cyclin B1 (Fig. 2r-6B and C). Ca^{2+} signaling has been shown to induce p53 via activation of cyclic AMPï responsive element binding protein [268]. In MCF-7 cells, p53 induction by E_2 is Ca^{2+} and calmodulin kinase IV dependent [269]. In addition, aberrant Ca^{2+} mobilization in response to anticancer/cytotoxic agents correlates with p53 induction

[267]. Thus, G-1 could lead to p53 induction via Ca^{2+} mobilization in MCF-7 cells. Then, p53 could induce p21 via a p53 response element to mediate arrest in G_1 phase of the cell cycle [268].

As a SERM, tamoxifen acts as an antiestrogen in ER-positive breast cancer but as an estrogen in the endometrium and bone [270]. Similarly, G-1 inhibits growth of ER-positive MCF-7 breast cancer cells but promotes growth of the endometrium [271] and plays an important role in promoting bone growth *in vivo* [272,273]. Thus, the tissue-specific proliferative effects of G-1 may parallel those of tamoxifen. It is interesting to speculate that 4OHT-induced Ca^{2+} mobilization (Fig. 2r-3A and B) may be involved in some of the tissue-specific effects of tamoxifen.

Taken together, GPR30 inhibits growth of ERŬ-positive breast cancer. Our studies also indicate that pharmacologic activation of GPR30 shows promise in combating ER-positive breast cancer. G-1 would also probably be well tolerated because it, like E_2, exerts beneficial effects against an animal model of multiple sclerosis but without E_2-associated side effects [274,275]. Thus, G-1 may represent the first in a new class of therapeutically relevant agents for use alone or in conjunction with conventional antihormonal therapeutics in breast cancer.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2s (Peng and Jordan) - Studies carried out by Dr. Jing Peng in the Jordan laboratory at Fox Chase Cancer Center

Expression of estrogen receptor alpha with a Tet-off adenoviral system induces G0/G1 cell cycle arrest in SKBr3 breast cancer cells

Introduction:

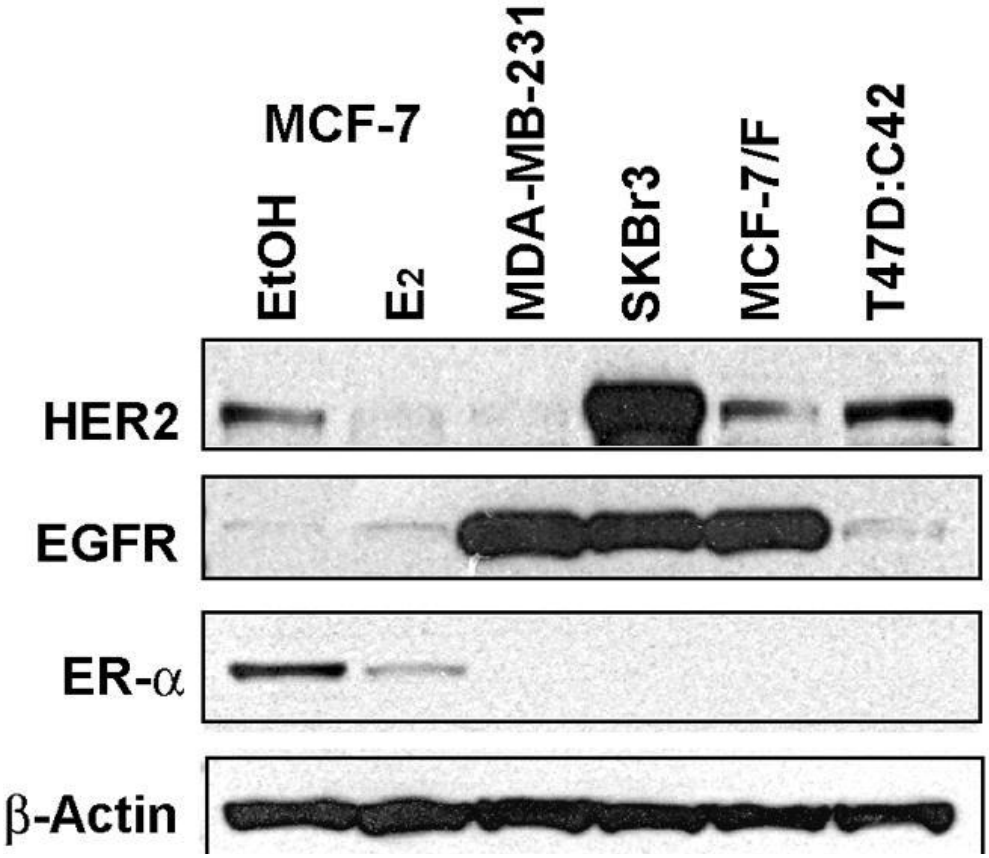
It is important to examine how ER-negative breast cancers cells with high HER2 react to estrogen when an exogenous ERŬis expressed. Potential new drug targets could be identified in ER-negative cancers if estrogen triggers apoptosis or growth inhibition through a common mechanism shared by different types of ER-negative cancer cells when an exogenous ERŬis introduced. In this study, a Tet-off adenoviral system was developed to deliver ERŬto ER-negative breast cancer SKBr3 cells that over-express both EGFR and HER2. The Tet-off adenoviral system is highly efficient and the expression level of ERŬis controlled by addition of doxycycline in a concentration-dependent manner. Using this system, we examined the function of ERŬand estradiol on cell proliferation. The results suggest that estrogen suppresses the proliferation of SKBr3 cells through a similar mechanism as estrogen does in MDA-MB-231 cells when an ectopic ERŬis expressed. The mechanism involves upregulation of $p21^{Cip1/Waf1}$ and downregulation of E2F1. The effect of estrogen on growth receptor expression was also examined in SKBr3 cells when exogenous ERŬwas expressed.

Work Accomplished:

Expression of ERα in SKBr3 breast cancer cells with Tet-off adenoviral system

Most studies expressing ectopic ERŬin ER-negative breast cancer cells have used MDA-MB-231 cells which have high levels of EGFR but low levels of HER2. Since about 20% breast cancers are HER2-positive, it is important to examine if hormone-responsiveness could be restored in ER-negative breast cancer cells that over-express HER2. Therefore, we chose SKBr3 cells which over-express both HER2 and EGFR for this study. The expression of HER2, EGFR and ERŬwere compared between SKBr3 and several other breast cancer cell lines as shown in Figure 2s-1. The ER-positive MCF-7 cells expressed low levels of EGFR and HER2, and estrogen treatment decreased HER2 expression. MDA-MB-231 cells had high levels of EGFR but little HER2. The ER-

negative MCF-7/F cells derived from MCF-7[276] highly expressed EGFR and moderately



e› **Fig. 2s-1**.

Another ER-negative T47D:C42 cells cloned from ER-positive T47D cells [187,188] had moderate expression of HER2 and little expression of EGFR. Only SKBr3 cell had high levels of both HER2 and EGFR. A Tet-off adenoviral delivery system was developed to express ERŬin SKBr3 cells. The

infection efficiency of adenoviruses in SKBr3 cells was analyzed using a green fluorescent protein

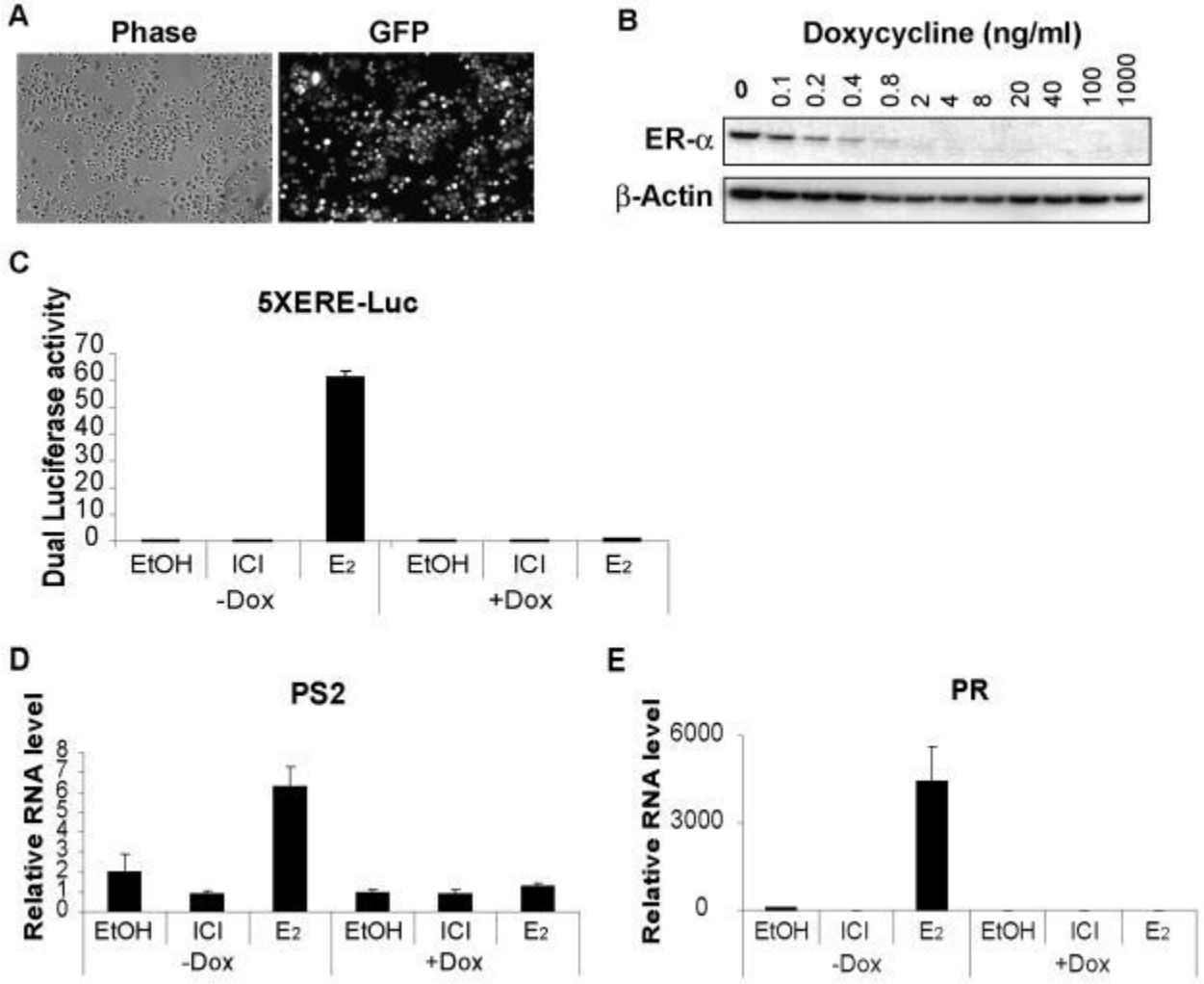
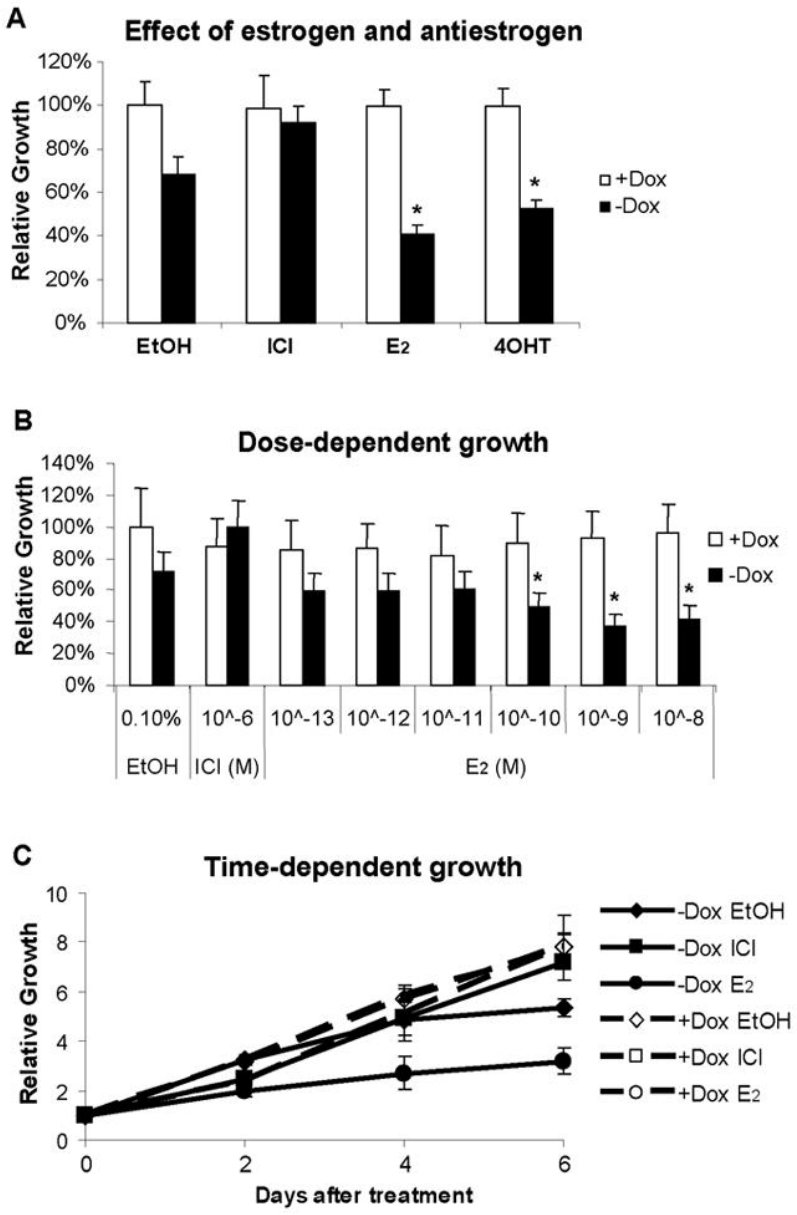


Fig.2s-2. *The Tet-off adenoviral system to express ERα in SKBr3 cells. (A) SKBr3 cells were infected with Ad-CMV-GFP and observed 24 hours after infection with a TE300 fluorescence microscope (Nikon Instruments, Melville, NY). (B) SKBr3 cells were co-infected by Adeno-X Tet-off and Ad-TRE-ERα in the presence of doxycycline at various concentrations. The cells were harvested 48 hours after infection and total protein was extracted for western blot. (C) SKBr3 cells infected by Adeno-X Tet-off and Ad-TRE-ERα in the presence (+Dox) or absence (−Dox) of 1 μg/ml doxycycline were transfected with 5xERE-firefly-luciferase and TA-Renilla-luciferase plasmids. The cells were harvested for dual luciferase activity assay after 48-hour treatment with the compounds as indicated. The ratio of firefly luciferase vs Renilla luciferase activities were plotted and the number of the +Dox/EtOH sample was arbitrarily set to be 1 for easy comparison. (D) SKBr3 cells infected by Adeno-X Tet-off and Ad-TRE-ERα in the presence (+Dox) or absence (−Dox) of 1 μg/ml doxycycline were treated with 0.1% EtOH, 1 μM fulvestrant (ICI) or 1 nM 17β-estradiol (E2) for 48 hours. The total RNA was extracted for real-time RT-PCR analysis of PS2 or PR (E) against endogenous control 36B4 using a relative standard curve generated by 10-fold serial dilution of MCF-7 cDNA. The value of the +Dox/EtOH sample was arbitrarily set to be 1 for easy comparison.*

(GFP) reporter adenovirus (Ad-CMV-GFP). As shown in Figure 2s-2A, >95% cells were infected and expressing GFP. The adenoviral system is more efficient than plasmid transfection which normally has <50% efficiency, thus a lengthy selection for stable-transfected cell colonies can be avoided using the adenoviral system since almost all the cells were infected and expressed the delivered gene of interest. The expression of ERŬcan be turned off by doxycycline when cells are co-infected with Adeno-X Tet-Off and Ad-TRE-ERŬadenovirus simultaneously. As shown in Figure 2s-2B, the expression level of ERŬdecreased as the concentration of doxycycline increased from 0 to 0.8 ng/ml, and ERŬexpression was almost undetectable as doxycycline concentration was above 2 ng/ml. The ERŬexpressed in SKBr3 cells by the adenovirus is fully functional. It activated luciferase reporter containing 5 estrogen receptor elements (5Ĭ ERE) in the presence of 1 nM E_2 while the luciferase reporter was not detected either when ERŬwas not expressed (+Dox) or when EtOH control or pure antiestrogen fulvestrant (ICI) was added (Figure 2s-2C). Real-time RT-PCR assay also indicated that the exogenous ERŬinduced the endogenous estrogen-responsive genes PS2 and progesterone receptor (PR) in response to E_2. The RNA level of PS2 was doubled by expression of ERŬitself (compare ī Dox/EtOH and +Dox/EtOH), and addition of 1 nM E_2 further increased PS2 RNA to 6 fold (compare ī Dox/E2 and +Dox/EtOH), but addition of fulvestrant did not change PS2 RNA expression (Figure 2s-2D). The induction of PR RNA was more dramatic, as PR RNA was barely detectable without ERŬexpression (+Dox) or with ERŬbut in the presence of EtOH control or antiestrogen fulvestrant. However, E_2 addition increased PR RNA level by thousands of folds when ERŬwas expressed (compare ī Dox/E2 and +Dox/EtOH, Figure 2s-2E).

Cell proliferation of SKBr3 cells after ERα expression

Fig.2s-3. *The effects of ERα expression and estrogen/antiestrogen treatment on the proliferation of SKBr3 cells. SKBr3 cells were infected by Adeno-X Tet-off and Ad-TRE-ERα in the presence (+Dox) or absence (−Dox) of 1 μg/ml doxycycline, treated by the 0.1% EtOH (v/v), 1 μM fulvestrant (ICI), 1 μM 4-hydroxytamoxifen (4OHT) or E_2 (at final concentration of 1 nM or as indicated in the graph) and harvested for DNA quantification. (A) Growth with different ER ligands treated for six days. (B) Dose-dependent growth with various E_2 concentrations treated for six days. (C) Time-dependent growth with cells harvested every 2 days after treatment. The samples with a statistically significant difference ($p<0.05$ by t-test) from the +Dox/EtOH control were marked with a "*".*



We next examined the effects of ERŬon SKBr3 cell proliferation by measuring the total cellular DNA content. As shown in Figure 2s-3A, growth of SKBr3 cells was irresponsive to fulvestrant, 4-hydoxytamoxifen or E_2 if no ERŬwas expressed. However, expression of ERŬitself reduced cell proliferation to about 70% (compare ī Dox/EtOH and +Dox/EtOH), although the reduction was not statistically significant, similar inhibition was repeatedly observed in independent experiments. The ERŬ-mediated growth suppression was abolished by fulvestrant, and addition of 1 nM E_2 or 1 ε M 4-hydroxytamoxifen inhibited SKBr3 cell proliferation to about 40% and 50% respectively, which was statistically significant (compare with the +Dox/EtOH control). With the ectopic expression of ERŬ, E_2 inhibited the growth of SKBr3 cells in a dose-dependent manner, as shown in Figure 2s-3B. Statistical difference was reached when E_2 concentration was Ó$10^{\bar{1}\,10}$ M (0.1 nM), comparing with the +Dox/EtOH control. Similar results were obtained in the time-dependent growth curve shown in Figure 2s-3C.

ERŬ expression arrests SKBr3 cells at G0/G1 cycle

Next, flow cytometry analysis was performed to examine cell cycle progression of SKBr3 cells when ERŬwas expressed. As shown in Figure 2s-4, about 50% cells were at G0/G1 cell cycle without ERŬ(+Dox) or with ERŬbut in the presence of fulvestrant (ī Dox/ICI). However, the population of cells at G0/G1 cell cycle increased to about 80% when ERŬwas expressed in the presence of EtOH control or 1 nM E_2. Apoptosis was not observed in SKBr3 cells as there was no significant cell accumulation at sub-G1 phase (cell debris) when ERŬwas expressed. Annexin V/PI staining, caspase activity assay or PARP-cleavage assay all confirmed that apoptosis did not occur (data not shown).

Modulation of E2F1 cell cycle checkpoint proteins by E_2 and ERŬ

The transcription factor E2F1 plays an important role in G1 to S cell cycle progression. Before cells enter S phase, hypo-phosphorylated pRb protein binds to E2F1 and prevents it from activating down-stream genes essential for DNA replication and cell proliferation. Activation of cyclin dependent kinases (CDKs) phosphorylates pRb and releases E2F1 for action. CDK inhibitory proteins such as $p21^{Cip1/Waf1}$, $p27^{Kip1}$ and $p16^{INK4A}$ inhibit CDKs activity thus lead to hypo-phosphorylation of pRb and inactivation of E2F1, which in turn undetectable without ERŬ expression (+Dox) or with ERŬexpression but in the presence of fulvestrant. The $p21^{Cip1/Waf1}$ protein level was increased by ERŬexpression and further increased by the addition of E_2, which coordinated with the phosphorylation status of pRb. Opposite regulation of E2F1 was observed by ERŬexpression and E_2 treatment. The RNA levels of $p21^{Cip1/Waf1}$and E2F1 were regulated in a similar patten as the protein levels (Figure 2s-5B). A moderate down-regulation of pRb at protein level was also observed in ERŬ-expressing samples but not at the RNA level. This might be resulted from the up-regulation of $p21^{Cip1/Waf1}$ because $p21^{Cip1/Waf1}$ mediates pRb protein degradation [277].

inds to E2F1 and prevents

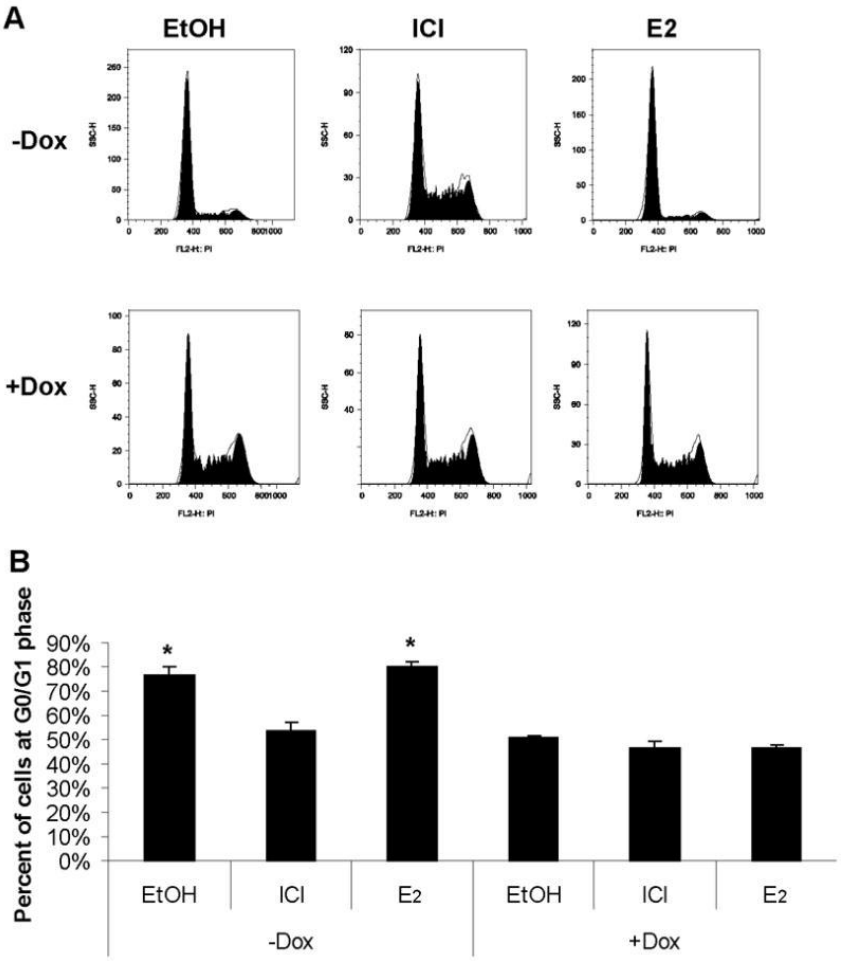


Fig.2s-4. *Cell cycle analysis of SKBr3 cells expressing ERα. SKBr3 cells were infected by Adeno-X Tet-off and Ad-TRE-ERα in the presence (+Dox) or absence (−Dox) of 1 μg/ml doxycycline, treated by 0.1% EtOH, 1 μM fulvestrant (ICI) or 1 nM E_2 for 2 days and harvested for cell cycle analysis. (A) Flow cytometry analysis of cell cycle distribution. (B) Percentage of cells at G0/G1 cell cycle from three independent experiments. The samples with a statistically significant difference ($p<0.05$ by t-test) from the +Dox/EtOH control were marked with a "*".*

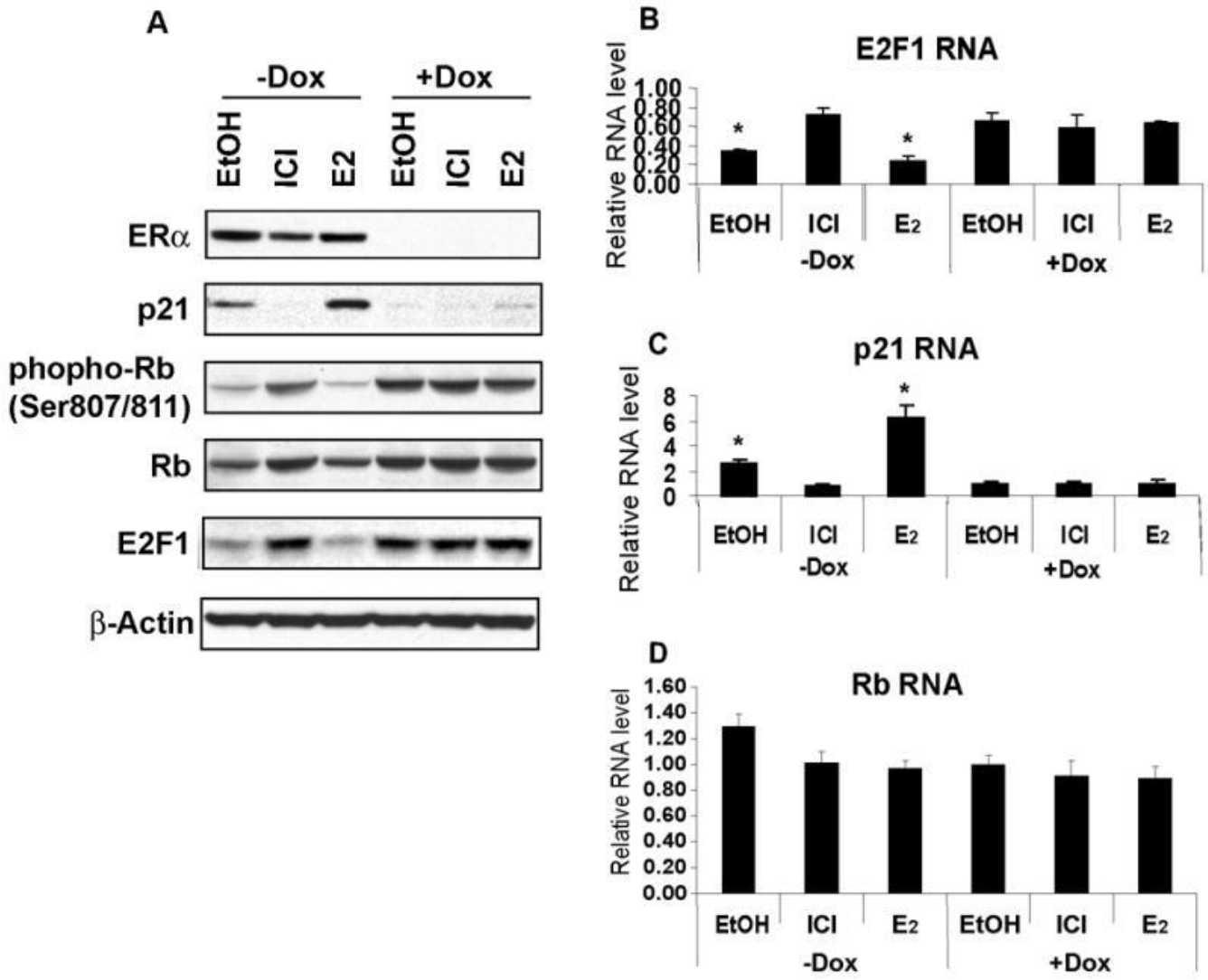


Fig.2s-5. *Modification of $p21^{Cip1/Waf1}$, pRb and E2F1 by ERα/E_2 in SKBr3 cells. SKBr3 cells were infected, treated and harvested as in Figure 4. Protein was extracted for western blot analysis (A) and RNA was prepared for real-time RT-PCR analysis to detect E2F1 (B), $p21^{Cip1/Waf1}$ (C) or pRb (D) as described in Figure 2. The samples with a statistically significant difference ($p<0.05$ by t-test) from the +Dox/EtOH control were marked with a "*****".*

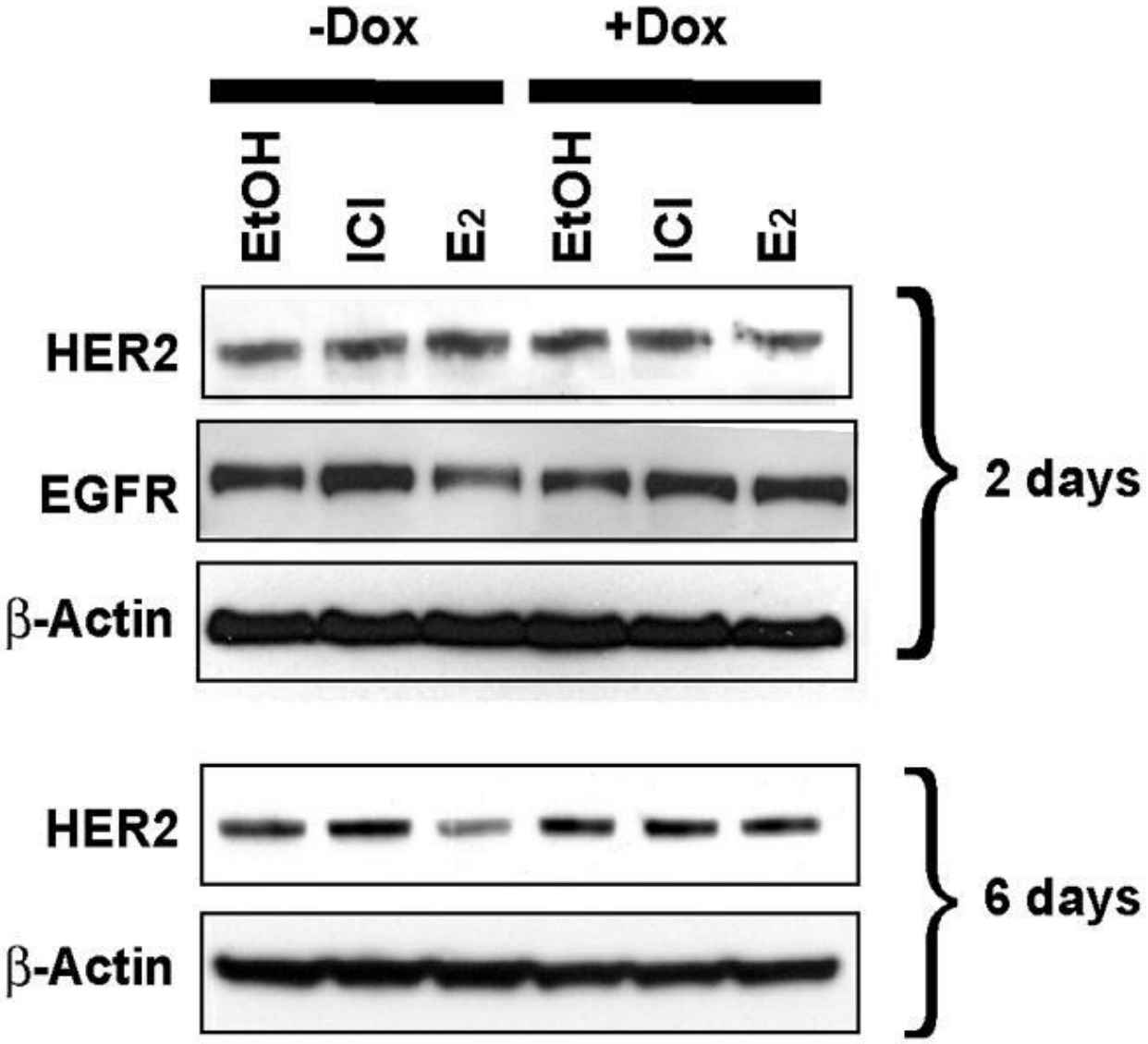


Fig.2s-6. *The effects of ERα expression and estrogen treatment on the expression of HER2 and EGFR in SKBr3 cells. SKBr3 cells were infected, treated for 2 days or 6 days then harvested for protein extraction and western blot analysis.*

causes cell cycle arrest at G0/G1 phase. Stender et al [278] found that E2F1 and p21 were differentially regulated by estrogen in ER-positive MCF-7 cells and ER-stably-transfected MDA-MB-231 cells. Therefore, we also examined modification of $p21^{Cip1/Waf1}$/pRb/E2F1 pathway proteins by E_2 and ERŬin SKBr3 cells. As shown in Figure 2s-5A, $p21^{Cip1/Waf1}$ was

The effects of estrogen on HER2 and EGFR expression

Intimate crosstalk between hormone receptor signaling and growth factor receptor signaling is a major contributor to breast cancer progression and endocrine resistance [279]. However, an inverse correlation is often found between ER and HER2 [280,281], and estrogen down-regulates HER2 expression in ER-positive MCF-7 cells [282] (Figure 2s-1). Growth factor signaling is essential for SKBr3 cell proliferation, therefore we examined the effects of estrogen and exogenous ERŬon the expression of HER2 and EGFR. As shown in Figure 2s-6, ERŬexpression itself had little effect on HER2 and EGFR expression (compare ī Dox/EtOH and +Dox/EtOH), however, 2-day treatment with E_2 decreased EGFR protein level and 6-day treatment of E_2 also reduced HER2 protein level. These results suggest that ectopic expression of ERŬand E_2 treatment might be a way to switch the more aggressive growth-factor-receptor-positive tumors to the prognostically more favorable hormone-sensitive type.

Discussion:

In this study, we developed a Tet-off adenovirus to express ERŬin ER-negative SKBr3 cells. Adenoviruses infect the cells and deliver the gene of interest with over 95% efficiency, thus can be used to study cellular effects of the interested gene in a ñtransient expressionò experiment. This is not always possible using the traditional plasmid transfection with less than 50% delivery efficiency because the background is high when most cells are not expressing the gene of interest. Instead, a stably-transfected clone has to be selected and expanded, which is a time-consuming process. In addition, the phenotype of a stably-transfected clone may not be the direct result of the interested gene expression but the result of the random gene insertion at the host genome. The inability of adenoviruses to integrate into the host genome minimizes the complications of destroying or activating other host genes, thus adenoviral vector is a valuable tool to express exogenous genes for gene therapy. Adenovirus-based therapy to express p53 tumor suppressor, AdvxinÈ (Introgen Therapeutics, Austin, TX), has demonstrated safety profile and clinic efficacy in several tumor types and approval is being sought in Europe and the United States to treat recurrent, refractory head and neck cancer [283]. Another similar adenoviral p53 transfer therapy, GendicineÈ (Benda Pharmaceutical, China), has been approved to treat head and neck cancer in China [284]. Therefore, adenovirus-based vectors could potentially be developed in the future to express ERŬin ER-negative breast cancers to restore hormone responsiveness.

The Tet-off system adds another advantage to the expression method by controlling the expression level of interested gene. As shown in Figure 2s-2B, the amount of ERŬexpressed is regulated by doxycycline. This provides a valuable approach to study gene function in a dose-dependent manner, which is not achievable using a constitutively-expressing vector. Moreover, expression of the interested gene can be turned on or off by removal or addition of doxycycline at any time, thus studying the gene function in a timely fashion is possible.

In MDA-MB-231 cells, ectopic ERŬexpression by adenovirus itself had no effect on cell proliferation, but treatment of E_2 suppressed cell proliferation [285]. However, in SKBr3 cells, the expression of ERŬitself inhibits cell proliferation and E_2 treatment amplifies the growth inhibitory effects, while pure antiestrogen fulvestrant abolished growth inhibitory effects of ERŬ(Figure 2s-3). It is possible that ERŬhas more ligand-independent activity in SKBr3 cells which over-express both HER2 and EGFR than in MDA-MB-231 cells that only over express EGFR. Estrogen exerts similar

growth inhibitory effects on MDA-MB-231 and SKBr3 cells when ERŬis expressed, but ERŬ-expressing MDA-MB-231 and SKBr3 cells respond differently to tamoxifen which is ineffective in MDA-MB-231 cells [285] but inhibitory in SKBr3 cells (Figure 3A). The mechanisms remain to be elucidated and could be that these two cell types have various cellular profile of transcription factors and different levels of nuclear receptor coregulators.

The proliferation inhibition mediated by ERŬand E_2 in SKBr3 cells is likely due to cell cycle arrest at G0/G1 phase (Figure 4), since significant apoptosis was not observed. Similar to MDA-MB-231 cells, E_2 and ERŬmodify the expression of G1 to S phase checkpoint proteins $p21^{Cip1/Waf1}$ and E2F1 in SKBr3 cells (Figure 5), suggesting an important role of E2F1 in hormone-mediated regulation of cell proliferation. E2F1 is critical to control cell cycle progression and apoptosis [286], and its overexpression is often linked to poor prognosis of breast cancer [287,288,289,290]. Therefore, E2F1 is a potential drug target for breast cancer. In addition, E_2 treatment down-regulates expression of HER2 and EGFR in ERŬ-expressing SKBr3 cells (Figure 6), suggesting that growth factor signalling could be diminished by E_2/ERŬand that a less aggressive hormone-responsive cancer type can be re-created.

Strategically, it is important to note that the ectopic E_2/ERŬcomplex is able to block cell cycle progression at G0/G1 phase. A similar effect occurs with endogenous E_2/ERŬcomplex in the MCF-7:5C cell line that is resistant to estrogen withdrawal [83]. However, in contrast to the MCF-7:5C cells that progress to apoptosis, SKBr3 cells with ectopic ERŬdo not. It will be important to discover the reason for the failure to trigger apoptosis because the ectopic ERŬcould be used to define and identify a common pathway for future drug discovery. In other words, a proportion of cancers that never had the ER may have a vestigial pathway that could be activated to provoke apoptosis. The Tet-off adenoviral ERŬsystem may be an approach to discover the veracity of this drug discovery strategy.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2t (Lewis-Wambi and Jordan) - Studies carried out by Dr. Joan Lewis-Wambi in the Jordan laboratory at Fox Chase Cancer Center

Potential of l-buthionine sulfoximine to enhance the apoptotic action of estradiol to reverse acquired antihormonal resistance in metastatic breast cancer

Introduction

In the current study, we investigated the in vitro effect of the combination of BSO and estradiol (E2) on MCF-7:2A cell viability in relation to apoptosis. We found that BSO or E2, as individual treatments, did not significantly alter the viability of MCF-7:2A cells nor induced apoptosis. However, the combined treatment of BSO and E2 depleted glutathione content and induced significant apoptosis in MCF-7:2A cells. In contrast, similar experiments performed in wild-type hormone responsive MCF-7 cells showed no apoptosis or growth inhibition following the combination treatment of BSO and E2. Our data indicates that glutathione participates in retarding apoptosis in antihormone-resistant human breast cancer cells and that depletion of this molecule by BSO may be critical in predisposing resistant cells to E2-induced apoptotic cell death. We suggest that these data may form the basis of improving therapeutic strategies for the treatment of antihormone-resistant ER-positive breast cancer.

Work Accomplished:

Glutathione levels are elevated in estrogen deprived MCF-7:2A breast cancer cells.

Previous studies have shown that GSH levels in primary breast tumors are more than twice the levels found in normal breast tissue, and levels in lymph node metastases are more than four times the levels in normal breast tissue [291]. Recently, we reported the development of an estrogen deprived breast cancer cell line MCF-7:2A that is resistant to estrogen-induced apoptosis and expresses high levels of the glutathione synthetase gene GSS. To determine whether GSH levels were elevated in our apoptosis-resistant MCF-7:2A breast cancer cell line glutathione assays were performed on these cells. Fig. 2t1-A shows that MCF-7:2A cells had significantly higher levels of GSH at 24, 48, and 72 h (11.9ï 15.8 nmol/mg protein) compared to wild-type MCF-7 cells (7.8ï 7.6 nmol/mg protein) and this trend continued up to day 7 (data not show). We next examined whether the GSH synthesis inhibitor BSO was capable of suppressing GSH levels in these cells. Fig. 2t-114B shows that treatment with 100Mof BSO for 48 h suppressed GSH levels by ~55% in MCF-7 cells and by ~75% in MCF-7:2A cells. Longer treatment with BSO (>48 h) yielded similar levels of inhibition (data not shown). These results indicate a possible link between elevated GSH levels and resistance to estrogen-induced apoptosis and they suggest that suppression of GSH by BSO has the ability to reverse the resistant phenotype of the MCF-7:2A cells.

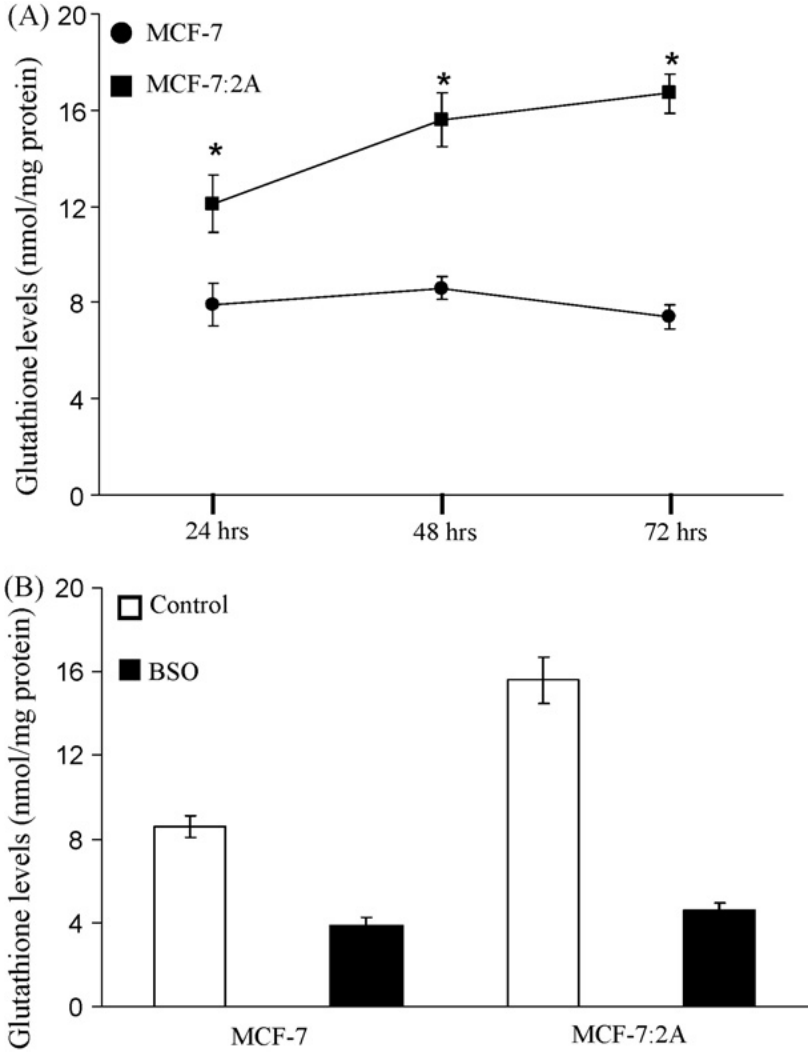


Figure 2t-1. *Intracellular glutathione levels in wild-type MCF-7 cells and antihormoneresistant MCF-7:2A breast cancer cells. (A) Cells were seeded at 2×106 cells per 100mm culture plates in estrogen-free media and total cellular glutathione was measured over a 72-h time period using a glutathione colorimetric assay kit, as described in Section 2. *P < .0001, with respect to MCF-7 cells. (B) BSO reduces glutathione levels in MCF-7 and MCF-7:2A cells. For experiment, cells were treated with 100µM BSO for 48 h and levels of glutathione. Bars±S.E.*

Glutathione suppression by BSO sensitizes antihormone-resistant MCF-7:2A cells to estrogen-induced apoptosis.

We next examined whether depletion of glutathione by BSO has the ability to sensitize MCF-7:2A cells to estrogen-induced apoptosis. Wild-type MCF-7 cells and estrogen deprivedMCF-7:2A cells were seeded in estrogen-free media, and after 24 h, were treated with nothing (control), 1 nM estradiol, or 10 ÕM to 10mM BSO in the presence or absence of 1 nM estradiol for 7 days. Fig.2t-2A shows that the growth of MCF-7 cells was stimulated 5-fold over the control cells by 1 nM estradiol during the course of the 7-day assay and that treatment with BSO, either alone or in combination with estradiol, did not significantly alter the growth of these cells except at very high concentrations (>1 mM). In contrast, MCF-7:2A cells treated with the combination of BSO and estradiol showed a significant concentration dependent decrease in cell growth relative to cells treated with estradiol or BSO alone (Fig. 2t-B). It is noteworthy that 100 ÕM BSO, as a single agent, did not cause growth

inhibition of MCF-7:2A cells. However, when combined with 1 nM estradiol the combination caused an 80ï 90% decrease in growth (Fig. 2t-2B). The cell killing effect of BSO and estradiol was observed as early as 48 h after treatment and persisted over the time course of the experiment with maximum cell death at the 7-day time point. The concentration of BSO used in this study is already known to be clinically achievable without significant side effects [292,293].

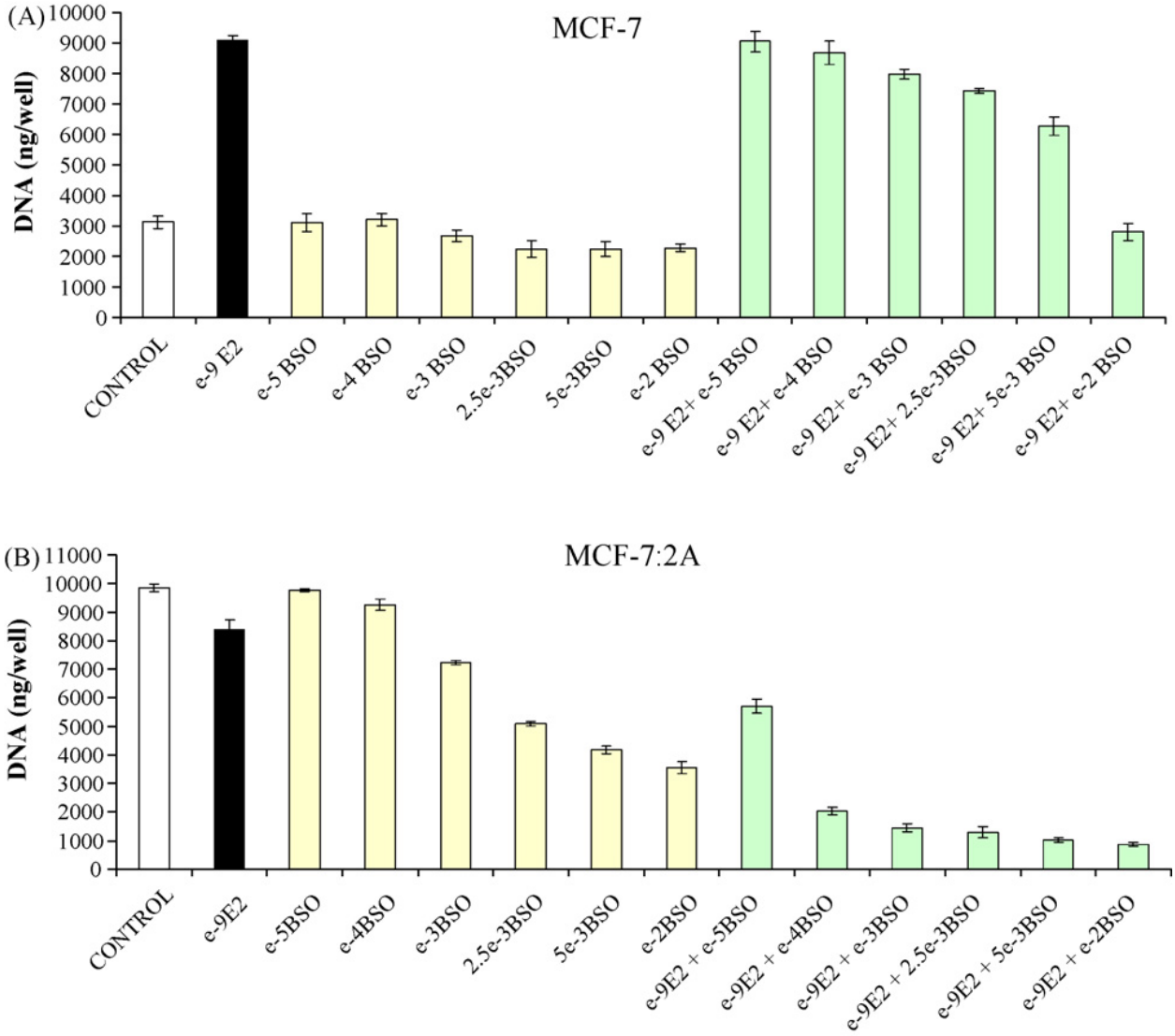


Figure 2t-2. *BSO enhances the growth inhibitory effect of estradiol in antihormone-resistant MCF-7:2A cells. (A) MCF-7 cells were grown in estrogen-free media for 3 days prior to the start of the growth assay. On the day of the experiment, cells were seeded in 24-well plates and after 24 h were treated with various concentrations (10 µM to 10mM) of BSO in the presence or absence of 1 nM (10^{-9} M) E2 for 7 days. At the indicated time points, cells were harvested and total DNA (ng/well) was quantitated as described in Section 2. (B) MCF-7:2A cells were treated similarly as described above. The data represents the mean of three independent experiments.*

Based on the above finding, we next determined whether MCF-7:2A cells underwent apoptotic cell death following BSO plus estradiol treatment. TUNEL assay was performed on cells treated with 100M BSO, 1 nM estradiol, or 100 ÕM BSO plus 1 nM estradiol for 72 h to detect

fragmentation of DNA, a characteristic of apoptotic cell death. Fig. 2t-3A shows that the percentage of TUNEL-positive cells significantly increased with the combination of BSO and estradiol but not with estradiol or BSO alone. After treatment with BSO and estradiol (72 h), as many as 53% of cells displayed TUNEL-positive staining, whereas, only 1% of the control cells and 5% of the estradiol treated cells were TUNEL-positive (Fig. 2t-3A). BSO treated cells looked similar to control cells. As expected, wild-type MCF-7 cells showed very little TUNEL-positive staining in the presence of estradiol alone or BSO plus estradiol combined (data not shown), thus indicating a lack of apoptosis in these cells. DAPI staining of MCF-7:2A cells treated with BSO and estradiol further confirmed that the cells were undergoing apoptosis (Fig. 2t-3B). In addition, phase contrast microscopy ofMCF-7:2A cells showed morphological changes associated with apoptosis following BSO and estradiol treatment (Fig. 2t-3C). Overall, these results indicate that BSO, as a single agent, causes neither growth inhibition nor cell death, but is capable of sensitizing antihormone-resistant MCF-7:2A cells to estradiol-induced apoptosis at clinically achievable concentrations.

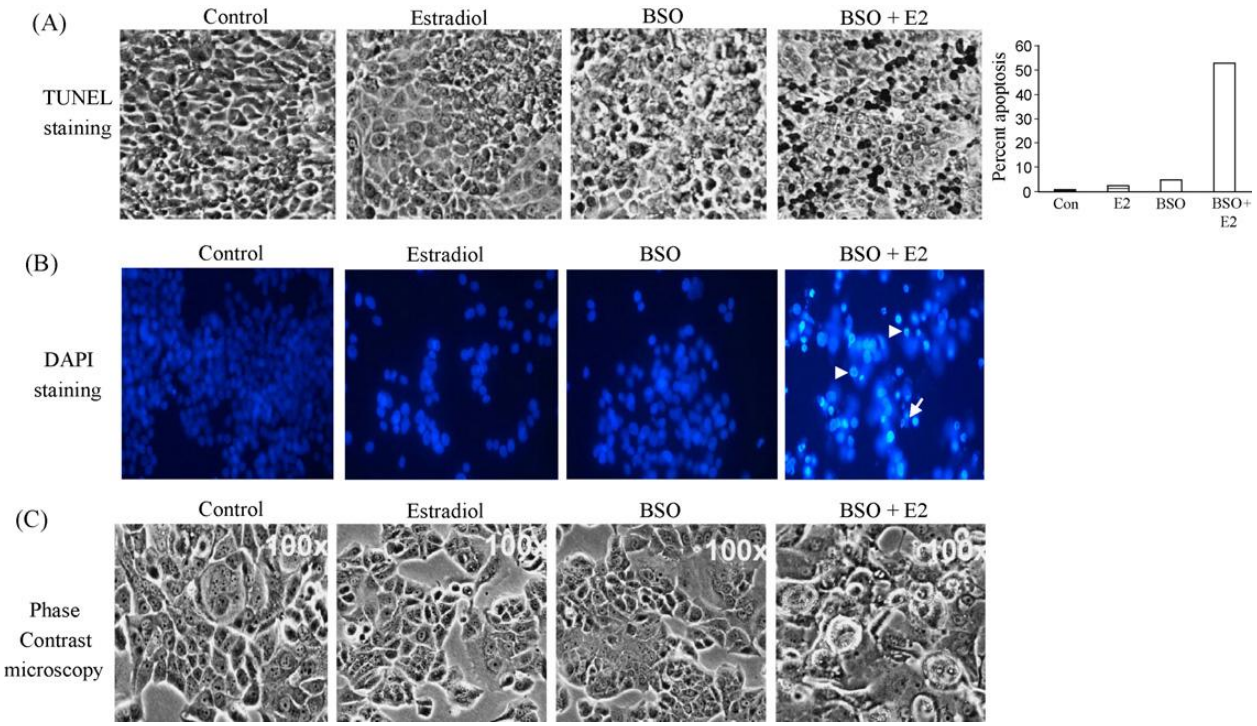


Figure 2t-3. *BSO enhances the apoptotic effect of estradiol in MCF-7:2A breast cancer cells. (A) Cells were treated with 1 nME2, 100MBSO, or 1 nME2 + 100 µM BSO for 72 h and TUNEL staining for apoptosis was performed as described in Section 2. Slides were photographed through bright field microscope under 100× magnification. TUNEL-positive cells were stained black (white arrows). Columns (right), mean percentage of apoptotic cells (annexin V-positive cells) from three independent experiments done in triplicate; bars, SEs. (B) Fluorescent microscopic analysis of apoptotic cells stained with 4,6-diamidino-2-phenylindole (DAPI). MCF-7:2A cells were treated with 1 nM E2, 100M BSO, or 1nME2+100M BSO as described above for 72 h. To assess the number of cells undergoing apoptosis, round and/or shrunken nuclei of DAPI-stained cells were counted (white arrows). At least 200 cells per slide were counted by two individuals to control for subjective variability. Experiments were repeated three times with similar results. Representative slides are shown. Scale bars = 50 µM. (C) Phase contrast microscopy of MCF-7:2A cells treated with 1 nM E2, 100 µM BSO, or 1 nM E2 + 100M BSO for 72 h.*

Discussion

In the current study, we investigated whether suppression of the antioxidant glutathione by BSO has the ability to sensitize antihormone-resistant MCF-7:2A breast cancer cells to estradiol induced apoptosis. Our results showed that glutathione levels were significantly elevated in antihormone-resistant MCF-7:2A breast cancer cells compared to wild-type MCF-7 cells and that the combination treatment of BSO and estradiol caused a dramatic increase in apoptosis whereas the individual treatments had no effect on growth. Noteworthy, the killing effect of BSO and estradiol occurred at clinically achievable concentrations and was observed as early as 48 h. These findings are consistent with previous studies which have shown that the cytotoxicity of a number of chemotherapeutic drugs, including melphalan [294], doxorubicin [295], and bleomycin [296], are significantly enhanced when glutathione is depleted by BSO.

Our laboratory has previously demonstrated that when estrogen receptor positive breast cancer cells are grown and maintained in LTED environments, they can ultimately develop enhanced responsiveness to greatly diminished levels of estrogen [83,132]. These pre-clinical animal models show that initially, estrogen receptor expressing tumors are stimulated by estrogen and respond appropriately to tamoxifen with tumor regression. However, with continued exposure to tamoxifen, the tumors become resistant and re-grow [132]. Additionally, treatment of these LTED tumors with post-menopausal levels of estrogen inhibits tumor growth as well as causes regression of established tamoxifen resistant tumors [83,84,93,132]. Mechanistic studies indicate that the apoptotic action of estrogen is due to its ability to either activate the fasR/FasL death receptor pathway [84,85] or to disrupt mitochondrial function through activation of the bcl-2 family proteins [83]. The paradoxical action of estrogen in these resistant cells is hypothesized to be due to increased sensitivity to estrogen due to adaptation to estrogen deprivation caused either by tamoxifen or an aromatase inhibitor [297]. It is believed that this ñestrogen hypersensitivityò helps to explain the effectiveness of high-dose estrogen in patients with extensive prior endocrine therapy [205].

Interestingly, our present findings indicate that the ability of estradiol to induce apoptosis in antihormone-resistant cells is influenced by the level of glutathione present in the cells. Glutathione levels were elevated ~1.4- to 1.6-fold in antihormone-resistant MCF-7:2A cells compared to wild-type MCF-7 cells and these cells failed to undergo apoptosis following 1 week of treatment with physiological concentrations of estradiol alone. In the presence of BSO, however, which depleted intracellular glutathione by ~60ï 70%, the combination treatment of BSO and estradiol caused a dramatic increase in apoptosis which was observed as early as 48 h with maximum induction observed at day 7. Previous studies have shown that glutathione is an important component of tumor drug resistance [293] and that depletion of intracellular glutathione by BSO significantly enhances the cytotoxicity of many cytotoxic agents, principally alkylating agents [292,298,299] and platinating compounds [300] but also irradiation [301] and anthracyclines [302]. The concentration of BSO used in our study was within the range of 10 ÕM to 1mM, which is similar to what has previously been reported in the literature. However, we did observe some toxicity at higher concentrations of BSO (>1mM) In wild-type MCF-7 and antihormone-resistant MCF-7:2A cells. It should be noted that BSO, at a clinically achievable concentration of 100 ÕM, was used for all of our combination experiments with estradiol since this concentration, as an individual treatment, did not significantly alter the growth of MCF-7:2A cells.

Glutathione, a sulfhydryl containing tripeptide, is involved in detoxifying cells from various toxins including chemotherapeutic agents [303,304]. Previous studies have demonstrated a strong correlation between elevated glutathione levels and increased resistance to chemotherapy in cancer cells [305]. This resistance was not limited to the particular chemotherapy agent used to induce

resistance, but was also evident when other chemotherapeutic agents were tested for cross-resistance [305]. Additionally, translational studies of in vitro cell lines derived from patients with chemorefractory disease were found to have elevated glutathione levels [306]. BSO inhibits ɔ-glutamylcysteine synthetase (ɔ-GCS), the rate limiting enzyme in the production of glutathione, thus depleting glutathione levels within the cell [307]. Both, GSH aswell as resultant increase in -GCS levels as a result of BSO treatment can be monitored peripherally in patients by analysis of peripheral mononuclear cells (PMNs) [308]. BSO also exhibits selectivity in that in vitro studies have demonstrated greater depletion of glutathione levels in tumor tissues than sampled normal tissues [303]. Based on its ability to target intracellular glutathione and reverse therapeutic resistance in refractory cancers, BSO is thought to be a potential antineoplastic agent and/or ñtherapeutic sensitizerò worthy of clinical evaluation.

Early phase clinical trials of BSO at doses resulting in both peripheral and tumor GSH depletion show that BSO can be safely administered to patients with refractory disease. BSO was administered intravenously twice daily either alone or together with chemotherapy to cancer patients whose disease who disease had progressed despite multiple lines of previous chemotherapy [308,309]. In these patients treated with escalating doses of BSO, nausea and vomiting amenable to anti-emetic therapy were the main toxicities. Bone marrow suppression correlating with extent of previous chemotherapy exposure was found to be the rate limiting toxicity in the combination studies. No other significant toxicities were noted. Intracellular glutathione levels measured in PMNs decreased in a linear manner with repeated doses of BSO to a maximum of approximately 10ï 40% of baseline values [308,309]. When tested in sequential tumor biopsies, glutathione was also found to be depleted to a variable extent in a similarly predictable pattern [309]. Additionally, BSO administration resulted in an initial rapid inhibition of -GCS activity followed by -GCS recovery during the intervening time between dosings. In fact, -GCS levels mirrored peripheral BSO concentrations in patients thus demonstrating targeted delivery of BSO. Clinically, responses to treatment, including complete responses, have been achieved [299,308,309].

In this present study, we demonstrated that glutathione depletion by BSO sensitized antihormone-resistant MCF-7:2A human breast cancer cells to estradiol-induced apoptosis in vitro. Taken together, it would be reasonable to incorporate this data into our working translational model for clinical evaluation (Fig. 2t-4). We therefore propose utilizing BSO together with estrogen in patients for a defined therapeutic course in patients with hormonally sensitive metastatic breast cancer whose disease has progressed on prior antihormonal therapies to significantly reduce their disease burden, while potentially reversing resistance to antihormonal therapies. This would then be followed by continuing treatment with an aromatase inhibitor for maintenance of additional clinical benefit for these patients (Fig.2t-4). Our future goal will be to address this hypothesis in the context of a clinical trial based on these new pre-clinical findings.

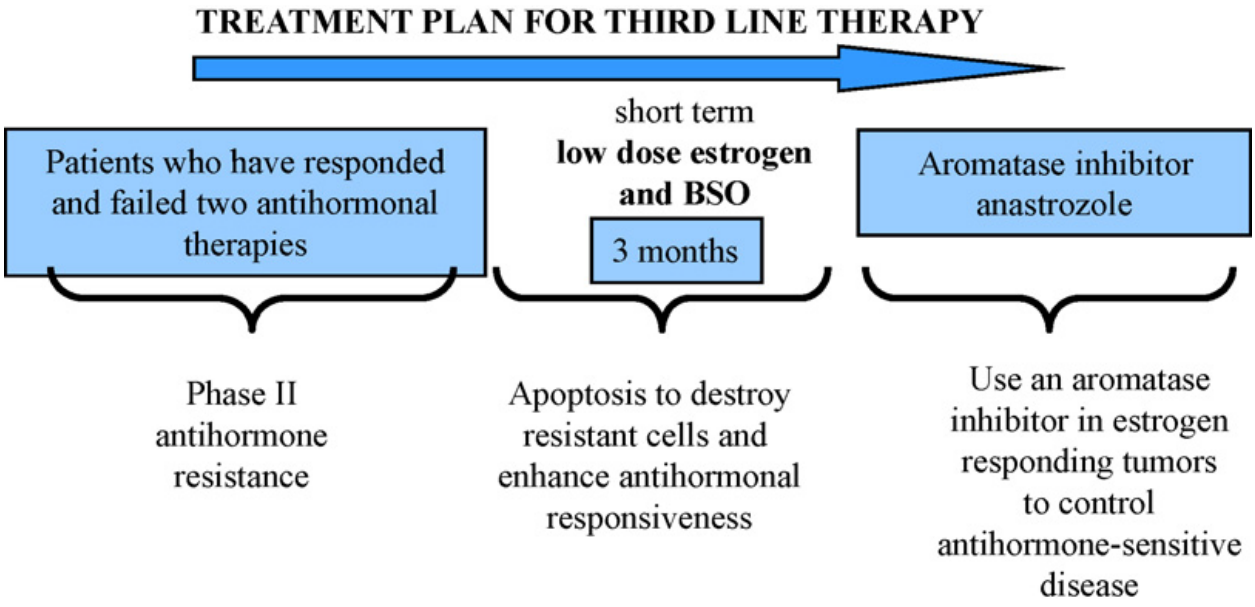


Figure 2t-4. *Clinical protocol to investigate the efficacy of estradiol plus BSO combination treatment to induce apoptosis in long-term endocrine refractory breast cancer. An anticipated treatment plan for third-line endocrine therapy. Patients must have responded and experience treatment failure with two successive antihormone therapies to be eligible for a course of low-dose estradiol combined with BSO therapy for 3 months. The anticipated response rate is 30% and responding patients will be treated with anastrozole until relapse. The overall goal is to increase response rates and maintain patients for longer on antihormone strategies before chemotherapy is required.*

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2u (Lewis-Wambi and Jordan) - Studies carried out by Dr. Lewis-Wambi in the Jordan laboratory at Fox Chase Cancer Center

Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis.

Introduction

L-Buthionine sulfoximine (BSO) is a specific ɔ-glutamylcysteine synthetase inhibitor that blocks the rate-limiting step of glutathionine (GSH) biosynthesis and in doing so depletes the intracellular GSH pool in both cultured cells and in whole animals [310]. Changes in GSH homeostasis have been implicated in the etiology and progression of a variety of human diseases, including breast cancer [311]. In particular, studies have shown that elevated levels of GSH prevent apoptotic cell death whereas depletion of GSH facilitates apoptosis [310,312]. BSO depletes cellular GSH [310] and sensitizes tumor cells to apoptosis induced by standard chemotherapeutic agents [300,313].

Based on microarray studies we found that the antihormone resistant MCF-7:2A cells express markedly elevated levels of glutathione synthetase (GS) and glutathione peroxidase 2 (GPx2); two enzymes that are involved in glutathione synthesis, which suggests that resistance to estrogen-induced apoptosis might be due to elevated levels of GSH present in the cells. If MCF-7:2A cells do indeed possess high levels of GSH, then it is possible that the use of BSO ï as a single agent ï might be able to sensitize these cells to estrogeninduced apoptosis. As mentioned before, there is current clinical interest in using low dose estradiol therapy to treat antihormone resistant breast cancer [314] however only a minimal 30% of patients respond to this therapeutic strategy. A combination of BSO and estradiol could possibly be used to improve the efficacy of estradiol as an apoptotic agent if glutathione depletion is fundamental to tumor cell survival. We have addressed the hypothesis that by altering glutathione levels we may be able to enhance apoptosis to estrogen and have employed BSO as our agent of choice because of earlier work clinically, which may provide a foundation for subsequent clinical trials.

In the present study, we show that depletion of cellular GSH by BSO sensitizes antihormone-resistant MCF-7:2A cells to estradiol-induced apoptosis that is mediated, in part, by the mitochondrial pathway and also activation of the c-Jun N-terminal kinase (JNK) signaling pathway. We further show that BSO, either alone or in combination with estradiol, causes tumor regression of MCF-7:2A cells in vivo.

Work Accomplished:

Estrogen deprivation increases glutathione levels in MCF-7:2A breast cancer cells.

Elevated glutathione levels and the activity of its related enzymes have been characterized as one of the factors which could render breast cancer cells resistant to apoptosis. We have previously shown that MCF-7:2A breast cancer cells are resistant to estrogen-induced apoptosis [99], therefore we measured glutathione levels in these cells along with parental MCF-7 cells. Figure 2u-1a showed

that glutathione levels were significantly higher in MCF-7:2A cells (11.9 ε M/mg protein) compared to MCF-7 cells (7.8 ε M/mg protein) and treatment with BSO (100 ε M), an inhibitor of glutathione synthesis, for 24 h depleted glutathione content by approximately 55% and 68% in MCF-7 and MCF-7:2A cells, respectively. It is worth noting that glutathione levels were consistently elevated in MCF-7:2A cells up to 7 days and the inhibitory effect of BSO persisted throughout that incubation period (Figure 2u-1a, insert).

We next examined whether the expression of glutathionerelated enzymes was altered in these cells. Using quantitative real-time PCR, we found a 6-fold increase in glutathione synthetase (GS) expression and a 40-fold increase in glutathione peroxidase 2 (GPx2) expressions in MCF-7:2A cells compared to parental MCF-7 cells (Figure 2u-1b). Western blot analysis also showed a marked increase in GS protein level in MCF-7:2A cells compared to parental MCF-7 cells (Figure 2u-1b, right panel).

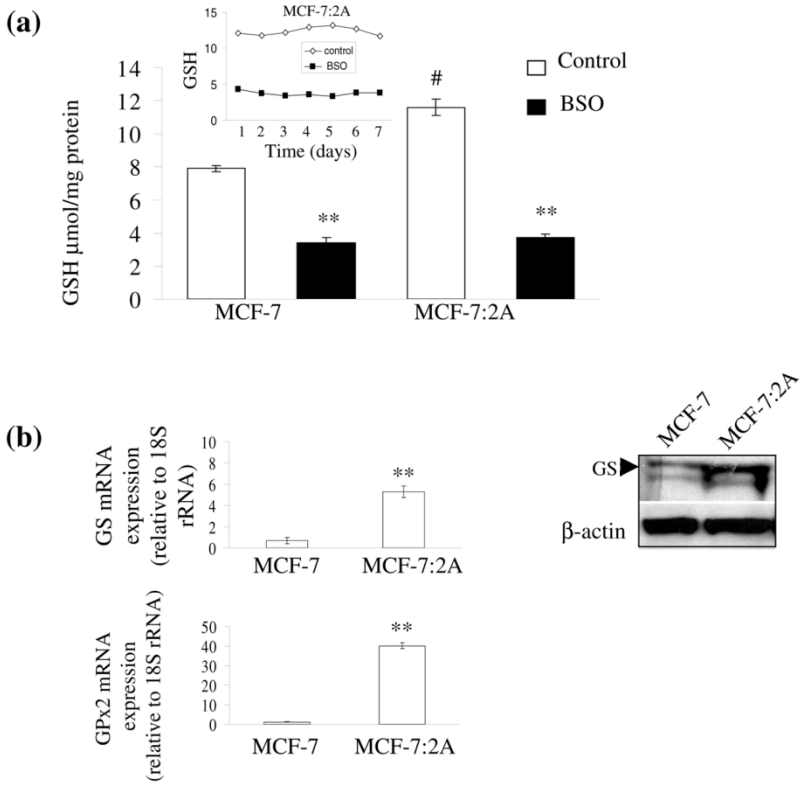


Figure 2u-1. *Intracellular glutathione (GSH) levels in wild-type MCF-7 cells and antihormone-resistant M MCF-7:2A breast cancer cells. (a) MCF-7 and MCF-7:2A cells were seeded at 2 × 106 cells per 100 mm culture plates in phenol red RPMI media containing 10% fetal bovine serum (FBS) and phenol red free RPMI media containing 10% 4× dextran coated charcoal-treated FBS (SFS), respectively, and after 24 h were treated with nothing (control) (white columns) or 100 µM buthionine sulfoximine (BSO) (black columns) for 24 h. Total cellular glutathione was measured using a Glutathione Colorimetric microplate assay kit, as described in Materials and methods. Columns, mean from three separate experiments; bars, ± standard error of the mean (SEM). **, p < 0.001 compared with control cells; #, p < 0.05 compared with MCF-7 control cells. Insert graph shows glutathione levels in MCF-7:2A cells over a 7-day period. (b) Quantitative real-time polymerase chain reaction (PCR) of glutathione sythetase (GS) (top left) and glutathione peroxidase 2 (GPx2) (bottom left) mRNA expression in MCF-7 and MCF-7:2A cells. **, p < 0.001 compared*

with MCF-7 control cells. Western blot analysis of GS protein expression in MCF-7:2A cells is also shown (top right).

BSO enhances the apoptotic effect of E2 in MCF-7:2A cells.

We next examined whether depletion of glutathione levels by BSO sensitizes MCF-7:2A cells to estrogen-induced apoptosis. For proliferation assays, MCF-7 and MCF-7:2A cells were seeded in estrogen-free media, and after 24 h, were treated with 100 ε M BSO, 1 nM E2, or 100 ε M BSO plus 1 nM estradiol for 2, 5, and 7 days. Figure 2u-2a shows that the growth of parental MCF-7 cells was stimulated sevenfold over the control cells by 1 nM estradiol during the course of the 7-day assay and that treatment with BSO, either alone or in combination with estradiol, did not significantly alter the growth of these cells. In contrast, MCF-7:2A cells treated with the combination of 100 ε M BSO and 1 nM estradiol showed a significant time-dependent decrease in cell growth relative to cells treated with either estradiol or BSO alone. The growth inhibitory effect of BSO and estradiol was observed as early as 48h after treatment and persisted over the time course of the experiment with maximum cell death at the 7-day time point. The combination of estradiol plus BSO also significantly reduced the proliferation of MCF-7:2A cells (Fig. 2u-2c, bottom) but it did not affect the growth of wild type MCF-7 cells (Figure 2u-2c, top). Furthermore, we found that treatment with the antiestrogen 4-hydroxytamoxifen (4-OHT) almost completely
reversed the growth inhibitory effect of estradiol and BSO in MCF-7:2A cells (see Additional data file 1) which suggests the involvement of the ER in this process.

Based on the above finding, we next determined whether MCF-7:2A cells underwent apoptotic cell death upon BSO and estradiol treatment. We performed a TUNEL assay, which detects the fragmentation of DNA, which is characteristic of cells undergoing apoptotic cell death. As shown in Figure 2u-2a, the percentage of TUNEL-positive cells significantly increased with the combination of BSO and estradiol but not with estradiol or BSO alone. After treatment with BSO and estradiol (96h), as many as 53% of cells displayed TUNEL-positive staining, whereas, only 1% of the control cells and 5% of the estradiol-treated cells were TUNEL-positive. BSO-treated cells looked similar to control cells. As expected, parental MCF-7 cells showed very little TUNEL-positive staining in the presence of estradiol alone or BSO plus estradiol combined (Figure 2u-2b, top panel), thus indicating a lack of apoptosis in these cells.

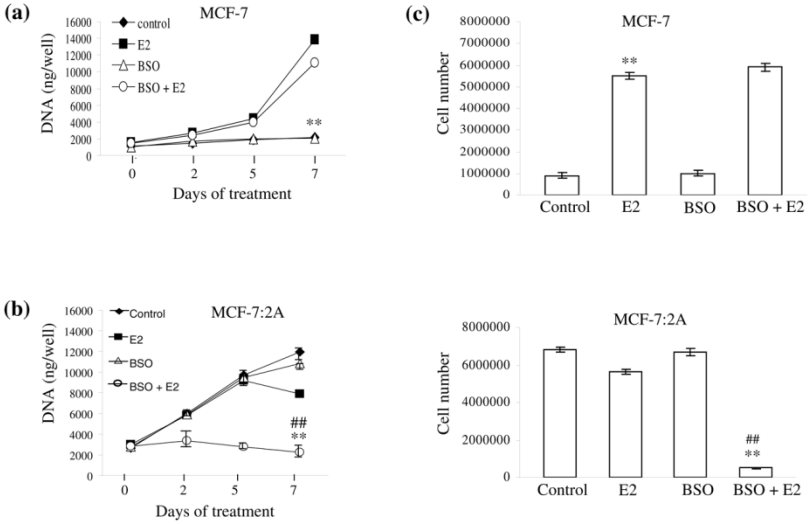


Figure 2u-2. *Effect of buthionine sulfoximine (BSO) plus estradiol on the growth of wild-type MCF-7 cells and antihormone-resistant MCF-7:2A cells. (a) MCF-7 cells were grown in estrogen-free*

*media for 3 days prior to the start of the growth assay. On the day of the experiment, 30,000 cells were seeded in 24-well plates and after 24 h were treated with < 0.1% ethanol vehicle (control), 1 nM 17β-estradiol (E2), 100 μM BSO, or 100 μM BSO plus 1 nM E2 for 7 days. At the indicated time points, cells were harvested and total DNA (ng/well) was quantitated as described in Materials and methods. The data represent the mean of three independent experiments; bars, ± standard error of the mean (SEM). **, p < 0.001 compared with control cells. (b) MCF-7:2A cells were seeded at the same density as MCF-7 cells and were treated similarly. The data represent the mean of three independent experiments; bars, ± SEM. **, p < 0.001 compared with control cells; ##, p < 0.001 compared with estradiol-treated cells. (c) The effect of BSO plus estradiol on cell proliferation was also determined by cell counting using a hemocytometer. For experiment, 0.5 × 106 MCF-7 (top) and MCF-7:2A (bottom) cells were seeded in 15-cm dishes and after 24 h were treated with 1 nM estradiol, 100 μM BSO, or E2 plus BSO combination for 7*
*days. Data shown represents the mean of three independent experiments; bars, ± SEM. **, p < 0.001 compared with control cells; ##, p < 0.001 compared with estradiol-treated cells.*

To further substantiate the apoptotic effect of BSO and estradiol in MCF-7:2A cells, annexin V-PI immunostaining was performed by flow cytometry. Figure 2u-3b shows that in the BSO plus estradiol-treated group, approximately 55.6% of cells stained positive for annexin V whereas in the control group and estradiol-treated group, approximately 7.4% and approximately 15.6%, respectively, of cells stained positive for annexin V. For the BSO-treated group, only 8.7% of cells stained positive for annexin.

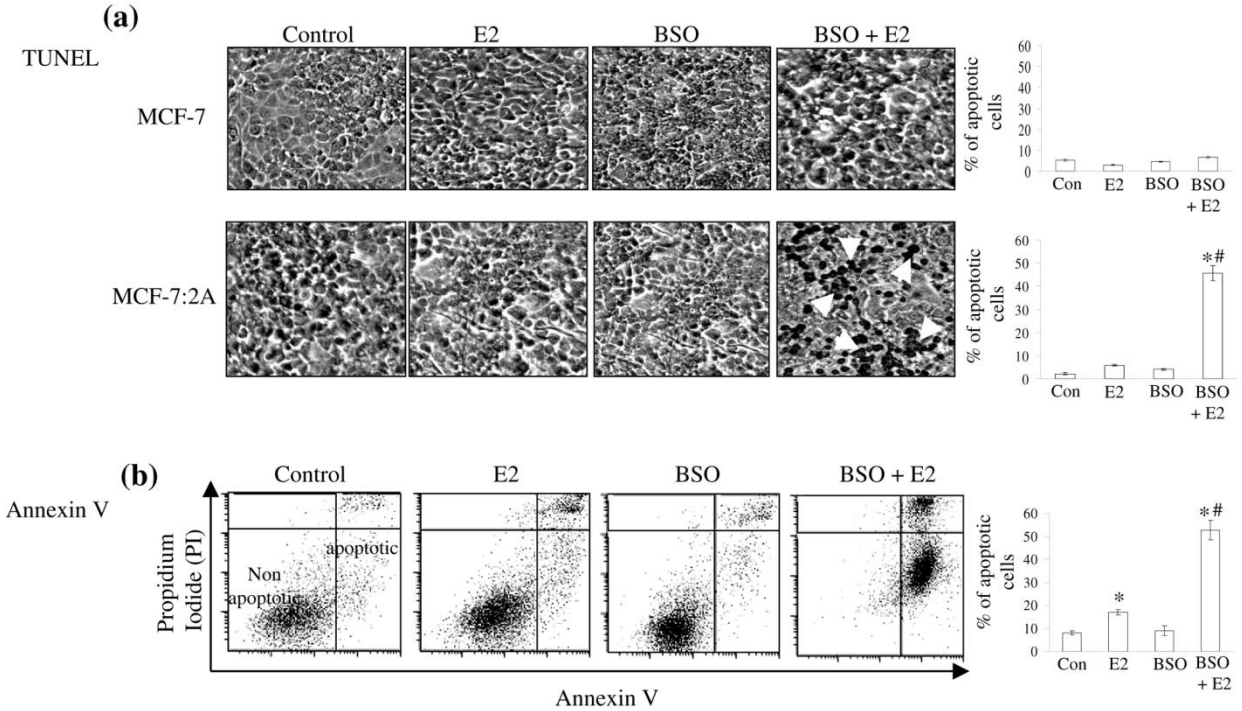


Figure 2u-3. *Buthionine sulfoximine (BSO) plus estradiol induce apoptosis in MCF-7-2A cells. (a) Terminal deoxynucleotidyl transferase-mediated dUTP nick endlabeling (TUNEL) staining for apoptosis in MCF-7:2A cells following BSO plus 17β-estradiol (E2) treatment for 96 h were performed as described in Materials and methods. Slides were photographed through a brightfield microscope under 100 × magnification. TUNEL-positive cells were stained black (white arrows). Columns (right), mean percentage of apoptotic cells (annexin V-positive cells) from three*

*independent experiments performed in triplicate; bars, ± standard error of the mean (SEM). *, p < 0.001 compared with control cells; #, p < 0.001 compared with estradiol-treated cells. (b) Annexin V staining for apoptosis. Cells were seeded in 100 mm plates at a density of 1 × 106 per plate and after 24 h were treated with ethanol vehicle (control), 1 nM E2, or BSO plus E2 for 72 h and then stained with fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (PI) and analyzed by flow cytometry. PI was used as a cell viability marker. Representative cytograms are shown for each group. Quantitation of apoptosis (percentage of control) in the different treatment groups is shown on the right. bars, ± SEM. *, p < 0.05 compared with control cells; #, p < 0.01 compared with estradiol-treated cells.*

Role of the mitochondrial pathway in BSO plus estradiol-induced apoptosis in MCF-7:2A cells.

To examine the role of the mitochondrial pathway in BSO plus estradiol-induced apoptosis, western blot analyses was used to measure Bax, Bcl-2, phosphorylated Bcl-2, and Bcl-xL protein levels in MCF-7:2A cells following treatment with 1 nM estradiol alone, 100 εM BSO, or BSO plus estradiol for 48 h. We found that Bcl-2, phospho-Bcl-2, and Bcl-xL protein levels were almost completely reduced in MCF-7:2A cells treated with BSO plus estradiol compared to control, BSO, or estradiol alone. In addition, a marked increase in Bax expression was also observed in MCF-7:2A cells following BSO plus estradiol combined treatment (Figure 2u-4a). In contrast, similar experiments performed with parental MCF-7 cells showed that BSO plus estradiol slightly increased Bcl-2 and phospho-Bcl-2 protein levels in these cells with a more dramatic effect observed with estradiol alone (Figure 2u-4a). It is worth noting that in MCF-7:2A cells endogenous levels of Bcl-2 and phosphorylated Bcl-2 were markedly elevated compared to parental MCF-7 cells. This finding is consistent with previous reports which show that overexpression of Bcl-2 increases glutathione levels and inhibits mitochondrial dysfunction and cell death elicited by glutathione-depleting reagents [315].

Although estradiol, as an individual treatment, did not significantly induce apoptosis in MCF-7:2A cells, it did decrease Bcl-2 protein level in these cells. We therefore tested whether siRNA knockdown of Bcl-2 expression would sensitize MCF-7:2A cells to estradiol-induced apoptosis. Expression of Bcl-2 following knockdown was analyzed by western blotting. As expected, Bcl-2 protein levels were significantly reduced following transfection of MCF-7:2A cells with Bcl-2 siRNA compared to control siRNA (Figure 2u-4b, top panel). Using annexin V staining, we found that apoptosis was increased by 20% in Bcl-2 siRNA transfected cells compared with cells transfected with the control siRNA (Figure 2u-4b, bottom panel), thus suggesting that suppression of antiapoptotic factors such as Bcl-2 has the ability to partially sensitize hormone-independent MCF-7:2A cells to apoptosis.

We next examined mitochondrial membrane integrity using the Rh123 retention assay. Cells were treated with nothing (control), estradiol, BSO, or BSO plus estradiol for 48 h. Figure 2u-3c shows that BSO plus estradiol treatment reduced Rh123 fluorescence in MCF-7:2A cells by approximately 50% compared to control, whereas, estradiol or BSO, as individual treatments, did not significantly alter Rh123 retention levels in these cells. BSO plus estradiol also enhanced cytochrome c release in MCF-7:2A cells. Figure 2u-4d shows that in the control cells, cytochrome c was detected primarily in the mitochondria and was undetectable in the cytosol; however, in the presence of BSO plus estradiol (48 h), all of cytochrome c was observed in the cytosol. BSO or estradiol, as individual treatments, did not significantly alter mitochondrial release of cytochrome c. The translocation of cytochrome c from the mitochondria to the cytosol following BSO plus estradiol treatment coincided with cleavage of caspase 7 and PARP (Figure 2u-3e), which is a

molecular signature of apoptosis. Cleavage of PARP and caspase 7 was blocked by the pan-caspase inhibitor z-VAD (data not shown).

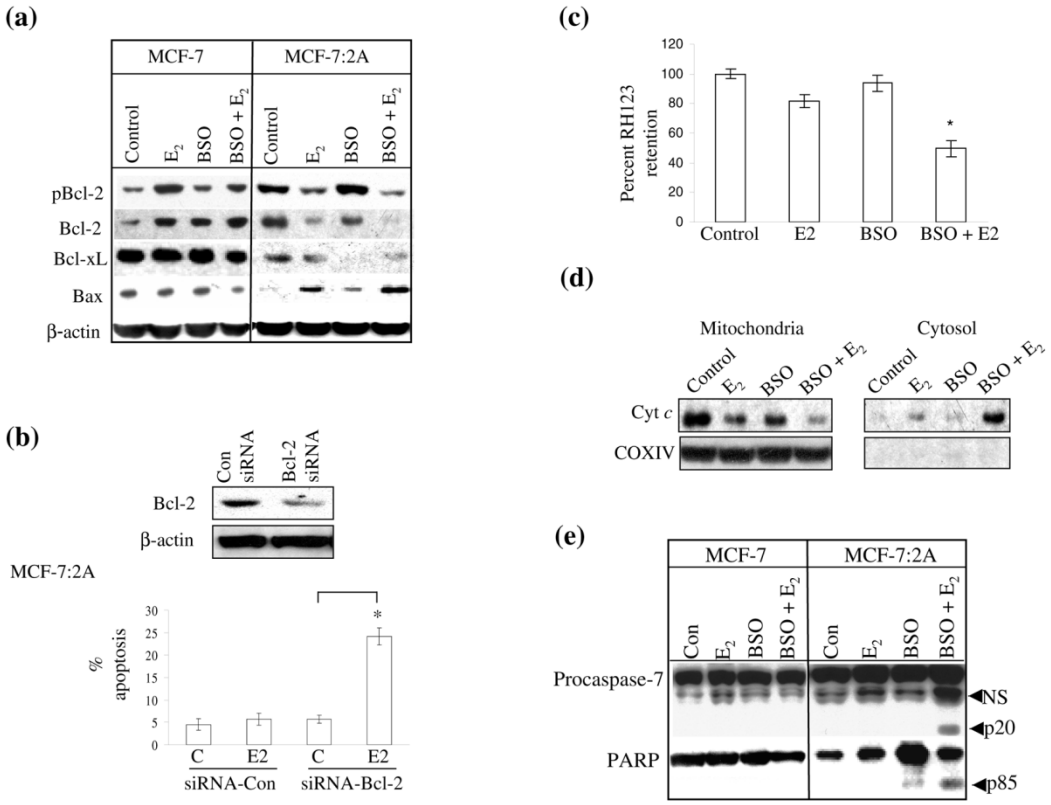


Figure 2u-4. *Effects of buthionine sulfoximine (BSO) and 17β-estradiol (Bcl-2 family protein expression and mitochondrial function in MCF-7 and MCF-7:2A cells. (a) Western blot analysis for pBcl-2, Bcl-2, Bcl-xL, and Bax protein expression in parental MCF-7 cells and MCF-7:2A cells following 48 h of treatment with ethanol vehicle (Control), 1 nM E2, 100 μM BSO, or E2 + BSO. Equal loading was confirmed by reprobing with an antibody against β-actin. (b) Small interfering RNA (siRNA) knockdown of Bcl-2 partially sensitizes MCF-7:2A cells to E2-induced apoptosis. Cells were transfected with 100 nM siRNA-Bcl-2 or siRNA-Con (control) and expression levels of Bcl-2 was determined by immunoblot analysis (top). Annexin V staining (bottom) showing the effects of siRNA-con and siRNA-Bcl-2 on apoptosis induced by estradiol treatment in MCF-7:2A cells. *, p < 0.001. (c) Loss of mitochondrial potential in MCF-7:2A cells was determined by rhodamine 123 (Rh123) retention assay. The percentage of cells retaining Rh123 in each treatment group was compared with untreated control. (d) Cytochrome c release from the mitochondria to the cytosol after treatment with E2 alone or BSO and E2 for 48 h was determined as described in Materials and methods. Anti-Cox IV antibody was used as a control to demonstrate that mitochondrial protein fractionation was successfully achieved. (e) Cleavage of caspase 7 and poly(ADP-ribose) polymerase (PARP) (72 h) was assessed by western blot using specific antibodies. The upper band of caspase 7 represents the full-length protein and the lower band (p20, arrow) represents the cleaved activated product; NS, nonspecific. Full length PARP is approximately 116 kDa; cleaved (active) PARP is 85 kDa (arrow). The results are representative of three independent experiments.*

The apoptotic effect of BSO and estradiol in MCF-7:2A cells is regulated, in part, by JNK signaling.

Emerging evidence supports a role for JNK in stress-induced mitochondrial apoptotic pathways in a variety of cell systems [316]. Therefore, we examined the possible involvement of c-Jun/JNK pathway in BSO plus estradiol-induced apoptosis in MCF-7:2A cells. JNK activation was determined by western blot analysis after 48-h treatment of cells with BSO plus estradiol. A profound induction of the p54 and p46 isoforms of phosphorylated JNK as well as a significant increase in phospho-c-Jun and c-Jun were observed in MCF-7:2A cells treated with BSO plus estradiol compared to BSO alone or control (Figure 10a). Interestingly, treatment with estradiol alone also significantly increased phosphorylated JNK in MCF-7:2A cells. We also found that pretreatment of MCF-7:2A cells with the JNK inhibitor, SP600125 (20 εM) markedly reduced the apoptotic effect of BSO plus estradiol in these cells (Figure 2u-5b). Overall, these results suggest a possible involvement of the c-Jun/JNK signaling pathway in BSO plus estradiol-induced apoptosis in MCF-7:2A cells.

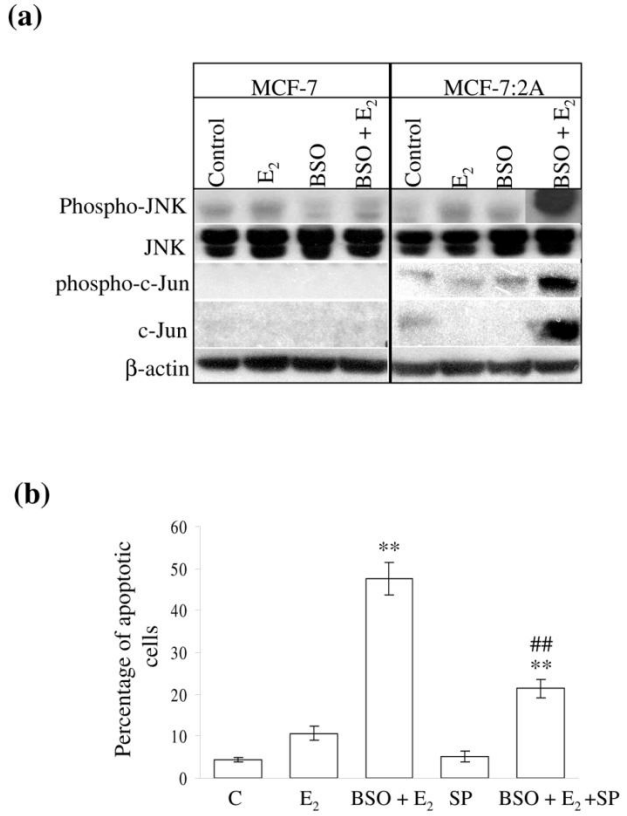


Figure 2u-5. *Activation of c-Jun N-terminal kinase (JNK) signaling pathway in MCF-7:2A cells in response to buthionine sulfoximine (BSO) and 17β-estradiol (E2) treatment. (a) MCF-7 and MCF-7:2A cells were treated with ethanol vehicle (control), 1 nM E2 or 100 μM BSO plus E2 for 48 h and protein levels of phosphorylated JNK, JNK, phosphorylated c-Jun, and c-Jun were analyzed by western blotting. β-Actin was used as a control. (b) Inhibition of JNK activation by SP600125 (SP) partially reverses the apoptotic effect of BSO and estradiol in MCF-7:2A cells. Cells were pretreated with 20 μM SP600125 or vehicle for 24 h, then further incubated for 48 h with 1 nM E2, E2 + 100 μM BSO, 20 μM SP, or E2 + BSO + SP and apoptosis was determined by annexin V-propidium iodide (PI) staining as described in Materials and methods. Columns, mean percentage of apoptotic cells from three independent experiments performed in triplicate; bars, ± standard error of*

*the mean (SEM). **, p < 0.001 compared with control (C) cells; ##, p < 0.01 compared with E2 plus BSO-treated cells.*

BSO inhibits the growth of MCF-7:2A cells in vivo.

To determine whether the effect of BSO plus estradiol was relevant in vivo, we used a xenograft model in which MCF-7:2A cells were injected into CrTac:NCr-Foxn1nu athymic mice (n =20). At 20 days post injection, tumors grew to a mean crosssectional area of 0.30 cm2 and mice were randomized to four groups; placebo (saline), estradiol, BSO, or the combination of BSO plus estradiol, as described in materials and methods. After 7 days of treatment, tumor growth was reduced by 25% in mice treated with estradiol alone whereas in the BSO and BSO plus estradiol group tumor growth was reduced by 40% and 60%, respectively, compared to the placebo group which showed a 7% increase in growth (Figure 2u-6a). Interestingly, we found that BSO in vitro had a relatively small effect on growth, however, in vivo its effect was very pronounced, thus suggesting the possibility of altered glutathione metabolism in vivo. We performed histology on tumors taken from placebo, estradiol, BSO, or BSO plus estradiol groups at day 27. H&E staining of the BSO plus estradiol-treated tumors revealed less tumor cells and more intercellular matrix, significantly less mitoses, chromatin clumping and dark staining which are associated with apoptosis, and enhanced abnormalities in shape and size, compared to tumors from placebo or BSO or estradiol-treated groups. We also characterized the proliferative status of these cells by staining tumors for the expression of Ki67, a marker of cell proliferation. We observed a 32% decrease ($p < 0.001$) in the number of Ki67 stained tumors from the BSO plus estradiol-treated group and a 21% decrease in the BSO-treated group compared to the placebo group whereas estradiol treatment caused an 8% increase in Ki67 staining (Figure 2u-6). Immunohistochemistry of paraffin-embedded tumor sections of mice treated with the combination of BSO and estradiol showed increased immunostaining for proteolytically cleaved PARP (marker for apoptosis) compared to control, estradiol, or BSO-treated groups (Figure 2u-6d). Overall, these data show that BSO either alone or in combination with estradiol, reduces tumor growth by inhibiting proliferation and increasing apoptosis.

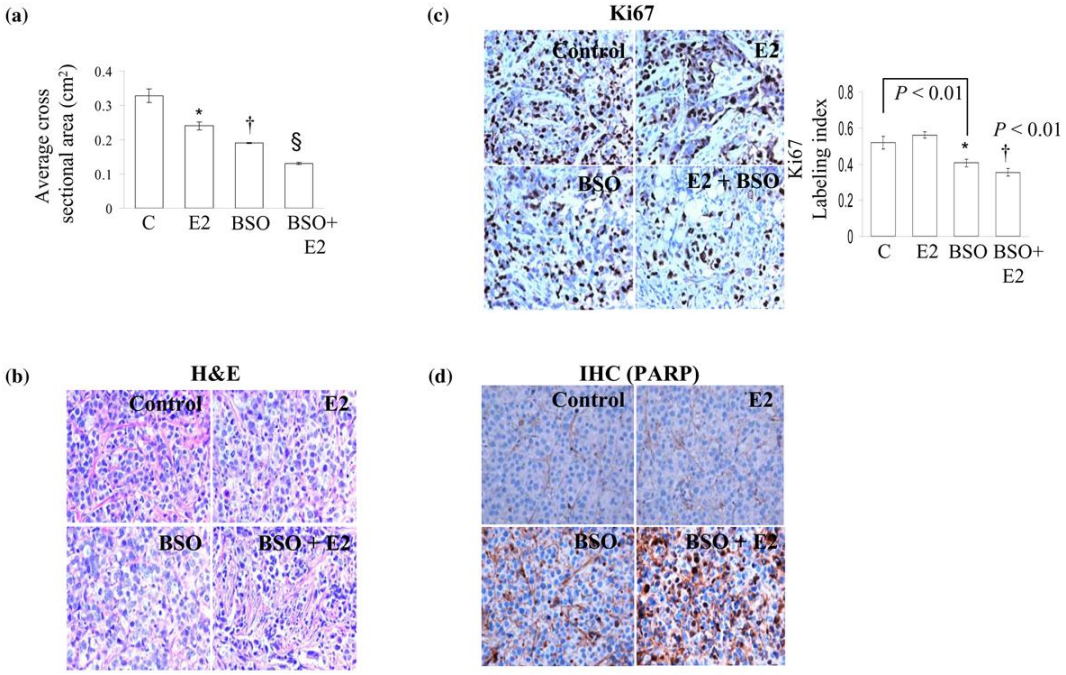


Figure 2u-6. *Buthionine sulfoximine (BSO) inhibits the growth of MCF-7:2A tumors in vivo. Athymic nude mice (4 to 5 weeks old, n = 20) were injected with MCF-7:2A breast cancer cells and after 20 days when tumors had reached a mean cross-sectional area of 0.3 cm2, animals were randomized into 4 groups and were treated with placebo (saline), 17β-estradiol (E2), BSO, or BSO plus E2 for 7 days as described in Materials and methods. BSO (4 mmol/kg weight) was diluted in saline and was injected intraperitoneally daily. (a) Tumor size was measured every day and cross-sectional area was calculated by multiplying the length (l) by the width (w) by π and dividing the product by 4 (lwπ/4). Data is shown as mean ± standard error of the mean (SEM). *, $p < 0.05$, control group compared with the E2 group; †, $p < 0.002$ control group compared with BSO group; § $p < 0.001$ control group compared with BSO + E2 group. (b) Microscopy of hematoxylin and eosin (H&E)-stained histological sections of MCF-7:2A tumors treated with placebo, E2, BSO, or BSO plus E2. (c) Immunohistochemical analysis of the proliferation marker Ki-67 in MCF-7:2A tumors treated with placebo, E2, BSO, or BSO plus E2. (d) Paraffin-embedded tumor sections of mice treated with E2, BSO, or BSO plus E2 were immunostained for proteolytically cleaved poly(ADP-ribose) polymerase (PARP), which exists only when cells undergo apoptosis. Three to four tumors per treatment group were analyzed.*

Discussion

In the current study, we investigated whether suppression of the antioxidant glutathione by BSO has the ability to sensitize antihormone resistant MCF-7:2A breast cancer cells to estradiol-induced apoptosis. Our results showed that glutathione levels and the enzymes involved in its synthesis, glutathione synthetase and glutathione peroxidase, were significantly elevated in MCF-7:2A cells compared to parental MCF-7 cells and that suppression of glutathione by BSO sensitized these cells to estrogen-induced apoptosis in vitro and in vivo. The BSO-mediated estradiol-induced apoptosis was associated with a marked decrease in the expression of antiapoptotic Bcl-2 and Bcl-xL proteins and a significant increase in proapoptotic Bax protein. It is worth noting that high-dose estrogen was generally considered the endocrine therapy of choice for postmenopausal women with breast cancer prior to the introduction of tamoxifen, however, due to undesirable side effects, the use of high-dose estrogen was largely abandoned [74]. Here, we show that the killing effect of estradiol in antihormone resistant cells can be achieved at physiological concentrations when it is combined with non-toxic concentrations of BSO. Our present findings are consistent with previous studies which have shown that the cytotoxicity of a number of chemotherapeutic drugs, including melphalan [294], doxorubicin [295], and bleomycin [296], are significantly enhanced when glutathione is depleted by BSO.

An important target of BSO plus estradiol-induced apoptosis appears to be Bcl-2, whose protein expression was dramatically decreased in MCF-7:2A cells following glutathione depletion. Previous studies have shown that Bcl-2 functions as an antioxidant to block apoptosis and that Bcl-2 protein levels and glutathione intracellular concentration is coordinately regulated with a decrease in either favoring cell death [317,318]. It is believed that one mechanism by which Bcl-2 may function as an antioxidant is through upregulation of glutathione, leading to rapid detoxification of reactive oxygen species and inhibition of free radical-mediated mitochondrial damage. Bcl-2 also has the ability to shift the entire cellular redox potential to a more reduced state, which is independent of its effect on glutathione levels [318]. It is worth noting that glutathione levels and Bcl-2 protein expression were significantly elevated in MCF-7:2A cells compared to parental MCF-7 cells. In phase I trials [299,309], the concentration of BSO in blood has been shown to reach 0.5 to 1 mM, whereas, in mice [319,320] the concentration has been estimated to be 5 to 6 mM following an in

vivo treatment of 4 mmol/kg. In our study, we showed that 100 εM BSO decreased glutathione concentrations by approximately 60% after 24 h and that BSO enhanced the apoptotic effect of estradiol in MCF-7:2A breast cancer cells as early as 48 h after treatment. Interestingly, treatment with BSO alone did not cause apoptosis in MCF-7:2A cells, indicating that glutathione depletion alone may not trigger apoptosis in these cells. This finding is consistent with previous studies by Mirkovic et al. [321] which showed that inhibition of glutathione by BSO did not increase susceptibility of mouse lymphoma cells to radiation-induced apoptosis even under conditions where glutathione levels were lowered by 50%. Other groups have made similar observations using BSO [322]. One possible explanation for this apparent contradiction might be the fact that BSO does not lower glutathione levels in mitochondria as effectively as it does in the cytoplasm [323]. Mitochondrial glutathione concentrations are regulated and have been implicated in apoptotic cell death [324], hence, it would be of interest to evaluate relative glutathione concentrations in the mitochondrial matrix of MCF-7:2A cells following treatment with BSO either alone or in combination with estradiol. Another possibility could be that cellular thiols other than glutathione may play important roles in regulating apoptosis [322]. The flavoprotein thioredoxin has been shown to be upregulated in several human tumors and is implicated in both cancer cell growth and apoptotic resistance [325]. However, it is not known whether Bcl-2 or other apoptotic regulators can influence the levels of thioredoxin or whether such modulation may contribute to resistance in human tumor cells. Apart from Bcl-2, we also found that proapoptotic Bax protein was markedly increased in MCF-7:2A cells by the combination of BSO plus estradiol and this induction coincided with a loss of mitochondrial membrane integrity and cytochrome c release. Bax is normally found as a monomer in the cytosol of non-apoptotic cells and it oligomerizes and translocates to the outer mitochondrial membrane in response to apoptotic stimuli and induces mitochondrial membrane permeabilization and cytochrome c release [326]. In MCF-7:2A cells, Bax protein was induced as early as 24 h after BSO plus estradiol treatment (Figure 2u-4) and suppression of Bax expression using siRNA was able to partially reverse the apoptotic effect of the combination treatment (data not shown). The induction of Bax coincided with cytochrome c release from the mitochondria into the cytosol, which was followed by activation of caspase 7, and PARP cleavage. It is worth noting that pretreatment of cells with the universal caspase inhibitor z-VAD almost completely blocked the apoptotic effect of BSO plus estradiol. It is also worth noting that antiapoptotic Bcl-2 and Bcl-xL proteins were also markedly decreased in MCF-7:2A cells following the combination treatment of BSO plus estradiol (Figure 2u-4) and overexpression of Bcl-xL partially blocked the apoptotic effect of BSO plus estradiol (data not shown). This finding is important because there is evidence that suggests that the ratio rather than the amount of antiapoptotic vs proapoptotic proteins determines whether apoptosis will proceed [327]. Thus, it is reasonable to suggest that the apoptotic effect of BSO plus estradiol is mediated, in part, by the mitochondrial pathway through their ability to alter the ratio between proapoptotic and antiapoptotic proteins in target cells.

In addition to the mitochondrial pathway, BSO plus estradiol appears to induce apoptosis, in part, through activation of the JNK signaling pathway. JNKs are a group of mitogen-activated protein kinases (MAPKs) that bind the N-terminal activation domain of the transcription factor c-Jun and phosphorylate c-Jun on amino acid residues Ser63 and Ser73 [328]. JNKs are stimulated by multiple factors including cytokines, DNA-damaging agents, and environmental stresses and are important in controlling programmed cell death or apoptosis. The inhibition of JNKs has been shown to enhance chemotherapy-induced inhibition of tumor cell growth, suggesting that JNKs may provide a molecular target for the treatment of cancer[328]. We found that JNK activation (as measured by the increased levels of phospho-JNK1/2 and the JNK substrate phospho-c-Jun) correlated well with BSO plus estradiol-induced apoptosis in MCF-7:2A cells and pharmacologic

disruption of this pathway using the JNK inhibitor SP600125 significantly attenuated this effect. Previously, Chen and coworkers [329] reported that BSO enhanced the apoptotic effect of arsenic (As_2O_3) in leukemia and lymphoma cells through activation of JNK and upregulation of death receptor (DR)5 and that inhibition of JNK by SP600125 decreased DR5 upregulation and apoptotic induction in U937 leukemia cells treated with arsenic plus BSO. While the exact mechanism by which JNK promotes apoptosis is not currently known, the phosphorylation of transcription factors such as c-Jun and p53, as well as pro- and antiapoptotic Bcl-2 family members [330] has been suggested to be of importance. It is worth noting that treatment with BSO plus estradiol markedly increased phosphorylated c-Jun in MCF-7:2A cells and decreased phosphorylated Bcl-2 in these cells. These findings thus suggest that BSO plus estradiol might mediate their apoptotic effect, in part, through activation of JNK.

Discussion

We have demonstrated that glutathione depletion by BSO sensitizes hormone-resistant MCF-7:2A human breast cancer cells to estradiol-induced apoptosis in vitro and in vivo. This finding has important clinical implications; particularly for the use of estrogen deprivation as long-term therapy, and it suggest that, if and when resistance develops, a strategy of treatment with estrogen combined with BSO may be effective in sensitizing resistant cells to apoptosis. It is worth noting that recently, Lonning and coworkers [205] reported a 33% complete response (that is, stable disease) with high dose diethylstilbestrol (DES) in postmenopausal patients with advanced breast cancer who were heavily pretreated with endocrine agents. However, 67% of the patients showed partial or no response [205] so the key to future clinical progress in the treatment of antihormone resistant breast cancer is to improve current treatment strategies. We are currently evaluating the optimal dose of daily estradiol therapy to reverse antihormonal resistance [45] but the goal is to enhance the estradiol-induced apoptotic response. The present findings suggest that BSO is indeed capable of enhancing the apoptotic effect of estradiol in antihormone resistant breast cancer cells. It is worth noting that a phase I study of BSO administered with the anticancer drug melphalan showed that continuous-infusion of BSO was relatively nontoxic and resulted in depletion of tumor glutathione [299,308]. Thus it is possible that future clinical studies of BSO infusions combined with low dose estrogen hold the promise of improving disease control for patients with antihormone resistant ER-positive metastatic breast cancer.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2v (Lewis-Wambi and Jordan) - Studies carried out by Dr. Lewis-Wambi in the Jordan laboratory at Fox Chase Cancer Center

Overexpression of CEACAM6 promotes migration and invasion of estrogen-deprived cancer cells.

Introduction

Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is a glycosylphosphatidylinositol-anchored cell surface protein that functions as a homotypic intercellular adhesion molecule [331]. It is overexpressed in a number of human malignancies including pancreatic cancer, gastrointestinal cancer and breast cancers [332,333], and increased levels of CEACAM6 are inversely correlated to the differentiation state of cancer cells. Previous studies have shown that CEACAM6 is overexpressed in pancreatic adenocarcinoma cells, and its overexpression is associated with greater in vivo metastatic ability and increased invasiveness and migration [334,335].

In this study, we investigated the role of CEACAM6 in cellular migration and invasion of breast cancer cells that have acquired resistance to estrogen deprivation. We found that CEACAM6 was significantly overexpressed in estrogen-deprived MCF-7:5C and MCF-7:2A breast cancer cells and that these cells were markedly more migratory and invasive than parental MCF-7 cells. Suppression of CEACAM6 expression by small interfering RNA (siRNA) completely reversed the invasive phenotype of MCF-7:5C and MCF-7:2A cells. E-cadherin and b-catenin were also significantly reduced in these cells. The mechanism of action of CEACAM6 appears to involve, in part, the c-Src and Akt signaling pathways.

Work Accomplished:

Characterisation of long-term estrogen-deprived breast cancer cells

The growth of estrogen-deprived MCF-7:5C and MCF-7:2A cells is compared to parental MCF-7 cells in Fig. 2v-1A. Both MCF-7:5C and MCF-7:2A cells grew robustly in the absence of estrogen whereas MCF-7 cells grew minimally without estrogen. The doubling times were 2.7, 3.4, and 6 d for MCF-7:5C, MCF-7:2A and MCF-7 cells, respectively. We also examined cell morphology changes associated with resistance to long term estrogen deprivation using phase-contrast microscopy. Fig. 2v-1B shows that MCF-7 cells grew as a uniform monolayer of tightly associated cells with limited cell spreading but distinct cellular boundaries, whereas estrogen-deprived MCF-7:5C and MCF-7:2A cells grew in a less uniform monolayer with cellular boundaries that were obscured. ERa mRNA and protein expression were also significantly increased in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells and treatment with estradiol or the pure anti-estrogen fulvestrant significantly down-regulated its expression (Fig. 2v-1C and D) in all three cell lines. Overall, these results show that estrogen deprivation increases ERa expression and alters the morphology of MCF-7:5C and MCF-7:2A cells.

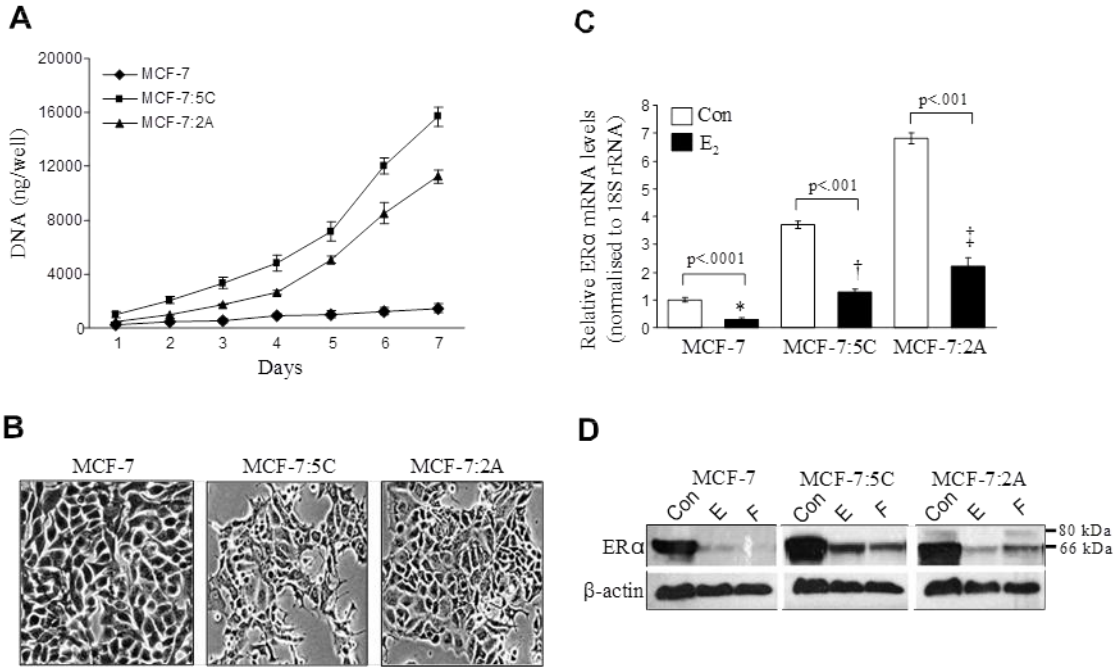


Fig. 2v-1 – *Characterisation of long-term estrogen-deprived breast cancer cells. (A) For proliferation assays, cellswere seeded in 24-well dishes (30,000 per well) in estrogen-free RPMI media and total DNAwas quantitated at the indicated time points. (B) Phase-contrast microscopy pictures of MCF-7, MCF-7:5C and MCF-7:2A cells. Images were produced by the Olympus DP-3030 camera and Olympus IX-70 software. Magnification, ·10. (C) ERa mRNA level was determined by quantitative RT-PCR. Relative expression of the target gene was calculated using the 2 delta CT method, where 18S rRNA was used as the endogenous control gene. All reactions were performed in triplicates and the error bar represents the standard deviation. (D) ERa protein levels were determined by immunoblotting with a specific ERa antibody. Cells were treated with 1 nM estradiol or 1 μM fulvestrant for 48 h and 50 lg of protein lysates was analysed. β-Actin was used as a loading control.*

Global gene expression profiles of estrogen-deprived breast cancer cells.

Transcriptional profiling of parental MCF-7 cells and estrogen-deprived MCF-7:5C and MCF- :2A cells was performed using Affymetrix Human Genome U133 Plus 2.0 Array. Twodimensional hierarchical clustering was performed to analyse differences in gene expression patterns between MCF-7 cells and MCF-7:5C and MCF-7:2A cells. Data filtering identified 904 genes that were significantly altered between MCF-7:5C and MCF-7:2A cells and parental MCF-7 cells (Fig. 2v-2A). The sample dendogram showed that MCF-7:2A cells and MCF-7 cells clustered more closely, whereas MCF-7:5C cells clustered on a more distant branch, suggesting that MCF-7:2A cells are more similar to parental MCF-7 cells than MCF-7:5C cells (Fig. 2v-2A). In order to define cell signaling mechanisms that differed significantly between parental MCF-7 and MCF-7:5C and MCF-7:2A cells, random permutation weighted gene analysis was performed as described in Section 2. A comparison of MCF-7 expression data with that of MCF-7:5C and MCF-7:2A revealed that 4068 genes were highly differentially expressed (Supplementary Table 1). Gene Ontology analysis showed a significant number of genes associated with cell cycle control, proliferation,

growth factor signaling, cell adhesion and motility and invasion. In particular, we found that CEACAM6 was overexpressed
by 27-fold in MCF-7:5C cells and 12-fold in MCF-7:2A cells (Fig. 2v-2B), and it was highly weighted in our random permutation analysis (p-value < .0001) (Supplementary Table 1).

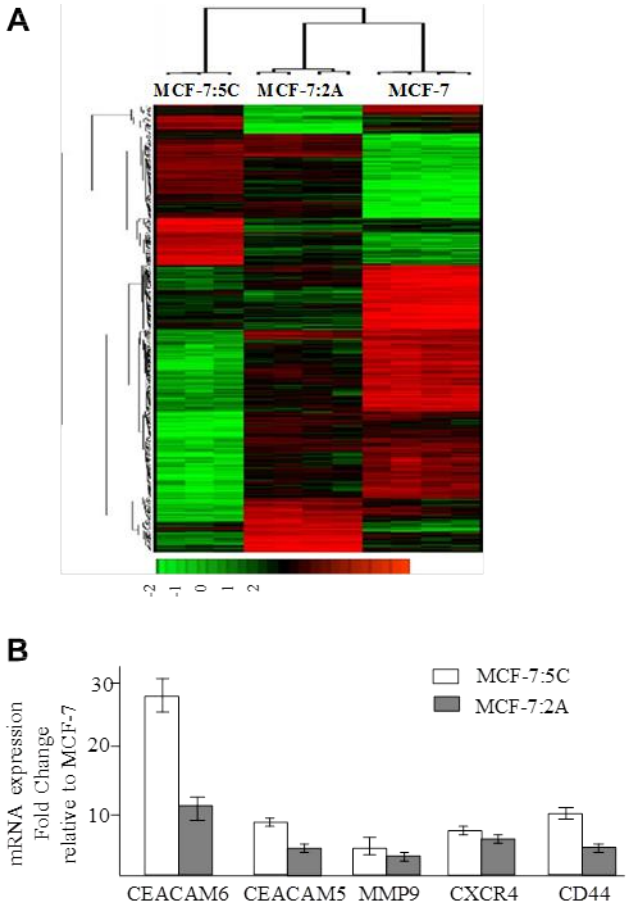


Fig. 2v-2 – *Overview of global gene expression patterns in wildtype MCF-7 cells and estrogen-deprived MCF-7:5C and MCF-7:2A variant clones. (A) Unsupervised hierarchical clustering dendogram of 904 genes most differentially expressed across the three cell lines. Each row represents a single gene. Red, genes with high expression levels and green, genes with low expression levels. The similarities in the expression pattern amongst the three cell lines are presented as a ‘‘condition tree’’ on the top of the matrix. (B) Expression levels of invasion genes in MCF-7:5C and MCF-7:2A cells compared to parental MCF-7 cells, as identified by microarray analysis.*

Estrogen deprivation increases CEACAM6 expression and enhances migration and invasion of estrogen-deprived breast cancer cells.

To confirm our microarray data, CEACAM6 mRNA expression was determined by quantitative RT-PCR. Fig. 2v-3A shows that CEACAM6 mRNA was significantly upregulated in estrogendeprived MCF-7:5C and MCF-7:2A cells compared with parental MCF-7 cells. Similarly, by Western blotting, CEACAM6 protein was undetectable in MCF-7 cells but was strongly expressed in MCF-7:5C and MCF-7:2A cells (Fig. 2v-3B). Other invasion proteins such as CEACAM5, MMP9, CXCR4 and CD44 were also markedly elevated in MCF-7:5C and MCF- 7:2A

cells compared to MCF-7 cells (Fig. 2v-3B). This finding is consistent with a recent study by Mackay and coworkers [336] which revealed that many genes associated with extracellular matrix remodelling were significantly upregulated following aromatase inhibitor treatment of primary breast tumors.

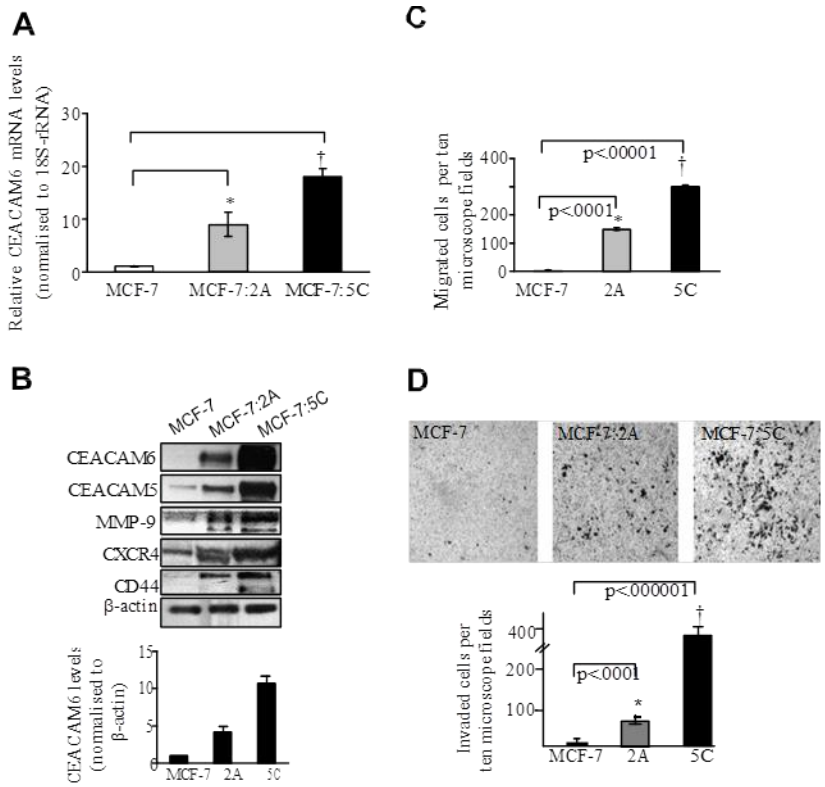


Fig. 2v-3 – *CEACAM6 promotes cell migration and invasion of estrogen-deprived breast cancer cells. (A) CEACAM6 mRNA levels in parental MCF-7 cells and estrogen-deprived MCF-7:5C and MCF-7:2A cells were measured by qRT-PCR. Relative expression of the target gene was calculated using the 2 delta CT method, where 18S rRNA was used as the endogenous control gene. All reactionswere performed in triplicates, and the error bar represents the standard deviation. (B)Western blot analysis of CEACAM6 and other invasion proteins in MCF-7, MCF-7:5C and MCF-7:2A cells. The relative ratio of CEACAM6 was calculated by densitometry (bottom). The bar graph (bottom) depicts the averages of the data obtained from three individual experiments, and data are expressed as means ± SE. (C) Quantification of cells migrating across Transwell filters. (D) Cells that invaded through the Matrigel-coated transwells were fixed, stained, visualised at 20x magnification by light microscopy and photographed. Each panel represents an example of three replicates. Ten random fields were counted per insert at 20x.*

Estrogen deprivation increases migration and invasion of breast cancer cells.

Since MCF-7:5C and MCF-7:2A cells overexpressed several invasion genes, we next assessed the migratory and invasive potential of these cells in vitro. Cell migration was measured using amodified Boyden chamber assay with 10% SFS as a chemoattractant. As shown in Fig. 2v-3C, MCF-7:5C and MCF-7:2A cells had the highest numbers of migrating cells compared to MCF-7 cells; a phenotype that correlated with CEACAM6 expression. Similar results were obtained when

the different cell lines were tested for their ability to invade through membranes coated with Matrigel. Fig. 2v-3D shows that MCF-7:5C and MCF-7:2A cells had the highest number of invading cells, while MCF-7 cells were non-invasive. The invasive ability of the cell lines was as follows: MCF-7:5C > MCF-7:2A > MCF-7.

CEACAM6 suppression inhibits invasion and migration of MCF-7:5C cells

To test the hypothesis that CEACAM6 is required for cell migration and invasion, we used iRNA to suppress CEACAM6 expression. MCF-7:5C cells were transfected with CEACAM6-specific or control (scrambled sequence) siRNA, and Western blot analysis was performed 72 h post-transfection. Fig. 2v-4A (top) shows that CEACAM6 protein was significantly suppressed (75ï 85%) in MCF-7:5C cells transfected with the CEACAM6- specific siRNA but not the control siRNA. siRNA suppression of CEACAM6 expression was also confirmed at the transcript level using qRT-PCR at 48 h following transfection (Fig. 2v-4A, bottom). To clarify the role of CEACAM6 in cell invasion, MCF-7:5C cells were pretreated with CEACAM6 siRNA or control siRNA for 48 h and invasion was measured over the subsequent 48 h. Fig. 2v-4B shows that CEACAM6 siRNA almost completely reversed the invasiveness of MCF-7:5C cells, whereas control siRNA did not affect cell invasion. The invasiveness of MCF-7:5C cells was inhibited by nearly 80% when CEACAM6 expression was suppressed. A similar trend was observed for cell migration (data not shown). Suppression of CEACAM6 also significantly reduced phosphorylated Akt and phosphorylated c-Src in MCF-7:5C cells (Fig. 2v-4C). E-cadherin and b-catenin were also significantly reduced in MCF-7:5C and MCF-7:2A cells, whereas pAkt and N-cadherin were significantly upregulated in these cells compared to parental MCF-7 cells (Fig. 2v-4D). Similar experiments performed in MCF-7:2A cells also showed a dramatic reduction (60%) in invasion following CEACAM6 suppression (data not shown).

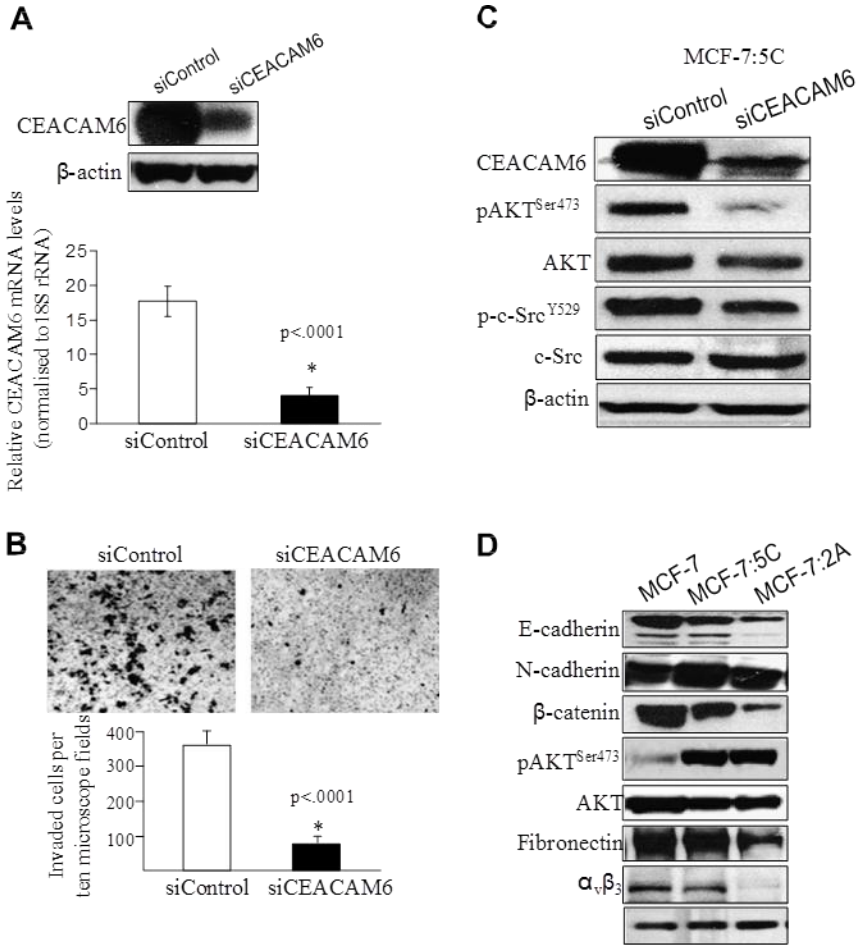


Fig. 2v-4 – *CEACAM6 suppression completely blocks invasion of MCF-7:5C breast cancer cells. (A) siRNA-mediated gene knockdown of CEACAM6 was verified by Western blot (top panel) and qRT-PCR (bottom panel). For qRT-PCR experiments, relative expression of CEACAM6 gene was calculated using the 2 delta CT method, where 18S rRNA was used as the endogenous control gene. All reactions were performed in triplicates and the error bar represents the standard deviation. (B) Matrigel invasion assay of siControl and siCEACAM6-transfected MCF-7:5C cells. (C) Immunoblot analysis of MCF-7:5C cells transfected with CEACAM6 siRNA or control siRNA for 72 h. b-Actin was used as a loading control. (D) Western blot analyses of E-cadherin, b-catenin, N-cadherin, Akt and pAKT protein expression in MCF-7, MCF-7:5C and MCF-7:2A cells.*

Estradiol down-regulates CEACAM6 expression and blocks migration and invasion of MCF-7:5C cells.

We also examined whether CEACAM6 expression is hormonally regulated in MCF-7:5C and MCF-7:2A cells. As shown in Fig. 2v-5A and B, estradiol completely down-regulated CEACAM6 mRNA and protein expression in MCF-7:5C and MCF-7:2A cells. This down-regulation was an ERa-mediated event since pretreatment with the anti-estrogen fulvestrant, which is known to degrade ERŭ[337,338], was able to reverse the inhibitory effect of estradiol on CEACAM6 protein in both cell lines (Fig. 2v-5B). Fulvestrant also completely counteracted the anti-invasive effects of estradiol in MCF-7:5C cells (Fig. 2v-5C). Interestingly, estradiol enhanced the invasiveness of parental MCF-7 cells (Fig. 2v-5D) without significantly changing CEACAM6 protein level in these cells (Fig. 2v-5B).

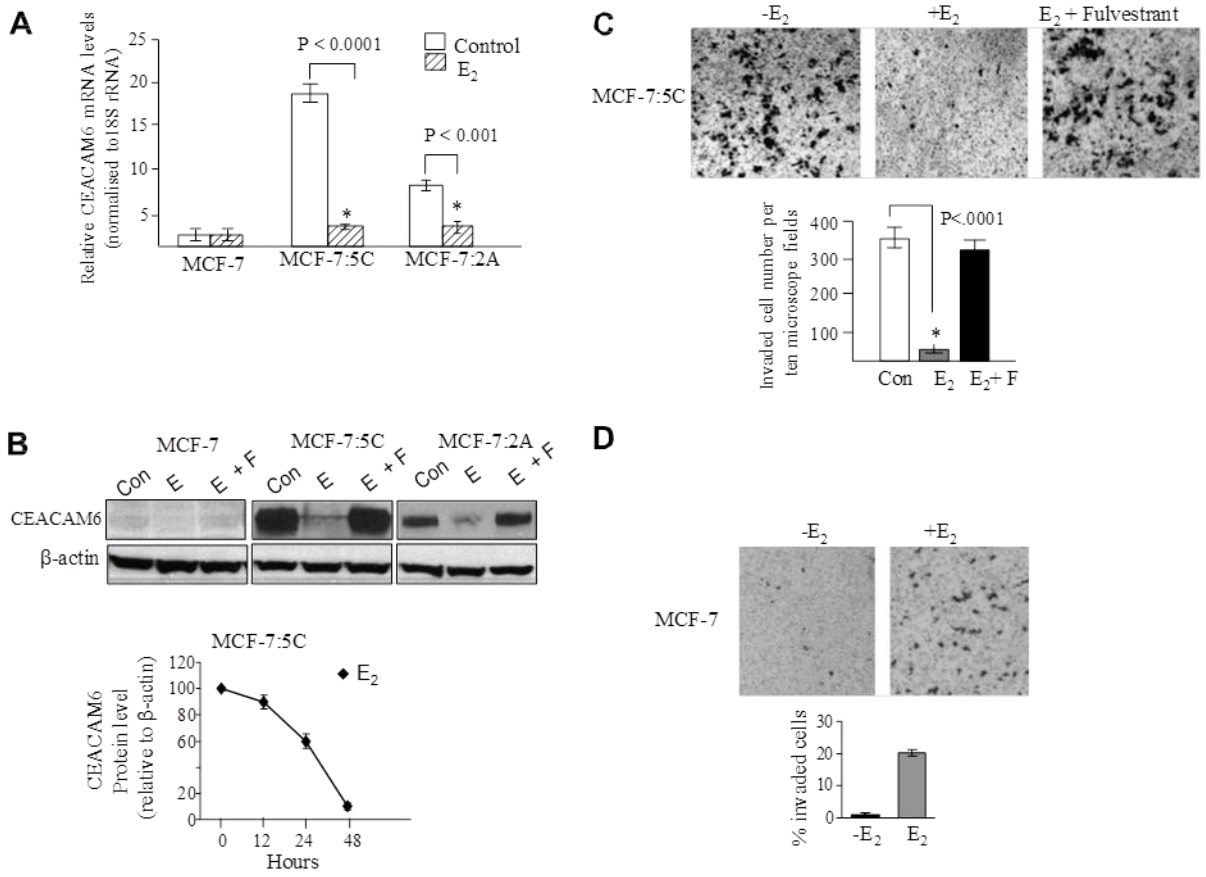


Fig. 2b-5 – *17β-Estradiol suppresses CEACAM6 expression and blocks invasion of estrogen-deprived breast cancer cells. (A) Quantitative RT-PCR analyses of CEACAM6 mRNA expression in MCF-7:5C and MCF-7:2A cells following treatment with 1 nM estradiol (E2) for 48 h. Expression levels were internally normalised to the housekeeping gene 18S rRNA (error bars, SE) (B) Western blot analysis of CEACAM6 protein expression in MCF-7, MCF-7:2A and MCF-7:5C cells. Line graph shows the timedependent effect of E2 on CEACAM6 protein level in MCF-7:5C cells. (C) Invasion of MCF-7:5C cells is blocked by E2 but not the pure anti-estrogen fulvestrant. Invasion assay was performed as previously described in Fig. 3. (D) Effect of estradiol on the invasiveness of wild-type MCF-7 cells. Each panel represents an example of three replicates.*

Inhibition of c-Src reduces the invasiveness of MCF-7:5C and MCF-7:2A cells.

Previous studies have reported that CEACAM6 cross-linking initiates c-Src-dependent cross-talk between CEACAM6 and ŭvb3 integrin, leading to increased ECM-adhesion and invasion [339]. We therefore determined c-Src kinase activity in estrogen-deprived MCF-7:2A and MCF-7:5C cells by measuring phosphorylation of c-Src at Tyr529. Both MCF-7:5C and MCF-7:2A cells showed significantly elevated levels of phosphorylated c-SrcY529 compared to parental MCF-7 cells, and treatment with the c-Src kinase inhibitor PP2 significantly reduced the invasiveness of MCF-7:5C and MCF-7:2A cells. Inhibition of Akt phosphorylation using the PI3K inhibitor LY294002 also significantly reduced cell growth and invasion of these cells, thus suggesting an important role for the c-Src and Akt signaling pathways in invasion.

Discussion

Despite advances in detection and treatment of metastatic breast cancer, mortality from this disease remains high because current therapies are limited by the emergence of therapy-resistant cancer cells. In this study, we showed that estrogen deprivation significantly increased the motility and invasiveness of two ERŮ-positive human breast cancer cell lines that have acquired resistance to estrogen deprivation, and that these cells overexpressed the invasive gene CEACAM6. Furthermore, knockdown of CEACAM6 expression completely inhibited the invasiveness of MCF-7:5C and MCF- 7:2A cells and caused a reduction in phosphorylated c-Src
and pAkt expression. A significant reduction in E-cadherin and b-catenin was also observed in MCF-7:5C and MCF-7:2A cells compared to parental MCF-7 cells. To our knowledge, this study is the first to demonstrate a critical role for CEACAM6 in migration and invasion of breast cancer cells that have acquired resistance to estrogen deprivation.

Previous studies have reported that overexpression of CEACAM6 in pancreatic adenocarcinoma cells is associated with enhanced cellular invasiveness and increased metastatic potential in vivo, and that this effect is completely attenuated by suppression of CEACAM6 expression [334]. Recently, Scott and coworkers [340] reported that CEACAM6 was upregulated by 20-fold in tamoxifen-resistant MCF-7 cells compared to tamoxifen-sensitive cells, and that hormone sensitivity could be partially restored in the tamoxifen-resistant cells by siRNA silencing of CEACAM6. This in vitro data were substantiated in clinical breast cancer where it was demonstrated that CEACAM6 was overexpressed in primary breast tumors that subsequently relapsed following adjuvant tamoxifen and in a multivariate analysis, only CEACAM6 remained a significant predictor of recurrence [341]. These findings are consistent with our present study which shows that CEACAM6 is significantly upregulated in estrogen-deprived breast cancer cells that have acquired resistance to estrogen suppression, and knockdown of CEACAM6 expression reverses the invasive phenotype of these cells. The fact that CEACAM6 is identified independently in two model systems using endocrine agents with distinct modes of action suggests that it may play an important role in endocrine resistance. Currently, the mechanism by which CEACAM6 facilitates invasion is not fully understood. However, there is evidence that CEACAM6, along with other GPI-anchored proteins, is capable of modulating the activity of intracellular tyrosine kinases such as c-Src [342,343]. In particular, studies by Duxbury and coworkers [339,344] showed that c-Src activity was increased in CEACAM6-overexpressing BxPC3 human pancreatic cancer cells and decreased following suppression of CEACAM6 expression, and that inhibition of c-Src activity significantly suppressed CEACAM6-mediated cellular invasiveness. We found that phosphorylated c-Src was significantly elevated in MCF-7:5C and MCF-7:2A cells, and that suppression of CEACAM6 expression reduced its level in these cells. Pharmacological blockade of c-Src using the Src tyrosine kinase inhibitor pyrazolopyrimidine (PP2) also inhibited the invasiveness of MCF-7:5C and MCF-7:2A cells. In addition, we found markedly elevated levels of phosphorylated Akt^{ser473} in MCF-7:5C and MCF-7:2A cells, which were dramatically reduced following CEACAM6 suppression. Akt is a serine/threonine protein kinase that mediates cell survival, proliferation [345,346], tumor cell migration and invasion and metastasis [347], and previous studies have shown that c-Src activates the PI3K/Akt signaling pathway [348]. Thus, it is possible that activation of both c-Src and Akt might play a role in mediating CEACAM6-induced migration and invasion.

The epithelial-to-mesenchymal transition (EMT) plays a key role in metastasis and is characterised by the conversion of epithelial cancer cells to a more motile phenotype that facilitates invasion. A critical molecular feature of EMT is the down-regulation of E-cadherin [349], a cell adhesion molecule present in the plasma membrane of most normal epithelial cells. E-cadherin acts

de facto as a tumor suppressor inhibiting invasion and metastasis and is frequently repressed or degraded during transformation. In our study, E-cadherin and b-catenin were significantly decreased, whereas N-cadherin was markedly increased in invasive MCF-7:5C and MCF-7:2A cells compared to non-invasive MCF-7 cells. In addition, our cell morphology studies showed EMT-like changes in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells. A variety of signal transduction pathways impinge on the regulation of E-cadherin levels or subcellular distribution. In particular, Akt/PKB has been shown to repress transcription of the E-cadherin gene, which leads to conversion of epithelial cells into invasive mesenchymal cells [350]. We have found that MCF-7:5C and MCF-7:2A both cells overexpress phosphorylated Akt, and gene ontology analysis of expression data obtained for MCF-7:5C and MCF-7:2A cells reveals that the P13K/Akt signaling pathway is significantly ($p = 0.002$) altered compared to parental MCF-7 cells.

In conclusion, we have identified CEACAM6 as a critical gene in the regulation of migration and invasion of breast cancer cells that have acquired resistance to estrogen deprivation. Since aromatase inhibitors are now considered the standard of care for the hormonal treatment of early breast cancer in postmenopausal women, this finding has important clinical implications for these patients because it suggests that extended use of aromatase inhibitors may potentially lead to the development of metastatic disease. CEACAM6 can thus serve as a powerful predictor of future recurrence and may also represent a promising new therapeutic target for breast cancer.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2w (Ariazi and Jordan) - Studies carried out by Dr. Eric Ariazi in the Jordan laboratory at Fox Chase Cancer Center

Exemestane's 17-hydroxylated metabolite exerts biological effects as an androgen

Introduction

A thorough investigation of exemestane and 17-hydroexemestane activities through ER and AR is warranted to provide evidence regarding whether exemestane could display a more favorable safety and toxicity profile than nonsteroidal AIs for long-term adjuvant use and as a chemopreventive of breast cancer in postmenopausal women. Therefore, we evaluated the pharmacologic actions of exemestane and its primary metabolite 17-hydroexemestane on ER- and AR-regulated activities in a range of cellular and molecular assays. First, we determined the relative binding affinity (RBA) of 17-hydroexemestane to ERŬ and AR. Next, using MCF-7 and T47D breast cancer cells, we examined the ability of 17-hydroexemestane to stimulate cell proliferation and cell cycle progression via ER and AR, to regulate ER- and AR-dependent transcription, and to modulate ERŬ and AR protein levels. Lastly, we investigated intermolecular interactions between 17-hydroexemestane and ERŬ and AR using molecular modeling.

Work Accomplished:

Experimentally Determined Binding of 17-Hydroexemestane and Exemestane to ERŬ and AR

Structures of the compounds relevant to these studies, the steroidal AI parent compound exemestane, its primary metabolite 17-hydroexemestane, E_2, and the synthetic non-aromatizable androgen R1881, are shown in Fig. 2w-1A. Importantly, the only difference between parental exemestane and its metabolite 17-hydroexemestane is a hydroxyl group in the metabolite in place of a ketone in the parent compound at the 17ß position, whereas both compounds share a 3-keto group. For steroidal estrogens, elimination or modification of the 17ß-OH group reduces binding to ERŬ, but that of the 3-OH group is much more dramatic [351]. For steroidal androgens, the trend is reversed; elimination or modification of the 17ß-OH group is more significant for AR binding than that of the 3-keto group ([351]. The 3-keto group found in both exemestane and 17-hydroexemestane also favors binding to AR [351].

We tested the binding of exemestane and 17-hydroexemestane to ERŬ and AR using fluorescence polarizationï based competitive hormone-binding assays (Fig. 2w-1B and 2w-1C). For purposes of comparison, compound affinities were arbitrarily categorized with respect to their RBAs as strong (100 to Ó1), moderate (<1 to Ó0.1), weak (<0.1 to Ó0.01), very weak (<0.01 to detectable binding defined as 50% competition), and inactive (compound did not compete for at least 50% binding). E_2 competitively bound ERŬ with an IC_{50} of 1.33 Ĭ $10^{ī 9}$ mol/L (RBA = 100;Fig. 2w-1B), and R1881 competitively bound AR with an IC_{50} of 1.34 Ĭ $10^{ī 8}$ mol/L (RBA = 100; Fig. 2w-1C). Considering ERŬ (Fig.2w- 1B), both R1881 and 17-hydroexemestane competed for binding to ERŬ with IC_{50}s of 1.02 Ĭ $10^{ī 6}$ mol/L (RBA = 0.130) and 2.12 Ĭ $10^{ī 5}$ mol/L (RBA = 0.006), respectively, which categorized R1881 as a moderate and 17-hydroexemestane as a very weak ERŬ ligand. Neither exemestane nor dexamethasone significantly competed for binding to ERŬ. Regarding AR (Fig. 2w-1C), 17-hydroexemestane and exemestane competed for binding to AR with IC_{50}s of 3.96 Ĭ $10^{ī 8}$ mol/L (RBA = 33.8) and 2.03 Ĭ $10^{ī 6}$ mol/L (RBA = 0.658), respectively, which classified 17-hydroexemestane as a strong and exemestane as a weak AR ligand. However, dexamethasone would also be categorized as a weak AR ligand. Hence, the observed

very weak ERŬ binding and strong AR binding of 17-hydroexemestane was consistent with what previously reported structure-activity relationships[351,352]would have predicted due to reduction of the 17-keto group in exemestane to a 17ƃ-OH in the metabolite.

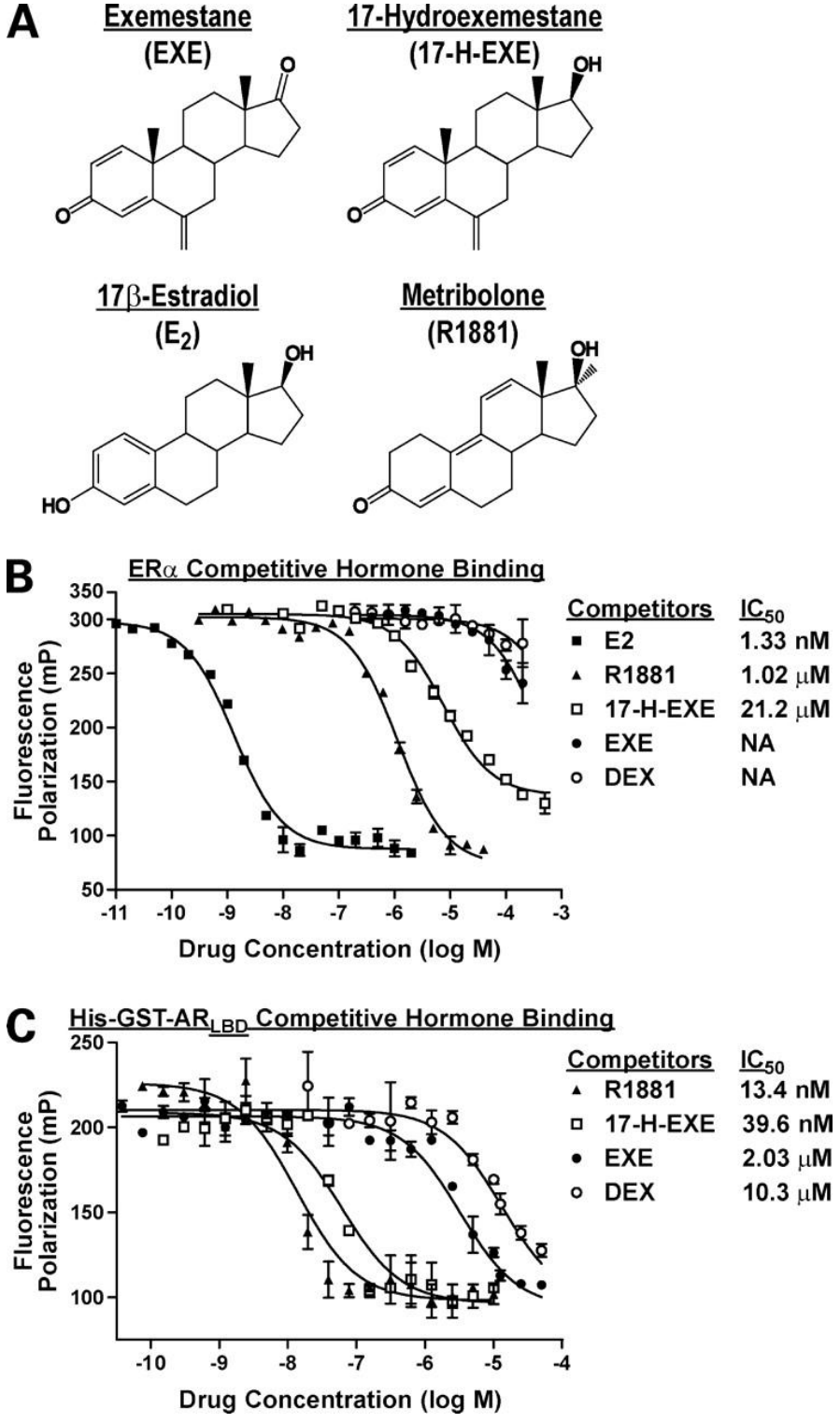


Figure 2w-1. *Compounds examined in this study and their RBAs for ERα and AR.* ***A,*** *structures of exemestane, its primary metabolite 17-hydroexemestane E_2, and R1881. ERα (**B**) and AR (**C**) fluorescence polarization-based competitive hormone-binding assays. Baculovirus-produced human ERα and rat AR ligand-binding domain tagged with a His-glutathione* S*-transferase epitope (His-GST-AR_{LBD}) were used at final concentrations of 15 and 25 nmol/L, respectively. The fluorescently labeled ERα and AR ligands, Fluormone ES2 and Fluormone AL Green, respectively, were both used at a final concentration of 1 nmol/L. The competing test compounds were E_2, R1881, 17-hydroexemestane, exemestane, and dexamethasone (DEX) as indicated.* Point, *mean of triplicate determinations;* bars, *95% confidence intervals. Curve fitting was done using GraphPad Prism software (version 4.03). IC_{50}s corresponding to a half-maximal shift in polarization values of the test compounds were determined using the maximum and minimum polarization values of the E_2-competitive binding curve for ERα or of the R1881-competitive binding curve for AR as appropriate.*

Proliferation Responses to 17-Hydroexemestane and Exemestane

We examined the effects of exemestane and 17-hydroexemestane on 7 days of proliferation in ERŭ- and AR-positive MCF-7 and T47D mammary carcinoma cells (Fig. 2w-2). As expected, both cell lines were growth stimulated by E_2, with growth EC_{50}s of 1.7 Ĭ $10^{ī 12}$ mol/L E_2 for MCF-7 cells (Fig. 2w-2A) and 7.1 Ĭ $10^{ī 12}$ mol/L E_2 for T47D cells (Fig. 2w-2B). These growth responses to E_2 were completely blocked by fulvestrant (all *P* values <0.001), validating the E_2 responsiveness via ER in these cell lines.

Both cell lines were also growth stimulated by R1881 (Fig. 2w-2A and B) and 17-hydroexemestane (Fig. 2w-2C and D), whereas exemestane did not exert any significant effect on proliferation (Fig. 2w-2C and D). Considering MCF-7 cells, R1881 exhibited a growth EC_{50} of 2.4 Ĭ $10^{ī 8}$ mol/L (Fig. 2w-2A), or approximately 4 orders of magnitude higher than that of E_2. Similarly, 17-hydroexemestane exhibited a growth EC_{50} of 2.7 Ĭ $10^{ī 6}$ mol/L in MCF-7 cells (Fig. 2w-2C) or approximately 6 orders of magnitude higher than that of E_2. These growth responses to R1881 and 17-hydroexemestane in MCF-7 cells were completely blocked by cotreatment with fulvestrant (Fig. 2w-2A and B; both *P* values <0.001). Therefore, whereas R1881, a non-aromatizable synthetic androgen, stimulated growth of MCF-7 cells, it did so by acting through ER. Hence, at high concentrations, R1881 exerted estrogenic activity. Similarly, at high concentrations, 17-hydroexemestane also exerted estrogenic activity and stimulated growth of MCF-7 cells by acting through ER.

Interestingly, in T47D cells, the growth response to R1881 and 17-hydroexemestane followed an apparent bimodal pattern, which was different than in MCF-7 cells. In T47D cells, proliferative effects of high concentrations of R1881 (5 Ĭ $10^{ī 6}$ mol/L;Fig. 2w-2B) and 17-hydroexemestane (5 Ĭ $10^{ī 6}$ mol/L; Fig. 2w-2D) were only partially blocked by fulvestrant (both *P* values <0.001), down to the level of growth observed at nanomolar concentrations of these compounds. However, proliferative effects of lower concentrations of R1881 ($10^{ī 9}$ mol/L) and 17-hydroexemestane ($10^{ī 8}$ mol/L) were completely blocked by the anti-androgen bicalutamide (both *P* values <0.001). Based on these observed levels of inhibition by bicalutamide and fulvestrant, maximal concentrations at which R1881 and 17-hydroexemestane stimulated growth through AR-dependent activities were $10^{ī 7}$ and $10^{ī 6}$ mol/L, respectively, and above these concentrations, R1881 and 17-hydroexemestane stimulated growth through ER-dependent activities. Using this information to define concentration ranges in which these compounds exert AR-mediated or ER-mediated effects in T47D cells, the growth EC_{50}s via AR of R1881 and 17-hydroexemestane were 1.0 Ĭ $10^{ī 10}$ mol/L (Fig. 2w-2B) and 4.3 Ĭ $10^{ī 10}$ mol/L (Fig. 2w-2D), respectively. Similarly, the growth EC_{50}s via ER of R1881 and 17-hydroexemestane in T47D cells were 3.1 Ĭ $10^{ī 7}$ mol/L (Fig. 2w-2B) and 1.5 Ĭ $10^{ī 6}$ mol/L (Fig. 2w-2D), respectively. Hence, in T47D cells, both R1881 and 17-hydroexemestane stimulated growth via AR at lower concentrations and via ER at higher concentrations. These results were

consistent with the observed binding affinities of these compounds to ERŬ(Fig. 2w-1B) and AR (Fig. 2w-1C).

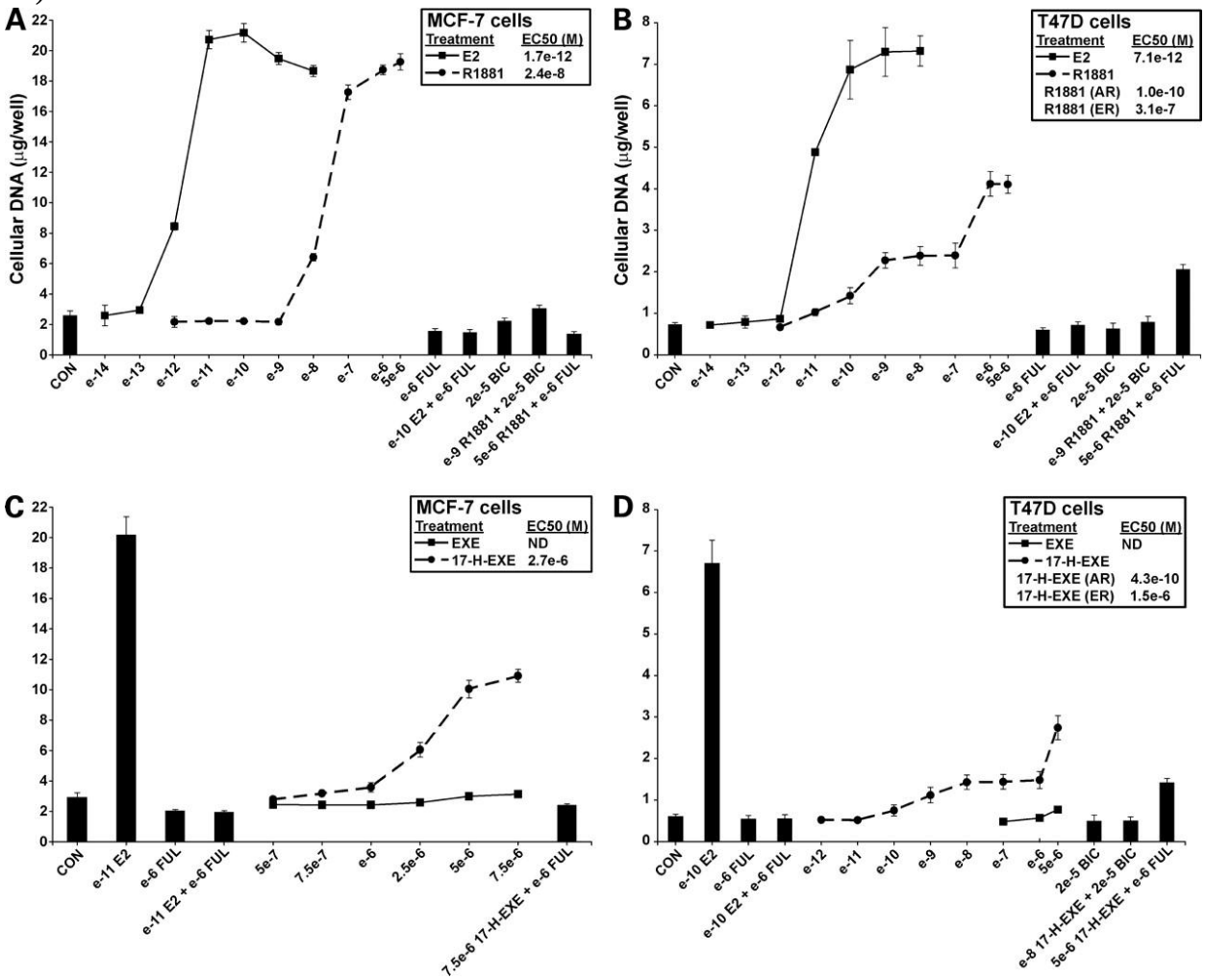


Figure 2w-2. ***17-Hydroexemestane and R1881 stimulate cellular proliferation.*** *DNA-based cellular proliferation assays of (**A**) MCF-7 cells treated with E_2 and R1881, (**B**) T47D cells treated with E_2 and R1881, (**C**) MCF-7 cells treated with exemestane and 17-hydroexemestane, and (**D**) T47D cells treated with exemestane and 17-hydroexemestane. Cells were cultured in steroid-free medium for 3 d before the assays. MCF-7 cells were seeded at 15,000 cells per well and T47D cells at 20,000 cells per well in 12-well plates. Cells were treated on days 0 (the day after seeding), 3, and 6, and then collected on day 7. Cellular DNA quantities were determined using the fluorescent DNA-binding dye Hoechst 33258 and compared against a standard curve. Data shown represent the mean of four replicates and SDs. DNA values were fitted to a sigmoidal dose-response curve and growth EC_{50}s calculated using GraphPad Prism 4.03 software. At high concentrations, 17-hydroexemestane and R1881 increased growth via ER in both cell lines but, at low concentrations, stimulated growth via AR selectively in T47D cells. Abbreviations: CON, control; FUL, fulvetsrant; BIC, bicalutamide.*

Regulation of ERα and AR Transcriptional Activities by 17-Hydroexemestane

Next, we investigated the ability of 17-hydroexemestane to regulate ER and AR transcriptional activity by transfecting cells with an ERE(5x)-regulated or ARE(5x)-regulated dual-luciferase plasmid set, treating cells with test compounds, and measuring dual-luciferase activity 44 h after treatment (Fig. 2w-3A-C). E_2 at $10^{\bar{1}\,10}$ mol/L induced ERE(5x)-regulated transcription by 19.4-fold in MCF-7 cells (Fig. 2w-3A; $P <$

0.001), and 11.3-fold in T47D cells (Fig. 2w-3B; $P < 0.001$) compared with control-treated cells; this E_2-induced transcriptional activity was blocked by fulvestrant (both P values <0.001), validating dependence on ER for ERE(5x)-regulated transcription. At high sub-micromolar and micromolar concentrations, R1881 stimulated ERE(5x)-regulated transcription in both cell lines, with maximal inductions of 22.7-fold at 5 Ĭ 10^{-6} mol/L in MCF-7 cells (Fig. 2w-3A; $P < 0.001$), and 7.9-fold at 5 Ĭ 10^{-6} mol/L in T47D cells (Fig. 2w-3B; $P < 0.001$) compared with control-treated cells. The ability of R1881 at 5 Ĭ 10^{-6} mol/L to induce ERE(5x)-regulated transcription was blocked by fulvestrant (Fig. 2w-3A and B; both P values <0.001), indicating that at high concentrations, R1881 acted as an estrogen. In a similar manner as R1881, 17-hydroxexemestane stimulated ERE(5x)-regulated transcription in a concentration-dependent manner at sub-micromolar and micromolar concentrations (Fig. 2w-3A and B). At 5 Ĭ 10^{-6} mol/L, 17-hydroxexemestane maximally induced ERE(5x)-regulated transcription by 7.7-fold in MCF-7 cells (Fig. 2w-3A; $P < 0.001$) and 3.3-fold in T47D cells (Fig. 2w-3B; $P < 0.001$) compared with control-treated cells; this transcriptional activation was blocked by fulvestrant (both P values <0.001). Therefore, at high concentrations, 17-hydroxexemestane acted as an estrogen and induced ER transcriptional activity.

In a similar manner, AR-dependent transcriptional activity was investigated. T47D cells showed a concentration-dependent induction of ARE(5x)-regulated transcription in response to R1881, with 10^{-9} mol/L R1881 inducing transcription by 8.5-fold and 10^{-6} mol/L R1881 maximally inducing transcription by 12.7-fold relative to control-treated cells (Fig. 2w-3C; both P values <0.001). Bicalutamide blocked 10^{-9} mol/L R1881-mediated induction of ARE(5x)-regulated transcription (Fig. 2w-3C; $P < 0.001$), confirming dependence on AR. MCF-7 cells failed to respond to 10^{-6} mol/L R1881 with induction of ARE(5x)-regulated transcription (data not shown), although these cells express AR protein. This supports our prior results that T47D cells were growth stimulated by R1881 through an AR-dependent mechanism (Fig. 2w-2B), but that MCF-7 cells were not (Fig. 2w-2A). As expected, 10^{-6} mol/L E_2 failed to induce ARE(5x)-regulated transcription (Fig. 2w-3C). Next, 17-hydroxexemestane was evaluated in T47D cells and, in a concentration-dependent manner, induced ARE(5x)-regulated transcription with maximal induction of 4.7-fold occurring at 5 Ĭ 10^{-6} mol/L relative to control treatment (Fig. 2w-3C; $P < 0.001$). However, because high concentrations of 17-hydroxexemestane were needed to induce this synthetic ARE(5x)-regulated promoter, we tested whether lower concentrations of 17-hydroxexemestane could modulate endogenous AR mRNA expression, which is known to be negatively feedback regulated by its gene product. Using real-time PCR, AR mRNA levels were determined in T47D cells following 24 h of treatment with test compounds (Fig. 2w-3D). R1881 at 10^{-9} mol/L significantly down-regulated AR mRNA expression by 48% ($P < 0.001$), whereas 10^{-9} mol/L E_2 did not (Fig. 2w-3D). Bicalutamide prevented R1881-mediated decrease in AR mRNA expression (Fig. 2w-3D), validating that AR mRNA levels were negatively feedback regulated. Similarly, a low 10^{-8} mol/L concentration of 17-hydroxexemestane led to a 41% decrease in AR mRNA levels ($P < 0.01$), with increased 17-hydroxexemestane concentrations further decreasing AR mRNA expression (Fig. 2w-3D). Bicalutamide blocked 17-hydroxexemestaneï mediated down-regulation of AR mRNA expression ($P < 0.01$), whereas fulvestrant did not (Fig. 2w-3D). Therefore, 17-hydroxexemestane acted as an androgen via AR to feedback-regulate the expression of endogenous AR mRNA in T47D cells.

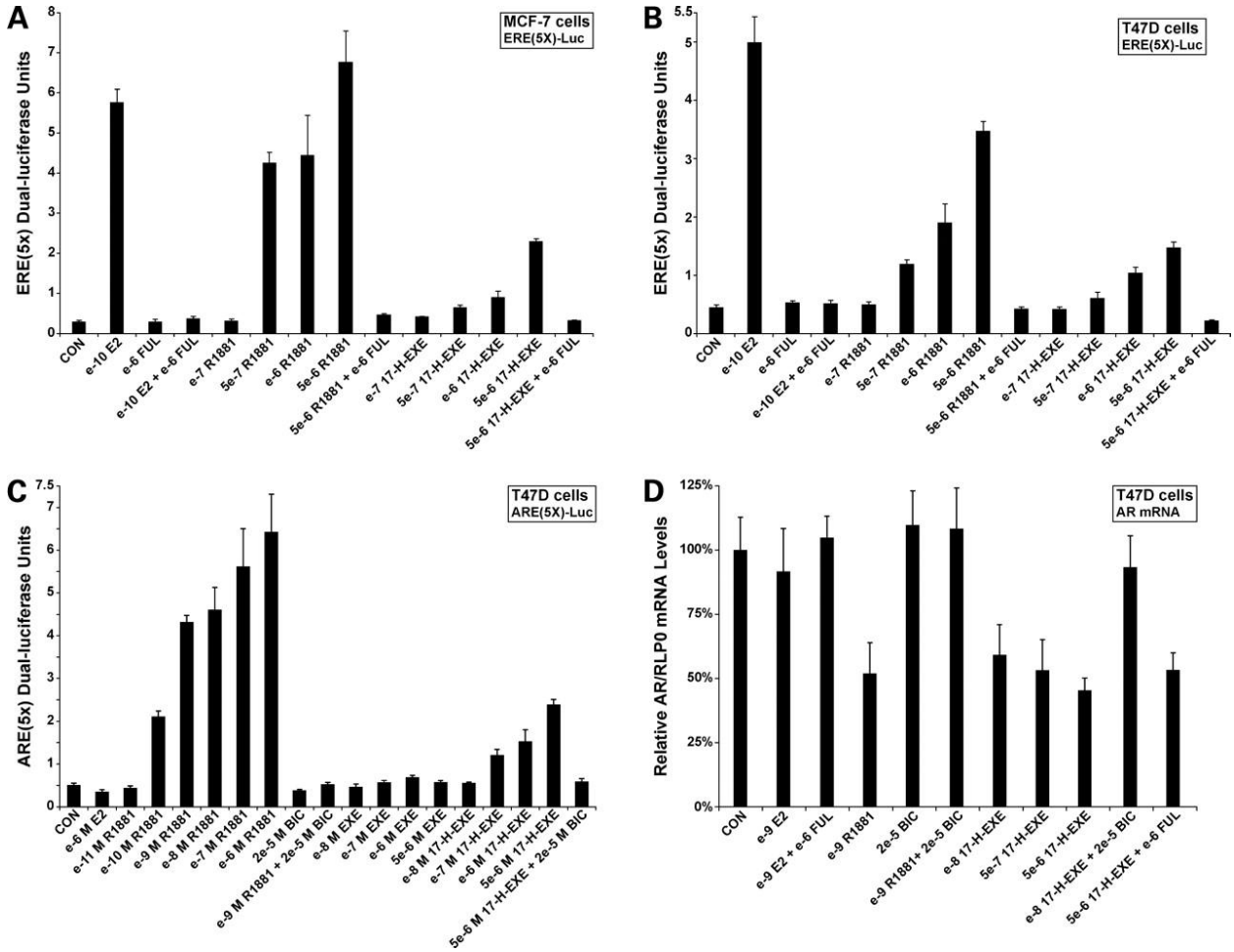


Figure 2w-3. ***17-Hydroexemestane and R1881 regulate ER transcriptional activity at high concentrations and AR transcriptional activity at low concentrations.*** *ERE(5x)-regulated dual-luciferase activity in (**A**) MCF-7 cells and (**B**) T47D cells. (**C**) ARE(5x)-regulated reporter gene activity in T47D cells.* Aï C*, Under steroid-free conditions, cells were transiently transfected with pERE(5x)TA-ffLuc or pARE(5x)-Luc (firefly luciferase reporter plasmids) and the internal normalization control pTA-srLuc (*Renilla *luciferase reporter plasmid). Four hours after transfection, cells were treated as indicated and then again the following day. Cells were assayed 44 h after transfection for dual-luciferase activity. Data shown are the mean of triplicate determinations and associated SDs. 17-Hydroexemestane and R881 stimulated ERE(5x)-regulated transcription in MCF-7 and T47D cells and ARE(5x)-regulated transcriptional activity in T47D cells.* ***D,*** *AR mRNA levels in T47D cells as determined by real-time PCR. T47D cells were treated as indicated for 24 h. RNA was isolated and converted to cDNA. Continuous accumulation of PCR products was monitored using the double strand-specific DNA dye SYBR Green. Quantitative measurements of AR mRNA and the endogenous normalization control RLP0 mRNA were determined by comparison to a standard curve of known quantities of serially diluted AR or RLP0 PCR product. The data represent the mean and SDs of three independent samples, each of which was measured in triplicate. 17-Hydroexemestane and R881 down-regulated AR mRNA levels at nanomolar concentrations in an AR-dependent manner.*

Modulation of AR and ERα Protein Levels by 17-Hydroexemestane

Androgens and estrogens modulate protein expression levels of their cognate receptors. R1881 stabilizes AR protein allowing its accumulation [353,354]), whereas E_2 promotes ERŬ degradation in a cell typeï

dependent manner[165]. Therefore, we investigated the effects of 17-hydroexemestane on AR and ERŬ protein levels by treating cells with test compounds for 24 h and analyzing receptor levels by immunoblotting. E_2 decreased ERŬ protein levels in MCF-7 (Fig. 2w-4A), but not T47D cells (Fig. 2w-4B), as we have previously shown [165]. As expected, fulvestrant promoted ERŬ protein degradation in both cell lines. E_2 did not significantly affect AR protein accumulation in MCF-7 cells (Fig. 2w-4A), but did down-regulate AR protein levels in T47D cells (Fig. 2w-4B). Also, fulvestrant and E_2 plus fulvestrant treatments did not significantly affect AR protein levels in MCF-7 cells (Fig. 2w-4A), but did modestly up-regulate AR protein levels in T47D cells (Fig. 2w-4B). As expected, R1881 caused an increase in accumulation of AR protein in both cell lines (Fig. 2w-4A and B), likely by stabilizing the protein. Next, we characterized the effects of low 10^{-8} mol/L and high 5×10^{-6} mol/L concentrations of 17-hydroexemestane on ERŬ and AR expression. The high 5×10^{-6} mol/L concentration of 17-hydroexemestane led to decreased ERŬ protein levels in MCF-7 (Fig. 2w-4A), but not in T47D cells (Fig. 2w-4B); this pattern indicates that 5×10^{-6} mol/L 17-hydroexemestane acted as an estrogen to regulate ERŬ protein in a cell typeï dependent manner. Similar to R1881, treatment with low 10^{-8} mol/L or high 5×10^{-6} mol/L concentrations of 17-hydroexemestane led to increased AR protein accumulation in both cell lines (Fig. 2w-4A and B), indicating that 17-hydroexemestane acted as an androgen likely by stabilizing AR protein. Therefore, 17-hydroexemestane modulated ERŬ and AR protein accumulation as would an estrogen and an androgen, respectively.

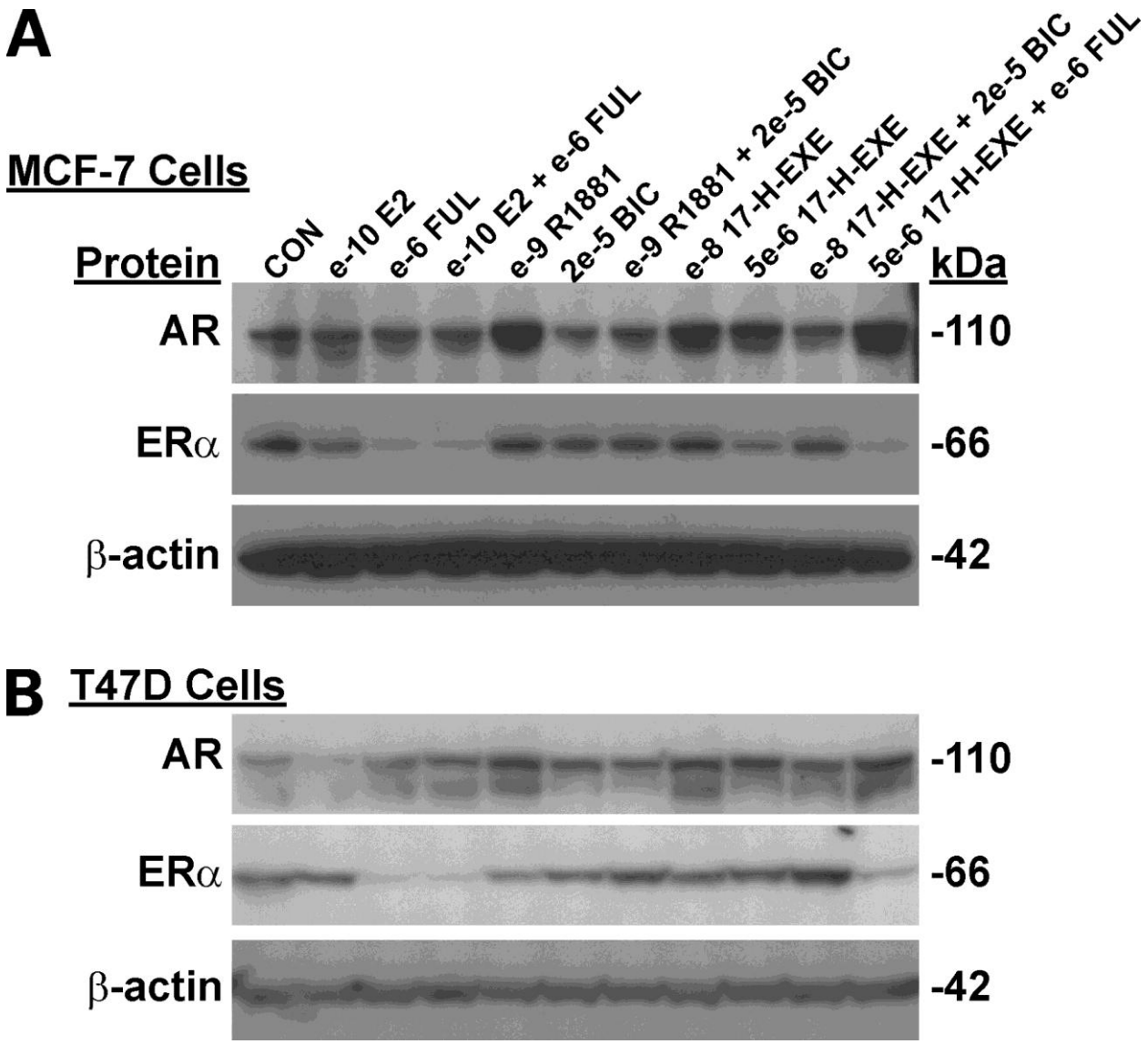


Figure 2w-4. ***17-Hydroexemestane modulates AR and ERα protein levels. Immunoblot analysis of AR and ERα in (A) MCF-7 cells and (B) T47D cells.*** *Cells were treated as indicated for 24 h, and 20 μg of cellular protein were resolved by 4% to 12% SDS-PAGE and then transferred to a nylon membrane. Membranes were probed for AR, ERα, and β-actin, and immunoreactive bands were visualized by chemiluminescence and autoradiography. Cropped blots are shown. 17-hydroexemestane up-regulated AR protein levels at 10^{-8} mol/L in both cell lines and down-regulated ERα in MCF-7 cells at 5×10^{-6} mol/L.*

Molecular Docking of 17-Hydroexemestane and Exemestane to ERα and AR

To investigate the mechanism by which 17-hydroexemestane binds ERŭ as a very weak ligand and AR as a strong ligand, molecular models were constructed *in silico*. The trends in the computed intermolecular interaction energies matched the experimentally determined RBAs. Superimposition of the docked and crystallographic structures of E_2 complexed with ERŭ (Fig. 2w-5A) and of R1881 complexed with AR (Fig. 2w-5B) showed that the docking models recapitulated the molecular recognition patterns of the crystal structures.

Considering ERŭ, the intermolecular interaction energies of R1881 and 17-hydroexemestane were less favorable than E_2 by 1.94 and 2.76 kJ/mol, respectively, due to decreased H-bond interactions and

increased steric clash. Exemestane was much less favorable than E_2 by 4.57 kJ/mol. Hence, the 17ß-OH group of 17-hydroexemestane compared with the 17-keto group of exemestane contributed ī 1.81 kJ/mol toward increased affinity for ERŭ Interestingly, the docking calculations suggested that the higher affinity of 17-hydroexemestane over exemestane for ERŭ was not due to increased H-bonding mediated by the 17ß-OH group, but rather increased lipophilic interactions due to a slight repositioning of the compound as a consequence of 17ß-OH group. In the E_2 docked to ERŭmodel, H-bonds between E_2 and Glu^{353}, Arg^{394}, and His^{524} side chains were observed (Fig. 2w-5A). In the docked 17-hydroexemestane to ERŭmodel (Fig. 2w-5C), the same Arg^{394} and His^{524} interactions were maintained, except that there was a loss of the Glu^{353} interaction.

Considering AR, the intermolecular interaction energy of 17-hydroexemestane was only 0.8 kJ/mol less favorable than R1881, whereas exemestane was significantly less favorable than R1881 by 6.27 kJ/mol. Docking of 17-hydroexemestane to AR, compared with the parent drug exemestane, indicated that 17-hydroexemestane exhibited improved lipophilic interactions by ī 2.11 kJ/mol, more favorable H-bonding interactions by ī 2.65 kJ/mol, and decreased steric clash by ī 1.08 kJ/mol. Hence, the 17ß-OH group in 17-hydroexemestane compared with the 17-keto group in exemestane contributed ī 5.47 kJ/mol toward higher affinity for binding AR. In the R1881 docked to AR model, H-bonds between R1881 and Asn^{705}, Gln^{711} and Arg^{752} were observed (Fig. 2w-5B). The OH side chain of Thr^{877} was in close proximity to both docked R1881 (Fig. 2w-5B) and 17-hydroexemestane (Fig. 2w-5D), but the angle was not favorable for H-bonding. Docking of 17-hydroexemestane to AR (Fig. 2w-5D) indicated a short 2.78-¡ H-bond between the 17ß-OH group of the ligand and Asn^{705}, but not between the 3-keto group of the ligand and Gln^{711} and Arg^{752}. Hence, the short 2.78-¡ H-bond observed in the 17-hydroexemestane docked to AR model was important in mediating high affinity binding.

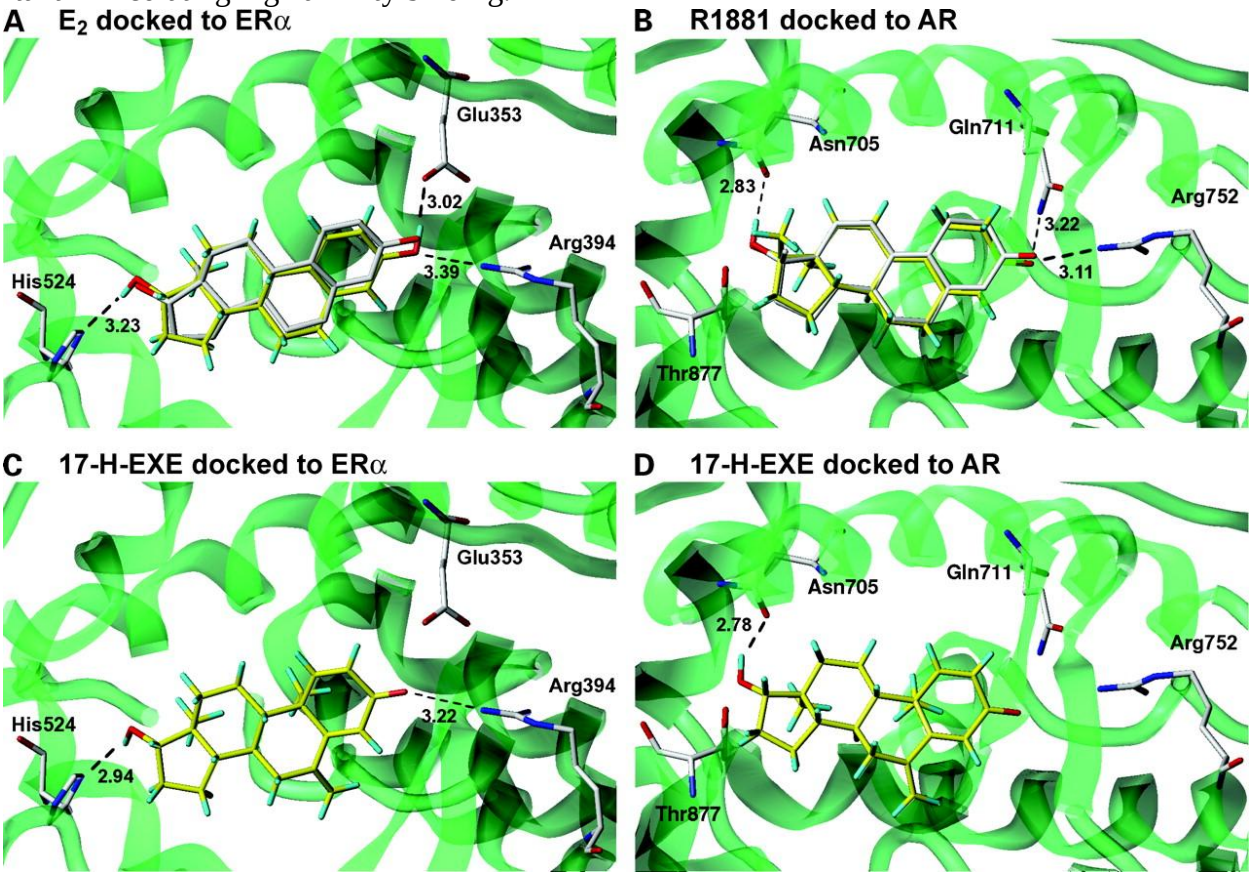


Figure 2w-5. ***Intermolecular interactions of ligands complexed with ERα and AR by computer docking. A,*** *superposition of E_2 from the X-ray crystal structure (gray) and modeled E_2 (yellow) docked to ERα.* ***B,***

superposition of R1881 from the crystal structure (gray) and modeled R1881 (yellow) docked to AR. ***C,*** *modeled 17-hydroexemestane docked to ERα.* ***D,*** *modeled 17-hydroexemestane docked to AR. Cyan, red, and blue, hydrogen, oxygen, and nitrogen atoms, respectively. Green, carbon backbone of the protein. Hydrogens from the X-ray crystal conformations of* E_2 *(****A****) and R1881 (****C****) were omitted. H-bonds were shown to the modeled compound conformations only. Dashed lines, intermolecular H-bonds up to 3.5 Å; their length in angstroms is indicated.*

Discussion

We observed that 17-hydroexemestane, the primary metabolite of exemestane, bound to ERŭ as a very weak ligand and acted through ER at high sub-micromolar and micromolar concentrations to stimulate growth, promote cell cycle progression, induce ERE-regulated reporter gene expression, and down-modulate ERŭ protein levels in breast cancer cells. However, we also observed that 17-hydroexemestane bound to AR as a strong ligand and found in T47D cells that 17-hydroexemestane stimulated growth, induced cell cycle progression, down-modulated AR mRNA expression, and stabilized AR protein levels, with all of these effects occurring at low nanomolar concentrations and blocked by bicalutamide. Moreover, computer docking indicated that the 17ß-OH group of 17-hydroexemestane versus the 17-keto group of exemestane contributed significantly more toward increasing affinity to AR than to ERŭ. Molecular modeling also indicated that 17ß-OH group of 17-hydroexemestane interacted with AR through an important H-bond of Asn^{705}, a conserved recognition motif employed by R1881. Therefore, we propose that the primary mechanism of action of exemestane *in vivo* is mediated by 17-hydroexemestane regulating AR activities.

The Food and Drug Administration label for exemestane (Aromasin; Pfizer) reports that in postmenopausal women with advanced breast cancer, the mean AUC (area under the curve) values of exemestane following repeated doses was 75.4 ngIh/mL (254 nmolIh/L), which was almost twice that in healthy postmenopausal women (41.4 ngIh/mL; 140 nmolIh/L;). Because circulating levels of 17-hydroexemestane can reach about 1/10 the level of the parent compound (Evans TR, et al. Cancer Res 1992), we hypothesize that circulating levels of 17-hydroexemestane are sufficient to bind AR and regulate AR-dependent activities. Furthermore, a subpopulation of patients may exist who metabolize exemestane at higher rates, leading to correspondingly higher circulating 17-hydroexemestane levels. For instance, one of three patients administered 800 mg of exemestane, the highest dose evaluated, achieved 17-hydroexemestane plasma levels approximately one-half the level of the parent compound (Evans TR, et al. Cancer Res 1992). Based on our results, we would predict that higher circulating levels of 17-hydroexemestane would associate with decreased rates of BMD loss and risk of bone fractures in postmenopausal women. We suggest that circulating levels of 17-hydroexemestane and exemestane should be determined in clinical trials and correlated to disease outcome and toxicity profiles such as BMD loss.

Although the clinical studies reported thus far were not designed to directly compare one AI versus another, comparisons in the rate of BMD loss from baseline to year 1, and from year 1 to 2 can be made. In the bone safety subprotocol of the IES (Intergroup Exemestane Study) trial, the rate of BMD loss was greatest within 6 months of switching from tamoxifen to exemestane at ī 2.7% in the lumbar spine and ī 1.4% in the hip, but thereafter, BMD loss progressively slowed in months 6 to 12 and again in months 12 to 24 to only ī 1.0% and ī 0.8% in the lumbar spine and hip, respectively [355], which is in the same range as would be expected for postmenopausal women in general. However, in the bone safety substudy of the MA.17 trial, patients administered letrozole experienced a relatively constant rate of BMD loss for 2 years: at 12 months, the rate of BMD loss from baseline was ī 3.3% and ī 1.43% in lumbar spine and hip, respectively, and from year 1 to year 2, ī 2.05% and ī 2.17% in lumbar spine and hip, respectively[356]. In the bone substudy of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial, the rate of BMD loss from baseline to year 1 was ī 2.2% in lumbar spine and ī 1.5% in hip and from year 1 to year 2, ī 1.8%

in lumbar spine and ī 1.9% in hip. Collectively, these results suggest that after the initial 12 months of AI therapy, exemestane may be associated with slower rates of BMD loss compared with nonsteroidal AIs. Furthermore, although not directly comparable, the fracture rate per 1,000 woman-years in the ATAC trial was 22.6 for anastrozole and 15.6 for tamoxifen [357], whereas in the IES trial, the incidence rate per 1,000 woman-years for multiple fractures was 19.2 for exemestane and 15.1 for tamoxifen [355] These results show that although both anastrozole and exemestane were associated with higher fracture rates than tamoxifen, they also suggest that exemestane may be associated with a lower fracture rate than anastrozole. Clinical trials now under way to directly compare the different AIs will hopefully provide clear results.

Androgens regulate growth of normal and neoplastic mammary cells in a cell type-specific manner, either by inhibiting or stimulating growth. However, the mechanisms by which androgens via AR regulate breast cancer growth remain elusive. Female AR knock-out mice exhibit decreased ductal branching and terminal end buds in prepubertal animals and retarded lobuloalveolar development in adult animals[358]. Likewise, targeted disruption of AR in MCF-7 cells also leads to severe inhibition of proliferation[358]. Epidemiologic analyses indicate a positive correlation between androgen levels and the incidence of breast cancer; meta-analysis from nine prospective studies showed that a doubling in testosterone concentrations in postmenopausal women translated into an increased relative risk of 1.42 unadjusted and 1.32 adjusted for E_2. AR status in breast cancer associates with both positive and negative indicators and clinical outcome. AR expression has been found in 84% to 91% of clinical breast cancers, and associated with ER status, but has also been found in 49% of ER-negative tumors. Patients with tumors that coexpress AR with ER and progesterone receptor have shown longer disease-free survival (DFS) than patients whose tumors were negative for all three receptors, but AR protein levels have also served as an independent predictor of axillary metastases in multivariate analysis. Furthermore, AR expression has correlated with decreased histopathologic grade, greater age, and postmenopausal status, but also lymph nodeï positive status [359]. In AR-positive/ER-negative tumors, AR expression again associated with positive and negative indicators/outcome such as increased age, postmenopausal status, and longer DFS but also tumor grade, tumor size, and HER-2/neu overexpression [360].

Patients who fail AI therapy, whether the AI was steroidal or nonsteroidal, likely harbor tumor cells that have been selected for growth in an estrogen-depleted environment and, hence, are not dependent on ER activity for survival. Not all androgens are metabolized by aromatase to estrogens; for instance, dihydrotestosterone cannot be converted to an estrogen by aromatase. Thus, a possible mechanism for failure of AI therapy in the clinic is androgen-stimulated breast cancer growth, a largely unrecognized alternative mechanism. We observed cellular proliferation of T47D cells in response to R1881 and 17-hydroexemestane, and these effects were blocked by bicalutamide. Therefore, T47D cells contain a functional AR signaling pathway that promoted growth in the absence of estrogen. Because functional AR signaling could be etiologically involved in a subpopulation of clinical breast cancers, those patients who have AR-positive tumors and achieve high circulating levels of 17-hydroexemestane, yet whose disease progresses while on exemestane therapy, may respond to AR-based therapy such as the antiandrogen bicalutamide.

TASK 2. GU/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2x (Ariazi and Jordan) - Studies carried out by Dr.Eric Ariazi in the Jordan laboratory at Fox Chase Cancer Center

Emerging principles for the development of resistance to antihormonal therapy: implications for the clinical utility of fulvestrant

Introduction

Over the past two decades, we have developed unique MCF-7 breast cancer xenograft models of long-term SERM (TAM and RAL) treatment and models of long-term estrogen withdrawal that could reasonably mimic resistance to AIs. These tumor models were developed *in vivo* and *in vitro*. The *in vivo* tumor models were designed to mimic the selection process needed over years to develop acquired resistance in the clinic by serially implanting MCF-7 tumors into SERM-treated and ovariectomized immunodeficient mice also over a period of years. The *in vitro* tumor models were developed by culturing MCF-7 cells in estrogen-free conditions, with or without SERM treatment if appropriate, for over 1 year to develop antihormone resistance, and then injecting these cells into ovariectomized athymic mice treated with the SERM, if appropriate, and allowing tumors to grow. We now have in hand a panel of breast cancer xenograft and tissue culture models that have allowed us to define the evolution of resistance to antihormonal therapy into at least two phases, each of which exhibits distinct growth responses to E_2 and FUL. We found that the growth of Phase I SERM-resistant tumors is stimulated by E_2, while growth of Phase II SERM or estrogen withdrawn-resistant tumors is, paradoxically, inhibited by E_2 treatment. Previous studies conducted by our group have shown that E_2 not only inhibits growth of Phase II SERM and estrogen withdrawn-resistant tumors; it also induces apoptosis, leading to tumor regression. However, a fraction of these Phase II tumors eventually re-grow after E_2-induced regression occurs, but these tumors are again re-sensitized to antihormonal therapy. We also found that while FUL does not support the growth of Phases I and II SERM-resistant tumors, it does allow growth of Phase II estrogen withdrawn-resistant tumors. Further, we found that while E_2 blocked growth of Phase II antihormone-resistant tumors, the combination of E_2 plus FUL resulted in robust growth. Phase II antihormonal resistance has not yet been widely recognized, but could be exploited by developing a novel third-line treatment plan based on short-term low-dose estrogen to debulk patientsô tumors who fail exhaustive endocrine therapy, followed by the combination of FUL plus an AI to maintain low tumor burden and avoid a negative interaction between physiologic E_2 and FUL.

Work Accomplished:

Growth of MCF-7/E2 tumors and responsiveness to FUL

MCF-7/E2 xenograft tumors are propagated *in vivo* by serial transplantation into 0.3 cm E_2 capsule-implanted ovariectomized athymic mice. To explore the sensitivity of MCF-7/E2 tumors to FUL, MCF-7/E2 tumor cores were implanted into 30 ovariectomized athymic mice and separated into 6 groups of 5 mice each, or 10 tumors per group. The treatment groups were control (no treatment), 0.3 cm E_2 capsule *sc*, or 0.3 cm E_2 capsule *sc* plus 1 of 4 different formulations and dosing schedules of FUL totaling 10 mg/week *sc* (Fig. 2x-1). Two of the FUL formulations were suspensions made with peanut oil, differing by whether FUL was initially dissolved in EtOH or in DMSO. These FUL (EtOH/peanut oil or DMSO/peanut oil) suspensions were administered as 5 mg *sc* injections given 2 days per week, totaling

10 mg/week. The third formulation was FUL dissolved in only DMSO, and was administered as a 2 mg *sc* injection given 5 days per week, totaling 10 mg/week. The fourth FUL formulation corresponded to the clinical Faslodex preparation, which is a proprietary solution of primarily EtOH and some castor oil as a slow release-rate modifier. The clinical Faslodex preparation was administered as a 2 mg *sc* injection given five times per week, totaling 10 mg/week.

The MCF-7/E2 tumors grew robustly when treated with the 0.3 cm E_2 capsule, but did not grow in the control group (Fig. 2x-1, E_2 versus control, $P < 0.0001$), demonstrating that these tumors were dependent on E_2. The implanted capsules produce E_2 levels that are in the physiologic range observed in perimenopausal women. The cross-sectional areas of each of the four groups of MCF-7/E2 tumors treated with E_2 plus FUL was significantly smaller than those tumors treated with E_2 alone (Fig. 2x-1, all *P*-values < 0.0001). Therefore, FUL inhibited E_2-stimulated growth of MCF-7/E2 tumors. However, the degree of growth inhibition varied depending upon the formulation. Comparing the FUL suspensions in peanut oil given two times per week, FUL initially dissolved in DMSO inhibited tumor growth significantly better than FUL initially dissolved in EtOH (Fig. 2x-1, $P = 0.001$). Comparing the FUL formulations given five times per week, FUL dissolved in only DMSO inhibited tumor growth significantly better than the clinical Faslodex preparation (Fig. 2x-1, $P = 0.004$). Although we did not measure circulating FUL levels, we hypothesize that circulating levels of FUL were higher when using DMSO-based formulations, leading to more potent inhibition of E_2-stimulated tumor growth.

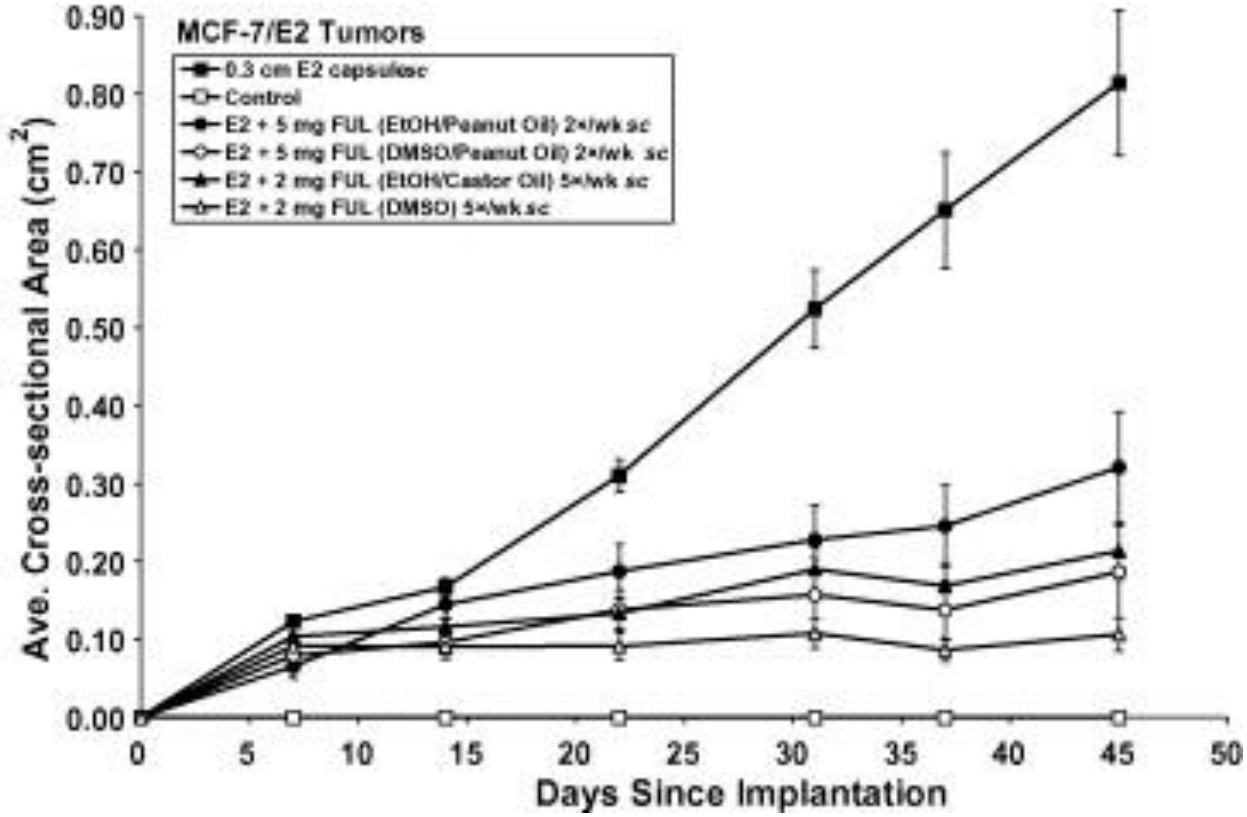


Figure 2x-1. *Growth inhibition of MCF-7/E2 tumors in response to different FUL formulations and dosing schedules. Thirty ovariectomized athymic nude mice were bitransplanted in the axillary mammary fat pads with MCF-7/E2 tumor pieces 1 mm^3 in size. At the time of tumor implantation, the mice were separated into 6 treatment groups of 5 mice each, or 10 tumors per group. The treatment groups were control (no treatment), 0.3 cm E_2 silastic capsule implanted* sc, *and four groups of different formulations/dosing schedules of 10 mg total FUL per week plus the 0.3 cm E_2 capsule* sc. *The 4 FUL formulations/dosing schedules corresponded to: (1) a 50 mg/ml suspension of FUL dissolved first in EtOH*

and then mixed with peanut oil, and administered two times per week as a 5 mg sc *injection; (2) the clinically used Faslodex preparation consisting of a 50 mg/ml solution of FUL in EtOH and castor oil, and administered five times per week as a 2 mg* sc *injection; (3) a 50 mg/ml suspension of FUL dissolved first in DMSO and then mixed with peanut oil, and administered two times per week as a 5 mg* sc *injection; or (4) a 50 mg/ml solution of FUL in 100% DMSO, and administered daily five times per week as a 2 mg* sc *injection. Tumor growth was tracked by weekly measurements using Vernier calipers and calculating the tumor cross-sectional area according to the formula: (length/2 × width/2 × ´). The data shown represent the average tumor cross-sectional area (cm^2) per group ± S.E. The cross-sectional area of E_2-treated tumors was statistically different from that of each of the four E_2 + FUL groups (all* P*-values < 0.0001). Also, the cross-sectional area of tumors in the E_2 + 5 mg FUL (EtOH/peanut oil suspension given 2 days per week) was statistically different from those in the E_2 + 5 mg FUL (DMSO/peanut oil suspension given 2 days per week) group (*P *= 0.0013). Likewise, the cross-sectional area of tumors in the E_2 + 2 mg FUL (EtOH/castor oil solution given 5 days per week) group was statistically different from that of the E_2 + 2 mg FUL (100% DMSO solution given 5 days per week) group (*P *= 0.0038).*

Growth of MCF-7/RAL1 tumors

MCF-7/RAL1 tumors are maintained *in vivo* by serial transplantation into 1.5 mg/day RAL-treated ovariectomized athymic mice. To illustrate the phase of SERM resistance the MCF-7/RAL1 tumor should be categorized into, MCF-7/RAL1 tumor cores were implanted into 20 ovariectomized athymic mice and separated into 4 treatment groups of 5 mice each (10 tumors/group) corresponding 1.5 mg/day RAL *po*, 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc* (Faslodex preparation), and control (no treatment). The MCF-7/RAL1 tumors were significantly stimulated to grow by RAL treatment ($P < 0.0001$) and by E_2 treatment ($P < 0.0001$) compared to control treatment (Fig. 2x-2). However, a modest amount of growth was observed in the control-treated group, indicating that these tumors are not absolutely dependent upon an ER ligand with partial agonist activity. We have previously shown that primary cultures of MCF-7/RAL1 tumors exhibit equivalent levels of estrogen response element (ERE)-regulated reporter gene activity in the absence of E_2 as did primary cultures of MCF-7/E2 tumors when treated with E_2. Thus, the unliganded ER activity in MCF-7/RAL1 tumors is high and probably contributed to the modest growth of these tumors without the need of RAL or E_2. FUL did not significantly effect the growth of MCF-7/RAL1 tumors (Fig.2x-2). Thus, either a SERM or E_2, but not FUL, supports the growth of these MCF-7/RAL1 xenografts. Therefore, these tumors are categorized as Phase I SERM-resistant.

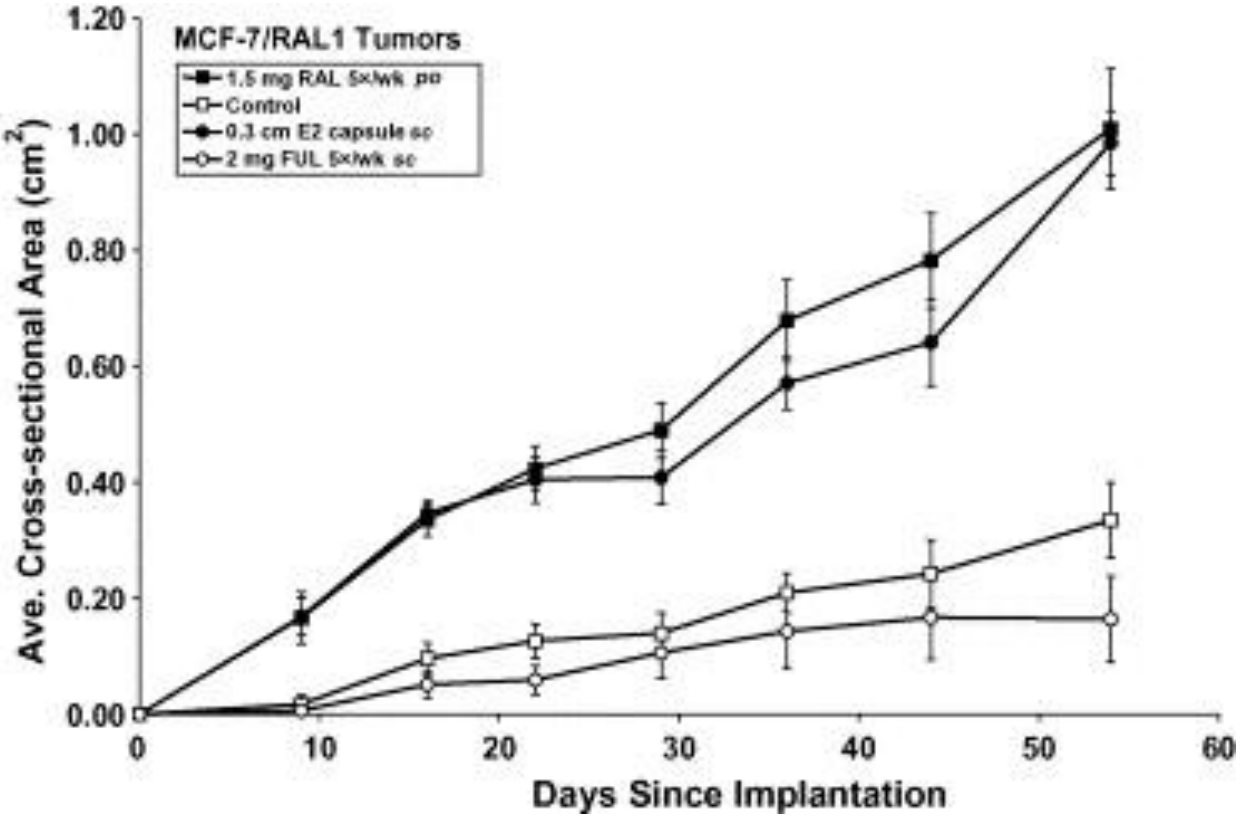


Figure 2x-2. Growth stimulation of MCF-7/RAL1 tumors in response to E_2, and inhibition by FUL. Twenty ovariectomized athymic nude mice were implanted in the axillary mammary fat pads with 1 mm^3 MCF-7/RAL1 tumor pieces and separated into 4 treatment groups of 5 mice each (10 tumors per group) corresponding to 1.5 mg/day RAL *po*, 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc*, and control (no treatment). The data shown represent the average tumor cross-sectional area (cm^2) per group ÑS.E. The cross-sectional area of RAL-treated ($P < 0.0001$) and E_2-treated MCF-7/RAL1 tumors ($P < 0.0001$) was significantly different from control-treated tumors.

Growth of MCF-7/RAL2 tumors

MCF-7/RAL2 tumor cells are maintained *in vitro* by culture in media containing 1 εM RAL. To study the growth properties of MCF-7/RAL2 cells *in vivo*, the cells were grown in culture and injected into 20 ovariectomized athymic mice, which were separated into 4 groups of 5 mice (10 tumors/group) and treated with 1.5 mg/day RAL *po*, 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc* (Faslodex preparation), or control (not treated). The MCF-7/RAL2 tumors only grew when treated with RAL (RAL versus control, $P < 0.0001$), and did not form any palpable tumors by day 42 when treated with E_2, FUL or not treated (control) (Fig. 2x-3). We have previously shown that when MCF-7/RAL2 tumors are allowed to grow by treating with TAM until they are established and then switching treatments to E_2, E_2 causes tumor regression by inducing apoptosis as measured by TUNEL staining. Therefore, growth of the MCF-7/RAL2 tumors was dependent on RAL, but inhibited by E_2 and FUL, which categorizes these tumors as Phase II SERM-resistant.

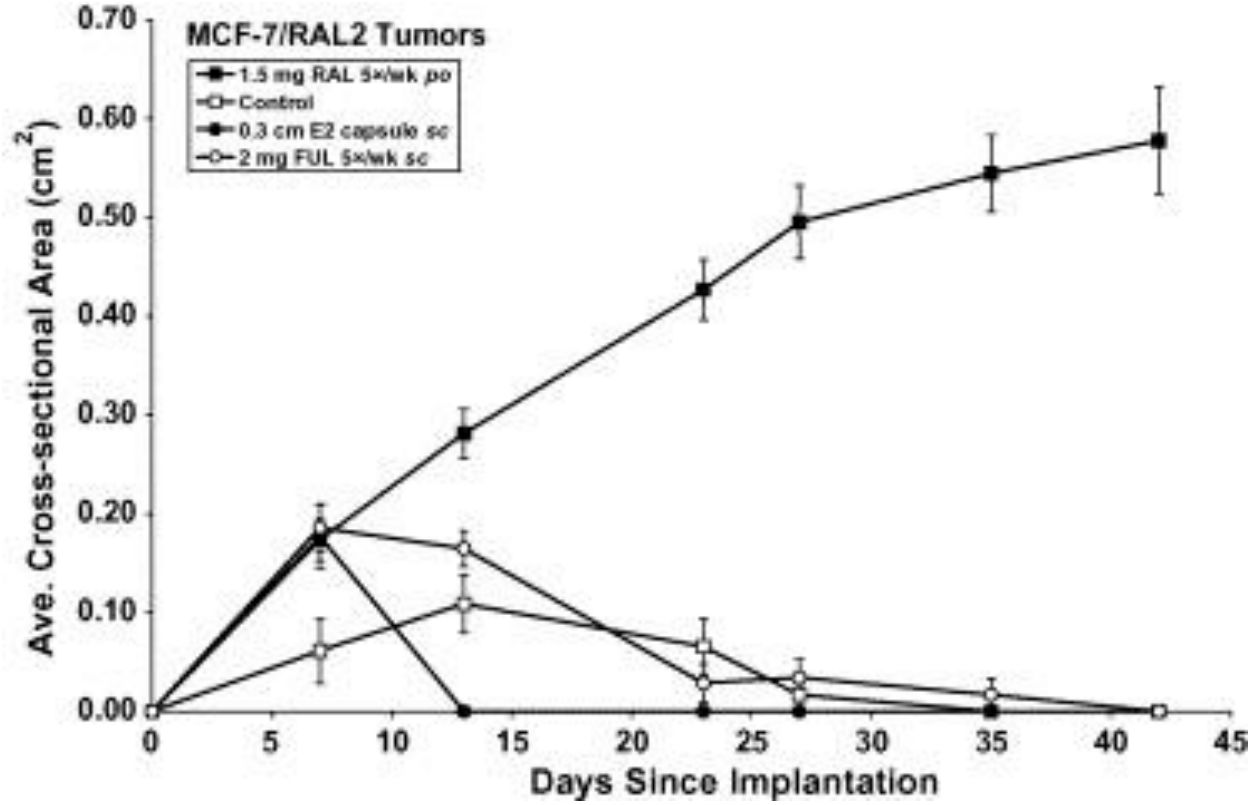


Figure 2x-3. ***Growth inhibition of MCF-7/RAL2 tumors in response to E_2 and FUL.*** *Twenty ovariectomized athymic nude mice were bilaterally injected in the axillary mammary fat pads with 10^7 MCF-7/RAL2 cells grown in culture and separated into 4 treatment groups of 5 mice each (10 tumors per group) corresponding to 1.5 mg/day RAL* po, *0.3 cm E_2 capsule* sc, *2 mg/day FUL* sc, *and control (no treatment). The data shown represent the average tumor cross-sectional area (cm^2) per group ± S.E. The cross-sectional area of RAL-treated MCF-7/RAL2 tumors was significantly different from E_2-treated, FUL-treated and control-treated tumors (all* P*-values < 0.0001).*

Growth of MCF-7/TAM2 tumors

MCF-7/TAM2 tumors are propagated *in vivo* by serial transplantation into 1.5 mg/day TAM-treated ovariectomized athymic mice. To characterize the growth properties of this tumor type, MCF-7/TAM2 tumor cores were implanted into 20 ovariectomized athymic mice, which were separated into 4 groups of 5 mice (10 tumors/group) and treated with 1.5 mg/day TAM *po*, 0.3 cm E_2 capsule *sc*, 2 mg/day FUL (Faslodex) *sc*, or not treated (control). MCF-7/TAM2 tumors were stimulated to grow by TAM compared to the control group (Fig. 2x=4, $P < 0.0001$). The control group did show a minimal amount of growth (Fig. 2x-4), which is hypothesized to be due to substantial unliganded ER activity as in the MCF-7/RAL1 model. FUL did not significantly effect growth of the MCF-7/TAM2 tumors versus control treatment. Interestingly, E_2 did significantly inhibit tumor growth compared to the control group (Fig. 2x-4, $P = 0.0004$). As with the MCF-7/RAL2 tumors, we have previously demonstrated that E_2 treatment leads to regression of MCF-7/TAM2 tumors [84,361]by inducing apoptosis as detected by TUNEL staining [132]. Therefore, TAM stimulated growth, FUL did not support growth, and E_2 inhibited growth of MCF-7/TAM2 tumors, defining this model as Phase II SERM-resistant.

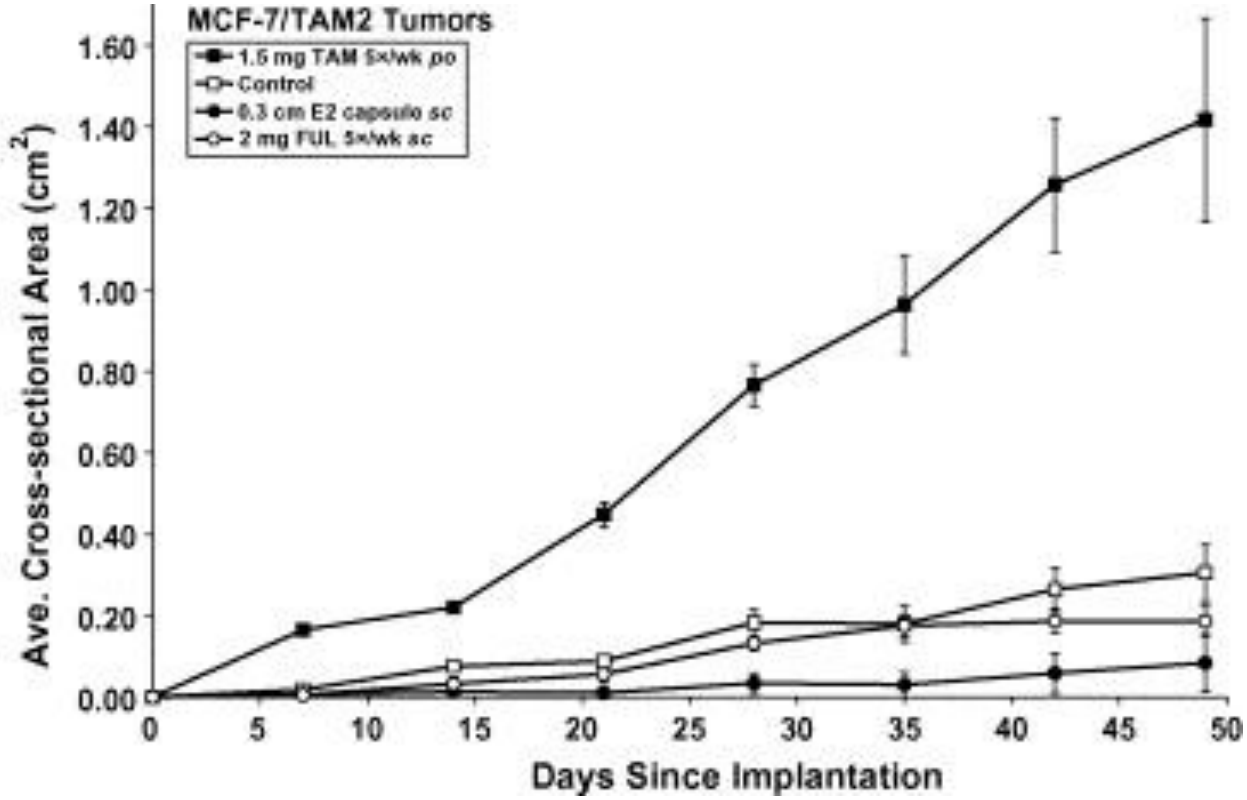


Figure 2x-4. *Growth inhibition of MCF-7/TAM2 tumors in response to E_2 and FUL. Twenty ovariectomized athymic nude mice were implanted in the axillary mammary fat pads with 1 mm^3 MCF-7/TAM2 tumor pieces and separated into 4 treatment groups of 5 mice each (10 tumors per group) corresponding to 1.5 mg/day TAM* po, *0.3 cm E_2 capsule* sc, *2 mg/day FUL* sc, *and control (no treatment). The data shown represent the average tumor cross-sectional area (cm^2) per group ± S.E. The cross-sectional area of TAM-treated (*$P < 0.0001$*) and E_2-treated MCF-7/TAM2 tumors (*$P = 0.0004$*) was significantly different from control tumors.*

Growth of long-term estrogen withdrawn-resistant models

Since having categorized each of the SERM-resistant tumor models as Phase I or II resistant, we characterized the growth properties of cells which have been cultured long-term under estrogen-free conditions to determine whether resistance to estrogen withdrawal (as a surrogate for AI resistance) also evolves through distinct stages. Initially, we compared the proliferation of parental MCF-7 cells with two cell lines resistant to long-term estrogen withdrawal, MCF-7/ED (estrogen-deprived) and MCF-7/5C cells. MCF-7/ED cells were originally selected by culture of parental MCF-7 in estrogen-free medium for >1 year, but were not cloned as a subline, rather they remain a population of cells. In a similar manner, MCF-7/5C cells were also derived from parental MCF-7 cells following long-term estrogen withdrawal, but were cloned as a subline[362]. Notably, MCF-7/ED and MCF-7/5C cells were generated independently in different studies, that is, MCF-7/5C cells were not subcloned from the MCF-7/ED cells.

Growth of parental MCF-7, MCF-7/ED and MCF-7/5C cells was determined by measuring DNA amounts after 7 days in culture. Before beginning the experiment, parental MCF-7 cells were cultured for 4 days in estrogen-free media, since they had been maintained in fully-estrogenized medium. The experiment was started by seeding each of the cell lines in 24-well plates in estrogen-free medium. The cells were treated every 2 days with EtOH (vehicle control), 1 nM E_2, 10 nM FUL, and 1 nM E_2 plus 10 nM FUL. After 7 days, DNA quantities per well were determined using a fluorescence-based DNA assay. As expected in

parental MCF-7 cells, E_2 induced growth by 6.9-fold (E_2 versus control treatment), and this E_2-stimulated proliferation was completely blocked by the addition of FUL (E_2 + FUL versus control) (Fig. 2x-5). Hence, E_2 stimulated proliferation of parental MCF-7 cells in an ER-dependent manner.

Next, MCF-7/ED cells representing a population of cells resistant to estrogen withdrawal were characterized. MCF-7/ED cells grew maximally under estrogen-free conditions (control treatment, 100% growth) and nearly maximally when treated with E_2 (80% of control) (Fig. 2x-5). However, FUL and E_2 plus FUL treatment inhibited growth of MCF-7/ED cells (18% and 29%, respectively, of control) (Fig. 2x-5). Thus, MCF-7/ED cell proliferation was largely unaffected by E_2, but dependence on the ER was demonstrated by the sensitivity of the cells to FUL.

Finally, we evaluated MCF-7/5C cells, which were a clonal derivative of long-term estrogen-withdrawn cells. MCF-7/5C cells grew maximally under estrogen-free conditions (control treatment, 100% growth), but E_2 treatment almost completely blocked proliferation (9% of control) (Fig. 2x-5). Interestingly, FUL-treated MCF-7/5C cells exhibited significant growth (52% of control) (Fig. 2x-5). Further, MCF-7/5C cells treated with E_2 plus FUL showed still greater amounts of proliferation (85% of control) (Fig. 2x-5). In prior studies, we have demonstrated that MCF-7/5C cells undergo apoptosis when treated with E_2, and that co-treatment with FUL blocks this effect of E_2. Hence, MCF-7/5C cells required ER to be unliganded for maximal proliferation and survival, whereas E_2-bound ER led to cytostasis and apoptosis. Further, FUL reversed the apoptotic signal of E_2 and promoted proliferation.

We next verified that the MCF-7/5C cells behaved similarly *in vivo* as a xenograft tumor as they did *in vitro* in cell culture. MCF-7/5C cells were grown in culture and injected into 20 ovariectomized athymic mice. The animals were separated into 4 treatment groups of 5 mice each (10 tumors/group), corresponding to control (not treated), 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc* (Faslodex), and 0.3 cm E_2 capsule *sc* plus 2 mg/day FUL *sc* (Faslodex). MCF-7/5C cells rapidly formed substantial tumors at every injection site (10 out of 10) in control-treated mice by 21 days after inoculation, but only 1 small yet palpable tumor formed out of 10 injection sites in mice treated with E_2, resulting in a highly significant difference in the average tumor cross-sectional area between the two treatment groups (Fig. 2x-6, $P < 0.0001$). In a prior report, we have shown that E_2 induces tumor regression and apoptosis in established MCF-7/5C xenograft tumors. Importantly, MCF-7/5C xenograft tumors showed robust growth in the presence of FUL or E_2 plus FUL, which was not significantly different than growth of the control (no treatment) group, but was significantly greater than in the E_2 treatment group (Fig. 2x-6, FUL versus E_2, $P < 0.0001$; E_2 + FUL versus E_2, $P < 0.0001$). Hence, the MCF-7/5C xenograft tumor model was resistant to growth inhibition by FUL, and FUL treatment abrogated E_2-mediated growth inhibition.

Considering these varied growth responses together, parental MCF-7 cells model the therapeutic stage of antihormonal therapy; MCF-7/ED cells represent Phase I resistance to estrogen withdrawal since they grew independent of E_2 yet remained sensitive to FUL; and MCF-7/5C tumors/cells were classified as Phase II resistant to estrogen withdrawal since E_2 inhibited their growth, but were resistant to growth inhibition by FUL or E_2 plus FUL.

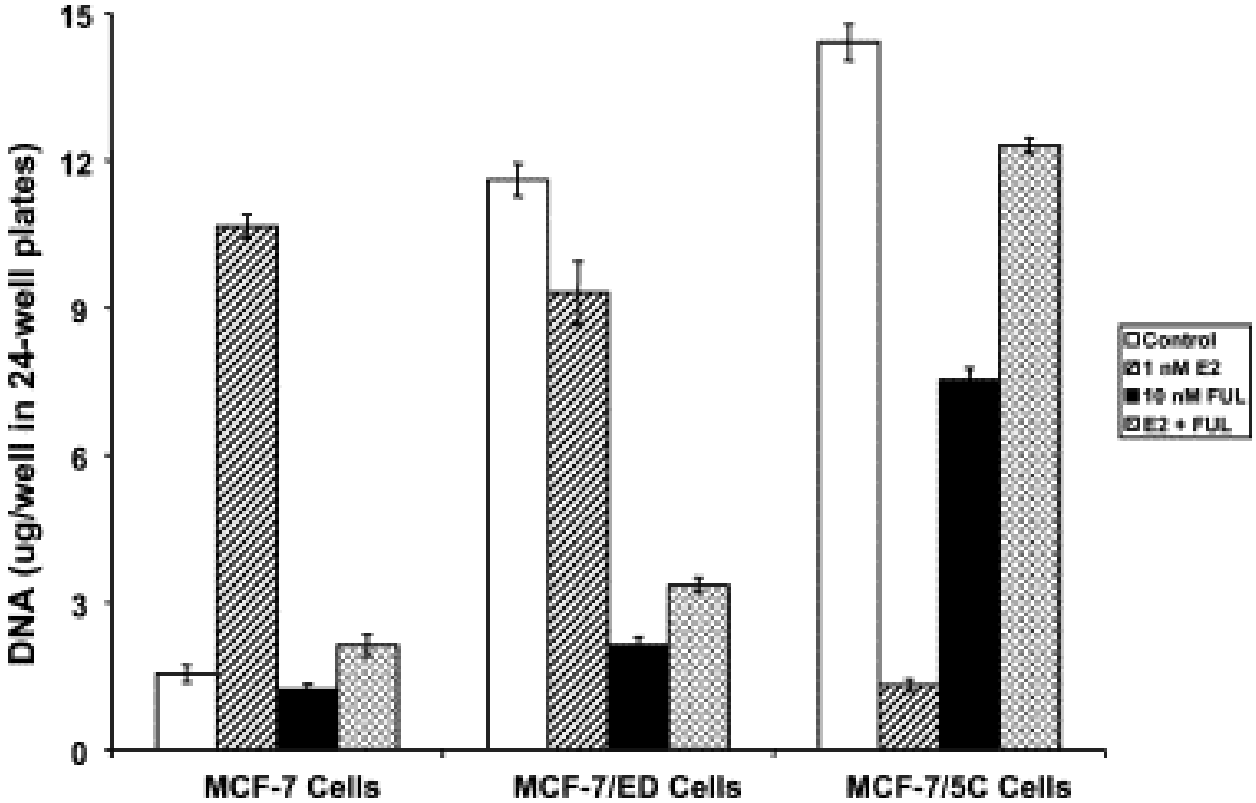


Figure 2x-5. ***Differential proliferation of MCF-7 long-term estrogen withdrawn cell culture models in response to E_2, FUL, and E_2 plus FUL for 7 days.*** *Cells were cultured under estrogen-free conditions for 4 days, and then seeded at 2×10^4 cells per well in a 24-well plate. Beginning 24 h after seeding (day 0) and every 2 days thereafter up to 6 days (days 2, 4, and 6), the cells were treated with 1 nM E_2, 10 nM FUL, 1 nM E_2 + 10 nM FUL, or control (0.1% EtOH)-treated. The experiment was stopped on day 7. As a measure of proliferation, the amount of DNA per well was determined using a fluorescence-based DNA quantitation assay. Data are shown as the mean of 6 replicate wells per group ± S.D. The experiment was performed three times independently, and one representative experiment is shown.*

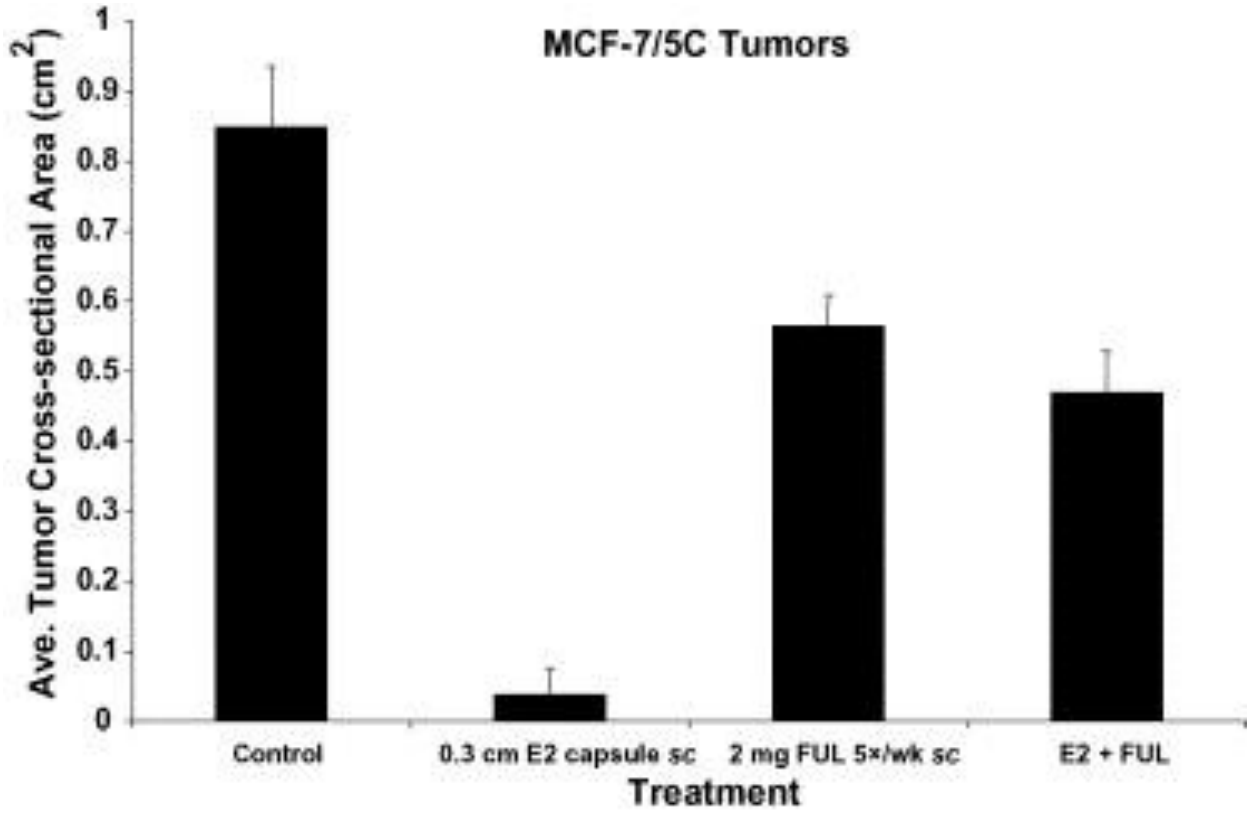


Figure 2x-6. ***Growth inhibition of MCF-7/5C tumors in response to E_2 treatment, and resistance to FUL, and E_2 plus FUL.*** *Twenty ovariectomized athymic nude mice were bilaterally injected in the axillary mammary fat pads with 10^7 MCF-7/5C cells grown in culture and separated into 4 treatment groups of 5 mice each (10 tumors per group) corresponding to control (no treatment), 0.3 cm E_2 capsule* sc, *2 mg/day FUL* sc, *and 0.3 cm E_2 capsule* sc *+ 2 mg/day FUL* sc. *The data are shown as a histogram on day 21 of the average tumor cross-sectional area (cm^2) per group ± S.E. The cross-sectional areas of control-treated, FUL-treated, and E_2 plus FUL-treated MCF-7/5C tumors were each significantly different from E_2-treated (all* P*-values < 0.0001). The cross-sectional area of both FUL-treated and E_2 plus FUL-treated MCF-7/5C tumors were not significantly different from that of control-treated MCF-7/5C tumors.*

Response of Phase II SERM-resistant tumor models to E_2 plus FUL

Since we observed that MCF-7/5C cells grew better when treated with E_2 plus FUL than with E_2 alone, we examined the effect of FUL in a background of physiologic E_2 in the Phase II SERM-resistant tumor models. The data from the MCF-7/RAL2 experiment depicted in Fig. 2x-3 was re-evaluated with an additional group of 5 animals (10 tumors) treated with a 0.3 cm E_2 capsule *sc* plus 2 mg/day FUL (Faslodex). MCF-7/RAL2 tumors treated with E_2 plus FUL showed robust growth compared to no palpable tumors in the E_2 alone ($P < 0.0001$), FUL alone ($P < 0.0001$), or control groups ($P < 0.0001$) (Fig. 2x-7). Therefore, E_2 plus FUL, when combined, negated the growth inhibitory effects of either compound by itself.

We then tested whether this interaction between physiologic E_2 and FUL also occurred in the MCF-7/TAM2 tumor model of Phase II SERM resistance. However, this experiment was designed to evaluate effects of different treatments on tumors once they are established by allowing tumors to grow in the presence of TAM until they were palpable, and then randomized to different treatment groups. MCF-7/TAM2 tumor cores were implanted into 25 ovariectomized athymic mice. All animals were treated with 1.5 mg/day TAM *po* until tumors grew to an average cross-sectional area of 0.24 cm^2, at which time TAM

treatment was withdrawn for 1 week to allow time for this drug to be completely metabolized and clear the animalsô systems. Following the 1 week of TAM withdrawal, the average cross-sectional area of all tumors was 0.37 cm^2, and the animals were randomized into 5 groups of 5 mice each (10 tumors/group) corresponding to continuing 1.5 mg/day TAM *po*, 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc*, 0.3 cm E_2 capsule *sc* plus 2 mg/day FUL *sc*, and control (no treatment). As would be predicted from the MCF-7/TAM2 experiment depicted in Fig.2x-4, TAM treatment significantly stimulated growth ($P = 0.0026$) and E_2 significantly inhibited growth ($P = 0.0098$) compared to the control group on day 52 (Fig. 2x-8). The size of FUL treated tumors was not significantly different than the control group. In contrast, we noted that tumors treated with the combination of E_2 + FUL did exhibit significantly greater growth than the control group (Fig. 2x-8, $P = 0.018$). Thus, in a second model of Phase II SERM resistance, growth inhibition by E_2 alone was negated in the presence of FUL, leading to growth stimulation.

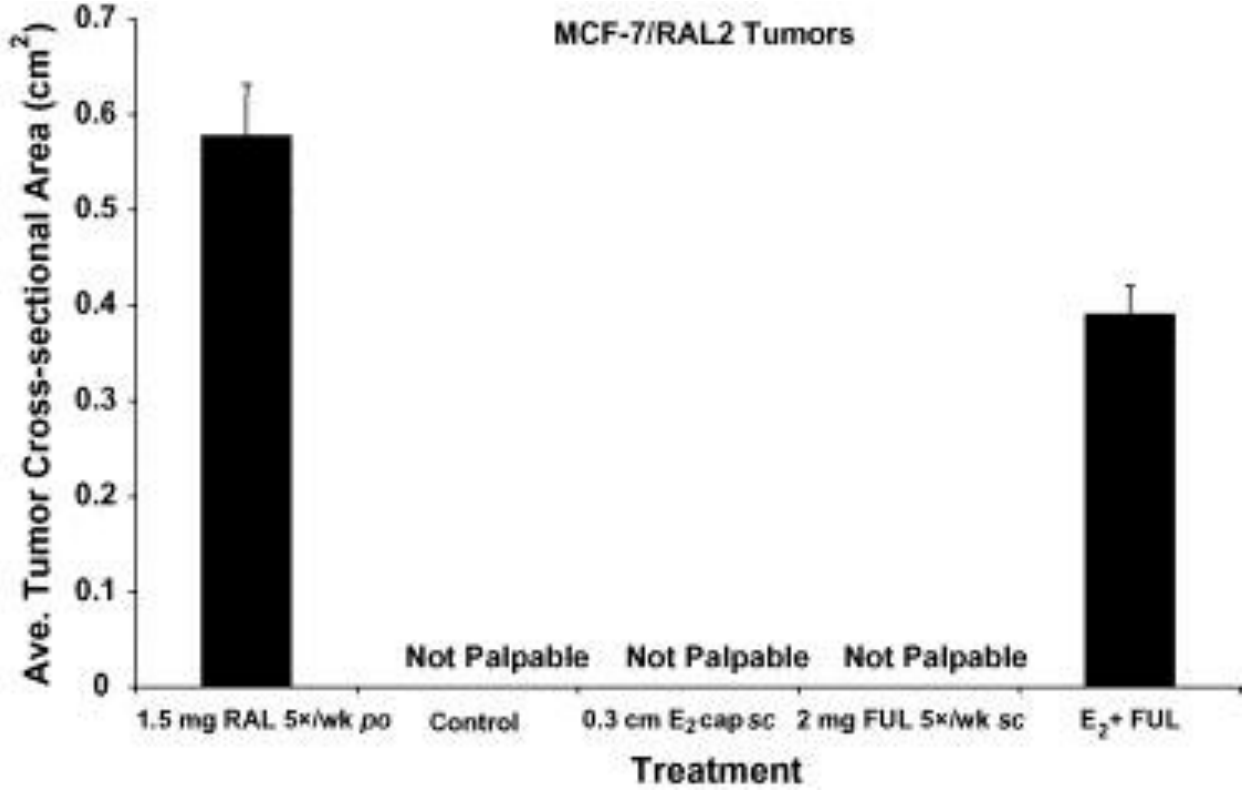


Figure 2x-7. ***E_2 plus FUL-stimulated growth of MCF-7/RAL2 tumors.*** *Data are from Fig. 2x-3 on day 42 and shown as a histogram, but supplemented with the additional group of five ovariectomized athymic mice (10 tumors) treated with 0.3 cm E_2 capsule* sc *plus 2 mg/day FUL* sc. *The cross-sectional area of E_2 plus FUL-treated MCF-7/RAL2 tumors was significantly different from that of control-treated, E_2-treated, and FUL-treated MCF-7/RAL2 tumors (all* P*-values < 0.0001).*

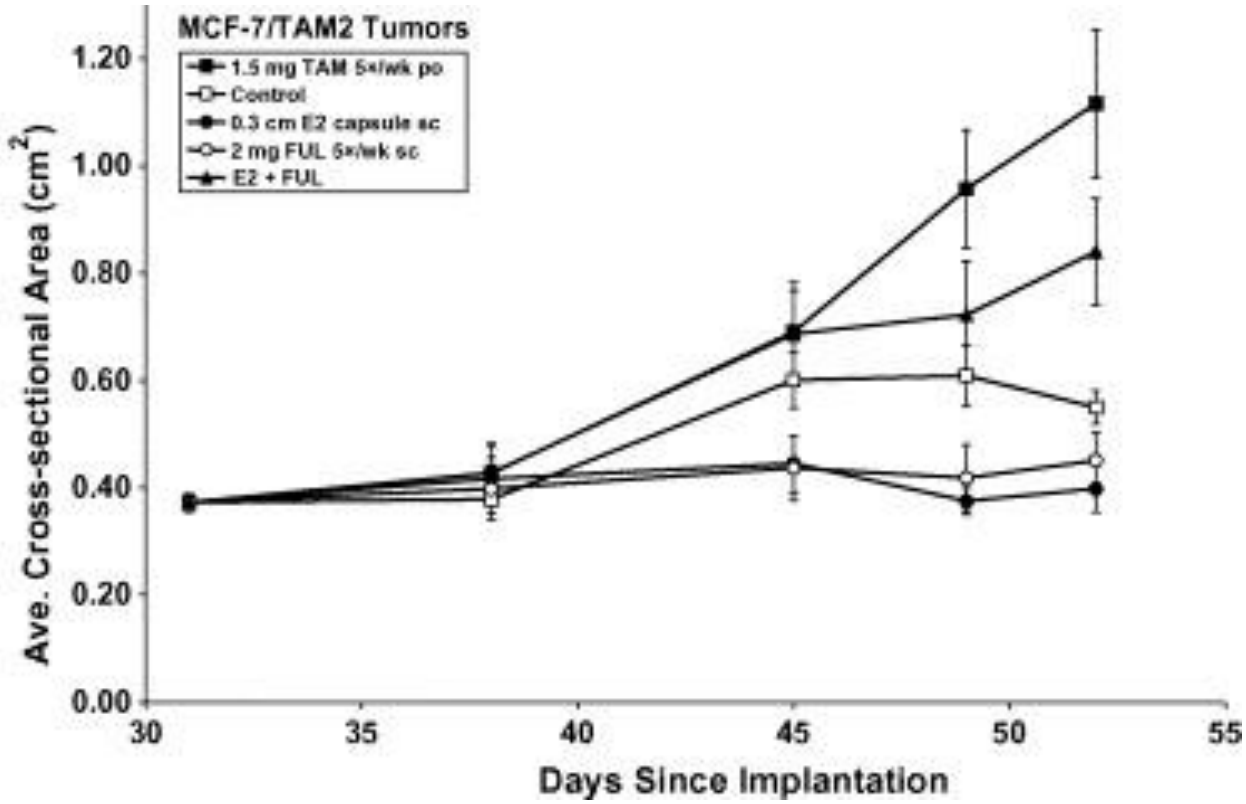


Figure 2x-8. ***E_2 plus FUL-stimulated growth of MCF-7/TAM2 tumors.*** *Twenty-five ovariectomized athymic mice were implanted in the axillary mammary fat pads with 1 mm^3 MCF-7/TAM2 tumor pieces, and then treated with 1.5 mg/day TAM* po *until the tumors were established at 0.24 cm^2, then TAM was withdrawn for 1 week. Following 1 week of TAM withdrawal, the tumors reached an average cross-sectional area of 0.37 cm^2 and the animals were separated into 5 treatment groups of 5 mice each (10 tumors per group) corresponding to 1.5 mg/day TAM* po*, 0.3 cm E_2 capsule* sc*, 2 mg/day FUL* sc*, 0.3 cm E_2 capsule* sc *plus 2 mg/day FUL* sc*, and control (no treatment). The data shown represent the average tumor cross-sectional area (cm^2) per group ± S.E. The cross-sectional areas of MCF-7/TAM2 tumors at day 52 were compared by one-way ANOVA. The cross-sectional areas of TAM-treated (*P = 0.0026*), E_2-treated (*P = 0.0098*), and E_2 plus FUL-treated MCF-7/TAM2 tumors (*P = 0.018*) were significantly different from control-treated tumors.*

Discussion

We sought to discover unifying principles involved in the development of antihormone resistance by systematically studying the growth properties of a panel of antihormonally resistant MCF-7-based breast cancer xenograft tumor models. We have confirmed and extended prior observations that have allowed the categorization of these tumor models as either Phase I or Phase II antihormone resistant. Phase I SERM resistance was characterized by growth stimulation in response to both a SERM or E_2 (MCF-7/RAL1 tumors, Fig. 2x-2), while in Phase II SERM resistance, only the SERM stimulated growth, whereas E_2 inhibited growth (MCF-7/RAL2 and MCF-7/TAM2, Fig. 2x-3 and Fig. 2x-4, respectively). Phase I long-term estrogen withdrawn (AI)ð resistant cells in culture grew independently of E_2 (MCF-7/ED cells, Fig. 2x-5), but Phase II resistant tumors were growth inhibited by E_2 (MCF-7/5C tumors, Fig. 2x-6). Hence, long-term blockade of ER activity by either SERMs or estrogen withdrawal can lead to selection of cells in which E_2 signals no longer proliferation, but rather inhibition, of growth, and as we have previously reported, apoptosis [132,234].

We also found that growth of Phase II SERM resistant tumors was not supported by FUL, but Phase II estrogen withdrawn-resistant tumors were cross-resistant to FUL (MCF-7/5C tumors, Fig. 2x-6). Further, FUL combined with physiologic E_2 nullified the inhibitory effects of either compound alone and led to stimulation of growth in Phase II SERM-resistant tumors (MCF-7/RAL2 and MCF-7/TAM2 tumors, Fig. 2x-7 and Fig. 2x-8, respectively), and supported growth in Phase II estrogen withdrawn-resistant tumors (MCF-7/5C tumors, Fig.2x- 6).

Noteworthy, the concentrations of FUL used in the cell culture proliferation experiments was 10 nM. We chose this concentration of FUL to reflect the circulating levels that are achieved clinically. In the clinic, FUL is not administered orally because of low bioavailability; rather it is given intra muscularly (as a single 250 mg dose once per month) to achieve slow constant release of the drug. In two independent multi-national Phase III clinical efficacy trials evaluating FUL in advanced breast cancer patients, the steady state circulating concentrations of FUL were determined to be approximately 6ï 7 ε g/l (9.9ï 11.5 nM) in the European trial and 9 ε g/l (14.8 nM) in the North American trial. Hence, we used FUL at 10 nM in cell culture, reflecting the circulating concentrations of FUL achieved in women, but this concentration was much lower than the 100 nM to 1 ε M FUL concentrations used in most cell culture studies. We hypothesize that the low circulating concentrations of FUL in patients may contribute to the lower than expected response rates in the clinic as would be predicted by the effectiveness of FUL in cell culture. In support of this hypothesis, we have found that while MCF-7/5C cells proliferate in the presence of 10 nM FUL, they do not in 1 ε M FUL (unpublished, JS Lewis-Wambi and VC Jordan).

The distinct growth responses of the tumor and cell culture models studied here illustrate that resistance to hormonal blockade therapy continually evolves but can be separated into at least two phases. Antihormonal resistance develops from selection of specific cell types that survive and proliferate when the ER is bound by a partial antiestrogen (Phase I SERM resistance) or unliganded (Phase I estrogen withdrawn-resistance). Prolonged hormonal blockade therapy maintains selective pressure, such that Phase I resistant cells continue to evolve to a Phase II resistant phenotype, and likely undefined additional phases. However, the study of Phase II antihormonal resistance has revealed a new biology of E_2 action involved in apoptosis that could be exploited to benefit breast cancer patients who have been exhaustively treated with SERMs and AIs. Moreover, the finding that FUL in a background of physiologic E_2 stimulated growth of Phase II resistant cells has important clinical implications. This knowledge can be implemented to optimize the application of third-line antihormonal therapy. We propose that patients who have responded and then failed two antihormone therapies may exhibit Phase II resistant characteristics, and therefore respond to low-dose short-term estrogen therapy. The estrogen therapy would lead to apoptosis in the Phase II resistant cells and thereby debulk the tumor. Prior laboratory studies indicate that cells which remain are re-sensitized to first-line or second-line antihormonal therapy. Hence, the low-dose short-term estrogen therapy would be followed by FUL plus an AI, to avoid the possible selection of cells that could grow in response to FUL plus physiologic E_2.

TASK 3: (GU/Riegel and Wellstein) - To decipher cellular signaling pathways using proteomics and to mesh proteomics and mRNA analysis.

Task 3a (Riegel and Wellstein) - Studies carried out by Drs. Reigel and Wellstein in the Reigel Wellstein laboratory at Georgetown University

Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells.

Introduction

To identify pathways that initiate estrogen-induced apoptosis versus growth, we used a combined proteomics and systems biology approach to elucidate triggering events and associated signaling pathways. We focused on changes of AIB1 interacting proteins, because of its central role in estrogen control of phenotypic behavior of breast cancer cells outlined above. AIB1 also coactivates IGF1R, EGFR and HER2 through modulation of tyrosine phosphorylation of these transmembrane receptors and phosphorylation of their subsequent signaling intermediaries [67,363,364,365]. Thus, to complement the analysis of direct AIB1 interacting proteins, we also monitored changes of phosphotyrosine (pY)-containing protein complexes, that are most likely regulated by growth factor signaling, as a means of discovering global intersecting pathways. As a model system, we used MCF-7 cells that proliferate in response to E2 [366], but also respond to EGF and heregulin [367] and have high levels of AIB1 protein due to gene amplification [368]. Wild-type MCF-7 cells were compared with MCF-7:5C cells that had been isolated under estrogen-free growth conditions [83,195]. MCF-7:5C cells were derived following long-term culture of MCF-7 cells in phenol red-free media. MCF-7:5C cells are ER-positive and undergo apoptosis after exposure to physiological concentrations of E2. In contrast, wild-type parental MCF-7 cells proliferate in the presence of the same concentration range of E2 [83,195]. The MCF-7:5C cells represent many of the characteristics of Phase II SERM resistant cells [369]. A parallel analysis after estrogen stimulation of these isogenic breast cancer cell lines served as a basis for the comparisons of signaling responses.

Here, we show that RNAi-mediated depletion of AIB1 reduces E2-induced growth of MCF-7 cells, and reverses the estrogen-induced apoptosis in MCF-7:5C cells. AIB1-interacting and pY-containing protein complexes were immunoprecipitated from short-term E2-treated cells, and the complexed proteins were identified by mass spectrometry (MS) analysis (Fig. 3a-1A). From a comparison of the data sets obtained with MCF-7 versus MCF-7:5C cells treated with or without E2, and from a computationally-derived global AIB1-interacting network prediction, we identified pathways that participate in the differential response to E2 in these breast cancer cells. We found that a limited number of major cellular signaling pathways i.e. GPCR, PI3 kinase, Wnt, Notch and their associated molecules were involved in the control of estrogen induced proliferative or apoptotic responses. This information will be useful for determining appropriate targets to induce apoptosis in endocrine resistant human breast cancer.

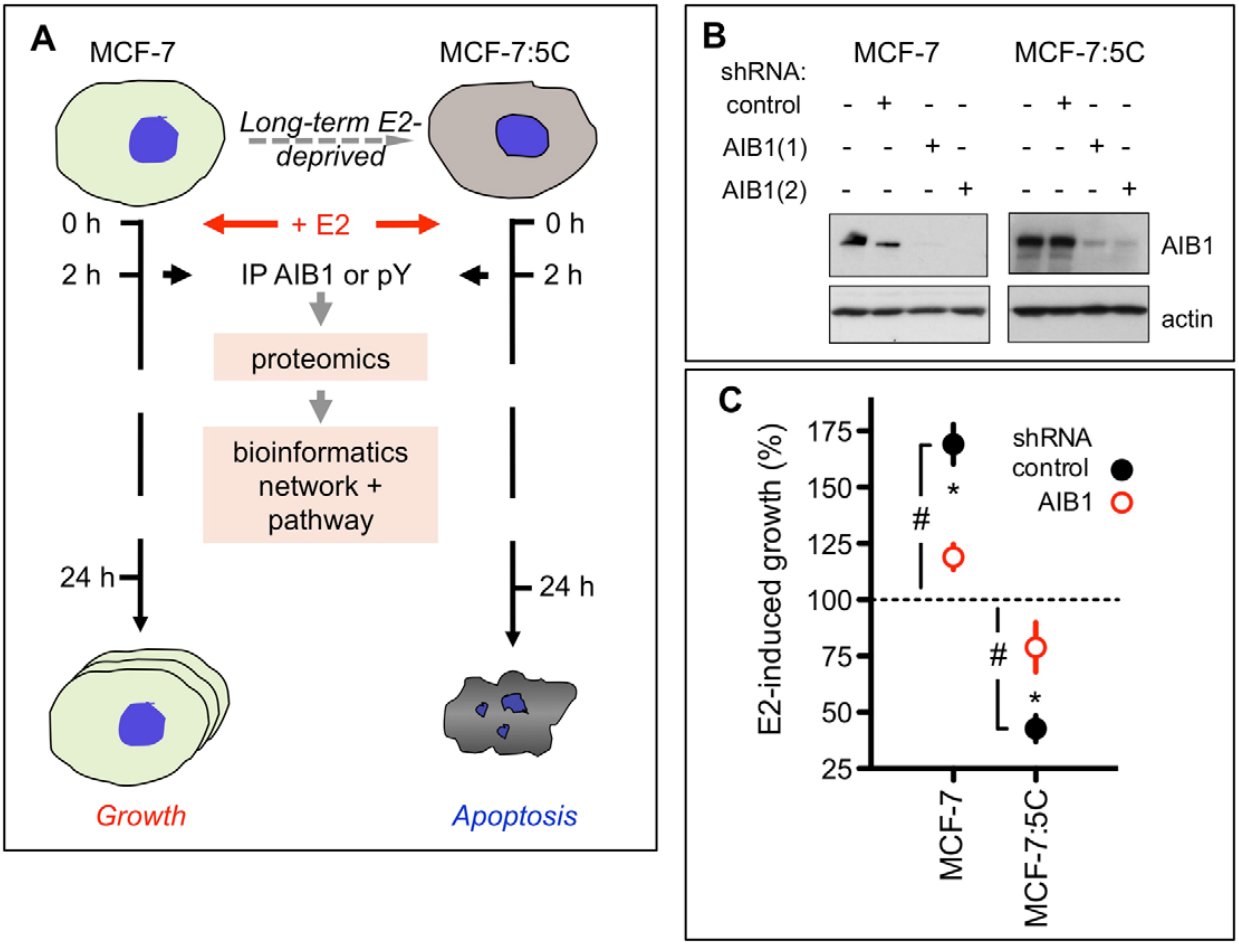


Figure 3a-1. *Phenotypic impact of AIB1 depletion on estradiol (E2) growth response in MCF-7 or MCF-7:5C cells (A) The experimental paradigm. The differential responses to estradiol (E2) treatment of MCF-7 (cell growth) and long-term estrogen deprived MCF-7:5C cells (apoptosis) are indicated. Proteomics profiles of the two cell lines at baseline and after a brief (2 h) E2 treatment were generated using immunoprecipitations (IP). Proteins interacting with AIB1 or phosphotyrosine containing protein complexes were isolated by IP followed by mass spectrometry. Data were then subjected to an integrated bioinformatics analysis of signaling pathways and protein networks. (B,C) Reversal of E2-dependent effects on MCF-7 and MCF-7:5C after depletion of endogenous AIB1 protein using two different lentiviral shRNAs. MCF-7 or MCF-7:5C cells were infected with lentiviral particles expressing control or AIB1-targeting shRNAs. (B) RNAi-mediated knockdown was assayed by Western blot analysis for AIB1 relative to an actin loading control. (C) Cell growth was assayed 6 days after plating without or with E2. The E2 effect is shown relative to the respective untreated controls (mean +S.E.M.). Closed circles: control shRNA; Open circles (red): AIB1 shRNA. #, $p<0.05$ E2 treatment effect vs. no treatment in control shRNA cells; *, $p<0.05$ E2 treatment effect in control shRNA cells vs. E2 treatment in AIB1 depleted cells. Representative data from one of at least three independent experiments are shown.*

Work Accomplished:

Impact of AIB1 depletion on E2-induced growth effects in MCF-7 and MCF-7:5C cells

To determine the role of AIB1 in the E2-induced, distinct growth phenotypes of MCF-7:5C and wild-type MCF-7 cells, both cell lines were infected with lentiviral vectors that express control or two distinct AIB1-targeted shRNAs, and selected in puromycin for stable integrants. Both MCF-7 and MCF-7:5C cells were depleted of AIB1 protein, compared to uninfected and control shRNA infected cells with either of the shRNAs (Fig. 3a-1B). Treatment with E2 significantly induced growth of control shRNA-infected MCF-7 cells and reduced the growth of MCF-7:5C cells (Fig. 3a-1C, black symbols). In contrast to this, in AIB1-depleted, wild-type MCF-7 cells, E2 did not stimulate growth significantly above baseline and in AIB1 depleted MCF-7:5C, E2 lost its apoptosis-inducing effect (Fig. 3a-1C, red symbols). These data suggest that AIB1 is a significant control hub of the E2-controlled growth phenotype in these ER-positive breast cancer cells.

Global analysis of AIB1- and phosphotyrosine-complexed proteins

Because AIB1 is rate-limiting for the E2-induced changes in the growth phenotype of MCF-7 and MCF-7:5C cells, we performed AIB1-specific immunoprecipitations of lysates from untreated and E2-treated (2 hrs) MCF-7 and MCF-7:5C cells to fractionate the respective proteome. Immunoprecipitation of phosphotyrosine-containing protein complexes was also performed to complement the AIB1-specific proteome fractionation (Fig. 3a-1A). The immunoprecipitates were released from the beads, separated by denaturing gel electrophoreses (SDS-PAGE) and followed by Coomassie Blue staining of proteins in the gels [54]. Visible bands and the same region in parallel gel lanes were harvested and proteins present identified by mass spectrometry (MS). Stringent filtering of the initial proteomic data resulted in a subset of 101 proteins that either interacted with AIB1 (n=58, Table S1 in [54]) or are present in pY-protein complexes (n=56, Table S2 in [54]), with 13 proteins common to both.

The analytical approach emphasizes reliable identification of proteins by correlating mass spectrometry ID with the apparent molecular mass obtained from the SDS-PAGE [54]. This approach mimics Western blotting without having to rely on the availablility of antibodies, appropriate sensitivity, suitability for Western blotting and specificity. Still, we used Western blotting of some proteins identified by MS and show two examples in [54] (see below). To validate the mass spectrometry findings, separate experiments with independent mass spectrometry analyses were run. We found 48% of the proteins reported here in two and 16% in three or more independent experiments. This compares favorably with a recent HUPO study where only 7 of 27 laboratories identified all 20 proteins present at equimolar concentrations in a test sample [370]. In our experiments, the abundance of individual endogenous proteins captured in the immunoprecipitates covers a wide range [54]. Thus, we expected that lower abundance proteins may drop below detection in repeat experiments. A combination of bioinformatics and mass spectrometry analysis was thus applied to meet this challenge as also described elsewhere [371,372].

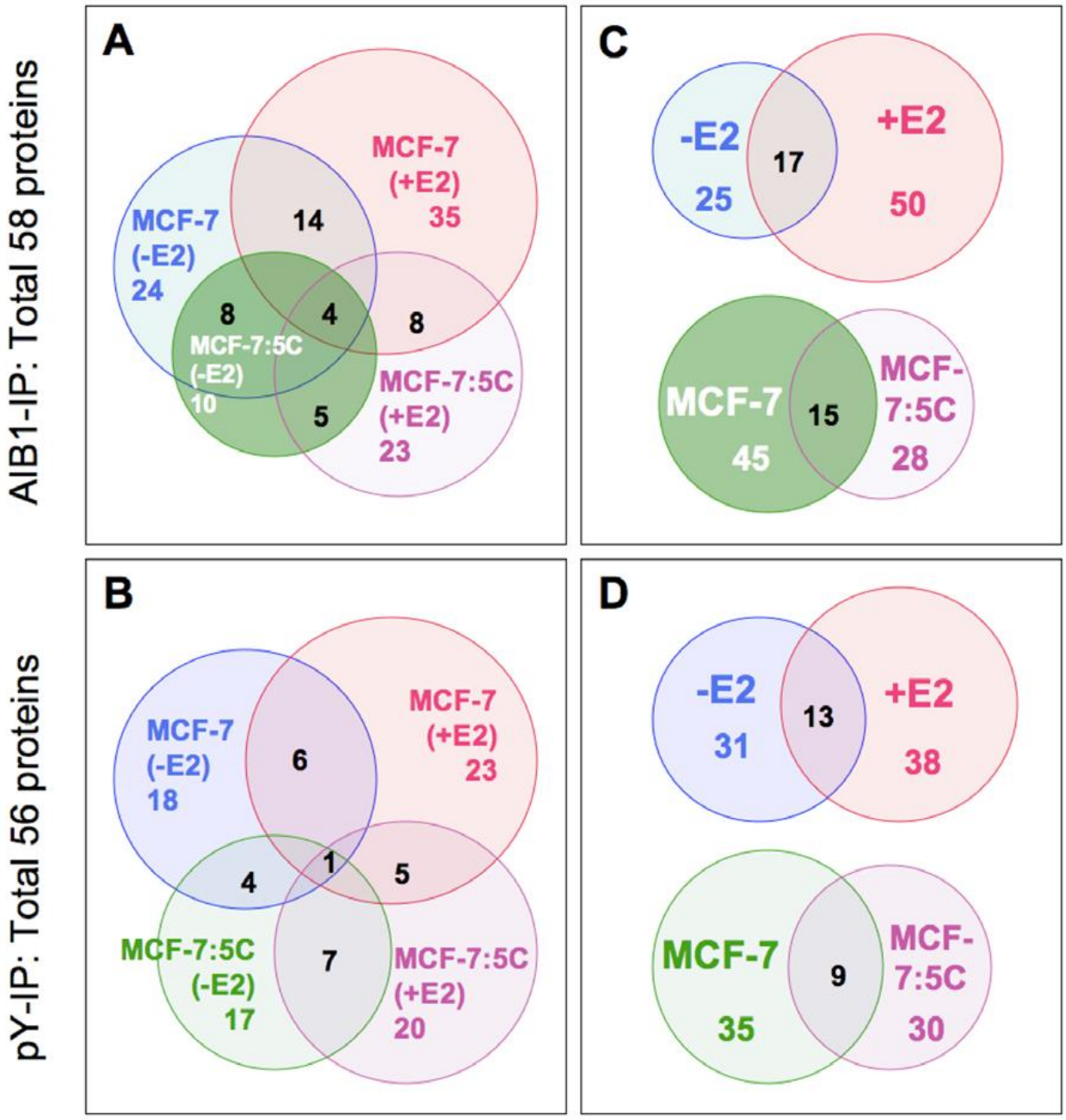
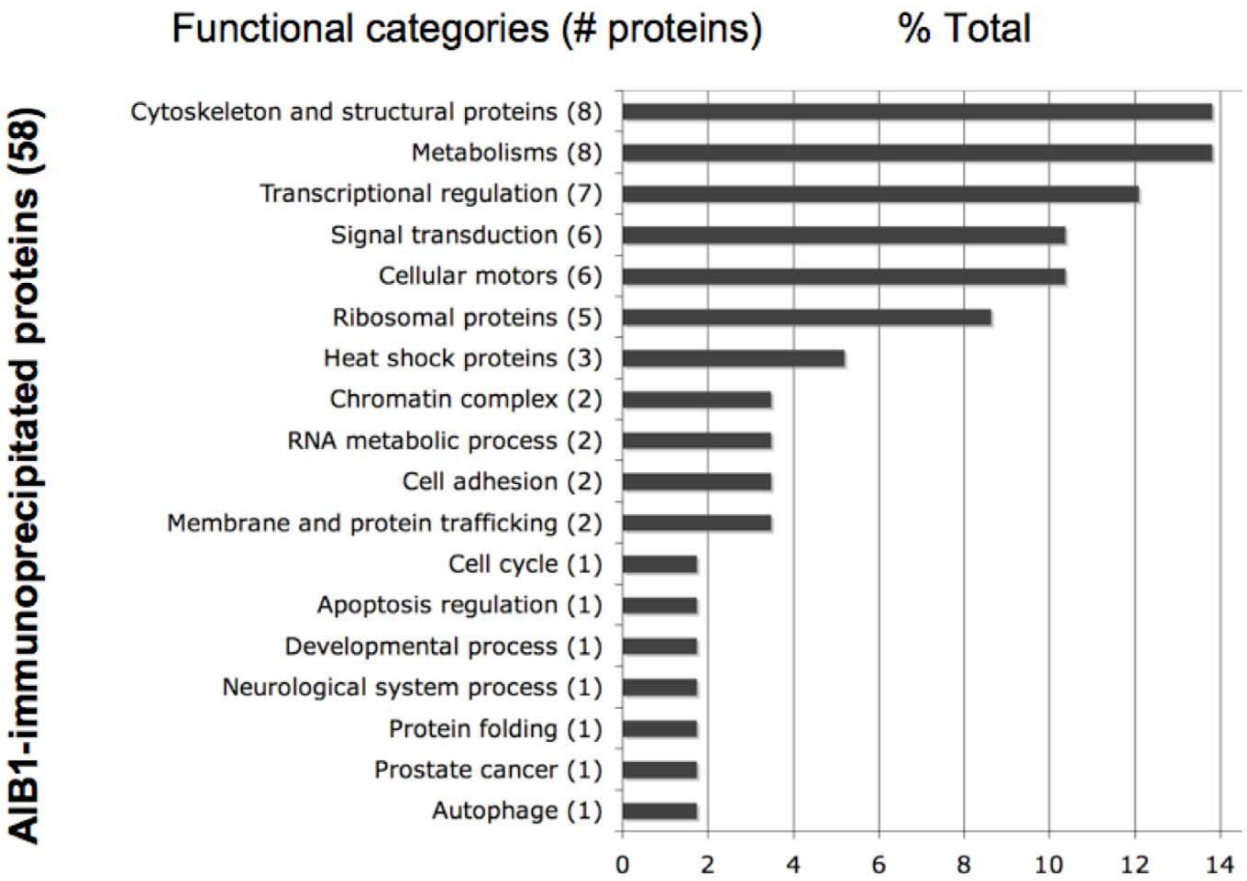


Figure 3a-2. *Summary of proteins identified under different conditions. Venn diagrams of proteins identified from anti-AIB1 (A,C) or anti-pY IP (B,D) experimental groups. (C,D) Proteins in combined AIB1-IP or pY-IP data sets. Individual proteins and subgroups are shown in Tables S1 & S2 in [54].*

The Venn diagrams of proteins pulled down with anti-AIB1 or anti-pY (Fig. 3a-2) show the distribution of proteins between E2-treated and untreated, as well as wild-type MCF-7 versus MCF-7:5C cells (A and B), or between E2-treated and untreated cells regardless of cell type (C, *top*; and D, *top*), or between MCF-7 and MCF-7:5C cells regardless of treatment (C, *bottom*; and D, *bottom*). The number of pY-complexed proteins identified was affected very little by E2 treatment (18 vs. 25 proteins) with 13 proteins in either treatment group (Fig. 3a-2D). In contrast, there was a significant,

4-fold higher number of AIB1-interacting proteins in the E2-treatment group (8 vs. 33 proteins; $p<0.05$, chi-square test; Fig.3a-2C) with 17 proteins not impacted in their interaction with AIB1. This suggests that AIB1-mediated protein-protein interactions are more responsive to E2 treatment, and new protein complexes are induced by E2 (Fig. 24A,C). In addition, the total number of proteins in complexes with AIB1 that overlap between MCF-7 and MCF-7:5C cells was not altered by the treatment, although the fraction of proteins per cell line that overlap decreases by 1/2 with E2-treatment (31% to 16%; Fig. 24A). Finally, while pathways activated by E2 gave rise to different sets of pY-containing protein complexes in both MCF-7 and MCF-7:5C cells, the percentage of proteins that overlap between cell lines remain almost constant regardless of treatment (4 vs. 5 in Fig. 24B).

Figure 3a-3 shows the functional categories ascribed to the AIB1-associated (top) and pY-complexed (bottom) proteins. Tables S1 and S2 in [54] identify the proteins in each of these categories, cell lines (MCF-7 versus MCF-7:5C), and conditions (+/- E2) under which they were identified. Nearly half of the AIB1-interacting proteins fall into four categories, i.e. cytoskeleton and structural proteins, metabolism, transcription regulation, and signal transduction. Most of the pY-complexed proteins fall into four major functional categories: cytoskeleton and structural proteins, transcription regulation, signal transduction, and protein transport and vesicle trafficking. Thirteen proteins were found to be both AIB1-interacting and pY-complexed in MCF-7 and MCF-7:5C cells (Table S1 in [54]).


Functional categories (# proteins)
% Total
AIB1-immunoprecipitated proteins (58)
Cytoskeleton and structural proteins (8)
Metabolisms (8)
Transcriptional regulation (7)
Signal transsduction (6)
Cellular motors (6)
Ribosomal proteins (5)
Heat shock proteins (3)
Chromatin complex (2)
RNA metabolic process (2)
Cell adhesion (2)
Membrane and protein trafficking (2)
Cell cycle (1)
Apoptosis regulation (1)
Developmental process (1)
Neurological system process (1)
Protein folding (1)
Prostate cancer (1)
Autophage (1)
0
2
4
6
8
10
12
14

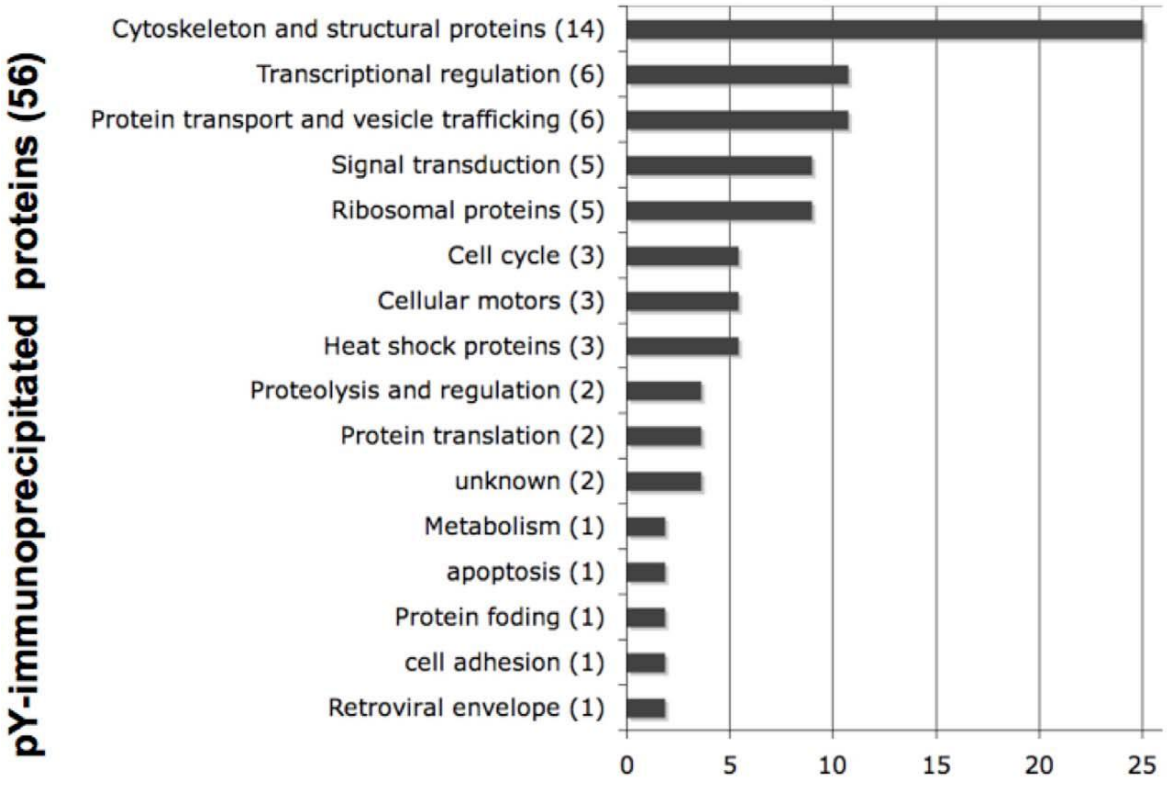

pY-immunoprecipitated proteins (56)
Cytoskeleton and structural proteins (14)
Transcriptional regulation (6)
Protein transport and vesicle trafficking (6)
Signal transsduction (5)
Ribosomal proteins (5)
Cell cycle (3)
Cellular motors (3)
Heat shock proteins (3)
Proteolysis and regulation (2)
Protein translation (2)
unknown (2)
Metabolism (1)
apoptosis (1)
Protein foding (1)
cell adhesion (1)
Retroviral envelope (1)
0
5
10
15
20
25

Figure 3a-3. *Functional categories of anti-AIB1 (upper) and anti-pY immunoprecipitated proteins (lower) from MCF-7 and MCF-7:5C breast cancer cells. Numbers in parenthesis are the number of proteins belonging to the respective category. Proteins profiled are those with CI values ≥95% from mass spectrometry.*

Distinct profiles were observed for metabolism-related proteins between AIB1- and pY-complexed proteins, where the AIB1 complexes contained eight different enzymes in contrast to only one in the anti-pY group. This is consistent with studies demonstrating that AIB1 plays a role in the control of basal metabolic processes [373,374] that resulted in growth retardation and reduced hormonal responses in AIB1 knock-out mice [375]. Quite strikingly, all of these proteins were identified in E2 treated cells (e.g. 5-oxoprolinase in MCF-7:5C and fatty acid synthase in MCF-7 cells), whereas only three were identified in untreated as well as E2 treated cells. Seven AIB1-interacting proteins were detected in the categories of transcriptional regulation and chromatin complex, consistent with the role of AIB1 as a transcriptional coactivator. Interestingly, several proteins were found with pY immunoprecipitation that were unique to E2-treated MCF-7:5C cells, one of which was FAK1 (PTK2; Table S2 in [54]). FAK1 is known to complex with EGFR as well as with an isoform of AIB1 and thus contribute to cellular signaling in breast cancer cells [376]. The MS based identification of FAK1 in the anti-pY immunoprecipitates was also seen by Western blot (Fig. S8A in [54]).

AIB1-containing protein complexes in E2-treated MCF-7:5C cells

We identified 18 proteins (CI >95%) that interact with AIB1 in E2-treated but not in untreated MCF-7:5C cells, 10 of which are also unique to MCF-7:5C cells (Table S1 in [54]; Fig. 3a-2A). These E2-induced AIB1-interacting proteins in MCF-7:5C cells mainly segregate in the category ñtranscriptional regulationò (6 of 18), several of which are also known to be involved in the control of apoptosis. For example, PRDM5, a PR domain and zinc-finger transcriptional regulator is a putative tumor suppressor and has been linked to cancer cell apoptosis [377]. TLE3, a transcriptional corepressor that binds to a number of transcription factors [378], can form a transcriptional repressor complex with RUNX3 [379], a known tumor suppressor that has been shown to be involved in apoptosis in gastric and colon cancer [380]. TLE3 has also been associated with the development of anti-estrogen resistance [381]. The MS identification of the 83 kDa TLE3 in AIB1 immunoprecipitations (IP) by was also seen by Western blot analysis (Fig. S8B in [54]). IASPP was identified in complex with AIB1 in both E2-treated MCF-7 and MCF-7:5C cells, but not in untreated cells. IASPP, a member of ASPP family of proteins, exerts anti-apoptosis effects through modulation of p53 [382,383,384]. Interestingly PRPF6, identified here as AIB1-interacting, is an U5 snRNP-associated protein involved in pre-mRNA splicing and has been shown to be a coactivator of the androgen receptor and mediates its ligand-independent AF-1 activation [385]. TLE3, PRDM5 and PRPF6 were all uniquely identified in E2-treated MCF-7:5C cells.

Potential pathways involved in E2-induced growth and apoptosis

To increase the potential of identifying pathways participating in E2-induced growth and apoptosis from the MS data sets, we not only analyzed proteins identified from MS with high confidence (CI Ó95%), but also took a global approach to include all proteins identified at various CI levels (see http://pir.georgetown.edu/iproxpress/coe2) by MS before filtering for pathway mapping with the IngenuityÊ and GeneGOÊ pathway tools [372]. We hypothesized that if proteins

identified at lower-level confidence by MS are found in known pathways that are consistent with the cellular phenotypes, they may provide valuable mechanistic insights. Also, supporting this approach are data from a recent study [386] with immunoprecipitation of nuclear extracts from MCF-7 cells that identified 13 of the 15 proteins we had seen at CI values in the lower range of 42-90%. The canonical pathway mapping analyses of all identified proteins suggest that several pathways are significantly represented both for proteins immunoprecipitated with anti-AIB1 and for those with anti-pY, including GPCRs, apoptosis, PI3K/AKT, and Wnt/ϐ-catenin and Notch signaling pathways (Fig. S1-S4 in [54]):

GPCR and growth factor signaling. Figure S1 in [54] depicts the GPCR-induced cell growth pathway, in which a number of proteins were identified in both AIB1 and pY-associated complexes. Gŭŏŏŏ

member of armadillo/ϐ-catenin superfamily [395], was identified in the AIB1 immunoprecipitates of E2-treated MCF-7 cells (Table S1 in [54]).

Our results suggest that multiple proteins found in AIB1 associated complexes, that function in Wnt signaling, also crosstalk with Notch and growth factor-induced signaling in response to E2 treatment in breast cancer cells. TLE3 was detected only in E2-treated MCF-7:5C cells, and Notch1, Notch3, and Numb-like protein were identified only in E2-treated MCF-7 cells (Table S3 in [54]). TLE3, the mammalian homolog of Gro [396], is a global corepressor mediating transcriptional repression targeted by a number of signal pathways. As shown in Fig. S4D [54], TLE3 connects the Notch and Wnt pathways [397,398]. In addition to the apoptosis related proteins discussed above (TLE3, PRDM5, CDK1), DBC1 was isolated from anti-pY immunoprecipitates in E2 treated MCF-7:5C cells (Table S2 in [54]). Interestingly, DBC1 was recently reported to increase p53 mediated apoptosis in breast cancer cells [399]. Taken together, proteins from GPCR and PI3K/AKT-mediated growth signaling pathways were more prevalent in E2-stimulated MCF-7 cells, whereas proteins related to apoptosis pathways were more prevalent in E2-stimulated MCF-7:5C cells. The respective connectivity of the pathways is depicted in Figure 3a-4.

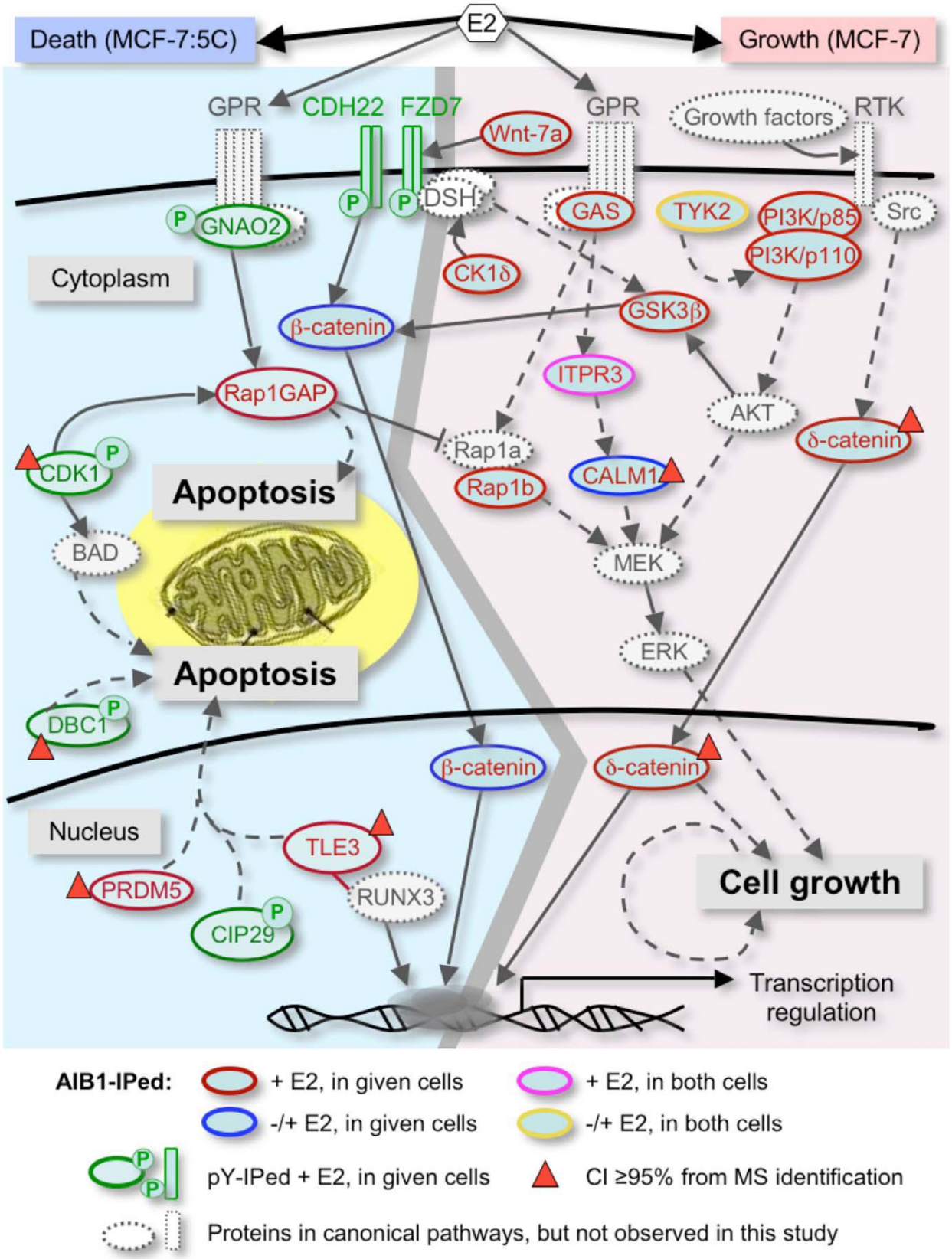


Figure 3a-4. *Pathway overview map of proteins involved in E2-induced cell growth or apoptosis in MCF-7 versus MCF-7:5C breast cancer cells. The thick grey line in the middle provides an*

arbitrary boundary between the pathways. Anti-AIB1 immunoprecipitated (AIB1-IPed) and anti-pY-immunoprecipitated proteins (pY-IPed) are indicated by red or green circles respectively (keys at the bottom). The blue circled proteins are AIB1-IPed proteins from MCF-7 (CALM1) or MCF-7:5C cells (β-catenin) under both E2- treated and untreated conditions; the purple circled one (ITPR3) is an AIB1-IPed protein from both cells only under E2 treated condition, while the yellow circled one (TYK2) is an AIB1-IPed protein from both cells under both E2 treated and untreated conditions. Proteins circled in grey are from known canonical pathways (e.g. ERK in cell growth or BAD in apoptosis) but not identified here. Solid line arrows indicate direct interactions (e.g. CDK1 phosphorylates Rap1GAP) or translocations (e.g. catenins) of proteins, while dashed arrows indicate indirect actions of proteins (e.g. AKT activate MEK through several steps). Hammer-ended lines indicate inhibitory effects on the target. Detailed pathways are shown in Fig. S1 - S4 in [54].

Global AIB1 interaction networks

To extract further information from these experimental data, they were linked with an AIB1 interaction network generated from published data [372]. A computational global AIB1 protein interaction network can be constructed from 91 AIB1 interaction partners (first neighbors) based on the literature published since AIB1 was first described in 1997 [368]. These 91 proteins belong to several major functional categories that include transcription, cell communication, developmental processes and cell cycle regulation. The initial network was expanded to secondary interaction neighbors, based on protein-protein interaction data in the public domain. At this level, the network is composed of 1150 proteins, including 21 highly connected nodes that form major hubs (Fig. 3a-5). These hubs include p53, BRCA1, BCL2, ABL1, CDK2, CDK4, EGFR, ER (=ESR1), p38, and MYC (Fig. 3a-5 and Fig. S5 in [54]). Closely related subnetworks of AIB1 (=NCOA3) shown in Figure S5 in [54] (*lower panel*), contain four hub proteins: BRCA1, MYC, CDK2 and PSME3. In the present study we identified 26 proteins that are part of the global AIB1 interaction network and function in signal transduction, transcriptional regulation, the cytoskeleton, and the heat shock response.

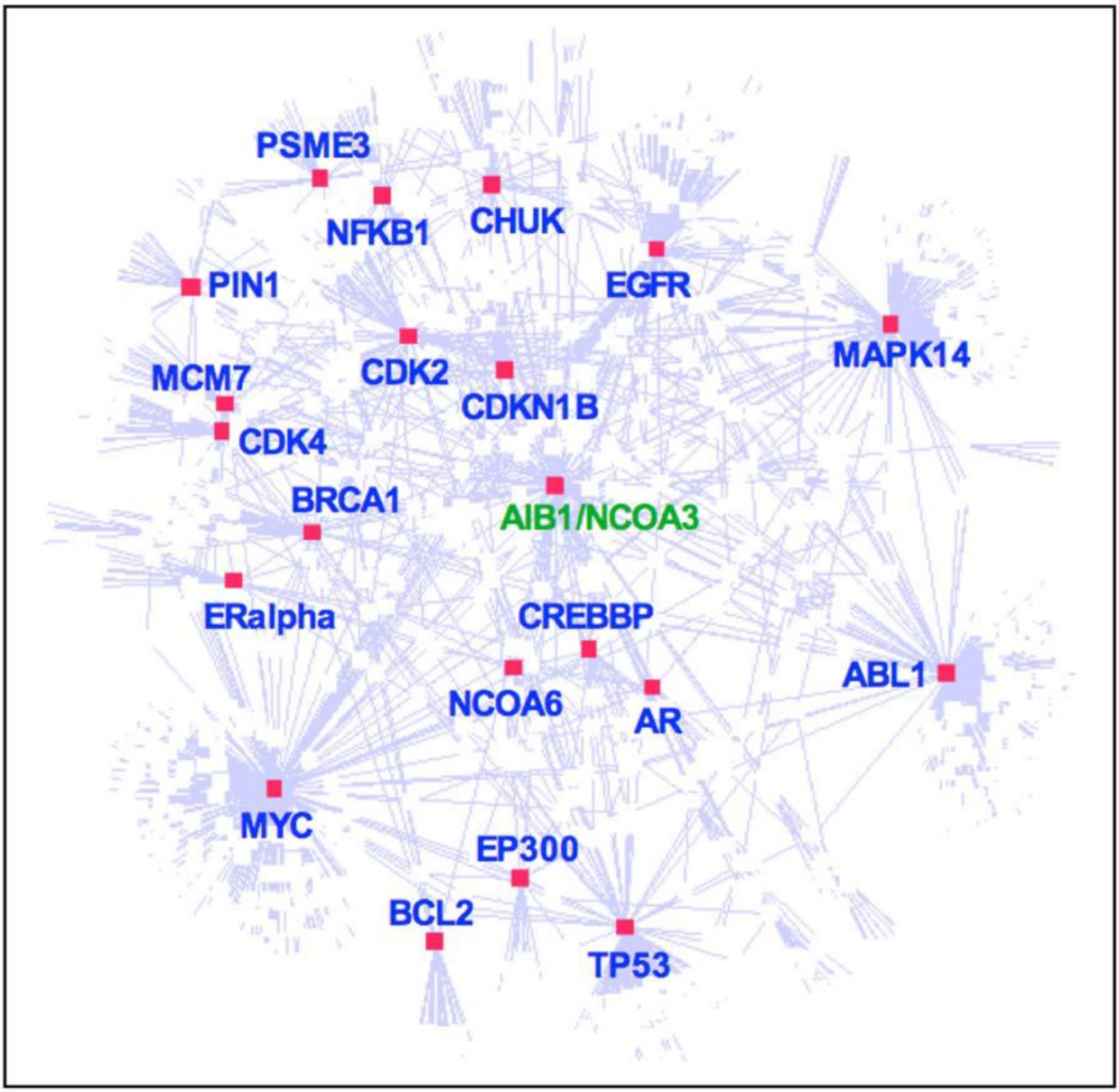


Figure 3a-5. *A global AIB1 interaction network showing the major hub proteins. Twenty-one hubs were identified using a cutoff of 20 node degrees. The full names of the respective gene symbols are provided in Table S8 in [54]. Detailed nodes in the network are shown in Fig. S5 in [54].*

Eighteen of the proteins experimentally associated with tyrosine-phosphorylated protein complexes are also part of the global AIB1-interaction network. Of these, seven were identified as interacting with AIB1, including CALM1, ACTB, ACTG1, TUBGCP2, MYH9, HSPA1B, and HSPA9. These proteins correspond to interacting hubs, such as CDK4, MYC, PSME3 and CHUK. We conclude that these hubs may participate in the differential cellular responses to E2.

Connection of E2 transcriptome and proteome effects

An interesting question is to what extent the proteomic pathway mapping parallels mRNA expression profiling in MCF-7 and MCF-7:5C cells. Baseline mRNA expression profiles of these cell lines have been posted earlier (GSE10879; ncbi.nlm.nih.gov/). An analysis of mRNA expression regulation after 48 hrs of E2 treatment of the cells was analyzed and published recently [400]. In MCF-7 cells Bcl-2, a major anti-apoptosis gene, was found upregulated by E2 treatment whereas no change of bcl-2 was seen in MCF-7:5C cells. In our analysis Bcl-2 is one of the major hubs in the AIB1 interaction networks (Fig. 3a-5 and Fig. S5 in [54]). On the other hand, the pro-apototic Bcl-2 antagonists Bak, Bax and Bim mRNAs were found upregulated 2- to 7-fold after E2 treatment of MCF-7:5C cells whereas no mRNA expression change was seen in the MCF-7 cells. Our analysis shows that upstream regulators of the canonical intrinsic mitochondrial pathway such as RSKs, were identified in the proteomics approach (Fig. 3a-4 and Fig. S2 in [54]).

The most differentially regulated mRNA after E2 treatment was Gadd45beta that was found up-regulated 5-fold in MCF-7:5C cells but down-regulated 5-fold in MCF-7 cells [400]. Gadd45beta was described earlier as a hub of the MAP kinase signaling cascade and connects to relA, the NFkappaB p65 subunit (see e.g. Ref. [401]) as well as cell survival in apoptosis resistant cells [402]. We isolated components of GPCR signaling in our proteomics analysis (Fig. 3a-4and Fig. S1 in [54]) that can connect to these downstream effectors and can thus serve as trigger mechanisms. Interestingly, GPR30 mRNA was found upregulated in MCF-7:5C cells after estradiol treatment [369] and GPR30 was shown to rapidly transmit non-genomic effects of E2 in breast cancer cells [189]. Overall, the mRNA expression analyses and proteomics data show some interesting convergences especially in apoptotic regulatory pathways which may be functionally relevant as initiators of estradiolï induced apoptosis or cell survival.

Discussion

The estrogen induced apoptotic response is most strongly associated with early signaling changes in G-protein coupled receptors, PI3 kinase, Wnt and Notch signaling and are integrated here into a global AIB1 signaling network that controls qualitatively distinct responses to estrogen.

TASK 4: (FCCC/Ariazi; TGen/Cunliffe; Jordan/GU) – To analyze E_2-induced survival and apoptotic pathways using gene arrays and siRNAs.

Task 4a (Ariazi and Jordan) - Studies carried out by Dr. Eric Ariazi in the Jordan laboratory at Fox Chase Cancer Center

Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time

Introduction

A pivotal study of high-dose DES therapy (15 mg daily) in 32 patients with metastatic breast cancer who had been treated exhaustively with antihormonal therapies produced a 30% objective response rate [12]. There were 4 of 32 complete responses, and one patient maintained a complete response for an additional 7 y, even after stopping estrogen[205]. A recent study in patients whose breast cancer had responded but then failed AI treatment[12] showed that low-dose E_2 treatment (6 mg daily) would produce the same clinical benefit as high-dose E_2 (30 mg daily) but with fewer toxic side effects. Thus, laboratory observations with low doses of estrogen treatment translate to clinical practice, and a mechanism is now emerging to explain the original observations by Haddow [74]. The goal of future translational research is to discover molecular mechanisms to amplify the estrogen-induced apoptotic trigger.

The question arises as to the precise sequence of events that lead to E_2-induced apoptosis. By describing and defining these molecular events, refractory cells may be manipulated to respond to estrogen-induced apoptosis. To begin to address the question, we have developed a series of MCF-7 variants that are either estrogen-dependent for growth (MCF-7:WS8 cells) [98,99] or resistant to estrogen deprivation (ED) and refractory (MCF-7:2A) [99,165] or sensitive (MCF-7:5C) [195] to E_2-induced apoptosis. We previously reported changes in gene expression among these cell lines by Affymetrix-based microarray analysis under estrogen-free conditions. Thus, these identified differentially expressed genes were associated with progression to an ED-resistant phenotype. We have also recently reported a proteomic analysis of 5C compared with WS8 cells after 2 h of E_2 exposure to identify proteins that may initiate apoptosis [54]. Here, we seek to identify genes differentially regulated by E_2 over a 2ï 96 h time course, which overlaps with actively occurring apoptosis. Therefore, we interrogated these models for changes in E_2-regulated global gene expression as a function of time using Agilent 4 Ǐ 44 K oligonucleotide microarrays. We developed a method termed differential area under the curve (dAUC) analysis to identify genes that exhibited significantly altered regulation by E_2 across time specifically in the apoptosis-sensitive 5C cells compared with both the estrogen-dependent WS8 and apoptosis-refractory 2A cells. Examination of the identified 5C-specific genes and functional testing indicated that E_2-elicited endoplasmic reticulum stress (ERS) and inflammatory stress responses that led to apoptosis.

Work Accomplished:

Cell Line Characterization.

Before gene expression microarray studies were carried out, the estrogen-dependent WS8, ED-resistant but apoptosis-refractory 2A, and apoptosis-sensitive 5C cells were characterized to confirm previously reported growth responses, biomarker status, and estrogen response element (ERE) - regulated transcriptional activity (Fig. 4a-1). The apoptotic responses of 5C cells to E_2 were also characterized according to loss of plasma membrane integrity (Fig. 4a-2). The 5C cells exhibited an

EC_{50} for apoptosis of 3 Ĭ 10^{-11} M E_2 after 96 h of exposure (Fig. 4a-2B). Additionally, 10^{-9} M E_2, the concentration used for the microarray studies, caused apoptosis ranging from ~30% to 42% of the 5C cells, depending on the experiment (Fig. 4a-2B and C). The pure antiestrogen fulvestrant completely blocked apoptosis induced by E_2 and DES, showing that apoptosis was ER-dependent (Fig. 4a-2C).

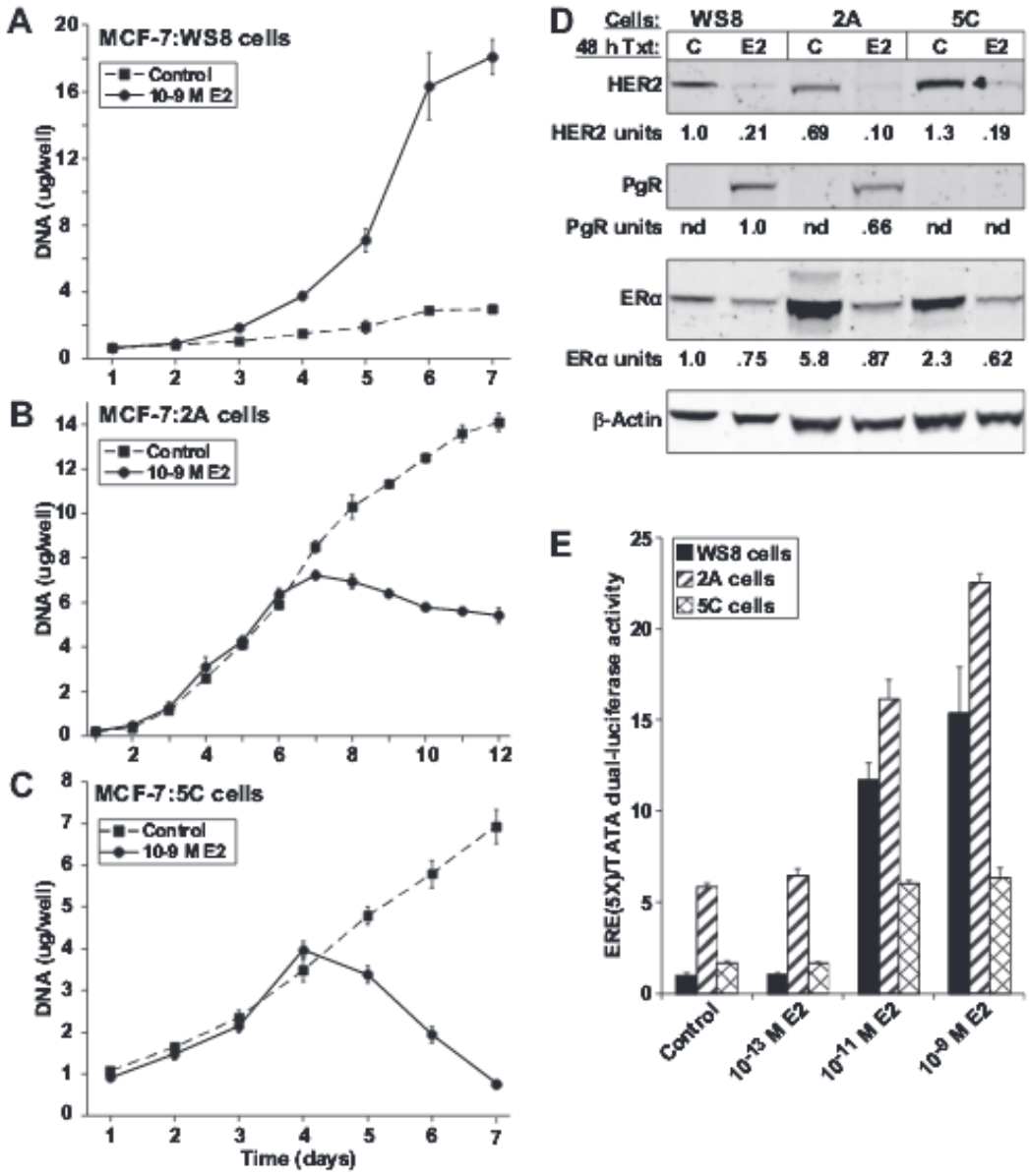
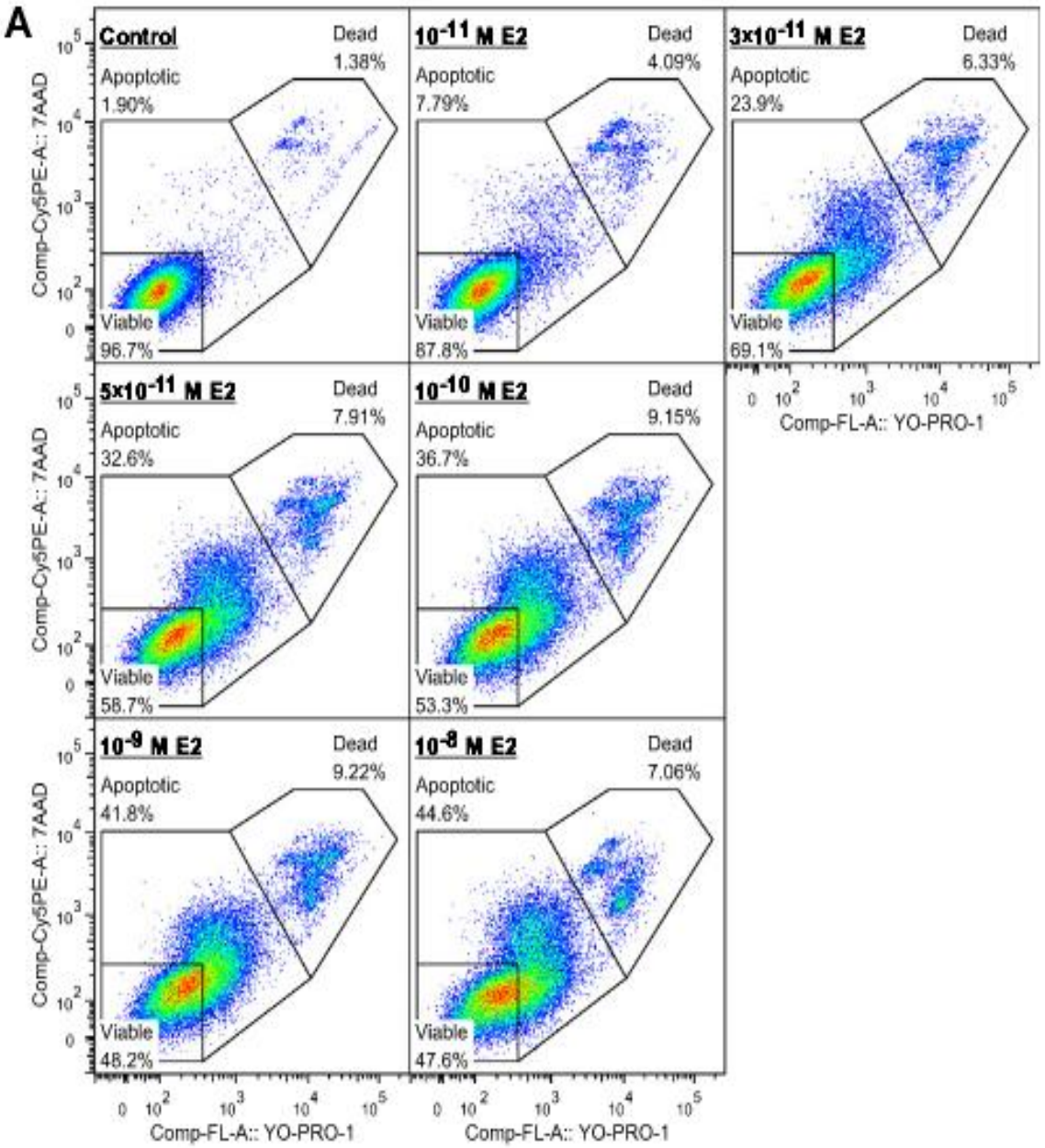


Figure 4a-1. ***Characterization of estrogen-dependent MCF-7:WS8 and estrogen deprivation-resistant MCF-7:2A and MCF-7:5C cell lines.*** *E_2-regulated growth of (A)MCF-7:WS8, (B) MCF-7:2A, and (C) MCF-7:5C cell lines. Cells were seeded in 24- (WS8 and 5C cells) or 6-well plates (2A cells) and allowed to grow in the presence or absence of E_2 over 7 (WS8 and 5C cells) or 12 d (2A cells). DNA mass per well was measured daily using the DNA binding fluorescent dye Hoechst 33258 compared with a standard curve. Data shown represent eight replicate wells and associated SDs per condition and time point. (D)*

Protein expression levels of ERα, PgR, and HER2. Protein levels were analyzed by immunoblotting. (E) ERE-regulated transcriptional activity using dual-luciferase assays. Cells were treated with the indicated E_2 concentrations for 24 h. Data shown represent triplicates and associated SDs.


A
Control
Dead
1.38%
Apoptotic
1.90%
Viable
96.7%
10-11 M E2
Dead
4.09%
Apoptotic
7.79%
Viable
87.8%
3x10-11 M E2
Dead
6.33%
Apoptotic
23.9%
Viable
69.1%
5x10-11 M E2
Dead
7.91%
Apoptotic
32.6%
Viable
58.7%
10-10 M E2
Dead
9.15%
Apoptotic
36.7%
Viable
53.3%
10-9 M E2
Dead
9.22%
Apoptotic
41.8%
Viable
48.2%
10-8 M E2
Dead
7.06%
Apoptotic
44.6%
Viable
47.6%
Comp-Cy5PE-A:: 7AAD
Comp-FL-A:: YO-PRO-1

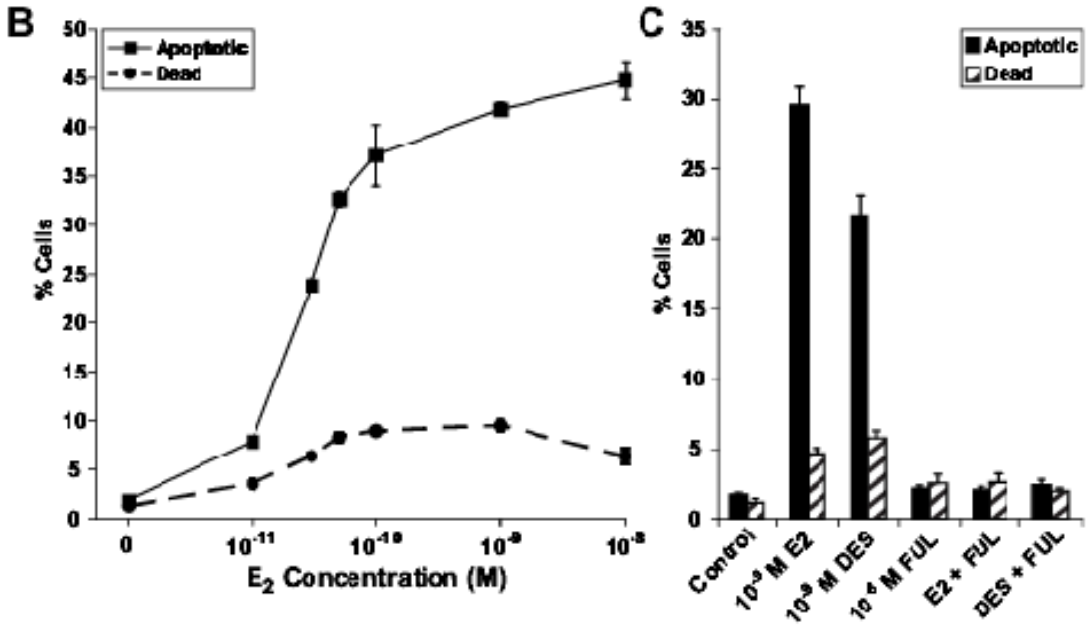


Figure 4a-2. ***E_2 induces apoptosis in 5C cells in a concentration-dependent manner through ER. (A and B) Concentration response of E_2-induced apoptosis.*** *Apoptosis was determined by flow cytometric analysis of cells stained with the DNA-specific binding dyes YO-PRO-1 and 7-aminoactinomycin D. Double-negative staining cells were defined as viable, double-positive staining cells were de fined as dead, and intermediately staining cells were defined as apoptotic. Cells were not treated (control) or treated with the indicated increasing E_2 concentrations for 96h. Examples of flow cytometry data are shown in A, and quan-titation of apoptotic and dead cells is shown in B.**(C) ER-dependent apoptosis in 5C cells.*** *E_2 and DES-induced apoptosis in 5Cs was completely blocked by fulvestrant. 5C cells were treated with the indicated ER ligands for 96 h and analyzed for apoptotic and dead cells as in A. Data shown in B and C represent triplicates and associated SDs.*

Global Gene Expression Across Time.

To identify genes and pathways/processes associated with E_2-induced apoptosis, differential regulation of global gene expression in response to E_2 was interrogated in ED-resistant/apoptotic-sensitive 5C cells vs. estrogen-dependent WS8 and ED-resistant/apoptotic-refractory 2A cells. Each cell line was treated with 10^{-9} M E_2 or vehicle control over a 96-h time course consisting of seven time points (2, 6, 12, 24, 48, 72, and 96 h) using six biological replicates per condition. cRNA probes from individual E_2-treated samples were competitively hybridized against time-matched pooled control probes using two-color Agilent 4 Ï 44 K human oligonucleotide microarrays. The resulting gene expression values were $\log_2$ ratios of mRNA levels in E_2/control-treated cells that, when plotted across time, form a curve. A measure of change in E_2-mediated regulation of expression over a defined time interval was then calculated as the difference in AUCs or dAUCs for a given gene between two cell lines. Genes that showed a 50% change in AUCs between two cell lines (corresponding to a dAUC = 0.58 on a $\log_2$ scale) at a P value < 0.00005 were defined as significantly different. The dAUC method was applied to identify differentially regulated genes at 2ï 96, 2ï 24, and 24ï 96 h to identify overall, relatively early, and late-responding genes, respectively.

To identify genes specifically associated with E_2-induced apoptosis, genes were selected with regulation that differed significantly with E_2 in the 5C cells vs. both the WS8 and 2A cells. A total of 1,142 genes

were identified as significantly differentially regulated by E_2 specifically in the 5C cells. These genes were examined for overrepresentation of those genes mapping to a particular curated pathway/network (Fig. 4a-3A). As expected, estrogen signaling and apoptosis genes were significantly enriched. Within the apoptosis category, ERS was the most enriched apoptosis subcategory (Fig. 4a-3B). Inflammatory response genes were also enriched. The overlapping distribution of genes mapping to estrogen signaling, apoptosis, and inflammatory responses is shown in the Venn diagram in Fig. 4a-3C.

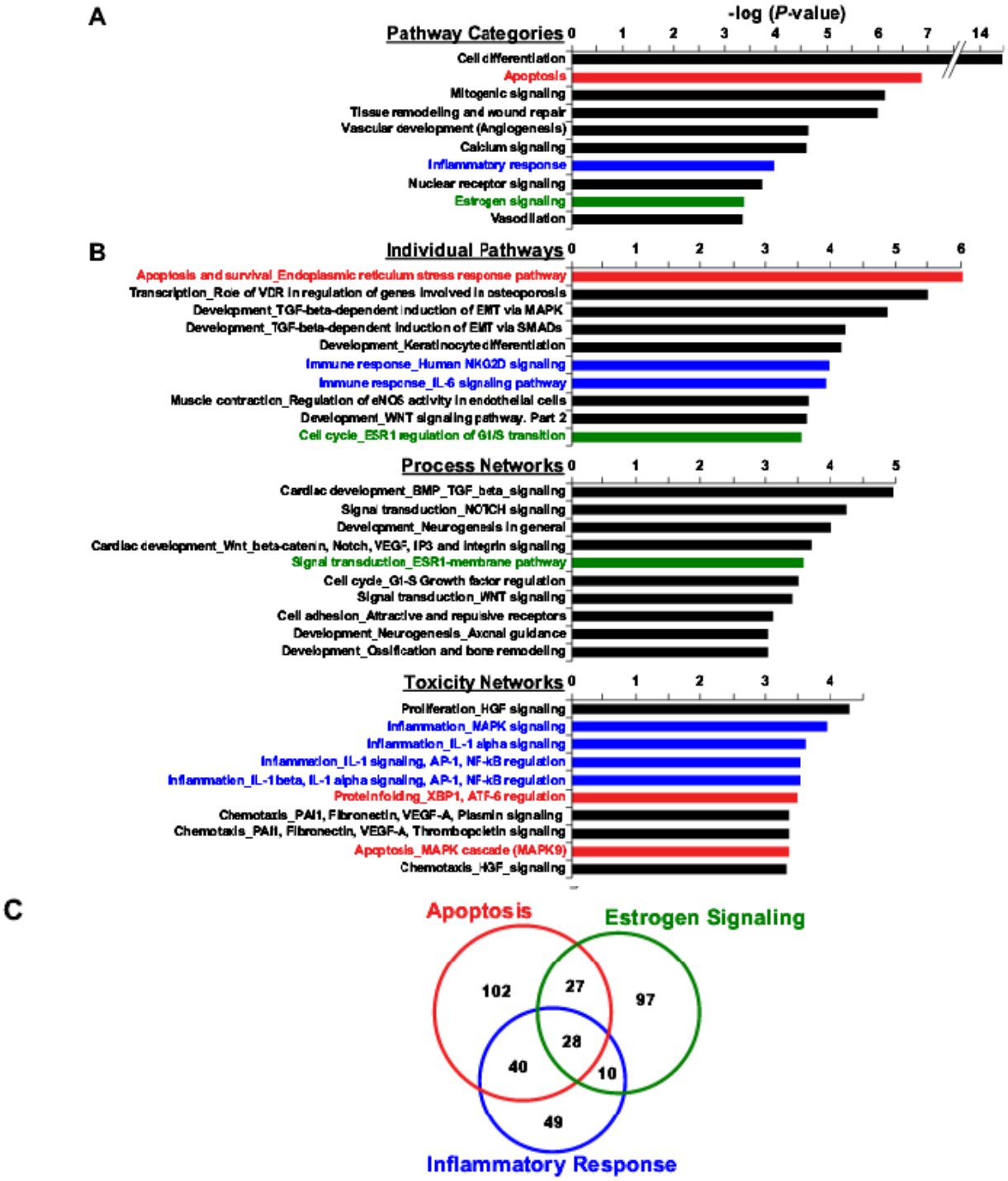


Figure 4a-3. ***Enrichment analysis of genes differentially regulated by E_2 in 5C cells relative to both WS8 and 5C cells.*** *(A) Broad pathway categories. (B) Individual pathways and networks. (A and B) The*

top 10 scoring pathways/processes are shown for each group. (C) Venn diagram showing the total and overlapping number of genes involved in estrogen signaling, in flammatory response, and apoptosis. (A–C) Estrogen signaling pathways are shown in green, apoptosis-related pathways are shown in red, and inflammatory pathways are shown in blue.

Estrogen Signaling Genes.

Estrogen signaling genes selectively regulated by E_2 in 5C cells relative to both WS8 and 2A cells are listed in Database S2 [10] and examples discussed are shown in Fig. 4a-4. Multiple genes were differentially regulated by E_2 in 5C cells compared with WS8 and 2A cells, which would diminish ERŬ activity and hence, the apoptotic stimulus. For example, genes that negatively modulate ERŬ activity (i.e., AR, CYP1B1, FHL2, HSD17B11, INHBA, NR2F1/COUP-TF1, SNAI1/Snail 1, and THRA/TRŬ) were selectively up-regulated, whereas those genes that promote ERŬ activity were selectively down-regulated (AREG, CAV1, and PIK3CB) by E_2 in 5C cells. The up-regulated estrogen metabolizing enzymes CYP1B1 and HSD17B11 would decrease intracellular E_2 pools. SETD7/SET7/SET9 methylates ER to stabilize the protein; hence, its down-regulation by E_2 in 5Cs would accelerate ERŬ protein degradation. ERŬ activity would be suppressed by up-regulation of transcription factors that repress ERŬ RNA expression (i.e., FHL2 and Snail-1) or compete with ERŬ for binding-extended ERE half-sites, which overlap with many natural EREs (i.e., COUP-TF1 and TRŬ). AR failed to down-regulate in response to E_2 in 5C cells, allowing greater AR activity. AR and ERŬ interact in complexes, and androgens inhibit E_2-stimulated growth of MCF-7 cells; thus, AR can oppose ERŬ. ERŬ activity can also be suppressed by activin-A, a TGFb superfamily ligand, in a SMAD3-dependent manner in MCF-7 cells. Both INHBA and SMAD3 were selectively induced in 5C cells, and INHBA homodimerizes to form activin-A, which signals to SMAD3; SMAD3 interacts with ERŬ at promoters to repress transcription. AREG and PIK3CB failed to increase in response to E_2 in 5C cells. This failure to increase may have prevented increased ERŬ activity, because AREG activates EGFR, which leads to ERŬ-Ser118 phosphorylation, and PIK3CB is the catalytic subunit of PI3K, which through Akt, targets ERŬ-Ser167 phosphorylation. CAV1 expression also failed to increase, which again prevents increased ERŬ activity, because CAV1 interacts with and promotes activity of membrane-localized ERŬ. However, not all of ERŬs activities were suppressed. In particular, ERŬ interacts with and directs transcription through AP-1 transcription complexes in addition to EREs. AP-1 complexes consist of FOS, JUN, and JUND subunits, which were all selectively induced by E_2 in 5C cells [FOS and JUN were verified by quantitative PCR (qPCR) in Fig. 4a-5]. Importantly, AP-1 complexes play important roles in apoptosis and inflammatory responses (discussed later), and thus, ERŬ interaction with AP-1 provides a mechanism for E_2 to target such genes.

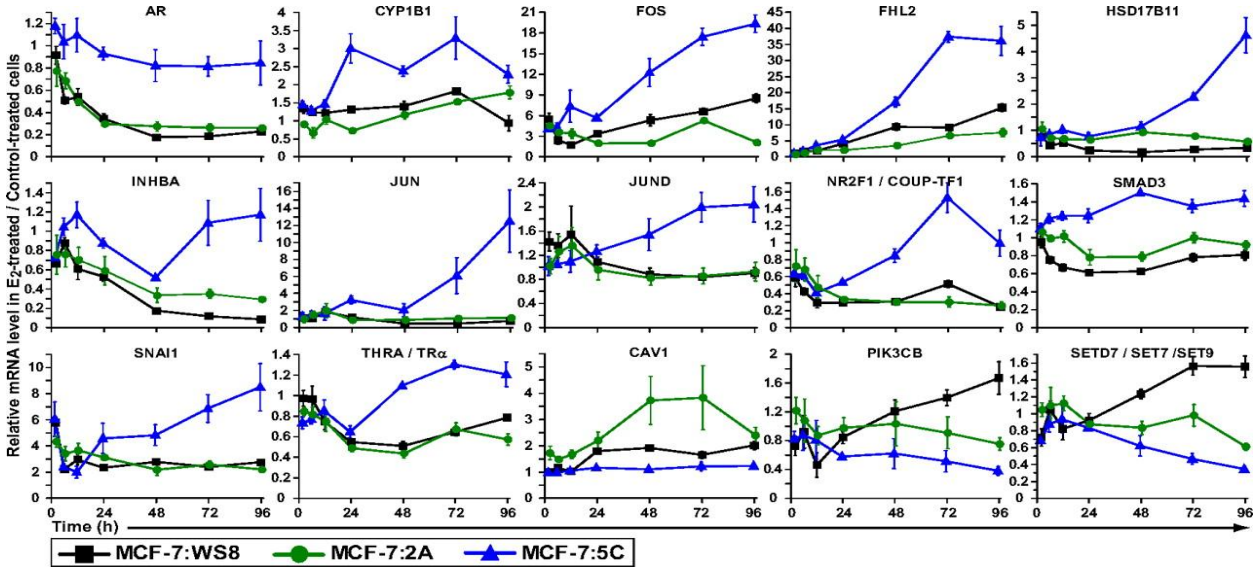


Figure 4a-4. *Examples of estrogen signaling genes.*

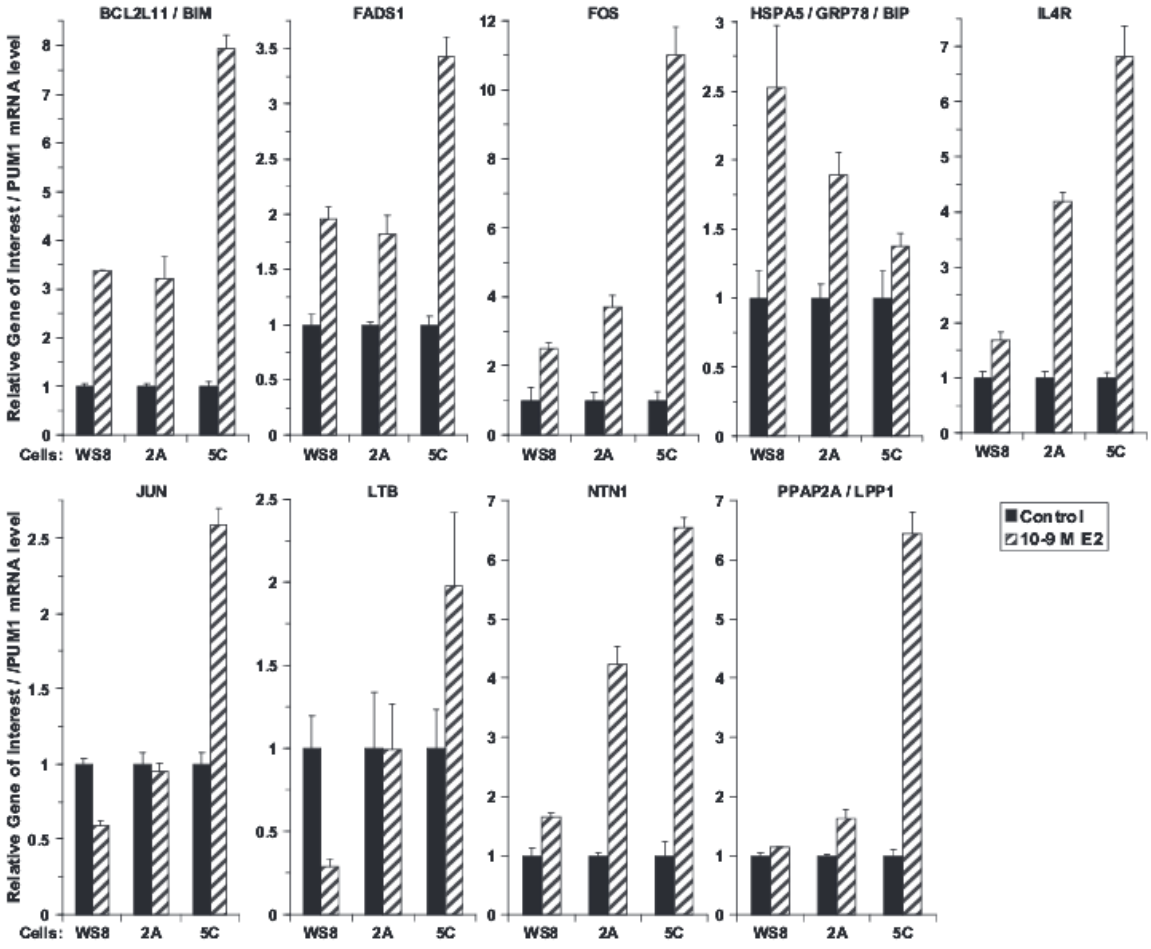


Figure 4a-5. ***Genes verified by qPCR to show differential expression in 5C cells compared with WS8 and 2A cells.*** *Cells were treated with or without 10^{-9}M E_2 for 72 h. RNA expression levels of the indicated genes were determined by real-time qPCR. Data shown represent quadruplicates and associated SDs.*

Apoptosis Genes.

The identified apoptosis genes are listed in Dataset S3 [10], and discussed examples are shown in Fig. 4a-6. Enrichment analysis indicated ERS-mediated apoptosis as the top-scoring individual pathways within the apoptosis category (Fig. 4a-3). The endoplasmic reticulum is a key site for protein folding. When cellular stresses perturb energy levels, the redox state, or Ca^{2+} concentrations, unfolded proteins accumulate and protein aggregation occurs; this condition is referred to as ERS. To relieve ERS, an unfolded protein response (UPR) is triggered by the chaperone HSPA5/GRP78/BiP. In addition to binding unfolded proteins, GRP78 binds and prevents oligomerization of the endoplasmic reticulum transmembrane receptors EIF2AK3/PERK, IRE1Ŭ/ERN1, and ATF6. When unfolded proteins accumulate, GRP78 is released from binding the transmembrane receptors, allowing them to oligomerize and autophosphorylate to initiate a UPR signal. The UPR signals to attenuate protein translation, induce expression of additional chaperones, and export misfolded proteins to the cytosol for degradation. If the UPR fails to relieve the stress, the function of the UPR switches from promoting cell survival to promoting cell death. Thus, excessive or prolonged ERS typically induces apoptosis.

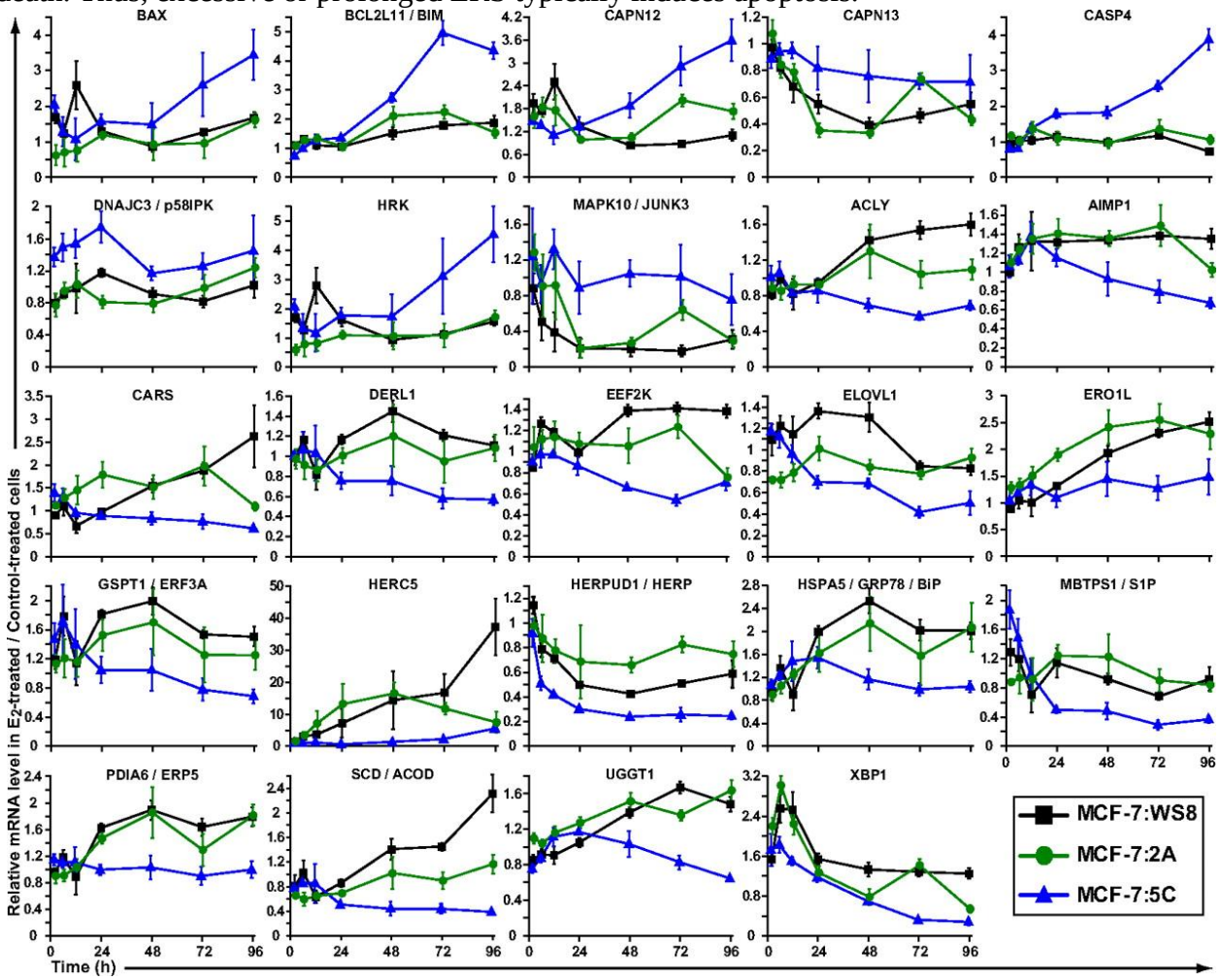


Figure 4a-6. ***Examples of apoptosis genes.*** *Full annotation, dAUC values, and* P *values of all apoptosis genes are given in Dataset 3.*

Growth stimulation of hormonally responsive cells by E_2 leads to increases in requirements for folding nascent polypeptides and clearance of malfolded proteins. However, in 5C cells compared with WS8 and 2A cells, E_2-regulated expression changes indicated a deficiency in these functions. In 5C cells, E_2 failed

to sufficiently up-regulate endoplasmic reticulum-localized protein folding genes, including GRP78 (verified by qPCR in Fig. 4a-5), ERO1L, PDIA6, and UGGT1. Cytoplasmic protein folding genes, including HSP90AB1/HSP90B, PPIAL4A, and PPIF (also FKBP10), also failed to up-regulate. Additionally, in 5C cells, E_2 preferentially down-regulated HERPUD1/HERP1 and DERL1, factors that promote degradation of endoplasmic reticulum-resident proteins. A deficiency in up-regulating UPR genes in 5C cells may have resulted in part by the pronounced E_2-mediated repression of MBTPS1/S1P, which cleaves ATF6, activating its translocation to the nucleus to induce transcription of UPR genes, including XBP1. Thus, decreased S1P may have led to decreased ATF6 and XBP1 activity, thereby preventing induction of multiple UPR genes.

E_2-mediated gene expression alterations in 5C cells indicated widespread inhibition of protein translation compared with E_2-treated WS8 and 2A cells. Within 2 h, E_2 had up-regulated DNAJC3/p58^{IPK}, which binds to and inactivates EIF2AK3/PERK, leading to reduced global translational initiation (Hotamisligi GS. Cell 2010). The aminoacyl tRNA synthetase interacting protein AIMP1 and tRNA synthetases, including CARS (also LARS, SARS, and YARS) failed to increase in response to E_2 in 5Cs. Other translational factors that failed to induce in 5C cells include EEF2K and GSPT1/ERF3A (also EEF1A1, ETF1, and PABPC4).

Under severe ERS, the UPR can shut down lipogenesis as cells commit to death ([403]. This was likely the case in E_2-treated 5C cells since they showed a lack of induction of critical genes involved in fatty acid synthesis, including ACLY, SCD/ACOD, and ELOVL1. ACLY is the primary enzyme responsible for synthesis of acetyl-CoA, the basic building block of fatty acids. SCD introduces a C-C double bond in fatty acyl-CoA substrates, including stearoyl-CoA and palmitoyl-CoA, a key step in producing monounsaturated fatty acids. ELOVL1 condenses both saturated and monounsaturated fatty acids. Notably, SCD and ELOVL1 are localized to the endoplasmic reticulum membrane.

In response to severe ERS, specific BCL2 and Bcl-2 homology domain 3 (BH3) -only family members are targeted to initiate apoptosis [404]. Prototypical BCL2 inhibits cell death by binding and inactivating proapoptotic members such as BAX. BH3 only-containing proteins like BCL2L11/BIM indirectly activate BAX by binding BCL2 (through the BH3 motif), thereby releasing BAX from the complex. BAX then permeabolizes the mitochondrial outer membrane, allowing cytochrome C release to the cytoplasm. Under ERS, BAX also interacts with and activates IRE1Ŭ. IRE1Ŭthen signals to JNK to simultaneously activate BIM and inhibit BCL2 [404]. A variety of ERS inducers stimulate BIM expression, and BIM is essential in ERS-induced apoptosis in a wide range of cell types [405]. This apoptotic pathway was likely activated by E_2 in 5C cells. E_2 failed to repress MAPK10 (JNK3) in 5C cells, indicating higher JNK3 activity. Meanwhile, E_2 selectively up-regulated BAX, BIM (verified by qPCR in Fig. 4a-5), and another BH3-only proapoptotic factor, HRK (also BBC3/PUMA but PUMA did not make the significance cutoff. Importantly, E_2 repressed BCL2 in 5C cells but induced it in WS8 cells. However, E_2 also repressed BCL2 in 2A cells, and therefore, it was not a 5C-specific gene. We previously verified the importance of BAX and BIM by showing that they were selectively induced by E_2 at the protein level in 5C vs. WS8 cells and that their depletion by RNAi blocked E_2-induced apoptosis [83]. Therefore, ERS may have triggered mitochondrial-mediated apoptotic cell death in E_2-treated 5C cells.

After prolonged ERS, specific caspases are activated to enact cell death. Examination of the caspases revealed that only CASP4 met the stringent statistical significance criteria in the microarray data. CASP1, CASP5, and CASP8 also showed up-regulation in 5C cells but did not meet our significance threshold. CASP4 along with CASP1 and CASP5 are inflammatory caspases, because they are involved in cytokine maturation [406]. CASP4 specifically localizes to the endoplasmic reticulum and undergoes cleavage in response to ERS-inducing agents/proteins but not other apoptotic agents, and its blockade using z-LEVD-fmk or depletion by RNAi can prevent endoplasmic stress-induced apoptosis in multiple model systems[407,408] Importantly, CASP4 autoactivates by dimerizing and undergoing interdomain

cleavage[409], and thus, simply overexpressing CASP4 is sufficient to induce cleavage of downstream caspases[410] and cause apoptosis[93]. Under ERS, CASP4 can also be activated by calpain [411], and CAPN12 and CAPN13 were selectively up-regulated in 5C cells.

Inflammatory Response Genes.

The inflammatory response genes are listed in Dataset 4 ([10], and discussed examples are shown in Fig. 4a-7. In 5C cells, E_2 elicited up-regulation of many proinflammatory cytokine/cytokine receptors, including IL-4R (verified by qPCR in Fig. 4a-5), IL-6R, IL-6ST/gp130, IL-17RD/Sef, and VEGFA. IL-4R was induced with early kinetics, indicating that it may be a primary response. IL-6R was up-regulated shortly after IL-4R, whereas IL-6ST/gp130, also an IL-4R subunit, was already up-regulated by 2 h. Hence, IL-6 signaling was likely activated in 5Cs. IL-17RD/Sef not only mediates IL-17 signaling, but its overexpression also leads to JNK activation and apoptosis[241], which links inflammatory responses and ERS. VEGFA also leads to activation of JNK in tamoxifen-resistant MCF-7 cells. An IFN response was likely activated, because the IFN IFNL1 and the IFN-responsive genes IFI6 and IFI16 were up-regulated. CASP4 can also be induced by IFN [412].

A number of other proinflammatory genes, such as CEBPB, NTN1 (verified by qPCR in Fig. 4a-5), and UNC5C, were selectively up-regulated in E_2-treated 5C cells with relatively early kinetics, indicating possible mechanistic roles. CEBPB is important in induction of IL-6, is activated by ERS, is required for nuclear import of the key ERS protein CHOP/GADD153, and enhances NF-əB signaling. NTN1 is a secreted inflammatory marker, but it protects tissues from inflammatory injury by suppressing cytokine production, repulsing leukocyte infiltration, and acting as an antiinflammatory and antiapoptotic ligand of its receptors DCC and the UNC-5 family members. In the context of E_2-induced apoptosis, NTN1 may have been up-regulated to limit or resolve the inflammatory response. Interestingly, E_2 rapidly down-regulated UNC5C in WS8 and 2A cells within 6 h but failed to do so in 5C cells, resulting in higher UNC5C expression. UNC5C may have a proinflammatory role, because synovial cells from patients with rheumatoid arthritis and osteoarthritis dramatically overexpress UNC5C (769-fold) compared with those cells of healthy donors.

Arachidonic acid (AA; 20:4n-6) is a polyunsaturated fatty acid that plays a key role as an inflammatory mediator. Enzymes involved in AA biosynthesis were up-regulated by E_2 in 5C cells, including FADS1 (verified by qPCR in Fig. 4a-5), FADS3, PLA2G10, PLCD3, MGLL/MAGL, PPAP2A/LPP1 (verified by qPCR in Fig. 4a-5), and SGMS1/SMS1. FADS3 and FADS1 catalyze the first and last steps in AA biosynthesis by introducing C-C double bonds in linoleic acid, producing ɔ-linolenic acid (18:3n-6), and dihomo-ɔ-linolenic acid (20:3n-6), producing AA. PLA2s hydrolyze phospholipids, releasing AA, whereas PLCD3 cleaves AA from diacylglycerol. MGLL converts monoacylglycerides such as 2-arachidonoylglycerol to free fatty acids including AA. PPAP2A/LPP1 converts phosphatidic acid to diacylglycerol, providing increased substrate levels for PLCD3 to release AA. As an inflammatory mediator, AA is used as a precursor by cyclooxygenase and lipoxygenase to generate inflammatory prostaglandins and leukotrienes, respectively. However, the cyclooxygenase pathway was unlikely to have been involved in E_2-induced apoptosis, because induction of PTGES failed in 5C cells compared with WS8 and 2A cells. In hormone-dependent breast cancer cells, E_2 is known to induce PTGES expression through an ERE, which may promote breast cancer proliferation, because the increased prostaglandin E_2 may enhance aromatase expression and also promote local productions of estrogens[413]. Thus, a failure to induce PTGES may, ultimately, have served to prevent any potential increases in estrogen concentrations in 5C cells. Considering that ERS likely led to a block of fatty acid synthesis and conversion to monounsaturated fatty acids (i.e., no induction of ACLY and SCD), the selective increases in AA-related genes likely indicate the importance of AA in promoting an inflammatory response in E_2-induced apoptosis.

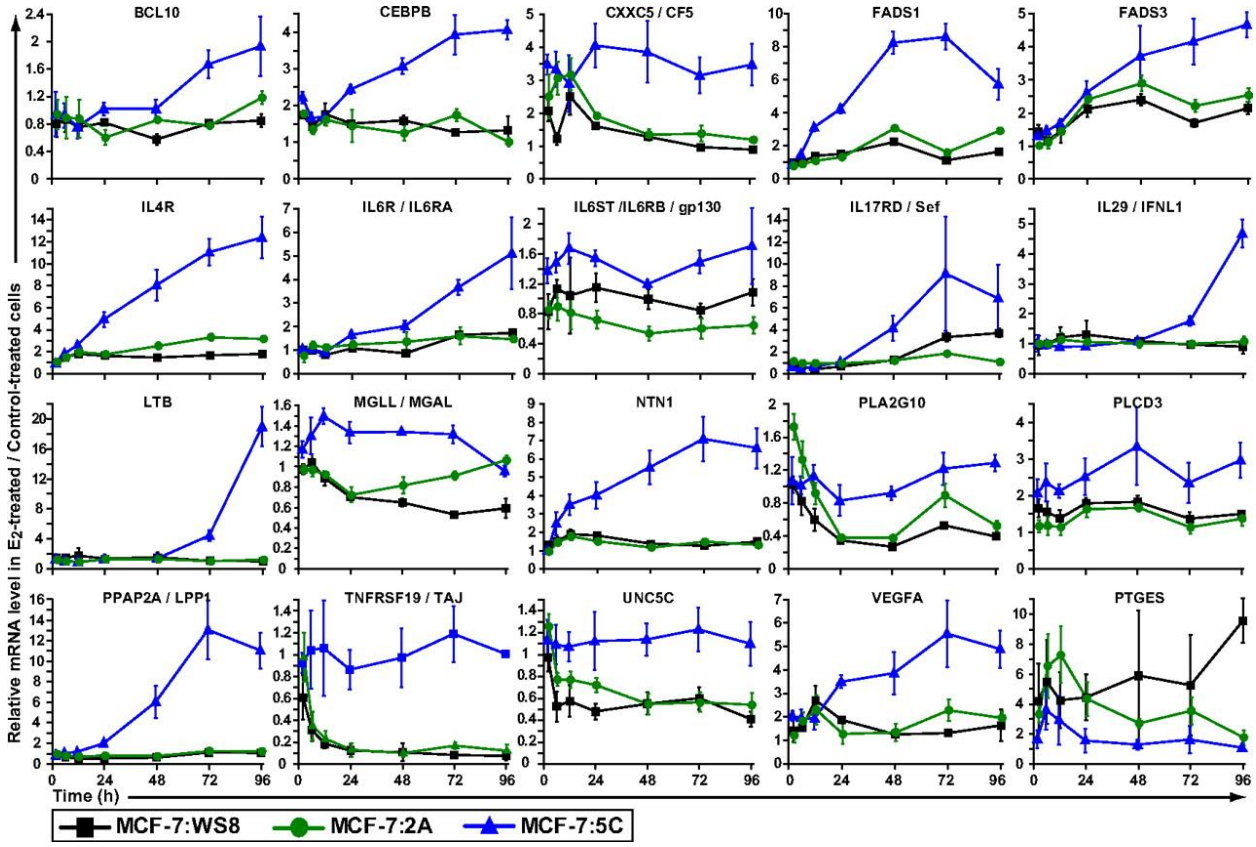


Figure 4a-7. ***Examples of inflammatory response genes.***

Cross-Talk Between ERS and Inflammatory Stress.

As mentioned previously, ERS and inflammatory pathways intersect. The key ERS genes IRE1Ŭ, ATF6, and PERK can all activate NF-əB, which serves as a master regulator of inflammatory response gene transcription [405,414]. Many of the identified cytokine/cytokine receptors signal through NF-əB pathways. Other genes selectively induced by E_2 in 5C cells, including BCL10 [415], , CXXC5 [416], LTB (verified by qPCR in Fig. 4a-5), and ITGB2 [417] activate NF-əB signaling as well. Additionally, SETD7/SET7/SET9, which negatively regulates NF-əB activity by methylating the RelA subunit to induce its degradation, was down-regulated by E_2 in 5Cs (Fig. 4a-4). Furthermore, multiple 5C-specific genes are NF-əBï responsive, including BIM [418], CASP4 ([419], CEBPB , CP, NTN1, and VEGFA. Moreover, ERŬ and NF-əB can interact to transcriptionally regulate promoters, providing a direct mechanism for E_2 to target a diverse array of inflammatory and apoptotic genes. Therefore, NF-əB signaling was very likely involved in E_2-induced apoptosis, and we are pursuing this hypothesis in future studies.

ERS also intersects with inflammatory responses through JNK. As mentioned, the ERS sensor IRE1Ŭ [403] and the IL receptor 17RD/Sef can activate JNK [420]. The orphan TNF receptor TNFRSF19/TAJ, which failed to down-regulate in response to E_2 in 5C cells, also activates JNK [421]. JNK then phosphorylates AP-1 complexes to induce expression of inflammatory response genes [403]. As mentioned earlier, the AP-1 subunits JUN, JUND, and FOS were selectively induced in E_2-treated 5C cells.

Functional Involvement of AA and CASP4 in E_2-Induced Apoptosis.

The involvement of ERS and inflammatory stress in E_2-induced apoptosis was functionally examined. We first tested whether E_2-induced apoptosis could be promoted by AA. AA was chosen, because (*i*) it is widely recognized as a proinflammatory agent; (*ii*) it induces apoptosis [422], at least in part by depleting

the endoplasmic reticulum of Ca^{2+} and inhibiting protein translation, thereby likely eliciting ERS [423]); (*iii*) it can activate NF-əB in mammary epithelial cells [424]; and (*iv*) several genes, which increase AA levels (e.g., FADS1 and PLA2G10), were up-regulated in response to E_2 in 5C vs. WS8 and 2A cells. 5C cells were exposed to varying concentrations of both AA and E_2 in a factorial design, and then, apoptosis was measured by flow cytometric analysis of YO-PRO-1 and 7-aminoactinomycin D staining (Fig. 4a-8A). Because E_2-induced apoptosis occurs maximally with 10^{-9} M E_2 after 96 h of exposure, E_2 was used at low concentrations of 2.5 and 5 Ĭ 10^{-11} M, and apoptosis was assayed at 72 h to allow observation of potential additional AA effects. The combination of AA plus E_2 at all varied concentrations increased the percentage of apoptotic plus dead cells in a greater than additive manner relative to either agent alone. Fitting the data to a multiple regression model showed the rate of increase (slope) in apoptotic plus dead cells progressively and significantly increased comparing E_2 alone with E_2 + 10 ε M AA or E_2 + 20 ε M AA. Therefore, AA and E_2 interacted to superadditively induce apoptosis, indicating that their pathways functionally intersect.

The importance of CASP4 was evaluated using a panel of irreversible caspase peptide inhibitors selectively targeting caspases-1 to -9 (except CASP3, which is not expressed in MCF-7 cells) [425]. 5C cells were treated with 10^{-9} M E_2 plus each caspase inhibitor as indicated for 96 h to induce apoptosis, which was measured by altered plasma membrane permeability (Fig. 4a-8B). The broad spectrum caspase inhibitor z-VAD-fmk was used as a positive control, because we previously reported that this inhibitor completely blocks E_2-induced apoptosis [165], whereas the inactive inhibitor z-FA-fmk was used as a negative control. In an effort to prevent off-target caspase inhibition, the blockers were used at 10 ε M, which was the concentration that reduced apoptosis by approximately one-half by the pan inhibitor z-VAD-fmk. The most active inhibitor was the CASP4 blocker z-LEVD-fmk, which was slightly more effective than the pan CASP inhibitor (Fig. 4a-8B). The CASP8 inhibitor z-IETD-fmk was the next most active blocker but was significantly less potent than z-LEVD-fmk (*P* value = 0.0026). Therefore, in an unbiased comparison of caspases-1 to -9, CASP4 was validated as functionally critical in E_2-induced apoptosis.

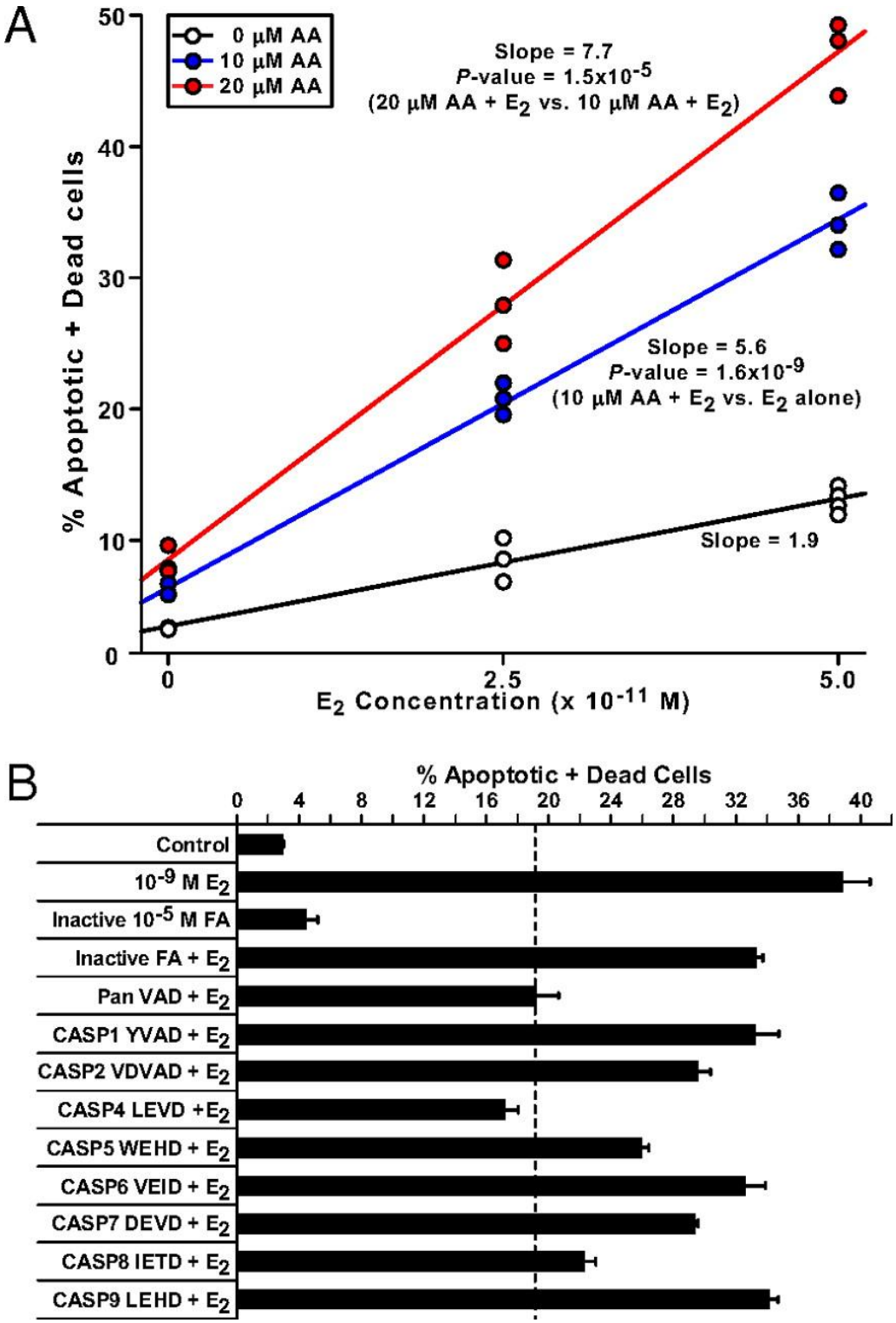


Figure 4a-8. ***Functional interrogation of E_2-induced apoptosis.*** *(*A*) AA and E_2 interact to superadditively induce apoptosis. 5C cells were treated with combinations of AA and E_2 as indicated for 72 h. (*B*) Screening of selective CASP inhibitors. The selectivity of the inhibitors for individual caspases is indicated according to the manufacturer. 5C cells were treated with 10^{-9} M E_2 and 10 µM of each CASP inhibitor as indicated for 96 h. (*A *and* B*) Apoptosis according to altered plasma membrane permeability was determined by flow cytometric analysis of cells stained with the DNA-specific binding dyes YO-PRO-1 and*

7-aminoactinomycin D. Double-negative staining cells were defined as viable, double-positive staining cells were defined as dead, and intermediately staining cells were defined as apoptotic. Data shown in B *represent triplicates and associated SDs.*

The functional activity of CASP4 was also studied. Real-time qPCR and immunoblotting confirmed induction of CASP4 expression at the mRNA and protein levels, respectively, occurred specifically in 5C cells in response to E_2 (Fig. 4a-9A and B). Importantly, in 5C cells, z-LEVD-fmk at 20 μM completely blocked E_2-induced PARP cleavage (Fig. 4a-9B), reversed E_2-inhibited growth (Fig. 4a-9C), and prevented morphologic alterations associated with apoptosis in 5C cells (Fig. 4a-9D). Because z-LEVD-fmk was used at 20 rather than 10 μM, we do not discount the possibility that some caspases in addition to CASP4 were also inhibited and that other caspases could still play an important role. Yet, our data establishes a critical role for CASP4 in E_2-induced apoptosis.

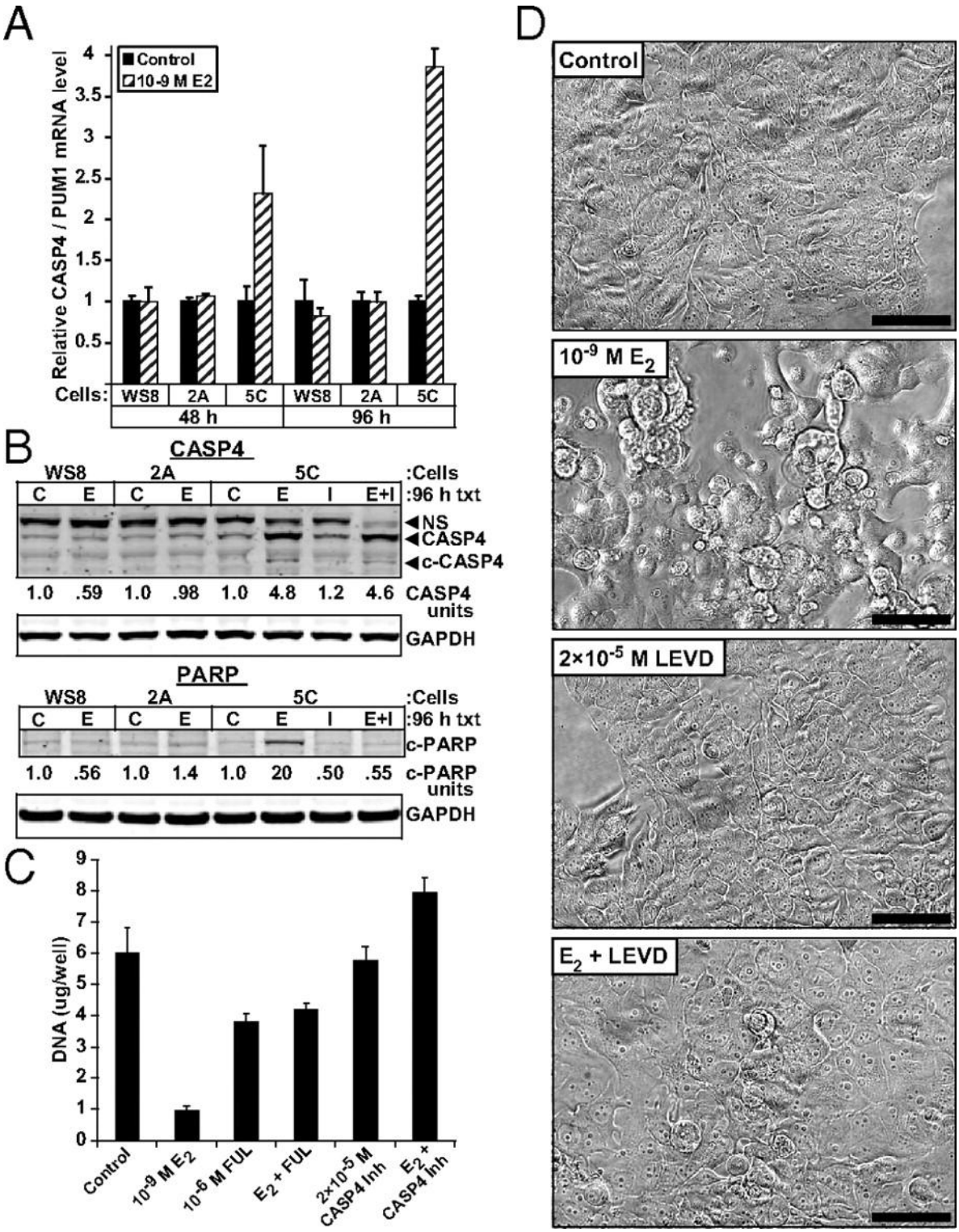


Figure 4a-9. ***Functional involvement of CASP4 in E_2-induced apoptosis.*** *E_2-induced CASP4 at the* (A) *mRNA and* (B) *protein levels in 5C cells but not in WS8 or 2A cells. CASP4 mRNA and protein levels were measured by qPCR and immunoblotting, respectively.* (B) *In 5C cells, E_2 led to cleavage of the apoptotic marker PARP, which was blocked by the CASP4 inhibitor z-LEVD-fmk. C, control; E, E_2; I, inhibitor (z-LEVD-fmk); c-CASP4, cleaved CASP4; NS, nonspecific band.* (C) *E_2-inhibited growth of 5C cells was completely reversed by z-LEVD-fmk. Proliferation was determined after 6 d of 10^{-9} M E_2 exposure and*

*measured by DNA mass per well. CASP4 Inh, CASP4 inhibitor z-LEVD-fmk. (*D*) Morphologic alterations after 96 h of 10^{-9} M E_2 in 5C cells were completely reversed by z-LEVD-fmk (LEVD). (Scale bar: 100 microns.) (*Aï D*) E_2 was used at 10^{-9} M and z-LEVD-fmk at 2×10^{-5} M. Data in* A *and* C *represent the average and SDs of four and eight replicates, respectively.*

Discussion

We have interrogated E_2-induced apoptosis by identifying differentially regulated genes across time associated with this process compared with E_2-stimulated and -independent growth using a method we developed termed dAUC analysis. Overrepresentation analysis of the identified genes indicated that 5C cells respond to E_2 by suppressing ERŬ signaling and producing endoplasmic reticulum and inflammatory stress. Estrogen signaling was suppressed by metabolically reducing intracellular E_2 concentrations (increased CYP1B1 and HDS17B11) and up-regulating genes that antagonize ERŬ activity (SETD7, FHL2, Snail 1, COUP-TF1, TRŬ, AR, INHBA, and SMAD3) or repressing genes that promote ERŬ activity (AREG, PIK3CB, and CAV1). ERS was indicated by a deficiency in up-regulating genes involved in initiating a UPR (GRP78, XBP1, and S1P), protein folding (GRP78, PDIA6, and UGGT1), and degradation of malfolded proteins (HERP1 and DERL1), which would lead to accumulation of unfolded/misfolded proteins. Meanwhile, expression profiles indicated a widespread inhibition of protein translation (increased $p58^{IPK}$ and decreased aminoacyl tRNA synthetases, EEF2K, and ERF3A) and fatty acid synthesis (decreased ACLY and SCD), which combined with accumulation of unfolded proteins, would also promote stress and apoptosis. ERS was also indicated by induction of BIM, BAX, and the inflammatory caspase CASP4. We previously showed that depletion of BIM or BAX blocked E_2-induced apoptosis, and here, we showed that blocking CASP4 with z-LEVD-fmk also blocked E_2-induced apoptosis. Inflammatory stress was indicated by up-regulation of cytokines/cytokine receptors (IL-4R, IL-6R, IL-6ST/gp130, IL-17RD, and LTB), IFN/IFN responsive genes (IFNL1, IFI6, and IFI16), AA biosynthetic genes (FADS1 and PLA2G10), and other inflammatory markers (CEBPB, NTN1, and UNC5C). These findings indicate that inflammatory and ERS responses leading to apoptosis are highly interrelated and may cross-talk in part through NF-əB, JNK, and AP-1. Thus, additional stimulation of ERS and inflammatory responses by AA interacted with E_2 to superadditively induce apoptosis.

It should be noted that the differentially expressed genes identified here are associated with E_2-induced apoptosis; hence, their causal role in apoptosis needs to be functionally validated. Additional characterization and functional validation of genes and pathways regulating E_2-mediated apoptosis in 5C cells is currently being investigated using genome-wide, high-throughput RNAi profiling. Also, the results presented here are based on only MCF-7 derivative cell lines; hence, the findings may have limited applicability to the clinic. However, the MCF-7 cell line has accurately predicted clinical responses to antihormonal therapy in breast cancer [426]. We and others have reported antihormonal-resistant MCF-7ï based models besides 5C cells that exhibit E_2-induced apoptosis in vitro and in vivo [85,93,132,133]. We are aware of only one other breast cancer model not derived from MCF-7 cells that exhibits this behavior (i.e., T47D cells stably expressing PKCŬ), but only when grown in vivo as xenograft tumors[427]. Therefore, molecular markers of ERS and inflammatory stress need to be confirmed in low-dose E_2 responding compared with nonresponding tumors in patients with estrogen-deprived metastatic breast cancer.

The identified 5C-specific genes may serve as biomarkers to predict response to estrogen therapy (e.g., the secreted factors IFNL1, LTB, and NTN1 could be readily measured in patients). The identified 5C-specific genes also provide the basis for potentially improving clinical response rates to estrogen by combining it with agents that promote ERS and/or tumor-specific inflammation. For example, neutralizing NTN1 antibodies, AA, or its precursor, conjugated LA, may increase response rates without engaging systemic

inflammatory responses. Furthermore, these findings lead to the hypothesis that antiinflammatory agents prescribed for ancillary clinical problems should not be used during antitumor estrogen therapy.

KEY RESEARCH ACCOMPISHMENTS

Task 1

Task 1a (LCCC, Isaacs)

- The creation of a new low-dose estrogen protocol to treat women who have been exhaustively treated with antihormone therapy.
- The consolidation of the Georgetown Lombardi Comprehensive Cancer Center vision of clinical trials testing with Hackensack Hospital in New Jersey.

Task 2

Task 2a (*GU - Jordan/Obiorah)*

- Steroidal and phytoestrogens induce proliferation of MCF-7 cells at physiologic concentrations but inhibit the growth and induce apoptosis of MCF7:5C cells.
- Although steroidal and phytoestrogens induce estrogen responsive genes, their anti-proliferative and apoptotic effects are mediated through the estrogen receptor. Knockdown of ERŬusing siRNA blocks all estrogen induced apoptosis and growth inhibition.
- Phytoestrogens induce endoplasmic reticulum stress and inflammatory response stress related genes in a comparable manner as the steroidal estrogens.

Task 2b (*GU - Jordan/Maximov)*

- We have synthesized fixed ring (FR) analogs of 4OHT and endoxifen as well as FR E and Z isomers with a methoxy and ethoxy side chains.
- Pharmacologic properties were documented in the MCF-7 cell line, and prolactin synthesis assessed in GH3 rat pituitary tumor cells.
- The FR Z-isomers of 4OHT and endoxifen were equivalent to 4OHT and endoxifen. Other test compounds used possessed partial estrogenic activity.
- The E-isomers of FR 4OHT and endoxifen had no estrogenic activity at therapeutic serum concentrations.
- None of the newly synthesized compounds were able to down regulate ER levels.
- Molecular modelling demonstrated that some compounds would each create a best fit with a novel agonist conformation of the ER.
- The results demonstrate modulation by the ER complex of cell replication or gene transcription in cancer.

Task 2c (*GU- Jordan/Obiorah)*

- Bisphenol induced cell cycle genes similar to those induced by 17ƃ oestradiol (E_2).
- Unlike the trigger by E_2 that occurs after 24h the trigger of apoptosis for bisphenol occurs at 4 days with quantifiable apoptotic changes noted at 6 days.
- A prolonged induction of endoplasmic reticulum stress and inflammatory stress response genes was observed with subsequent activation of apoptosis related genes in the second week of treatment with bisphenol.

Task 2d (*GU - Jordan/ Obiorah)*

- We classified the conformation of planar estrogens or angular TPE (tri-phenyl ethylene) complexes as ñestrogen-likeò or ñantiestrogen-likeò complexes, respectively.
- The TPE:ER complexes did not readily recruit the co-activator steroid receptor coactivator-3 (SRC3) or ER to the PS2 promoter in MCF-7 and MCF7:5C cells.
- Molecular modeling showed that the TPE prefer to bind to the ER in an antagonistic fashion, which did not seal the ligand binding domain (LBD) effectively, therefore reduced SRC3 binding ensued.
- The fully activated ER complex with helix 12 sealing the LBD is suggested to be the appropriate trigger to initiate rapid estrogen induced apoptosis.

Task 2e (*GU- Jordan/Sengupta/Obiorah)*

- E_2-induced apoptosis is a delayed process, whereas paclitaxel immediately inhibits the growth and induces death of MCF7:5C cells.
- The cellular commitment for E_2-triggered apoptosis occur after 24 h. Activation of the intrinsic pathway was observed by 36 h of E_2 treatment with subsequent induction of the extrinsic apoptotic pathway by 48 h.
- Paclitaxel exclusively activated extra mitochodrial apoptotic genes and caused rapid G2/M blockade by 12 h of treatment. By contrast, E_2 causes an initial proliferation with elevated S phase of cell cycles followed by apoptosis of the MCF7:5C cells.
- We report that E_2-induced apoptosis involves a novel, multidynamic process that is distinctly different from that of a classic cytotoxic chemotherapeutic drug used in breast cancer.

Task 2f (*GU- Jordan/Sweeney)*

- The data suggest that MCF-7:2A cells employ stronger antioxidant defense mechanisms than do MCF-7:5C cells, and that oxidative stress is ultimately required for MCF-7:2A cells to die in response to E2 treatment.
- Tumor necrosis factor (TNF) family member activation is also essential for E(2)-induced apoptosis to occur in MCF-7:2A cells; upregulation of TNFǓ occurs simultaneously with oxidative stress activation.
- The unfolded protein response (UPR) signaling pattern is similar to that in MCF-7:5C cells, it is not sufficient to cause cell death in MCF-7:2A cells.
- Additionally, increased insulin-like growth factor receptor ƀ (IGF-1Rƀ) confers a mechanism of growth and anti-apoptotic advantage in MCF-7:2A cells.

Task 2g (*GU- Jordan/Sengupta)*

- Basal expression of cMYC transcripts and protein level is 3-4 fold higher in a panel of ER+ breast cancer cell model representing the endocrine therapy resistance compared to parental counterpart MCF7 cells.
- Pharmacological inhibition of cMYC and Knock-down of cMYC gene in MCF7:5C cells inhibited the estrogen independent growth of the cells by reducing the óSô phase cells.
- High cMYC expression correlated with poor relapse free survival in patients treated with endocrine therapy but not chemotherapy.
- High levels of serine-2-phosphorylated RNA polymerase II (a marker of elongation of RNA synthesis) was recruited at the cMYC promoter in MCF7:5C cells, as compared to parental MCF7 cells, are most likely responsible for the higher levels of cMYC transcripts.

- Pharmacological inhibition of CDK9 not only blocked the estrogen-independent growth of the MCF-7:5C cells but also inhibited the transcription of cMYC gene and the protein levels.

Task 2h (*GU- Jordan/Fan)*

- Long-term treatment with PP2 and E_2 blocked apoptosis and the resulting cell line (MCF-7:PF) was unique, as they grew vigorously in culture with physiological levels of E_2 in an ER-dependent manner.
- One major change was that PP2 collaborated with E2 to increase the level of insulin-like growth factor-1 receptor beta (IGF-1Rϐ). Blockade of IGF-1Rϐ completely abolished E2-stimulated growth in MCF-7:PF cells.
- Furthermore, combination treatment up-regulated transcription factors, Twist1 and Snail, and repressed E-cadherin expression which made MCF-7:PF cells display a characteristic phenotype of epithelial-mesenchymal transition (EMT).

Task 2i (*GU- Jordan/Fan)*

- Estrogen (E_2) widely activated apoptosis-related genes detected by RNA-sequence analysis in MCF-7:5C cells. Majority of these genes were related with stress.
- We further found that E_2 activated sensors of unfolded protein response (UPR) after 24 hours treatment in MCF-7:5C cells. It indicated that E_2 caused extra unfolded protein accumulating in the endoplasmic reticulum.
- E_2 activated oxidative stress indicator HMOX1 and increased the production of reactive oxygen species (ROS).
- We confirmed that E_2-induced apoptosis utilized c-Src tyrosine kinase as an important signaling pathway. c-Src tyrosine kinase acted as an important signal transducer in the process of stress induced by E_2.

Task 2j (*GU- Jordan/Sengupta)*

- Bisphenol (BP) and bisphenol-A (BPA) induced the proliferation of MCF7 breast cancer cells, however, unlike BPA, BP failed to induce apoptosis in MCF7:5C cells
- BPA consistently acted as an agonist in our studies but BP exhibited mixed agonistic/antagonistic properties.
- Molecular docking revealed agonistic and antagonistic mode of binding for BPA and BP respectively. BPA treatment resembled E2 treatment in terms of PCR-based regulation of apoptotic genes whereas BP was similar to 4OHT treatment.
- The chemical structure of ERŬ ligand determined the agonistic or antagonistic biological responses by the virtue of their binding mode, conformation of the liganded-ERŬ complex and the context of the cellular function.

Task 2k (*GU- Jordan/Fan)*

- A specific c-Src inhibitor, 4-amino-5-(4-chlorophenyl)-7(t-butyl)pyrazolo[3,4-d]pyrinidine (PP2), was utilised to block c-Src activity to identify targeted vulnerabilities affected by ER and HER2 in a panel of breast cancer cell lines.
- The antiproliferative effect of PP2 closely correlated with the inhibition of c-Src mediated extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and/or phosphoinositide 3-kinase (PI3K)/Akt growth pathways.

- Inhibition of c-Src tyrosine kinase predominantly blocked ER negative breast cancer cell growth, particularly the triple (i.e. ER, progesterone receptor (PR), and HER2) negative cells.

Task 2l (*FCCC- Jordan/Lewis-Wambi)*

- We characterized the effects of BZA and several other SERMs on the proliferation of hormone-independent MCF-7 and T47D breast cancer cells and hormone-independent MCF-7:5C and MCF-7:2A cells and examined their mechanism of action in these cells.
- We found that all of the SERMs inhibited the growth of MCF-7 and T47D cells, however, BZA was the only SERM that inhibited the growth of hormone-independent MCF-7:5C and MCF-7:2A cells.
- Consistent with these growth results, we found that BZA induced G1 blockade in MCF- 7:5C and MCF-7:2A cells and it significantly downregulated ERŬand cyclin D1 which were constitutively overexpressed in these cells.
- siRNA knockdown of ERŬand/or cyclin D1 significantly reduced the inhibitory effect of BZA in MCF-7:5C cells.
- Further analysis revealed that BZA downregulated ERŬprotein by increasing its degradation and it suppressed cyclin D1 protein and promoter activity in MCF-7:5C cells. Lastly, molecular modeling studies demonstrated that BZA bound to ERŬin an orientation similar to raloxifene and had the tendency to form the same contacts with the aminoacids lining the binding cavity.
- The manuscript entitled ñThe Selective Estrogen Receptor Modulator Bazedoxifene Inhibits Hormone-Independent Breast Cancer Cell Growth and Downregulates Estrogen Receptor Ŭ and Cyclin D1ò is under consideration in Molecular Pharmacology.

Task 2m (*FCCC- Jordan/Maximov)*

- TPEs were not only unable to induce efficient apoptosis in MCF7:5C cells but also reversed the E2-induced apoptosis similar to 4OHT within the first week of treatment.
- The TPEs and 4OHT did not reduce the ERŬprotein levels unlike E2.
- The ChIP assay confirmed very weak recruitment of SRC3 despite modest recruitment of ERŬin the presence of TPEs.
- Moleular modeling suggests that TPE would bind in antagonistic mode with ERŬ, thus cannot efficiently recruit co-activator SRC3. As a result, the TPE complex cannot induce apoptosis of MCF-7:5C cells, although it can cause growth of MCF-7:WS8 cells. The conformation of the estrogen-ER complex differentially controls growth and apoptosis.

Task 2n (*FCCC - Jordan/Balaburski)*

- MCF-7 cells cultured under estrogen-deprived conditions in the presence of 1 ÕM RAL for more than a year develop RAL resistance resulting in an independent cell line, MCF7-RAL.
- The MCF7-RAL cells grow in response to both estradiol (E_2) and RAL. Fulvestrant (FUL) blocks RAL and E2-mediated growth.
- Continuous re-transplantation of the tumors growing in RAL-treated mice indicated that RAL stimulated tumor growth.
- MCF7-RAL tumors grew with RAL and not E2, a characteristic of phase II resistance.
- The MCF7-RAL tumors that initially were inhibited by E2 grew in the presence of E2 and subsequently grew with either RAL or E2.

- Subsequent transplantation of E2 stimulated tumors and evaluations of the actions of RAL, demonstrated robust E2-stimulated growth that was blocked by RAL.

Task 2o (*FCCC - Jordan/Sengupta)*

- Estradiol (E_2) induced the transcription of X-Box Binding Protein-1 (XBP1) in both MCF7 and ECC1 cells.
- E2 dependent recruitment of ERŬ, steroid receptor co-activator (SRC) 1 and 3, and RNA polymerase II were observed at the promoter and/or enhancer region of XBP1 gene.
- Depletion of XBP1 markedly inhibited the E_2-induced growth in MCF7 and ECC1 cells.
- ERE-mediated transcription was not altered in XBP1 over-expressing or XBP1 depleted MCF7 cells.

Task 2p (*FCCC - Jordan/Maximov)*

- 1,1,2-Triphenylethylene (TPE) derivatives were synthesized and evaluated against 17ɓ-estradiol (E2) for their estrogenic activity in MCF-7:WS8 human breast cancer cells.
- All TPEs were estrogenic and, unlike 4-hydroxytamoxifen (4OHTAM) and Endoxifen, induced cell growth to a level comparable to that of E2.
- All the TPEs increased ERE activity in MCF-7:WS8 cells.
- Transient transfection of the ER-negative breast cancer cell line T47D:C4:2 with wild-type ER or D351G ER mutant revealed that all of the TPEs increased ERE activity in the cells expressing the wild-type ER but not the mutant, thus confirming the importance of Asp351 for ER activation by the TPEs.
- Using available conformations of the ER liganded with 4OHTAM or diethylstilbestrol, the TPEs optimally occupy the 4OHTAM ER conformation that expresses Asp351.

Task 2q (*FCCC - Jordan/Sengupta)*

- Combination of low doses of Brivanib alaninate and tamoxifen was as effective as higher doses of either drug used alone as an anti-tumor for SERM sensitive (MCF-7 E2) xenograft.
- The combination of the low dose of Brivanib alaninate and tamoxifen is successful at decreasing tumor growth of established tumors, while neither agent alone is effective.
- A decreased phosphorylation of VEGFR-2 in the tumors that were treated with brivanib alaninate was observed while an increase in VEGFA transcription to compensate for the blockade of VEGFR-2 was seen.
- Tamoxifen increased the phosphorylation of VEGFR-2 and this effect was abrogated by brivanib alaninate. There was also increased necrosis in tumors treated with brivanib alaninate.

Task 2r (*FCCC - Jordan/Ariazi)*

- Gene expression microarray data from five cohorts comprising 1,250 breast carcinomas showed an association between increased GPR30 expression and ERŬ-positive status.
- E2 and DES, but not G-1, transiently downregulated both ER and GPR30, indicating that this was ER mediated.
- GPR30, but not ERŬ, mediated E2-induced Ca2+ responses because E2, 4-hydroxytamoxifen (activates GPR30), and G-1, but not DES, elicited cytosolic Ca2+ increases not only in MCF-7 cells but also in ER-negative SKBr3 cells.

- Proliferation studies involving GPR30 depletion indicated that the role of GPR30 was to promote SKBr3 cell growth but reduce MCF-7 cell growth.
- G-1 profoundly inhibited MCF-7 cell growth, potentially via p53 and p21 induction.

Task 2s (*FCCC - Jordan/Peng)*

- ERŬexpression inhibited the growth of SKBr3 cells which was further enhanced in the presence of 1 nM 17ɓ-estradiol.
- Upon ERŬexpression the SKBr3 cells were arrested at G0/G1 cell cycle, which corresponded to an increase of $p21^{Cip1/Waf1}$, hypo-phosphorylation of pRb and decrease of E2F1.
- Reduced EGFR and HER2 expression was observed after estrogen treatment in SKBr3 cells with ERŬexpression.

Task 2t (*FCCC - Jordan/Lewis-Wambi)*

- Treatment of MCF-7:2A cells with 1 nM E2 plus 100 ÕM BSO combination for 1week reduced the growth of these cells by almost 80ï 90% whereas the individual treatments had no significant effect on growth.
- TUNEL and 4ǫ6-diamidino-2-phenylindole (DAPI) staining showed that the inhibitory effect of the combination treatment was due to apoptosis.
- Our data indicates that glutathione participates in retarding apoptosis in antihormone-resistant human breast cancer cells.
- Depletion of glutathione by BSO may be critical in predisposing resistant cells to estrogen-induced apoptosis.

Task 2u (*FCCC - Jordan/Lewis-Wambi)*

- Exposure of MCF-7:2A cells to 1 nM E2 plus 100 ε M BSO combination for 48 to 96 h produced a sevenfold increase in apoptosis whereas the individual treatments had no significant effect on growth.
- Induction of apoptosis by the combination treatment of E2 plus BSO was evidenced by changes in Bcl-2 and Bax expression.
- The E2 plus BSO combination treatment also markedly increased phosphorylated c-Jun Nterminal kinase (JNK) levels in MCF-7:2A cells and blockade of the JNK pathway attenuated the apoptotic effect this treatment.
- Our in vitro findings corroborated in vivo data from a mouse xenograft model in which daily administration of BSO either as a single agent or in combination with E2 significantly reduced tumor growth of MCF-7:2A cells.
- Our data indicates that GSH participates in retarding apoptosis in antihormone-resistant human breast cancer cells and that depletion of this molecule by BSO may be critical in predisposing resistant cells to E2-induced apoptotic cell death.

Task 2v (*FCCC - Jordan/Lewis-Wambi)*

- We showed that CEACAM6 was overexpressed in a panel of oestrogen receptor (ERa)-positive human breast cancer cell lines (MCF-7:5C and MCF-7:2A). Expression array

analysis revealed that MCF-7:5C and MCF-7:2A cells overexpressed CEACAM6 mRNA by 27-fold and 12-fold, respectively, and were 6ï 15-times more invasive compared to non-invasive wild-type MCF-7 cells which expressed low levels of CEACAM6.

- This CEACAM6 overexpression was associated with a more aggressive invasive phenotype in vitro.
- Suppression of CEACAM6 expression using small interfering RNA (siRNA) completely reversed migration and invasion of MCF-7:5C and MCF-7:2A cells and it significantly reduced phosphorylated Akt and c-Src expression in these cells.
- Our findings establish CEACAM6 as a unique mediator of migration and invasion of drug resistant estrogen-deprived breast cancer cells and suggest that this protein could be an important biomarker of metastasis.

Task 2w (*FCCC - Jordan/Ariazi)*

- We investigated aromatase inhibitors (AI) metabolite, 17-hydroexemestane 1 and observed it bound estrogen receptor alpha (ERalpha) very weakly and androgen receptor (AR) strongly.
- 17-Hydroexemestane induced proliferation, stimulated cell cycle progression and regulated transcription at high sub-micromolar and micromolar concentrations through ER in both MCF-7 and T47D cell lines, but through AR at low nanomolar concentrations selectively in T47D cells.
- 17-Hydroexemestane down-regulated ERalpha protein levels at high concentrations in a cell type-specific manner similarly as 17beta-estradiol, and increased AR protein accumulation at low concentrations in both cell types similarly as R1881.

Task 2x (*FCCC - Jordan/Ariazi)*

- Endocrine-resistance are divided into Phase I and Phase II stages.
- Estrogen inhibits growth of Phase II antihormone-resistant tumors.
- Based on these and prior laboratory findings, we propose a clinical strategy for optimal third-line therapy: patients who have responded to and then failed at least two antihormonal treatments may respond favorably to short-term low-dose estrogen due to E_2-induced apoptosis.

Task 3

Task 3a (GU - Riegel/Wellstein)

- We have completed our proteomics analysis to identify AIB1 and pY-complex proteins that are regulated differentially in response to E2 in MCF-7 versus MCF-7/5C cells.
- We have built a pathway model that integrates this information into the differential response of these cells to E2 (growth vs. apoptosis) and can now interrogate the relative contributions of these pathways revealed by the initial analysis.
- We have built an AIB1 interaction network that allows us to find functional association with pathways that have druggable targets
- We have established MS analysis of the ER and found differential posttranslational modifications in the ER when comparing MCF-7 and MCF-7/5C cells. This should reveal additional signaling mechanisms that impact on ER activity and will be connected to the findings already in place.

Task 4

Task 4a (FCCC- Ariazi; TGen – Azorsa/Balagurunathan/Cunliffe)

- We interrogated E(2)-induced apoptosis by analysis of gene expression across time (2-96 h) in MCF-7 cell variants that were estrogen-dependent (WS8) or resistant to estrogen deprivation and refractory (2A) or sensitive (5C) to E(2)-induced apoptosis.
- We developed a method termed differential area under the curve analysis that identified genes uniquely regulated by E(2) in 5C cells compared with both WS8 and 2A cells and hence, were associated with E(2)-induced apoptosis.
- The up-regulated proinflammatory genes included IL, IFN, and arachidonic acid-related genes. Functional testing showed that arachidonic acid and E(2) interacted to superadditively induce apoptosis.

REPORTABLE OUTCOMES

Publications

Refereed Research Publications

1. Ariazi, E., Lewis-Wambi, J.S., Gill, S.D., Pyle, J.R., Ariazi, J.L., Kim, H.R., Sharma, C.G.N., Cordera, F., Shupp, H.A., Li, T. and Jordan, V.C. (2006). Emerging principles for the development of resistance to antihormonal therapy: Implications for the clinical utility of fulvestrant. Journal of Steroid Biochemistry and Molecular Biology 102:128-138.
2. Ariazi, E.A., Kraus, R.J., Farrell, M.L., Jordan, V.C. and Mertz, J.E. (2006). Estrogen-related receptor Ǔ1's transcriptional activities are regulated in part via the ErbB2 (HER2) signaling pathway. Molecular Cancer Research 5:71-85.
3. Pannier, A.K., Ariazi, E.A., Bellis, A.D., Bengali, Z., Jordan, V.C. and Shea, L.D. (2007). Bioluminesence imaging for assessment and normalization in trasfected cell arrays. Biotechnology Bioengineering 98:486-497.
4. Ariazi, E.A., Leit«o, A., Oprea, T.I., Chen, B., Louis, T., Bertucci, A.M., Sharma, C.G.N., Gill, S.D., Kim, H.R., Shupp, H.A., Pyle, J.R., Madrack, A., Donato, A.L., Cheng, D., Paige, J.R. and Jordan, V,C. (2007). Exemestaneôs 17-hydroxylated metabolite exerts biological effects as an androgen. Molecular Cancer Therapeutics 6:2817-2827.
5. Lu, M., Strohecker, A., Chen, F., Kwan, T., Bosman, J., Jordan, V.C. and Cryns, V.L. (2008). Aspirin sensitizes cancer cells to TRAIL-induced apoptosis by reducing survivin levels. Clinical Cancer Research 14:3168-3176.
6. Lewis-Wambi, J., Cunliffe, H.E., Kim, H.R., Willis, A.L. and Jordan, V.C. (2008). Overexpression of CEACAM6 promotes migration and invasion of estrogen deprived breast cancer cells. European Journal of Cancer 44:1770-1779.
7. Lewis-Wambi, J.S., Kim, H.R., Wambi, C., Patel, R., Pyle, J.R., Klein-Szanto, A.J. and Jordan, V.C. (2008). Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. Breast Cancer Research 10:R104.
8. Lewis-Wambi, J.S., Swaby, R.R., Kim, H. and Jordan, V.C. (2009). Potential of L-buthionine sulfoximine to enhance the apoptotic action of estradiol to reverse acquired antihormonal resistance in metastatic breast cancer. J Steroid Biochemistry and Molecular Biology 114:33-39.
9. Yu, J.J., Robb, V.A., Morrison, T.A., Ariazi, E.A., Karbowniczek, M., Astrinidis, A., Wang, C., Hernandez-Cuebas, L., Seeholzer, L.F., Nicolas, E., Hensley, H., Jordan, V.C., Walker, C.L. and Henske, E.P. (2009). Estrogen promotes the survival and pulmonary metastasis of tuberin-null cells. Proceedings of the National Academy of Sciences U S A 106:2635-2640.

10. Morrow, M., Chatteron, R.T. Jr, Rademaker, A.W., Hou, N., Jordan, V.C., Hendrick, R.E. and Khan, S.A. (2009). A prospective study of variability in mammographic density during the menstrual cycle. Breast Cancer Research Treatment 121:565-574.

11. Peng, J. and Jordan, V.C. (2010). Expression of estrogen receptor alpha with a Tet-off adenoviral system induces G0/G1 cell cycle arrest in SKBr3 breast cancer cells. International Journal of Oncology 36:451-458.

12. Ariazi, E.A., Brailoiu, E., Yerrum, S., Shupp, H.A., Slifker, M.J., Cunliffe, H.E., Black, M.A., Donato, A.L., Arterburn, J.B., Oprea, T.I., Prossnitz, E.R., Dun, N.J. and Jordan, V.C. (2010). The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. Cancer Research 70:1184-1194. (Selected for Faculty of 1000 Medicine, identified as an important article published in Medicine for its scientific merit and positive contribution to the medical literature).

13. Patel, R.R., Sengupta, S., Kim, H.R., Klein-Szanto, A.J., Pyle, J.R., Zhu, F., Li, T., Ross, E.A., Oseni, S., Fargnoli, J. and Jordan, V.C. (2010). Experimental treatment of estrogen receptor (ER) positive breast cancer with tamoxifen and brivanib alaninate, a VEGFR-2/FGFR-1 kinase inhibitor: a potential clinical application of angiogenesis inhibitors. European Journal of Cancer 46:1537-1553.

14. Maximov, P.Y., Myers, C.B., Curpan, R.F., Lewis-Wambi, J.S. and Jordan, V.C. (2010). Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. Journal of Medicinal Chemistry 53:3273-3283.

15. Sengupta, S.S., Sharma, C.G.N. and Jordan, V.C. (2010). Estrogen regulation of X-Box binding protein-1 and its role in estrogen induced crowth of breast and endometrial cancer cells. Hormone Molecular Biology and Clinical Investigation 2:235-243.

16. Balaburski, G., Dardes, R.C., Johnson, M., Haddad, B., Zhu, F., Ross, E.A., Sengupta, S., Klein-Szanto, A., Liu, H., Kim, H. and Jordan, V.C. (2010). Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: A unifying concept in anti-hormone resistance. International Journal of Oncology 37:387-398.

17. Vogel, V.G., Costantino, J.P., Wickerham, D.L., Cronin, W.M., Cecchini, R.S., Atkins, J.N., Bevers, T.B., Fehrenbacher, L., Pajon, E.R., Wade, J.L., Robidoux, A., Margolese, R.G., James, J., Runowicz, C.D., Ganz, P.A., Reis, S.E., McCaskill-Stevens, W., Ford, L.G., Jordan, V.C. and Wolmark, N. (2010). Update of the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing Breast Cancer. Cancer Prevention Research 3:696-706.

18. Maximov, P., Sengupta, S., Lewis-Wambi, J.S., Kim, H.R., Curpan, R.F. and Jordan, V.C. (2011). The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: a hypothesis. Hormone Molecular Biology and Clinical Investigation 5:27-34.

19. Yang, C.Z., Yaniger, S.I., Jordan, V.C., Klein, D.J. and Bittner, G.D. (2011). Most plastic products release estrogenic chemicals: a potential health problem that can be solved. Environmental Health Perspectives 119:989-996.

20. Hu, Z., Kagan, B.L., Ariazi, E., Rosenthal, D.S., Zhang, L., Li, J.V., Huang, H., Wu, C., Jordan, V.C., Riegel, A.T. and Wellstein, A. (2011). Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. PLoS ONE Epub 6:e20410.

21. Lewis-Wambi, J.S., Kim, H., Curpan, R., Grigg, R., Sarker, M.A. and Jordan, V.C. (2011). The selective estrogen receptor modulator, bazedoxifene, inhibits hormone-independent breast cancer cell growth and downregulates estrogen receptor Ŭand cyclin D1. Molecular Pharmacology 80:610-620.

22. Ariazi, E.A., Cunliffe, H.E., Lewis-Wambi, J.S., Slifker, M.J., Willis, A.L., Ramos, P., Tapia, C., Kim, H.R., Yerrum, S., Sharma, C.G.N., Nicolas, E., Balagurunathan, Y., Ross, E.A. and Jordan, V.C. (2011). Estrogen-induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proceedings of the National Academy of Sciences U.S.A. 108:18879-18886.

23. He, H.H., Meyer, C.A., Chen, M.W., Jordan, V.C., Brown, M. and Liu, X.S. (2012). Differential DNase I hypersensitivity reveals factor-dependent chromatic dynamics. Genome Research 22:1015-25.

24. Fan, P., McDaniel, R.E., Kim, H.R., Clagett, D., Haddad, B., Jordan, V.C. (2012). Modulating Therapeutic Effects of the c-Src Inhibitor via Estrogen Receptor and HER2 in Breast Cancer Cell Lines. European Journal of Cancer 48:3488-98.

25. Gass, M.L.S., Manson, J.E., Cosman, F., Grodstein, F., Jordan, V.C., Karas, R.H., Kaunitz, A.M., Maki, P.M., Schmidt, P.J., Shifren, J.L., Stuenkel, C.A. and Utian, W.H. (2012). Position statement: the 2012 hormone therapy position statement of The North American Menopause Society. Menopause: The Journal of The North American Menopause Society 19:257-271.

26. Korch C., Spillman, M.A., Jackson, T.A., Jacobsen, B.M., Murphy S.K., Lessey, B.A., Jordan, V.C. and Bradford, A.P. (2012). DNA profiling analysis of endometrial and ovarian cell lines reveals misidentification, redundancy and contamination. Gynecologic Oncology 127:241-248.

27. Sengupta, S. S., Obiorah, I. O., Maximov, P. Y., Curpan, R., and Jordan, V. C. (2013) Molecular mechanism of action of bisphenol and bisphenol-A mediated by estrogen receptor alpha in growth and apoptosis of breast cancer cells. British Journal of Pharmacology 169:167-78.

28. Segala G., de Medina P., Iuliano L., Zerbinati C., Paillasse M. R., Noguer E., Dalenc F., Payre B., Jordan V. C., Record M., Silvente-Poirot S., Poirot M (2013).5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of tamoxifen in breast cancer cells. Biochemical Pharmacology 88:175-189.

29. Fan, P., Griffith, O.L., Agboke, F., Anur, P., Zou, X., McDaniel, R.E., Creswell, K., Kim, S.H., Katzenellenbogen, J.A., Gray, J.W. and Jordan, V.C. (2013). c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. Cancer Research 73:4510-4520.

30. Baniwal, S.K., Chimge, N.O., Jordan, V.C., Tripathy, D., Frenkel, B. (2013). Prolactin-induced protein (PIP) regulates proliferation of luminal A type breast cancer cells in an estrogen independent maner. PLoS One. 8: e62361.

31. Fan, P., Agboke, F.A., McDaniel, R.E., Sweeney, E.E., Zou, X., Creswell, K., Jordan, V.C. (2014). Inhibition of c-Src blocks estrogen-induced apoptosis and restores estrogen-stimulated growth in long-term estrogen-deprived breast cancer cells. European Journal of Cancer. 50:457-468

32. Sengupta, S., Biarnes, M.C., and Jordan, V.C. (2014). Cyclin dependent kinase-9 mediated transcriptional deregulation of cMYC as a critical determinant of endocrine therapy resistance in breast cancers. Breast Cancer Research and Treatment. 143:113-124.

33. Sweeney, E.E., Fan, P., Jordan, V.C. (2014) Mechanisms underlying differential response to estrogen-induced apoptosis in long-term estrogen-deprived breast cancer cells. International Journal of Oncology. 44:1529-1537.

34. Obiorah, I.O., Sengupta, S., Fan, P., Jordan, V.C. (2014) Delayed triggering of oestrogen induced apoptosis that contrasts with rapid paclitaxil induced breast cancer cell death. British Journal of Cancer. 110:1488-1496.

35. Obiorah, I.O., Sengupta, S., Curpan, R., Jordan, V.C (2014) Defining the conformation of the estrogen receptor complex that controls estrogen induced apoptosis in breast cancer. Molecular Pharmacology. *In press*.

36. Obiorah, I.O., Jordan, V.C. (2014) Differences in the rate of oestrogen induced apoptosis by oestradiol and the triphenylethylene bisphenol. British Journal of Pharmacology. *In press*.

37. Maximov, P.Y. Fernandes, D.J., McDaniel, R.E., Myers, C., Curpan, R., Jordan, V.C. (2014) Influence of the length and positioning of the antiestrogenic side chain of endoxifen and 4-hydroxytamoxifen on gene activation and growth of estrogen receptor positive cancer cells. Journal of Medicinal Chemistry. *In press*.

38. Obiorah, I.O., Fan, P., Jordan, V.C. Breast cancer cell apoptosis with phytoestrogens is dependent on an estrogen deprived state. Cancer Prevention Research. *In press.*

39. Maximov, P.Y., McDaniel, R.E., Fernandes, D.J., Bhatta, P., Korostyshevskiy, V, Curpan, R., Jordan, V.C. Pharmacological relevance of endoxifen in a laboratory simulation of breast cancer in postmenopausal patients. Journal of the National Cancer Institute. *In Press*.

40. Maximov, P.Y., McDaniel, R.E., Fernandes, D.J., Korostyshevskiy, V., Bhatta, P., Jordan, V.C. In vitro simulation of tamoxifen treatment in premenopausal breast cancer patients with different CYP2D6 genotypes. British Journal of Pharmacology. *In press.*

Books

1. Jordan, V.C. (2013). Estrogen Action, SERMs and Womenôs Health. Imperial College Press, London.
2. Maximov, P.Y., McDaniel, R. E., and Jordan, V.C. (2013). Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basal, Switzerland.

Published Named Lectures and Special Journal Issues

1.Jordan, V.C. and Brodie, A.M.H. (2006). Development and evolution of targeted endocrine therapies for the treatment of breast cancer. (Charles F. Kettering Prize, General Motors Cancer Research Foundation) Steroids 72:7-25.

2.Jordan, V.C. (2007). Successful translation research with selective oestrogen receptor modulators to treat and prevent breast cancer. Geburtshilfe und Frauenheilkunde (German Journal of Obstetrics and Gynecology) 67:443-450.

3.Jordan, V.C. (2008). The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer--survival or death? Journal of Clinical Oncology: 26:3073-3082.

4.Jordan, V.C. (2009). A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention . Cancer Research: 69:1243-1254.

5.Jordan, V.C. (2009). Jephcott Lecture: Estrogen action and the life or death of breast cancer. In: the Jephcott Symposium and Lecture Series: Current Innovations in the Treatment and Prevention of Breast Cancer. Z. Raytor (ed.). Royal Society of Medicine, pp. 55-63.

6.Jordan, V.C., Obiorah, I., Fan, P., Kim, H.R., Ariazi, E., Cunliffe, H. and Brauch, H. (2011). Evolution of long-term adjuvant antihormone therapy: Consequences and Opportunities. The St. Gallen Prize Lecture. Breast 20(Suppl 3):S1-S11.

7.Obiorah, I. and Jordan, V.C. (2013) The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. North American Menopause Society/Pfizer-Wulf H. Utian Endowed Lecture. Menopause 20:372-382.

Biographical Contributions

1. Poirot, M. (2011). Four decades of discovery in breast cancer research and treatment ï an interview with V. Craig Jordan. International Journal of Developmental Biology 55:703-712.
2. Gupta, S. (2011). Profile of V. Craig Jordan. Profile of a recently elected member of the National Academy of Sciences to accompany the memberôs Inaugural Article. Proceedings of the National Academy of Sciences U.S.A. 108:18876-18878.
3. Jordan, V.C. (2014) Tamoxifen the first targeted long term adjuvant therapy for breast cancer. Endocrine Related Cancer. 21:R235-246.
4. Jordan, V.C. (2014) Profile - Who do you think you are? Endocrine Related Cancer. 21:P13-17.

5. Jordan, V.C. (2014) Tamoxifen as the first successful targeted therapy in cancer: the gift that kept on giving. Breast Cancer Management. *In press*.

6. Jordan, V.C. (2014) Proven value of translational research with appropriate animal models to advance breast cancer treatment and save lives: the tamoxifen tale. British Jounal of Clinical Pharmacology. *In press.*

Contributions to Reference Works

1. Swaby, R.F., Sharma, C.G.N., Jordan, V.C. (2007). SERMs for the treatment and prevention of breast cancer. In: Reviews in Endocrine and Metabolic Disorders. (A. Manni, Ed), Springer, Norwell, MA, 8:229-239.

2. Jordan, V.C. (2008). A personal account of the Chemoprevention of Breast Cancer: Possible or not possible? In: Principles of Molecular Oncology, Third Edition. (Miquel H. Bronchud, MD, PhD, MaryAnn Foote, PhD, Guiseppe Giaccone, MD, PhD, Olufunmilayo Olopade, MD and Paul Workman, PhD (eds)) Humana Press NJ, pp 391-398.

3. Peng, J. and Jordan, V.C. (2008). Estrogen receptor pathways and breast cancer. In: Principles of Molecular Oncology, Third Edition. (Miquel H. Bronchud, MD, PhD, MaryAnn Foote, PhD, Guiseppe Giaccone, MD, PhD, Olufunmilayo Olopade, MD and Paul Workman, PhD (eds)) Humana Press NJ, pp 189-206.

4. Ariazi, E.A. and Jordan, V.C. (2008). Estrogen Receptors as Therapeutic Targets in Breast Cancer. In: Methods and Principles of Medicinal Chemistry (E. Ottow and H. Weinmann (eds)) Wiley-VCH Verlag GmbH & Co. KgaA, Weinheim, Berlin, Germany, pp. 127-199.

5. Davydov, A. and Jordan, V.C. (2009). Endometrial Cancer. In: Encylopedia of Cancer, 2nd Edition (M. Schwab (Ed), Springer, Heidelberg, Germany.

6. Jordan, V.C. (2009) Tamoxifen. In: Encyclopedia of Cancer, 2nd Edition (M. Schwab (Ed), Springer, Heidelberg, Germany.

7. Buzdar, A.U., Dawood, S., Harvey, H.A., and Jordan, V.C. (2010). Antiestrogens,Progestinsand Aromatase Inhibitors In: Cancer Medicine 8th Edition. Kufe,Bast,Hait,Hong,Pº llock, Weichselbaum, Holland ,and Frei(Eds) B.C. Decker Inc. Hamilton, London, pp 737-749.

8. Fan, P., and Jordan, V.C. (2014) Antiestrogens and Selective Estrogen Receptor Modulators. In: Molecular Oncology (Eds: Gelmann, Sawyers, and Rauscher). Cambridge University Press, pp.884-892. Cambridge, UK.

Refereed Review Articles

1. Jordan, V.C. (2007). Chemoprevention of Breast Cancer with Selective Oestrogen Receptor Modulators. Nature Reviews Cancer 7:46-53.

2. Jordan, V.C. (2007). (Commentary) SERMS: meeting the promise of multifunctional medicines. Journal of the National Cancer Institute 99:350-356.

3. Jordan, V.C. (2007). New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer. Steroids 72:829-842.

4. Jordan, V.C. and O'Malley, B.W. (2007). Biology of Neoplasia: Selective estrogen receptor modulators and antihormonal resistance in breast cancer. Journal of Clinical Oncology 25:5815-5824.

5. Jordan, V.C. (2008). Tamoxifen: catalyst for the change to targeted therapy. European Journal of Cancer 44:30-38.

6. Swaby, R.F. and Jordan, V.C. (2008). Low dose estrogen therapy to reverse acquired antihormonal resistance in the treatment of breast cancer. Clinical Breast Cancer 8:125-133.

7.Oseni, T., Patel, R., Pyle, J.R. and Jordan, V.C. (2008). Selective estrogen receptor modulators and phytoestrogens. Planta Medica 74:1656-1665.

8.Benson, J., Jatoi, I., Keisch, M., Esteva, F., Makris, A. and Jordan, V.C. (2009). Early Breast Cancer. The Lancet 373:1463-1479.

9.Maximov, P.Y., Lewis-Wambi, J.S. and Jordan, V.C. (2009). The Paradox of Oestradiol-Induced Breast Cancer Cell Growth and Apoptosis. Current Signal Transduction Therapy 4:88-102.

10. Lewis-Wambi, J.S. and Jordan, V.C. (2009). Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? Breast Cancer Research 11:206.

11. Peng, J., Sengupta, S. and Jordan, V.C. (2009). Potential of selective estrogen receptor modulators as treatments and preventives of breast cancer. Anti-Cancer Agents in Medicinal Chemistry 9:481-499.

12. Brauch, H. and Jordan, V.C. (2009). Targeting of tamoxifen to enhance antitumour action for the treatment and prevention of breast cancer: the ópersonalisedô approach? European Journal of Cancer 45:2274-2283.

13. Patel, R. and Jordan, V.C. (2009). Chemoprevention of breast cancer with selective estrogen receptor modulators. Current Medical Literature Breast Cancer 21:81-88.

14. Obiorah, I.E. and Jordan, V.C. (2011). Progress in endocrine approaches to the treatment and prevention of breast cancer. Maturitas 70:315-321.

15. Sweeney, E.E., McDaniel, R.E., Maximov, P.Y., Fan, P. and Jordan, V.C. (2012). Models and Mechanisms of Acquired Antihormone Resistance in Breast Cancer: Significant Clinical Progress Despite Limitations. Hormone Molecular Biology and Clinical Investigation 9:143-163.

16. Maximov, P.Y., Lee, T.M., and Jordan, V.C. (2013). The discovery and development of Selective Estrogen Receptor Modulators (SERMs) for clinical practice. Current Clinical Pharmacology 8:135-155

17. McDaniel, R.E., Maximov, P.Y., and Jordan, V.C. (2013). Estrogen-mediated mechanisms to control the growth and apoptosis of breast cancer cells: a translational research success story. In: Vitamins and Hormones vol. 93 (ed. Gerald Litwack) Elsevier pp. 1-49.

18. Jordan, V.C., Agboke, F.A., McDaniel, R.E., and Maximov, P.Y. A synthesis of thirty years of progress in selective steroid modulation. Steroids: Special Issue. *In press.*

19. Obiorah, I.O., and Jordan, V.C. Selective estrogen induced apoptosis. Steroids: Special Issue. *In press*.

20. Fan, P., and Jordan, V.C. Acquired resistance to selective estrogen receptor modulators (SERMs) in clinical practice (tamoxifen and raloxifen) by selection pressure in breast cancer cell populations. Steroids: Special Issue. *In press*.

21. Jordan, V.C. Linking estrogen-induced apoptosis with decreases in mortality following long term adjuvant tamoxifen therapy. Journal of the National Cancer Institute. *In press.*

Editorials

1. Jordan, V.C. and Gelber, R.D. (2007). Problems with progesterone receptor in practice? Journal of Clinical Oncology 25:1957-1959.
2. Jordan, V.C. (2007). Estrogen Receptors in BRCA1-Mutant Breast Cancer: Now You See Them, Now You Donõt. Journal of the National Cancer Institute 99:1655-1657.
3. Jordan, V.C. (2008). The rise of raloxifene and the fall of invasive breast cancer. Journal of the National Cancer Institute 100:831-833.
4. Jordan, V.C. and Ford, L.S. (2011). Paradoxical Clinical Effect of Estrogen on Breast Cancer Risk: A ñNewò Biology of Estrogen-Induced Apoptosis. Cancer Prevention Research 4:633-637.
5. Jordan, V.C. (2011). Decades of Discovery: The Selective Estrogen Receptor Modulator (SERM) Story: The St. Gallen Prize. *References en Gynecologie Obstetrique* 14:385-392.
6. Jordan, V.C. (2013). Targeted Therapies: Any Surprises from Selective Oestrogen Receptor Modulators? Nature Reviews Clinical Oncology 10:432-4.
7. Jordan, V.C. (2014). A(nother) scientific strategy to prevent breast cancer in post-menopausal women by enhancing estrogen-induced apoptosis? Menopause *ePub ahead of print*.
8. Jordan, V.C. (2014). Timing is key to avoid the bad and enhance the good of soy supplements. Journal of the National Cancer Institite. *In Press.*

Papers Published in Conference Proceedings and Invited Chapters in Books.

1. Patel, R.R., Sharma, C.G. and Jordan, V.C. (2007). Optimising the antihormonal treatment and prevention of breast cancer. Breast Cancer 14:113-122.
2. Patel, R.R., Pyle, J.R. and Jordan, V.C. (2007). The Current Clinical Indications for Fulvestrant. Clinical Updates in Cancer 2:4-6.

3. Jordan, V.C., Lewis-Wambi, J., Kim, H., Cunliffe, H., Ariazi, E., Sharma, C.G.N., Shupp, H.A. and Swaby, R. (2007). Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients. Breast 16:S105-S113.

4. Balaburski, G. and Jordan, V.C. (2008). Practical progress in the chemoprevention of breast cancer with selective estrogen receptor modulators (SERMs). In: Breast Cancer: Prognosis, Treatment and Prevention (2nd edition), Jorge R. Pasqualini (Ed), Informa Healthcare, USA, Inc., New York, NY, pp. 253-266.

5. Sengupta, S. and Jordan, V.C. (2008). Selective estrogen receptor modulators as an anticancer tool: Mechanisms of efficiency and resistance. In: Innovative Endocrinology of Cancer (Lev M. Bernstein and Richard J. Santen (eds), Landes Bioscience 630: pp. 206-219.

6. Davydov, A. and Jordan, V.C. (2008). Endometrial Cancer. In: Encylopedia of Cancer, 2nd Edition (M. Schwab (Ed)), Springer, Heidelberg, Germany, pp. 971-973.

7. Jordan, V.C. (2008). Tamoxifen. In: Encylopedia of Cancer, 2nd Edition (M. Schwab (Ed)), Springer, Heidelberg, Germany, pp. 2897-2898.

8. Jordan, V.C., Patel, R., Lewis-Wambi, J.S. and Swaby, R.F. (2008). By looking back we can see the way forward: enhancing the gains achieved with antihormone therapy. Breast Cancer Research 10 Suppl 4:S16.

9. Jordan, V.C., Lewis-Wambi, J.S., Patel, R.R., Kim, H. and Ariazi, E.A. (2009). New hypotheses and opportunities in endocrine therapy: amplication of oestrogen-induced apoptosis. Breast 18(S3):S10-S17.

10. Maximov, P.Y. and Jordan, V.C. (2012). Estrogen-Induced Apoptosis in Breast Cancer Cells: Translation to Clinical Relevance. In: Targeting New Pathways and Cell Death in Breast Cancer (Rebecca Aft (Ed)). InTech, Rijeka, Croatia, pp. 3-22.

11. Jordan, V.C. (2013). A Century of Deciphering the Control Mechanisms of Estrogen Action in Breast Cancer: the origins of targeted therapy and chemoprevention. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp1-30.

12. Lewis-Wambi, J.S. and Jordan, V.C. (2013). Diethylstilbestrol: The Pioneering Cancer Treatment and Tragedy in Reproductive Endocrinology. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp. 31-54.

13. Castracane, V.D. and Jordan, V.C. (2013). Contraception and Womenôs Health. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp. 55-80.

14. Eng-Wong, J., Obiorah, I. and Jordan, V.C. (2013). Hormone Replacement Therapy and Womenôs Health. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp. 81-110.

15. Obiorah, I. and Jordan, V.C. (2013). Induction of Ovulation by Non-Steroidal Antiestrogens. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp. 111-132.

16. Greene, G. and Jordan, V.C. (2013). Clues About Estrogen and Antiestrogen Action from X-Ray Crystallography. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp. 133-152.
17. Howell, A. and Jordan, V.C. (2013). Adjuvant Antihormone Therapy. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp. 229-254.
18. Brauch, H. and Jordan, V.C. (2013). Pharmacogenomics of Tamoxifen. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp. 255-294.
19. Clarke, R. and Jordan, V.C. (2013). Antihormone Drug Resistance. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp. 295-324.
20. Sengupta, S. and Jordan, V.C. (2013). Novel Selective Estrogen Receptor Modulators. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp. 325-360.
21. Powles, T., McDaniel, R.E. and Jordan, V.C. (2013). Chemoprevention and Breast Cancer. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp. 361-390.
22. Fan, P. and Jordan, V.C. (2013). Emering Principle: Selective Nuclear Receptor Modulators. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp 431- 456.
23. Jordan, V.C. (2013) Scientific Survival Suggestions. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp. 459 ï 464.
24. Jordan, V.C. (2013) Decades of discovery: the selective estrogen receptor modulator (SERM) story: the St. Gallen Prize. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp. 465-484.
25. Jordan, V.C. (2013) An account of students obtaining a PhD degree (or MD for physicians in the British system) in the Tamoxifen Team over the last 30 years. In: Estrogen Action, SERMs and Womenôs Health (Ed: Jordan, V.C.). Imperial College Press, London, pp. 485-502.
26. Maximov, P. Y., McDaniel, R. E., and Jordan, V.C. (2013). Discovery and early pharmacology of non-steroidal estrogens and antiestrogens. In: Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basel, Switzerland, pp. 1-30.
27. Maximov, P. Y., McDaniel, R. E., and Jordan, V.C. (2013). Tamoxifen goes forward alone. In: Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basel, Switzerland, pp. 31-46.
28. Maximov, P. Y., McDaniel, R. E., and Jordan, V.C. (2013). Metabolism of tamoxifen: the basis for future drug development. In: Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basel, Switzerland, pp. 47-68.

29. Maximov, P. Y., McDaniel, R. E., and Jordan, V.C. (2013). Adjuvant therapy: the breakthrough in survival. In: Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basal, Switzerland, pp. 69-84.
30. Maximov, P. Y., McDaniel, R. E., and Jordan, V.C. (2013). Selective Estrogen Receptor Modulation (SERM): The Wisconsin story. In: Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basel, Switzerland, pp. 85-100.
31. Maximov, P. Y., McDaniel, R. E., and Jordan, V.C. (2013). Tamoxifen and Carcinogenesis. In: Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basel, Switzerland, pp. 101-114.
32. Maximov, P. Y., McDaniel, R. E., and Jordan, V.C. (2013). Chemoprevention: Cinderella waiting for the ball. In: Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basel, Switzerland, pp. 115-134.
33. Maximov, P. Y., McDaniel, R. E., and Jordan, V.C. (2013). Tamoxifen and raloxifene head to head: the STAR trial. In: Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basal, Switzerland, pp. 135-142.
34. Maximov, P. Y., McDaniel, R. E., and Jordan, V.C. (2013). Acquired resistance to tamoxifen: back to the beginning. In: Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basel, Switzerland, pp. 143-164.
35. Maximov, P. Y., McDaniel, R. E., and Jordan, V.C. (2013). The legacy of Tamoxifen. In: Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basel, Switzerland, pp. 165-178.
36. Leick, M. B. and Jordan, V. C. (2013) Evolution of Estrogen Action in Breast Cancer: From Culprit to Killer. In: Estrogen Prevention for Breast Cancer (Ed: Suba, Z.) Nova Science Publishers, pp. 127-150.
37. Sweeney, E. and Jordan, V.C. (2013) Estrogen Receptor (ER). In: Encyclopedia of Cancer Therapeutic Targets. Springer Science + Business Media, LLC, New York, pp. 1-8.
38. McDaniel, R. and Jordan, V.C. (2013) Endocrine Prevention of Breast Cancer. In: Early Breast Cancer: From Screening to Multidisciplinary Management (Ed: Benson, J.). Informa Healthcare, London, England, pp. 39-49.
39. Peng, J., Sengupta, S., and Jordan V.C. (2013) Selective Estrogen Receptor Modulators as Treatments and Preventives of Breast Cancer. In: Advances in Anticancer Agents in Medicinal Chemistry (Ed: Prudhomme, M.). Bentham Science Publishers, Oak Park, IL, pp. 344-395.

Abstracts

1. *Abstract # 5710 was selected for an oral presentation in a mini symposium session at the 2007 American Association for Cancer Research Annual Meeting in Los Angeles, CA, and was published in the 2007 proceedings of the American Association for Cancer Research.*
   Overexpression of CEACAM6 promotes invasion and migration of aromatase inhibitor resistant breast cancer cells.
   Joan Lewis-Wambi, Helen R. Kim, Heather Cunliffe, and V. Craig Jordan.
2. *Abstract #2687 was published in the 2008 Proceedings of the 99th Annual Meeting of the American Association for Cancer Research.*
   Glutathione depletion sensitizes hormone-independent human breast cancer cells to estrogen-induced apoptosis.
   Joan S. Lewis-Wambi, Helen R. Kim, Chris Wambi, V. Craig Jordan
3. *Abstract #5452 was published in the 2008 Proceedings of the 99th Annual Meeting of the American Association for Cancer Research.*
   GPR30 modulates estrogen-stimulated proliferation of breast and endometrial cancer cells by regulating estrogen receptor alpha homeostasis.
4. Eric A. Ariazi, Heather A. Shupp, Jing Peng, Anne L. Donato, Surojeet Sengupta, Catherine G.N. Sharma, Helen R. Kim, Heather E. Cunliffe, Eric R. Prossnitz, V. Craig Jordan.
5. *Abstract #P2-2 was published in the 2008 Program for the 5th Era of Hope Department of Defense Breast Cancer Research Program Meeting.*
   Glutathione depletion sensitizes hormone-independent human breast cancer cells to estrogen-induced apoptosis.
   Joan S. Lewis-Wambi, Helen R. Kim, Chris Wambi, V. Craig Jordan
6. *Abstract #P2-17 was published in the 2008 Program for the 5th Era of Hope Department of Defense Breast Cancer Research Program Meeting.*
   Genomic Evolution of Endocrine-Resistant Breast Cancer Cell Lines Reveals Molecular Aberrations Consistent with Biological Phenotype
   Catherine M. Mancini, Coya Tapia, Amanda L. Willis, Joan S. Lewis-Wambi, Eric A. Ariazi, Helen R. Kim, Heather E. Cunliffe, V. Craig Jordan
7. *Abstract #P2-18 was published in the 2008 Program for the 5th Era of Hope Department of Defense Breast Cancer Research Program Meeting.*
   Comparative Gene Expression Profiling to Identify Unifying and Selective Pathways Involved in Tamoxifen, Raloxifene, and Aromatase Inhibitor-Resistant Breast Cancer Xenograft Tumors
   Eric Ariazi, Heather E. Cunliffe, Michael S. Slifker, Suraj Peri, Amanda L. Willis, Catherine M. Mancini, Heather A. Shupp, Surojeet Sengupta, Jing Peng, Anne L. Donato, Catherine G.N. Sharma, Shaun D. Gill, Jennifer R. Pyle, Karthik Devarajan, Yoganand Balagurunathan, Eric A. Ross, V. Craig Jordan
8. *Abstract #P2-19 was published in the 2008 Program for the 5th Era of Hope Department of Defense Breast Cancer Research Program Meeting.*

The Evolution of Drug Resistance to Antihormonal Therapy Exposes A Vulnerability in Breast Cancer

V. Craig Jordan, Eric A. Ariazi, Joan S. Lewis-Wambi, Ramona F. Swaby, Anton Wellstein, Anna T. Riegel, Heather E. Cunliffe

9. *Abstract #P2-20 was published in the 2008 Program for the 5th Era of Hope Department of Defense Breast Cancer Research Program Meeting.*
   Single Arm Phase 2 Study of Pharmacologic Dose Estrogen in Postmenopausal Women With Hormone Receptor-Positive Metastatic Breast Cancer After Failure of Sequential Endocrine Therapies
   Ramona Swaby, Mary B. Daly, Nancy E. Davidson, Eric A. Ross, Lori J. Goldstein, V. Craig Jordan
10. *Abstract #P2-27 was published in the 2008 Program for the 5th Era of Hope Department of Defense Breast Cancer Research Program Meeting.*
    Proteomic Analysis of Phosphotyrosine-Containing Protein Complexes During Estrogen-Induced Proliferation and Apoptosis in MCF-7 Human Breast Cancer Cells
    Anton Wellstein, Benjamin Kagan, Zhang-Zhi Hu, Cathy Wu, Hongzhan Huang, Lihua Zhang, Habtom W. Ressom, Francoise Seillier-Moiseiwitsch, Anna T. Riegel, V. Craig Jordan

11. *Abstract #2502 was published in the 2009 Proceedings of the 100th Annual Meeting of the American Association for Cancer Research, Denver, CO, April 18-22, 2009.*
    Cross-talk between GPR30 and estrogen receptor alpha (ERα): ERα mediates estrogen-induced down-regulation of GPR30 expression and the GPR30 agonist G-1 induces ERα-Ser118 phosphorylation.
    Eric A. Ariazi, Smitha Yerrum, Surojeet Sengupta, Heather A. Shupp, Anne L. Donato, Heather E. Cunliffe, V. Craig Jordan.

12. *Abstract #2338 was published in the 2009 Proceedings of the 100th Annual Meeting of the American Association for Cancer Research, Denver, CO, April 18-22, 2009.*
    17β-Estradiol Impairs the Growth of Selective Estrogen Receptor Modulator (SERM) resistant endometrial tumors.
    Gregor M. Balaburski, Rita Dardes, V. Craig Jordan.

13. *Abstract #4606 was published in the 2009 Proceedings of the 100th Annual Meeting of the American Association for Cancer Research, Denver, CO, April 18-22, 2009.*
    Bazedoxifene (TSE-424) is a potent inhibitor of long-term estrogen deprived breast cancer cells.
    Joan S. Lewis-Wambi, Helen R. Kim, V. Craig Jordan

14. *Abstract #3285 was published in the 2009 Proceedings of the 100th Annual Meeting of the American Association for Cancer Research, Denver, CO, April 18-22, 2009.*
    Pathway and network analysis of E2-induced apoptosis in breast cancer cells.
    Zhangzhi Hu, Benjamin Kagan, Hongzhan Huang, Hongfang Liu, V. Craig Jordan, Anna T. Riegel, Anton Wellstein, Cathy Wu.

15. *Abstract was presented during the IMPAKT Breast Cancer Conference, European Society for Medical Oncology, Brussels, Belgium, May 7-9, 2009 and published in Annals of Oncology* 2009: 20(2):11-12.
    Back to Basics: Oestrogen To Kill Anti-Hormone Resistant Breast Cancer.
    V. Craig Jordan, Joan Lewis-Wambi, Helen Kim, Ping Fan, Eric Ariazi.

16. *Abstract was presented at the TGEN Annual Scientific Retreat, June 4, 2009.*
    Genomic Evolution of Endocrine-Resistant Breast Cancer Cell Lines Reveals Molecular Aberrations Consistent with Biological Phenotype.
    Catherine M. Mancini, Pilar Ramos, Amanda L. Willis, Megan L. Russell, Ping Fan, Joan S. Lewis-Wambi, Eric A. Ariazi, Helen R. Kim, Coya Tapia, Michael Bittner, V. Craig Jordan, Heather E. Cunliffe.

17. *Abstract was presented at the TGEN Annual Scientific Retreat, June 4, 2009.*
    A New Therapeutic Paradigm for Breast Cancer: Exploiting Low-Dose Estrogen-Induced Apoptosis.
    Pilar Ramos, Eric A. Ariazi, Amanda L. Willis, Yoganand Balagurunathan, David Azorsa, Meredith Henderson, Seungchan Kim, Michael Bittner, V. Craig Jordan, Heather E. Cunliffe.

18. *Abstract #P3-72 was presented during the Endocrine Society Annual Meeting, Washington, D.C., June 10-13, 2009.*
    The roles of AIB/SRC-3 and erbB-2/HER2 in the estrogen-induced apoptosis of human breast cancer cells.
    Benjamin L. Kagan, Zhangzhi Hu, V. Craig Jordan, Anna T. Riegel, Anton Wellstein.

19. *Abstract was presented at the 27th Annual Miami Breast Cancer Conference, Miami, FL, March 3-6, 2010.*
    Challenges to improve adjuvant endocrine therapy.
    V. Craig Jordan.
20. *Abstract was presented at the 27th Annual Miami Breast Cancer Conference, Miami, FL, March 3-6, 2010.*
    Chemoprevention in the high risk patient.
    V. Craig Jordan.
21. *Abstract was presented at the 27th Annual Miami Breast Cancer Conference, Miami, FL, March 3-6, 2010.*
    Predictor of permanent menopause after chemotherapy.
    V. Craig Jordan.
22. *Abstract #607 was published in the 2010 Proceedings of the 101st Annual Meeting of the American Association for Cancer Research, Washington, DC, April 17-21, 2010.*
    Loss of pigment epithelium derived-factor (PEDF) is associated with breast cancer progression and antihormone drug resistance.
    Joan S. Lewis-Wambi, Helen Kim, V. Craig Jordan.
23. *Abstract #609 was published in the 2010 Proceedings of the 101st Annual Meeting of the American Association for Cancer Research, Washington, DC, April 17-21, 2010.*
    Paradoxical actions of a c-Src inhibitor on estradiol-induced apoptosis in long-term estrogen deprivation breast cancer cells.

Ping Fan, Helen Kim, V. Craig Jordan.

24. *Abstract #1279 was published in the 2010 Proceedings of the 101st Annual Meeting of the American Association for Cancer Research, Washington, DC, April 17-21, 2010.*

    Caspase-4 is critical in estrogen-induced apoptosis in antihormone-resistant breast cancer.

    V. Craig Jordan, Eric A. Ariazi, Heather E. Cunliffe, Joan S. Lewis-Wambi, Smitha Yerrum, Helen R. Kim, Catherine G.N. Sharma, Amanda Willis, Pilar Ramos, Coya Tapia, Michael J. Slifker, Suraj Peri, Eric A. Ross.

25. *Abstract was presented at the Cold Spring Harbor Laboratory/Wellcome Trust Scientific Conference, Cambridge UK, August 11-15, 2010.*

    Proteomics and systems analysis of estrogen-induced cell growth or cell death in breast cancer cells.

    Zhang-Zhi Hu, Benjamin L. Kagan, Lihua Zhang, V. Craig Jordan, Anna T. Riegel, Anton Wellstein.

26. *Abstract was presented at the 2010 Taipei International Breast Cancer Symposium, Taiwan, September 4, 2010.*

    Selective Oestrogen Receptor Modulators: Concept to Reality, Drugs and Medicines to Prevent Multiple Diseases in Women.

    V. Craig Jordan.

27. *Abstract was presented at the 2010 Taipei International Breast Cancer Symposium, Taiwan, September 4, 2010.*

    Antihormone Resistance: The New Biology of Low Dose Estrogen Treatment for Breast Cancer.

    V. Craig Jordan.

28. *Abstract was presented at the 1st International Symposium of the Journal: Hormone Molecular Biology and Clinical Investigation, Seefeld, Tyrol, Austria, September 11-14, 2010.*

    Mechanisms of oestrogen-induced apoptosis in breast cancer.

    V. Craig Jordan.

29. *Abstract was presented at the European Molecular Biology Organization Conference Series Chemical Biology 2010: From Functional Genomics to Systems Biology, Heidelberg, Germany, September 22-25, 2010.*

    Proteomics and systems analysis of estrogen-induced cell growth or cell death in breast cancer cells.

    Zhangzhi Hu, Benjamin Kagan, Lihua Zhang, Cathy Wu, Eric Ariazi, V. Craig Jordan, Anna T. Riegel, Anton Wellstein.

30. *Abstract #B61 was published in the 2010 Proceedings of the 3rd Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved Conference of the American Association for Cancer Research, Miami, FL, September 30 – October 3, 2010.*

    Phospholipid scramblase is a critical mediator of estrogen-induced apoptosis in antihormone resistant breast cancer cells.

    Joan S. Lewis-Wambi, Tabitha King, Annalese Smith.

31. *Abstract was presented at the TGen Annual Scientific Retreat, Phoenix Convention Center, September 4, 2009.*

Investigating mechanisms of PKC signaling deregulation in endocrine-resistant breast cancer: The role of RACK7.
Pilar Ramos, Amanda L. Willis, JM Chatigny, Megan L. Russell, Joan S. Lewis-Wambi, Eric A. Ariazi, V. Craig Jordan, Heather E. Cunliffe.

32. *Abstract was presented at the Amazon Project 8th Conference, Palermo, Italy, November 15-20, 2010.*

Estrogen action in the life and death of breast cancer cells.
V. Craig Jordan.

33. *Abstract was presented at the 12th St. Gallen International Breast Cancer Conference, St. Gallen, Switzerland, March 16-19, 2011.*

Evolution of long-term adjuvant antihormone therapy: Consequences and opportunities.
V. Craig Jordan.

34. *Abstract #774 was published in the 2011 Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research, Orlando, FL, April 2-6, 2011.*

PEDF silencing a novel mechanism of antihormone resistance in breast cancer.
Joan S. Lewis-Wambi, Min Huang.

35. *Abstract #2291 was published in the 2011 Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research, Orlando, FL, April 2-6, 2011.*

Inhibition of c-Src restores estrogen-stimulated growth in estrogen-deprivation resistant MCF-7 breast cancer cells.
Ping Fan, Helen Kim, Russell E. McDaniel, V. Craig Jordan.

36. *Abstract was presented at the Cologne Cancer Club, Cologne, Germany, May 4, 2011.*

Tamoxifen: a pioneering medicine that gave us SERMs to prevent multiple diseases in women's health.
V. Craig Jordan.

37. *Abstract #955 was published in the 2012 Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research, Chicago, IL, March 31-April 4, 2012.*

Role of cMYC as a critical determinant of estrogen-independent growth of estrogen deprivation-resistant breast cancer cells.
Surojeet Sengupta, Michael Biarnes, V. Craig Jordan.

38. *Abstract #956 was published in the 2012 Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research, Chicago, IL, March 31-April 4, 2012.*

The liver-X-receptor-β is involved in the induction by Tamoxifen of breast cancer cell differentiation and death.
Gregory Segala, Philippe de Medina, Michael Paillasse, V. Craig Jordan, Sandrine Silvente-Poirot, Marc Poirot.

39. *Abstract #2921 was published in the 2012 Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research, Chicago, IL, March 31-April 4, 2012. Additionally, this abstract was selected as an oral presentation in a Minisymposium session.*

Critical mediation of E_2-induced apoptosis through c-Src in long-term estrogen deprived breast cancer cells.
Ping Fan, Obi L. Griffith, Pavana Anur, Helen R. Kim, Joe W. Gray, V. Craig Jordan.

40. *Abstract #3924 was published in the 2012 Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research, Chicago, IL, March 31-April 4, 2012.*

Additionally, this abstract was selected as newsworthy for the AACR Annual Meeting, and partook in the media outreach process, including press release/conference activities.

Alteration of the shape of the ligand estrogen receptor complex controls estrogen-induced apoptosis in breast cancer.

Ifeyinwa Obiorah, Surojeet Sengupta, Ramona Curpan, V. Craig Jordan.

41. *Abstract #5597 was published in the 2012 Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research, Chicago, IL, March 31-April 4, 2012.*

    Modulating therapeutic effects of c-Src inhibitor via estrogen receptor and HER2 in breast cancer cell lines.

    Philipp Y. Maximov, Russell E. McDaniel, V. Craig Jordan, Hiltrud Brauch.

42. *Abstract #5682 was published in the 2012 Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research, Chicago, IL, March 31-April 4, 2012.*

    Modeling the pharmacological importance of endoxifen for the treatment of ER-positive breast cancer in premenopausal patients.

    Ping Fan, Russell E. McDaniel, Helen R. Kim, V. Craig Jordan
    Philipp Y. Maximov, Cynthia Myers, Daphne J. Fernandes, V. Craig Jordan.

43. Abstract # P2-13-09. Was presented at the San Antonio Breast Cancer Symposium, December 4-8, 2012 and was published in Cancer Research 2012;72(24 Suppl).

    Pharmacological impact of endoxifen in a laboratory simulation of tamoxifen therapy in postmenopausal breast cancer patients

    PY Maximov, RE McDaniel, P Bhatta, H Brauch, and VC Jordan

44. *Abstract # 5642 was published in the 2013 Proceedings of the 104th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 6-10 2013.*

    Transcriptional deregulation of cMYC as a critical determinant of estrogen independence and aromatase inhibitor resistance in breast cancers.

    Surojeet Sengupta, Michael C. Biarnes, V. Craig Jordan.

45. *Abstract # 2939 was published in the 2013 Proceedings of the 104th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 6-10 2013.*

    Novel molecular mechanism for estradiol-induced apoptosis that contrasts with cytotoxic chemotherapy-induced apoptosis in breast cancer.

    Ifeyinwa Obiorah, Surojeet Sengupta, V. Craig Jordan

46. *Abstract # 828 was published in the 2013 Proceedings of the 104th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 6-10 2013.*

    Mechanisms underlying differential response to estrogen-induced apoptosis in long-term estrogen-deprived breast cancer cell lines.

    Elizabeth E. Sweeney, Ping Fan, V. Craig Jordan.

47. *Abstract # 830 was published in the 2013 Proceedings of the 104th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 6-10 2013.*

    Transcriptional modulation of estrogen-induced apoptosis through activation of c-Fos/c-Jun in long-term estrogen deprived breast cancer cells.

    Ping Fan, Fadeke Agboke, Obi L. Grifith, Russell E. McDaniel, V. Craig Jordan.

48. *Abstract # 4409 was published in the 2013 Proceedings of the 104th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 6-10 2013.*
The characterization of isomerically stable fixed ring derivatives of tamoxifen metabolites 4-hydroxytamoxifen and 4-hydroxy-N-desmethyltamoxifen (endoxifen) in vitro.
Philipp Y. Maximov, Cynthia Meyers, Daphne Fernandes, V. Craig Jordan

Presentations

2006

1. Successful translational research with SERMs, Frankfurt Germany, October, 2006.
2. Hormone therapies used in the treatment of breast cancer, Fox Chase Cancer Support Group, Fox Chase Cancer Center, Philadelphia, PA, November 2006
3. The role of estrogen in the life and death of breast cancer cells. Abramson Family Cancer ResearchCenter, Institute/Hematology-Oncology Series, University of Pennsylvania, Philadelphia, PA.November, 2006.
4. The new biology of estrogen action: can new knowledge help patients?, City of Hope, California, November, 2006.
5. The role of estrogen in the life and death of breast cancer cells, Genetech, California, November, 2006.

6 Clinical Application and development of SERMs. San Antonio Breast Cancer Conference, December 2006.

2007

1 . Update on Hormonal Therapies for the Prevention and Treatment of Breast Cancer, Northern California Breast Cancer Symposium, February, 2007.
2. Optimizing treatment and prevention of breast cancer: time for a change, Primary Therapy of Early Breast Cancer, 10th International Conference, St. Gallen, Switzerland, Anniversary Meeting, March, 2007.
3. A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis, Translational Science Lecture Series, Fox Chase Cancer Center, Philadelphia, PA, March, 2007.
4. What do wc do about antihormonal resistance in breast cancer?, AACR Annual Meeting, April, 2007.
5. Search for a novel apoptotic target in cancer, AstraZeneca Presentation, April 10, 2007.
6. Honnoncs and Women's Health in the 21st Century, Joint Steering Committee to Public Policy, April, 2007
7. Oestrogen in the life and death of breast cancer, University of Leeds Symposium, May, 2007.
8. Targeted breast cancer therapy: A change to aromatase inhibitors, National Surgery Oncology
   Conference, Antayla, Turkey, May, 2007.
9. Successful Translational Research for the targeted treatment and prevention of breast cancer, AnnualRetreat for Cancer Research, UMDNJ, New Jersey, May, 2007.
10. Optimizing treatment and prevention of breast cancer: time for a change, White Nights in St. Petersburg, Russia, June, 2007.
11. Selective estrogen receptor modulators for the treatment and prevention of breast cancer. Worceester Foundation for Biomedical Research, Pincus Award, June 14, 2007.
12. Oestrogen is bad for patients with breast cancer? Controversies in breast cancer, Edinburgh, September 3-4, 2007
13. What is new in chemoprevention for breast cancer? Aultman Cancer Conference, Ohio, September 14-15, 2007.

14. Background, scientific rational and organization, Center of Excellence External Advisory Board meeting, September 18, 2007
15. Estrogens and antiestrogens in the life and death of rbeast cancer cells: new treatment opportunities. Ohio State University Grand Rounds, September 21, 2007.
16. Tamoxifen, the first targeted therapy for the treatment of breast cancer. Medical Oncology Fellows Conference, September 24, 2007.
17. Practical issues in endocrine prevention, 9th Lynn Sage Meeting, Chicago, IL, September, 2007.

2008

1. Jordan, VC. The Science of Selective Estrogen Receptor Modulators and their Clinical Application, AAAS Annual Meeting, Boston, February 14-18, 2008
2. Jordan, VC. Current Status of Breast Cancer Prevention, 25th Annual Miami Breast Cancer Conference, Orlando, Florida, February 20-23 2008
3. Jordan, VC. New Insights into the role of estrogen in the life and death of breast cancer cells, Visiting Professor Seminar Speaker Series, University of New Mexico Cancer Center, March 3, 2008
4. Jordan, VC. Protein-Centric Integration and Functional Analysis of Cancer Omics Data, USHUPO 4th Annual Conference, Bethesda, MD, March 16-19, 2008
5. Jordan, VC. Tamoxifen and Raloxifene: the First Selective Estrogen Receptor Modulators, 12th Annual Interdisciplinary Womenôs Health Research Symposium, March 28, 2008
6. Jordan, VC. Development and current role of tamoxifen and raloxifene in the treatment and prevention of breast cancer and osteoporosis, Institut f¿r Klinische Pharmakologie, Stuttgart, Germany, April 29, 2008
7. Jordan, VC. Defeating Drug Resistance to SERMs: Building on the Success of Tamoxifen and Raloxifene, Institut f¿r Klinische Pharmakologie, Stuttgart, Germany, May 2008
8. Jordan, VC. Targeting Oestrogen to Kill Breast Cancer Cells, First International Conference, Forli, Italy, May 16, 2008
9. Jordan, VC. Translational Research in Breast Cancer at the UWCCC that changed Medical Practice, Department of Medicine Research Day, Madison WI, May 29, 2008
10. Jordan, VC. The Paradoxical Actions of Estrogen in Breast Cancer: Survival or Death!, 38th Karnofsky Award Lecture, The American Society of Clinical Oncology, May 31, 2008
11. Jordan, VC. Model Systems of Anti-hormonal Therapy to Evaluate New Targeted Treatments for Breast and Endometrial Cancer, Pfizer, June, 2008
12. Cunliffe, H. Genomic Evolution of Endocrine-Resistant Breast Cancer Cell Lines Reveals Molecular Aberrations Consistent with Biological Phenotype, Session 8-1, 5th Era of Hope Meeting, Baltimore, MD, June 26, 2008
13. Ariazi EA. Comparative Gene Expression Profiling to Identify Unifying and Selective Pathways Involved in Tamoxifen, Raloxifene, and Aromatase Inhibitor-Resistant Breast Cancer Xenograft Tumors, Session 8-2, 5th Era of Hope Meeting, Baltimore, MD, June 26, 2008

14. Jordan, VC. Estrogen in the life and death of breast cancer cells, Loyola University Medical Center, Cardinal Bernardin Cancer Center, October 9, 2008.
15. Jordan, VC. Estrogen in The Life and Death of Breast Cancer Cells, 10th Annual Lynn Sage Memorial Lecture, 10th Annual Lynn Sage Breast Cancer Symposium, October 24, 2009.
16. Jordan, VC. Estrogen in the life and death of breast cancer cells, Division of Cancer Medicine, Grand Rounds, M.D. Anderson Cancer Center, Houston, TX, November 4, 2008.
17. Cunliffe, H. A New Therapeutic Paradigm for Breast Cancer, Exploiting Low-Dose Estrogen-Induced Apoptosis, Breast Cancer Genetics and Genomics Symposium, Auckland Medical School, New Zealand, November 18, 2008.
18. Jordan, VC. Evolving Hormonal Strategies of the Treatment and Prevention of Breast Cancer, 12th Annual Cancer Congress, Moscow, Russia, November 19, 2008.
19. Jordan, VC. Opportunities to Reverse Antihormonal Drug Resistance and Advance Molecular Medicine, Madison, Wisconsin, November 2008.
20. Jordan, VC. Cancers vs. DES & Other (Anti-)Oestrogens (Cellular Aspects), The State of the Art on DES in 2008, UCB Pharma, Paris, France, December 8, 2008.
21. Jordan, VC. Cancers vs. DES & Other Anti-Oestrogens: Clinical Aspects, The State of the Art on DES in 2008, UCB Pharma, Paris, France, December 8, 2008.
22. Jordan, VC. The Evolution of Drug Resistance to Anti-hormonal Therapy Exposes a Vulnerability in Breast Cancer, Session 36-5, 5th Era of Hope Meeting, Baltimore, MD, June 28, 2008
23. Ariazi EA, Jordan, VC. Estrogen receptors as therapeutic targets in breast cancer, 236th American Chemical Society National Meeting, Division of Computers in Chemistry, Final paper number 27, Philadelphia, PA, August 17-21, 2008
24. Brailoiu E, Brailoiu GC, Dun SL, Deliu E, Ariazi EA, Arterburn JB, Prossnitz ER, Oprea TI, Jordan VC, Dun NJ. Localization, distribution, and pharmacology of G protein-coupled estrogen receptor GPR30, 236th American Chemical Society National Meeting, Division of Computers in Chemistry, Final paper number 27, Philadelphia, PA, August 17-21, 2008
25. Jordan, VC. Modulation of Oestrogen Action: By Looking Back We Can See The Way Forward, Controversies Meeting, Edinburgh, September 2, 2008
26. Jordan, VC. Defeating Drug Resistance to SERMs: Building on the Success of Tamoxifen and Raloxifene, Lund, Sweden, September 4, 2008
27. Jordan, VC. Oestrogen in the Life and Death of Breast Cancer Cells: The Consequences of Anti-Hormonal Therapy, Nobel Symposium, Svartsjº , Sweden, September 9, 2008
28. Jordan, VC. Oestrogen-induced apoptotic mechanisms and their potential application in breast cancer therapy, 18th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, Seefeld, Austria, September 20, 2008
29. Jordan, VC. Breast Cancer Research and Treatment by Blocking Estrogen Action, National Breast Cancer Coalition, Washington D.C, 2008

2009

1. Jordan, VC. Are there personalized strategies for overcoming resistance to endocrine therapy?, 2nd Annual Symposium on Personalized Therapies for Breast Cancer, Miami, FL, January 25, 2009.

2. Jordan, VC. Defeating Drug Resistance to SERMs: Building on the Success of Tamoxifen and Raloxifene, Dr. Margarete Fischer-Bosch-Institut f¿r Klinische Pharmakologie, Stuttgart, Germany, February 5, 2009.
3. Jordan, VC. New Hypotheses and Opportunities in Endocrine Therapy: Amplification of Oestrogen-Induced Apoptosis, Primary Therapy of Early Breast Cancer 11th International Conference, St. Gallen, Switzerland, March 11, 2009.
4. Jordan, VC. Oestrogen Action and the Life or Death of Breast Cancer, Royal Society of Medicine, Jephcott Lecture, London, England, April 7, 2009.
5. Cunliffe, H. A New Therapeutic Paradigm for Breast Cancer: Exploiting Low-Dose Estrogen-Induced Apoptosis, TGEN Annual Cancer and Cell Biology Division Retreat, Civic Center, Phoenix, AZ, April 25, 2009.
6. Jordan, VC. The Consequence of Exhaustive Antihormone Therapy: Estrogen Kills Breast Cancer Cells, New York Metropolitan Breast Cancer Group, Glenn Robbins Award, New York, NY, April 28, 2009.
7. Jordan, VC. The Consequence of Exhaustive Antihormone Therapy: Estrogen Kills Breast Cancer Cells, Vincent T. Lombardi Comprehensive Cancer Center, Georgetown University, Grand Rounds, Washington, D.C., May 1, 2009.
8. Jordan, VC. Back to Basics: Oestrogen to Kill Anti-Hormone Resistant Breast Cancer: Build on That, IMPAKT Breast Cancer Conference, European Society for Medical Oncology, Brussels, Belgium, May 7, 2009.
9. Jordan, VC. Tamoxifen, Raloxifene, SERMs, and Beyond, University of Crete, Heraklion, Crete, Greece, May 14, 2009.
10. Jordan, VC. A Solution to the Clinical Problem of Antihormone Drug Resistance in Breast Cancer. 29th Annual Conference of the German Society of Senology, Dusseldorf, Germany, June 11, 2009.
11. Jordan, VC. Deciphering the Control Mechanisms of Estrogen Action in Breast Cancer, Keynote Forum: Scientific Innovations for Future Anticancer Medicine, 2nd World Cancer Congress, Gateway to Future Medicine, Beijing, China, June 22, 2009.
12. Jordan, VC. Evolving Understanding of Estrogen Action and its Application for Breast Cancer Treatment, Midwest Breast Cancer Symposium, University of Iowa, Iowa City, IA, July 17, 2009.
13. Jordan, VC. Improving Refractory Endocrine Therapy with Agents to Inhibit Angiogenesis, Bristol-Myers Squibb, Princeton, New Jersey, August 19, 2009.
14. Jordan, VC. Estrogen Action for Growth and Apoptosis in Breast Cancer, World Class University Symposium, Dankoo University, Seoul, South Korea, August 25-26, 2009.

2010:

1. Ramos P. ñInvestigating mechanisms of PKC signaling deregulation in endocrine-resistant breast cancer: The role of RACK7.ò Glendale Community College Lecture Series, Glendale, AZ, February 19, 2010.
2. Jordan VC. ñChemoprevention in the High Risk Patient.ò 27th Annual Miami Breast Cancer Conference, Prevention Strategies, Miami, FL, March 3, 2010.
3. Jordan VC. ñPredictor of Permanent Menopause After Chemotherapy.ò 27th Annual Miami Breast Cancer Conference, Emerging and Controversial Topics in Systemic Adjuvant Therapy, Miami, FL, March 5, 2010.
4. Jordan VC. ñChallenges to Improve Adjuvant Endocrine Therapy.ò 27th Annual Miami Breast Cancer Conference, Future Challenges, Miami, FL, March 6, 2010.

5. Jordan VC. ñOvercoming Hormone Therapy Resistance.ò 5th Annual Georgetown Breast Cancer Update: Emerging Trends in Management of Breast Cancer, Washington, DC, March 13, 2010.
6. Jordan VC. ñAmplifying the apoptotic actions of oestrogen to treat metastatic breast cancer.ò Translational Cancer Medicine, American Association for Cancer Research, Amsterdam, Netherlands, March 22, 2010.
7. Jordan VC. ñTargeted Therapy to Steroid Hormone Receptors: Evolution of Ideas Into Lives Saved.ò AACR 101st Annual Meeting, Walter E. Washington Convention Center, Washington, DC, April 17-21, 2010.
8. Jordan VC. ñEstrogen in the life and death of breast cancer cells.ò NIH/NCI Distinguished Speaker, Distinguished Scientist Lecture Series, National Cancer Institute, Frederick, MD, May 4, 2010.
9. Jordan VC. ñThe discovery and application of selective estrogen receptor modulators.ò Department of Pharmacology seminar, Georgetown University Medical Center, Washington DC, May 21, 2010.
10. Obiorah I. ñEstrogen regulated growth and apoptosis.ò Cancer Research Data Meeting, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, June 24, 2010.
11. Jordan VC. ñThe quest for multi-functional medicines: path for progress: the tamoxifen story.ò Moscow, Russia, July 14-18, 2010.
12. Jordan VC. ñThe quest for multi-functional medicines: path for progress: the SERM story.ò Moscow, Russia, July 14-18, 2010.
13. Hu ZZ, Kagan BL, Zhang L, Jordan VC, Riegel AT, Wellstein A. ñProteomics and systems analysis of estrogen-induced cell growth or cell death in breast cancer cells.ò Systems Biology: Networks Plenary Lecture. Cold Spring Harbor Laboratory/Wellcome Trust Scientific Conference, Cambridge UK, August 11-15, 2010.
14. Jordan VC. ñAnti-hormone resistance: the new biology of low dose oestrogen treatment for breast cancer.ò 2010 Taipei International Breast Cancer Symposium, The Breast Cancer Society of Taiwan, Taipei, Taiwan, September 4, 2010.
15. Jordan VC. ñSelective oestrogen receptor modulators: concept to reality. Drugs and medicines to prevent multiple diseases in women.ò 2010 Taipei International Breast Cancer Symposium, The Breast Cancer Society of Taiwan, Taipei, Taiwan, September 4, 2010.
16. Jordan VC. ñMechanisms of oestrogen-induced apoptosis in breast cancer.ò 1st International Symposium of the Journal: Hormone Molecular Biology and Clinical Investigation, Seefeld, Tyrol, Austria, September 11-14, 2010.
17. Jordan VC. ñHormone receptor positive disease subgroup update (recent progress).ò Stand Up 2 Cancer (SU2C), 2nd AACR Breast Cancer Dream Team (BCDT) Update Meeting, Dallas, TX, September 25-26, 2010.
18. Jordan VC. ñHormone receptor positive disease subgroup follow-on meeting (animal models).ò Stand Up 2 Cancer (SU2C), 2nd AACR Breast Cancer Dream Team (BCDT) Update Meeting, Dallas, TX, September 25-26, 2010.

19. Jordan VC. ñEstrogen-induced apoptosis in breast cancer.ò Research Update Seminar Series, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, September 29, 2010.
20. Ariazi EA. ñDeciphering Global Gene Expression Across Time by Differential Area-Under-the-Curve Analysis to Examine Estrogen-induced Apoptosis in Aromatase Inhibitor-resistant Breast Cancer Cells.ò Research Update Seminar Series, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, October 6, 2010.
21. Jordan VC. ñBreast Cancer Chemoprevention: Have We Made Progress?ò 12th Annual Lynn Sage Breast Cancer Symposium, Chicago, IL, October 28-31, 2010.
22. Jordan VC. ñOestrogen action in the life and death of breast cancer cells.ò Amazon Project, 8th Conference, Palermo, Italy, November 15-20, 2010.
23. Fan P. ñModulation of Estrogen-Induced Apoptosis by Inhibiting c-Src in Estrogen-Deprived Resistant MCF-7 Breast Cancer Cells: Potential Clinical Implications.ò Research Update Seminar Series, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, November 17, 2010.
24. Riegel AT. ñCo-activators in Malignant Progression.ò Research Update Seminar Series, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, December 8, 2010.
25. Jordan VC. ñEvolution of hormone therapy for the prevention and treatment of breast cancer: new opportunities.ò A New Era In The Diagnosis and Treatment of Breast Cancer, Bangkok, Thailand, December 10-19, 2010.

2011:

1. Jordan VC. ñDeciphering the molecular pharmacology of tamoxifen treatment for breast cancer: the critical role of the graduate student.ò Uniformed Services University, Molecular & Cell Biology Graduate Program, Bethesda, MD, January 13,2011.
2. Jordan VC. ñModulation of Estrogen-Induced Apoptosis in Breast Cancer.ò Think Tank 21, Breast Cancer Symposium, Montego Bay, Jamaica, January 16-22, 2011.
3. Wellstein A. ñUnderstanding and targeting cancer/stromal interactions.ò Research Update Seminar Series, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, January 26, 2011.
4. Jordan VC. ñEvolution of long-term adjuvant anti-hormone therapy: consequences and opportunities.ò 12th St. Gallen International Breast Cancer Conference, St. Gallen, Switzerland, March 16-19, 2011.
5. Obiorah I. ñModulation of estrogen-induced apoptosis in breast cancer.ò Cancer Research Data Meeting, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, April 14, 2011.
6. Jordan VC. ñTamoxifen: A Pioneering Medicine That Gave Us SERMs to Prevent Multiple Diseases in Womenôs Health.ò Cologne Cancer Club, Cologne, Germany, May 4, 2011.
7. Jordan VC ñEvolution of long-term adjuvant anti-hormone therapy: consequences and opportunities. The 2011 St. Gallen Prize in Clinical Breast Cancer Research Lecture.ò

Research Update Seminar Series, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, July 13, 2011.

8. Weiner L, Jordan VC. ñMission and Mandate for the Conquest of Cancer in the United States of America.ò Research Update Seminar Series, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, September 14, 2011.
9. Jordan VC. ñImproving the Response Rates to Endocrine Therapy.ò 13th Annual Lynn Sage Breast Cancer Symposium, Chicago, IL, September 15-18, 2011.
10. Jordan VC. ñThe consequences of oestrogen-deprivation in breast cancer treatment: oestrogen-induced apoptosis.ò Karolinska Institute, Stockholm, Sweden, September 26, 2011.
11. Jordan VC. ñUnanticipated Opportunities for the Treatment and Prevention of Breast Cancer with Physiologic Estradiol Therapy.ò North Coast Breast Cancer Conference 2011, Sandusky, OH, October 7-8, 2011.
12. Jordan VC. ñDecades of Discovery in the Treatment and Prevention of Breast Cancer by the Modulation of Estrogen Action.ò North Coast Breast Cancer Conference 2011, Sandusky, OH, October 7-8, 2011.
13. Jordan VC. ñInvestigation into the modulation of estrogen-induced apoptosis. Clinical Relevanceò Stand Up 2 Cancer (SU2C), 2nd AACR Breast Cancer Dream Team (BCDT) Update Meeting, Dallas, TX, October 21-22, 2011.
14. Jordan VC. ñHormone receptor positive disease subgroup update (recent progress).ò Stand Up 2 Cancer (SU2C), 2nd AACR Breast Cancer Dream Team (BCDT) Update Meeting, Dallas, TX, October 21-22, 2011.
15. Jordan VC. ñThen and Now: Four Decades of Discovery in Breast Cancer Treatment and Prevention.ò Sarah Stewart Lecture Series, Georgetown University School of Medicine, Georgetown University Medical Center, Washington, DC, November 17, 2011.

2012:

1. Jordan VC ñModulating Estrogen Receptor Action in Breast Cancer: The Goodman & Gilman Award for Receptor Pharmacology. The American Society of Experimental Pharmacology and Therapeutics (ASPET).ò Research Update Seminar Series, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, January 18, 2012.
2. Jordan VC ñThe SERM Story for Breast Cancer & Beyond (Something Out of Nothing).ò Link Lecture, The Institute of Cancer Research, Royal Marsden Hospital, London, England, March 15, 2012.
3. Jordan VC ñScientific Survival Suggestions after Four Decades of Discovery.ò University of Liverpool, Liverpool, UK, July 25 2012.
4. Jordan VC ñThe Science of SERMs.ò University of Arkansas Medical School, Little Rock, AK, September 13, 2012.

5. Jordan VC ñScientific Survival Suggestions after Four Decades of Discovery.ò National Center for Toxicological Research Food and Drug Administration, Jefferson, AK, September 14, 2012
6. Jordan VC ñThe treatment and prevention of breast cancer by selective estrogen receptor modulation- something from nothing.ò Robert M. Hearin Distinguished Lecture University of Mississippi Medical Center. Jackson, MS, September 27, 2012
7. Jordan VC ñChemoprevention of Breast Cancer: Lessons Learned.ò 14TH Annual Lynn Sage Breast Cancer Symposium, Chicago IL, October 5, 2012.
8. Jordan VC ñThe Pfizer-Wulf H. Utian Endowed Lecture:The Paradox of Estradiol-Induced Breast Cancer Cell Growth and Apoptosis.ò North American Menopause Society, Orlando, FL, October 6, 2012.
9. Jordan VC ñThe Paradox of Estradiol-Induced Breast Cancer Cell Growth and Apoptosis: a Change in Clinical Practice?.ò Georgetown University - Wednesday Research Update Seminar Series. Washington, DC, October 17, 2012.
10. Jordan VC ñThe New Biology of Estrogen Induced Apoptosis for the Treatment and Prevention of Breast Cancer.ò Robert H. Lurie Comprehensive Cancer Center Northwestern University. Chicago, IL, November 2, 2012.
11. Jordan VC ñScientific Survival Suggestions to Graduate Students and Postdoctoral Fellows.ò Robert H. Lurie Comprehensive Cancer Center Northwestern University. Chicago, IL, November 2, 2012.
12. Jordan VC ñScientific Survival Suggestions to Medical and Surgical Fellows.ò Robert H. Lurie Comprehensive Cancer Center Northwestern University. Chicago, IL, November 3, 2012.
13. Jordan VC ñTHE SCIENCE OF SERMs: FOUR DECADES OF DISCOVERY Something from Nothing.ò Southern California Clinical and Translational Science Institute. USC Health Sciences Campus, November 7, 2012.
14. Jordan VC ñImproving The Response Rate to Endocrine Therapy.ò 19th Annual Multi-Disciplinary Cancer Symposium New Perspectives on Breast Cancer 2012Washington Adventist Hospital (11-2012). Bethesda, MD, November 9, 2012.

2013

1. Jordan VC ñMolecular Mechanism of Estrogen-Induced Apoptosis in Breast Cancer:Paradox to Paradigmò Georgetown University - Wednesday Research Update Seminar Series. Washington, DC, October 17, 2012.
2. Jordan VC ñFour Decades of Discovery for the Treatment and Prevention of Breast Cancer: The SERM Story The 38th Sir Edward Mellanby Memorial Orationò. CSIR-CDRI Lucknow, India, February 11, 2013.
3. Jordan VC ñThe Origins of the New Biology of Estrogen-Induced Apoptosis in Breast Cancer Research, Treatment, and Prevention Inaugural Dr. Gary D. Kruh Memorial Lectureò University of Illinois, Chicago, IL, May 9, 2013.

4. Jordan VC ñOmics to Solve the Mechanism of Estrogen -Induced Apoptosis in Breast Cancer to Aid Women's Healthò ting Drug Failure, Dr. Margarete Fischer-Bosch - Institut f¿r Klinische Pharmakologie, Stuttgart 26-29 June 2013
5. Jordan VC ñEvolution of Endocrine therapy for Breast cancer - the Paradox of Estrogen Induced Apoptosisò Fort Detrick Frederick MD October 24th, 2013
6. Jordan VC ñEvolution of Endocrine therapy for Breast cancer - the Paradox of Estrogen Induced ApoptosisòThe Weatherhall Institute Oxford University 3 November 2013
7. Jordan VC ñImproving The Response Rate to Endocrine Therapyò Lynn Sage Breast Cancer SymposiumChicago, IL Saturday September 28th, 2013
8. Jordan VC ñEvolution of Endocrine therapy for Breast cancer - the Paradox of Estrogen Induced Apoptosisò 9¯mes Journ®es Canc®rop¹ le Grand Sud-Ouest Limoges, France, 16-18 October 2013.
9. Jordan VC ñRepurposing Failed Drugs to Create Successful Medicines in Womenôs Healthò New York University ñDeveloping New Drugsò December 4 2013.

2014

1. Jordan VC ñEvolution of Endocrine therapy for Breast cancer - the Paradox of Estrogen Induced Apoptosisò, Ceders-Sinai Hospital, January 16 2014.
2. Jordan VC ñWhy It Is The Right Thing To Do: Invest In Young Cancer Research Scientistsò Yorkshire Cancer Research Annual Scientific Meeting Pavilions of Harrogate June 25th 2014.

Cell lines developed from work Supported by this award

MCF-7:PF cell line developed by Dr. Ping Fan in Georgetown, derived from long-term treatment of MCF-7:5C with estrogen plus the src inhibitor PP2. Cell response to estrogen was converted from induction of apoptosis to stimulation.

Informatics developed from work supported by this award

The data reported in [10] have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE29917).

Funding applied for based on work supported by this award

Joan Lewis-Wambi, PhD
Award document number: KCA120051A
Grant title: The new biology of estrogen action in aromatase resistant breast cancer cells.
Project period begin: 8/07/2007
Project period end: 7/31/2012

V. Craig Jordan (Principal Investigator)
Eric A. Ariazi (Co-Principal Investigator)
Submitted: Pre-application to the Susan G. Komen for the Cure 2009 Grants Program

Application Title**:** The G protein-coupled estrogen receptor GPR30ôs role in antihormone-sensitive to antihormone-resistant breast cancer.
Mechanism: Investigator Initiated Research
Pre-application Submission Date: 07/01/08
The Pre-application was found responsive to the Request for Applications (RFA), and consequently, invited for a full application.
Full-application Submission Date: 09/04/08

Anna Riegel (Principal Investigator)
Anton Wellstein (Co-Principal Investigator)
Submitted: Pre-application to NIH Challenge Grants in Health and Science Research (RC1)
Application Title**:** Role of A1B1 in hormone refractory cancer.
Mechanism: [0D09-003]-Recovery Act Limited Competition: NIH Challenge Grants in Health and Science Research (RC1)
Pre-application Submission Date: 05/08/09
Full-application Submission Date: The application was reviewed and has scored in the 10th percentile, and JIT has been submitted as of 07/27/09

V. Craig Jordan (Principal Investigator)
Susan G. Komen For The Cure Award # SAC100009 for the Susan G Komen For The Cure Foundation. ñMolecular modification of the estrogen receptor (ER) for estrogen action antagonism and apoptosis.ò Period: July 1, 2010 ï August 24, 2014

V. Craig Jordan (Principal Investigator)
SU2C (Stand Up 2 Cancer) Grant number SU2C-AACR-DT0409, subcontract under Stand Up 2 Cancer, American Association for Cancer Research (AACR). ñAn integrated approach to targeting breast cancer molecular subtypes and their ñresistanceò phenotypes.ò Period: October 1, 2009 ï September 30, 2012

Appointments

Eric A. Ariazi, PhD
Faculty Appointment (secondary appointment) - Assistant Professor, Department of Pharmacology, Temple University School of Medicine, Philadelphia, PA, June 2008

Eric A. Ariazi, PhD
July 1, 2009, Scientific Consultant to Dr. V. Craig Jordan and the Center of Excellence W81XWH-06-1-0590 - ñA New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosisò.

Ping Fan, PhD
July 1, 2009, Research Assistant Professor

V. Craig Jordan, OBE, PhD, DSc, FMedSci
July 1, 2009, Scientific Director at the Lombardi Comprehensive Cancer Center, Vice Chair

of Department of Oncology, Professor of Oncology and Pharmacology

Helen Kim, BS
July 1, 2009, Laboratory Manager

Joan Lewis-Wambi, PhD
July 1, 2009, Research Assistant Professor

Surojeet Sengupta, PhD
July 1, 2009, Research Assistant Professor

Julia J. Tijerina
March 17, 2010, Executive Assistant to Dr. V. Craig Jordan

Russell E. McDaniel
March 17, 2010, Research Assistant I

Philipp Y. Maximov, MD, PhD
February 28, 2011, Susan G. Komen Foundation For The Cure Postdoctoral Research Fellow

Michael C. Biarnes, BS
July 6, 2011, Research Assistant I

Fadeke Agboke, BS
August 31, 2011, Research Assistant I

Puspanjali Bhatta, BS
September 14, 2011, Research Assistant I

Amy Botello
April 30, 2012, Executive Assistant to Dr. V. Craig Jordan

Russell E. McDaniel
September 1, 2012, Laboratory Manager

Daphne Fernandes
October 1, 2012, Research Assistant I

CONCLUSIONS

It is important to stress at the outset of this section, that there is growing momentum within the clinical community that our work is an important new dimension in women's health.
This is illustrated by three, well-defined facts:

1) Our focus on the applicability of our laboratory results through the use of low dose estrogen for the treatment of metastatic breast cancer following antihormone drug resistance is now a general topic of discussion. Our work has been pivotal for the publication of others; the concept we proposed from the laboratory data enhances the treatment of women with breast cancer[12,428].

2) Our concepts form the basis of a major clinical trial in Europe and around the world, described as the Study of Letrozole Extension (SOLE)[429]. The strategy for the study is to examine whether continuous long term antihormone therapy is better or worse for the adjuvant treatment of ER-positive breast cancer than therapy that has three months per year drug holidays, where the women's own estrogen can destroy the antihormone resistant breast cancer cells before drug resistance disease gets a hold.

3) The recent published findings of the Women's Health Initiative (WHI) of estrogen replacement therapy in hysterectomized postmenopausal women showed a reduction in the incidence of breast cancer that in fact continues for five years after estrogen therapy stops[1]. We are providing all of the scientific knowledge database to explain this apparently paradoxical finding (estrogen replacement reduces the risk of breast cancer!). We obviously take very seriously, the fact that we are the pioneering group scientifically in this area and through the investment of the DOD CoE grant via their visionary peer reviewed system, we have been given the responsibility to decipher the mechanisms involved in this new biology of estrogen-induced apoptosis in breast cancer.

It is clear from the aforementioned three broad applications in clinical medicine that we have an opportunity to revolutionize women's health. I will systematically create an executive summary conclusion for each of our ongoing Tasks.

A clinical trial comparing high-dose (30mg daily Estrace) versus low dose Estrace (6mg Estrace daily) has now been published (Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A, Jamalabadi-Majidi S, Crowder R, Siegel BA. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 2009;302(7):774-80.) by others based on our laboratory work. The study demonstrates that low-dose estrogen has lower side effects. The withdrawal of financial support from our industry partner prevented the implementation of clinical trails by our group. Nevertheless, the principle is now embedded in clinical research.

Our major accomplishment to date on the grant has been to create a map of the life and death of breast cancer cells in response to physiological estrogen. This is a new unique dataset that is invaluable, but the complexity of our dataset is currently a challenge to the best brains in bioinformatics in the world, with whom we are currently collaborating. It is important to realize that our visionary approach proposed the equivalent of creating a movie, of the life and death of breast cancer cells through gene activation and suppression, but every other research group in the world is studying only single photographs of cells and tumors at a single point in time. Nevertheless, it is our accomplishment that has been enhanced by considerable bioinformatics input and the development of new computer modeling systems to analyze gene dosing activation against time for the growth and death of human breast cancer cells in response to estrogen. We have taken all of our enormous

gene array data against time (96 hours) andprovided it to Dr. Joe Gray at the University of Oregon. He is working with us to create an unique pathway analysis ñmovieò. This has never been done before. With our database, we have already identified the sequence of events for estrogen-induced apoptosis in our endocrine resistant breast cancer cells. Estrogen induces a stress response and activates inflammatory genes. This discovery now allows us to interrogate this mechanism of inflammation-mediated cell death through its modulation with anti-inflammatory agents such as glucocorticoids. Our publications show that glucocorticoid actively prevents estrogen-induced apoptosis. The other major finding from our database is a description of the caspase cascade that provokes cell death and destruction following estrogen-induced apoptosis. We have precisely defined and identified caspase 4 as the trigger caspase in the initiation of estrogen-induced apoptosis. However, we now seek to build upon our database and use molecular pharmacology to define and refine the input signal through the estrogen receptor that modulates estrogen-induced apoptosis. We identified activation of AP-1 as are the basic estrogen-ER related event that triggers estrogen induced apoptosis.

The triggering mechanism of estrogen induced apoptosis in especially prepared estrogen deprived populations of breast cancer cells requires the ER. We have interrogated the vital importance of the ER signaling transduction pathway in a series of interlocking and interconnected cell studies involving ligands that bind to the ER to modulate apoptosis. These data have significant implications for modulating apoptosis in the clinic; and we will illustrate our conclusions systematically in the following paragraphs (**Task 2**).

Phytoestrogens have been investigated as natural alternatives to hormone replacement therapy and their potential as chemopreventive agents. We investigated the effects of equol, genestein, and coumesterol on estrogen deprived MCF-7:5C cells, which simulate the postmenopausal state of a women after years of estrogen deprivation, and compared the effects of steroidal estrogens E_2 and equilinen present in conjugated equine estrogen. Steroidal and phytoestrogens induce proliferation of MCF-7 cells at physiologic concentrations, but inhibit the growth and induce apoptosis in MCF-7:5C cells. Although steroidal and phytoestrogens induce estrogen responsive genes, there antiproliferative and apoptotic effects are mediated through the ER. Knockdown of ERŬusing siRNA blocks all estrogen induced apoptosis and growth inhibition. Phytoestrogens induce endoplasmic reticulum stress and inflammatory response stress-related genes in a comparable manner as the steroidal estrogens. Inhibition of inflammation using dexamethasone blocked both steroidal and phytoestrogen induced apoptosis and growth inhibition as well as their ability to induce apoptotic genes. Together, this suggests that phytoestrogens can potentially be used as chemoprevention agents in older postmenopausal women, but caution should be excercised when used in conjunction with steroidal anti-inflammatory agents due to their antiapoptitic effects. In an editorial in the Journal of the National Cancer Institute, we have recently cautioned about the use of phytoestrogens in soy products by women with breast cancer (**Task 2**).

We have identified an unusual feature of apoptosis induced by E_2. Wheras the commonly used cancer chemotherapy, paclitaxel, causes immediate inhibiotion of breast cancer cell growth, estrogen induced apoptosis is a delayed process occurring over days. There is a cellular commitment to E_2 trigered apoptosis that occurs after 24 hours. This involves first the activation of the intrinsic (mitochondrial) pathway with the subsequent induction of the extrinsic (death receptor) pathway by 48 hours. By contrast, paclitaxel excluxively initiates a rapid G2/M blocade by 12 hours, and exclusively activates extra-mitochondral apoptotic genes. By contrast E_2 causes initial proliferation with elevated S phase of cell cycles followed by apoptosis. These data illustrate that E_2 induced apoptosis involves a novel, multidynamic process that is distinctly different from that of a classic cytotoxic chemotherapeutic drug used in breast cancer treatment (**Task 2**). We have followed up the

observation that E_2 causes delayed apoptosis through the ER target by defining the conformation of the ER complex that controls estrogen induced apoptosis in breast cancer.

Triphenylethylenes (TPEs) were used for mechanistic studies on estrogen induced apoptosis. These TPEs all stimulate growth in MCF-7 cells, but unlike the planar estrogens they initially block estrogen induced apoptosis in the long term estrogen deprived MCF-7:5C cells. To define the conformation of the TPE:ER complex, we employed a previously validated assay using the induction of transforming growth factor ŭmRNA *in situ* in MDA-MB-231 cells stably transfected with either wild-type ER or a mutant D351 gene ER. The assays discriminate ligand fit in the ER based on the published chrystallography of planar estrogenes or TPE antiestrogens. The conformation of planar estrogens or angular TPE complexes were classified as ñestrogen likeò or ñantiestrogen likeò complexes respectively. The TPE:ER complexes did not readily recruit SRC-3 or ER to the pS2 promoter in MCF-7 and MCF-7:5C cells. Molecular modeling showed TPEs preferred to bind to the ER in and antagonistic fashion by helix 12 not sealing the ligand binding domain effectively. As a result, SRC-3 cannot bind. We noted however, that the TPE antiestrogens, though they initially block apoptosis with planar estrogens, will cause apoptosis over a two week time course. We subsequently studied this phenomenon in more detail comparing and contrasting the planar estrogen estradiol with the angular estrogen TPE bisphenol (**Task 2**).

Bisphenol induced cell cycle genes similar to those induced by E_2. Unlike the trigger by E_2 that occurs after 24 hours, the trigger of apoptosis for bisphenol occurred at 4 days with quantifiable apoptotic changes noted at 6 days. A prolonged induction in endoplasmic reticulum stress and inflammatory response genes was observed with subsequent action of apoptosis related genes in the second week of treatment with bisphenol. We conclude, that both the shape of the complex and duration of treatment control the initiation of estrogen induced apoptosis (**Task 2)**.

Our publications demonstrate that physiological concentrations of estrogen (E_2) induce endoplasmic reticulum and oxidative stress which finally result in apoptosis in E_2-deprived breast cancer cells, MCF-7:5C. c-Src is involved in the process of E_2-induced stress. To mimic the clinical administration of c-Src inhibitors, we treated cells with either E_2, a c-Src inhibitor PP2, or the combination for 8 weeks to further explore the apoptotic potential of the c-Src inhibitor and E_2 on MCF-7:5C cells. Protein levels of receptors and signaling pathways were examined by immunoblotting. Expression of mRNA was detected through real-time PCR. Cell cycles were analyzed by flow cytometry. Long-term treatment with PP2 alone or E_2 alone decreased cell growth. In contrast, a combination of PP2 and E_2 blocked apoptosis and the resulting cell line (MCF-7:PF) was unique, as they grew vigorously in culture with physiological levels of E_2, which could be blocked by the pure antiestrogen ICI182,780. One major change was that PP2 collaborated with E_2 to increase the level of insulin-like growth factor-1 receptor beta (IGF-1Rß). Blockade of IGF-1Rß completely abolished E_2-stimulated growth in MCF-7:PF cells. Furthermore, combination treatment up-regulated transcription factors Twist1 and Snail and repressed E-cadherin expression which made MCF-7:PF cells display a characteristic phenotype of epithelial-mesenchymal transition (EMT). These data illustrate the role of the c-Src inhibitor to block E_2-induced apoptosis and enhance E_2-stimulated growth. These data demonstrate that a cytostatic therapy can rapidly result in changing cell populations through selection pressure. The fact that a new cell population emerges that is SERM stimulated for growth through growth factor pathways, but genomic pathways remain blocked, is an important advance(**Task 2**).

We have shown that estrogen (E_2) induces apoptosis in long-term E_2 deprived breast cancer cells (MCF-7:5C) through stress responses, but the molecular mechanism underlying E_2-induced stress remains to be elucidated. Here, we report that the oncogene c-Src acts as an important adapter protein of estrogen receptor (ER) involved in stress responses induced by E_2 in MCF-7:5C cells. E_2

elevated c-Src phosphorylation in MCF-7:5C cells and 4-hydroxytamoxifen (4-OHT) blocked this stimulation which suggested that E_2 activated c-Src through ER. E_2 activated the sensors of unfolded protein response (UPR) inositol-requiring protein 1 alpha (IRE1Ŭ) and PRK-like endoplasmic reticulum kinase (PERK)/eukaryotic translation initiation factor-2Ŭ (eIF2Ŭ). The indicator of oxidative stress, heme oxygenase 1 gene (HMOX1), was dramatically up-regulated by E_2. Further examination showed that E_2 significantly increased reactive oxygen species (ROS) production in MCF-7:5C cells. And the energy stress sensor adenosine monophosphate (AMP)-activated protein kinase (AMPK) was activated by E_2. The specific inhibitor of c-Src, PP2, was able to abolish the phosphorylation of eIF2Ŭand AMPK and reduced the production of ROS induced by E_2. Therefore, PP2 blocked E_2-induced apoptosis, which was confirmed by knockdown of c-Src with a specific small interferon RNA. All of these data illustrate that c-Src functions as a critical transducer in E_2-initiated endoplasmic reticulum stress and oxidative stress which trigger apoptotic cascades in MCF-7:5C cells. This study provides an important rationale for further exploration of the stress responses in endocrine resistant breast cancer to improve clinical benefit (**Task 2**).

Overall, we have shown that estrogen-induced apoptosis of MCF7:5C cells is mediated by ER-stress and UPR. PERK mediated eIF2Ŭphosphorylation is the key pathway by which it activates the apoptotic signaling. Moreover, pharmacological intervention which increases the phosphorylated status of eIF2Ŭ, is sufficient to induce apoptosis in the MCF7:5C cells. We have also studied the downstream effectors of this pathway and determined that ATF4, CHOP and BIM play important roles in implementing the ER-stress mediated apoptosis in these cells (**Task 2**)

Development of resistance to existing endocrine-therapies in estrogen receptor alpha positive (ERŬ+) breast cancers is the major obstacle for maintaining efficacy of targeted therapy. Recent clinical studies have indicated over-expression of cMYC oncogene is associated with aromatase inhibitor (AI) resistant breast cancers. To understand the mechanisms involved in acquiring resistance we investigated the significance of cMYC over-expression in an endocrine-therapy resistant breast cancer cell model, MCF7:5C cells, which have been cultured long-term in estrogen-deprived media. Compared to the parental counterpart MCF7 cells, cMYC mRNA and protein was 3 fold over-expressed in MCF7:5C cell, which was found to be driving the estrogen-independent growth of these cells. Further investigation suggested transcriptional de-regulation of cMYC gene was responsible for its over-expression in the MCF7:5C cells. Chromatin immuno-precipitation assay revealed markedly higher recruitment of phosphorylated serine-2 carboxy-terminal domain (CTD) of RNA polymerase-II at the proximal promoter of cMYC gene in MCF7:5C cells as compared to its parental cells. The level of phospho-CDK9, a factor responsible for phosphorylation of serine-2 of RNA polymerase II CTD, was found to be elevated in MCF7:5C cells. Pharmacological inhibition of CDK9 not only reduced the transcripts and the protein levels of cMYC in MCF7:5C cells but also selectively inhibited its growth. This study describes the molecular events involved in the transcriptional over-expression of cMYC gene in AI-resistant breast cancer cells and identifies CDK9 as a potential novel drug target for therapeutic intervention in endocrine-resistant breast cancers (**Task 2**).

Estrogen receptor alpha (ERŬ) binds to different ligand which can function as complete / partial estrogen-agonist or antagonist. This depends on the chemical structure of the ligands which modulates the transcriptional activity of the estrogen-responsive genes by altering the conformation of the liganded-ERŬcomplex. This study determined the molecular mechanism of estrogen-agonistic / antagonistic action of structurally similar ligands, bisphenol (BP) and bisphenol-A (BPA) on cell proliferation and apoptosis of ERŬ+ve breast cancer cells. DNA was measured to assess the proliferation and apoptosis of breast cancer cells. RT- PCR and ChIP assays were performed to quantify the transcripts of TFF1 gene and recruitment of ERŬand SRC3 at the promoter of TFF1

gene, respectively. Molecular docking was used to delineate the binding modes of BP and BPA with the ERŭ. PCR-based arrays were used study the regulation of the apoptotic genes. BP and BPA induced the proliferation of breast cancer cells, however, unlike BPA, BP failed to induce apoptosis. BPA consistently acted as an agonist in our studies but BP exhibited mixed agonistic/antagonistic properties. Molecular docking revealed agonistic and antagonistic mode of binding for BPA and BP respectively. BPA treatment resembled E2 treatment in terms of PCR based regulation of apoptotic genes whereas BP was similar to 4OHT treatment. The chemical structure of ERŭligand determines the agonistic or antagonistic biological responses by the virtue of their binding mode, conformation of the liganded-ERŭcomplex and the context of the cellular function.

The administration of high dose synthetic estrogens was the first successful chemical therapy used in the treatment of metastatic breast cancer in postmenopausal women and this approach became the standard of care in postmenopausal women with metastatic breast cancer between 1950s and the end of the 1970s. The most recent analysis of the Womenôs Health Initiative estrogen alone trial in hysterectomised women revealed a persistent significant decrease in the incidence of breast cancer as well as breast cancer mortality. Although estrogens are known to induce proliferation of breast cancer cells, we have shown that physiologic concentrations induce apoptosis in long term estrogen deprived breast cancer cells. We have developed laboratory models that illustrate the new biology of estrogen induced apoptosis or growth to explain the effects of estrogen replacement therapy. The key to the success of estrogen therapy lies in a sufficient period of withdrawal of physiological estrogens (5-10years) and the subsequent regrowth of nascent breast tumor cells that survive under estrogen deprived conditions. These nascent tumors are now vulnerable to estrogen induced apoptosis.

The clinical basis for the use of physiologic estrogen treatment of metastatic anti hormone resistant breast tumors is based on laboratory studies which show that resistance to long- term antihormone breast cancer therapy evolves over 5 years. Taxanes have been used extensively in the treatment of early and advanced breast cancer and play an active role in the survival of breast cancer patients. We have interrogated the sequence of events that involve the apoptotic signaling pathway induced by estradiol (E_2) in comparison to paclitaxel. Cell culture studies show estrogen induced apoptosis to be a slow process, while paclitaxel rapidly inhibits the growth and induces death of long term estrogen deprived MCF7 cells (MCF7:5C). Using 4-hydroxytamoxifen to block E_2 induced apoptosis at different times, we established that the cellular commitment for E_2 triggered apoptosis occur only after 24 h. Activation of the intrinsic pathway was observed by 36h of E_2 treatment with subsequent induction of the extrinsic apoptotic pathway by 48h. Apoptosis was induced by paclitaxel exclusively through the extramitochodrial pathway and caused rapid G2/M blockade by 12 h of treatment. By contrast, E_2 causes an initial proliferation, then apoptosis of the MCF7:5C cells. These data indicate that E_2 induced apoptosis involves a novel, multidynamic process that is distractedly different from that of a classic cytotoxic chemotherapeutic drug used in breast cancer (**Task 2**).

The cis and trans isomers of the tested metabolites of tamoxifen have different estrogenic properties in wild type breast cancer cells.The cis and trans isomers of the tested compounds have a different ability to induce estrogen-induced apoptosis and with a different timecourse (delayed apoptosis), when compared to estradiol in antiestrogen resistant breast cancer cells. The tested compounds with various structures were evaluated for their estrogenic/antiestrogenic properties in human breast cancer cell lines to induce growth or estrogen-induced apoptosis. The compounds properties were evaluated using a DNA cell proliferation assay. The results of the assays demonstrate that there is a different biology of the isomers of the same antiestrogen FR4OHT and that the conformation of the ER that is dictated by the structure of the ligand is crucial in inducing

apoptosis in hormone independent breast cancer cells. Further experiments are needed to confirm the hypothesis (**Task 2**).

The oncogene nuclear receptor coactivator amplified in breast cancer 1 (AIB1) is a transcriptional coactivator that is overexpressed in various types of human cancers. However, the molecular mechanisms controlling AIB1 expression in the majority of cancers remain unclear. In this study,we identified a novel interacting protein of AIB1, forkhead-box protein G1 (FoxG1), which is an evolutionarily conserved forkhead-box transcriptional corepressor. We show that FoxG1 expressionis low in breast cancer cell lines, and that low levels of FoxG1 are correlated with a worse prognosis in breast cancer. We also demonstrate that transient overexpression of FoxG1 can suppress endogenous levels of AIB1 mRNA and protein in MCF-7 breast cancer cells. Exogenously expressed FoxG1 in MCF-7 cells also leads to apoptosis that can be rescued in part by AIB1 overexpression. Using chromatin immunoprecipitation (ChIP), we determined that FoxG1 is recruited to a region of the AIB1 gene promoter previously characterized to be responsible for AIB1-induced, positive auto-regulation of transcription through the recruitment of an activating, multiprotein complex, involving AIB1, E2F1 and Sp1. Increased FoxG1 expression significantly reduces the recruitment of AIB1, E2F1 and p300 to this region of the endogenous AIB1 gene promoter. Our data imply that FoxG1 can function as a pro-apoptotic factor in part through suppression of AIB1 coactivator transcription complex formation, thereby reducing the expression of the AIB1 oncogene (**Task 3**).

Dr. Heather Cunliff earlier completed the CGH for MCF-7:WS8 (estrogen deprived), MCF-7:5C and MCF-7:2A (two estrogen deprived clones that grow spontaneously). Results for these studies were presented as an oral presentation by Dr. Cunliff in 2007 at the Era of Hope Meeting in Baltimore. Dr. Cunliff has now completed the Agilent gene array for MCF-7:2A and MCF-7:5C hybridized against MCF-7:WS8 (estrogen deprived) RNAs. These data will be amalgamated with the prior CGH data and are currently being prepared for publication.

We have discovered that long-term treatment of MCF-7:5C cells with physiologic estrogen and the c-Src inhibitor, PP2, results in the inhibition of estrogen-induced apoptosis causes the evolution of a new cell population that is ER positive, PgR positive, and now grows in response to a panel of SERMs (**Task 4**). The fact that MCF-7:PF cells unexpectedly were stimulated to grow with either 4OHT and E_2 demanded an indepth examination and pathway analysis. The global gene microarray showed a remarkable overlap in genes regulated in the same direction by E_2 and 4OHT. Pathway enrichment analysis of the 280 genes commonly deregulated by 4OHT and E_2 revealed functions mainly related to membrane, cytoplasm, and metabolic processes. Further analysis of 98 upregulated genes by both 4OHT and E_2 uncovered a significant enrichment in genes associated with membrane remodeling, cytoskeleton reorganization, cytoplasmic adaptor proteins, cytoplasm organelle proteins, and related processes. Furthermore, 4OHT was more potent than E_2 to upregulate some membrane remodeling molecules such as *EHD2, FHL2, HOMER3,* and *RHOF.* In conrtrast, 4OHT acted as an antagonist to inhibit expression of the majority of enriched membrane-associated genes. The opposing E_2 and 4OHT transcriptome profiles were observed in wild-type MCF-7 cells. We conclude that long term selection pressure has changed the cell population responses to 4OHT. Membrane-associated signaling is critical for 4OHT-stimulated cell growth in MCF-7:PF cells. We have followed up our array studies to evaluate the biological significance of the growth factor pathways in MCF-7:PF cells. We addressed the question of whether these selective ER modulator (SERM) 4OHT or othere SERMs could target ER to prevent E_2 stimulated growth in MCF-7:PF cells. Though both 4OHT and and other SERMs stimulate cell growth of MCF-7:PF cells in an ER dependent manner, unlike E_2, 4OHT suppresses classical ER target genes, as does the pure antiestrogen fulvestrant. 4OHT did not recruit ER or SRC-3 to the promoter of an ER target gene

pS2. Paradoxically, 4OHT reduced total IGF-1Rϐ, but increased phosphorylation of IGF-1Rϐ. Mechanistic studies revealed that 4OHT functioned as an agonist to enhance the non genomic activity of ER and activate focal adhesion molecules to further increase phosphorylation of IGF-1Rϐ. Disruption of membrane associated signaling, IGF-1R and focal adhesion kinase (fak), completely abolished 4OHT stimulated cell growth. This study is the first to recapitulate a cellular model *in vitro* of acquired tamoxifen resistance, previously developed in athymic mice *in vivo*.

REFERENCES

1. Anderson GL, Chlebowski RT, Aragaki AK, *et al.* Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *Lancet Oncol* 2012; 13:476-486.
2. Chlebowski RT, Anderson GL. Changing concepts: Menopausal hormone therapy and breast cancer. *J Natl Cancer Inst* 2012; 104:517-527.
3. Obiorah I, Jordan VC. 2012 NAMS/PFIZER- Wulf H. Utian endowed lecture. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. *Menopause* 2013; 20:372-382.
4. Fritz WA, Coward L, Wang J, Lamartiniere CA. Dietary genistein: perinatal mammary cancer prevention, bioavailability and toxicity testing in the rat. *Carcinogenesis* 1998; 19:2151-2158.
5. Thompson LU, Chen JM, Li T, Strasser-Weippl K, Goss PE. Dietary flaxseed alters tumor biological markers in postmenopausal breast cancer. *Clin Cancer Res* 2005; 11:3828-3835.
6. Andrade JE, Ju YH, Baker C, Doerge DR, Helferich WG. Long-term exposure to dietary sources of genistein induces estrogen-independence in the human breast cancer (MCF-7) xenograft model. *Mol Nutr Food Res* 2014.
7. Peterson G, Barnes S. Genistein inhibits both estrogen and growth factor-stimulated proliferation of human breast cancer cells. *Cell Growth Differ* 1996; 7:1345-1351.
8. Diel P, Geis RB, Caldarelli A, *et al.* The differential ability of the phytoestrogen genistein and of estradiol to induce uterine weight and proliferation in the rat is associated with a substance specific modulation of uterine gene expression. *Mol Cell Endocrinol* 2004; 221:21-32.
9. Kostelac D, Rechkemmer G, Briviba K. Phytoestrogens modulate binding response of estrogen receptors alpha and beta to the estrogen response element. *J Agric Food Chem* 2003; 51:7632-7635.
10. Ariazi EA, Cunliffe HE, Lewis-Wambi JS, *et al.* Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *Proc Natl Acad Sci U S A* 2011; 108:18879-18886.
11. Fan P, Griffith OL, Agboke FA, *et al.* c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. *Cancer Res* 2013; 73:4510-4520.
12. Ellis M, Gao F, Dehdashti F, *et al.* Lower-dose vs high-dose oral estradiol therapy of hormone receptorï positive, aromatase inhibitorï resistant advanced breast cancer: A phase 2 randomized study. *JAMA* 2009; **302**:774-780.
13. Haddow A. David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer* 1970; 26:737-754.
14. Stoll B (1977) Palliation by castration or hormone ablation. In: Stoll BA, editor. Breast Cancer Management Early and Late. London: William Herman Medical Books Ltd. pp. 135-149.
15. Maximov PY, Myers CB, Curpan RF, Lewis-Wambi JS, Jordan VC. Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. *J Med Chem* 2010; 53:3273-3283.
16. McCague R, Leclercq G, Jordan VC. Nonisomerizable analogues of (Z)- and (E)-4-hydroxytamoxifen. Synthesis and endocrinological properties of substituted diphenylbenzocycloheptenes. *J Med Chem* 1988; 31:1285-1290.
17. Lal B, Khanna JM, Anand N. Phenethylamine in a rigid framework. 2,3-Substituted cis- and trans-6-amino-6,7,8,9-tetrahydro-5H-benzocyclohepten-5-ols. *J Med Chem* 1972; 15:23-27.
18. Kahn AM, Proctor GR, Rees L. Novel aromatic systems. Part IV. Synthesis and dehydrogenation of 4ô-Hydroxy-1,2-benzocycloheptatrienes. *J Chem Soc* 1966:990-994.
19. Jarman M, McCague R. Heptafluoro-p-tolyl and tetrafluoro-4-pyridyl as novel and selective protecting groups for phenolic and alcoholic functions: synthesis and cleavage of perfluoroaryl ethers of steroids. *J Chem Res, Synop* 1985:114-115.
20. Fauq AH, Maharvi GM, Sinha D. A convenient synthesis of (Z)-4-hydroxy-N-desmethyltamoxifen (endoxifen). *Bioorg Med Chem Lett* 2010; 20:3036-3038.
21. Stearns V, Johnson MD, Rae JM, *et al.* Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. *J Natl Cancer Inst* 2003; 95:1758-1764.
22. Murahashi SI, Naota T, Miyaguchi N, Nakato T. Ruthenium-catalyzed oxidation of tertiary-amines with hydrogen-peroxide in the presence of methanol. *Tetrahedron Letters* 1992; 33:6991-6994.
23. Lee HW, Ahn JB, Lee JH, Kang SK, Ahn SK, Ha DC. Selective N-demethylation of tertiary aminofumagillols with selenium dioxide via a non-classical Polonovski type reaction. *Heterocycles* 2006; 68:915-932.
24. Murdter TE, Schroth W, Bacchus-Gerybadze L, *et al.* Activity levels of tamoxifen metabolites at the estrogen receptor and the impact of genetic polymorphisms of phase I and II enzymes on their concentration levels in plasma. *Clin Pharmacol Ther* 2011; 89:708-717.

25. Jordan VC, Fritz NF, Tormey DC. Endocrine effects of adjuvant chemotherapy and long-term tamoxifen administration on node-positive patients with breast cancer. *Cancer Res* 1987; 47:624-630.
26. Ravdin PM, Fritz NF, Tormey DC, Jordan VC. Endocrine status of premenopausal node-positive breast cancer patients following adjuvant chemotherapy and long-term tamoxifen. *Cancer Res* 1988; 48:1026-1029.
27. Warnmark A, Treuter E, Gustafsson JA, Hubbard RE, Brzozowski AM, Pike AC. Interaction of transcriptional intermediary factor 2 nuclear receptor box peptides with the coactivator binding site of estrogen receptor alpha. *J Biol Chem* 2002; 277:21862-21868.
28. Shiau AK, Barstad D, Loria PM, *et al.* The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 1998; 95:927-937.
29. Rajan SS, Kim Y, Vanek K, Joachimiak A, Jordan C, Greene GL. Crystal structure of human estrogen receptor alpha LBD in complex with GRIP peptide and two ismoers of Ethoxy triphenylethylene. to be published.
30. Renaud J, Bischoff SF, Buhl T, *et al.* Estrogen receptor modulators: identification and structure-activity relationships of potent ERalpha-selective tetrahydroisoquinoline ligands. *J Med Chem* 2003; 46:2945-2957.
31. Vajdos FF, Hoth LR, Geoghegan KF, *et al.* The 2.0 A crystal structure of the ERalpha ligand-binding domain complexed with lasofoxifene. *Protein Sci* 2007; 16:897-905.
32. Maximov PY, Fernandes DJ, McDaniel RE, Myers CB, Curpan RF, Jordan VC. Influence of the length and positioning of the antiestrogenic side chain of endoxifen and 4-hydroxytamoxifen on gene activation and growth of estrogen receptor positive cancer cells. *J Med Chem* 2014; 57:4569-4583.
33. Jordan VC, Koch R, Langan S, McCague R. Ligand interaction at the estrogen receptor to program antiestrogen action: a study with nonsteroidal compounds in vitro. *Endocrinology* 1988; 122:1449-1454.
34. Murphy CS, Langan-Fahey SM, McCague R, Jordan VC. Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. *Mol Pharmacol* 1990; 38:737-743.
35. Lieberman ME, Gorski J, Jordan VC. An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. *J Biol Chem* 1983; 258:4741-4745.
36. Jordan VC, Lieberman ME. Estrogen-stimulated prolactin synthesis in vitro. Classification of agonist, partial agonist, and antagonist actions based on structure. *Mol Pharmacol* 1984; 26:279-285.
37. Jordan VC, Lieberman ME, Cormier E, Koch R, Bagley JR, Ruenitz PC. Structural requirements for the pharmacological activity of nonsteroidal antiestrogens in vitro. *Mol Pharmacol* 1984; 26:272-278.
38. Jordan VC, Koch R, Mittal S, Schneider MR. Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. *Br J Pharmacol* 1986; 87:217-223.
39. Murphy CS, Parker CJ, McCague R, Jordan VC. Structure-activity relationships of nonisomerizable derivatives of tamoxifen: importance of hydroxyl group and side chain positioning for biological activity. *Mol Pharmacol* 1991; 39:421-428.
40. Lonard DM, Nawaz Z, Smith CL, O'Malley BW. The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. *Mol Cell* 2000; 5:939-948.
41. Obiorah IE, Sengupta S, Curpan R, Jordan VC. Defining the conformation of the estrogen receptor complex that controls estrogen induced apoptosis in breast cancer. *Mol Pharmacol* 2014; epub ahead of print.
42. Nicholson RI, Gee JM, Manning DL, Wakeling AE, Montano MM, Katzenellenbogen BS. Responses to pure antiestrogens (ICI 164384, ICI 182780) in estrogen-sensitive and -resistant experimental and clinical breast cancer. *Ann N Y Acad Sci* 1995; 761:148-163.
43. Wu X, Hawse JR, Subramaniam M, Goetz MP, Ingle JN, Spelsberg TC. The tamoxifen metabolite, endoxifen, is a potent antiestrogen that targets estrogen receptor alpha for degradation in breast cancer cells. *Cancer Res* 2009; 69:1722-1727.
44. Liu H, Lee ES, Deb Los Reyes A, Zapf JW, Jordan VC. Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor alpha complex. *Cancer Res* 2001; 61:3632-3639.
45. Jordan VC. The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer--survival or death? *J Clin Oncol* 2008; 26:3073-3082.
46. Obiorah I, Sengupta S, Fan P, Jordan VC. Delayed triggering of oestrogen induced apoptosis that contrasts with rapid paclitaxel-induced breast cancer cell death. *Br J Cancer* 2014; 110:1488-1496.
47. Lewis-Wambi J, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? *Breast Cancer Res* 2009; **11**:206.
48. Lieberman ME, Jordan VC, Fritsch M, Santos MA, Gorski J. Direct and reversible inhibition of estradiol-stimulated prolactin synthesis by antiestrogens in vitro. *J Biol Chem* 1983; 258:4734-4740.
49. Maximov P, Sengupta S, Lewis-Wambi JS, Kim HR, Curpan RF, Jordan VC. The Conformation of the Estrogen Receptor Directs Estrogen-Induced Apoptosis in Breast Cancer: A Hypothesis. *Horm Mol Biol Clin Investig* 2011; 5:27-34.

50. Sengupta S, Obiorah I, Maximov P, Curpan R, Jordan VC. Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. *Br J Pharmacol* 2013; 169:167-178.
51. Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ* 2004; 11:381-389.
52. Akira S, Isshiki H, Sugita T, *et al.* A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J* 1990; 9:1897-1906.
53. Bourgoin-Voillard S, Gallo D, Laios I, *et al.* Capacity of type I and II ligands to confer to estrogen receptor alpha an appropriate conformation for the recruitment of coactivators containing a LxxLL motif-Relationship with the regulation of receptor level and ERE-dependent transcription in MCF-7 cells. *Biochem Pharmacol* 2010; 79:746-757.
54. Hu ZZ, Kagan BL, Ariazi EA, *et al.* Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. *PLoS One* 2011; 6:e20410.
55. Hitomi J, Katayama T, Eguchi Y, *et al.* Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. *J Cell Biol* 2004; 165:347-356.
56. Jordan VC, Schafer JM, Levenson AS, *et al.* Molecular classification of estrogens. *Cancer Res* 2001; 61:6619-6623.
57. McDonnell DP, Clemm DL, Hermann T, Goldman ME, Pike JW. Analysis of estrogen receptor function in vitro reveals three distinct classes of antiestrogens. *Mol Endocrinol* 1995; 9:659-669.
58. Brzozowski AM, Pike ACW, Dauter Z, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 1997; 389:753-758.
59. Shiau AK, Barstad D, Loria P, et al. The Structural Basis of Estrogen Receptor/Coactivator Recognition and the Antagonism of This Interaction by Tamoxifen. *Cell* 1998; 95:927-937.
60. Levenson AS, MacGregor Schafer JI, Bentrem DJ, Pease KM, Jordan VC. Control of the estrogen-like actions of the tamoxifen-estrogen receptor complex by the surface amino acid at position 351. *J Steroid Biochem Mol Biol* 2001; 76:61-70.
61. Levenson AS, Tonetti DA, Jordan VC. The oestrogen-like effect of 4-hydroxytamoxifen on induction of transforming growth factor alpha mRNA in MDA-MB-231 breast cancer cells stably expressing the oestrogen receptor. *Br J Cancer* 1998; 77:1812-1819.
62. MacGregor Schafer J, Liu H, Bentrem DJ, Zapf JW, Jordan VC. Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. *Cancer Res* 2000; 60:5097-5105.
63. Merenbakh-Lamin K, Ben-Baruch N, Yeheskel A, *et al.* D538G Mutation in Estrogen Receptor-alpha: A Novel Mechanism for Acquired Endocrine Resistance in Breast Cancer. *Cancer Res* 2013; 73:6856-6864.
64. Toy W, Shen Y, Won H, *et al.* ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nat Genet* 2013; 45:1439-1445.
65. Liu H, Park WC, Bentrem DJ, *et al.* Structure-function relationships of the raloxifene-estrogen receptor-alpha complex for regulating transforming growth factor-alpha expression in breast cancer cells. *J Biol Chem* 2002; 277:9189-9198.
66. Font de Mora J, Brown M. AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Mol Cell Biol* 2000; 20:5041-5047.
67. Lahusen T, Henke RT, Kagan BL, Wellstein A, Riegel AT. The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer. *Breast Cancer Res Treat* 2009; 116:225-237.
68. List HJ, Lauritsen KJ, Reiter R, Powers C, Wellstein A, Riegel AT. Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells. *J Biol Chem* 2001; 276:23763-23768.
69. Borras M, Hardy L, Lempereur F, *et al.* Estradiol-induced down-regulation of estrogen receptor. Effect of various modulators of protein synthesis and expression. *J Steroid Biochem Mol Biol* 1994; 48:325-336.
70. Borras M, Laios I, el Khissiin A, *et al.* Estrogenic and antiestrogenic regulation of the half-life of covalently labeled estrogen receptor in MCF-7 breast cancer cells. *J Steroid Biochem Mol Biol* 1996; 57:203-213.
71. Reid G, Hubner MR, Metivier R, *et al.* Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. *Mol Cell* 2003; 11:695-707.
72. Wijayaratne AL, Nagel SC, Paige LA, *et al.* Comparative analyses of mechanistic differences among antiestrogens. *Endocrinology* 1999; 140:5828-5840.
73. Lewis-Wambi JS, Kim H, Curpan R, Grigg R, Sarker MA, Jordan VC. The selective estrogen receptor modulator bazedoxifene inhibits hormone-independent breast cancer cell growth and down-regulates estrogen receptor alpha and cyclin D1. *Mol Pharmacol* 2011; 80:610-620.

74. Haddow A, Watkinson JM, Paterson E, Koller PC. Influence of synthetic oestrogens on advanced malignant disease. *Br Med J* 1944; 2:393-398.
75. Brzozowski AM, Pike AC, Dauter Z, *et al.* Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 1997; 389:753-758.
76. Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* 2003; 114:181-190.
77. Thorburn A. Death receptor-induced cell killing. *Cell Signal* 2004; 16:139-144.
78. De Paepe B, Creus KK, De Bleecker JL. The tumor necrosis factor superfamily of cytokines in the inflammatory myopathies: potential targets for therapy. *Clin Dev Immunol* 2012; 2012:369432.
79. Hu P, Han Z, Couvillon AD, Kaufman RJ, Exton JH. Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression. *Mol Cell Biol* 2006; 26:3071-3084.
80. Zhang K, Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. *Nature* 2008; 454:455-462.
81. Baldwin AS, Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 1996; 14:649-683.
82. Dobrovolskaia MA, Kozlov SV. Inflammation and cancer: when NF-kappaB amalgamates the perilous partnership. *Curr Cancer Drug Targets* 2005; 5:325-344.
83. Lewis JS, Meeke K, Osipo C, *et al.* Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 2005; 97:1746-1759.
84. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* 2003; 95:1597-1608.
85. Liu H, Lee ES, Gajdos C, *et al.* Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. *J Natl Cancer Inst* 2003; 95:1586-1597.
86. Kameda T, Mano H, Yuasa T, *et al.* Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. *J Exp Med* 1997; 186:489-495.
87. Hughes DE, Dai A, Tiffee JC, Li HH, Mundy GR, Boyce BF. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-beta. *Nat Med* 1996; 2:1132-1136.
88. Kutuk O, Letai A. Displacement of Bim by Bmf and Puma rather than increase in Bim level mediates paclitaxel-induced apoptosis in breast cancer cells. *Cell Death Differ* 2010; 17:1624-1635.
89. Ajabnoor GM, Crook T, Coley HM. Paclitaxel resistance is associated with switch from apoptotic to autophagic cell death in MCF-7 breast cancer cells. *Cell Death Dis* 2012; 3:e260.
90. Czernick M, Rieger A, Goping IS. Bim is reversibly phosphorylated but plays a limited role in paclitaxel cytotoxicity of breast cancer cell lines. *Biochem Biophys Res Commun* 2009; 379:145-150.
91. Ellis MJ, Gao F, Dehdashti F, *et al.* Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *JAMA* 2009; 302:774-780.
92. Obiorah I, Jordan VC. Scientific rationale for postmenopause delay in the use of conjugated equine estrogens among postmenopausal women that causes reduction in breast cancer incidence and mortality. *Menopause* 2013; 20:372-382.
93. Song RX, Mor G, Naftolin F, *et al.* Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *J Natl Cancer Inst* 2001; 93:1714-1723.
94. Doroshow JH. Glutathione peroxidase and oxidative stress. *Toxicol Lett* 1995; 82-83:395-398.
95. Siemankowski LM, Morreale J, Briehl MM. Antioxidant defenses in the TNF-treated MCF-7 cells: selective increase in MnSOD. *Free Radic Biol Med* 1999; 26:919-924.
96. Fan P, Agboke FA, McDaniel RE, *et al.* Inhibition of c-Src blocks oestrogen-induced apoptosis and restores oestrogen-stimulated growth in long-term oestrogen-deprived breast cancer cells. *Eur J Cancer* 2014; 50:457-468.
97. Lee JY, Han CY, Yang JW, *et al.* Induction of glutathione transferase in insulin-like growth factor type I receptor-overexpressed hepatoma cells. *Mol Pharmacol* 2007; 72:1082-1093.
98. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* 1992; 90:77-86.
99. Pink JJ, Jiang SY, Fritsch M, Jordan VC. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* 1995; 55:2583-2590.
100. Brunner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R. Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. *Cancer Res* 1993; 53:283-290.
101. Brunner N, Frandsen TL, Holst-Hansen C, *et al.* MCF7/LCC2: a 4-hydroxytamoxifen resistant human breast cancer variant that retains sensitivity to the steroidal antiestrogen ICI 182,780. *Cancer Res* 1993; 53:3229-3232.

102. Brunner N, Boysen B, Jirus S, *et al.* MCF7/LCC9: an antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen. *Cancer Res* 1997; 57:3486-3493.
103. Sweeney EE, McDaniel RE, Maximov PY, Fan P, Jordan VC. Models and Mechanisms of Acquired Antihormone Resistance in Breast Cancer: Significant Clinical Progress Despite Limitations. *Horm Mol Biol Clin Investig* 2012; 9:143-163.
104. Yin X, Giap C, Lazo JS, Prochownik EV. Low molecular weight inhibitors of Myc-Max interaction and function. *Oncogene* 2003; 22:6151-6159.
105. Follis AV, Hammoudeh DI, Wang H, Prochownik EV, Metallo SJ. Structural rationale for the coupled binding and unfolding of the c-Myc oncoprotein by small molecules. *Chem Biol* 2008; 15:1149-1155.
106. Gyorffy B, Lanczky A, Eklund AC, *et al.* An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast Cancer Res Treat* 2010; 123:725-731.
107. Saunders A, Core LJ, Lis JT. Breaking barriers to transcription elongation. *Nat Rev Mol Cell Biol* 2006; 7:557-567.
108. Shim EY, Walker AK, Shi Y, Blackwell TK. CDK-9/cyclin T (P-TEFb) is required in two postinitiation pathways for transcription in the C. elegans embryo. *Genes Dev* 2002; 16:2135-2146.
109. Larochelle S, Amat R, Glover-Cutter K, *et al.* Cyclin-dependent kinase control of the initiation-to-elongation switch of RNA polymerase II. *Nat Struct Mol Biol* 2012; 19:1108-1115.
110. Sims RJ, 3rd, Belotserkovskaya R, Reinberg D. Elongation by RNA polymerase II: the short and long of it. *Genes Dev* 2004; 18:2437-2468.
111. Krystof V, Cankar P, Frysova I, *et al.* 4-arylazo-3,5-diamino-1H-pyrazole CDK inhibitors: SAR study, crystal structure in complex with CDK2, selectivity, and cellular effects. *J Med Chem* 2006; 49:6500-6509.
112. Ellis MJ, Ding L, Shen D, *et al.* Whole-genome analysis informs breast cancer response to aromatase inhibition. *Nature* 2012; 486:353-360.
113. Miller TW, Balko JM, Ghazoui Z, *et al.* A gene expression signature from human breast cancer cells with acquired hormone independence identifies MYC as a mediator of antiestrogen resistance. *Clin Cancer Res* 2011; 17:2024-2034.
114. McNeil CM, Sergio CM, Anderson LR, *et al.* c-Myc overexpression and endocrine resistance in breast cancer. *J Steroid Biochem Mol Biol* 2006; 102:147-155.
115. Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. *Annu Rev Med* 2011; 62:233-247.
116. Venditti M, Iwasiow B, Orr FW, Shiu RP. C-myc gene expression alone is sufficient to confer resistance to antiestrogen in human breast cancer cells. *Int J Cancer* 2002; 99:35-42.
117. Jeng MH, Shupnik MA, Bender TP, *et al.* Estrogen receptor expression and function in long-term estrogen-deprived human breast cancer cells. *Endocrinology* 1998; 139:4164-4174.
118. Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell* 1995; 81:323-330.
119. Bentley DL, Groudine M. A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL60 cells. *Nature* 1986; 321:702-706.
120. Krumm A, Meulia T, Brunvand M, Groudine M. The block to transcriptional elongation within the human c-myc gene is determined in the promoter-proximal region. *Genes Dev* 1992; 6:2201-2213.
121. Wang C, Mayer JA, Mazumdar A, *et al.* Estrogen induces c-myc gene expression via an upstream enhancer activated by the estrogen receptor and the AP-1 transcription factor. *Mol Endocrinol* 2011; 25:1527-1538.
122. Musgrove EA, Sergio CM, Loi S, *et al.* Identification of functional networks of estrogen- and c-Myc-responsive genes and their relationship to response to tamoxifen therapy in breast cancer. *PLoS One* 2008; 3:e2987.
123. Kininis M, Isaacs GD, Core LJ, Hah N, Kraus WL. Postrecruitment regulation of RNA polymerase II directs rapid signaling responses at the promoters of estrogen target genes. *Mol Cell Biol* 2009; 29:1123-1133.
124. Nechaev S, Adelman K. Pol II waiting in the starting gates: Regulating the transition from transcription initiation into productive elongation. *Biochim Biophys Acta* 2011; 1809:34-45.
125. Buratowski S. Progression through the RNA polymerase II CTD cycle. *Mol Cell* 2009; 36:541-546.
126. Peterlin BM, Price DH. Controlling the elongation phase of transcription with P-TEFb. *Mol Cell* 2006; 23:297-305.
127. Baumli S, Hole AJ, Noble ME, Endicott JA. The CDK9 C-helix exhibits conformational plasticity that may explain the selectivity of CAN508. *ACS Chem Biol* 2012; 7:811-816.
128. Zhang W, Prakash C, Sum C, *et al.* Bromodomain-containing protein 4 (BRD4) regulates RNA polymerase II serine 2 phosphorylation in human CD4+ T cells. *J Biol Chem* 2012; 287:43137-43155.
129. Jang MK, Mochizuki K, Zhou M, Jeong HS, Brady JN, Ozato K. The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription. *Mol Cell* 2005; 19:523-534.

130. Delmore JE, Issa GC, Lemieux ME, *et al.* BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* 2011; 146:904-917.
131. Zuber J, Shi J, Wang E, *et al.* RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature* 2011; 478:524-528.
132. Yao K, Lee ES, Bentrem DJ, *et al.* Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000; 6:2028-2036.
133. Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res* 1993; 127:23-33.
134. Fan P, McDaniel RE, Kim HR, Clagett D, Haddad B, Jordan VC. Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines. *Eur J Cancer* 2012; 48:3488-3498.
135. Fan P, Wang J, Santen RJ, Yue W. Long-term treatment with tamoxifen facilitates translocation of estrogen receptor alpha out of the nucleus and enhances its interaction with EGFR in MCF-7 breast cancer cells. *Cancer Res* 2007; 67:1352-1360.
136. Hiscox S, Morgan L, Green TP, Barrow D, Gee J, Nicholson RI. Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. *Breast Cancer Res Treat* 2006; 97:263-274.
137. Hiscox S, Jordan NJ, Smith C, *et al.* Dual targeting of Src and ER prevents acquired antihormone resistance in breast cancer cells. *Breast Cancer Res Treat* 2009; 115:57-67.
138. Chen Y, Guggisberg N, Jorda M, *et al.* Combined Src and aromatase inhibition impairs human breast cancer growth in vivo and bypass pathways are activated in AZD0530-resistant tumors. *Clin Cancer Res* 2009; 15:3396-3405.
139. Lange CA, Shen T, Horwitz KB. Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. *Proc Natl Acad Sci U S A* 2000; 97:1032-1037.
140. Jeng MH, Langan-Fahey SM, Jordan VC. Estrogenic actions of RU486 in hormone-responsive MCF-7 human breast cancer cells. *Endocrinology* 1993; 132:2622-2630.
141. Nam JS, Ino Y, Sakamoto M, Hirohashi S. Src family kinase inhibitor PP2 restores the E-cadherin/catenin cell adhesion system in human cancer cells and reduces cancer metastasis. *Clin Cancer Res* 2002; 8:2430-2436.
142. Balzer EM, Whipple RA, Thompson K, *et al.* c-Src differentially regulates the functions of microtentacles and invadopodia. *Oncogene* 2010; 29:6402-6408.
143. Li CW, Xia W, Huo L, *et al.* Epithelial-mesenchymal transition induced by TNF-alpha requires NF-kappaB-mediated transcriptional upregulation of Twist1. *Cancer Res* 2012; 72:1290-1300.
144. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; 281:1309-1312.
145. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. *Cell* 2005; 120:483-495.
146. Tsai YC, Weissman AM. The Unfolded Protein Response, Degradation from Endoplasmic Reticulum and Cancer. *Genes Cancer* 2010; 1:764-778.
147. Chu I, Arnaout A, Loiseau S, *et al.* Src promotes estrogen-dependent estrogen receptor alpha proteolysis in human breast cancer. *J Clin Invest* 2007; 117:2205-2215.
148. Santen RJ, Song RX, Zhang Z, *et al.* Adaptive hypersensitivity to estrogen: mechanisms and clinical relevance to aromatase inhibitor therapy in breast cancer treatment. *J Steroid Biochem Mol Biol* 2005; 95:155-165.
149. Nakagawa T, Zhu H, Morishima N, *et al.* Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 2000; 403:98-103.
150. Harrington WR, Kim SH, Funk CC, *et al.* Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. *Mol Endocrinol* 2006; 20:491-502.
151. Zhang Y, Zhao H, Asztalos S, Chisamore M, Sitabkhan Y, Tonetti DA. Estradiol-induced regression in T47D:A18/PKCalpha tumors requires the estrogen receptor and interaction with the extracellular matrix. *Mol Cancer Res* 2009; 7:498-510.
152. Levenson AS, Catherino WH, Jordan VC. Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. *J Steroid Biochem Mol Biol* 1997; 60:261-268.
153. Wolf DM, Jordan VC. The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. *Breast Cancer Res Treat* 1994; 31:129-138.
154. Bentrem D, Fox JE, Pearce ST, *et al.* Distinct molecular conformations of the estrogen receptor alpha complex exploited by environmental estrogens. *Cancer Res* 2003; 63:7490-7496.
155. Routledge EJ, White R, Parker MG, Sumpter JP. Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) alpha and ERbeta. *J Biol Chem* 2000; 275:35986-35993.
156. Metivier R, Penot G, Hubner MR, *et al.* Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* 2003; 115:751-763.

157. Metivier R, Stark A, Flouriot G, *et al.* A dynamic structural model for estrogen receptor-alpha activation by ligands, emphasizing the role of interactions between distant A and E domains. *Mol Cell* 2002; 10:1019-1032.
158. Labhart P, Karmakar S, Salicru EM, *et al.* Identification of target genes in breast cancer cells directly regulated by the SRC-3/AIB1 coactivator. *Proc Natl Acad Sci U S A* 2005; 102:1339-1344.
159. Shao W, Keeton EK, McDonnell DP, Brown M. Coactivator AIB1 links estrogen receptor transcriptional activity and stability. *Proc Natl Acad Sci U S A* 2004; 101:11599-11604.
160. Gould JC, Leonard LS, Maness SC, *et al.* Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. *Mol Cell Endocrinol* 1998; 142:203-214.
161. Kitamura S, Suzuki T, Sanoh S, *et al.* Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. *Toxicol Sci* 2005; 84:249-259.
162. Kuiper GG, Lemmen JG, Carlsson B, *et al.* Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* 1998; 139:4252-4263.
163. Gao L, Tu Y, Agren H, Eriksson LA. Characterization of Agonist Binding to His524 in the Estrogen Receptor alpha Ligand Binding Domain. *J Phys Chem B* 2012:4823-4830.
164. Mayer EL, Baurain JF, Sparano J, *et al.* A phase 2 trial of dasatinib in patients with advanced HER2-positive and/or hormone receptor-positive breast cancer. *Clin Cancer Res* 2011; 17:6897-6904.
165. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 1996; 56:2321-2330.
166. Luttrell DK, Lee A, Lansing TJ, *et al.* Involvement of pp60c-src with two major signaling pathways in human breast cancer. *Proc Natl Acad Sci U S A* 1994; 91:83-87.
167. Okamoto W, Okamoto I, Yoshida T, *et al.* Identification of c-Src as a potential therapeutic target for gastric cancer and of MET activation as a cause of resistance to c-Src inhibition. *Mol Cancer Ther* 2010; 9:1188-1197.
168. Faivre EJ, Lange CA. Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells. *Mol Cell Biol* 2007; 27:466-480.
169. Zhao Y, Planas-Silva MD. Mislocalization of cell-cell adhesion complexes in tamoxifen-resistant breast cancer cells with elevated c-Src tyrosine kinase activity. *Cancer Lett* 2009; 275:204-212.
170. Tan M, Li P, Klos KS, *et al.* ErbB2 promotes Src synthesis and stability: novel mechanisms of Src activation that confer breast cancer metastasis. *Cancer Res* 2005; 65:1858-1867.
171. Finn RS, Dering J, Ginther C, *et al.* Dasatinib, an orally active small molecule inhibitor of both the src and abl kinases, selectively inhibits growth of basal-type/"triple-negative" breast cancer cell lines growing in vitro. *Breast Cancer Res Treat* 2007; 105:319-326.
172. Eckert LB, Repasky GA, Ulku AS, *et al.* Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoikis. *Cancer Res* 2004; 64:4585-4592.
173. Huang F, Reeves K, Han X, *et al.* Identification of candidate molecular markers predicting sensitivity in solid tumors to dasatinib: rationale for patient selection. *Cancer Res* 2007; 67:2226-2238.
174. Lewis-Wambi JS, Kim HR, Wambi C, *et al.* Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* 2008; 10:R104.
175. Berry M, Nunez AM, Chambon P. Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence. *Proc Natl Acad Sci U S A* 1989; 86:1218-1222.
176. Cuzick J, Powles T, Veronesi U, *et al.* Overview of the main outcomes in breast-cancer prevention trials. *Lancet* 2003; 361:296-300.
177. Fisher B, Costantino JP, Wickerham DL, *et al.* Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 1998; 90:1371-1388.
178. Vogel VG, Costantino JP, Wickerham DL, *et al.* Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. *Cancer Prev Res (Phila)* 2010; 3:696-706.
179. Archer DF, Pinkerton JV, Utian WH, *et al.* Bazedoxifene, a selective estrogen receptor modulator: effects on the endometrium, ovaries, and breast from a randomized controlled trial in osteoporotic postmenopausal women. *Menopause* 2009; 16:1109-1115.
180. Kagan R, Williams RS, Pan K, Mirkin S, Pickar JH. A randomized, placebo- and active-controlled trial of bazedoxifene/conjugated estrogens for treatment of moderate to severe vulvar/vaginal atrophy in postmenopausal women. *Menopause* 2010; 17:281-289.
181. Pinkerton JV, Utian WH, Constantine GD, Olivier S, Pickar JH. Relief of vasomotor symptoms with the tissue-selective estrogen complex containing bazedoxifene/conjugated estrogens: a randomized, controlled trial. *Menopause* 2009; 16:1116-1124.

182. Komm BS, Kharode YP, Bodine PV, Harris HA, Miller CP, Lyttle CR. Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity. *Endocrinology* 2005; 146:3999-4008.
183. Miller CP, Collini MD, Tran BD, *et al.* Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. *J Med Chem* 2001; 44:1654-1657.
184. Garcia-Closas M, Chanock S. Genetic susceptibility loci for breast cancer by estrogen receptor status. *Clin Cancer Res* 2008; 14:8000-8009.
185. Garcia-Closas M, Hall P, Nevanlinna H, *et al.* Heterogeneity of breast cancer associations with five susceptibility loci by clinical and pathological characteristics. *PLoS Genet* 2008; 4:e1000054.
186. Sommer S, Fuqua SA. Estrogen receptor and breast cancer. *Semin Cancer Biol* 2001; 11:339-352.
187. Murphy CS, Pink JJ, Jordan VC. Characterization of a receptor-negative, hormone-nonresponsive clone derived from a T47D human breast cancer cell line kept under estrogen-free conditions. *Cancer Res* 1990; 50:7285-7292.
188. Pink JJ, Bilimoria MM, Assikis J, Jordan VC. Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. *Br J Cancer* 1996; 74:1227-1236.
189. Ariazi EA, Brailoiu E, Yerrum S, *et al.* The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. *Cancer Res* 2010; 70:1184-1194.
190. Lammie GA, Peters G. Chromosome 11q13 abnormalities in human cancer. *Cancer Cells* 1991; 3:413-420.
191. Sutherland RL, Musgrove EA. Cyclins and breast cancer. *J Mammary Gland Biol Neoplasia* 2004; 9:95-104.
192. Said TK, Conneely OM, Medina D, O'Malley BW, Lydon JP. Progesterone, in addition to estrogen, induces cyclin D1 expression in the murine mammary epithelial cell, in vivo. *Endocrinology* 1997; 138:3933-3939.
193. Wilcken NR, Prall OW, Musgrove EA, Sutherland RL. Inducible overexpression of cyclin D1 in breast cancer cells reverses the growth-inhibitory effects of antiestrogens. *Clin Cancer Res* 1997; 3:849-854.
194. Zwijsen RM, Wientjens E, Klompmaker R, van der Sman J, Bernards R, Michalides RJ. CDK-independent activation of estrogen receptor by cyclin D1. *Cell* 1997; 88:405-415.
195. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 2005; 94:131-141.
196. Wijayaratne AL, McDonnell DP. The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. *J Biol Chem* 2001; 276:35684-35692.
197. Carroll JS, Liu XS, Brodsky AS, *et al.* Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* 2005; 122:33-43.
198. Jordan VC, MacGregor Schafer, J.I., Levenson, A.S., Liu, H., Pease, K.M., Simons, L.A. and Zapf, J.W. Molecular classification of estrogens. *Cancer Res* 2001; 61:6619-6623.
199. Rae JM, Creighton CJ, Meck JM, Haddad BR, Johnson MD. MDA-MB-435 cells are derived from M14 melanoma cells--a loss for breast cancer, but a boon for melanoma research. *Breast Cancer Res Treat* 2007; 104:13-19.
200. O'Regan RM, Cisneros A, England GM, *et al.* Effects of the antiestrogens tamoxifen, toremifene, and ICI 182,780 on endometrial cancer growth. *J Natl Cancer Inst* 1998; 90:1552-1558.
201. Jiang SY, Langan-Fahey SM, Stella AL, McCague R, Jordan VC. Point mutation of estrogen receptor (ER) in the ligand-binding domain changes the pharmacology of antiestrogens in ER-negative breast cancer cells stably expressing complementary DNAs for ER. *Mol Endocrinol* 1992; 6:2167-2174.
202. Brooks SC, Locke ER, Soule HD. Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. *J Biol Chem* 1973; 248:6251-6253.
203. Martino S, Cauley JA, Barrett-Connor E, *et al.* Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J Natl Cancer Inst* 2004; 96:1751-1761.
204. Lonning PE. Additive endocrine therapy for advanced breast cancer - back to the future. *Acta Oncol* 2009; 48:1092-1101.
205. Lonning PE, Taylor PD, Anker G, *et al.* High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 2001; 67:111-116.
206. Robertson JF, Llombart-Cussac A, Rolski J, *et al.* Activity of fulvestrant 500 mg versus anastrozole 1 mg as first-line treatment for advanced breast cancer: results from the FIRST study. *J Clin Oncol* 2009; 27:4530-4535.
207. Kokontis J, Takakura K, Hay N, Liao S. Increased androgen receptor activity and altered c-myc expression in prostate cancer cells after long-term androgen deprivation. *Cancer Res* 1994; 54:1566-1573.
208. Umekita Y, Hiipakka RA, Kokontis JM, Liao S. Human prostate tumor growth in athymic mice: inhibition by androgens and stimulation by finasteride. *Proc Natl Acad Sci U S A* 1996; 93:11802-11807.

209. Chuu CP, Hiipakka RA, Fukuchi J, Kokontis JM, Liao S. Androgen causes growth suppression and reversion of androgen-independent prostate cancer xenografts to an androgen-stimulated phenotype in athymic mice. *Cancer Res* 2005; 65:2082-2084.
210. Abrahamsson PA. Potential benefits of intermittent androgen suppression therapy in the treatment of prostate cancer: a systematic review of the literature. *Eur Urol* 2010; 57:49-59.
211. Maximov PY, Lewis-Wambi JS, Jordan VC. The Paradox of Oestradiol-Induced Breast Cancer Cell Growth and Apoptosis. *Curr Signal Transduct Ther* 2009; 4:88-102.
212. Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. *Cancer Res* 2009; 69:1243-1254.
213. Cunliffe HE, Ringner M, Bilke S, *et al.* The gene expression response of breast cancer to growth regulators: patterns and correlation with tumor expression profiles. *Cancer Res* 2003; 63:7158-7166.
214. DeNardo DG, Kim HT, Hilsenbeck S, Cuba V, Tsimelzon A, Brown PH. Global gene expression analysis of estrogen receptor transcription factor cross talk in breast cancer: identification of estrogen-induced/activator protein-1-dependent genes. *Mol Endocrinol* 2005; 19:362-378.
215. Creighton CJ, Cordero KE, Larios JM, *et al.* Genes regulated by estrogen in breast tumor cells in vitro are similarly regulated in vivo in tumor xenografts and human breast tumors. *Genome Biol* 2006; 7:R28.
216. Lacroix M, Leclercq G. About GATA3, HNF3A, and XBP1, three genes co-expressed with the oestrogen receptor-alpha gene (ESR1) in breast cancer. *Mol Cell Endocrinol* 2004; 219:1-7.
217. Laganiere J, Deblois G, Lefebvre C, Bataille AR, Robert F, Giguere V. From the Cover: Location analysis of estrogen receptor alpha target promoters reveals that FOXA1 defines a domain of the estrogen response. *Proc Natl Acad Sci U S A* 2005; 102:11651-11656.
218. Rae JM, Johnson MD, Scheys JO, Cordero KE, Larios JM, Lippman ME. GREB 1 is a critical regulator of hormone dependent breast cancer growth. *Breast Cancer Res Treat* 2005; 92:141-149.
219. Eeckhoute J, Keeton EK, Lupien M, Krum SA, Carroll JS, Brown M. Positive cross-regulatory loop ties GATA-3 to estrogen receptor alpha expression in breast cancer. *Cancer Res* 2007; 67:6477-6483.
220. Bertucci F, Houlgatte R, Benziane A, *et al.* Gene expression profiling of primary breast carcinomas using arrays of candidate genes. *Hum Mol Genet* 2000; 9:2981-2991.
221. Bertucci F, Nasser V, Granjeaud S, *et al.* Gene expression profiles of poor-prognosis primary breast cancer correlate with survival. *Hum Mol Genet* 2002; 11:863-872.
222. Fujimoto T, Onda M, Nagai H, Nagahata T, Ogawa K, Emi M. Upregulation and overexpression of human X-box binding protein 1 (hXBP-1) gene in primary breast cancers. *Breast Cancer* 2003; 10:301-306.
223. Tozlu S, Girault I, Vacher S, *et al.* Identification of novel genes that co-cluster with estrogen receptor alpha in breast tumor biopsy specimens, using a large-scale real-time reverse transcription-PCR approach. *Endocr Relat Cancer* 2006; 13:1109-1120.
224. Wang DY, Fulthorpe R, Liss SN, Edwards EA. Identification of estrogen-responsive genes by complementary deoxyribonucleic acid microarray and characterization of a novel early estrogen-induced gene: EEIG1. *Mol Endocrinol* 2004; 18:402-411.
225. Barnett DH, Sheng S, Charn TH, *et al.* Estrogen receptor regulation of carbonic anhydrase XII through a distal enhancer in breast cancer. *Cancer Res* 2008; 68:3505-3515.
226. Deschenes J, Bourdeau V, White JH, Mader S. Regulation of GREB1 transcription by estrogen receptor alpha through a multipartite enhancer spread over 20 kb of upstream flanking sequences. *J Biol Chem* 2007; 282:17335-17339.
227. Sun J, Nawaz Z, Slingerland JM. Long-range activation of GREB1 by estrogen receptor via three distal consensus estrogen-responsive elements in breast cancer cells. *Mol Endocrinol* 2007; 21:2651-2662.
228. Gomez BP, Riggins RB, Shajahan AN, *et al.* Human X-box binding protein-1 confers both estrogen independence and antiestrogen resistance in breast cancer cell lines. *Faseb J* 2007; 21:4013-4027.
229. Fang Y, Yan J, Ding L, *et al.* XBP-1 increases ERalpha transcriptional activity through regulation of large-scale chromatin unfolding. *Biochem Biophys Res Commun* 2004; 323:269-274.
230. Gust R, Keilitz R, Schmidt K. Investigations of new lead structures for the design of selective estrogen receptor modulators. *J Med Chem* 2001; 44:1963-1970.
231. Lubczyk V, Bachmann H, Gust R. Investigations on estrogen receptor binding. The estrogenic, antiestrogenic, and cytotoxic properties of C2-alkyl-substituted 1,1-bis(4-hydroxyphenyl)-2-phenylethenes. *J Med Chem* 2002; 45:5358-5364.
232. Lubczyk V, Bachmann H, Gust R. Antiestrogenically active 1,1,2-tris(4-hydroxyphenyl)alkenes without basic side chain: synthesis and biological activity. *J Med Chem* 2003; 46:1484-1491.
233. Cai ZW, Zhang Y, Borzilleri RM, *et al.* Discovery of brivanib alaninate ((S)-((R)-1-(4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[2,1-f][1,2,4] triazin-6-yloxy)propan-2-yl)2-aminopropanoate), a novel prodrug

of dual vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1 kinase inhibitor (BMS-540215). *J Med Chem* 2008; 51:1976-1980.
234. O'Regan RM, Osipo C, Ariazi E, *et al.* Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. *Clin Cancer Res* 2006; 12:2255-2263.
235. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 1988; 48:5183-5187.
236. Dardes RC, Schafer JM, Pearce ST, Osipo C, Chen B, Jordan VC. Regulation of estrogen target genes and growth by selective estrogen-receptor modulators in endometrial cancer cells. *Gynecol Oncol* 2002; 85:498-506.
237. Rosenbaum-Dekel Y, Fuchs A, Yakirevich E, *et al.* Nuclear localization of long-VEGF is associated with hypoxia and tumor angiogenesis. *Biochem Biophys Res Commun* 2005; 332:271-278.
238. Huynh H, Ngo VC, Fargnoli J, *et al.* Brivanib alaninate, a dual inhibitor of vascular endothelial growth factor receptor and fibroblast growth factor receptor tyrosine kinases, induces growth inhibition in mouse models of human hepatocellular carcinoma. *Clin Cancer Res* 2008; 14:6146-6153.
239. Bhide RS, Cai ZW, Zhang YZ, *et al.* Discovery and preclinical studies of (R)-1-(4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5- methylpyrrolo[2,1-f][1,2,4]triazin-6-yloxy)propan- 2-ol (BMS-540215), an in vivo active potent VEGFR-2 inhibitor. *J Med Chem* 2006; 49:2143-2146.
240. Qu Z, Van Ginkel S, Roy AM, *et al.* Vascular endothelial growth factor reduces tamoxifen efficacy and promotes metastatic colonization and desmoplasia in breast tumors. *Cancer Res* 2008; 68:6232-6240.
241. Aesoy R, Sanchez BC, Norum JH, Lewensohn R, Viktorsson K, Linderholm B. An autocrine VEGF/VEGFR2 and p38 signaling loop confers resistance to 4-hydroxytamoxifen in MCF-7 breast cancer cells. *Mol Cancer Res* 2008; 6:1630-1638.
242. Takei H, Lee ES, Jordan VC. In vitro regulation of vascular endothelial growth factor by estrogens and antiestrogens in estrogen-receptor positive breast cancer. *Breast Cancer* 2002; 9:39-42.
243. Burstein HJ, Elias AD, Rugo HS, *et al.* Phase II study of sunitinib malate, an oral multitargeted tyrosine kinase inhibitor, in patients with metastatic breast cancer previously treated with an anthracycline and a taxane. *J Clin Oncol* 2008; 26:1810-1816.
244. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004; 25:581-611.
245. Kranz A, Mattfeldt T, Waltenberger J. Molecular mediators of tumor angiogenesis: enhanced expression and activation of vascular endothelial growth factor receptor KDR in primary breast cancer. *Int J Cancer* 1999; 84:293-298.
246. Scherbakov AM, Lobanova YS, Shatskaya VA, Onopchenko OV, Gershtein ES, Krasil'nikov MA. Activation of mitogenic pathways and sensitization to estrogen-induced apoptosis: two independent characteristics of tamoxifen-resistant breast cancer cells? *Breast Cancer Res Treat* 2006; 100:1-11.
247. Weigand M, Hantel P, Kreienberg R, Waltenberger J. Autocrine vascular endothelial growth factor signalling in breast cancer. Evidence from cell lines and primary breast cancer cultures in vitro. *Angiogenesis* 2005; 8:197-204.
248. Ryden L, Linderholm B, Nielsen NH, Emdin S, Jonsson PE, Landberg G. Tumor specific VEGF-A and VEGFR2/KDR protein are co-expressed in breast cancer. *Breast Cancer Res Treat* 2003; 82:147-154.
249. Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* 2009; 15:232-239.
250. Paez-Ribes M, Allen E, Hudock J, *et al.* Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 2009; 15:220-231.
251. Reynolds AR, Hart IR, Watson AR, *et al.* Stimulation of tumor growth and angiogenesis by low concentrations of RGD-mimetic integrin inhibitors. *Nat Med* 2009; 15:392-400.
252. Ebos JM, Lee CR, Kerbel RS. Tumor and host-mediated pathways of resistance and disease progression in response to antiangiogenic therapy. *Clin Cancer Res* 2009; 15:5020-5025.
253. Jordan VC, Lewis-Wambi JS, Patel RR, Kim H, Ariazi EA. New hypotheses and opportunities in endocrine therapy: amplification of oestrogen-induced apoptosis. *Breast* 2009; 18 Suppl 3:S10-17.
254. Ryden L, Stendahl M, Jonsson H, Emdin S, Bengtsson NO, Landberg G. Tumor-specific VEGF-A and VEGFR2 in postmenopausal breast cancer patients with long-term follow-up. Implication of a link between VEGF pathway and tamoxifen response. *Breast Cancer Res Treat* 2005; 89:135-143.
255. Doisneau-Sixou SF, Sergio CM, Carroll JS, Hui R, Musgrove EA, Sutherland RL. Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. *Endocr Relat Cancer* 2003; 10:179-186.
256. Guo Y, Yang K, Harwalkar J, *et al.* Phosphorylation of cyclin D1 at Thr 286 during S phase leads to its proteasomal degradation and allows efficient DNA synthesis. *Oncogene* 2005; 24:2599-2612.
257. Pines J, Hunter T. Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. *Cell* 1989; 58:833-846.

258. Filardo EJ, Graeber CT, Quinn JA, *et al.* Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression. *Clin Cancer Res* 2006; 12:6359-6366.
259. Kuo WH, Chang LY, Liu DL, *et al.* The interactions between GPR30 and the major biomarkers in infiltrating ductal carcinoma of the breast in an Asian population. *Taiwan J Obstet Gynecol* 2007; 46:135-145.
260. Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, Hathaway HJ. Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. *Annu Rev Physiol* 2008; 70:165-190.
261. Prossnitz ER, Barton M. Signaling, physiological functions and clinical relevance of the G protein-coupled estrogen receptor GPER. *Prostaglandins Other Lipid Mediat* 2009; 89:89-97.
262. Bologa CG, Revankar CM, Young SM, *et al.* Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* 2006; 2:207-212.
263. Pandey DP, Lappano R, Albanito L, Madeo A, Maggiolini M, Picard D. Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF. *EMBO J* 2009; 28:523-532.
264. Ahola TM, Manninen T, Alkio N, Ylikomi T. G protein-coupled receptor 30 is critical for a progestin-induced growth inhibition in MCF-7 breast cancer cells. *Endocrinology* 2002; 143:3376-3384.
265. Pinton P, Giorgi C, Siviero R, Zecchini E, Rizzuto R. Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis. *Oncogene* 2008; 27:6407-6418.
266. Lee AH, Chu GC, Iwakoshi NN, Glimcher LH. XBP-1 is required for biogenesis of cellular secretory machinery of exocrine glands. *Embo J* 2005; 24:4368-4380.
267. Olofsson MH, Havelka AM, Brnjic S, Shoshan MC, Linder S. Charting calcium-regulated apoptosis pathways using chemical biology: role of calmodulin kinase II. *BMC Chem Biol* 2008; 8:2.
268. Lipskaia L, Lompre AM. Alteration in temporal kinetics of Ca2+ signaling and control of growth and proliferation. *Biol Cell* 2004; 96:55-68.
269. Qin C, Nguyen T, Stewart J, Samudio I, Burghardt R, Safe S. Estrogen up-regulation of p53 gene expression in MCF-7 breast cancer cells is mediated by calmodulin kinase IV-dependent activation of a nuclear factor kappaB/CCAAT-binding transcription factor-1 complex. *Mol Endocrinol* 2002; 16:1793-1809.
270. Ariazi EA, Ariazi JL, Cordera F, Jordan VC. Estrogen receptors as therapeutic targets in breast cancer. *Curr Top Med Chem* 2006; 6:181-202.
271. Dennis MK, Burai R, Ramesh C, *et al.* In vivo effects of a GPR30 antagonist. *Nat Chem Biol* 2009; 5:421-427.
272. Chagin AS, Savendahl L. GPR30 estrogen receptor expression in the growth plate declines as puberty progresses. *J Clin Endocrinol Metab* 2007; 92:4873-4877.
273. Martensson UE, Salehi SA, Windahl S, *et al.* Deletion of the G protein-coupled receptor 30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice. *Endocrinology* 2009; 150:687-698.
274. Blasko E, Haskell CA, Leung S, *et al.* Beneficial role of the GPR30 agonist G-1 in an animal model of multiple sclerosis. *J Neuroimmunol* 2009; 214:67-77.
275. Wang C, Dehghani B, Li Y, *et al.* Membrane estrogen receptor regulates experimental autoimmune encephalomyelitis through up-regulation of programmed death 1. *J Immunol* 2009; 182:3294-3303.
276. Liu H, Cheng D, Weichel AK, *et al.* Cooperative effect of gefitinib and fumitremorgin c on cell growth and chemosensitivity in estrogen receptor alpha negative fulvestrant-resistant MCF-7 cells. *Int J Oncol* 2006; 29:1237-1246.
277. Broude EV, Swift ME, Vivo C, *et al.* p21(Waf1/Cip1/Sdi1) mediates retinoblastoma protein degradation. *Oncogene* 2007; 26:6954-6958.
278. Stender JD, Frasor J, Komm B, Chang KC, Kraus WL, Katzenellenbogen BS. Estrogen-regulated gene networks in human breast cancer cells: involvement of E2F1 in the regulation of cell proliferation. *Mol Endocrinol* 2007; 21:2112-2123.
279. Arpino G, Wiechmann L, Osborne CK, Schiff R. Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. *Endocr Rev* 2008; 29:217-233.
280. Huang HJ, Neven P, Drijkoningen M, *et al.* Hormone receptors do not predict the HER2/neu status in all age groups of women with an operable breast cancer. *Ann Oncol* 2005; 16:1755-1761.
281. Schiff R, Massarweh SA, Shou J, *et al.* Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators. *Cancer Chemother Pharmacol* 2005; 56 Suppl 1:10-20.
282. Read LD, Keith D, Jr., Slamon DJ, Katzenellenbogen BS. Hormonal modulation of HER-2/neu protooncogene messenger ribonucleic acid and p185 protein expression in human breast cancer cell lines. *Cancer Res* 1990; 50:3947-3951.

283. Senzer N, Nemunaitis J. A review of contusugene ladenovec (Advexin) p53 therapy. *Curr Opin Mol Ther* 2009; 11:54-61.
284. Patil SD, Rhodes DG, Burgess DJ. DNA-based therapeutics and DNA delivery systems: a comprehensive review. *AAPS J* 2005; 7:E61-77.
285. Lazennec G, Katzenellenbogen BS. Expression of human estrogen receptor using an efficient adenoviral gene delivery system is able to restore hormone-dependent features to estrogen receptor-negative breast carcinoma cells. *Mol Cell Endocrinol* 1999; 149:93-105.
286. DeGregori J, Johnson DG. Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis. *Curr Mol Med* 2006; 6:739-748.
287. Baldini E, Camerini A, Sgambato A, *et al.* Cyclin A and E2F1 overexpression correlate with reduced disease-free survival in node-negative breast cancer patients. *Anticancer Res* 2006; 26:4415-4421.
288. Han S, Park K, Bae BN, *et al.* E2F1 expression is related with the poor survival of lymph node-positive breast cancer patients treated with fluorouracil, doxorubicin and cyclophosphamide. *Breast Cancer Res Treat* 2003; 82:11-16.
289. Vuaroqueaux V, Urban P, Labuhn M, *et al.* Low E2F1 transcript levels are a strong determinant of favorable breast cancer outcome. *Breast Cancer Res* 2007; 9:R33.
290. Zhang SY, Liu SC, Al-Saleem LF, *et al.* E2F-1: a proliferative marker of breast neoplasia. *Cancer Epidemiol Biomarkers Prev* 2000; 9:395-401.
291. Perry RR, Mazetta JA, Levin M, Barranco SC. Glutathione levels and variability in breast tumors and normal tissue. *Cancer* 1993; 72:783-787.
292. Anderson CP, Seeger RC, Satake N, *et al.* Buthionine sulfoximine and myeloablative concentrations of melphalan overcome resistance in a melphalan-resistant neuroblastoma cell line. *J Pediatr Hematol Oncol* 2001; 23:500-505.
293. Bailey HH. L-S,R-buthionine sulfoximine: historical development and clinical issues. *Chem Biol Interact* 1998; 111-112:239-254.
294. Kramer RA, Greene K, Ahmad S, Vistica DT. Chemosensitization of L-phenylalanine mustard by the thiol-modulating agent buthionine sulfoximine. *Cancer Res* 1987; 47:1593-1597.
295. Dusre L, Mimnaugh EG, Myers CE, Sinha BK. Potentiation of doxorubicin cytotoxicity by buthionine sulfoximine in multidrug-resistant human breast tumor cells. *Cancer Res* 1989; 49:511-515.
296. Russo A, DeGraff W, Friedman N, Mitchell JB. Selective modulation of glutathione levels in human normal versus tumor cells and subsequent differential response to chemotherapy drugs. *Cancer Res* 1986; 46:2845-2848.
297. Masamura S, Santner SJ, Heitjan DF, Santen RJ. Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. *J Clin Endocrinol Metab* 1995; 80:2918-2925.
298. Anderson ME. Glutathione: an overview of biosynthesis and modulation. *Chem Biol Interact* 1998; 111-112:1-14.
299. Bailey HH, Ripple G, Tutsch KD, *et al.* Phase I study of continuous-infusion L-S,R-buthionine sulfoximine with intravenous melphalan. *J Natl Cancer Inst* 1997; 89:1789-1796.
300. Rudin CM, Yang Z, Schumaker LM, *et al.* Inhibition of glutathione synthesis reverses Bcl-2-mediated cisplatin resistance. *Cancer Res* 2003; 63:312-318.
301. Rosi A, Grande S, Luciani AM, *et al.* Role of glutathione in apoptosis induced by radiation as determined by 1H MR spectra of cultured tumor cells. *Radiat Res* 2007; 167:268-282.
302. Benderra Z, Trussardi A, Morjani H, Villa AM, Doglia SM, Manfait M. Regulation of cellular glutathione modulates nuclear accumulation of daunorubicin in human MCF7 cells overexpressing multidrug resistance associated protein. *Eur J Cancer* 2000; 36:428-434.
303. Calvert P, Yao KS, Hamilton TC, O'Dwyer PJ. Clinical studies of reversal of drug resistance based on glutathione. *Chem Biol Interact* 1998; 111-112:213-224.
304. Renschler MF. The emerging role of reactive oxygen species in cancer therapy. *Eur J Cancer* 2004; 40:1934-1940.
305. Hamilton TC, Winker MA, Louie KG, *et al.* Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. *Biochem Pharmacol* 1985; 34:2583-2586.
306. Ozols RF, Louie KG, Plowman J, *et al.* Enhanced melphalan cytotoxicity in human ovarian cancer in vitro and in tumor-bearing nude mice by buthionine sulfoximine depletion of glutathione. *Biochem Pharmacol* 1987; 36:147-153.
307. Griffith OW, Meister A. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). *J Biol Chem* 1979; 254:7558-7560.
308. Bailey HH, Mulcahy RT, Tutsch KD, *et al.* Phase I clinical trial of intravenous L-buthionine sulfoximine and melphalan: an attempt at modulation of glutathione. *J Clin Oncol* 1994; 12:194-205.

309. O'Dwyer PJ, Hamilton TC, LaCreta FP, *et al.* Phase I trial of buthionine sulfoximine in combination with melphalan in patients with cancer. *J Clin Oncol* 1996; 14:249-256.
310. Anderson CP, Tsai JM, Meek WE, *et al.* Depletion of glutathione by buthionine sulfoxine is cytotoxic for human neuroblastoma cell lines via apoptosis. *Exp Cell Res* 1999; 246:183-192.
311. Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. *Biomed Pharmacother* 2003; 57:145-155.
312. Hammond CL, Lee TK, Ballatori N. Novel roles for glutathione in gene expression, cell death, and membrane transport of organic solutes. *J Hepatol* 2001; 34:946-954.
313. Schnelldorfer T, Gansauge S, Gansauge F, Schlosser S, Beger HG, Nussler AK. Glutathione depletion causes cell growth inhibition and enhanced apoptosis in pancreatic cancer cells. *Cancer* 2000; 89:1440-1447.
314. Swaby RF, Jordan VC. Low-dose estrogen therapy to reverse acquired antihormonal resistance in the treatment of breast cancer. *Clin Breast Cancer* 2008; 8:124-133.
315. Kane DJ, Sarafian TA, Anton R, *et al.* Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* 1993; 262:1274-1277.
316. Bossy-Wetzel E, Bakiri L, Yaniv M. Induction of apoptosis by the transcription factor c-Jun. *EMBO J* 1997; 16:1695-1709.
317. Voehringer DW. BCL-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity. *Free Radic Biol Med* 1999; 27:945-950.
318. Ellerby LM, Ellerby HM, Park SM, *et al.* Shift of the cellular oxidation-reduction potential in neural cells expressing Bcl-2. *J Neurochem* 1996; 67:1259-1267.
319. Kang YJ, Uthus EO. Suppression of plasma estradiol and progesterone concentrations by buthionine sulfoximine in female rats. *Biochem Pharmacol* 1996; 51:567-570.
320. Morrison JP, Coleman MC, Aunan ES, Walsh SA, Spitz DR, Kregel KC. Aging reduces responsiveness to BSO- and heat stress-induced perturbations of glutathione and antioxidant enzymes. *Am J Physiol Regul Integr Comp Physiol* 2005; 289:R1035-1041.
321. Mirkovic N, Voehringer DW, Story MD, McConkey DJ, McDonnell TJ, Meyn RE. Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is reversed by depleting cellular thiols. *Oncogene* 1997; 15:1461-1470.
322. Sato N, Iwata S, Nakamura K, Hori T, Mori K, Yodoi J. Thiol-mediated redox regulation of apoptosis. Possible roles of cellular thiols other than glutathione in T cell apoptosis. *J Immunol* 1995; 154:3194-3203.
323. Griffith OW, Meister A. Origin and turnover of mitochondrial glutathione. *Proc Natl Acad Sci U S A* 1985; 82:4668-4672.
324. Martensson J, Meister A. Mitochondrial damage in muscle occurs after marked depletion of glutathione and is prevented by giving glutathione monoester. *Proc Natl Acad Sci U S A* 1989; 86:471-475.
325. Baker A, Payne CM, Briehl MM, Powis G. Thioredoxin, a gene found overexpressed in human cancer, inhibits apoptosis in vitro and in vivo. *Cancer Res* 1997; 57:5162-5167.
326. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 2008; 9:47-59.
327. Jacobson MD, Raff MC. Programmed cell death and Bcl-2 protection in very low oxygen. *Nature* 1995; 374:814-816.
328. Vasilevskaya I, O'Dwyer PJ. Role of Jun and Jun kinase in resistance of cancer cells to therapy. *Drug Resist Updat* 2003; 6:147-156.
329. Chen D, Chan R, Waxman S, Jing Y. Buthionine sulfoximine enhancement of arsenic trioxide-induced apoptosis in leukemia and lymphoma cells is mediated via activation of c-Jun NH2-terminal kinase and up-regulation of death receptors. *Cancer Res* 2006; 66:11416-11423.
330. Park J, Kim I, Oh YJ, Lee K, Han PL, Choi EJ. Activation of c-Jun N-terminal kinase antagonizes an anti-apoptotic action of Bcl-2. *J Biol Chem* 1997; 272:16725-16728.
331. Kuroki M, Matsuo Y, Kinugasa T, Matsuoka Y. Three different NCA species, CGM6/CD67, NCA-95, and NCA-90, are comprised in the major 90 to 100-kDa band of granulocyte NCA detectable upon SDS-polyacrylamide gel electrophoresis. *Biochem Biophys Res Commun* 1992; 182:501-506.
332. Scholzel S, Zimmermann W, Schwarzkopf G, Grunert F, Rogaczewski B, Thompson J. Carcinoembryonic antigen family members CEACAM6 and CEACAM7 are differentially expressed in normal tissues and oppositely deregulated in hyperplastic colorectal polyps and early adenomas. *Am J Pathol* 2000; 156:595-605.
333. Blumenthal RD, Leon E, Hansen HJ, Goldenberg DM. Expression patterns of CEACAM5 and CEACAM6 in primary and metastatic cancers. *BMC Cancer* 2007; 7:2.
334. Duxbury MS, Ito H, Benoit E, Ashley SW, Whang EE. CEACAM6 is a determinant of pancreatic adenocarcinoma cellular invasiveness. *Br J Cancer* 2004; 91:1384-1390.

335. Duxbury MS, Matros E, Clancy T, *et al.* CEACAM6 is a novel biomarker in pancreatic adenocarcinoma and PanIN lesions. *Ann Surg* 2005; 241:491-496.
336. Mackay A, Urruticoechea A, Dixon JM, *et al.* Molecular response to aromatase inhibitor treatment in primary breast cancer. *Breast Cancer Res* 2007; 9:R37.
337. Gibson MK, Nemmers LA, Beckman WC, Jr., Davis VL, Curtis SW, Korach KS. The mechanism of ICI 164,384 antiestrogenicity involves rapid loss of estrogen receptor in uterine tissue. *Endocrinology* 1991; 129:2000-2010.
338. Dauvois S, Danielian PS, White R, Parker MG. Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover. *Proc Natl Acad Sci U S A* 1992; 89:4037-4041.
339. Duxbury MS, Ito H, Ashley SW, Whang EE. c-Src-dependent cross-talk between CEACAM6 and alphavbeta3 integrin enhances pancreatic adenocarcinoma cell adhesion to extracellular matrix components. *Biochem Biophys Res Commun* 2004; 317:133-141.
340. Scott DJ, Parkes AT, Ponchel F, Cummings M, Poola I, Speirs V. Changes in expression of steroid receptors, their downstream target genes and their associated co-regulators during the sequential acquisition of tamoxifen resistance in vitro. *Int J Oncol* 2007; 31:557-565.
341. Maraqa L, Cummings M, Peter MB, *et al.* Carcinoembryonic antigen cell adhesion molecule 6 predicts breast cancer recurrence following adjuvant tamoxifen. *Clin Cancer Res* 2008; 14:405-411.
342. Stefanova I, Horejsi V, Ansotegui IJ, Knapp W, Stockinger H. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* 1991; 254:1016-1019.
343. Skubitz KM, Campbell KD, Ahmed K, Skubitz AP. CD66 family members are associated with tyrosine kinase activity in human neutrophils. *J Immunol* 1995; 155:5382-5390.
344. Duxbury MS, Ito H, Ashley SW, Whang EE. CEACAM6 cross-linking induces caveolin-1-dependent, Src-mediated focal adhesion kinase phosphorylation in BxPC3 pancreatic adenocarcinoma cells. *J Biol Chem* 2004; 279:23176-23182.
345. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev* 1999; 13:2905-2927.
346. Bellacosa A, Kumar CC, Di Cristofano A, Testa JR. Activation of AKT kinases in cancer: implications for therapeutic targeting. *Adv Cancer Res* 2005; 94:29-86.
347. Sekharam M, Zhao H, Sun M, *et al.* Insulin-like growth factor 1 receptor enhances invasion and induces resistance to apoptosis of colon cancer cells through the Akt/Bcl-x(L) pathway. *Cancer Res* 2003; 63:7708-7716.
348. Jones RJ, Brunton VG, Frame MC. Adhesion-linked kinases in cancer; emphasis on src, focal adhesion kinase and PI 3-kinase. *Eur J Cancer* 2000; 36:1595-1606.
349. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 2003; 15:740-746.
350. Cheng GZ, Chan J, Wang Q, Zhang W, Sun CD, Wang LH. Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel. *Cancer Res* 2007; 67:1979-1987.
351. Fang H, Tong W, Shi LM, *et al.* Structure-activity relationships for a large diverse set of natural, synthetic, and environmental estrogens. *Chem Res Toxicol* 2001; 14:280-294.
352. Fang H, Tong W, Branham WS, *et al.* Study of 202 natural, synthetic, and environmental chemicals for binding to the androgen receptor. *Chem Res Toxicol* 2003; 16:1338-1358.
353. Zhou ZX, Kemppainen JA, Wilson EM. Identification of three proline-directed phosphorylation sites in the human androgen receptor. *Mol Endocrinol* 1995; 9:605-615.
354. Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM. Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. *Mol Endocrinol* 1995; 9:208-218.
355. Coleman RE, Banks LM, Girgis SI, *et al.* Skeletal effects of exemestane on bone-mineral density, bone biomarkers, and fracture incidence in postmenopausal women with early breast cancer participating in the Intergroup Exemestane Study (IES): a randomised controlled study. *Lancet Oncol* 2007; 8:119-127.
356. Perez EA, Josse RG, Pritchard KI, *et al.* Effect of letrozole versus placebo on bone mineral density in women with primary breast cancer completing 5 or more years of adjuvant tamoxifen: a companion study to NCIC CTG MA.17. *J Clin Oncol* 2006; 24:3629-3635.
357. Howell A, Cuzick J, Baum M, *et al.* Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* 2005; 365:60-62.
358. Yeh S, Hu YC, Wang PH, *et al.* Abnormal mammary gland development and growth retardation in female mice and MCF7 breast cancer cells lacking androgen receptor. *J Exp Med* 2003; 198:1899-1908.
359. Bieche I, Parfait B, Tozlu S, Lidereau R, Vidaud M. Quantitation of androgen receptor gene expression in sporadic breast tumors by real-time RT-PCR: evidence that MYC is an AR-regulated gene. *Carcinogenesis* 2001; 22:1521-1526.

360. Agoff SN, Swanson PE, Linden H, Hawes SE, Lawton TJ. Androgen receptor expression in estrogen receptor-negative breast cancer. Immunohistochemical, clinical, and prognostic associations. *Am J Clin Pathol* 2003; 120:725-731.
361. Wolf DM, Langan-Fahey SM, Parker CJ, McCague R, Jordan VC. Investigation of the mechanism of tamoxifen-stimulated breast tumor growth with nonisomerizable analogues of tamoxifen and metabolites. *J Natl Cancer Inst* 1993; 85:806-812.
362. Gottardis MM, Wagner RJ, Borden EC, Jordan VC. Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro. *Cancer Res* 1989; 49:4765-4769.
363. Tilli MT, Reiter R, Oh AS, *et al.* Overexpression of an N-terminally truncated isoform of the nuclear receptor coactivator amplified in breast cancer 1 leads to altered proliferation of mammary epithelial cells in transgenic mice. *Mol Endocrinol* 2005; 19:644-656.
364. Lahusen T, Fereshteh M, Oh A, Wellstein A, Riegel AT. Epidermal growth factor receptor tyrosine phosphorylation and signaling controlled by a nuclear receptor coactivator, amplified in breast cancer 1. *Cancer Res* 2007; 67:7256-7265.
365. Fereshteh MP, Tilli MT, Kim SE, *et al.* The nuclear receptor coactivator amplified in breast cancer-1 is required for Neu (ErbB2/HER2) activation, signaling, and mammary tumorigenesis in mice. *Cancer Res* 2008; 68:3697-3706.
366. Lippman ME, Bolan G. Oestrogen-responsive human breast cancer in long term tissue culture. *Nature* 1975; 256:592-593.
367. Konecny GE, Pegram MD, Venkatesan N, *et al.* Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. *Cancer Res* 2006; 66:1630-1639.
368. Anzick SL, Kononen J, Walker RL, *et al.* AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* 1997; 277:965-968.
369. Jordan VC, Lewis-Wambi J, Kim H, *et al.* Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients. *Breast* 2007; 16 Suppl 2:S105-113.
370. Bell AW, Deutsch EW, Au CE, *et al.* A HUPO test sample study reveals common problems in mass spectrometry-based proteomics. *Nat Methods* 2009; 6:423-430.
371. Aebersold R. A stress test for mass spectrometry-based proteomics. *Nat Methods* 2009; 6:411-412.
372. Hu Z-Z, Huang H, Wu CH, *et al.* Omics-based molecular target and biomarker identification. *Methods Mol Biol* 2011; 719:547-571.
373. Louet JF, Coste A, Amazit L, *et al.* Oncogenic steroid receptor coactivator-3 is a key regulator of the white adipogenic program. *Proc Natl Acad Sci U S A* 2006; 103:17868-17873.
374. Coste A, Louet JF, Lagouge M, *et al.* The genetic ablation of SRC-3 protects against obesity and improves insulin sensitivity by reducing the acetylation of PGC-1{alpha}. *Proc Natl Acad Sci U S A* 2008; 105:17187-17192.
375. Xu J, Liao L, Ning G, Yoshida-Komiya H, Deng C, O'Malley BW. The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. *Proc Natl Acad Sci USA* 2000; 97:6379-6384.
376. Long W, Yi P, Amazit L, *et al.* SRC-3Delta4 Mediates the Interaction of EGFR with FAK to Promote Cell Migration. *Molecular Cell* 2010; 37:321-332.
377. Deng Q, Huang S. PRDM5 is silenced in human cancers and has growth suppressive activities. *Oncogene* 2004; 23:4903-4910.
378. Brinkmeier ML, Potok MA, Cha KB, *et al.* TCF and Groucho-related genes influence pituitary growth and development. *Mol Endocrinol* 2003; 17:2152-2161.
379. Nagahama Y, Ishimaru M, Osaki M, *et al.* Apoptotic pathway induced by transduction of RUNX3 in the human gastric carcinoma cell line MKN-1. *Cancer Sci* 2008; 99:23-30.
380. Tong DD, Jiang Y, Li M, *et al.* RUNX3 inhibits cell proliferation and induces apoptosis by TGF-beta-dependent and -independent mechanisms in human colon carcinoma cells. *Pathobiology* 2009; 76:163-169.
381. van Agthoven T, Sieuwerts AM, Meijer-van Gelder ME, *et al.* Relevance of breast cancer antiestrogen resistance genes in human breast cancer progression and tamoxifen resistance. *J Clin Oncol* 2009; 27:542-549.
382. Ahn J, Byeon IJ, Byeon CH, Gronenborn AM. Insight into the structural basis of pro- and antiapoptotic p53 modulation by ASPP proteins. *J Biol Chem* 2009; 284:13812-13822.
383. Liu ZJ, Cai Y, Hou L, *et al.* Effect of RNA interference of iASPP on the apoptosis in MCF-7 breast cancer cells. *Cancer Invest* 2008; 26:878-882.
384. Sullivan A, Lu X. ASPP: a new family of oncogenes and tumour suppressor genes. *Br J Cancer* 2007; 96:196-200.
385. Zhao Y, Goto K, Saitoh M, *et al.* Activation function-1 domain of androgen receptor contributes to the interaction between subnuclear splicing factor compartment and nuclear receptor compartment. Identification of the p102

U5 small nuclear ribonucleoprotein particle-binding protein as a coactivator for the receptor. *J Biol Chem* 2002; 277:30031-30039.
386. Lanz RB, Bulynko Y, Malovannaya A, *et al.* Global characterization of transcriptional impact of the SRC-3 coregulator. *Mol Endocrinol* 2010; 24:859-872.
387. Jordan JD, Carey KD, Stork PJ, Iyengar R. Modulation of rap activity by direct interaction of Galpha(o) with Rap1 GTPase-activating protein. *J Biol Chem* 1999; 274:21507-21510.
388. Zhang J, Liu J, Yu C, Lin A. BAD Ser128 is not phosphorylated by c-Jun NH2-terminal kinase for promoting apoptosis. *Cancer Res* 2005; 65:8372-8378.
389. Klumpp S, Krieglstein J. Serine/threonine protein phosphatases in apoptosis. *Curr Opin Pharmacol* 2002; 2:458-462.
390. Fernando RI, Wimalasena J. Estradiol abrogates apoptosis in breast cancer cells through inactivation of BAD: Ras-dependent nongenomic pathways requiring signaling through ERK and Akt. *Mol Biol Cell* 2004; 15:3266-3284.
391. Roux PP, Richards SA, Blenis J. Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity. *Mol Cell Biol* 2003; 23:4796-4804.
392. Bundy DL, McKeithan TW. Diverse effects of BCL3 phosphorylation on its modulation of NF-kappaB p52 homodimer binding to DNA. *J Biol Chem* 1997; 272:33132-33139.
393. Mathas S, Johrens K, Joos S, *et al.* Elevated NF-kappaB p50 complex formation and Bcl-3 expression in classical Hodgkin, anaplastic large-cell, and other peripheral T-cell lymphomas. *Blood* 2005; 106:4287-4293.
394. Castellano L, Giamas G, Jacob J, *et al.* The estrogen receptor-alpha-induced microRNA signature regulates itself and its transcriptional response. *Proc Natl Acad Sci USA* 2009; 106:15732-15737.
395. Zimmer A, Reynolds K. Gene targeting constructs: effects of vector topology on co- expression efficiency of positive and negative selectable marker genes. *Biochem Biophys Res Commun* 1994; 201:943-949.
396. Hoffman BG, Zavaglia B, Beach M, Helgason CD. Expression of Groucho/TLE proteins during pancreas development. *BMC Dev Biol* 2008; 8:81.
397. Liu Y, Dehni G, Purcell KJ, *et al.* Epithelial expression and chromosomal location of human TLE genes: implications for notch signaling and neoplasia. *Genomics* 1996; 31:58-64.
398. Cuevas IC, Slocum AL, Jun P, *et al.* Meningioma transcript profiles reveal deregulated Notch signaling pathway. *Cancer Res* 2005; 65:5070-5075.
399. Hiraike H, Wada-Hiraike O, Nakagawa S, *et al.* Identification of DBC1 as a transcriptional repressor for BRCA1. *Br J Cancer* 2010; 102:1061-1067.
400. Lewis-Wambi JS, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? *Breast Cancer Res* 2009; 11:206.
401. Papa S, Zazzeroni F, Bubici C, *et al.* Gadd45ϐ mediates the NF-əB suppression of JNK signalling by targeting MKK7/JNKK2. *Nat Cell Biol* 2004; 6:146-153.
402. Engelmann A, Speidel D, Bornkamm GW, Deppert W, Stocking C. Gadd45 beta is a pro-survival factor associated with stress-resistant tumors. *Oncogene* 2008; 27:1429-1438.
403. Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell* 2010; 140:900-917.
404. Razandi M, Alton G, Pedram A, Ghonshani S, Webb P, Levin ER. Identification of a structural determinant necessary for the localization and function of estrogen receptor alpha at the plasma membrane. *Mol Cell Biol* 2003; 23:1633-1646.
405. Puthalakath H, O'Reilly LA, Gunn P, *et al.* ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* 2007; 129:1337-1349.
406. Berry DA, Inoue L, Shen Y, *et al.* Modeling the impact of treatment and screening on U.S. breast cancer mortality: a Bayesian approach. *J Natl Cancer Inst Monogr* 2006:30-36.
407. Dowsett M, Cuzick J, Ingle J, *et al.* Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. *J Clin Oncol* 2010; 28:509-518.
408. Osborne CK, Coronado EB, Robinson JP. Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol* 1987; 23:1189-1196.
409. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res* 1987; 47:4355-4360.
410. Welshons WV, Jordan VC. Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *Eur J Cancer Clin Oncol* 1987; 23:1935-1939.
411. Matsuzaki S, Hiratsuka T, Kuwahara R, Katayama T, Tohyama M. Caspase-4 is partially cleaved by calpain via the impairment of Ca2+ homeostasis under the ER stress. *Neurochem Int* 2010; 56:352-356.

412. Chawla-Sarkar M, Lindner DJ, Liu YF, *et al.* Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis* 2003; 8:237-249.
413. Frasor J, Weaver AE, Pradhan M, Mehta K. Synergistic up-regulation of prostaglandin E synthase expression in breast cancer cells by 17beta-estradiol and proinflammatory cytokines. *Endocrinology* 2008; 149:6272-6279.
414. Perkins ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* 2007; 8:49-62.
415. Bhattacharyya S, Borthakur A, Tyagi S, *et al.* B-cell CLL/lymphoma 10 (BCL10) is required for NF-kappaB production by both canonical and noncanonical pathways and for NF-kappaB-inducing kinase (NIK) phosphorylation. *J Biol Chem* 2010; 285:522-530.
416. Matsuda A, Suzuki Y, Honda G, *et al.* Large-scale identification and characterization of human genes that activate NF-kappaB and MAPK signaling pathways. *Oncogene* 2003; 22:3307-3318.
417. Perera PY, Mayadas TN, Takeuchi O, *et al.* CD11b/CD18 acts in concert with CD14 and Toll-like receptor (TLR) 4 to elicit full lipopolysaccharide and taxol-inducible gene expression. *J Immunol* 2001; 166:574-581.
418. Sarnico I, Lanzillotta A, Boroni F, *et al.* NF-kappaB p50/RelA and c-Rel-containing dimers: opposite regulators of neuron vulnerability to ischaemia. *J Neurochem* 2009; 108:475-485.
419. Lakshmanan U, Porter AG. Caspase-4 interacts with TNF receptor-associated factor 6 and mediates lipopolysaccharide-induced NF-kappaB-dependent production of IL-8 and CC chemokine ligand 4 (macrophage-inflammatory protein-1). *J Immunol* 2007; 179:8480-8490.
420. Yang X, Kovalenko D, Nadeau RJ, *et al.* Sef interacts with TAK1 and mediates JNK activation and apoptosis. *J Biol Chem* 2004; 279:38099-38102.
421. Eby MT, Jasmin A, Kumar A, Sharma K, Chaudhary PM. TAJ, a novel member of the tumor necrosis factor receptor family, activates the c-Jun N-terminal kinase pathway and mediates caspase-independent cell death. *J Biol Chem* 2000; 275:15336-15342.
422. Serini S, Piccioni E, Merendino N, Calviello G. Dietary polyunsaturated fatty acids as inducers of apoptosis: implications for cancer. *Apoptosis* 2009; 14:135-152.
423. Rotman EI, Brostrom MA, Brostrom CO. Inhibition of protein synthesis in intact mammalian cells by arachidonic acid. *Biochem J* 1992; 282 (Pt 2):487-494.
424. Martinez-Orozco R, Navarro-Tito N, Soto-Guzman A, Castro-Sanchez L, Perez Salazar E. Arachidonic acid promotes epithelial-to-mesenchymal-like transition in mammary epithelial cells MCF10A. *Eur J Cell Biol* 2010; 89:476-488.
425. Kurokawa H, Nishio K, Fukumoto H, Tomonari A, Suzuki T, Saijo N. Alteration of caspase-3 (CPP32/Yama/apopain) in wild-type MCF-7, breast cancer cells. *Oncol Rep* 1999; 6:33-37.
426. Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Res* 1997; 57:3071-3078.
427. Chisamore MJ, Ahmed Y, Bentrem DJ, Jordan VC, Tonetti DA. Novel antitumor effect of estradiol in athymic mice injected with a T47D breast cancer cell line overexpressing protein kinase Calpha. *Clin Cancer Res* 2001; 7:3156-3165.
428. Munster PN, Carpenter JT. Estradiol in breast cancer treatment: reviving the past. *JAMA* 2009; 302:797-798.
429. Jordan VC, Ford LG. Paradoxical clinical effect of estrogen on breast cancer risk: a "new" biology of estrogen-induced apoptosis. *Cancer Prev Res (Phila)* 2011; 4:633-637.

APPENDIX

1. Ariazi, E., Lewis-Wambi, J.S., Gill, S.D., Pyle, J.R., Ariazi, J.L., Kim, H.R., Sharma, C.G.N., Cordera, F., Shupp, H.A., Li, T. and Jordan, V.C. (2006). Emerging principles for the development of resistance to antihormonal therapy: Implications for the clinical utility of fulvestrant. Journal of Steroid Biochemistry and Molecular Biology 102:128-138.
2. Ariazi, E.A., Kraus, R.J., Farrell, M.L., Jordan, V.C. and Mertz, J.E. (2006). Estrogen-related receptor Ǔ1's transcriptional activities are regulated in part via the ErbB2 (HER2) signaling pathway. Molecular Cancer Research 5:71-85.
3. Pannier, A.K., Ariazi, E.A., Bellis, A.D., Bengali, Z., Jordan, V.C. and Shea, L.D. (2007). Bioluminesence imaging for assessment and normalization in trasfected cell arrays. Biotechnology Bioengineering 98:486-497.
4. Ariazi, E.A., Leit«o, A., Oprea, T.I., Chen, B., Louis, T., Bertucci, A.M., Sharma, C.G.N., Gill, S.D., Kim, H.R., Shupp, H.A., Pyle, J.R., Madrack, A., Donato, A.L., Cheng, D., Paige, J.R. and Jordan, V,C. (2007). Exemestaneôs 17-hydroxylated metabolite exerts biological effects as an androgen. Molecular Cancer Therapeutics 6:2817-2827.
5. Lu, M., Strohecker, A., Chen, F., Kwan, T., Bosman, J., Jordan, V.C. and Cryns, V.L. (2008). Aspirin sensitizes cancer cells to TRAIL-induced apoptosis by reducing survivin levels. Clinical Cancer Research 14:3168-3176.
6. Lewis-Wambi, J., Cunliffe, H.E., Kim, H.R., Willis, A.L. and Jordan, V.C. (2008). Overexpression of CEACAM6 promotes migration and invasion of estrogen deprived breast cancer cells. European Journal of Cancer 44:1770-1779.
7. Lewis-Wambi, J.S., Kim, H.R., Wambi, C., Patel, R., Pyle, J.R., Klein-Szanto, A.J. and Jordan, V.C. (2008). Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. Breast Cancer Research 10:R104.
8. Lewis-Wambi, J.S., Swaby, R.R., Kim, H. and Jordan, V.C. (2009). Potential of L-buthionine sulfoximine to enhance the apoptotic action of estradiol to reverse acquired antihormonal resistance in metastatic breast cancer. J Steroid Biochemistry and Molecular Biology 114:33-39.
9. Yu, J.J., Robb, V.A., Morrison, T.A., Ariazi, E.A., Karbowniczek, M., Astrinidis, A., Wang, C., Hernandez-Cuebas, L., Seeholzer, L.F., Nicolas, E., Hensley, H., Jordan, V.C., Walker, C.L. and Henske, E.P. (2009). Estrogen promotes the survival and pulmonary metastasis of tuberin-null cells. Proceedings of the National Academy of Sciences U S A 106:2635-2640.
10. Morrow, M., Chatteron, R.T. Jr, Rademaker, A.W., Hou, N., Jordan, V.C., Hendrick, R.E. and Khan, S.A. (2009). A prospective study of variability in mammographic density during the menstrual cycle. Breast Cancer Research Treatment 121:565-574.
11. Peng, J. and Jordan, V.C. (2010). Expression of estrogen receptor alpha with a Tet-off adenoviral system induces G0/G1 cell cycle arrest in SKBr3 breast cancer cells. International Journal of Oncology 36:451-458.

12. Ariazi, E.A., Brailoiu, E., Yerrum, S., Shupp, H.A., Slifker, M.J., Cunliffe, H.E., Black, M.A., Donato, A.L., Arterburn, J.B., Oprea, T.I., Prossnitz, E.R., Dun, N.J. and Jordan, V.C. (2010). The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. Cancer Research 70:1184-1194. (Selected for Faculty of 1000 Medicine, identified as an important article published in Medicine for its scientific merit and positive contribution to the medical literature).

13. Patel, R.R., Sengupta, S., Kim, H.R., Klein-Szanto, A.J., Pyle, J.R., Zhu, F., Li, T., Ross, E.A., Oseni, S., Fargnoli, J. and Jordan, V.C. (2010). Experimental treatment of estrogen receptor (ER) positive breast cancer with tamoxifen and brivanib alaninate, a VEGFR-2/FGFR-1 kinase inhibitor: a potential clinical application of angiogenesis inhibitors. European Journal of Cancer 46:1537-1553.

14. Maximov, P.Y., Myers, C.B., Curpan, R.F., Lewis-Wambi, J.S. and Jordan, V.C. (2010). Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. Journal of Medicinal Chemistry 53:3273-3283.

15. Sengupta, S.S., Sharma, C.G.N. and Jordan, V.C. (2010). Estrogen regulation of X-Box binding protein-1 and its role in estrogen induced crowth of breast and endometrial cancer cells. Hormone Molecular Biology and Clinical Investigation 2:235-243.

16. Balaburski, G., Dardes, R.C., Johnson, M., Haddad, B., Zhu, F., Ross, E.A., Sengupta, S., Klein-Szanto, A., Liu, H., Kim, H. and Jordan, V.C. (2010). Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: A unifying concept in anti-hormone resistance. International Journal of Oncology 37:387-398.

17. Vogel, V.G., Costantino, J.P., Wickerham, D.L., Cronin, W.M., Cecchini, R.S., Atkins, J.N., Bevers, T.B., Fehrenbacher, L., Pajon, E.R., Wade, J.L., Robidoux, A., Margolese, R.G., James, J., Runowicz, C.D., Ganz, P.A., Reis, S.E., McCaskill-Stevens, W., Ford, L.G., Jordan, V.C. and Wolmark, N. (2010). Update of the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing Breast Cancer. Cancer Prevention Research 3:696-706.

18. Maximov, P., Sengupta, S., Lewis-Wambi, J.S., Kim, H.R., Curpan, R.F. and Jordan, V.C. (2011). The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: a hypothesis. Hormone Molecular Biology and Clinical Investigation 5:27-34.

19. Yang, C.Z., Yaniger, S.I., Jordan, V.C., Klein, D.J. and Bittner, G.D. (2011). Most plastic products release estrogenic chemicals: a potential health problem that can be solved. Environmental Health Perspectives 119:989-996.

20. Hu, Z., Kagan, B.L., Ariazi, E., Rosenthal, D.S., Zhang, L., Li, J.V., Huang, H., Wu, C., Jordan, V.C., Riegel, A.T. and Wellstein, A. (2011). Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. PLoS ONE Epub 6:e20410.

21. Lewis-Wambi, J.S., Kim, H., Curpan, R., Grigg, R., Sarker, M.A. and Jordan, V.C. (2011). The selective estrogen receptor modulator, bazedoxifene, inhibits hormone-

independent breast cancer cell growth and downregulates estrogen receptor Ŭand cyclin D1. Molecular Pharmacology 80:610-620.

22. Ariazi, E.A., Cunliffe, H.E., Lewis-Wambi, J.S., Slifker, M.J., Willis, A.L., Ramos, P., Tapia, C., Kim, H.R., Yerrum, S., Sharma, C.G.N., Nicolas, E., Balagurunathan, Y., Ross, E.A. and Jordan, V.C. (2011). Estrogen-induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proceedings of the National Academy of Sciences U.S.A. 108:18879-18886.

23. He, H.H., Meyer, C.A., Chen, M.W., Jordan, V.C., Brown, M. and Liu, X.S. (2012). Differential DNase I hypersensitivity reveals factor-dependent chromatic dynamics. Genome Research 22:1015-25.

24. Fan, P., McDaniel, R.E., Kim, H.R., Clagett, D., Haddad, B., Jordan, V.C. (2012). Modulating Therapeutic Effects of the c-Src Inhibitor via Estrogen Receptor and HER2 in Breast Cancer Cell Lines. European Journal of Cancer 48:3488-98.

25. Gass, M.L.S., Manson, J.E., Cosman, F., Grodstein, F., Jordan, V.C., Karas, R.H., Kaunitz, A.M., Maki, P.M., Schmidt, P.J., Shifren, J.L., Stuenkel, C.A. and Utian, W.H. (2012). Position statement: the 2012 hormone therapy position statement of The North American Menopause Society. Menopause: The Journal of The North American Menopause Society 19:257-271.

26. Korch C., Spillman, M.A., Jackson, T.A., Jacobsen, B.M., Murphy S.K., Lessey, B.A., Jordan, V.C. and Bradford, A.P. (2012). DNA profiling analysis of endometrial and ovarian cell lines reveals misidentification, redundancy and contamination. Gynecologic Oncology 127:241-248.

27. Sengupta, S. S., Obiorah, I. O., Maximov, P. Y., Curpan, R., and Jordan, V. C. (2013) Molecular mechanism of action of bisphenol and bisphenol-A mediated by estrogen receptor alpha in growth and apoptosis of breast cancer cells. British Journal of Pharmacology 169:167-78.

28. Segala G., de Medina P., Iuliano L., Zerbinati C., Paillasse M. R., Noguer E., Dalenc F., Payre B., Jordan V. C., Record M., Silvente-Poirot S., Poirot M (2013).5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of tamoxifen in breast cancer cells. Biochemical Pharmacology 88:175-189.

29. Fan, P., Griffith, O.L., Agboke, F., Anur, P., Zou, X., McDaniel, R.E., Creswell, K., Kim, S.H., Katzenellenbogen, J.A., Gray, J.W. and Jordan, V.C. (2013). c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. Cancer Research 73:4510-4520.

30. Baniwal, S.K., Chimge, N.O., Jordan, V.C., Tripathy, D., Frenkel, B. (2013). Prolactin-induced protein (PIP) regulates proliferation of luminal A type breast cancer cells in an estrogen independent maner. PLoS One. 8: e62361.

31. Fan, P., Agboke, F.A., McDaniel, R.E., Sweeney, E.E., Zou, X., Creswell, K., Jordan, V.C. (2014). Inhibition of c-Src blocks estrogen-induced apoptosis and restores

estrogen-stimulated growth in long-term estrogen-deprived breast cancer cells. European Journal of Cancer. 50:457-468

32. Sengupta, S., Biarnes, M.C., and Jordan, V.C. (2014). Cyclin dependent kinase-9 mediated transcriptional deregulation of cMYC as a critical determinant of endocrine therapy resistance in breast cancers. Breast Cancer Research and Treatment. 143:113-124.

33. Sweeney, E.E., Fan, P., Jordan, V.C. (2014) Mechanisms underlying differential response to estrogen-induced apoptosis in long-term estrogen-deprived breast cancer cells. International Journal of Oncology. 44:1529-1537.

34. Obiorah, I.O., Sengupta, S., Fan, P., Jordan, V.C. (2014) Delayed triggering of oestrogen induced apoptosis that contrasts with rapid paclitaxil induced breast cancer cell death. British Journal of Cancer. 110:1488-1496.

35. Obiorah, I.O., Sengupta, S., Curpan, R., Jordan, V.C (2014) Defining the conformation of the estrogen receptor complex that controls estrogen induced apoptosis in breast cancer. Molecular Pharmacology. *In press*.

36. Obiorah, I.O., Jordan, V.C. (2014) Differences in the rate of oestrogen induced apoptosis by oestradiol and the triphenylethylene bisphenol. British Journal of Pharmacology. *In press*.

37. Maximov, P.Y. Fernandes, D.J., McDaniel, R.E., Myers, C., Curpan, R., Jordan, V.C. (2014) Influence of the length and positioning of the antiestrogenic side chain of endoxifen and 4-hydroxytamoxifen on gene activation and growth of estrogen receptor positive cancer cells. Journal of Medicinal Chemistry. *In press*.

38. Obiorah, I.O., Fan, P., Jordan, V.C. Breast cancer cell apoptosis with phytoestrogens is dependent on an estrogen deprived state. Cancer Prevention Research. *In press.*

39. Maximov, P.Y., McDaniel, R.E., Fernandes, D.J., Bhatta, P., Korostyshevskiy, V, Curpan, R., Jordan, V.C. Pharmacological relevance of endoxifen in a laboratory simulation of breast cancer in postmenopausal patients. Journal of the National Cancer Institute. *In Press*.

40. Maximov, P.Y., McDaniel, R.E., Fernandes, D.J., Korostyshevskiy, V., Bhatta, P., Jordan, V.C. In vitro simulation of tamoxifen treatment in premenopausal breast cancer patients with different CYP2D6 genotypes. British Journal of Pharmacology. *In press.*

41. Jordan, V.C. (2013). Estrogen Action, SERMs and Womenôs Health. Imperial College Press, London.

42. Maximov, P.Y., McDaniel, R. E., and Jordan, V.C. (2013). Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basal, Switzerland.

43. Jordan, V.C. and Brodie, A.M.H. (2006). Development and evolution of targeted endocrine therapies for the treatment of breast cancer. (Charles F. Kettering Prize, General Motors Cancer Research Foundation) Steroids 72:7-25.

44. Jordan, V.C. (2007). Successful translation research with selective oestrogen receptor modulators to treat and prevent breast cancer. Geburtshilfe und Frauenheilkunde (German Journal of Obstetrics and Gynecology) 67:443-450.

45. Jordan, V.C. (2008). The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer--survival or death? Journal of Clinical Oncology: 26:3073-3082.

46. Jordan, V.C. (2009). A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention . Cancer Research: 69:1243-1254.

47. Jordan, V.C., Obiorah, I., Fan, P., Kim, H.R., Ariazi, E., Cunliffe, H. and Brauch, H. (2011). Evolution of long-term adjuvant antihormone therapy: Consequences and Opportunities. The St. Gallen Prize Lecture. Breast 20(Suppl 3):S1-S11.

48. Obiorah, I. and Jordan, V.C. (2013) The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. North American Menopause Society/Pfizer-Wulf H. Utian Endowed Lecture. Menopause 20:372-382.

49. Poirot, M. (2011). Four decades of discovery in breast cancer research and treatment ï an interview with V. Craig Jordan. International Journal of Developmental Biology 55:703-712.

50. Gupta, S. (2011). Profile of V. Craig Jordan. Profile of a recently elected member of the National Academy of Sciences to accompany the memberôs Inaugural Article. Proceedings of the National Academy of Sciences U.S.A. 108:18876-18878.

51. Jordan, V.C. (2014) Tamoxifen the first targeted long term adjuvant therapy for breast cancer. Endocrine Related Cancer. 21:R235-246.

52. Jordan, V.C. (2014) Profile - Who do you think you are? Endocrine Related Cancer. 21:P13-17.

53. Jordan, V.C. (2014) Tamoxifen as the first successful targeted therapy in cancer: the gift that kept on giving. Breast Cancer Management. *In press*.

54. Jordan, V.C. (2014) Proven value of translational research with appropriate animal models to advance breast cancer treatment and save lives: the tamoxifen tale. British Jounal of Clinical Pharmacology. *In press.*

55. Swaby, R.F., Sharma, C.G.N., Jordan, V.C. (2007). SERMs for the treatment and prevention of breast cancer. In: Reviews in Endocrine and Metabolic Disorders. (A. Manni, Ed), Springer, Norwell, MA, 8:229-239.

56. Ariazi, E.A. and Jordan, V.C. (2008). Estrogen Receptors as Therapeutic Targets in Breast Cancer. In: Methods and Principles of Medicinal Chemistry (E. Ottow and H. Weinmann (eds)) Wiley-VCH Verlag GmbH & Co. KgaA, Weinheim, Berlin, Germany, pp. 127-199.

57. Buzdar, A.U., Dawood, S., Harvey, H.A., and Jordan, V.C. (2010). Antiestrogens,Progestinsand Aromatase Inhibitors In: Cancer Medicine 8th Edition. Kufe,Bast,Hait,Hong,Pollock, Weichselbaum, Holland ,and Frei(Eds) B.C. Decker Inc. Hamilton, London, pp 737-749.

58. Fan, P., and Jordan, V.C. (2014) Antiestrogens and Selective Estrogen Receptor Modulators. In: Molecular Oncology (Eds: Gelmann, Sawyers, and Rauscher). Cambridge University Press, pp.884-892. Cambridge, UK.

59. Jordan, V.C. (2007). Chemoprevention of Breast Cancer with Selective Oestrogen Receptor Modulators. Nature Reviews Cancer 7:46-53.

60. Jordan, V.C. (2007). (Commentary) SERMS: meeting the promise of multifunctional medicines. Journal of the National Cancer Institute 99:350-356.

61. Jordan, V.C. (2007). New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer. Steroids 72:829-842.

62. Jordan, V.C. and OôMalley, B.W. (2007). Biology of Neoplasia: Selective estrogen receptor modulators and antihormonal resistance in breast cancer. Journal of Clinical Oncology 25:5815-5824.

63. Jordan, V.C. (2008). Tamoxifen: catalyst for the change to targeted therapy. European Journal of Cancer 44:30-38.

64. Swaby, R.F. and Jordan, V.C. (2008). Low dose estrogen therapy to reverse acquired antihormonal resistance in the treatment of breast cancer. Clinical Breast Cancer 8:125-133.

65. Oseni, T., Patel, R., Pyle, J.R. and Jordan, V.C. (2008). Selective estrogen receptor modulators and phytoestrogens. Planta Medica 74:1656-1665.

66. Benson, J., Jatoi, I., Keisch, M., Esteva, F., Makris, A. and Jordan, V.C. (2009). Early Breast Cancer. The Lancet 373:1463-1479.

67. Maximov, P.Y., Lewis-Wambi, J.S. and Jordan, V.C. (2009). The Paradox of Oestradiol-Induced Breast Cancer Cell Growth and Apoptosis. Current Signal Transduction Therapy 4:88-102.

68. Lewis-Wambi, J.S. and Jordan, V.C. (2009). Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? Breast Cancer Research 11:206.

69. Peng, J., Sengupta, S. and Jordan, V.C. (2009). Potential of selective estrogen receptor modulators as treatments and preventives of breast cancer. Anti-Cancer Agents in Medicinal Chemistry 9:481-499.

70. Brauch, H. and Jordan, V.C. (2009). Targeting of tamoxifen to enhance antitumour action for the treatment and prevention of breast cancer: the ópersonalisedô approach? European Journal of Cancer 45:2274-2283.

71. Patel, R. and Jordan, V.C. (2009). Chemoprevention of breast cancer with selective estrogen receptor modulators. Current Medical Literature Breast Cancer 21:81-88.

72. Obiorah, I.E. and Jordan, V.C. (2011). Progress in endocrine approaches to the treatment and prevention of breast cancer. Maturitas 70:315-321.

73. Sweeney, E.E., McDaniel, R.E., Maximov, P.Y., Fan, P. and Jordan, V.C. (2012). Models and Mechanisms of Acquired Antihormone Resistance in Breast Cancer: Significant Clinical Progress Despite Limitations. Hormone Molecular Biology and Clinical Investigation 9:143-163.

74. Maximov, P.Y., Lee, T.M., and Jordan, V.C. (2013). The discovery and development of Selective Estrogen Receptor Modulators (SERMs) for clinical practice. Current Clinical Pharmacology 8:135-155

75. McDaniel, R.E., Maximov, P.Y., and Jordan, V.C. (2013). Estrogen-mediated mechanisms to control the growth and apoptosis of breast cancer cells: a translational research success story. In: Vitamins and Hormones vol. 93 (ed. Gerald Litwack) Elsevier pp. 1-49.

76. Jordan, V.C., Agboke, F.A., McDaniel, R.E., and Maximov, P.Y. A synthesis of thirty years of progress in selective steroid modulation. Steroids: Special Issue. *In press.*

77. Obiorah, I.O., and Jordan, V.C. Selective estrogen induced apoptosis. Steroids: Special Issue. *In press*.

78. Fan, P., and Jordan, V.C. Acquired resistance to selective estrogen receptor modulators (SERMs) in clinical practice (tamoxifen and raloxifen) by selection pressure in breast cancer cell populations. Steroids: Special Issue. *In press*.

79. Jordan, V.C. and Gelber, R.D. (2007). Problems with progesterone receptor in practice? Journal of Clinical Oncology 25:1957-1959.

80. Jordan, V.C. (2007). Estrogen Receptors in BRCA1-Mutant Breast Cancer: Now You See Them, Now You Donât. Journal of the National Cancer Institute 99:1655-1657.

81. Jordan, V.C. (2008). The rise of raloxifene and the fall of invasive breast cancer. Journal of the National Cancer Institute 100:831-833.

82. Jordan, V.C. and Ford, L.S. (2011). Paradoxical Clinical Effect of Estrogen on Breast Cancer Risk: A ñNewò Biology of Estrogen-Induced Apoptosis. Cancer Prevention Research 4:633-637.

83. Jordan, V.C. (2011). Decades of Discovery: The Selective Estrogen Receptor Modulator (SERM) Story: The St. Gallen Prize. *References en Gynecologie Obstetrique* 14:385-392.

84. Jordan, V.C. (2013). Targeted Therapies: Any Surprises from Selective Oestrogen Receptor Modulators? Nature Reviews Clinical Oncology 10:432-4.

85. Jordan, V.C. (2014). A(nother) scientific strategy to prevent breast cancer in post-menopausal women by enhancing estrogen-induced apoptosis? Menopause *ePub ahead of print*.

86. Jordan, V.C. (2014). Timing is key to avoid the bad and enhance the good of soy supplements. Journal of the National Cancer Institite. *In Press.*

87. Patel, R.R., Sharma, C.G. and Jordan, V.C. (2007). Optimising the antihormonal treatment and prevention of breast cancer. Breast Cancer 14:113-122.

88. Patel, R.R., Pyle, J.R. and Jordan, V.C. (2007). The Current Clinical Indications for Fulvestrant. Clinical Updates in Cancer 2:4-6.

89. Jordan, V.C., Lewis-Wambi, J., Kim, H., Cunliffe, H., Ariazi, E., Sharma, C.G.N., Shupp, H.A. and Swaby, R. (2007). Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients. Breast 16:S105-S113.

90. Jordan, V.C., Patel, R., Lewis-Wambi, J.S. and Swaby, R.F. (2008). By looking back we can see the way forward: enhancing the gains achieved with antihormone therapy. Breast Cancer Research 10 Suppl 4:S16.

91. Jordan, V.C., Lewis-Wambi, J.S., Patel, R.R., Kim, H. and Ariazi, E.A. (2009). New hypotheses and opportunities in endocrine therapy: amplication of oestrogen-induced apoptosis. Breast 18(S3):S10-S17.

92. Maximov, P.Y. and Jordan, V.C. (2012). Estrogen-Induced Apoptosis in Breast Cancer Cells: Translation to Clinical Relevance. In: Targeting New Pathways and Cell Death in Breast Cancer (Rebecca Aft (Ed)). InTech, Rijeka, Croatia, pp. 3-22.

93. Leick, M. B. and Jordan, V. C. (2013) Evolution of Estrogen Action in Breast Cancer: From Culprit to Killer. In: Estrogen Prevention for Breast Cancer (Ed: Suba, Z.) Nova Science Publishers, pp. 127-150.

94. McDaniel, R. and Jordan, V.C. (2013) Endocrine Prevention of Breast Cancer. In: Early Breast Cancer: From Screening to Multidisciplinary Management (Ed: Benson, J.). Informa Healthcare, London, England, pp. 39-49.

95. Peng, J., Sengupta, S., and Jordan V.C. (2013) Selective Estrogen Receptor Modulators as Treatments and Preventives of Breast Cancer. In: Advances in Anticancer Agents in Medicinal Chemistry (Ed: Prudhomme, M.). Bentham Science Publishers, Oak Park, IL, pp. 344-395.

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Emerging principles for the development of resistance to antihormonal therapy: Implications for the clinical utility of fulvestrant☆

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Abstract

We seek to evaluate the clinical consequences of resistance to antihormonal therapy by studying analogous animal xenograft models. Two approaches were taken: (1) MCF-7 tumors were serially transplanted into selective estrogen receptor modulator (SERM)-treated immunocompromised mice to mimic 5 years of SERM treatment. The studies *in vivo* were designed to replicate the development of acquired resistance to SERMs over years of clinical exposure. (2) MCF-7 cells were cultured long-term under SERM-treated or estrogen withdrawn conditions (to mimic aromatase inhibitors), and then injected into mice to generate endocrine-resistant xenografts. These tumor models have allowed us to define Phase I and Phase II antihormonal resistance according to their responses to E_2 and fulvestrant. Phase I SERM-resistant tumors were growth stimulated in response to estradiol (E_2), but paradoxically, Phase II SERM and estrogen withdrawn-resistant tumors were growth inhibited by E_2. Fulvestrant did not support growth of Phases I and II SERM-resistant tumors, but did allow growth of Phase II estrogen withdrawn-resistant tumors. Importantly, fulvestrant plus E_2 in Phase II antihormone-resistant tumors reversed the E_2-induced inhibition and instead resulted in growth stimulation. These data have important clinical implications. Based on these and prior laboratory findings, we propose a clinical strategy for optimal third-line therapy: patients who have responded to and then failed at least two antihormonal treatments may respond favorably to short-term low-dose estrogen due to E_2-induced apoptosis, followed by treatment with fulvestrant plus an aromatase inhibitor to maintain low tumor burden and avoid a negative interaction between physiologic E_2 and fulvestrant.
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Keywords: Breast cancer; Estradiol; Tamoxifen; Raloxifene; Fulvestrant

1. Introduction

The target for endocrine therapy is the estrogen receptor (ER), and the translation of laboratory findings on the control of estrogen-regulated tumor growth has established the current treatment strategies which have been validated in clinical trials [1–7]. Tamoxifen (TAM), the prototype selective estrogen receptor modulator (SERM), is a current standard adjuvant treatment used for 5 years in all stages of ER-positive breast cancer [8–11]. However, aromatase inhibitors (AIs) are becoming the leading choice for antihormonal treatment of ER-positive breast cancer in postmenopausal patients. Still, there is a need to study the long-term therapeutic consequences of TAM because of its use in premenopausal ER-positive breast cancer [5,9], and as a chemopreventive agent to reduce the risk of breast cancer in high-risk women [12]. There is also considerable interest in the use of raloxifene (RAL), a related SERM, as a chemopreventive agent [13,14], since it has recently been shown in the STAR trial (study of TAM and RAL) to exhibit equivalent efficacy as TAM in reducing the risk of breast cancer [15]. Additionally, RAL

Abbreviations: AI, aromatase inhibitor; ER, estrogen receptor; E_2, 17β-estradiol; FUL, fulvestrant; RAL, raloxifene; SERM, selective estrogen receptor modulator

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is used for the treatment and prevention of osteoporosis in postmenopausal women [16], has been noted to have endometrial safety [13,14,16], and reduces the risk of cardiovascular disease [17–19]. Since RAL may have to be given indefinitely to prevent osteoporosis, RAL-exposed breast cancer will almost certainly occur. Overall, there is a large and growing population of women at risk for developing endocrine therapy-resistant breast cancer.

It is important to emphasize that the successful treatment of patients with one endocrine agent and then failure, leads to exhaustive endocrine therapy with the succession of agents, each with decreasing efficacy. The failure of TAM as a first-line therapy forms the basis of the use of AIs or fulvestrant (ICI 182,780, Faslodex®) as second-line therapies for the treatment of breast cancer.

In postmenopausal women, the aromatase enzyme converts androgens to estrogens in peripheral tissues such as adipose tissue and in the breast cancer tissue itself [20,21]. AIs block activity of this enzyme and fall into two classes, steroidal and non-steroidal [22–24]. Exemestane (Aromasin®) [25,26], a steroidal AI, irreversibly binds aromatase at the catalytic site and inactivates the enzyme. Anastrozole (Arimidex®) [27,28] and letrozole (Femara®) [29,30], non-steroidal AIs, bind aromatase at a different site, a heme group, to reversibly inhibit the enzyme. AIs have been evaluated in advanced breast cancer and in the adjuvant setting [25–30]. In the largest adjuvant trial, the ATAC trial (Arimidex, TAM, alone or in combination), patients in the anastrozole arm versus the TAM arm showed significantly longer disease-free survival, reduced contralateral breast cancer, and reduced distant metastases [31,32]. Indeed, AIs are now recommended and may replace TAM as the standard first-line antihormonal adjuvant therapy in postmenopausal ER-positive breast cancer patients. Further, due to the success of this and of other trials evaluating AIs for extended adjuvant therapy, AIs are also indicated after 5 years [33] and even 2 years of TAM [25,28].

FUL is an analogue of E_2 and the first in a new class of drugs that are complete antiestrogens, that is, they display no agonist activity via AF-1 or AF-2 of the ER [34,35]. FUL also leads to potent downregulation of ER protein expression because FUL binding to ER induces an abnormal conformation that results in accelerated ubiquitylation and shuttling of the ER to the proteasome for degradation [34,36]. Two large Phase III clinical trials have been conducted to evaluate FUL versus the AI anastrazole in postmenopausal advanced ER-positive breast cancer patients who have failed TAM. Both of these trials showed that FUL was equally effective as anastrazole in terms of time to progression and objective response rates [37,38]. Hence, FUL has been approved as a second-line therapy. FUL is also currently being evaluated in combination with AIs [35].

Over the past two decades, we have developed unique MCF-7 breast cancer xenograft models of long-term SERM (TAM and RAL) treatment and models of long-term estrogen withdrawal that could reasonably mimic resistance to AIs. These tumor models were developed *in vivo* and *in vitro*. The *in vivo* tumor models were designed to mimic the selection process needed over years to develop acquired resistance in the clinic by serially implanting MCF-7 tumors into SERM-treated and ovariectomized immunodeficient mice also over a period of years [39–49]. The *in vitro* tumor models were developed by culturing MCF-7 cells in estrogen-free conditions, with or without SERM treatment if appropriate, for over 1 year to develop antihormone resistance, and then injecting these cells into ovariectomized athymic mice treated with the SERM, if appropriate, and allowing tumors to grow [50–52]. We now have in hand a panel of breast cancer xenograft and tissue culture models that have allowed us to define the evolution of resistance to antihormonal therapy into at least two phases, each of which exhibits distinct growth responses to E_2 and FUL. We found that the growth of Phase I SERM-resistant tumors is stimulated by E_2, while growth of Phase II SERM or estrogen withdrawn-resistant tumors is, paradoxically, inhibited by E_2 treatment. Previous studies conducted by our group have shown that E_2 not only inhibits growth of Phase II SERM and estrogen withdrawn-resistant tumors; it also induces apoptosis, leading to tumor regression. However, a fraction of these Phase II tumors eventually re-grow after E_2-induced regression occurs, but these tumors are again re-sensitized to antihormonal therapy. We also found that while FUL does not support the growth of Phases I and II SERM-resistant tumors, it does allow growth of Phase II estrogen withdrawn-resistant tumors. Further, we found that while E_2 blocked growth of Phase II antihormone-resistant tumors, the combination of E_2 plus FUL resulted in robust growth. Phase II antihormonal resistance has not yet been widely recognized, but could be exploited by developing a novel third-line treatment plan based on short-term low-dose estrogen to debulk patients' tumors who fail exhaustive endocrine therapy, followed by the combination of FUL plus an AI to maintain low tumor burden and avoid a negative interaction between physiologic E_2 and FUL.

2. Materials and methods

2.1. Athymic mice, tumor inoculation, and tumor tracking

All procedures involving animals have been approved by the Fox Chase Cancer Center's Internal Animal Care and Use Committee.

All animal studies employed female ovariectomized athymic BALB/c nude (*nu/nu*) mice (Taconic, Hudson, NY, USA) that were inoculated with tumor cells at 5–6 weeks of age. For experiments employing tumor models which were generated and serially propagated as xenografts (*in vivo*), 1 mm^3 tumor sections were bilaterally transplanted using a trochar into the axillary mammary fat pads. For studies using tumor models which were generated and maintained in tissue culture (*in vitro*), cells were suspended in phosphate-buffered

saline and bilaterally injected into axillary mammary fat pads at 10^7 cells per site.

Tumor growth was tracked by weekly measurements of tumor length (l) and width (w) using Vernier calipers, from which the tumor cross-sectional area was calculated using the equation: $l/2 \times w/2 \times \pi$. Tumor growth curves are expressed as the average cross-sectional tumor area per treatment group ± standard error (S.E.).

2.2. Drug treatments

Mice were treated with estrogen by implanting a 0.3 cm E_2 silastic capsule subcutaneously into the intrascapular region on the back of the mouse at the time of tumor cell inoculation. The capsules were prepared by filling silicone tubing (0.078 in. inner diameter/0.125 in. outer diameter; Fisher) 0.3 cm in length with a 1:3 (w/w) mixture of E_2 (Sigma–Aldrich, St. Louis, MO, USA) and silastic elastomer (Dow Corning, Midland, MI, USA), and then sealing the ends with silicone adhesive (Dow Corning) and sterilized by gamma irradiation. Athymic mice implanted with these capsules achieve mean serum levels of 83.8 pg/ml (308 pM) E_2 [53], which approximates perimenopausal E_2 levels in women. RAL and TAM were orally administered by gastric intubation at 1.5 mg/day 5 days per week. Evista tablets (Eli Lilly Pharmaceuticals, Indianapolis, IN, USA; purchased from the Fox Chase Cancer Center's pharmacy), the clinically available form of RAL (60 mg/tablet), were initially dissolved in water, and then suspended at 10 mg/ml in 10% polyethylene glycol 400/Tween 80 (99.5% polyethylene glycol 400, 0.5% Tween 80) and 0.9% carboxymethyl cellulose. TAM (Sigma) was initially dissolved in ethanol (EtOH), and then suspended at 10 mg/ml in 10% polyethylene glycol 400/Tween 80 (99.5% polyethylene glycol 400, 0.5% Tween 80) and 0.9% carboxymethyl cellulose. FUL was administered by *sc* injection in the scruff of the neck at a total of 10 mg/week. For the experiment depicted in Fig. 1, four different FUL formulations and dosing schedules were used: FUL was initially dissolved in (1) EtOH or (2) dimethylsulfoxide (DMSO), and then made into a suspension with peanut oil at 50 mg/ml and administered as a 5 mg injection twice per week; (3) FUL was dissolved in only DMSO at 50 mg/ml and administered as a 2 mg injection 5 days per week; or (4) the clinical Faslodex preparation, a 50 mg/ml proprietary solution of FUL in primarily EtOH supplemented with castor oil as a release rate modifier, was administered as 2 mg injections 5 days per week. For all other experiments, only the clinical Faslodex preparation was used and administered as 2 mg injections 5 days per week. FUL powder was a kind gift of AstraZeneca (Macclesfield, United Kingdom), and the clinical Faslodex preparation was purchased from the Fox Chase Cancer Center's pharmacy.

2.3. Generation of MCF-7/E2 xenograft tumors

The MCF-7/E2 xenograft tumor model, representing the antihormonal-sensitive stage of breast cancer, was originally developed by bilateral injection of 10^7 MCF-7 cells, grown in tissue culture, into the axillary mammary fat pads of female ovariectomized athymic BALB/c *nu/nu* mice implanted with a 0.3 cm E_2 capsule [39]. The resulting MCF-7/E2 tumors have been propagated *in vivo* by serial transplantation into likewise E_2-treated ovariectomized athymic mice.

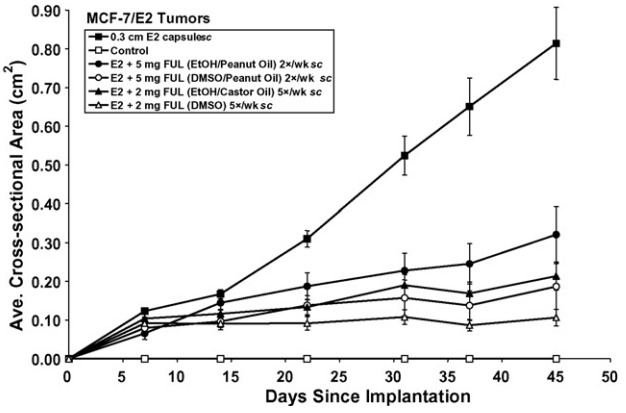

Fig. 1. Growth inhibition of MCF-7/E2 tumors in response to different FUL formulations and dosing schedules. Thirty ovariectomized athymic nude mice were bitransplanted in the axillary mammary fat pads with MCF-7/E2 tumor pieces 1 mm^3 in size. At the time of tumor implantation, the mice were separated into 6 treatment groups of 5 mice each, or 10 tumors per group. The treatment groups were control (no treatment), 0.3 cm E_2 silastic capsule implanted *sc*, and four groups of different formulations/dosing schedules of 10 mg total FUL per week plus the 0.3 cm E_2 capsule *sc*. The 4 FUL formulations/dosing schedules corresponded to: (1) a 50 mg/ml suspension of FUL dissolved first in EtOH and then mixed with peanut oil, and administered two times per week as a 5 mg *sc* injection; (2) the clinically used Faslodex preparation consisting of a 50 mg/ml solution of FUL in EtOH and castor oil, and administered five times per week as a 2 mg *sc* injection; (3) a 50 mg/ml suspension of FUL dissolved first in DMSO and then mixed with peanut oil, and administered two times per week as a 5 mg *sc* injection; or (4) a 50 mg/ml solution of FUL in 100% DMSO, and administered daily five times per week as a 2 mg *sc* injection. Tumor growth was tracked by weekly measurements using Vernier calipers and calculating the tumor cross-sectional area according to the formula: (length/2 × width/2 × π). The data shown represent the average tumor cross-sectional area (cm^2) per group ± S.E. The cross-sectional area of E_2-treated tumors was statistically different from that of each of the four E_2 + FUL groups (all P-values < 0.0001). Also, the cross-sectional area of tumors in the E_2 + 5 mg FUL (EtOH/peanut oil suspension given 2 days per week) was statistically different from those in the E_2 + 5 mg FUL (DMSO/peanut oil suspension given 2 days per week) group ($P = 0.0013$). Likewise, the cross-sectional area of tumors in the E_2 + 2 mg FUL (EtOH/castor oil solution given 5 days per week) group was statistically different from that of the E_2 + 2 mg FUL (100% DMSO solution given 5 days per week) group ($P = 0.0038$).

2.4. Generation of MCF-7/RAL1 xenograft tumors

The MCF-7/RAL1 (Phase I) SERM-resistant tumor model was derived by transplantation of MCF-7/E2 tumors into RAL-treated ovariectomized athymic mice. After extended RAL treatment, a small percentage of these tumors showed minimal but significant growth, and following repeated transplantation into new RAL-treated ovariectomized athymic mice, these tumors exhibited robust RAL-stimulated growth [49]. MCF-7/RAL1 tumors have been propagated *in vivo*

for over 3 years by serial transplantation into RAL-treated ovariectomized athymic mice.

2.5. Generation of MCF-7/RAL2 xenograft tumors

The MCF-7/RAL2 (Phase II) SERM-resistant tumor model was developed *in vitro* by tissue culture of MCF-7 cells in estrogen-free medium supplemented with 1 μM RAL for over 1 year [50]. For every experiment involving MCF-7/RAL2 tumors, cells were grown in culture, and then bilaterally injected into the axillary mammary fat pads of ovariectomized athymic mice at 10^7 cells per site. MCF-7/RAL2 cells were maintained in culture in phenol red-free media MEM plus 5% dextran-coated charcoal-treated calf serum (DCC-CS), 2 mM glutamine, 6 ng/ml bovine insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1× non-essential amino acids (all media components from Invitrogen, Carlsbad, CA, USA).

2.6. Generation of MCF-7/TAM2 xenograft tumors

The MCF-7/TAM2 (Phase II) SERM-resistant tumor model was developed in a similar manner as the MCF-7/RAL1 tumor model, by initial transplantation of MCF-7/E2 tumor pieces into TAM-treated ovariectomized athymic mice, and repeated transplantation of minimally growing tumors into new TAM-treated mice until robust TAM-stimulated growth occurred. These MCF-7/TAM tumors passed through different phases of SERM resistance; they were initially stimulated to grow by both TAM and E_2 [40,54], but have evolved over 5 years of serial propagation *in vivo* to a stage in which only TAM, but not E_2, stimulates growth [46–48].

2.7. Generation of MCF-7 long-term estrogen-deprived cell culture models

MCF-7:ED cells were derived by maintaining a population of MCF-7 cells under estrogen-deprived conditions for >1 year to mimic AI treatment, and represent Phase I resistance to long-term estrogen withdrawal. MCF-7:ED cells were originally selected in phenol red-free MEM plus 5% dextran-coated charcoal-treated calf serum, but have more recently been maintained (this report) in phenol red-free RPMI, 10% dextran-coated charcoal-treated fetal bovine serum, 2 mM glutamine, 6 ng/ml bovine insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1× non-essential amino acids.

MCF-7/5C cells were clonally isolated from a population of MCF-7 cells grown under long-term estrogen-free conditions [55] and represent Phase II estrogen withdrawn resistance. MCF-7/5C cells were originally cultured in phenol red-free MEM plus 5% dextran-coated charcoal-treated calf serum, and under these conditions, MCF-7/5C cells exhibited estrogen and SERM independent growth [51,55]. However, we have observed that when MCF-7/5C cells are switched to phenol red-free RPMI plus 10% dextran-coated charcoal-treated fetal bovine serum, the cells undergo rapid apoptosis when treated with 1 nM E_2 [51,52]. In all experiments described in this report, MCF-7/5C cells were maintained under the latter estrogen-free media conditions (phenol red-free RPMI plus 10% dextran-coated charcoal-treated fetal bovine serum, 2 mM glutamine, 6 ng/ml bovine insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1× non-essential amino acids).

2.8. Generation of MCF-7/5C estrogen-withdrawn xenograft tumors

The MCF-7/5C (Phase II) long-term estrogen withdrawn-resistant tumors were generated by bilateral injection of these cells grown in culture into the axillary mammary fat pads of ovariectomized athymic mice at 10^7 cells per site.

2.9. Cell proliferation assays

Wild-type MCF-7 cells were switched from fully-estrogenized medium to estrogen-free medium (phenol red-containing RPMI medium supplemented with 10% FBS switched to phenol red-free RPMI medium supplemented with 10% dextran-coated charcoal-treated fetal bovine serum) for 4 days prior to beginning the proliferation assay. Since MCF-7/ED and MCF-7/5C cells are routinely maintained in estrogen-free media, no media switch was required. MCF-7, MCF-7/ED, and MCF-7/5C cells were seeded in phenol red-free RPMI containing 10% DCC-FBS at a density of 2×10^4 cells per well in 24-well plates. After 24 h (day 0), the medium was replaced with fresh estrogen-free RPMI medium and cells were treated with 0.1% ethanol vehicle (control), 1 nM E_2, 10 nM FUL, or 1 nM E_2 + 10 nM FUL. Cells were retreated with the drugs on days 2, 4, and 6 and the experiment was stopped on day 7. As a measure of proliferation, the DNA content of the cells was determined using a Fluorescent DNA Quantitation kit (Bio-Rad Laboratories, Hercules, CA). For each analysis, six replicate wells were used, and at least three independent experiments were performed. Proliferation of cell lines following 7 days of growth are shown as the mean DNA amount per well per treatment ± standard deviation (S.D.).

2.10. Statistical analyses

In tumor growth experiments in which treatments were started at the time of tumor innoculation, tumors were analyzed longitudinally with two-factor analysis of variance (ANOVA) to determine significant differences in cross-sectional areas between all tumors in each treatment group in a time-dependent manner (data in Figs. 1–4, 6 and 7). In the tumor growth experiments in which treatments were started after the tumors were established, one-factor ANOVA was used to determine significant differences in cross-sectional areas between all tumors in each treatment group on the last

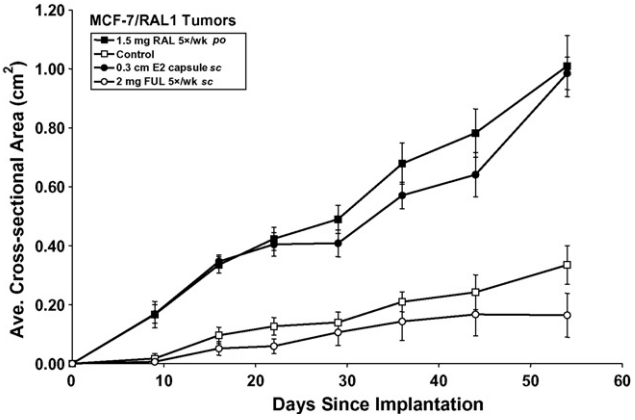

Fig. 2. Growth stimulation of MCF-7/RAL1 tumors in response to E_2, and inhibition by FUL. Twenty ovariectomized athymic nude mice were implanted in the axillary mammary fat pads with 1 mm^3 MCF-7/RAL1 tumor pieces and separated into 4 treatment groups of 5 mice each (10 tumors per group) corresponding to 1.5 mg/day RAL *po*, 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc*, and control (no treatment). The data shown represent the average tumor cross-sectional area (cm^2) per group ± S.E. The cross-sectional area of RAL-treated ($P < 0.0001$) and E_2-treated MCF-7/RAL1 tumors ($P < 0.0001$) was significantly different from control-treated tumors.

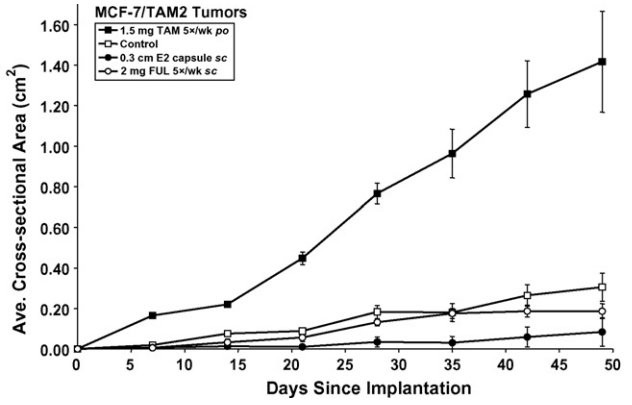

Fig. 4. Growth inhibition of MCF-7/TAM2 tumors in response to E_2 and FUL. Twenty ovariectomized athymic nude mice were implanted in the axillary mammary fat pads with 1 mm^3 MCF-7/TAM2 tumor pieces and separated into 4 treatment groups of 5 mice each (10 tumors per group) corresponding to 1.5 mg/day TAM *po*, 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc*, and control (no treatment). The data shown represent the average tumor cross-sectional area (cm^2) per group ± S.E. The cross-sectional area of TAM-treated ($P < 0.0001$) and E_2-treated MCF-7/TAM2 tumors ($P = 0.0004$) was significantly different from control tumors.

day of the experiment (data in Fig. 8, day 52). All statistical tests were two-sided and calculated using SAS (SAS Institute, Cary, NC, USA).

3. Results

3.1. Growth of MCF-7/E2 tumors and responsiveness to FUL

MCF-7/E2 xenograft tumors are propagated *in vivo* by serial transplantation into 0.3 cm E_2 capsule-implanted ovariectomized athymic mice. To explore the sensitivity of MCF-7/E2 tumors to FUL, MCF-7/E2 tumor cores were implanted into 30 ovariectomized athymic mice and separated into 6 groups of 5 mice each, or 10 tumors per group. The treatment groups were control (no treatment), 0.3 cm E_2 capsule *sc*, or 0.3 cm E_2 capsule *sc* plus 1 of 4 different formulations and dosing schedules of FUL totaling 10 mg/week *sc* (Fig. 1). Two of the FUL formulations were suspensions made with peanut oil, differing by whether FUL was initially dissolved in EtOH or in DMSO. These FUL (EtOH/peanut oil or DMSO/peanut oil) suspensions were administered as 5 mg *sc* injections given 2 days per week, totaling 10 mg/week. The third formulation was FUL dissolved in only DMSO, and was administered as a 2 mg *sc* injection given 5 days per week, totaling 10 mg/week. The fourth FUL formulation corresponded to the clinical Faslodex preparation, which is a proprietary solution of primarily EtOH and some castor oil as a slow release-rate modifier. The clinical Faslodex preparation was administered as a 2 mg *sc* injection given five times per week, totaling 10 mg/week.

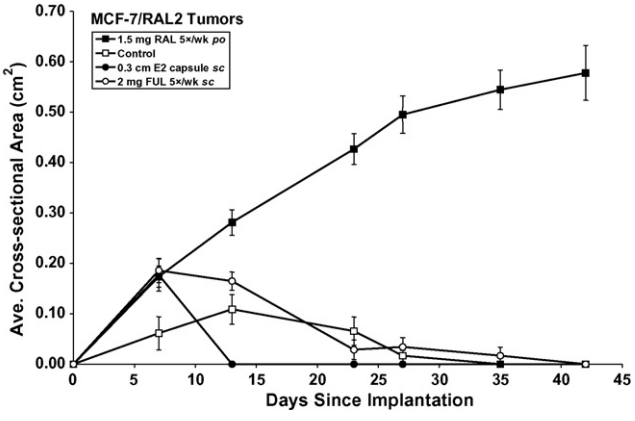

Fig. 3. Growth inhibition of MCF-7/RAL2 tumors in response to E_2 and FUL. Twenty ovariectomized athymic nude mice were bilaterally injected in the axillary mammary fat pads with 10^7 MCF-7/RAL2 cells grown in culture and separated into 4 treatment groups of 5 mice each (10 tumors per group) corresponding to 1.5 mg/day RAL *po*, 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc*, and control (no treatment). The data shown represent the average tumor cross-sectional area (cm^2) per group ± S.E. The cross-sectional area of RAL-treated MCF-7/RAL2 tumors was significantly different from E_2-treated, FUL-treated and control-treated tumors (all *P*-values < 0.0001).

The MCF-7/E2 tumors grew robustly when treated with the 0.3 cm E_2 capsule, but did not grow in the control group (Fig. 1, E_2 versus control, $P < 0.0001$), demonstrating that these tumors were dependent on E_2. The implanted capsules produce E_2 levels that are in the physiologic range observed in perimenopausal women. The cross-sectional areas of each of the four groups of MCF-7/E2 tumors treated with E_2 plus FUL was significantly smaller than those tumors treated with E_2 alone (Fig. 1, all *P*-values < 0.0001). Therefore, FUL inhibited E_2-stimulated growth of MCF-7/E2 tumors. However, the degree of growth inhibition varied depending upon the formulation. Comparing the FUL suspensions in peanut oil given two times per week, FUL initially dissolved in DMSO inhibited tumor growth significantly better than FUL

initially dissolved in EtOH (Fig. 1, $P = 0.001$). Comparing the FUL formulations given five times per week, FUL dissolved in only DMSO inhibited tumor growth significantly better than the clinical Faslodex preparation (Fig. 1, $P = 0.004$). Although we did not measure circulating FUL levels, we hypothesize that circulating levels of FUL were higher when using DMSO-based formulations, leading to more potent inhibition of E_2-stimulated tumor growth.

3.2. Growth of MCF-7/RAL1 tumors

MCF-7/RAL1 tumors are maintained *in vivo* by serial transplantation into 1.5 mg/day RAL-treated ovariectomized athymic mice. To illustrate the phase of SERM resistance the MCF-7/RAL1 tumor should be categorized into, MCF-7/RAL1 tumor cores were implanted into 20 ovariectomized athymic mice and separated into 4 treatment groups of 5 mice each (10 tumors/group) corresponding 1.5 mg/day RAL *po*, 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc* (Faslodex preparation), and control (no treatment). The MCF-7/RAL1 tumors were significantly stimulated to grow by RAL treatment ($P < 0.0001$) and by E_2 treatment ($P < 0.0001$) compared to control treatment (Fig. 2). However, a modest amount of growth was observed in the control-treated group, indicating that these tumors are not absolutely dependent upon an ER ligand with partial agonist activity. We have previously shown that primary cultures of MCF-7/RAL1 tumors exhibit equivalent levels of estrogen response element (ERE)-regulated reporter gene activity in the absence of E_2 as did primary cultures of MCF-7/E2 tumors when treated with E_2 [49]. Thus, the unliganded ER activity in MCF-7/RAL1 tumors is high and probably contributed to the modest growth of these tumors without the need of RAL or E_2. FUL did not significantly effect the growth of MCF-7/RAL1 tumors (Fig. 2). Thus, either a SERM or E_2, but not FUL, supports the growth of these MCF-7/RAL1 xenografts. Therefore, these tumors are categorized as Phase I SERM-resistant.

3.3. Growth of MCF-7/RAL2 tumors

MCF-7/RAL2 tumor cells are maintained *in vitro* by culture in media containing 1 μM RAL. To study the growth properties of MCF-7/RAL2 cells *in vivo*, the cells were grown in culture and injected into 20 ovariectomized athymic mice, which were separated into 4 groups of 5 mice (10 tumors/group) and treated with 1.5 mg/day RAL *po*, 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc* (Faslodex preparation), or control (not treated). The MCF-7/RAL2 tumors only grew when treated with RAL (RAL versus control, $P < 0.0001$), and did not form any palpable tumors by day 42 when treated with E_2, FUL or not treated (control) (Fig. 3). We have previously shown that when MCF-7/RAL2 tumors are allowed to grow by treating with TAM until they are established and then switching treatments to E_2, E_2 causes tumor regression by inducing apoptosis as measured by TUNEL staining [50]. Therefore, growth of the MCF-7/RAL2 tumors was dependent on RAL, but inhibited by E_2 and FUL, which categorizes these tumors as Phase II SERM-resistant.

3.4. Growth of MCF-7/TAM2 tumors

MCF-7/TAM2 tumors are propagated *in vivo* by serial transplantation into 1.5 mg/day TAM-treated ovariectomized athymic mice. To characterize the growth properties of this tumor type, MCF-7/TAM2 tumor cores were implanted into 20 ovariectomized athymic mice, which were separated into 4 groups of 5 mice (10 tumors/group) and treated with 1.5 mg/day TAM *po*, 0.3 cm E_2 capsule *sc*, 2 mg/day FUL (Faslodex) *sc*, or not treated (control). MCF-7/TAM2 tumors were stimulated to grow by TAM compared to the control group (Fig. 4, $P < 0.0001$). The control group did show a minimal amount of growth (Fig. 4), which is hypothesized to be due to substantial unliganded ER activity as in the MCF-7/RAL1 model. FUL did not significantly effect growth of the MCF-7/TAM2 tumors versus control treatment. Interestingly, E_2 did significantly inhibit tumor growth compared to the control group (Fig. 4, $P = 0.0004$). As with the MCF-7/RAL2 tumors, we have previously demonstrated that E_2 treatment leads to regression of MCF-7/TAM2 tumors [46,48] by inducing apoptosis as detected by TUNEL staining [47]. Therefore, TAM stimulated growth, FUL did not support growth, and E_2 inhibited growth of MCF-7/TAM2 tumors, defining this model as Phase II SERM-resistant.

3.5. Growth of long-term estrogen withdrawn-resistant models

Since having categorized each of the SERM-resistant tumor models as Phase I or II resistant, we characterized the growth properties of cells which have been cultured long-term under estrogen-free conditions to determine whether resistance to estrogen withdrawal (as a surrogate for AI resistance) also evolves through distinct stages. Initially, we compared the proliferation of parental MCF-7 cells with two cell lines resistant to long-term estrogen withdrawal, MCF-7/ED (estrogen-deprived) and MCF-7/5C cells. MCF-7/ED cells were originally selected by culture of parental MCF-7 in estrogen-free medium for >1 year, but were not cloned as a subline, rather they remain a population of cells. In a similar manner, MCF-7/5C cells were also derived from parental MCF-7 cells following long-term estrogen withdrawal, but were cloned as a subline [55]. Notably, MCF-7/ED and MCF-7/5C cells were generated independently in different studies, that is, MCF-7/5C cells were not subcloned from the MCF-7/ED cells.

Growth of parental MCF-7, MCF-7/ED and MCF-7/5C cells was determined by measuring DNA amounts after 7 days in culture. Before beginning the experiment, parental MCF-7 cells were cultured for 4 days in estrogen-free media, since they had been maintained in fully-estrogenized medium. The experiment was started by seeding each of the cell lines in 24-well plates in estrogen-free medium. The cells were treated

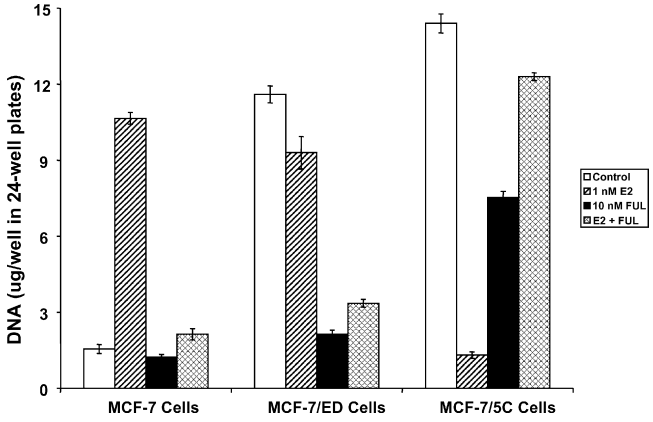

Fig. 5. Differential proliferation of MCF-7 long-term estrogen withdrawn cell culture models in response to E_2, FUL, and E_2 plus FUL for 7 days. Cells were cultured under estrogen-free conditions for 4 days, and then seeded at 2×10^4 cells per well in a 24-well plate. Beginning 24 h after seeding (day 0) and every 2 days thereafter up to 6 days (days 2, 4, and 6), the cells were treated with 1 nM E_2, 10 nM FUL, 1 nM E_2 + 10 nM FUL, or control (0.1% EtOH)-treated. The experiment was stopped on day 7. As a measure of proliferation, the amount of DNA per well was determined using a fluorescence-based DNA quantitation assay. Data are shown as the mean of 6 replicate wells per group ± S.D. The experiment was performed three times independently, and one representative experiment is shown.

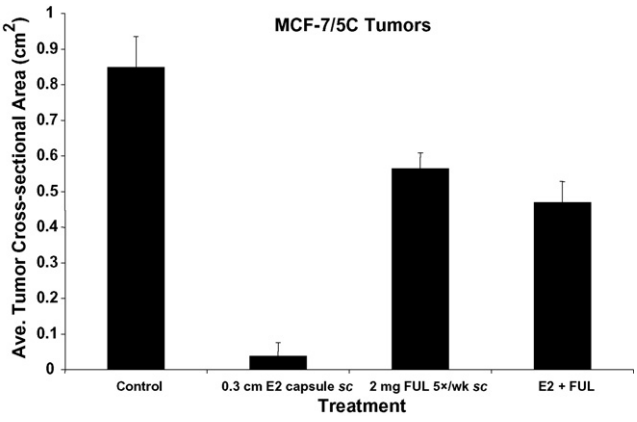

Fig. 6. Growth inhibition of MCF-7/5C tumors in response to E_2 treatment, and resistance to FUL, and E_2 plus FUL. Twenty ovariectomized athymic nude mice were bilaterally injected in the axillary mammary fat pads with 10^7 MCF-7/5C cells grown in culture and separated into 4 treatment groups of 5 mice each (10 tumors per group) corresponding to control (no treatment), 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc*, and 0.3 cm E_2 capsule *sc* + 2 mg/day FUL *sc*. The data are shown as a histogram on day 21 of the average tumor cross-sectional area (cm^2) per group ± S.E. The cross-sectional areas of control-treated, FUL-treated, and E_2 plus FUL-treated MCF-7/5C tumors were each significantly different from E_2-treated (all *P*-values < 0.0001). The cross-sectional area of both FUL-treated and E_2 plus FUL-treated MCF-7/5C tumors were not significantly different from that of control-treated MCF-7/5C tumors.

every 2 days with EtOH (vehicle control), 1 nM E_2, 10 nM FUL, and 1 nM E_2 plus 10 nM FUL. After 7 days, DNA quantities per well were determined using a fluorescence-based DNA assay. As expected in parental MCF-7 cells, E_2 induced growth by 6.9-fold (E_2 versus control treatment), and this E_2-stimulated proliferation was completely blocked by the addition of FUL (E_2 + FUL versus control) (Fig. 5). Hence, E_2 stimulated proliferation of parental MCF-7 cells in an ER-dependent manner.

Next, MCF-7/ED cells representing a population of cells resistant to estrogen withdrawal were characterized. MCF-7/ED cells grew maximally under estrogen-free conditions (control treatment, 100% growth) and nearly maximally when treated with E_2 (80% of control) (Fig. 5). However, FUL and E_2 plus FUL treatment inhibited growth of MCF-7/ED cells (18% and 29%, respectively, of control) (Fig. 5). Thus, MCF-7/ED cell proliferation was largely unaffected by E_2, but dependence on the ER was demonstrated by the sensitivity of the cells to FUL.

Finally, we evaluated MCF-7/5C cells, which were a clonal derivative of long-term estrogen-withdrawn cells. MCF-7/5C cells grew maximally under estrogen-free conditions (control treatment, 100% growth), but E_2 treatment almost completely blocked proliferation (9% of control) (Fig. 5). Interestingly, FUL-treated MCF-7/5C cells exhibited significant growth (52% of control) (Fig. 5). Further, MCF-7/5C cells treated with E_2 plus FUL showed still greater amounts of proliferation (85% of control) (Fig. 5). In prior studies, we have demonstrated that MCF-7/5C cells undergo apoptosis when treated with E_2, and that co-treatment with FUL blocks this effect of E_2 [52]. Hence, MCF-7/5C cells required ER to be unliganded for maximal proliferation and survival, whereas E_2-bound ER led to cytostasis and apoptosis. Further, FUL reversed the apoptotic signal of E_2 and promoted proliferation.

We next verified that the MCF-7/5C cells behaved similarly *in vivo* as a xenograft tumor as they did *in vitro* in cell culture. MCF-7/5C cells were grown in culture and injected into 20 ovariectomized athymic mice. The animals were separated into 4 treatment groups of 5 mice each (10 tumors/group), corresponding to control (not treated), 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc* (Faslodex), and 0.3 cm E_2 capsule *sc* plus 2 mg/day FUL *sc* (Faslodex). MCF-7/5C cells rapidly formed substantial tumors at every injection site (10 out of 10) in control-treated mice by 21 days after inoculation, but only 1 small yet palpable tumor formed out of 10 injection sites in mice treated with E_2, resulting in a highly significant difference in the average tumor cross-sectional area between the two treatment groups (Fig. 6, $P < 0.0001$). In a prior report, we have shown that E_2 induces tumor regression and apoptosis in established MCF-7/5C xenograft tumors [52]. Importantly, MCF-7/5C xenograft tumors showed robust growth in the presence of FUL or E_2 plus FUL, which was not significantly different than growth of the control (no treatment) group, but was significantly greater than in the E_2 treatment group (Fig. 6, FUL versus E_2, $P < 0.0001$; E_2 + FUL versus E_2, $P < 0.0001$). Hence, the MCF-7/5C xenograft tumor model was resistant to growth inhibition by FUL, and FUL treatment abrogated E_2-mediated growth inhibition.

Considering these varied growth responses together, parental MCF-7 cells model the therapeutic stage of antihormonal therapy; MCF-7/ED cells represent Phase I resistance

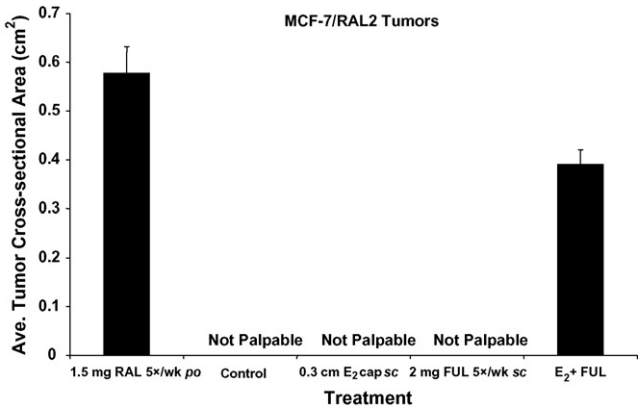

Fig. 7. E_2 plus FUL-stimulated growth of MCF-7/RAL2 tumors. Data are from Fig. 3 on day 42 and shown as a histogram, but supplemented with the additional group of five ovariectomized athymic mice (10 tumors) treated with 0.3 cm E_2 capsule *sc* plus 2 mg/day FUL *sc*. The cross-sectional area of E_2 plus FUL-treated MCF-7/RAL2 tumors was significantly different from that of control-treated, E_2-treated, and FUL-treated MCF-7/RAL2 tumors (all P-values < 0.0001).

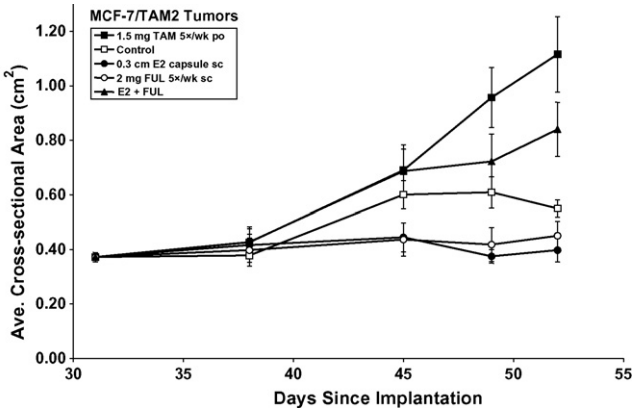

Fig. 8. E_2 plus FUL-stimulated growth of MCF-7/TAM2 tumors. Twenty-five ovariectomized athymic mice were implanted in the axillary mammary fat pads with 1 mm^3 MCF-7/TAM2 tumor pieces, and then treated with 1.5 mg/day TAM *po* until the tumors were established at 0.24 cm^2, then TAM was withdrawn for 1 week. Following 1 week of TAM withdrawal, the tumors reached an average cross-sectional area of 0.37 cm^2 and the animals were separated into 5 treatment groups of 5 mice each (10 tumors per group) corresponding to 1.5 mg/day TAM *po*, 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc*, 0.3 cm E_2 capsule *sc* plus 2 mg/day FUL *sc*, and control (no treatment). The data shown represent the average tumor cross-sectional area (cm^2) per group ± S.E. The cross-sectional areas of MCF-7/TAM2 tumors at day 52 were compared by one-way ANOVA. The cross-sectional areas of TAM-treated ($P = 0.0026$), E_2-treated ($P = 0.0098$), and E_2 plus FUL-treated MCF-7/TAM2 tumors ($P = 0.018$) were significantly different from control-treated tumors.

to estrogen withdrawal since they grew independent of E_2 yet remained sensitive to FUL; and MCF-7/5C tumors/cells were classified as Phase II resistant to estrogen withdrawal since E_2 inhibited their growth, but were resistant to growth inhibition by FUL or E_2 plus FUL.

3.6. Response of Phase II SERM-resistant tumor models to E_2 plus FUL (Figs. 7 and 8)

Since we observed that MCF-7/5C cells grew better when treated with E_2 plus FUL than with E_2 alone, we examined the effect of FUL in a background of physiologic E_2 in the Phase II SERM-resistant tumor models. The data from the MCF-7/RAL2 experiment depicted in Fig. 3 was re-evaluated with an additional group of 5 animals (10 tumors) treated with a 0.3 cm E_2 capsule *sc* plus 2 mg/day FUL (Faslodex). MCF-7/RAL2 tumors treated with E_2 plus FUL showed robust growth compared to no palpable tumors in the E_2 alone ($P < 0.0001$), FUL alone ($P < 0.0001$), or control groups ($P < 0.0001$) (Fig. 7). Therefore, E_2 plus FUL, when combined, negated the growth inhibitory effects of either compound by itself.

We then tested whether this interaction between physiologic E_2 and FUL also occurred in the MCF-7/TAM2 tumor model of Phase II SERM resistance. However, this experiment was designed to evaluate effects of different treatments on tumors once they are established by allowing tumors to grow in the presence of TAM until they were palpable, and then randomized to different treatment groups. MCF-7/TAM2 tumor cores were implanted into 25 ovariectomized athymic mice. All animals were treated with 1.5 mg/day TAM *po* until tumors grew to an average cross-sectional area of 0.24 cm^2, at which time TAM treatment was withdrawn for 1 week to allow time for this drug to be completely metabolized and clear the animals' systems. Following the 1 week of TAM withdrawal, the average cross-sectional area of all tumors was 0.37 cm^2, and the animals were randomized into 5 groups of 5 mice each (10 tumors/group) corresponding to continuing 1.5 mg/day TAM *po*, 0.3 cm E_2 capsule *sc*, 2 mg/day FUL *sc*, 0.3 cm E_2 capsule *sc* plus 2 mg/day FUL *sc*, and control (no treatment). As would be predicted from the MCF-7/TAM2 experiment depicted in Fig. 4, TAM treatment significantly stimulated growth ($P = 0.0026$) and E_2 significantly inhibited growth ($P = 0.0098$) compared to the control group on day 52 (Fig. 8). The size of FUL treated tumors was not significantly different than the control group. In contrast, we noted that tumors treated with the combination of E_2 + FUL did exhibit significantly greater growth than the control group (Fig. 8, $P = 0.018$). Thus, in a second model of Phase II SERM resistance, growth inhibition by E_2 alone was negated in the presence of FUL, leading to growth stimulation.

4. Discussion

We sought to discover unifying principles involved in the development of antihormone resistance by systematically studying the growth properties of a panel of antihormonally resistant MCF-7-based breast cancer xenograft tumor models. We have confirmed and extended prior observations [39–52] that have allowed the categorization of these tumor models as either Phase I or Phase II antihormone resistant. Phase I SERM resistance was characterized by growth stimulation in response to both a SERM or E_2 (MCF-7/RAL1

tumors, Fig. 2), while in Phase II SERM resistance, only the SERM stimulated growth, whereas E_2 inhibited growth (MCF-7/RAL2 and MCF-7/TAM2, Figs. 3 and 4, respectively). Phase I long-term estrogen withdrawn (AI)—resistant cells in culture grew independently of E_2 (MCF-7/ED cells, Fig. 5), but Phase II resistant tumors were growth inhibited by E_2 (MCF-7/5C tumors, Fig. 6). Hence, long-term blockade of ER activity by either SERMs or estrogen withdrawal can lead to selection of cells in which E_2 signals no longer proliferation, but rather inhibition, of growth, and as we have previously reported, apoptosis [47,50,52].

We also found that growth of Phase II SERM resistant tumors was not supported by FUL, but Phase II estrogen withdrawn-resistant tumors were cross-resistant to FUL (MCF-7/5C tumors, Fig. 6). Further, FUL combined with physiologic E_2 nullified the inhibitory effects of either compound alone and led to stimulation of growth in Phase II SERM-resistant tumors (MCF-7/RAL2 and MCF-7/TAM2 tumors, Figs. 7 and 8, respectively), and supported growth in Phase II estrogen withdrawn-resistant tumors (MCF-7/5C tumors, Fig. 6).

Noteworthy, the concentrations of FUL used in the cell culture proliferation experiments was 10 nM. We chose this concentration of FUL to reflect the circulating levels that are achieved clinically. In the clinic, FUL is not administered orally because of low bioavailability; rather it is given intra muscularly (as a single 250 mg dose once per month) to achieve slow constant release of the drug. In two independent multi-national Phase III clinical efficacy trials evaluating FUL in advanced breast cancer patients, the steady state circulating concentrations of FUL were determined to be approximately 6–7 μg/l (9.9–11.5 nM) in the European trial and 9 μg/l (14.8 nM) in the North American trial [56]. Hence, we used FUL at 10 nM in cell culture, reflecting the circulating concentrations of FUL achieved in women, but this concentration was much lower than the 100 nM to 1 μM FUL concentrations used in most cell culture studies. We hypothesize that the low circulating concentrations of FUL in patients may contribute to the lower than expected response rates in the clinic as would be predicted by the effectiveness of FUL in cell culture. In support of this hypothesis, we have found that while MCF-7/5C cells proliferate in the presence of 10 nM FUL, they do not in 1 μM FUL (unpublished, JS Lewis-Wambi and VC Jordan).

The distinct growth responses of the tumor and cell culture models studied here illustrate that resistance to hormonal blockade therapy continually evolves but can be separated into at least two phases (Fig. 9). Antihormonal resistance develops from selection of specific cell types that survive and proliferate when the ER is bound by a partial antiestrogen (Phase I SERM resistance) or unliganded (Phase I estrogen withdrawn-resistance). Prolonged hormonal blockade therapy maintains selective pressure, such that Phase I resistant

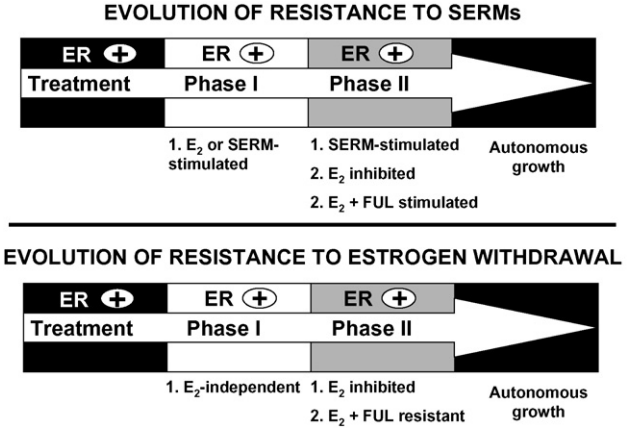

Fig. 9. Evolution of antihormonal resistance in laboratory models of breast cancer. Long-term antihormonal therapy leads to selection of resistant cells that are stimulated to grow by a SERM or grow in an estrogen-depleted environment (AI resistance). Based on prior laboratory studies (4–10, 12, 13) and data presented here, the progression of antihormonal resistance can be separated into at least two phases defined by different growth responses to E_2 and FUL. In Phase I resistant disease, tumor cells are either growth stimulated by E_2 (as in SERM resistance) or grow independently of E_2 (as in estrogen withdrawn/AI resistance). However, these Phase I resistant cells remain dependent on ER since they are sensitive to growth inhibition by FUL. Selection of tumor cells continues during exhaustive antihormonal therapy until Phase II resistance develops, which is characterized by a new biology of E_2 action. Both Phase II SERM and estrogen withdrawn-resistant tumors respond to E_2 with growth inhibition and apoptosis. FUL still inhibits growth of Phase II SERM-resistant tumors, but not of Phase II estrogen withdrawn-resistant tumors. Moreover, FUL interacts with E_2 at physiologic concentrations to promote growth of both Phase II SERM and estrogen withdrawn-resistant disease. These emerging concepts on the evolution of antihormonal resistance based on laboratory studies have important implications for the utility of estrogen and FUL in the clinic.

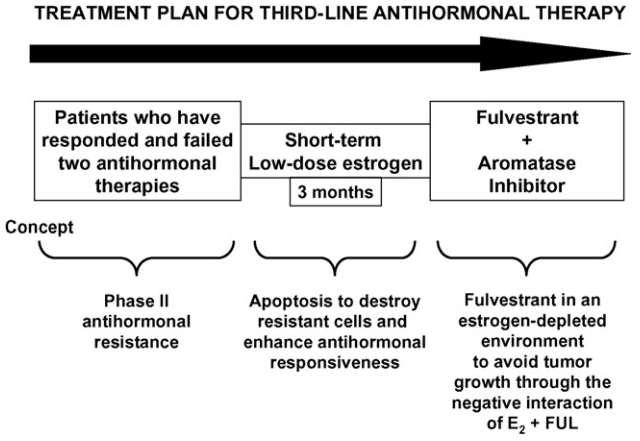

Fig. 10. A proposed clinical strategy for the optimal application of third-line antihormonal therapy. We propose that patients who have initially responded to, and then failed, two previous antihormonal therapies may exhibit Phase II antihormonal resistance. These patients would benefit from short-term low-dose estrogen, since estrogen would induce apoptosis in the Phase II resistant cells and debulk the tumor. Prior laboratory evidence indicates that the small percentage of Phase II tumors which revert to an estrogen-stimulated stage after estrogen-induced regression, are also re-sensitized to antihormonal therapy [47,49]. A low tumor burden would be maintained by FUL in an estrogen-depleted environment, *i.e.* FUL plus an aromatase inhibitor, to avoid the possible emergence of tumor growth through a negative interaction between FUL and physiologic estrogen.

cells continue to evolve to a Phase II resistant phenotype, and likely undefined additional phases. However, the study of Phase II antihormonal resistance has revealed a new biology of E_2 action involved in apoptosis that could be exploited to benefit breast cancer patients who have been exhaustively treated with SERMs and AIs. Moreover, the finding that FUL in a background of physiologic E_2 stimulated growth of Phase II resistant cells has important clinical implications. This knowledge can be implemented to optimize the application of third-line antihormonal therapy (Fig. 10). We propose that patients who have responded and then failed two antihormone therapies may exhibit Phase II resistant characteristics, and therefore respond to low-dose short-term estrogen therapy. The estrogen therapy would lead to apoptosis in the Phase II resistant cells and thereby debulk the tumor. Prior laboratory studies indicate that cells which remain are re-sensitized to first-line or second-line antihormonal therapy [46,48]. Hence, the low-dose short-term estrogen therapy would be followed by FUL plus an AI, to avoid the possible selection of cells that could grow in response to FUL plus physiologic E_2.

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References

[1] E.V. Jensen, G.E. Block, S. Smith, K. Kyser, E.R. DeSombre, Estrogen receptors and breast cancer response to adrenalectomy, Natl. Cancer Inst. Monogr. 34 (1971) 55–70.
[2] W.L. McGuire, P.P. Carbone, E.P. Vollmer, Oestrogen receptors in human breast cancer: an overview, in: W.L. McGuire, P.P. Carbone, E.P. Vollmer (Eds.), Oestrogen Receptors in Human Breast Cancer, vol. 6, Raven Press, New York, 1975.
[3] L.J. Lerner, V.C. Jordan, The development of antiestrogens for the treatment of breast cancer: eighth Cain memorial award lecture, Cancer Res. 50 (1990) 4177–4189.
[4] V.C. Jordan, Fourteenth gaddum memorial lecture. A current view of tamoxifen for the treatment and prevention of breast cancer, Br. J. Pharm. 110 (1993) 507–517.
[5] Early Breast Cancer Trialists' Collaborative Group, Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials, Lancet 365 (2005) 1687–1717.
[6] E.V. Jensen, V.C. Jordan, The estrogen receptor: a model for molecular medicine, Clin. Cancer Res. 9 (2003) 1980–1989.
[7] E.A. Ariazi, J.L. Ariazi, F. Cordera, V.C. Jordan, Estrogen receptors as therapeutic targets in breast cancer, Curr. Top. Med. Chem. 6 (2006) 181–202.
[8] K. Strasser-Weippl, P.E. Goss, Advances in adjuvant hormonal therapy for postmenopausal women, J. Clin. Oncol. 23 (2005) 1751–1759.
[9] Early Breast Cancer Trialists' Collaborative Group, Tamoxifen for early breast cancer: an overview of the randomised trials, Lancet 351 (1998) 1451–1467.
[10] B. Fisher, J. Dignam, J. Bryant, N. Wolmark, Five versus more than five years of tamoxifen for lymph node-negative breast cancer: updated findings from the national surgical adjuvant breast and bowel project b-14 randomized trial, J. Natl. Cancer Inst. 93 (2001) 684–690.
[11] I. Smith, Breast cancer: adjuvant treatment for early breast cancer, Ann. Oncol. 16 (2005) ii182–ii187.
[12] J. Cuzick, T. Powles, U. Veronesi, J. Forbes, R. Edwards, S. Ashley, P. Boyle, Overview of the main outcomes in breast-cancer prevention trials, Lancet 361 (2003) 296–300.
[13] S.R. Cummings, S. Eckert, K.A. Krueger, D. Grady, T.J. Powles, J.A. Cauley, L. Norton, T. Nickelsen, N.H. Bjarnason, M. Morrow, M.E. Lippman, D. Black, J.E. Glusman, A. Costa, V.C. Jordan, The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the more randomized trial, JAMA 281 (1999) 2189–2197.
[14] S. Martino, J.A. Cauley, E. Barrett-Connor, T.J. Powles, J. Mershon, D. Disch, R.J. Secrest, S.R. Cummings, Continuing outcomes relevant to evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene, J. Natl. Cancer Inst. 96 (2004) 1751–1761.
[15] V.G. Vogel, J.P. Costantino, D.L. Wickerham, W.M. Cronin, R.S. Cecchini, J.N. Atkins, T.B. Bevers, L. Fehrenbacher, E.R. Pajon Jr., J.L. Wade III, A. Robidoux, R.G. Margolese, J. James, S.M. Lippman, C.D. Runowicz, P.A. Ganz, S.E. Reis, W. McCaskill-Stevens, L.G. Ford, V.C. Jordan, N. Wolmark, Effects of tamoxifen vs. raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP study of tamoxifen and raloxifene (star) p-2 trial, JAMA 295 (2006) 2727–2741.
[16] A. Cranney, P. Tugwell, N. Zytaruk, V. Robinson, B. Weaver, J. Adachi, G. Wells, B. Shea, G. Guyatt IV, Meta-analysis of raloxifene for the prevention and treatment of postmenopausal osteoporosis, Endocr. Rev. 23 (2002) 524–528.
[17] E. Barrett-Connor, D.A. Cox, P.W. Anderson, The potential of serms for reducing the risk of coronary heart disease, Trends Endocr. Metab. 10 (1999) 320–325.
[18] N.K. Wenger, E. Barrett-Connor, P. Collins, D. Grady, M. Kornitzer, L. Mosca, A. Sashegyi, S.K. Baygani, P.W. Anderson, E. Moscarelli, Baseline characteristics of participants in the raloxifene use for the heart (RUTH) trial, Am. J. Cardiol. 90 (2002) 1204–1210.
[19] L. Mosca, E. Barrett-Connor, N.K. Wenger, P. Collins, D. Grady, M. Kornitzer, E. Moscarelli, S. Paul, T.J. Wright, J.D. Helterbrand, P.W. Anderson, Design and methods of the raloxifene use for the heart (RUTH) study, Am. J. Cardiol. 88 (2001) 392–395.
[20] C.T. Evans, D.B. Ledesma, T.Z. Schulz, E.R. Simpson, C.R. Mendelson, Isolation and characterization of a complementary DNA specific for human aromatase-system cytochrome p-450 mRNA, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 6387–6391.
[21] L.R. Nelson, S.E. Bulun, Estrogen production and action, J. Am. Acad. Dermatol. 45 (2001) S116–S124.
[22] I.E. Smith, M. Dowsett, Aromatase inhibitors in breast cancer, N. Engl. J. Med. 348 (2003) 2431–2442.
[23] H. Joensuu, B. Ejlertsen, P.E. Lonning, L.E. Rutqvist, Aromatase inhibitors in the treatment of early and advanced breast cancer, Acta Oncol. 44 (2005) 23–31.
[24] J. Geisler, P.E. Lonning, Aromatase inhibition: translation into a successful therapeutic approach, Clin. Cancer Res. 11 (2005) 2809–2821.
[25] R.C. Coombes, E. Hall, L.J. Gibson, R. Paridaens, J. Jassem, T. Delozier, S.E. Jones, I. Alvarez, G. Bertelli, O. Ortmann, A.S. Coates, E. Bajetta, D. Dodwell, R.E. Coleman, L.J. Fallowfield, E. Mickiewicz, J. Andersen, P.E. Lonning, G. Cocconi, A. Stewart, N. Stuart, C.F. Snowdon, M. Carpentieri, G. Massimini, J.M. Bliss, The Intergroup Exemestane Study, A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer, N. Engl. J. Med. 350 (2004) 1081–1092.

[26] H.T. Mouridsen, Exemestane following tamoxifen in postmenopausal women with primary breast cancer, J. Clin. Oncol. 22 (2004) 3833–3834.
[27] J. Bonneterre, B. Thurlimann, J.F.R. Robertson, M. Krzakowski, L. Mauriac, P. Koralewski, I. Vergote, A. Webster, M. Steinberg, M. von Euler, Anastrozole versus tamoxifen as first-line therapy for advanced breast cancer in 668 postmenopausal women: results of the tamoxifen or arimidex randomized group efficacy and tolerability study, J. Clin. Oncol. 18 (2000) 3748–3757.
[28] F. Boccardo, A. Rubagotti, M. Puntoni, P. Guglielmini, D. Amoroso, A. Fini, G. Paladini, M. Mesiti, D. Romeo, M. Rinaldini, S. Scali, M. Porpiglia, C. Benedetto, N. Restuccia, F. Buzzi, R. Franchi, B. Massidda, V. Distante, D. Amadori, P. Sismondi, Switching to anastrozole versus continued tamoxifen treatment of early breast cancer: preliminary results of the Italian tamoxifen anastrozole trial, J. Clin. Oncol. 23 (2005) 5138–5147.
[29] H. Mouridsen, M. Gershanovich, Y. Sun, R. Perez-Carrion, C. Boni, A. Monnier, J. Apffelstaedt, R. Smith, H.P. Sleeboom, F. Jaenicke, A. Pluzanska, M. Dank, D. Becquart, P.P. Bapsy, E. Salminen, R. Snyder, H. Chaudri-Ross, R. Lang, P. Wyld, A. Bhatnagar, Phase iii study of letrozole versus tamoxifen as first-line therapy of advanced breast cancer in postmenopausal women: analysis of survival and update of efficacy from the international letrozole breast cancer group, J. Clin. Oncol. 21 (2003) 2101–2109.
[30] H. Mouridsen, M. Gershanovich, Y. Sun, R. Perez-Carrion, C. Boni, A. Monnier, J. Apffelstaedt, R. Smith, H.P. Sleeboom, F. Janicke, A. Pluzanska, M. Dank, D. Becquart, P.P. Bapsy, E. Salminen, R. Snyder, M. Lassus, J.A. Verbeek, B. Staffler, H.A. Chaudri-Ross, M. Dugan, Superior efficacy of letrozole versus tamoxifen as first-line therapy for postmenopausal women with advanced breast cancer: results of a phase III study of the international letrozole breast cancer group, J. Clin. Oncol. 19 (2001) 2596–2606.
[31] M. Baum, A.U. Budzar, J. Cuzick, J. Forbes, J.H. Houghton, J.G. Klijn, T. Sahmoud, Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial, Lancet 359 (2002) 2131–2139.
[32] A. Howell, J. Cuzick, M. Baum, A. Buzdar, M. Dowsett, J.F. Forbes, G. Hoctin-Boes, J. Houghton, G.Y. Locker, J.S. Tobias, Results of the ATAC (arimidex, tamoxifen, alone or in combination) trial after completion of 5 years' adjuvant treatment for breast cancer, Lancet 365 (2005) 60–62.
[33] P.E. Goss, J.N. Ingle, S. Martino, N.J. Robert, H.B. Muss, M.J. Piccart, M. Castiglione, D. Tu, L.E. Shepherd, K.I. Pritchard, R.B. Livingston, N.E. Davidson, L. Norton, E.A. Perez, J.S. Abrams, D.A. Cameron, M.J. Palmer, J.L. Pater, Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17, J. Natl. Cancer Inst. 97 (2005) 1262–1271.
[34] R.W. Carlson, The history and mechanism of action of fulvestrant, Clin. Breast Cancer 6 (Suppl. 1) (2005) S5–S8.
[35] W.J. Gradishar, T. Sahmoud, Current and future perspectives on fulvestrant, Clin. Breast Cancer 6 (Suppl. 1) (2005) S23–S29.
[36] A.C. Pike, A.M. Brzozowski, J. Walton, R.E. Hubbard, A.G. Thorsell, Y.L. Li, J.A. Gustafsson, M. Carlquist, Structural insights into the mode of action of a pure antiestrogen, Structure (Camb) 9 (2001) 145–153.
[37] A. Howell, J.F.R. Robertson, J. Quaresma Albano, A. Aschermannova, L. Mauriac, U.R. Kleeberg, I. Vergote, B. Erikstein, A. Webster, C. Morris, Fulvestrant, formerly ici 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment, J. Clin. Oncol. 20 (2002) 3396–3403.
[38] C.K. Osborne, J. Pippen, S.E. Jones, L.M. Parker, M. Ellis, S. Come, S.Z. Gertler, J.T. May, G. Burton, I. Dimery, A. Webster, C. Morris, R. Elledge, A. Buzdar, Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial, J. Clin. Oncol. 20 (2002) 3386–3395.
[39] M.M. Gottardis, S.P. Robinson, V.C. Jordan, Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumoristatic action of tamoxifen, J. Steroid Biochem. 30 (1988) 311–314.
[40] M.M. Gottardis, V.C. Jordan, Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration, Cancer Res. 48 (1988) 5183–5187.
[41] M.M. Gottardis, S.Y. Jiang, M.H. Jeng, V.C. Jordan, Inhibition of tamoxifen-stimulated growth of an mcf-7 tumor variant in athymic mice by novel steroidal antiestrogens, Cancer Res. 49 (1989) 4090–4093.
[42] D.M. Wolf, V.C. Jordan, A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy, Recent Results Cancer Res. 127 (1993) 23–33.
[43] D.M. Wolf, V.C. Jordan, L. William, Mcguire memorial symposium. Drug resistance to tamoxifen during breast cancer therapy, Breast Cancer Res. Treat. 27 (1993) 27–40.
[44] D.M. Wolf, V.C. Jordan, Characterization of tamoxifen stimulated MCF-7 tumor variants grown in athymic mice, Breast Cancer Res. Treat. 31 (1994) 117–127.
[45] D.M. Wolf, S.M. Langan-Fahey, C.J. Parker, R. McCague, V.C. Jordan, Investigation of the mechanism of tamoxifen-stimulated breast tumor growth with nonisomerizable analogues of tamoxifen and metabolites, J. Natl. Cancer Inst. 85 (1993) 806–812.
[46] K. Yao, E.S. Lee, D.J. Bentrem, G. England, J.I. Schafer, R.M. O'Regan, V.C. Jordan, Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice, Clin. Cancer Res. 6 (2000) 2028–2036.
[47] C. Osipo, C. Gajdos, H. Liu, B. Chen, V.C. Jordan, Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer, J. Natl. Cancer Inst. 95 (2003) 1597–1608.
[48] C. Osipo, C. Gajdos, D. Cheng, V.C. Jordan, Reversal of tamoxifen resistant breast cancer by low dose estrogen therapy, J. Steroid Biochem. Mol. Biol. 93 (2005) 249–256.
[49] R.M. O'Regan, C. Osipo, E. Ariazi, E.S. Lee, K. Meeke, C. Morris, A. Bertucci, M.A. Sarker, R. Grigg, V.C. Jordan, Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice, Clin. Cancer Res. 12 (2006) 2255–2263.
[50] H. Liu, E.-S. Lee, C. Gajdos, S.T. Pearce, B. Chen, C. Osipo, J. Loweth, K. McKian, A. De Los Reyes, L. Wing, V.C. Jordan, Apoptotic action of 17{beta}-estradiol in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*, J. Natl. Cancer Inst. 95 (2003) 1586–1597.
[51] J.S. Lewis, C. Osipo, K. Meeke, V.C. Jordan, Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal, J. Steroid Biochem. Mol. Biol. 94 (2005) 131–141.
[52] J.S. Lewis, K. Meeke, C. Osipo, E.A. Ross, N. Kidawi, T. Li, E. Bell, N.S. Chandel, V.C. Jordan, Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation, J. Natl. Cancer Inst. 97 (2005) 1746–1759.
[53] R.M. O'Regan, A. Cisneros, G.M. England, J.I. MacGregor, H.D. Muenzner, V.J. Assikis, M.M. Bilimoria, M. Piette, Y.P. Dragan, H.C. Pitot, R. Chatterton, V.C. Jordan, Effects of the antiestrogens tamoxifen, toremifene, and ICI 182,780 on endometrial cancer growth, J. Natl. Cancer Inst. 90 (1998) 1552–1558.
[54] M.M. Gottardis, R.J. Wagner, E.C. Borden, V.C. Jordan, Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth *in vivo* and *in vitro*, Cancer Res. 49 (1989) 4765–4769.
[55] S.Y. Jiang, D.M. Wolf, J.M. Yingling, C. Chang, V.C. Jordan, An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol, Mol. Cell. Endocrinol. 90 (1992) 77–86.
[56] J.F. Robertson, B. Erikstein, K.C. Osborne, J. Pippen, S.E. Come, L.M. Parker, S. Gertler, M.P. Harrison, D.A. Clarke, Pharmacokinetic profile of intramuscular fulvestrant in advanced breast cancer, Clin. Pharmacokinet. 43 (2004) 529–538.

Estrogen-Related Receptor α1 Transcriptional Activities Are Regulated in Part via the ErbB2/HER2 Signaling Pathway

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Abstract

We previously showed that (*a*) estrogen-related receptor α1 (ERRα1) down-modulates estrogen receptor (ER)–stimulated transcription in low ErbB2–expressing MCF-7 mammary carcinoma cells, and (*b*) ERRα and ErbB2 mRNA levels positively correlate in clinical breast tumors. We show here that ERRα1 represses ERα-mediated activation in MCF-7 cells because it failed to recruit the coactivator glucocorticoid receptor interacting protein 1 (GRIP1) when bound to an estrogen response element. In contrast, ERRα1 activated estrogen response element– and ERR response element–mediated transcription in ERα-positive, high ErbB2–expressing BT-474 mammary carcinoma cells, activation that was enhanced by overexpression of GRIP1. Likewise, regulation of the endogenous genes *pS2, progesterone receptor*, and *ErbB2* by ERRα1 reflected the cell type–specific differences observed with our reporter plasmids. Importantly, overexpression of activated ErbB2 in MCF-7 cells led to transcriptional activation, rather than repression, by ERRα1. Two-dimensional PAGE of radiophosphate-labeled ERRα1 indicated that it was hyperphosphorylated in BT-474 relative to MCF-7 cells; incubation of these cells with anti-ErbB2 antibody led to reduction in the extent of ERRα1 phosphorylation. Additionally, mitogen-activated protein kinases (MAPK) and Akts, components of the ErbB2 pathway, phosphorylated ERRα1 *in vitro*. ERRα1-activated transcription in BT-474 cells was inhibited by disruption of ErbB2/epidermal growth factor receptor signaling with trastuzumab or gefitinib or inactivation of downstream components of this signaling, MAPK kinase/MAPK, and phosphatidylinositol-3-OH kinase/Akt, with U0126 or LY294002, respectively. Thus, ERRα1 activities are regulated, in part, via ErbB2 signaling, with ERRα1 likely positively feedback-regulating ErbB2 expression. Taken together, we conclude that ERRα1 phosphorylation status shows potential as a biomarker of clinical course and antihormonal- and ErbB2-based treatment options, with ERRα1 serving as a novel target for drug development. (Mol Cancer Res 2007;5(1):71–85)

Introduction

The steroid nuclear receptor estrogen receptor α (ERα), officially termed NR3A1 (1), is pivotally involved in the etiology of breast cancer. ERα mediates the effects of estrogens on transcription and is expressed at high levels in approximately three fourths of human breast tumors. It thereby serves as a critical biomarker of clinical course and target for therapy (reviewed in ref. 2). The orphan nuclear receptors estrogen-related receptor α (ERRα; NR3B1), ERRβ (NR3B2), and ERRγ (NR3B3; ref. 1) exhibit a high degree of sequence similarity with ERα (reviewed in ref. 3). They do not bind naturally occurring estrogens, but share other biochemical activities with ERs, including binding to estrogen response elements (ERE; refs. 4-8). ERRs also bind to extended nuclear receptor half-site sequences resembling 5′-TNAAGGTCA-3′, referred to as ERR response elements (ERRE; refs. 5-7, 9, 10).

ERRα mRNA levels are similar or greater than ERα mRNA levels in approximately one fourth of unselected human breast cancers, with the highest levels occurring in tumors lacking functional ERα (11). Additionally, ERRα mRNA levels correlate in breast cancers with those of ErbB2 (also called HER2/*neu*; ref. 11), a marker of tumor aggressiveness. Suzuki et al. (12) reported that ~55% of human breast cancers are positive for ERRα by immunohistochemistry, with ERRα-positive status being associated with greatly increased risk of recurrence and adverse clinical outcome. Thus, ERRα shows potential as a prognosticator and target for breast cancer therapy, with ERRα possibly playing an important role by substituting for ERα activities, especially in ErbB2-positive, ERα-negative, and tamoxifen-resistant tumors.

Whereas ERα usually regulates gene expression in a ligand-inducible manner, ERRα1, the 423-amino-acid major isoform encoded by the *ESRRA* gene (National Center for

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Biotechnology Information accession NP_004442.3; refs. 4, 5), can constitutively activate transcription in the absence of ligand. ERRα1 interacts with peroxisome proliferator-activated receptor coactivator-1α (13, 14) and the p160 family of coactivators, including glucocorticoid receptor interacting protein 1 (GRIP1/SRC2; ref. 15), via a carboxyl-terminal coactivator-binding inverted LxLxxL motif (16). Bulky amino acid side chains almost completely fill the ERRα1 putative ligand-binding pocket (14, 17), with residue Phe^{329} recapitulating interactions analogous to ones provided by ligands, thereby promoting binding of coactivators (17).

ERRα1 has been shown to bind the promoter of the estrogen-inducible *pS2* gene (also called *TFF1*; ref. 18) and to modulate transcription of the estrogen precursor metabolizing genes *aromatase* (*CYP19*; ref. 19) and *DHEA sulfotransferase* (*SULT2A1*; ref. 20). It also modulates expression of the estrogen-responsive genes *lactoferrin* (4), *osteopontin* (21), and even *ERRα* itself (also called *ESRRA*; refs. 22, 23). The effect of ERRα1 binding to a transcriptional response element can be either negative or positive depending on the specific cell type (8) and promoter context (24). For example, ERRα1 down-modulates E_2-induced transcription in ERα-positive human mammary carcinoma MCF-7 cells by an active mechanism (8), yet activates gene transcription in ERα-negative human mammary carcinoma SK-BR-3 cells (19) and a variety of other cell lines, including human cervical carcinoma HeLa cells (8), human endometrial RL95-2 cells (4), human embryonic kidney 293 cells (21), and rat ROS 17/2.8 osteosarcoma cells (21).

The factors determining whether ERRα1 activates or down-modulates transcription have yet to be fully identified. Epidermal growth factor receptor (EGFR) and ErbB2, members of the ErbB family of transmembrane receptor tyrosine kinases, signal in part through the MAPK and phosphatidylinositol-3-OH kinase (PI3K)/Akt signaling pathways (25). Stimulation of these pathways can lead to activation of unliganded ERα (26), with overexpression of EGFR and ErbB2 implicated in the failure of antiestrogen therapy in both model systems (27-29) and clinical breast cancers (30-32). Thus, by analogy with ERα, we hypothesized that signaling via ErbB2 leads to phosphorylation of ERRα1, thereby modulating its activities. Findings in support of this hypothesis include the following: (*a*) ERRα1 can exist as a phosphoprotein (9); (*b*) human breast tumors that express high lev els of ErbB2 mRNA also frequently express high levels of ERRα mRNA (11); and (*c*) SK-BR-3 cells, in which ERRα1 functions as a constitutive activator (19), contain 2 orders of magnitude more ErbB2 mRNA than MCF-7 cells (33) in which it functions as a down-modulator of transcription (8).

To test the validity of this hypothesis, we examined the effects of ErbB2 signaling on the transcriptional activity and phosphorylated state of ERRα1 in MCF-7 versus BT-474 cells, another mammary carcinoma cell line with very high ErbB2 levels (33). We found that overexpression of ERRα1 led to increased accumulation of pS2, progesterone receptor (PgR), and ErbB2 mRNAs in BT-474 cells and decreased accumulation of pS2 and ErbB2 mRNAs in MCF-7 cells. ERRα1 was hyperphosphorylated in BT-474 cells compared with MCF-7 cells and could be phosphorylated by MAPKs and Akts *in vitro*. Strikingly, ERRα1 transcriptional activity was stimulated by overexpression of activated ErbB2 in MCF-7 cells and inhibited in BT-474 cells treated with (*a*) the ErbB2 inhibitor trastuzumab (Herceptin; ref. 34), (*b*) the EGFR tyrosine kinase inhibitor gefitinib (Iressa, ZD1839; ref. 35), (*c*) the MAPK kinase (MEK) inhibitor U0126 (36), or (*d*) the PI3K inhibitor LY294002 (37). Thus, we conclude that ErbB2 signaling can modulate ERRα1 activities.

Results

Effects of E_2 on Expression of ERRα in MCF-7 and BT-474 Cells

ERRα1 can either repress or activate ERE-regulated expression (8), a target sequence to which it binds exclusively as a homodimer (38). Given our finding that ERRα mRNA levels positively correlate with those of ErbB2 and ErbB3 (11), we speculated that posttranslational modifications mediated by the ErbB2-directed pathway might contribute to regulation of ERRα1 activities. We studied here two ERα-positive breast cancer cell lines, MCF-7 and BT474, known to express low and high levels of ErbB2, respectively (39), to examine the effects of ErbB2 on ERRα1 activities.

First, we measured endogenous ERRα1 and ERα protein levels and the effects of estrogen on these levels in these two cell lines. Lysates were prepared from MCF-7 and BT-474 cells cultured in estrogen-free medium and treated for 24 h with 100 pmol/L 17β-estradiol (E_2), its vehicle ethanol as a control, 1 nmol/L E_2, or 1 nmol/L E_2 plus 1 μmol/L of the complete antiestrogen fulvestrant. The proteins in the lysates were separated by SDS-PAGE, blotted to a filter, and probed with antibodies specific for ERα, ERRα, and β-actin as an internal control (Fig. 1). As expected, ERα levels were similar in the two ERα-positive cell lines (Fig. 1, *lane 1* versus *lane 5*), down-regulated following treatment with E_2 (Fig. 1, *lanes 2* and *3* versus *lane 1* and *lanes 6* and *7* versus *lane 5*), and further down-regulated in the presence of fulvestrant (Fig. 1, *lane 4* versus *lane 3* and *lane 8* versus *lane 7*), a drug known to promote proteasome-mediated degradation of ERα (40). On the

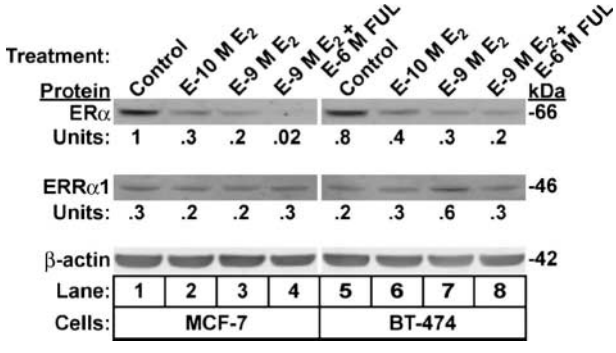

FIGURE 1. Immunoblot analysis showing endogenous expression of ERα and ERRα1 in MCF-7 and BT474 cells. Cells were cultured for 24 h in estrogen-free medium supplemented with ethanol, 100 pmol/L E_2, 1 nmol/L E_2, or 1 nmol/L E_2 plus 1 μmol/L fulvestrant. Endogenous ERα, ERRα1, and β-actin were detected using primary antibodies specific for these proteins followed by IR fluorescent dye–conjugated secondary antibodies. ERα and ERRα1 protein levels normalized to β-actin are shown as units relative to the ERα level in the control-treated MCF-7 cells (*lane 1*).

other hand, ERRα1 levels were not significantly affected by the presence of either of these ligands of ERα, except at the higher concentration of E_2 in BT-474 cells (Fig. 1). Furthermore, the ERα/ERRα protein ratios were not significantly different between the MCF-7 and BT-474 cells under either the estrogen-free (3.3 versus 4.0, respectively) or 100 pmol/L E_2 (1.5 versus 1.3, respectively) growth conditions. Thus, we can assume in the experiments presented below that differential effects on transcription were due to changes in the activities of ERRα1, not in its levels within the cells.

ERRα1 Represses ERE-Regulated Transcription in MCF-7 Cells but Activates Transcription in BT-474 Cells

We initially studied ERRα1-regulated transcription using minimal synthetic reporter genes containing (*a*) a palindromic ERE or an ERRE, (*b*) a TATA box, and (*c*) an initiator element rather than complex natural promoters. We did so to ensure observed effects were not due to indirect influences of other factors binding to other regulatory elements such as AP1- or Sp1-binding sites. Cells were cotransfected with an ERE(5×)-regulated or ERRE(5×)-regulated dual-luciferase reporter gene set, with the *Renilla* luciferase plasmid serving as an internal control for the firefly luciferase plasmid (Fig. 2A). Concurrently, cells were cotransfected in parallel with a TATA-regulated dual-luciferase reporter set (Fig. 2A), which served as an external control for experimental conditions, the physiologic state of the cells, and non–specific effects on the basal transcriptional machinery. The effect and specificity of ERRα1 was evaluated by cotransfecting the cells with a plasmid expressing wild-type ERRα1, mutant $ERR\alpha1_{L413A/L418A}$, a variant defective in the carboxyl-terminal inverted LxLxxL motif that serves as a coactivator docking site (8), or their parental empty vector. The cells were also cotransfected with a plasmid that expressed GRIP1, a member of the p160 family of coactivators, or its empty parental plasmid. Afterward, the cells were cultured for 40 h in estrogen-free medium supplemented with the indicated compounds, harvested, and assayed for firefly and *Renilla* luciferase activity.

As expected, treatment of the ERα-positive MCF-7 cells with 100 pmol/L E_2 induced ERE-regulated transcription ~19-fold (Fig. 2B, *lane 7* versus *lane 1*). Introduction of exogenous wild-type ERRα1 or mutant $ERR\alpha1_{L413A/L418A}$ led to a 68% and 71% reduction, respectively, in E_2-stimulated transcription in these cells (Fig. 2B, *lanes 9* and *11* versus *lane 7*). The finding that addition of $ERR\alpha1_{L413A/L418A}$ did not lead to complete loss of the E_2-stimulated activity indicates that some of the promoter sites probably remained occupied by ERα in these high ERα–expressing cells. Overexpression of the coactivator GRIP1 enhanced E_2-stimulated transcription an additional 1.8-fold (Fig. 2B, *lane 8* versus *lane 7*), most likely by stimulating the activity of ERα bound to the EREs. This finding indicates that GRIP1 was limiting in these cells. Nevertheless, overexpression of GRIP1 failed to overcome the down-modulation by ERRα1 of the ERα-stimulated transcription (Fig. 2B, *lane 10* versus *lane 8*). Confirming and extending prior findings (8), we conclude that ERRα1 acted as a repressor in MCF-7 cells, down-modulating ERα-stimulated transcription. Importantly, whereas overexpression of the coactivator GRIP1 enhanced ERα activity, it failed to convert ERRα1 to an activator.

To examine ERRα1 activity via EREs in the absence of ERα-stimulated transcription, the cotransfected cells were cultured in estrogen-free medium supplemented with (*a*) only the drug vehicle, ethanol (Fig. 2B, *lanes 1-6*), or (*b*) the complete antiestrogen fulvestrant along with 100 pmol/L E_2 (Fig. 2B, *lanes 13-18*). Under either of these conditions, overexpression of wild-type ERRα1 led to a barely significant increase in transcription (Fig. 2B, *lanes 3, 4, 15, 16* versus *lanes 1, 2, 13, 14*, respectively). Thus, ERRα1 exhibited a very low level of activator activity in MCF-7 cells when ERα is absent.

We likewise examined the ability of ERRα1 to modulate ERE-regulated transcription in BT-474 cells (Fig. 2D). In the absence of ERα-stimulated transcription, overexpression of wild-type ERRα1 led to an ~5-fold increase in ERE-regulated transcription (Fig. 2D, *lane 3* versus *lane 1* and *lane 15* versus *lane 13*). Overexpression of GRIP1 led to an additional 2-fold enhancement in ERRα1-stimulated transcription (Fig. 2D, *lane 4* versus *lane 3* and *lane 16* versus *lane 15*) as well as a 2-fold enhancement in ERα-stimulated transcription (Fig. 2D, *lane 8* versus *lane 7*). Thus, GRIP1 was limiting in the BT-474 cells. The $ERR\alpha1_{L413A/L418A}$ mutant variant failed to enhance expression (Fig. 2D, *lanes 5*, *6, 17*, and *18* versus *lanes 1, 2, 13*, and *14*, respectively). Overexpression of wild-type ERRα1 did not significantly alter the level of ERE-regulated transcriptional activity when the BT-474 cells were incubated in the presence of 100 pmol/L E_2 (Fig. 2D, *lanes 9* and *10* versus *lanes 7* and *8*). This finding was likely due to ERRα1 simply substituting for ERα as another activator of transcription when it displaced ERα for binding the EREs. By contrast, the $ERR\alpha1_{L413A/L418A}$ mutant led instead to a 50% to 60% reduction in E_2-stimulated transcription (Fig. 2D, *lanes 11* and *12* versus *lanes 7* and *8*). This reduction in expression was similar to the one observed in E_2-stimulated ERE-regulated transcription by wild-type ERRα1 in MCF-7 cells. Hence, a mutant defective in docking coactivators mimicked in BT-474 cells the repressor activity of wild-type ERRα1 seen in MCF-7 cells. Thus, in contrast to the results observed in MCF-7 cells, ERRα1 activated ERE-regulated transcription in BT-474 cells, likely doing so in part via GRIP1 interaction with its carboxyl-terminal coactivator binding motif.

ERRα1 also regulates transcription via ERREs, including the sequence 5′-TCAAGGTCA-3′. In sharp contrast to the effects observed on ERE-regulated expression, ERRE-regulated expression in MCF-7 cells was only slightly affected by either incubation with E_2 (Fig. 2C, *lanes 7* and *8* versus *lanes 1* and *2*) or overexpression of wild-type ERRα1 (Fig. 2C). Hence, neither ERα nor ERRα1 significantly affect ERRE-regulated transcription in MCF-7 cells.

ERRE-regulated expression was also unaffected by incubation with 100 pmol/L E_2 in BT-474 cells (Fig. 2E, *lanes 7* and *8* versus *lanes 1* and *2*). However, independent of E_2, ERRα1 activated ERRE-regulated transcription ~2.7- to 3-fold and 5.5- to 6-fold in the absence and presence of GRIP1, respectively (Fig. 2E, *lanes 3* and *4* versus *lanes 1* and *2*; *lanes 9* and *10* versus *lanes 7* and *8*; *lanes 15* and *16* versus *lanes 13* and *14*). Again, the $ERR\alpha1_{L413A/L418A}$ mutant failed to induce transcription (Fig. 2E, *lanes 5* and *6* versus *lanes 1* and *2*; *lanes 11* and *12* versus *lanes 7* and *8*; *lanes 17* and *18*

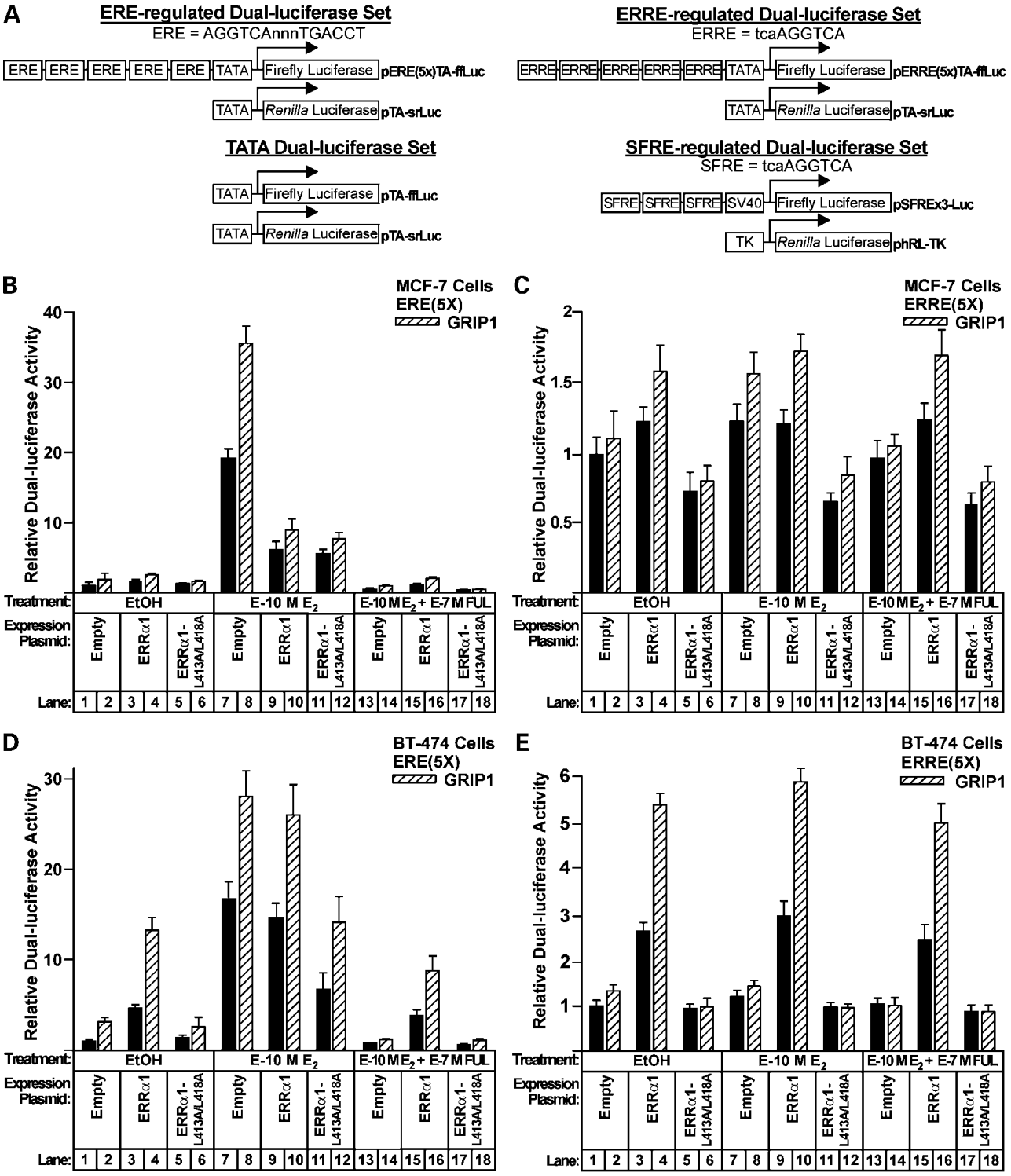

FIGURE 2. Differential transcriptional activity of ERRα1 in low ErbB2–expressing MCF-7 cells versus high ErbB2–expressing BT-474 cells. **A.** Reporter gene sets used in this study. **B** to **E.** MCF-7 and BT-474 cells were cotransfected with the ERE(5×) or ERRE(5×)-regulated dual luciferase reporter gene sets along with ERRα1, ERRα1$_{L413A/L418A}$, GRIP1, or empty parental plasmids as indicated. As an external normalization control, cells were also cotransfected in parallel for each condition indicated with the TATA-regulated dual-luciferase reporter set in place of the ERE(5×) or ERRE(5×) reporter sets. Cells were incubated for 40 h in estrogen-free medium supplemented with ethanol (*EtOH*), 100 pmol/L E_2, or 100 pmol/L E_2 plus 100 nmol/L fulvestrant as indicated. Columns, mean of samples processed in triplicate; bars, SE. Data are presented relative to the luciferase activity present in the cells assayed in lane 1 of each figure. Hatched columns, cells cotransfected with the GRIP1 expression plasmid; solid columns, cells cotransfected with its empty parental plasmid.

versus *lanes 13* and *14*), indicating dependence of ERRα1 activation via ERREs on coactivator docking. Thus, ERRα1 activated both ERE- and ERRE-regulated transcription in BT-474 cells.

Vanacker et al. (7) previously reported that ERα can efficiently bind to and activate transcription via an ERRE. We observed here that E_2 very weakly induced ERRE-regulated transcription. To address this discrepancy, we examined the responsiveness to E_2 of the ERE- and ERRE-regulated reporter gene sets studied above in parallel with a previously described ERRα1-responsive reporter, pSFRE×3-Luc, which contains three copies of the same core extended ERE half-site driving an SV40 minimal early promoter (Fig. 2A; ref. 7). As expected, the ERE(5×)-regulated reporter efficiently responded to E_2 in a concentration-dependent manner, with E_2 maximally inducing ERE-regulated activity 14-fold in MCF-7 (Fig. 3A) and 12-fold in BT-474 cells (Fig. 3B). However, supraphysiologic concentrations of E_2 up to 1 μmol/L maximally induced the ERRE (5×)-regulated reporter set by 2.1-fold and the SFRE×3-regulated reporter set by 1.5-fold in MCF-7 cells (Fig. 3A). Similarly, the maximum level of ERRE(5×)- and SFRE×3-regulated reporter activity was 1.6-fold in BT-474 cells (Fig. 3B). Hence, ERREs exhibited only minimal responses to E_2-stimulated ERα, effects that could have been indirect.

To understand the reason for the lack of responsiveness of the ERRE/SFRE–regulated promoters to E_2, we also examined whether ERα could bind to this ERRE sequence. Competition electrophoretic mobility shift assays were done using a radiolabeled, double-stranded ERE-containing oligodeoxynucleotide as probe; whole-cell extracts obtained from COS cells containing overexpressed ERα or ERRα1 as protein source; and unlabeled, double-stranded oligodeoxynucleotide containing an ERE, mutant ERE, or ERRE sequence as competitor (Fig. 3C). As expected, ERα efficiently bound the ERE (Fig. 3C, *lanes 5-7* versus *lane 4*), but not the mutant ERE (Fig. 3C, *lanes 8-10*). Contrary to a prior report (7), ERα also failed to significantly bind the ERRE (Fig. 3C, *lanes 11-13* versus *lane 4*). On the other hand, ERRα1 efficiently bound both the ERE (Fig. 3C, *lanes 16-18*, versus *lane 15*) and the ERRE (Fig. 3C, *lanes 22-24*), but not the mutant ERE (Fig. 3C, *lanes 19-21*). Thus, we conclude that ERα does not significantly bind to the extended half-site ERRE consensus sequence 5′-TCAAGGTCA-3′, whereas ERRα1 can efficiently bind both the consensus sequence and at least some EREs.

Differential Regulation of Endogenous Cellular pS2, PgR, and ErbB2 Genes by ERRα1 in MCF-7 versus BT-474 Cells

Does ERRα1 also differentially regulate expression of endogenous cellular genes in a cell type–dependent manner? To begin to answer this question, we examined the promoter regions of cellular genes implicated in breast cancer for potential ERREs by searching a eukaryotic promoter database[3] (41) and Genbank.[4] The binding affinities of ERRα1 for these putative sites relative to a consensus ERRE were determined by semiquantitative competition EMSAs done with the consensus ERRE-containing double-stranded oligonucleotide serving as the radiolabeled probe DNA. ERRα1 bound these sites with a variety of affinities (Table 1). Interestingly, ERRα1 bound the *PgR* site 1 with a higher relative binding affinity (RBA, 1.85) than the reference ERRE although they contain the same ERRE extended half-site sequence. Thus, the precise context of an ERRE can modulate ERRα1 binding affinity for it. ERRα1 also bound quite well to the *ErbB2* (RBA, 1.08), *PgR* site 2 (RBA, 0.90), and *pS2* site 2 (RBA, 0.50) sequences.

To examine the effects of ERRα1 on expression of the endogenous cellular genes *pS2, PgR*, and *ErbB2* in MCF-7 and BT-474 cells, cells were cotransfected in parallel with plasmids encoding enhanced green fluorescent protein (EGFP) and either wild-type ERRα1 or its empty parental vector. After incubation for 48 h in estrogen-free medium to avoid effects due to ERα, EGFP-positive cells were isolated by fluorescence-activated cell sorting. RNA was purified from these EGFP-positive cells and assayed by quantitative real-time PCR for amounts of pS2, PgR, ErbB2, and ERRα mRNA relative to cellular 18S rRNA as an internal control (Fig. 4). Consistent with this protocol having worked successfully, ERRα mRNA levels were found to be 50- to 67-fold higher in the cells (isolated by fluorescence-activated cell sorting) transfected with the ERRα1 expression plasmid compared with the ones transfected with the empty parental plasmid (data not shown). Whereas overexpression of ERRα1 in MCF-7 cells led to a modest decrease or no change in expression of these three genes (Fig. 4A-C, *lane 2* versus *lane 1*), it led to a 2- to 11-fold increase in their expression in BT-474 cells (Fig. 4A-C, *lane 4* versus *lane 3*). Furthermore, with the basal level of ErbB2 mRNA already 5-fold higher in BT-474 cells than in MCF-7 cells (Fig. 4C, *lane 3* versus *lane 1*), the resulting differential expression of ErbB2 increased to a highly significant 30-fold when ERRα1 was overexpressed (Fig. 4C, *lane 4* versus *lane 2*). Thus, we conclude that ERRα1 differentially regulates endogenous target genes as well as synthetic reporter ones in a cell type–dependent manner.

Extent of Phosphorylation of ERRα1 Correlates with its Ability to Activate Transcription

To begin to determine the mechanism(s) of ERRα1 cell type–dependent activity, we examined ERRα1 phosphorylation status. MCF-7 and BT-474 cells were incubated with [^{32}P]P_i; ERRα1 was immunoprecipitated from protein extracts prepared from these cells; and its phosphorylated isoforms were resolved by two-dimensional PAGE. Although BT-474 cells were found to contain predominantly one highly phosphorylated isoform of ERRα1, MCF-7 cells contained several differentially phosphorylated isoforms of ERRα1 (Fig. 5A). Given the ^{32}P label was roughly equally distributed among three isoforms of ERRα1 in the MCF-7 cells, the percentage of ERRα1 by moles in the most highly phosphorylated isoform in these cells was at most 15%. Thus, BT-474 cells contained a much larger percentage of their ERRα1 in a highly phosphorylated isoform than did the MCF-7 cells.

The monoclonal antibody (mAb) 4D5 is the murine precursor of the humanized antibody trastuzumab. They share the same epitope-reacting regions, disrupting the ErbB2

[3] http://www.epd.isb-sib.ch/.
[4] http://www.ncbi.nlm.nih.gov/.

signaling pathway without affecting the overall amount of ERRα1 per cell (Fig. 7). Incubation of BT-474 cells with antibody 4D5 led to a significant reduction in the extent of ERRα1 phosphorylation, with the appearance of several phosphorylated isoforms of ERRα1 in a pattern somewhat similar to the one observed with the MCF-7 cells (Fig. 5A). The lower amount of phospo-labeled ERRα1 observed in the 4D5-treated cells was due to this treatment being inhibitory to cell growth (data not shown). Thus, we confirmed prior reports that ERRα1 is a phosphoprotein (9, 38). We also conclude that ERRα1 was phosphorylated *in vivo* at several sites, with the extent of phosphorylation being cell type dependent and reduced by disruption of the ErbB2 signaling pathway. Importantly, the extent of phosphorylation correlated with the transcriptional activity of ERRα1: The partially phosphorylated isoforms present in MCF-7 cells likely functioned as repressors, whereas the highly phosphorylated isoform(s) present in BT-474 cells probably functioned as activators.

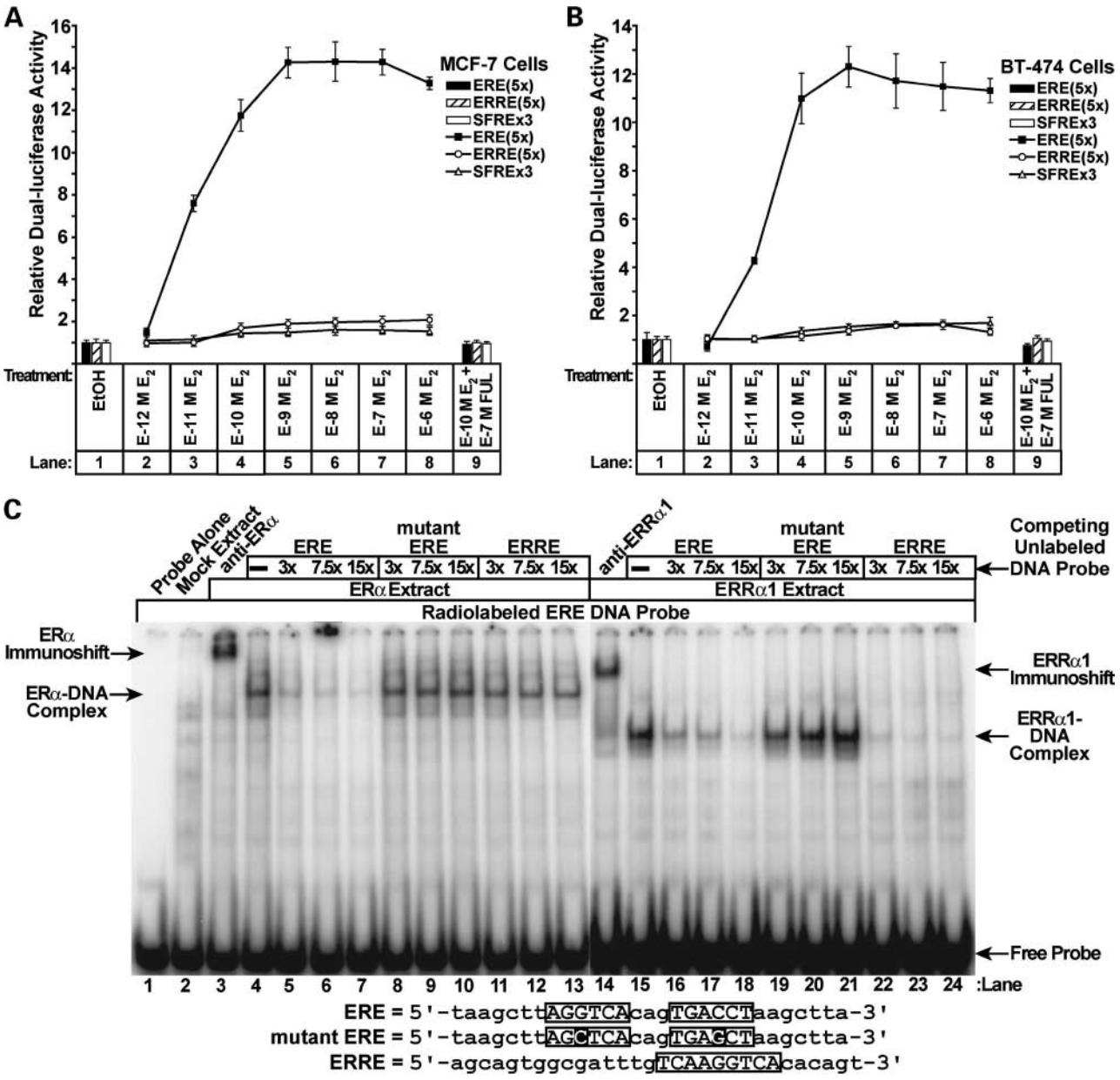

FIGURE 3. ERα activity on a palindromic ERE sequence compared with an extended half-site ERRE sequence. MCF-7 (**A**) and BT-474 (**B**) cells were cotransfected as described in Fig. 2 with the indicated dual-luciferase reporter gene sets and cultured in estrogen-free medium supplemented with ethanol. Concentrations of E_2 range from 1 pmol/L to 1 μmol/L, or 100 pmol/L E_2 plus 100 nmol/L fulvestrant as indicated. Cells were harvested 40 h later and assayed for luciferase activity, with normalization to both the internal and external reporter genes. Points, means of samples processed in triplicate; bars, SE. **C.** EMSAs showing ERRα1, but not ERα, binds the ERRE as well as the ERE with high affinity. Competition EMSAs were done with whole-cell extracts of COS cells transfected with plasmids expressing ERα or ERRα1 serving as protein source, a radiolabeled double-stranded oligonucleotide containing an ERE (5′-taagcttAGGTCAcagTGACCTaagctta-3′) serving as probe, and unlabeled double-stranded oligonucleotides corresponding to the sequences indicated below the gel serving as competitors. Capitalized letters within boxes, ERE and ERRE extended half-site sequences. White letters on black, mutations.

Table 1. ERRα1 Relative Binding of Affinities (RBAs) for Sequences in Promoters of Human Genes Implicated in Breast Cancer

Gene	Location*	Oligonucleotide Sequence	RBA
PgR site 1	−3,294	tcctaaggactg**TCAAGGTCA**tcaaatacaagg	1.85
ErbB2	−3,441	aaaggaactttc**CCAAGGTCA**cagagctgagct†	1.08
Reference ERRE	NA	agcagtggcgatttg**TCAAGGTCA**cacagt	1.00
PgR site 2	−5,166	tccttgctaaac**CCAAGGTCA**taaatcttttct†	0.90
Erβ	−559	ggtgctcccact**TAGAGGTCA**cgcgcggcgtcg	0.56
pS2 site 2	−407	tcccttcccct**GCAAGGTCA**cggtggccaccc	0.50
Cathepsin D	−3,635	tggcatattggg**TGAAGGTCA**agggagtggctt†	0.49
IGF1R	+272	gctccggctcgc**TGAAGGTCA**cagccgaggcga†	0.37
Human MDM2	+575	gggagttcaggg**TAAAGGTCA**cggggccggggc	0.35
Prolactin	−1,347	caaatttgaaac**TAAAGGTCA**caggctgcttta	0.32
IGF2 site 2	−6,479	ctgtcggcagga**ACAAGGTCA**ccccttggcgtt†	0.23
elk1	−2,185	ctcccatctcac**TTAAGGTCA**aagccagggtcc	0.21
BRCA1	−293	gtaattgctgta**CGAAGGTCA**gaatcgctacct†	0.19
aromatase	−99	cctgagactcta**CCAAGGTCA**gaaatgctgcaa	0.18
PgR site 3	−5,912	aaaattgttttg**TCTAGGTCA**tttgcattttca†	0.14
EGF	−396	caaataatgggc**TGAAGGTGA**actatctttact	0.14
pS2 site 1	−266	gtaggacctgga**TTAAGGTCA**ggttggaggaga	0.11
ERα	−865	atgtttggtatg**AAAAGGTCA**catttatattc	0.10

Abbreviation: NA, not applicable.
*Location of the ERRE sequence relative to the gene transcription start site.
† Sequence found in reverse orientation in the natural promoter.

Activated MAPKs and Akts Phosphorylate ERRα1 In vitro

We next tested whether ERRα1 can serve *in vitro* as a substrate of MAPKs and Akts, downstream kinases in the ErbB2 signaling pathway. We incubated equal amounts of *Escherichia coli*–produced, carboxyl-terminal 6×His-tagged ERRα1 (ERRα1-His) with activated MAPK1, MAPK2, Akt1, or Akt2 in the presence of [γ-^{32}P]ATP and resolved the resulting phosphorylated products by 4% to 12% gradient SDS-PAGE. Myelin basic protein was included in each reaction as an internal control. Each of these four kinases phosphorylated ERRα1 *in vitro* (Fig. 5B). Interestingly, differences were observed in the mobilities of the phosphorylated proteins, consistent with multiple sites on ERRα1 being phosphorylated by the MAPKs (Fig. 5B, *lanes 1* and *2*) and fewer sites being phosphorylated by the Akts (Fig. 5B, *lanes 3* and *4*).

To begin to localize sites of phosphorylation by 1-423 MAPK2, full-length glutathione *S*-transferase (GST)-ERRα1$_{1-423}$ and truncated variants of it were synthesized in and purified from *E. coli*. Equimolar amounts of each protein were incubated with activated p42 MAPK and [γ^{32}P]ATP and resolved by 12% SDS-PAGE (Fig. 5C). Phosphorylated heat- and acid-stable protein regulated by insulin (PHAS-I) and GST-β-globin$_{1-123}$

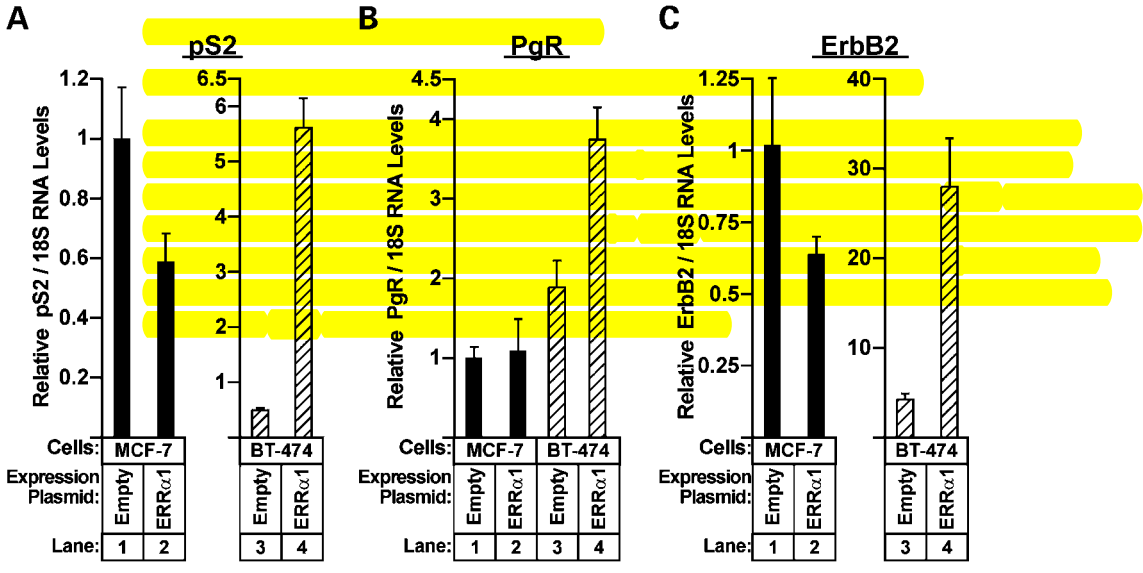

FIGURE 4. Effects of ERRα1 overexpression in MCF-7 (*black columns*) and BT-474 cells (*hatched columns*) on expression of the endogenous cellular genes encoding pS2 (**A**), PgR (**B**), and ErbB2 (**C**) mRNA. Cells were cotransfected with pEGFP and the ERRα1 expression plasmid or its empty parent plasmid, pcDNA3.1 and incubated under estrogen-free conditions. Twenty-four hours later, EGFP-positive cells were isolated by fluorescence-activated cell sorting. The pS2, PgR, and ErbB2 RNAs in cells were analyzed by quantitative real-time PCR, with normalization to the 18S rRNA present in the same RNA samples. Data are shown relative to empty parental plasmid transfected MCF-7 cells (*lane 1*). Columns, mean of samples processed in quadruplicate; bars, SE.

were assayed in parallel as positive and negative controls, respectively. As expected, activated MAPK efficiently phosphorylated PHAS-1, but not GST-β-globin$_{1-123}$ (Fig. 5C, *lane 1* versus *lane 5*, respectively). MAPK phosphorylated each of the GST-ERRα1 fusion proteins, with significantly more label incorporated into GST-ERRα1$_{1-423}$ than into GST-ERRα1$_{1-376}$ and GST-ERRα1$_{1-173}$ (Fig. 5C, *lane 4* versus *lanes 3* and *2*). Thus, ERRα1 can serve as a substrate of MAPK1/2 and Akt1/2, with multiple phosphorylation sites likely present within the protein, including at least one within the carboxyl-terminal domain.

Overexpression of ErbB2 in MCF-7 Cells Converts ERRα1 to an Activator

Given that ERRα1 transcriptional activity correlated with the cell ErbB2 status (Figs. 2 and 4), we desired to test more directly whether altering cellular ErbB2 signaling would affect ERRα1 transcriptional activity. One approach we used was to cotransfect MCF-7 cells in parallel with (*a*) the ERE (5×)-regulated and TATA-regulated dual luciferase reporter sets; (*b*) the expression plasmid encoding wild-type ERRα1; and (*c*) pErbB2$_{Act}$, an expression plasmid encoding an activated (oncogenic) form of rat ErbB2 (rat *neu*; ref. 42) or its empty vector as a control. The cells were cultured in estrogen-free medium in the absence or presence of 1 μmol/L fulvestrant to prevent complications from ERα. As observed above (Fig. 2B), overexpression of ERRα1 alone led to minimal activation of ERE-regulated transcription (Fig. 6A, *lane 2* versus *lane 1* and *lane 6* versus *lane 5*). Addition of ErbB2$_{Act}$ without exogenous ERRα1 stimulated ERE-regulated transcription ~2-fold under both estrogen-free conditions and in the presence of fulvestrant (Fig. 6A, *lane 3* versus *lane 1* and *lane 7* versus *lane 5*, respectively). Hence, this activation by ErbB2$_{Act}$ was probably mediated via endogenous ERRα1, not ERα. Strikingly, overexpression of ERRα1 together with ErbB2$_{Act}$ stimulated ERE-regulated transcription almost 4-fold (Fig. 6A, *lane 4* versus *lane 1* and *lane 8* versus *lane 5*). This effect of ErbB2$_{Act}$ occurred without a change in the level of ERRα1 (Fig. 6B, *lanes 2, 4, 6*, and *8*). Thus, we conclude that ERRα1 transcriptional activity can be altered by ErbB2 signaling.

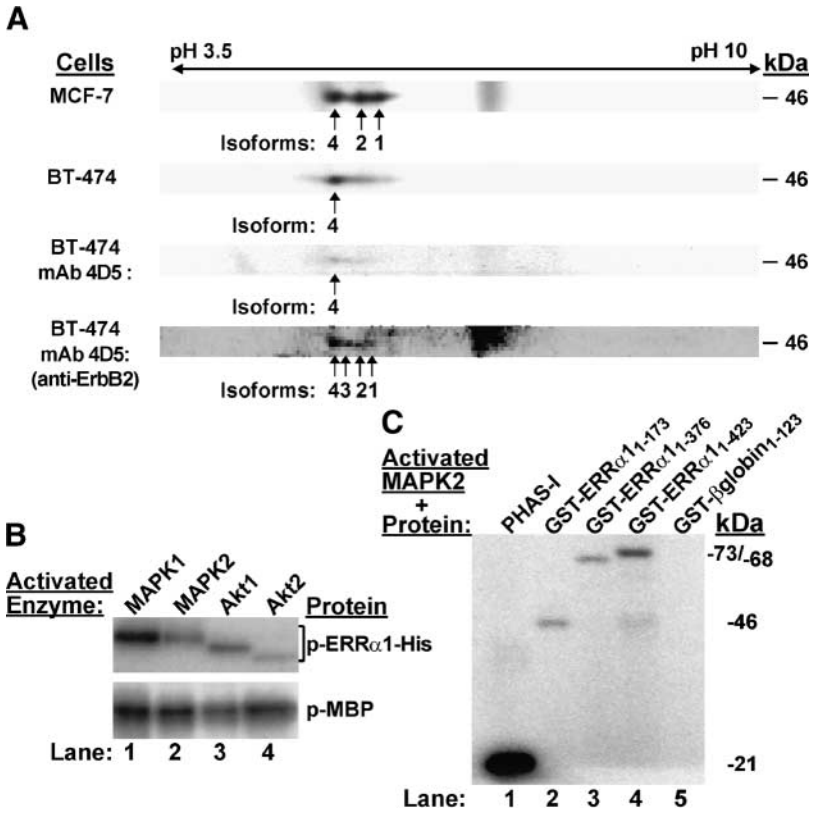

FIGURE 5. Phosphorylation of ERRα1 *in situ* and *in vitro*. **A.** Autoradiograms of two-dimensional gels showing the *in situ* phosphorylated states of ERRα1 in MCF-7 cells, BT-474 cells, and BT-474 cells incubated with a murine mAb to ErbB2 (HER2). MCF-7 and BT-474 cells were transfected in parallel with the wild-type ERRα1 expression plasmid. Twenty-seven hours later, the cells were metabolically labeled by incubation for 4 h in phosphate-free medium supplemented with [γ^{32}P]ATP. Afterward, whole-cell extracts were prepared. ERRα1 was immunoprecipitated with an anti-GST-hERRα1 polyclonal antiserum and resolved by two-dimensional PAGE. The mAb 4D5–treated cells incorporated less ^{32}P because they were growth inhibited; a second, longer exposure of this same gel is shown directly below the original one. **B.** *In vitro* phosphorylation of ERRα1 using activated MAPKs and Akts. Equal amounts of 6×His-tagged ERRα1 were incubated in parallel with activated MAPK1, MAPK2, Akt1, and Akt2 along with [γ^{32}P]ATP. The products were resolved by 4% to 12% gradient SDS-PAGE and visualized by autoradiography. Myelin basic protein (*MBP*) was included in the reactions as an internal positive control. **C.** Localization of sites of phosphorylation of ERRα1 *in vitro* by activated MAPK2. Equimolar amounts of the indicated GST-ERRα1 fusion proteins were incubated with activated MAPK2 and [γ^{32}P]ATP. PHAS-I and GST-β-globin (molecular weight 41 kDa) were incubated likewise in parallel as positive and negative controls, respectively. The products were resolved by 12% SDS-PAGE and visualized with a PhosphorImager.

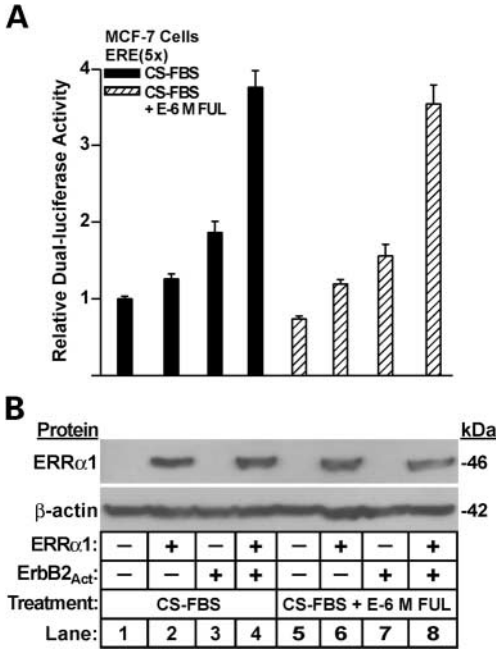

FIGURE 6. Overexpression of $ErbB2_{Act}$ (activated rat *neu* oncogene) leads to activation of ERRα1 in MCF-7 cells. **A.** MCF-7 cells were cotransfected with the ERE(5×)-regulated dual-luciferase sets described in Fig. 2, plasmids expressing ERRα1 or their empty expression plasmid, and $ErbB2_{Act}$ or its empty plasmid as indicated. Cells were harvested 48 h later. Columns, mean of samples processed in triplicate relative to the level present in the cells in lane 1; bars, SE. Solid columns, cells incubated in charcoal-stripped serum (*CS-FBS*); cross-hatched columns, cells incubated in charcoal-stripped serum supplemented with E−6 mol/L fulvestrant (*FUL*). **B.** Immunoblot analysis of ERRα1 present in MCF-7 cells treated as in **A**. The membrane was probed with antibodies specific to ERRα1 and β-actin followed by horseradish peroxidase–conjugated secondary antibodies and visualized by enhanced chemiluminescence and autoradiography.

Inhibition of ErbB2 Signaling Abrogates Transcriptional Activation by ERRα1

We next investigated whether blocking specific components within the ErbB2 signaling pathway led to inhibition of transcriptional activation by ERRα1. BT-474 cells were cotransfected with the reporter gene sets and expression plasmids as described in Fig. 2D (*lanes 1-4*). They were subsequently incubated for 40 h in estrogen-free medium supplemented with 20 μg/mL nonspecific murine IgG as a control, the humanized anti-ErbB2 mAb trastuzumab at 20 μg/mL, or the small-molecule EGFR inhibitor gefitinib at 1 μmol/L (Fig. 7A). Gefitinib blocks transphosphorylation of ErbB2 by EGFR, thereby indirectly inhibiting ErbB2 (35). As expected, overexpression of ERRα1 in the IgG-treated cells led to a 4- to 5-fold activation of ERE(5×)-regulated transcription (Fig. 7A, *lanes 3* and *4* versus *lanes 1* and *2*). Incubation with trastuzumab led to an ∼85% reduction in ERE-regulated transcription by ERRα1 (Fig. 7A, *lanes 7* and *8* versus *lanes 3* and *4*) to a level even below that observed in the presence of only endogenous ERRα1 in the absence of the drug (Fig. 7A, *lanes 1* and *2*). This large reduction was probably due to treatment with trastuzumab altering as well the transcriptional activity of the endogenous ERRα1 (Fig. 7A, *lanes 5* and *6* versus *lanes 1* and *2*). Incubation with gefitinib led to an even greater ∼90% reduction in ERE-regulated transcription by ERRα1 (Fig. 7A, *lanes 11* and *12* versus *lanes 3* and *4*). Overexpression of GRIP1 largely failed to reverse the effect of the drug treatments (Fig. 7A, *even-numbered lanes*). Immunoblot analysis with an ERRα1-specific antiserum showed that incubation with the drugs had not affected accumulation of ERRα1 in the cells (Fig. 7C, *lanes 1-3*). Immunoblot analysis with antisera specific to the phosphorylated versus unphosphorylated forms of MAPK and Akt confirmed that these drug treatments had, indeed, inhibited activation of the MEK/MAPK and PI3K/Akt signaling pathways in these cells (Fig. 7D, *lanes 1-3*). Thus, we conclude that disruption of the ErbB2 signaling pathway with either trastuzumab or gefitinib prevented ERRα1 from functioning as an activator of ERE-regulated transcription in BT-474 cells, likely doing so in part by inhibiting addition of specific posttranslational phosphorylations of ERRα1 necessary for it to exist in its activator form. Moreover, blocking the ErbB2-directed cascade of signaling events rendered ERRα1 unresponsive to GRIP1-mediated coactivation.

To test whether inhibition of ErbB2 signaling modulates ERRα1 activity by affecting the activities of downstream components in this pathway, we likewise examined the effects on ERRα1 activity of incubation of BT-474 cells with U0126 and LY294002, direct inhibitors of MEK and PI3K, respectively (see Fig. 8). In the cells treated with only DMSO, the solvent for these drugs, overexpression of ERRα1 led, as expected, to an ∼5-fold activation of ERE-regulated transcription (Fig. 7B, *lane 3* versus *lane 1*). Incubation with 20 μmol/L U0126 led to an ∼50% reduction in ERRα1-induced transcription regardless of whether GRIP1 was also overexpressed (Fig. 7B, *lanes 7* and *8* versus *lanes 3* and *4*). The effect of incubation with 20 μmol/L LY294002 was even greater, inhibiting ERRα1-mediated activation of ERE-regulated transcription by 75% to 85% (Fig. 7B, *lanes 11* and *12* versus *lanes 3* and *4*). Again, immunoblot analysis showed that the drug-treated cells still accumulated ERRα1 (Fig. 7B, *lanes 4* and *5*), with U0126 having led to inhibition of MAPK phosphorylation without affecting Akt status (Fig. 7D, *lane 4*) and LY294002 having led to inhibition of Akt phosphorylation without affecting MAPK status (Fig. 7D, *lane 5*). Therefore, the MEK/MAPK and PI3K/Akt signaling pathways contribute to the ability of ERRα1 to activate ERE-regulated transcription in BT-474 cells.

Discussion

We showed here that ERRα1 down-modulated E_2-induced ERE-regulated transcription in low ErbB2–expressing MCF-7 cells, doing so even when the coactivator GRIP1 was overexpressed (Fig. 2B). This inability of GRIP1 to overcome repression by ERRα1 was not due to lack of functionality because GRIP1 efficiently enhanced ERE-regulated expression mediated by an amino-terminal deleted variant of ERRα1 in these cells.[5] Thus, the failure of wild-type ERRα1 to respond to GRIP1 must lie with its intrinsic properties in this cell line. On the other hand, wild-type ERRα1 functioned instead as a

[5] E.H. Vu et al., submitted for publication.

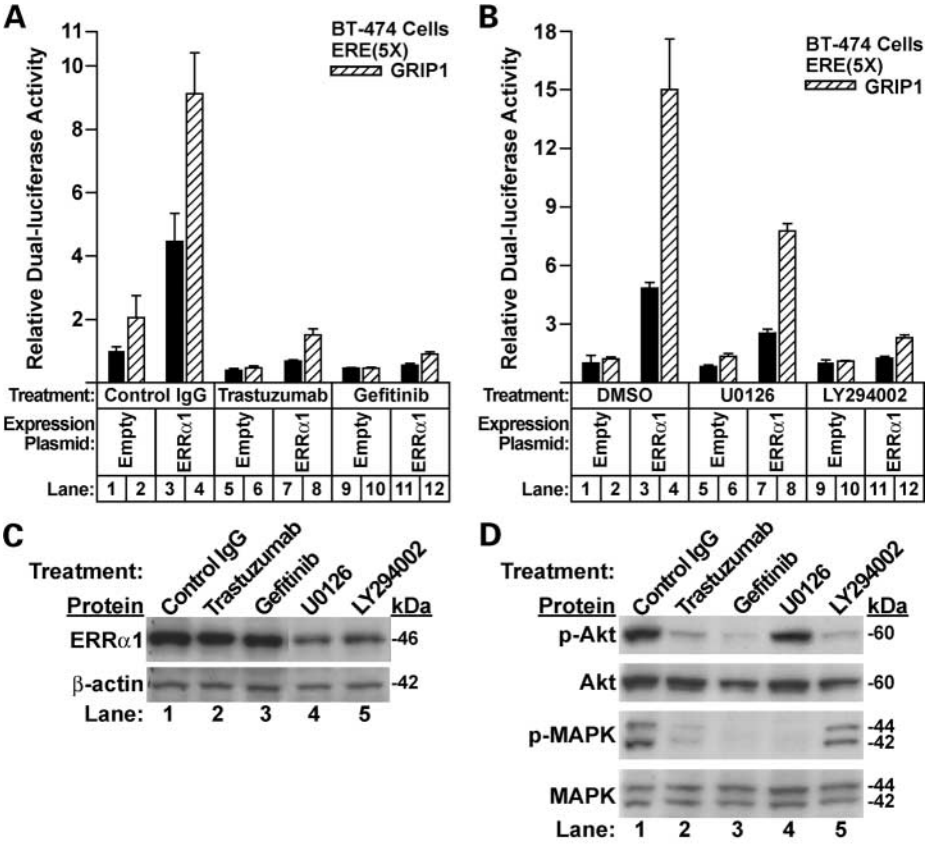

FIGURE 7. Effects of modulation of the ErbB2 signaling pathway on ERRα1-mediated activation of transcription. **A** and **B.** BT-474 cells were cotransfected as described in Fig. 2 with the ERE(5×)-regulated dual-luciferase reporter set along with the indicated expression plasmids and, likewise, in parallel with the TATA-regulated reporter set. They were incubated for 40 h in estrogen-free medium supplemented with (**A**) 20 μg/mL nonspecific mouse IgG, 20 μg/mL trastuzumab, or 1 μmol/L gefitinib as indicated; or (**B**) DMSO as the drug vehicle control, 20 μmol/L U0126, or 20 μmol/L LY294002 as indicated. Columns, mean of samples processed in triplicate relative to the levels present in the cells in lanes 1; bars, SE. Hatched columns, cells cotransfected with the GRIP1 expression plasmid; solid columns, cells cotransfected with its empty parental plasmid. **C.** Immunoblot analysis of overexpressed ERRα1 in BT-474 cells treated as in **A** and **B**. The membranes were probed with antibodies that react specifically with ERRα1 and β-actin as a control. Reactive proteins were visualized by enhanced chemiluminescence and autoradiography. Lanes 1 to 3, samples from **A**; lanes 4 and 5, samples from **B** done on a different day. **D.** Immunoblot analysis of the phosphorylation status of Akt and MAPK in BT-474 cells treated as in **A** and **B**. The membranes were probed with antibodies that react specifically with phosphorylated Akt (*p-Akt*), total Akt, phosphorylated p42/44 MAPK (*p-MAPK*), and total p42/44 MAPK.

ligand-independent activator of ERE-regulated transcription in BT-474 cells, activity that was further stimulated by overexpression of GRIP1 (Fig. 2D). Thus, ERRα1 transcriptional activity and ability to recruit GRIP1 is cell type dependent.

ERRα1 also bound to (Fig. 3C, *lanes 14-24*) and activated transcription via ERREs (Fig. 2E) in BT-474 cells. Putative ERREs were identified in the promoter regions of multiple cellular genes implicated in breast cancer (Table 1), three of which, *pS2, PgR*, and *ErbB2*, were shown here to be upregulated in response to ERRα1 in BT-474 cells, but not in MCF-7 cells (Fig. 4). This is the first report showing that ERRα1 affects *ErbB2* expression, with ErbB2 mRNA levels activated almost 30-fold above the level observed in MCF-7 cell. Given that ErbB2 is an indicator of aggressive tumor growth, its regulation by ERRα1 provides another potential link for ERRα1 playing a role in the development of some breast cancers.

We also showed here that ERRα1 can exist in multiple phosphorylated isoforms *in vitro* (Fig. 5B and C) and *in situ* (Fig. 5A). The extent of this phosphorylation was significantly greater, on average, in BT-474 cells than in MCF-7 cells and reduced by treatment of BT-474 cells with the murine version of trastuzumab (Fig. 5A). Furthermore, ERRα1 could serve directly as a substrate of activated MAPKs (Fig. 5B, *lanes 1* and *2*, and C) and Akts (Fig. 5B, *lanes 3* and *4*) *in vitro*. Importantly, ERRα1 was shown to be a target of ErbB2 signaling: (*a*) Overexpression of an activated ErbB2 oncogene converted ERRα1 from a repressor to an activator of ERE-regulated transcription in MCF-7 cells (Fig. 6); and (*b*) disruption of ErbB2 signaling in BT-474 cells with trastuzumab, gefitinib, U0126, or LY294002 converted ERRα1 from an activator to a repressor of ERE-regulated transcription (Fig. 7). Therefore, we conclude that ERRα1 transcriptional activity is regulated, in part, by the ErbB2 signaling pathway affecting the precise state of phosphorylation of ERRα1 (Fig. 8).

Cross-talk between ERα and ERRα1

ERα and ERRα1 can both bind EREs, competing for binding to them (refs. 6-8; Fig. 3C). Vanacker et al. (7) reported

that ERα can also bind to an ERRE, activating transcription 6- to 10-fold through this sequence in an E_2-stimulated manner. In contrast, we observed only minimal (i.e., 1.5- to 2-fold) activation of two different ERRE/SFRE–regulated reporters in MCF-7 and BT-474 cells following addition of E_2, conditions that led to 12- to 14-fold activation of our minimal ERE-regulated reporter (Fig. 3A and B). This finding was expected because ERα was unable to significantly bind a consensus ERRE (Fig. 3C, *lanes 1-13*). Differences between our experiments and the previous report include the following: (*a*) use of an ERα-negative rat osteosarcoma cell line transfected with an ERα expression plasmid for the transcription assays instead of breast carcinoma cell lines that endogenously express high levels of ERα; and (*b*) use of ERα-programmed reticulocyte lysates for the protein source for the EMSAs instead of whole-cell lysates of COS cells transfected with an ERα expression plasmid. Thus, the ERα protein levels in our reporter gene assays and EMSAs were probably significantly lower than they were in the previously reported experiments. We conclude that ERα probably does not significantly interact with an ERRE when present at physiologic concentrations; however, it remains possible that ERα exhibits a low affinity for some ERREs when its concentration is nonphysiologically high. Because ERRα1 exhibits a strong preference over ERα for binding to ERREs, there probably exists specific ERRα1-regulated genes that could serve as biomarkers of ERRα1 activities.

Coregulators of ERRα1

GRIP1 has been shown to recognize the nuclear receptor box within the COOH terminus of ERRα1, enhancing transcriptional activity (16). Why, then, did GRIP1 fail to significantly enhance ERRα1 transcriptional activity in MCF-7 cells (Fig. 2B and C)? Barry et al. (10) have reported that the exact sequence of the nine-nucleotide extended half-site sequence and the state of phosphorylation of ERRα1 (38) affect whether ERRα1 preferentially binds to an ERRE as a monomer or homodimer; ERRα1 acts as a repressor when bound as a monomer because it cannot recruit coactivators such as PGC-1α. However, the transcriptional activity of ERRα1 and its ability to recruit the coactivator GRIP1 to an ERE was shown here to be dependent on cell type (Fig. 2B versus D) and ErbB2 status (Fig. 6). Since ERRα1 binds to an ERE only as a homodimer (Fig. 3C; ref. 38), an alternative mechanism(s) must also exist by which ERRα1 transcriptional activity can be regulated. Based on the data presented here, we hypothesize that the recruitment of coactivators such as GRIP1 is determined, at least in part, by the phosphorylation status of specific amino acid residues within ERRα1 (Fig. 8).

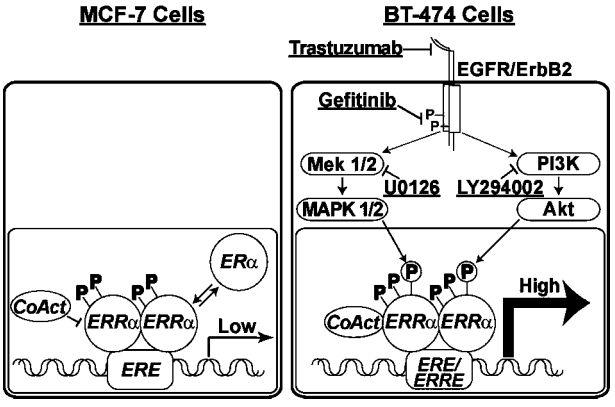

FIGURE 8. Model for modulation of transcription by ERRα1 via the ErbB2 signaling pathway. Only a few of the numerous players in the ErbB2 signaling pathways are indicated, along with the steps in these pathways blocked by the drugs used in this study. See text for details.

In addition to ERRα1, GRIP1 itself is also a phosphoprotein target of EGFR signaling via MAPK whose phosphorylation is required for full activity (43). Thus, the effects of activated ErbB2 and the drug inhibitors of EGFR/ErbB2 signaling on transcription (Figs. 6 and 7) could have been due to changes in the phosphorylation status of GRIP1 and ERRα1.

PGC-1α can also function as a strong coactivator of ERRα1 (13). However, it is not present in the mammary cell lines studied here (data not shown). SRC-1 and SRC-3/AIB1 have also been shown to stimulate ERRα1 activity in transient transfection assays in some mammalian cell lines, albeit only modestly (15, 16). Thus, GRIP1 is likely the major, physiologically relevant coactivator of ERRα1 in mammary cells. Hence, we hypothesize that ERRα1/GRIP1 complexes likely substitute for ERα/AIB1 complexes as the major activators driving ERE-regulated expression in some breast cancers, especially ERα-negative ones. In these cases, drugs that specifically disrupt these complexes may serve as a novel therapy.

Ligand-Independent Regulation of ERRα1 Activities via ErbB2 Signaling

Based on the results presented here, we hypothesize the following model for regulation of ERRα1 activities (Fig. 8). In cells expressing ErbB2 at low levels (e.g., MCF-7), ERRα1 exists, on average, in a minimally phosphorylated state in which it binds EREs as a homodimer, yet fails to respond to GRIP1-dependent coactivation. Thus, it inhibits transcription. In cells expressing ErbB2 at high levels (e.g., BT-474), ErbB2, as either a homodimer or heterodimer with other ErbB family members, signals additional or alternative phosphorylations of ERRα1, at least in part, through MEK/MAPK and PI3K/Akt signaling pathways. This highly phosphorylated form of ERRα1 binds to both EREs and ERREs as a homodimer, activating transcription via interactions with cellular coactivators such as GRIP1. Thus, changes in specific sites of phosphorylation of ERRα1 induced via the ErbB2 signaling pathway convert ERRα1 between repressor and activator of transcription.

The precise mechanism(s) that regulates the interaction between GRIP1 and ERRα1 is still unclear. Barry et al. (38) have proposed that ERRα1 can switch between monomer and homodimer, with only the homodimer form binding coactivators. Another possibility is that the repressor domain(s) of ERRα1 interacts with cellular corepressors, blocking binding of coactivators. Castet et al. (44) recently reported that the corepressor RIP140 inhibits ERRα-mediated trans-activation of ERE-dependent expression. Other data consistent with a corepressor(s) regulating the activity of ERRα1 include (*a*) identification of a repressor domain within the NH_2-terminal region of ERRα1 (45) and (*b*) overexpression of $ERR\alpha1_{E97G,A98S,A101V}$,

a variant of ERRα1 that fails to bind DNA due to mutations in its DNA-binding domain P-box, derepressing ERE-regulated transcription in MCF-7 cells, presumably by sequestering a corepressor (8). A third possibility is that changes in phosphorylation lead to changes in other posttranslational modifications in ERRα1, thereby affecting the coregulators with which it interacts. Consistent with this latter hypothesis is the recent finding that ERRα1 is also sumoylated; mutation of one of these sites of sumoylation also affects ERRα1 transcriptional activity.[5] These three mechanisms are not mutually exclusive.

Other kinase/phosphatase signaling pathways probably also target ERRα1, leading to changes in ERRα1 activities via alterations in its specific sites of phosphorylation. For example, Barry and Giguère (38) recently reported that protein kinase Cδ, an enzyme whose activity is stimulated by epidermal growth factor or phorbol 12-myristate 13-acetate, can phosphorylate ERRα1 within its DNA-binding domain, thereby enhancing both the binding of ERRα1 homodimers to ERREs and transcription. They further showed that stimulation of ERRα1 phosphorylation by incubation of their MCF-7 cells with phorbol 12-myristate 13-acetate can lead to an ~2-fold activation of transcription of the *pS2* gene via an ERRE present within its promoter. Likely, numerous cellular kinases and phosphatases activated through signaling pathways can affect specific sites of phosphorylation that exist within the A/B,[5] C (38), and E/F (Fig. 5C) domains of ERRα1. Depending on which of these multiple specific sites becomes phosphorylated, ERRα1 functions as a repressor or activator to modulate expression of numerous ERE- and ERRE-regulated cellular genes.

Role of ERRα in Breast Cancer

The ErbB family of tyrosine kinase receptors signals diverse pathways that play roles in the development of aggressive breast cancers and their resistance to antihormonal therapy. Hence, factors whose activities are both estrogen-independent and sensitive to disruptors of ErbB2 signaling likely contribute to some tamoxifen-resistant and ER-negative breast cancers. ERRα1 meets the following criteria: (*a*) the activator form of ERRα1 can functionally substitute for ERα in ErbB2-overexpressing cells (Figs. 2D and 6), and (*b*) blockade of ErbB2 signaling or its downstream effectors, e.g., MEK/MAPK or PI3K/Akt, leads to conversion of ERRα1 from an activator to a repressor, eliminating the ability of ERRα1 to substitute for ERα (Fig. 7). Thus, ER-positive breast tumors expressing high levels of ErbB2 along with the activator form of ERRα will likely not respond well to hormonal-blockade therapies; rather, they may respond well instead to ErbB2-based therapies such as trastuzumab. Given that ERRα1 likely down-modulates the activity of ERα in some ERα-positive tumors while it functionally substitutes for ERα in other tumors leading to estrogen-independent activation of key genes involved in breast cancer, ERRα1 and its phosphorylation status should be evaluated as biomarkers of prognosis and determinants of specific therapeutic treatments. Moreover, ERRα may have use, in itself, as a target for a new class of drugs, possibly for use in combination with some current therapies.

Materials and Methods

Cell Lines

MCF-7/WS8 mammary carcinoma cells were used in all studies in which MCF-7 cells are indicated; they were clonally derived from MCF-7 cells by selection for sensitivity to growth stimulation by E_2 (46, 47). BT-474 cells were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines were maintained in estrogenized medium (i.e., phenol red-containing RPMI 1640, 10% whole fetal bovine serum, 6 ng/mL insulin, 2 mmol/L glutamine, 100 μmol/L nonessential amino acids, and 100 units of penicillin and streptomycin per milliliter). Two days before reseeding of cells for an experiment, the medium was changed to phenol red- and estrogen-free medium containing charcoal-stripped fetal bovine serum (48). The monkey kidney COS-M6 cell line was cultured as previously described (8). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cellular Treatment Agents

E_2 (Sigma-Aldrich, St. Louis, MO) and the complete antiestrogen fulvestrant (ICI 182,780, Faslodex; a generous gift from AstraZeneca, Macclesfield, United Kingdom) were dissolved in ethanol. Control, nonspecific murine IgG (reagent grade, Sigma-Aldrich) was dissolved in PBS. Trastuzumab (Herceptin; purchased from the Lurie Cancer Center pharmacy) was dissolved in bacteriostatic water. A hybridoma cell line that secretes the mAb 4D5, a murine precursor of trastuzumab directed against the ectodomain of ErbB2 (HER2), was obtained from the American Type Culture Collection; IgG was purified from the ascites fluid of a mouse inoculated with this cell line. Gefitinib (Iressa, ZD1839; a generous gift from AstraZeneca) was initially dissolved in DMSO, followed by further dilution in ethanol. U0126 (Promega, Madison, WI) and LY294002 (Promega) were dissolved in DMSO. All test agents were added to the medium at a 1:1,000 (v/v) dilution.

Plasmids

Plasmid pcDNA3.1-hERRα1 (ERRα1), encoding the full-length, 423-amino-acid major human isoform of ERRα, and plasmid pcDNA3.1-hERRα1$_{L413A/L418A}$ (ERRα1$_{L413A/L418A}$), encoding a variant of ERRα1 defective in the carboxyl-terminal coactivator-binding LxLxxL motif, have been previously described (8). Plasmid pcDNA3.1-hERRα1$_{1-376}$ (ERRα1$_{1-376}$), generated by PCR-based subcloning, encodes a carboxyl-terminal truncated variant of ERRα1 lacking the coactivator-binding LxLxxL motif. Plasmid pcDNA3-GRIP1 encodes the coactivator GRIP1 (49). The replication-defective retrovirus pJRneu has been previously described (42); it encodes the activated form of the rat *neu* oncogene (ErbB2$_{Act}$). Plasmid pEGFP encodes enhanced green fluorescent protein (TaKaRa; Clontech, Palo Alto, CA).

Plasmids pTA-ffLuc and pTA-srLuc, containing TATA-box basal promoter firefly and *Renilla* luciferase reporter genes, respectively, were constructed by insertion via *Hin*dIII linkers of the nucleotides −31 to +31 region of the herpes simplex virus thymidine kinase promoter into pGL3-Basic and phRG-B (Promega), respectively. Plasmid pERE(5×)TA-ffLuc, containing five tandem copies of the consensus palindromic ERE, was

constructed by insertion into the *Xho*I and *Bgl*II sites of pTA-ffLuc of the oligodeoxynucleotides 5′-tcgagagAGGTCActgTGACCTctgagagAGGTCActgTGACCT ctctcagAGGTCActgTGACCTctgcgagAGGTCActgTGACCTctgcgagAGGTCActgTGACCTcta-3′ and 5′-gatctagAGGTCAcagTGACCTctcgcagAGGTCAcagTGACCTctcgcagAGGTCAcagTGACCTctgagagAGGTCAcagTGACCTctctcagAGGTCAcagTGACCT ctc-3′. Plasmid pERRE(5×)TAffLuc, containing five tandem copies of a consensus ERRE, was constructed by insertion into pTA-ffLuc of the oligodeoxynucleotides 5′-tcgagtcaAGGTCAgagctcgtcaAGGTCActgcgctcaAGGTCAgctagcgtcaAGGTCAgcatgcgtcaAGGTCAa-3′ and 5′-gatctTGACCTtgacgcatgcTGACCTtgacgctagcTGACCTtgagctgcagTGACCTtgacgagctcTGACCTtgac-3′. EREs and ERREs are underlined, with core ERE half-sites indicated in uppercase letters. The previously described plasmid pSFRE×3-Luc (refs. 6, 50; generous gift of Jean-Marc Vanacker) contains three copies of a consensus ERRE/SFRE (5′-TCAAGGTCA-3′) upstream of the SV40 minimal early promoter regulating firefly luciferase expression. Plasmid phRL-TK contains the herpes simplex virus thymidine kinase promoter driving expression of a humanized *Renilla* luciferase (Promega).

Transient Transfection Reporter Gene Assays

Transient transfection assays were done using a dual-luciferase system (Promega) in which pTA-srLuc served as an internal control (Fig. 2A). The basal TATA promoter–regulated set (Fig. 2A) was transfected in parallel with the ERE- or ERRE/SFRE–regulated sets as an external control for the physiologic state of the cells and effects of test agents on basal transcription activity. All data were normalized to both of these controls.

After culture for 2 days in estrogen-free medium, MCF-7 and BT-474 cells were seeded in estrogen-free medium into 24-well plates at densities of 100,000 and 150,000 per well, respectively. The amounts of the indicated DNAs cotransfected per well using TransIT LT1 reagent (Mirus, Madison, WI) were as follows: 150 ng of pcDNA3.1-ERRα1, pcDNA3.1-hERRα$1_{L413A/L418A}$, and pcDNA3.1; 200 ng of pGRIP1 or its empty parental plasmid, pcDNA3.1; 200 ng of pJRneu or its empty parental plasmid, pJR (42); 200 ng of pERE(5×)-TA-ffLuc, pERRE(5×)-TA-ffLuc, pTA-ffLuc, or pSFRE3×-Luc; and 50 ng of pTA-srLuc or phRLTK. Four hours later, the cells were placed in fresh medium containing the indicated treatment agents until harvested.

EMSAs

To assess the binding affinities of ERRα1 for putative ERREs located within promoter regions of cellular genes, competition EMSAs were done as previously described (5, 8). In brief, COS-M6 cells were transfected with pcDNA3.1-ERRα1 (4 μg per 100-mm-diameter dish) using TransIT LT1 reagent. Forty-eight hours later, whole-cell extracts were prepared as the source of ERRα1 protein. Twenty micrograms of ERRα1-containing protein extract was preincubated on ice for 15 min with the indicated unlabeled competitor oligonucleotides in reaction buffer (8). Afterward, 1 ng of radiolabeled double-stranded oligodeoxynucleotide containing the sequence 5′-agcagtggcgatttgTCAAGGTCAcacagt-3′ serving as probe DNA was added, and incubation was continued for 15 min at room temperature before electrophoresis in a nondenaturing 5% polyacrylamide gel at 200 V for 2 h at 4°C. Gels were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Immunoshift assays were done by addition of the rabbit polyclonal antiserum anti-GST-hERRα$1_{17\text{-}329}$ (5). Competition EMSAs were done likewise with whole-cell extracts prepared from COS-M6 cells transfected with pCMV-ERα or pcDNA3.1-hERRα1 serving as protein source, radiolabeled double-stranded oligonucleotide containing the sequence 5′-taagcttAGGTCAcagTGACCTaagctta-3′ serving as probe DNA, and unlabeled oligodeoxynucleotide containing the indicated sequence serving as competitor.

Cellular RNA Analyses

Cells were seeded in 15-cm plates at ~70% confluency, transfected with 8 μg pEGFP plus 14 μg of pcDNA3.1-hERRα1 or pcDNA3.1 using TransIT LT1 reagent, and incubated under estrogen-free conditions. Twenty-four hours later, EGFP-positive cells (100,000-500,000 per group) were sorted and collected by fluorescence-activated cell sorting (Beckman Coulter Epics Elite ESP equipped with Elite software; Beckman Coulter, Miami, FL). Total RNA, isolated from these EGFP-positive cells with an RNeasy Micro kit (Qiagen), was converted to first-strand cDNA using Super-Script III with a combination of random hexamers and oligo(dT) as primers (Invitrogen). Quantitative real-time PCR assays were done as previously described (11, 50) with the Taqman Universal or SYBR Green PCR Master Mixes and an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). The *pS2* forward primer was 5′-GAGGCCCAGACAGAGACGTG-3′, *pS2* reverse primer was 5′-CCCTGCAGAAGTGTCTAAAATTCA-3′ and the *pS2* probe was 5′-[FAM]-CTGCTGTTTCGACGACACCGT-TCG-[QSY7]-3′. The *PgR* forward primer was 5′-CGTGCC-TATCCTGCCTCTCAA-3′ and the *PgR* reverse primer was 5′-CCGCCGTCGTAACTTTCGT-3′. Abundances were determined by comparison with a standard curve generated by serial dilution of known quantities of the PCR product (11), with internal normalization to 18S rRNA.

Immunoblot Analyses

Proteins were extracted in Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA) supplemented with Protease Inhibitor Cocktail Set III and Phosphatase Inhibitor Cocktail Set II (Calbiochem, San Diego, CA). Protein amounts were quantified with Coomassie Protein Assay Reagent (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as the standard. Proteins (20 μg per extract) were separated by 12% SDS-PAGE and electroblotted onto an Immobilon-FL polyvinylidene fluoride membrane (Millipore, Billerica, MA). In Fig. 1, the membrane was probed with an ERα mAb (clone AER611; Lab Vision, Fremont, CA), an ERRα mAb (2ERR10, a gift of Nancy Thompson), and a β-actin antibody (Sigma-Aldrich) followed by IRDye 680– and IRDye 800CW–conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE). Quantitation was done with an Odyssey IR Imager and Odyssey Software v2.0 (LI-COR Biosciences). In Fig. 6B,

retained primary antibodies were detected using horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) in conjunction with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and autoradiography. In Fig. 7C and D, the membranes were probed sequentially with the rabbit polyclonal anti-GST-ERRα1$_{117\text{-}329}$ (5) and β-actin (Sigma), or with p44/42 MAPK polyclonal antibody, phosphospecific p44/42 MAPK (Thr^{202}/Tyr^{204}) mAb E10, Akt polyclonal antibody, and phosphospecific Akt (Ser^{473}) polyclonal antibody (Cell Signaling Technology), respectively. Reacting primary antibodies were detected using horseradish peroxidase–conjugated secondary antibodies, enhanced chemiluminescence, and autoradiography.

In situ ^{32}P-labeling and Two-Dimensional PAGE

MCF-7 and BT-474 cells at ~70% of confluency were transiently transfected with pcDNA3.1-hERRα1 (3 μg per 10-cm dish). The medium was supplemented with anti-ErbB2 mAb 4D5 (2.5 μg/mL) where indicated. Twenty-four hours later, the cells were washed twice with phosphate-free RPMI 1640 (Specialty Media, Phillipsburg, NJ), incubated in phosphate-free RPMI 1640 for 3 h at 37°C, and metabolically labeled by addition of 2.5 mCi/dish of ^{32}P-labeled orthophosphoric acid (9,000 Ci/mmol, NEN Life Science, East Greenwich, RI) and incubation for an additional 4 h. Afterward, the cells were lysed by incubation for 20 min at 4°C in 600 μL lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 0.1% SDS, 0.5 mmol/L phenylmethylsulfonyl fluoride]. After preclearing by incubation with protein-A agarose (Santa Cruz Biotechnology, Santa Cruz, CA), ERRα1 was immunoprecipitated with a rabbit polyclonal serum against GST-ERRα1$_{117\text{-}329}$ (5) and protein A–conjugated agarose beads, eluted by incubation at 100°C in 12 μL of 2× SDS loading buffer, and resolved by two-dimensional gel electrophoresis (Kendrick Laboratory, Inc., Madison, WI) done with isoelectric focusing (pH 3.5-10) and 12% SDS polyacrylamide gels.

Protein Kinase Assays

Activated forms of MAPK1, MAPK2, Akt1, and Akt2 (0.05 unit) were incubated in parallel with 1.5 μg ERRα1 and 1 μCi of [γ-^{32}P]ATP (3 mCi/μmol) at 30°C for 30 min in total reaction volumes of 25 μL. Human MAPK1 and human MAPK2, both containing an amino-terminal GST tag, were expressed and purified from *E. coli* followed by activation with MEK1 (Upstate Cell Signaling Solutions, Lake Placid, NY). Human Akt1 and human Akt2, each containing an amino-terminal 6×His tag and lacking amino acids 1 to 117 (pleckstrin homology domain), were expressed and purified from Sf21 cells (Upstate Cell Signaling Solutions). The Akt1 and Akt2 proteins contained activating mutations of Ser^{473} to aspartic acid and Ser^{474} to aspartic acid, respectively. Full-length human ERRα1 containing a carboxyl-terminal 6×His tag was expressed and purified from *E. coli* using Ni-NTA agarose. The MAPK phosphorylation assays were done in 20 mmol/L MOPS (pH 7.2), 25 mmol/L β-glycerol phosphate, 5 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 1 mmol/L DTT, 27 nmol/L $MgCl_2$, and 180 μmol/L ATP. The Akt phosphorylation assays were done in 50 mmol/L Tris-HCl (pH 7.5), 0.1 mmol/L EGTA, 15 mmol/L DTT, 27 nmol/L $MgCl_2$, and 180 μmol/L ATP. Myelin basic protein (20 μg, Upstate Cell Signaling Solutions) served as a positive control. The products were resolved by 4% to 12% gradient SDS-PAGE.

Rat p42 MAPK (extracellular signal-regulated kinase 2; Calbiochem) that had been phosphorylated *in vitro* by a constitutively active MEK1 mutant served as the activated MAPK for the experiment shown in Fig. 5C. Full-length and deleted variant GST-ERRα1 and GST-β-globin$_{1\text{-}123}$ fusion proteins were expressed and purified from *E. coli* as previously described (51). MAPK phosphorylation assays were done at 30°C for 30 min in 40 μL reactions containing 12 units (20 ng) of activated extracellular signal-regulated kinase 2; 1 μCi [γ-^{32}P]ATP (5 mCi/μmol); and 1.0 μg GST-ERRα1$_{1\text{-}423}$, 1.08 μg GST-ERRα1$_{1\text{-}376}$, 1.6 μg GST-ERRα1$_{1\text{-}173}$, 1 μg PHAS-I (Calbiochem), or 1 μg GST-β-globin$_{1\text{-}123}$ in 25 mmol/L HEPES (pH 7.5), 10 mmol/L MgOAc, and 50 μmol/L ATP. The products were resolved by 12% SDS-PAGE.

Statistical Analyses

Numbers shown are means ± SEs of experiments done in triplicate or quadruplicate. Significant differences in mRNA levels were determined using an unpaired *t* test with two-tailed *P* values and a 95% confidence interval. RBAs of naturally occurring ERREs compared with a reference ERRE shown in Table 1 were determined by calculation with GraphPad Prism (version 3.00 for Windows, GraphPad, San Diego, CA) of the amount of moles of unlabeled competitor oligonucleotide needed to reduce one mole of radiolabeled probe DNA-ERRα1 1 complex by 50%.

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References

1. Nuclear Receptors Nomenclature Committee. A unified nomenclature system for the nuclear receptor superfamily. Cell 1999;97:161–3.

2. Ariazi EA, Ariazi JL, Cordera F, Jordan VC. Estrogen receptors as therapeutic targets in breast cancer. Curr Top Med Chem 2006;6:195–216.

3. Ariazi EA, Jordan VC, Estrogen-related receptors as emerging targets in cancer and metabolic disorders. Curr Top Med Chem 2006;6:181–93.

4. Yang N, Shigeta H, Shi H, Teng CT, Estrogen-related receptor, hERR1, modulates estrogen receptor-mediated response of human lactoferrin gene promoter. J Biol Chem 1996;271:5795–804.

5. Johnston SD, Liu X, Zuo F, et al. Estrogen-related receptor α1 functionally binds as a monomer to extended half-site sequences including ones contained within estrogen-response elements. Mol Endocrinol 1997;11:342–52.

6. Vanacker JM, Bonnelye E, Chopin-Delannoy S, Delmarre C, Cavaillès V, Laudet V. Transcriptional activities of the orphan nuclear receptor ERRα (estrogen receptor-related receptor α). Mol Endocrinol 1999;13:764–73.

7. Vanacker JM, Pettersson K, Gustafsson JÅ, Laudet V. Transcriptional targets shared by estrogen receptor-related receptors (ERRs) and estrogen receptor (ER) α, but not by ERβ. EMBO J 1999;18:4270–9.

8. Kraus RJ, Ariazi EA, Farrell ML, Mertz JE. Estrogen-related receptor α1 actively antagonizes estrogen receptor-regulated transcription in MCF-7 mammary cells. J Biol Chem 2002;277:24826–34.

9. Sladek R, Bader JA, Giguère V. The orphan nuclear receptor estrogen-related receptor α is a transcriptional regulator of the human medium-chain acyl coenzyme A dehydrogenase gene. Mol Cell Biol 1997;17:5400–9.

10. Barry JB, Laganière J, Giguère V. A single nucleotide in an estrogen-related receptor α site can dictate mode of binding and peroxisome proliferator-activated receptor γ coactivator 1α activation of target promoters. Mol Endocrinol 2006;20:302–10.

11. Ariazi EA, Clark GM, Mertz JE. Estrogen-related receptor α and estrogen-related receptor γ associate with unfavorable and favorable biomarkers, respectively, in human breast cancer. Cancer Res 2002;62:6510–8.

12. Suzuki T, Miki Y, Moriya T, et al. Estrogen-related receptor α in human breast carcinoma as a potent prognostic factor. Cancer Res 2004;64:4670–6.

13. Huss JM, Kopp RP, Kelly DP. Peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor-α and -γ: identification of novel leucine-rich interaction motif within PGC-1α. J Biol Chem 2002;277:40265–74.

14. Kallen J, Schlaeppi JM, Bitsch F, et al. Evidence for ligand-independent transcriptional activation of the human estrogen-related receptor α (ERRα): crystal structure of ERRα ligand binding domain in complex with peroxisome proliferator-activated receptor coactivator-1α. J Biol Chem 2004;279:49330–7.

15. Xie W, Hong H, Yang NN, et al. Constitutive activation of transcription and binding of coactivator by estrogen-related receptors 1 and 2. Mol Endocrinol 1999;13:2151–62.

16. Zhang Z, Teng CT. Estrogen receptor-related receptor α1 interacts with coactivator and constitutively activates the estrogen response elements of the human lactoferrin gene. J Biol Chem 2000;275:20837–46.

17. Chen S, Zhou D, Yang C, Sherman M. Molecular basis for the constitutive activity of estrogen-related receptor α-1. J Biol Chem 2001;276:28465–70.

18. Lu D, Kiriyama Y, Lee KY, Giguère V. Transcriptional regulation of the estrogen-inducible pS2 breast cancer marker gene by the ERR family of orphan nuclear receptors. Cancer Res 2001;61:6755–61.

19. Yang C, Zhou D, Chen S. Modulation of aromatase expression in the breast tissue by ERRα-1 orphan receptor. Cancer Res 1998;58:5695–700.

20. Seely J, Amigh KS, Suzuki T, et al. Transcriptional regulation of dehydroepiandrosterone sulfotransferase (SULT2A1) by estrogen-related receptor α (ERRα). Endocrinology 2005;146:3605–13.

21. Vanacker JM, Delmarre C, Guo X, Laudet V. Activation of the osteopontin promoter by the orphan nuclear receptor estrogen receptor related α. Cell Growth Differ 1998;9:1007–14.

22. Liu D, Zhang Z, Gladwell W, Teng CT. Estrogen stimulates estrogen-related receptor α gene expression through conserved hormone response elements. Endocrinology 2003;144:4894–904.

23. Laganière J, Tremblay GB, Dufour CR, et al. A polymorphic autoregulatory hormone response element in the human estrogen-related receptor α (ERRα) promoter dictates peroxisome proliferator-activated receptor γ coactivator-1α control of ERRα expression. J Biol Chem 2004;279:18504–10.

24. Herzog B, Cardenas J, Hall RK, et al. Estrogen-related receptor α is a repressor of phosphoenolpyruvate carboxykinase gene transcription. J Biol Chem 2006;281:99–106.

25. Grant S, Qiao L, Dent P. Roles of ErbB family receptor tyrosine kinases, and downstream signaling pathways, in the control of cell growth and survival. Front Biosci 2002;7:d376–89.

26. Lannigan DA. Estrogen receptor phosphorylation. Steroids 2003;68:1–9.

27. McClelland RA, Barrow D, Madden TA, et al. Enhanced epidermal growth factor receptor signaling in MCF7 breast cancer cells after long-term culture in the presence of the pure antiestrogen ICI 182,780 (Faslodex). Endocrinology 2001;142:2776–88.

28. Benz CC, Scott GK, Sarup JC, et al. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/*neu*. Breast Cancer Res Treat 1992;24:85–95.

29. Coutts AS, Murphy LC. Elevated mitogen-activated protein kinase activity in estrogen-nonresponsive human breast cancer cells. Cancer Res 1998;58:4071–4.

30. Wright C, Nicholson S, Angus B, et al. Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer. Br J Cancer 1992;65:118–21.

31. Houston SJ, Plunkett TA, Barnes DM, Smith P, Rubens RD, Miles DW. Overexpression of c-erbB2 is an independent marker of resistance to endocrine therapy in advanced breast cancer. Br J Cancer 1999;79:1220–6.

32. Dowsett M, Harper-Wynne C, Boeddinghaus I, et al. HER-2 amplification impedes the antiproliferative effects of hormone therapy in estrogen receptor-positive primary breast cancer. Cancer Res 2001;61:8452–8.

33. Kraus MH, Popescu NC, Amsbaugh SC, King CR. Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J 1987;6:605–10.

34. Leyland-Jones B. Trastuzumab: hopes and realities. Lancet Oncol 2002;3:137–44.

35. Moulder SL, Yakes FM, Muthuswamy SK, Bianco R, Simpson JF, Arteaga CL. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/*neu* (erbB2)-overexpressing breast cancer cells *in vitro* and *in vivo*. Cancer Res 2001;61:8887–95.

36. Favata MF, Horiuchi KY, Manos EJ, et al. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 1998;273:18623–32.

37. Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002). J Biol Chem 1994;269:5241–8.

38. Barry JB, Giguère V. Epidermal growth factor-induced signaling in breast cancer cells results in selective target gene activation by orphan nuclear receptor estrogen-related receptor α. Cancer Res 2005;65:6120–9.

39. Szöllösi J, Balázs M, Feuerstein BG, Benz CC, Waldman FM. ERBB-2 (HER2/*neu*) gene copy number, $p185^{HER-2}$ overexpression, and intratumor heterogeneity in human breast cancer. Cancer Res 1995;55:5400–7.

40. Carlson RW. The history and mechanism of action of fulvestrant. Clin Breast Cancer 2005;6(Suppl 1):S5–8.

41. Périer RC, Praz V, Junier T, Bonnard C, Bucher P. The eukaryotic promoter database (EPD). Nucleic Acids Res 2000;28:302–3.

42. Wang B, Kennan WS, Yasukawa-Barnes J, Lindstrom MJ, Gould MN. Frequent induction of mammary carcinomas following *neu* oncogene transfer into *in situ* mammary epithelial cells of susceptible and resistant rat strains. Cancer Res 1991;51:5649–54.

43. Lopez GN, Turck CW, Schaufele F, Stallcup MR, Kushner PJ. Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. J Biol Chem 2001;276:22177–82.

44. Castet A, Herledan A, Bonnet S, Jalaguier S, Vanacker JM, Cavaillès V. Receptor-interacting protein 140 differentially regulates estrogen receptor-related receptor transactivation depending on target genes. Mol Endocrinology 2006;20:1035–47.

45. Zhang Z, Teng CT. Estrogen receptor α and estrogen receptor-related receptor α1 compete for binding and coactivator. Mol Cell Endocrinol 2001;172:223–33.

46. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 1992;90:77–86.

47. Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res 1997;57:3071–8.

48. Katzenellenbogen JA, Johnson HJ, Jr., Myers HN. Photoaffinity labels for estrogen binding proteins of rat uterus. Biochemistry 1973;12:4085–92.

49. Rogatsky I, Zarember KA, Yamamoto KR. Factor recruitment and TIF2/GRIP1 corepressor activity at a collagenase-3 response element that mediates regulation by phorbol esters and hormones. EMBO J 2001;20:6071–83.

50. Bauer JA, Thompson TA, Church DR, Ariazi EA, Wilding G. Growth inhibition and differentiation in human prostate carcinoma cells induced by the vitamin D analog 1α,24-dihydroxyvitamin D_2. Prostate 2003;55:159–67.

51. O'Reilly GH. Regulation of the SV40 late promoter by nuclear receptors and large T antigen. Genetics. Madison: University of Wisconsin-Madison; 2000.

ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Bioluminescence Imaging for Assessment and Normalization in Transfected Cell Arrays

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ABSTRACT: Transfected cell arrays (TCAs) represent a high-throughput technique to correlate gene expression with functional cell responses. Despite advances in TCAs, improvements are needed for the widespread application of this technology. We have developed a TCA that combines a two-plasmid system and dual-bioluminescence imaging to quantitatively normalize for variability in transfection and increase sensitivity. The two-plasmids consist of: (i) normalization plasmid present within each spot, and (ii) functional plasmid that varies between spots, responsible for the functional endpoint of the array. Bioluminescence imaging of dual-luciferase reporters (renilla, firefly luciferase) provides sensitive and quantitative detection of cellular response, with minimal post-transfection processing. The array was applied to quantify estrogen receptor α (ERα) activity in MCF-7 breast cancer cells. A plasmid containing an ERα-regulated promoter directing firefly luciferase expression was mixed with a normalization plasmid, complexed with cationic lipids and deposited into an array. ER induction mimicked results obtained through traditional assays methods, with estrogen inducing luciferase expression 10-fold over the antiestrogen fulvestrant or vehicle. Furthermore, the array captured a dose response to estrogen, demonstrating the sensitivity of bioluminescence quantification. This system provides a tool for basic science research, with potential application for the development of patient specific therapies.
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KEYWORDS: transfected cell array; bioluminescence imaging; substrate-mediated gene delivery; estrogen receptor; breast cancer

Introduction

Analysis of multiple pathways or genes in a parallel format can be achieved using a transfected cell array, a high-throughput technique to correlate gene expression with functional cell responses, based on gene delivery from a substrate that supports cell adhesion (Bengali et al., 2005; Pannier et al., 2005; Segura and Shea, 2002; Segura et al., 2003). While traditional microarrays can quantify the expression level of thousands of genes, they cannot accurately describe the functional activity of these genes in a cellular and physiological context (Pepperkok and Ellenberg, 2006). Transfected cell arrays present a powerful approach to study gene function in the context of a living cell, allowing proteins to be translated and folded correctly and to interact within the environment of the cell. Additionally, a large number of genes can be potentially screened in parallel for induction or repression of a given function (Palmer and Freeman, 2005). Transfected cell arrays offer compact, economical, and high-throughput analysis in living cells that provides greater consistency across assays and facilitates comparisons between condi-

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tions, while reducing the amount of reagents and cell numbers required, which is an important factor for difficult to prepare cell types (Hook et al., 2006; Palmer and Freeman, 2005).

Since the original report on transfected cell arrays (Ziauddin and Sabatini, 2001), reverse transfection has been employed in several high-throughput cell based microarrays to screen for gene function or activity (8–20). Reverse transfection involves printing mixtures of different plasmids and gelatin into specific domains onto a substrate. A lipid-based transfection agent is then floated over the array, and cells are subsequently seeded to form a living cell microarray of locally transfected cells in a lawn of nontransfected cells. The first transfected cell array was used to analyze genes for phosphotyrosine activity and identified six genes; five genes that encode known tyrosine kinase proteins and one that encodes a protein of unknown function (Ziauddin and Sabatini, 2001). Transfected cell arrays have since been applied to study signaling pathways (Webb et al., 2003), screen antibody fragments (Delehanty et al., 2004b), identify possible new lysophosphatidic acid receptors (Lee et al., 2006), perform protein localization studies (Hu et al., 2005, 2006), screen for proapoptotic genes (Mannherz et al., 2006; Palmer et al., 2006), and annotate protein function (Hodges et al., 2005). The transfected cell array has also been adapted to high-throughput RNAi studies (Mousses et al., 2003), specifically for the analysis of spindle formation (Silva et al., 2004), secretory pathways (Erfle et al., 2004), and chromosome segregation and nuclear structure in a time-lapse system (Neumann et al., 2006).

Technological improvements have enhanced the capabilities of the arrays, yet further advancements are required for widespread application of this system. Most efforts have focused on increasing transfection efficiency within the array by using preformed complexes (Delehanty et al., 2004a,b; Erfle et al., 2004; Hodges et al., 2005; Mousses et al., 2003; Pannier et al., 2005; Redmond et al., 2004; Silva et al., 2004; Yoshikawa et al., 2004), incorporating fibronectin (Yoshikawa et al., 2004), atelocollagen (Honma et al., 2001), and recombinant proteins (Redmond et al., 2004) with plasmid or DNA complexes, manipulating substrate hydrophobicity (Delehanty et al., 2004a), or coating cationic polymer and collagen onto surfaces prior to transfection (Chang et al., 2004). Micropatterning strategies have also been used to fabricate arrays with improved transfection, using self-assembled monolayers to pattern DNA (Pannier et al., 2005; Yamauchi et al., 2004a) or siRNA (Fujimoto et al., 2006) complex immobilization on gold slides or electrodes (Yamauchi et al., 2004b, 2005). Arrays have been formed with dendrimers (How et al., 2004) and viral vectors (Bailey et al., 2006; Hobson et al., 2003; Michiels et al., 2002) for enhanced gene delivery, magnetic beads (Isalan et al., 2005) or hydrogels (Peterbauer et al., 2006) to localize cells and vectors, and for alternative cell types, including Drosophila (Wheeler et al., 2004) and non-adherent cells (Kato et al., 2004). Further improvements are needed to accommodate issues with transfection efficiency, spot-to-spot variability, normalization, post-transfection processing, sensitivity, image acquisition and quantification, cell types that are difficult to transfect, as well as to expand the biological endpoints.

In this report, we combine a two-plasmid system and dual-bioluminescence imaging (Pichler et al., 2005; Rafiq et al., 1998; Rutter et al., 1998) to quantify array output, normalize for variability in transfection efficiency, and address sensitivity concerns to overcome known shortcomings of the transfected cell arrays. Soft lithography principles (Xia and Whitesides, 1998) were used to create the transfected cell array, in which a rubber mold was used to confine deposition of preformed DNA complexes to designated regions of the substrate and pattern transfection upon cell seeding. Larger spot sizes were employed in the array to provide sufficient numbers of transfected cells and increase the reliability and statistical relevance of quantitative data obtained from each spot (Fujimoto et al., 2006; Hodges et al., 2005). To account for inherent variances in transfection between spots, transfection efficiency and protein production were normalized with the addition of a second plasmid within all spots of the array, encoding renilla luciferase driven by a constitutive promoter, in addition to a primary regulated plasmid reporting on the activation of a transcription factor through firefly luciferase expression. Bioluminescence imaging of the two luciferase reporters allows for quick image acquisition with no post-transfection processing.

We illustrate the utility of the array to quantitatively assay for the activity of a transcription factor in response to various activators or inhibitors. The estrogen receptor α (ERα) pathway in ER-positive, estrogen-responsive breast cancer cells was analyzed in an array format, using an ERE-regulated promoter reporter system. ERα expression is an important biomarker for determining treatment course for clinical breast cancer (Ariazi et al., 2006; Pearce and Jordan, 2004). Estrogens, via ERα, act as potent mitogens of ER-positive breast cancer (Ikeda and Inoue, 2004). In our plasmid system, the ER-regulated promoter directs firefly luciferase expression in response to transcriptional activation by 17β-estradiol (E_2)-bound ERα. Bioluminescence imaging was employed to quantify luciferase-based light emission resulting from the ER-regulated and normalization plasmids. The array can thus be employed to analyze the induction and inhibition of the transcription factors, which could be used in a high-throughput format to elucidate gene function and cellular pathways responsible for diseases (Hook et al., 2006; Palmer and Freeman, 2005; Pepperkok and Ellenberg, 2006).

Materials and Methods

Cells

All studies used ER-positive MCF-7/WS8 mammary carcinoma cells, clonally derived from MCF-7 cells by selection for sensitivity to growth simulation by E_2 (Jiang

et al., 1992; Levenson and Jordan, 1997). Cells were cultured in fully estrogenized, phenol red-containing RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 μM non-essential amino acids, 100 U antibiotic/antimycotic, 2 mM L-glutamine, and 6 ng/ml insulin and maintained at 37°C in a humidified 5% CO_2 atmosphere. Prior to transfecting cells for an experiment, cells were cultured under estrogen-free conditions by substituting phenol red-free RPMI-1640 and dextran-coated charcoal-treated FBS in the medium. For experiments in which transfected cells were assayed in 24-well plates using a luminometer, or imaged in arrays using a CCD camera, cells were cultured under estrogen-free condition for 4 days or 18 h, respectively, prior to seeding. Culture in estrogen-free media for either time period allowed adequate time for upregulation of ER protein levels due to E2 withdrawal (data not shown), while the shorter culture period enhanced cell viability in the array. All media and media components were purchased from GIBCO/Invitrogen (Carlsbad, CA).

Plasmids

Plasmids were purified from bacteria culture using Qiagen (Valencia, CA) reagents and stored in Tris–EDTA buffer solution (10 mM Tris, 1 mM EDTA, pH 7.4) or water at −20°C. Plasmid pEGFP-LUC encodes both the enhanced green fluorescent protein (EGFP) and firefly luciferase protein, under the direction of a CMV promoter (Clontech, Mountain View, CA). Plasmid pLUC encodes the firefly luciferase gene in the pNGVL1 (National Gene Vector Labs, University of Michigan) vector backbone with a CMV promoter. Estrogen-responsive plasmid pERE(3×)TK-ffLUC (Catherino and Jordan 1995) contains three tandem copies of the palindromic estrogen response element (ERE) sequence, placed upstream of a minimal herpes simplex thymidine kinase (TK) promoter, directing expression of the firefly luciferase coding sequence in response to transcriptional activation by estradiol (E_2)-bound ERα, followed by recruitment of cofactor complexes and basal transcriptional machinery. Plasmid pTK-rLUC (phRL-TK, Promega, Madison, WI) contains the minimal TK promoter driving expression of a humanized renilla luciferase and was used for normalization of the firefly luciferase plasmids. Plasmid pβGAL encodes for nuclear-targeted β-galactosidase in the pNGVL1 (National Gene Vector Labs, University of Michigan) vector backbone with a CMV promoter and was used for control spots on the array.

DNA Complex Formation

DNA complexes were formed with Lipofectamine 2000 (Invitrogen), Lipofectamine LTX (Invitrogen) or Effectene (Qiagen), following manufacturer's instructions. Briefly, for both Lipofectamine 2000 and Lipofectamine LTX, DNA complexes were formed at a DNA/lipid ratio of 1:2 in serum-free, Opti-MEM media (Invitrogen), by adding transfection reagent diluted in media dropwise to DNA in media, mixing by gentle pipeting, and then incubating for 20 min. Effectene complexes were formed by diluting DNA into EC buffer, to which the Enhancer buffer was added at a DNA to Enhancer ratio of 1:8. After 2–5 min of incubation at room temperature, the Effectene transfection reagent was then added to the DNA/Enhancer mixture at a DNA to Transfection reagent ratio of 1:4. After incubation at room temperature for 10 min, complexes were diluted with serum-free media before addition to surfaces or cells. DNA in complexes containing multiple plasmids was extensively mixed prior to complex formation. For induction studies in estrogen-free media, phenol red-free Opti-MEM media was used for complex formation.

Multiwell Dish Format Reporter Gene Assays

Multiwell dish format reporter gene assays were performed to compare the ability of surface delivery of complexes to monitor ERα response in comparison to traditional bolus delivery. For surface delivery, the surface of wells of a 24-well plate (Becton Dickinson, Franklin Lakes, NJ) were serum-coated by incubation with dextran-coated charcoal-stripped FBS (10% in 1× PBS, pH 7.4, 380 μL) for 18 h at 4°C, followed by two wash steps with PBS (Bengali et al., 2005). Complexes were then immobilized following complex formation, as described above, by incubation of DNA complexes (475 μL) with the serum-coated wells for 2 h. After complex incubation, the wells were washed twice with Opti-MEM (for Lipofectamine 2000 complexes) or EC buffer (for Effectene complexes) and 250,000 MCF-7 cells (which had been cultured in estrogen-free media for 4 days) were seeded onto the immobilized DNA-lipid complexes in each well.

For bolus delivery, MCF-7 cells, which had been cultured in estrogen-free media for 4 days, were seeded in estrogen-free medium into 24-well plates at densities of 125,000 cells per well. Eighteen hours later, complexes, formed as described above, were diluted in antibiotic-free, estrogen-free media and then added to the cells.

For both surface and bolus delivery, complexes contained both the pERE(3×)TK-ffLUC plasmid and the normalization plasmid, pTK-rLUC, at a ratio of 4:1. Total DNA amounts added for surface delivery ranged from 0.13 to 1.32 μg/cm^2 (0.25–2.5 μg per well) and 0.05 to 0.26 μg/cm^2 (0.025–0.5 μg per well) for bolus delivery. Given binding profiles, these ranges result in approximately the same amount of DNA bound to the surface as delivered as a bolus (Bengali et al., 2005).

Immediately after complex addition for bolus delivery and 4 h after cell seeding for surface delivery, cells were treated with combinations of E_2 (Sigma–Aldrich, St. Louis, MO), the complete anti-estrogen fulvestrant [(FUL), also termed ICI 182,780, Tocris Bioscience, Ellisville, MO] or vehicle controls. E_2 and FUL were both dissolved in ethanol and diluted in estrogen-free media to obtain the indicated concentrations (10^{-12} to 10^{-9} M for E_2; 10^{-6} M for FUL) prior to addition to cells. Ethanol diluted in estrogen-free

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media served as the vehicle control. Cells were harvested and assayed for firefly and renilla luciferase reporter gene activities 48 h after transfection using the Dual-Luciferase Reporter assay system (Promega). In this dual-luciferase system, firefly and renilla luciferases are measured sequentially, in a single well. These measurements are accomplished by adding the firefly luciferase substrate first, measuring luminescence, and then adding reagents that quench the firefly luciferase reaction and simultaneously provide the renilla luciferase substrate, followed by measuring renilla luciferase activity. The dual-luciferase assays were carried out using an automated microplate luminometer equipped with dual-injection ports (Mithras LB 940, Berthold Technologies, Oak Ridge, TN). Relative dual-luciferase activity was calculated by dividing the luminescent signal from the firefly reporter gene by the renilla luminescent signal.

Array Fabrication

Soft lithography techniques were used to pattern DNA complex deposition. A polydimethylsiloxane (PDMS) mold was fabricated by curing PDMS into thin, flat disks. Briefly, PDMS was prepared in a 10:1 (v/v) ratio of Silicone Elastomer-184 and Silicone Elastomer Curing Agent-184 (Sylgard 184, Dow Corning, Midland, MI) by mixing the base and curing agent at least 50 times using a syringe mixing system. After allowing all air bubbles to escape, the PDMS was poured directly into a polystyrene tissue culture dish (100 mm, Corning, Corning, NY) and cured at 60°C for approximately 2 h. The cured PDMS was removed from the dish and rods of precise diameters were then used to punch holes into the PDMS, with diameters of 2.4 mm. The PDMS mold was rinsed in 70% ethanol, oxidized using oxygen plasma and then reversibly sealed to polystyrene microscope slides (Nunc, Rochester, NY), which were fitted into custom-fabricated Teflon slide holders. The holes in the PDMS mold, termed microwells, served as reservoirs for deposition of DNA complexes onto the polystyrene slide. After 2 h of complex deposition in humid conditions, the PDMS mold was peeled away from the polystyrene, and the slide was rinsed thoroughly with Opti-MEM. For all array studies, DNA concentrations ranged from 0.007 to 0.021 μg/μL, with 2.2 to 4 μL of complex volume added to the microwells of the PDMS mold.

To visualize DNA complex immobilization on the array and verify deposition replicated the pattern of the microwells in the PDMS mold, plasmid (pEGFP-LUC) was labeled with tetramethyl rhodamine (Label IT Nucleic Acid Labeling Kit, Mirus, Madison, WI), complexed as described above, and deposited in the microwells. After deposition, PDMS removal and rinsing, the resulting spots were visualized with fluorescence microscopy (see below).

Transfection of cells on the array was verified by depositing complexes formed with plasmid pEGFP-LUC in the microwells, as described above, and imaging with fluorescence microscopy. After complex deposition, PDMS removal and rinsing, MCF-7 cells were seeded onto the slide at a density of 10^6 cells per slide (18.75 cm^2). Transfection was analyzed after 24 and 48 h and characterized through GFP expression. Transfected cells were visualized using an epifluorescence microscope (Leica; Bannockburn, IL) with a FITC filter and equipped with a digital camera. Transfection, as assayed through bioluminescence imaging, was verified by depositing complexes containing both pLUC and pTK-rLUC plasmids, at a 1:1 ratio. After deposition, PDMS removal and rinsing, cells were seeded as described above. Transfection was analyzed after 24 h and characterized by dual-luciferase expression through light emission (see below).

For induction studies in the array, complexes formed with different plasmids were immobilized in different spots of the array, in triplicate. Briefly, complexes were formed with pLUC, pERE(3×)TK-ffLUC, pERE(3×)TK-ffLUC and pTK-rLUC (2:1 ratio), or pβGAL. After deposition, PDMS removal and rinsing, MCF-7 cells, which had been cultured in estrogen-free media for 18 h, were seeded in estrogen-free medium on arrays at a density of 10^6 cells per slide. Immediately after cell seeding, cells were treated with combinations E_2, FUL, or vehicle control, as described above. Dual-luciferase levels were analyzed 24 h later by bioluminescence imaging.

Bioluminescence Imaging

Expression of both luciferase reporter genes was assessed through imaging of light production upon sequential addition of the luciferase substrates to the bulk media. Bioluminescence imaging of the array was performed using an IVIS imaging system (Xenogen Corp., Alameda, CA), which utilizes a cooled CCD camera. For imaging, ViviRen (Promega), a modified renilla luciferase substrate, was diluted to 0.66 mM in serum-containing media and then added to the arrays at a final concentration of 10 μM. After 2 min, the arrays were placed into a light-tight chamber and bioluminescence images were acquired for a total exposure time of 1 min. Immediately following imaging with ViviRen, 1 mM D-luciferin (Molecular Therapeutics, Inc., Ann Arbor, MI, 20 mg/mL in PBS), the firefly luciferase substrate, was added into the media above the cells cultured on the array, and bioluminescence images were acquired 3 min later, with 1 min exposure. Gray scale and bioluminescence images were superimposed using the Living Image software (Xenogen Corp.). A constant size region of interest (ROI) was drawn over the spots of the array to calculate light signals. The signal intensity was reported as an integrated light flux (photons/sec), determined by IGOR software (WaveMetrics, Lake Oswego, OR). The signal due to firefly luciferase was determined by subtracting ViviRen signal from the luciferin signal. Normalization was accomplished by dividing the firefly luciferase signal (luciferin signal minus ViviRen signal, Promega) by the renilla luciferase

signal (ViviRen signal). A renilla signal threshold was set at 3.5E4 photon/sec (2X background) to distinguish spots of unreliable signals indicating insufficient transfection.

Statistics

Statistical analysis was performed using JMP software (SAS Institute, Inc., Cary, NC). Comparative analyses were completed using one-way ANOVA with Tukey post-tests, at a 95% confidence level. Mean values with standard deviation are reported and all experiments were performed with a minimum sample size of three, performed in replicate.

Results

Multiwell Dish Format ERE-Reporter Gene Induction Studies

Multiwell dish format reporter gene assays were performed to compare ERα-regulated, ERE-dependent transcriptional activity in MCF-7 cells transfected via surface-mediated delivery of DNA complexes in comparison to traditional bolus delivery (Fig. 1). DNA complexes, formed using an E_2-responsive firefly luciferase reporter plasmid pERE(3×)TK-ffLUC and a normalization plasmid pTK-rLUC encoding renilla luciferase, were delivered to cells via bolus or surface delivery. Transfected cells were treated with various combinations of the agonist E_2, the complete antiestrogen FUL, or ethanol. Surface delivery of the plasmids (Fig. 1B) resulted in E_2-stimulated responses similar to bolus delivery (Fig. 1A), with E_2 statistically inducing firefly luciferase expression six- to sevenfold ($P < 0.001$) over vehicle control or the addition of FUL. Hence, the physiologic state of the cells during surface-mediated delivery allowed the cells to transcriptionally respond to E_2. Further, the maximal induction of reporter gene activity was similar whether the DNA complexes were delivered via bolus or surface-mediated techniques.

The amount of transfected plasmid was subsequently investigated, which indicated a similar DNA mass-dependent effect in reporter gene activity for both surface and bolus-mediated transfection methods (Fig. 2). For bolus delivery (Fig. 2A), all DNA amounts resulted in significantly different responses ($P < 0.01$), except for 0.11 and 0.2 μg/cm^2, which were not statistically different from each other. Maximal induction was achieved at 0.13 μg/cm^2 (0.25 μg per well). For surface delivery (Fig. 2B), all DNA amounts resulted in significantly different responses ($P < 0.05$), with 1.05 μg/cm^2 (2 μg per well) corresponding to the highest induction by E_2. These results indicate that sufficient amounts of DNA must be transfected for optimal reporter gene activity, and excess amounts of DNA lead to less efficient reporter gene activity, possibly due to toxicity, for both delivery methods.

Assuming that approximately 20% of DNA added to the cell culture dish surface is immobilized (Bengali et al. 2005), the condition with the highest induction (1.05 μg/cm^2), would have presented approximately 0.21 μg/cm^2 of DNA to the cells, which is higher than the bolus condition with the highest induction (0.13 μg/cm^2), but still in the range of robust activity. Therefore, surface delivery required more

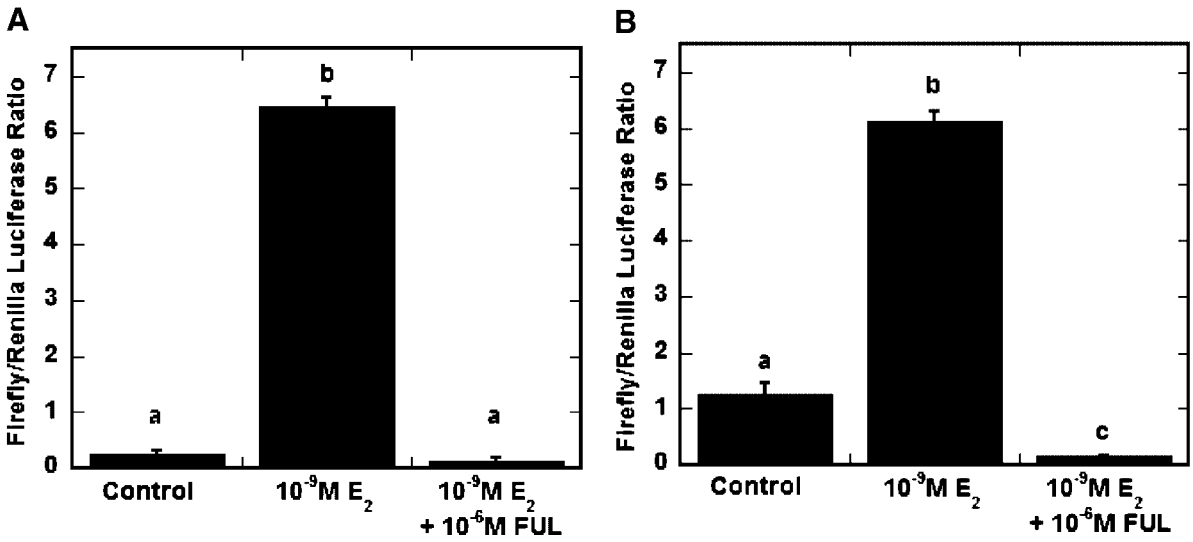

Figure 1. Multiwell dish format reporter gene assay to compare surface delivery to traditional bolus delivery. Surface delivery (**B**) of ERE reporter plasmid system (pERE(3×)TK-ffLUC and normalization plasmid pTK-rLUC) resulted in E_2-stimulated transcriptional responses in MCF-7 breast cancer cells similar to bolus delivery (**A**), reported as a ratio of firefly to renilla luciferase, with E_2 statistically inducing firefly luciferase expression six- to sevenfold over vehicle control or the addition of FUL. (Columns labeled with same letter designate conditions not statistically different; all other comparisons, $P < 0.001$).

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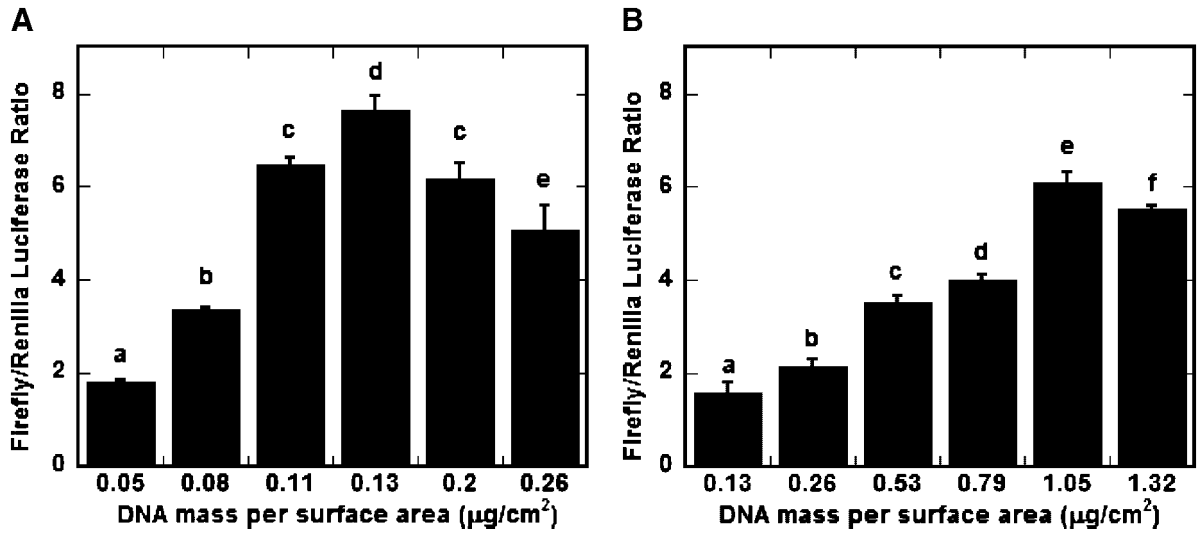

Figure 2. The effect of DNA amount on E_2 activation of ERE reporter plasmid system (pERE(3×)TK-ffLUC and normalization plasmid pTK-rLUC) delivered to MCF-7 breast cancer cells. Total amount of DNA added to the surface (**B**) or delivered as a bolus (**A**), in the presence of 10^{-9} M E_2, resulted in a similar dose-response effect. (Columns labeled with same letter designate conditions not statistically different; all other comparisons, $P < 0.01$ for (A), $P < 0.05$ for (B).)

DNA added to the surface than what would have been expected given binding profiles (Bengali et al. 2005). The requirement for more DNA may be due to lower than anticipated binding efficiencies (~10%, but still within the range of profiles reported).

The specific transfection reagent used to form DNA complexes, and E_2 concentration responses were subsequently investigated to determine the applicability and sensitivity of the reporter system (Fig. 3). For Lipofectamine 2000-DNA complexes (Fig. 3A), E_2-induction profiles were not significantly different using bolus versus surface delivery (Fig. 3A), with E_2 eliciting a concentration response from 10^{-12} to 10^{-10} M ($P < 0.05$), and maximal responsiveness observed from 10^{-10} to 10^{-9} M E_2 ($P > 0.05$) for both delivery methods. For Effectene complexes (Fig. 3B), bolus delivery resulted in statistically higher levels of ERE induction ($P < 0.05$) than surface delivery for all concentrations of E_2, except control. However, the level of

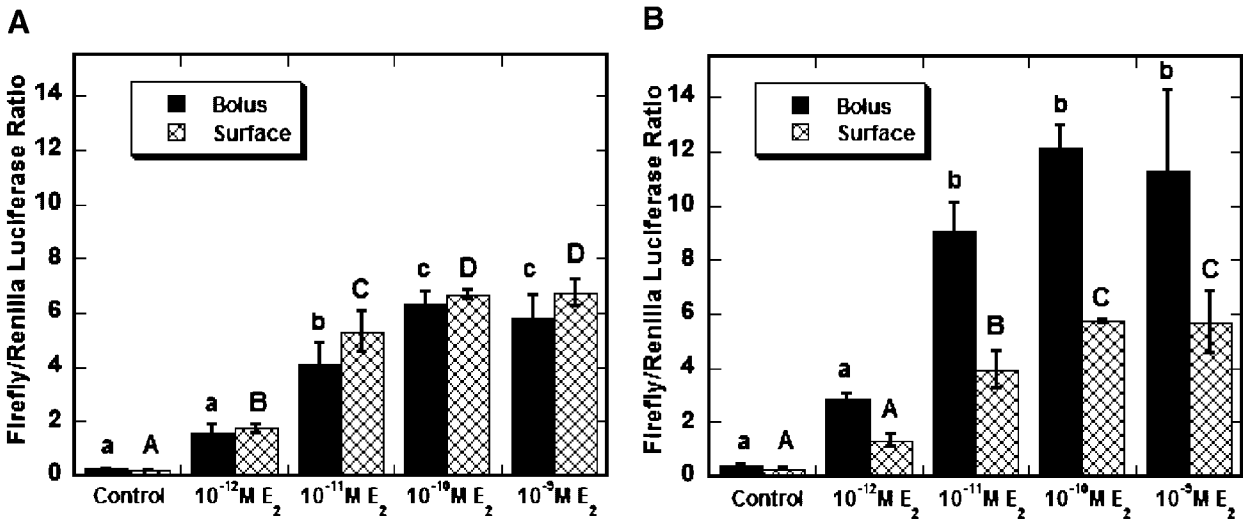

Figure 3. The effect of complexing agent and E_2 dose response on the ERE reporter plasmid system (pERE(3×)TK-ffLUC and normalization plasmid pTK-rLUC). Bolus and surface delivery of Lipofectamine 2000 complexes (**A**) resulted in induction profiles that were not statistically different from each other, for each concentration of E_2. Bolus delivery of Effectene complexes (**B**) resulted in statistically higher induction ($P < 0.05$) than surface delivery for all concentrations of E_2, except control, however surface delivery resulted in more statistically different induction responses. (Columns labeled with same letter designate conditions not statistically different; all other comparisons, $P < 0.05$.)

ERE induction for surface-mediated delivery was similar whether complexing DNA with Effectene (Fig. 3B) or with Lipofectamine 2000 (Fig. 2A). Therefore, the particular transfection reagent used affected transcriptional activity via the conventional bolus delivery, but not via surface delivery. None the less, these results demonstrate that either Effectene or Lipofectamine 2000 can be used to delivery plasmid via surface-mediated transfection. Further, other transfection reagents can likely be adapted for use in surface-mediated delivery.

Array Fabrication and Verification

An array was created using soft lithography techniques to pattern DNA-lipid complex deposition and subsequent transfection upon cell seeding (Fig. 4). Briefly, a PDMS mold with microwells (Fig. 4A) was reversibly sealed to polystyrene microscope slides (Fig. 4B), with the microwells serving as reservoirs for deposition of DNA complexes onto the polystyrene slide (Fig. 4C). Rhodamine-labeled DNA complexes deposited within microwells were immobilized to the slide in distinct regions, replicating the pattern of microwells in the PDMS mold (Fig. 4D–F). Transfection of MCF-7 cells seeded onto arrays of complexes was determined by GFP expression, and was also confined to the patterns (Fig. 4G–I).

Bioluminescence Imaging of the Array

Arrays formed with complexes containing plasmids encoding firefly and renilla luciferase reporter genes (pLUC and pTK-rLUC) were used to verify the ability of bioluminescence imaging to detect dual-luciferase expression (Fig. 5). Transfection of MCF-7 cells seeded onto these arrays was assayed after 24 h by sequentially adding the renilla and firefly luciferase substrates. Following ViviRen addition, spot intensities averaged $1.10 \times 10^5 \pm 2.56 \times 10^4$ photon/sec (Fig. 5A), which are similar to signals obtained with arrays of only pTK-rLUC plasmid (data not shown). D-Luciferin was subsequently added to the same array, which was then imaged to acquire a dual signal (Fig. 5B), with average spot intensities of $3.66 \times 10^6 \pm 4.34 \times 10^5$ photon/sec. Firefly luciferase expression was determined by subtracting the initial ViviRen signal from the signal obtained through imaging with the D-luciferin. Firefly expression averaged $3.55 \times 10^6 \pm 4.30 \times 10^5$ photon/sec, also similar to intensities obtained with arrays formed with only pLUC plasmid (data not shown). After normalization, the firefly luciferase signal was 34 ± 8 fold greater than the respective renilla expression. Timecourse studies revealed that the ViviRen signal remained constant for 10 min after substrate addition. Therefore the firefly luciferase signal could be obtained using this dual imaging strategy followed by subtraction techniques, given imaging was accomplished within 10 min of ViviRen addition (data not shown). Bioluminescence imaging was able to sensitively capture both luciferase signals, enabling the same cell population to be analyzed for the expression of multiple reporter genes.

Array Format ERE-Reporter Gene Induction Studies

To assess the ability of the arrays to monitor induction of ERα transcriptional activity (Fig. 6), complexes formed with different plasmids were immobilized as an array in triplicates as follows: 1. pLUC, 2. no DNA (mock), 3. pERE(3×)TK-ffLUC, 4. pERE(3×)TK-ffLUC and pTK-rLUC (2:1 ratio), and 5. pβGAL. Cells seeded on the arrays were treated with combinations of ethanol control (Fig. 6A,B), 10^{-9} M E_2 (Fig. 6C,D), or 10^{-9} M $E_2 + 10^{-6}$ M FUL (Fig. 6E,F). Dual-luciferase levels were analyzed 24 h

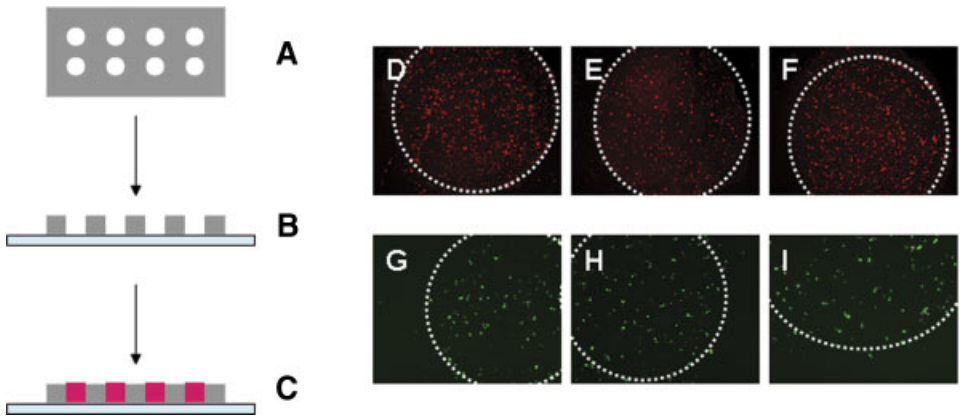

Figure 4. Array fabrication with soft lithography techniques to pattern DNA-lipid complex deposition and transfection. A polydimethylsiloxane (PDMS) mold (**A**) was reversibly sealed to polystyrene slides (**B**), so that the holes in the mold, termed microwells, served as reservoirs for deposition of DNA complexes onto the polystyrene (**C**). After complex deposition in the microwells, the PDMS mold was peeled away from the polystyrene slide, which was then rinsed thoroughly. Rhodamine-labeled DNA complexes were immobilized on the slide in distinct regions, replicating the pattern of microwells in the PDMS mold (**D–F**). Transfection of MCF-7 cells seeded onto these arrays of patterned complexes on polystyrene slides was also confined to the patterns, as determined by GFP expression (**G–I**). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

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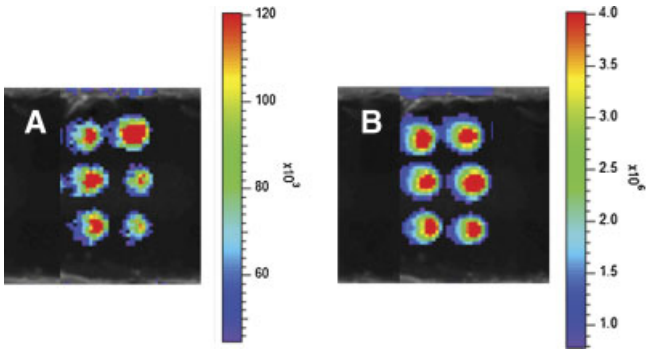

Figure 5. Bioluminescence imaging to detect dual-luciferase expression in an array format. Transfection of MCF-7 cells seeded onto arrays of complexes was assayed after 24 h by sequentially adding the renilla and firefly luciferase substrates. The renilla substrate, ViviRen (10 μM), was first added into the media and the array was imaged to determine pTK-rLUC expression (**A**). D-Luciferin (1 mM) was subsequently added to the same array, which was then imaged to acquire a dual signal (**B**). Firefly luciferase expression (pLUC) was determined by subtracting the ViviRen signal from the signal obtained through imaging with the D-luciferin. When normalized, the firefly luciferase signal was 34 ± 8 fold greater than the respective renilla expression. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

later using bioluminescence imaging, by first imaging with ViviRen (Fig. 6A,C,E), and then imaging each array with D-luciferin (Fig. 6B,E,F). Renilla luciferase activity was only detected in cells transfected with pTK-rLUC plasmid (Fig. 6A,C,E, column 4), and not in cells transfected with only firefly luciferase-encoding plasmids (Fig. 6A,C,E, columns 1 and 3), a control βGAL-encoding plasmid (Fig. 6A,C,E, column 5) or no DNA (Fig. 6A,C,E, column 2). Accordingly, firefly luciferase activity was only detected in cells transfected with pLUC (Fig. 6B,D,F, column 1) or pERE(3×)TK-ffLUC (Fig. 6B,D,F, columns 3 and 4), but not in mock or βGAL control transfected cells (Fig. 6B,D,F, columns 2 and 5) These results verify the specificity of renilla and firefly luciferase detection in this system.

As predicted, firefly luciferase activity was detected at substantially higher levels in cells transfected with pERE(3×)TK-ffLUC and treated with E_2 (Fig. 6D, columns 3 and 4) compared to those treated with ethanol (Fig. 6B, columns 3 and 4) or E_2 + FUL (Fig. 6 F, columns 3 and 4). In control-treated arrays, spots of highest intensity were visualized for pLUC (Fig. 6B, column 1), given its highly active CMV promoter. Cells transfected with both the pERE(3×)TK-ffLUC and pTK-rLUC plasmids (Fig. 6B, column 4) resulted in higher signal intensities in the presence of luciferin than cells transfected with only the pERE(3×)TK-ffLUC (Fig. 6B, column 3), as there was no carryover of ViviRen signal in the latter spots of transfected cells without pTK-rLUC. For E_2 addition to the array, signal intensities with luciferin increased as compared to the control condition for all cells transfected with pERE(3×)TK-ffLUC plasmids (Fig. 6D, columns 3 and 4), indicating ERα-dependent transcriptional activation of the ERE-regulated plasmid. Expression of the pLUC plasmid was largely unaffected by E_2 (Fig. 6D, column 1). Addition of the antiestrogen FUL to the arrays completely eliminated the signal in cells transfected with pERE(3×)TK-ffLUC alone (Fig. 6F, column 3), or substantially reduced signal intensities in cells transfected with both pERE(3×)TK-ffLUC and pTK-rLUC (Fig. 6F, column 4), in which the luminescence that was detected was again due to carryover of the ViviRen signal. Therefore, addition of 10^{-6} M FUL led to a complete blockade of ERα-stimulated activity by 10^{-9} M E_2. pLUC expression was also lowered in the presence of FUL (Fig. 6F, column 1) indicating that some transcriptional elements in the CMV may be indirectly regulated by ERα, possibly by ERα tethering to AP1 and SP1 proteins bound directly to DNA in this promoter.

Average renilla luminescence intensities in cells transfected with pERE(3×)TK-ffLUC and pTK-rLUC plasmids (Fig. 6A,C,E, column 4) were similar in control and E_2 + FUL treated cells, but lower in E_2 alone treated cells. This lower renilla luciferase activity is likely due to competition for transcriptional cofactors between the ERE(3×)TK and TK-only regulated promoters. Under E_2 stimulation conditions, ERα transcriptional coregulators and basal transcriptional machinery may be preferentially recruited to ERE-containing promoters rather than promoters lacking EREs. Hence, in cells treated with E_2, squelching likely occurs at the TK-renilla luciferase promoter due to titrating out of limiting transcription factors.

Induction of the ER-regulated plasmid system in the array mimicked results obtained through traditional assays methods. Firefly luciferase expression was determined by subtracting the ViviRen signal from the signal obtained through imaging with the D-luciferin, which was then normalized by the ViviRen signal (Fig. 6G). For cells transfected with both the pERE(3×)TK-ffLUC and pTK-rLUC plasmids (Fig. 6, column 4), E_2 statistically induced dual-luciferase activity 10-fold ($P < 0.001$) over control or FUL conditions (Fig. 6G). This robust induction verifies that the array can accurately report on the activity of the ERα transcription factor. The concentration response of E_2 was examined to determine the sensitivity of the reporter system in an array format (Fig. 7). For arrays with spots containing both the pERE(3×)TK-ffLUC and pTK-rLUC plasmids, increasing the concentration of E_2 statistically increased the induction of firefly luciferase expression ($P < 0.05$), capturing the concentration-response of E_2 in the induction of this plasmid system.

Discussion

Methods to use mammalian cells as suitable screening systems need to be developed to elucidate gene function and cellular pathways responsible for diseases (Grimm, 2004). Transfected cell arrays offer an advantage in their ability to analyze the expression of genes and the function of proteins in living cells, where the machinery is present to ensure correct function of the gene products. These live cell

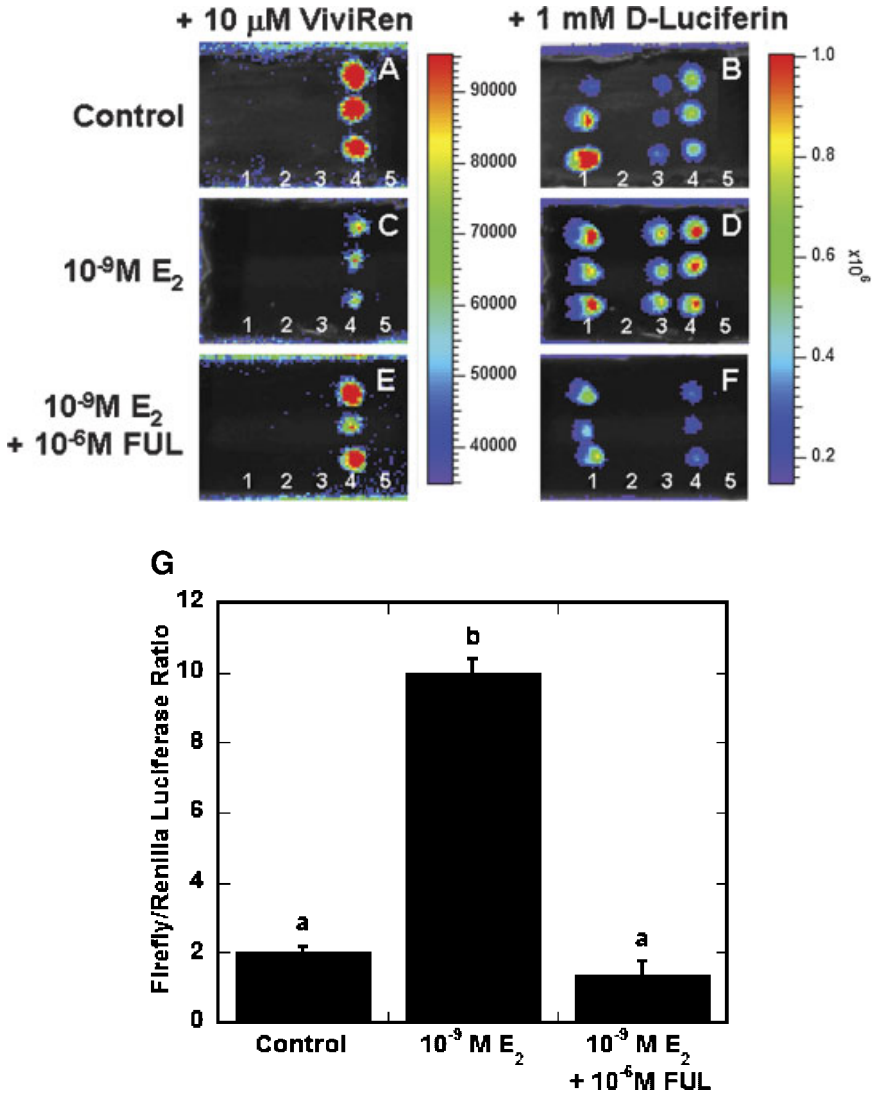

Figure 6. Arrays to monitor ERα induction of transcriptional activity. Complexes formed with different plasmids were immobilized in different spots of the array, in triplicate, as follows: (1) pLUC, (2) none, (3) pERE(3×)TK-ffLUC, (4) pERE(3×)TK-ffLUC and pTK-rLUC (2:1 ratio), and (5) pβGAL. Cells seeded on the arrays were treated with combinations of ethanol control (**A**,**B**), E_2 (**C**,**D**), or E_2 plus FUL (**E**,**F**). Dual-luciferase levels were analyzed 24 h later with bioluminescence imaging, by first imaging with the renilla luciferase substrate, ViviRen (A,C,E) and then imaging each array with D-luciferin, the firefly luciferase substrate (B,E,F). Induction of the ERE-regulated plasmid system was calculated by normalizing firefly luciferase expression to renilla luciferase expression (**G**). Firefly luciferase expression was determined by subtracting the ViviRen signal from the signal obtained through imaging with the D-luciferin. For spots containing both the pERE(3×)TK-ffLUC and pTK-rLUC plasmids (column 4), E_2 statistically induced firefly luciferase expression 10-fold over control or FUL conditions, reported as a ratio of firefly to renilla luciferase (G). (Columns labeled with same letter designate conditions not statistically different; all other comparisons, $P < 0.001$.) [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

microarrays could provide a method to link gene expression to functional cell responses, with the potential to impact many aspects of science and medicine. Transfected cell arrays have been primarily used for identification of gene function (Hodges et al., 2005) and discovery of novel genes and proteins (Ziauddin and Sabatini, 2001), and have potential utility in emerging applications such as detection of biological warfare agents and environmental toxins through surface receptors (Delehanty et al., 2004b), detection of tumor-associated antigens (Hoeben et al., 2006), and determination of molecular markers or targets (Palmer and Freeman, 2005), prior to the costly development of novel diagnostic and therapeutic strategies. With many possible applications for transfected cell arrays, technological advances are needed to improve array accuracy and consistency and to facilitate endpoint analysis (Hook et al., 2006; Palmer and Freeman, 2005). We have combined dual plasmid delivery and bioluminescence imaging to create a transfected cell array that allows for normalization of transfection, and provides rapid and sensitive quantification of the cellular response with minimal post-transfection processing.

In our transfected cell array, we employed a dual plasmid system to provide normalization, sensitivity, and quantifi-

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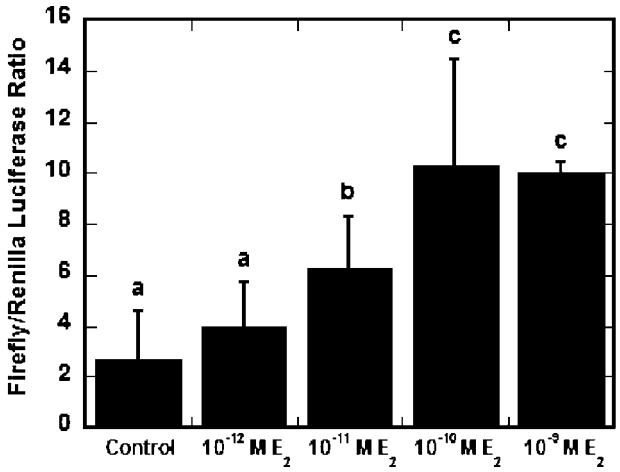

Figure 7. Concentration response of E_2 on the ERE reporter plasmid system in an array format. For spots containing both the pERE(3×)TK-ffLUC and pTK-rLUC plasmids, increasing the concentration of E_2 statistically increased the induction of firefly luciferase expression, verifying a true concentration-response of E_2 in the induction of this plasmid system in an array format. (Columns labeled with same letter designate conditions not statistically different; all other comparisons, $P < 0.05$.)

cation, which are all intricately related, in part, through the transfection efficiency. Spot to spot variability in transfection can compromise the ability to quantify a response within an array, as sub-maximal responses may indicate either a limited effect or simply inefficient or unequal delivery. A variance in fluorescence intensity of transfected cells (GFP) has been noted between spots of the array, which likely correlates with the number of plasmids internalized (Hook et al., 2006). Therefore, to enable normalization of transfection efficiency, a two-plasmid system consisting of: (i) a normalization plasmid that is present within each spot, and (ii) a functional plasmid that varies between spots and is responsible for the functional endpoint of the array, was deposited in each spot. Both plasmids contain the same TK promoter, which is important for normalization, and should allow comparison between cell lines on the array. Delivery of two plasmids has been shown to result in a majority of cells expressing both reporter genes (unpublished observations). To normalize with a second plasmid, the efficiency of delivery must be sufficient to obtain a signal from each plasmid. This issue was addressed using larger spot sizes relative to many previous reports. Small spot size can contribute to low transfection efficiencies (Palmer and Freeman, 2005), which are detrimental because each spot on the array may contain so few cells that an insufficient number of cells are transfected locally to be statistically informative (Hodges et al., 2005). Small spots with low transfection efficiency make image acquisition and quantification difficult and lower sensitivity, which can lead to high false positive and false negative rates (Palmer et al., 2006), further demonstrating a need to account for efficiency and normalization issues to increase the reliability of quantitative data obtained from each spot (Fujimoto et al., 2006). To further address issues with transfection efficiency, our array fabrication and normalization approach could be compatible with viral delivery (Bailey et al., 2006; Hobson et al., 2003; Michiels et al., 2002), however a plasmid system is more versatile due to the easier production and handling methods.

Bioluminescence imaging (Rutter et al., 1998) was employed to quantify the response of the dual plasmids within the array, with minimal post-transfection processing and high sensitivity. Endpoint analysis for the arrays often requires tagging or staining (Hook et al., 2006) to report gene function, which can require extensive post-transfection processing, such as fixation and immunostaining (Lee et al., 2006; Wheeler et al., 2005). The normalization and functional plasmids contain renilla and firefly luciferase reporters respectively, which can both be rapidly quantified in each spot by sequential addition of the respective substrates to the culture media followed by imaging of the array. Luciferase reporters are known to be more sensitive than GFP, without the issues of autofluorescence and background signals (Rutter et al., 1998). Luciferase is more quantitative and allows for small differences in expression to be determined, which enabled our system to determine a dose response to an external stimulus. An additional potential advantage, the short half-life of luciferase could allow for real-time imaging to follow the dynamics of gene activity (Rutter et al., 1998). However, alternative imaging systems requiring automated microscopy and image processing (Pepperkok and Ellenberg, 2006; Wheeler et al., 2005) can allow for detection of changes in cellular morphology and cellular level data, which is not possible with bioluminescence imaging.

The array was used to quantify the activity of the ERα in breast cancer cells with an ERE-regulated promoter reporter system, as an example of an inducible plasmid system in a cancer model. ERα, a member of the nuclear receptor superfamily of transcription factors, activates transcription through binding of its ligand, E_2. Expression of ERα is clinically used as a biomarker to determine treatment for breast cancer patients (Ariazi et al., 2006; Pearce and Jordan, 2004). However, simple expression of transcription factors like ERα does not necessarily reflect pathway activation, as transcription factor activity is regulated through diverse mechanisms (Levine and Tjian, 2003), including heteromeric complexes, ubiquitination, methylation, acetylation, and post-translational modifications such as phosphorylation. The transfected cell array allows for the determination of transcription factor activity. In the case of the ERα, we assayed for induction by E_2. Induction in the array mimicked results obtained through traditional luciferase assay methods, with E_2 inducing luciferase expression 10-fold over fulvestrant or vehicle controls. The array also captured the varying ER activity in response to a range of E_2 dosages, further demonstrating the sensitivity of the bioluminescence quantification system.

In summary, this report demonstrates the ability to quantitatively assess a transfected cell array using dual

bioluminescence imaging to enable normalization of transfection efficiency, while reducing post-transfection processing and increasing sensitivity. Additionally, ER activity was quantified in a physiologically relevant model of breast cancer, indicating the effectiveness of the array system, as many of the published arrays have only used HEK293T cells, a cell line known to be easily transfected and not applicable to many relevant biological endpoints or applications. The dual plasmid system and bioluminescence imaging are enabling technologies that, when combined with high-throughput arrays involving large numbers of plasmids, have the potential to impact basic research in cancer and other disciplines through investigation of fundamental biological processes (Hoeben et al., 2006). With further advancements in the transfection of primary cells, transfected cell arrays have the potential for use in cancer medicine, to classify clinical cancer samples through prognostic profiles (Chen and Davis, 2006), to provide novel information regarding disease progression, and to identify molecular targets for patient-specific therapy (Kozarova et al., 2006).

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References

Ariazi EA, Ariazi JL, Cordera F, Jordan VC. 2006. Estrogen receptors as therapeutic targets in breast cancer. Curr Top Med Chem 6(3):181–202.

Bailey SN, Ali SM, Carpenter AE, Higgins CO, Sabatini DM. 2006. Microarrays of lentiviruses for gene function screens in immortalized and primary cells. Nat Methods 3(2):117–122.

Bengali Z, Pannier AK, Segura T, Anderson BC, Jang JH, Mustoe TA, Shea LD. 2005. Gene delivery through cell culture substrate adsorbed DNA complexes. Biotechnol Bioeng 90(3):290–302.

Catherino WH, Jordan VC. 1995. Increasing the number of tandem estrogen response elements increases the estrogenic activity of a tamoxifen analogue. Cancer Lett 92(1):39–47.

Chang FH, Lee CH, Chen MT, Kuo CC, Chiang YL, Hang CY, Roffler S. 2004. Surfection: A new platform for transfected cell arrays. Nucleic Acids Res 32(3):e33.

Chen DS, Davis MM. 2006. Molecular and functional analysis using live cell microarrays. Curr Opin Chem Biol 10(1):28–34.

Delehanty JB, Shaffer KM, Lin B. 2004a. A comparison of microscope slide substrates for use in transfected cell microarrays. Biosens Bioelectron 20(4):773–779.

Delehanty JB, Shaffer KM, Lin B. 2004b. Transfected cell microarrays for the expression of membrane-displayed single-chain antibodies. Anal Chem 76(24):7323–7328.

Erfle H, Simpson JC, Bastiaens PI, Pepperkok R. 2004. siRNA cell arrays for high-content screening microscopy. Biotechniques 37(3):454–458, 460, 462.

Fujimoto H, Yoshizako S, Kato K, Iwata H. 2006. Fabrication of cell-based arrays using micropatterned alkanethiol monolayers for the parallel silencing of specific genes by small interfering RNA. Bioconjug Chem 17(6):1404–1410.

Grimm S. 2004. The art and design of genetic screens: Mammalian culture cells. Nat Rev Genet 5(3):179–189.

Hobson DA, Pandori MW, Sano T. 2003. In situ transduction of target cells on solid surfaces by immobilized viral vectors. BMC Biotechnol 3(1):4.

Hodges E, Redelius JS, Wu W, Hoog C. 2005. Accelerated discovery of novel protein function in cultured human cells. Mol Cell Proteomics 4(9):1319–1327.

Hoeben A, Landuyt B, Botrus G, De Boeck G, Guetens G, Highly M, van Oosterom AT, de Bruijn EA. 2006. Proteomics in cancer research: Methods and application of array-based protein profiling technologies. Analytica Chimica Acta 564(1):19–33.

Honma K, Ochiya T, Nagahara S, Sano A, Yamamoto H, Hirai K, Aso Y, Terada M. 2001. Atelocollagen-based gene transfer in cells allows high-throughput screening of gene functions. Biochem Biophys Res Commun 289(5):1075–1081.

Hook AL, Thissen H, Voelcker NH. 2006. Surface manipulation of biomolecules for cell microarray applications. Trends Biotechnol 24(10):471–477.

How SE, Yingyongnarongkul B, Fara MA, Diaz-Mochon JJ, Mittoo S, Bradley M. 2004. Polyplexes and lipoplexes for mammalian gene delivery: From traditional to microarray screening. Comb Chem High Throughput Screen 7(5):423–430.

Hu YH, Vanhecke D, Lehrach H, Janitz M. 2005. High-throughput subcellular protein localization using cell arrays. Biochem Soc Trans 33(Pt 6):1407–1408.

Hu YH, Warnatz HJ, Vanhecke D, Wagner F, Fiebitz A, Thamm S, Kahlem P, Lehrach H, Yaspo ML, Janitz M. 2006. Cell array-based intracellular localization screening reveals novel functional features of human chromosome 21 proteins. BMC Genomics 7:155.

Ikeda K, Inoue S. 2004. Estrogen receptors and their downstream targets in cancer. Arch Histol Cytol 67(5):435–442.

Isalan M, Santori MI, Gonzalez C, Serrano L. 2005. Localized transfection on arrays of magnetic beads coated with PCR products. Nat Methods 2(2):113–118.

Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. 1992. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 90(1):77–86.

Kato K, Umezawa K, Miyake M, Miyake J, Nagamune T. 2004. Transfection microarray of nonadherent cells on an oleyl poly(ethylene glycol) ether-modified glass slide. Biotechniques 37(3):444–448, 450, 452.

Kozarova A, Petrinac S, Ali A, Hudson JW. 2006. Array of informatics: Applications in modern research. J Proteome Res 5(5):1051–1059.

Lee CW, Rivera R, Gardell S, Dubin AE, Chun J. 2006. GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, L PA5. J Biol Chem 281(33):23589–23597.

Levenson AS, Jordan VC. 1997. MCF-7: The first hormone-responsive breast cancer cell line. Cancer Res 57(15):3071–3078.

Levine M, Tjian R. 2003. Transcription regulation and animal diversity. Nature 424(6945):147–151.

Mannherz O, Mertens D, Hahn M, Lichter P. 2006. Functional screening for proapoptotic genes by reverse transfection cell array technology. Genomics 87(5):665–672.

Michiels F, van Es H, van Rompaey L, Merchiers P, Francken B, Pittois K, van der Schueren J, Brys R, Vandersmissen J, Beirinckx F, et al. 2002. Arrayed adenoviral expression libraries for functional screening. Nat Biotechnol 20(11):1154–1157.

Mousses S, Caplen NJ, Cornelison R, Weaver D, Basik M, Hautaniemi S, Elkahloun AG, Lotufo RA, Choudary A, Dougherty ER, et al. 2003. RNAi microarray analysis in cultured mammalian cells. Genome Res 13(10):2341–2347.

Neumann B, Held M, Liebel U, Erfle H, Rogers P, Pepperkok R, Ellenberg J. 2006. High-throughput RNAi screening by time-lapse imaging of live human cells. Nat Methods 3(5):385–390.

Palmer E, Freeman T. 2005. Cell-based microarrays: Current progress, future prospects. Pharmacogenomics 6(5):527–534.

DOI 10.1002/bit

Palmer EL, Miller AD, Freeman TC. 2006. Identification and characterisation of human apoptosis inducing proteins using cell-based transfection microarrays and expression analysis. BMC Genomics 7(1):145.

Pannier AK, Anderson BC, Shea LD. 2005. Substrate-mediated delivery from self-assembled monolayers: Effect of surface ionization, hydrophilicity, and patterning. Acta Biomaterialia 1(5):511–522.

Pearce ST, Jordan VC. 2004. The biological role of estrogen receptors alpha and beta in cancer. Crit Rev Oncol Hematol 50(1):3–22.

Pepperkok R, Ellenberg J. 2006. High-throughput fluorescence microscopy for systems biology. Nat Rev Mol Cell Biol 7(9):690–696.

Peterbauer T, Heitz J, Olbrich M, Hering S. 2006. Simple and versatile methods for the fabrication of arrays of live mammalian cells. Lab Chip 6(7):857–863.

Pichler A, Zelcer N, Prior JL, Kuil AJ, Piwnica-Worms D. 2005. In vivo RNA interference-mediated ablation of MDR1 P-glycoprotein. Clin Cancer Res 11(12):4487–4494.

Rafiq I, Kennedy HJ, Rutter GA. 1998. Glucose-dependent translocation of insulin promoter factor-1 (IPF-1) between the nuclear periphery and the nucleoplasm of single MIN6 beta-cells. J Biol Chem 273(36):23241–23247.

Redmond TM, Ren X, Kubish G, Atkins S, Low S, Uhler MD. 2004. Microarray transfection analysis of transcriptional regulation by cAMP-dependent protein kinase. Mol Cell Proteomics 3(8):770–779.

Rutter GA, Kennedy HJ, Wood CD, White MR, Tavare JM. 1998. Real-time imaging of gene expression in single living cells. Chem Biol 5(11): R285–R290.

Segura T, Shea LD. 2002. Surface-tethered DNA complexes for enhanced gene delivery. Bioconjug Chem 13(3):621–629.

Segura T, Volk MJ, Shea LD. 2003. Substrate-mediated DNA delivery: Role of the cationic polymer structure and extent of modification. J Control Release 93(1):69–84.

Silva JM, Mizuno H, Brady A, Lucito R, Hannon GJ. 2004. RNA interference microarrays: High-throughput loss-of-function genetics in mammalian cells. Proc Natl Acad Sci USA 101(17):6548–6552.

Webb BL, Diaz B, Martin GS, Lai F. 2003. A reporter system for reverse transfection cell arrays. J Biomol Screen 8(6):620–623.

Wheeler DB, Bailey SN, Guertin DA, Carpenter AE, Higgins CO, Sabatini DM. 2004. RNAi living-cell microarrays for loss-of-function screens in Drosophila melanogaster cells. Nat Methods 1(2):127–132.

Wheeler DB, Carpenter AE, Sabatini DM. 2005. Cell microarrays and RNA interference chip away at gene function. Nat Genet 37(Suppl): S25–S30.

Xia Y, Whitesides GM. 1998. Soft lithography. Angew Chem Int Ed 37:550–575.

Yamauchi F, Kato K, Iwata H. 2004a. Micropatterned, self-assembled monolayers for fabrication of transfected cell microarrays. Biochim Biophys Acta 1672(3):138–147.

Yamauchi F, Kato K, Iwata H. 2004b. Spatially and temporally controlled gene transfer by electroporation into adherent cells on plasmid DNA-loaded electrodes. Nucleic Acids Res 32(22):e187.

Yamauchi F, Kato K, Iwata H. 2005. Layer-by-layer assembly of poly-(ethyleneimine) and plasmid DNA onto transparent indium-tin oxide electrodes for temporally and spatially specific gene transfer. Langmuir 21(18):8360–8367.

Yoshikawa T, Uchimura E, Kishi M, Funeriu DP, Miyake M, Miyake J. 2004. Transfection microarray of human mesenchymal stem cells and on-chip siRNA gene knockdown. J Control Release 96(2):227–232.

Ziauddin J, Sabatini DM. 2001. Microarrays of cells expressing defined cDNAs. Nature 411(6833):107–110.

Exemestane's 17-hydroxylated metabolite exerts biological effects as an androgen

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Abstract

Aromatase inhibitors (AI) are being evaluated as long-term adjuvant therapies and chemopreventives in breast cancer. However, there are concerns about bone mineral density loss in an estrogen-free environment. Unlike nonsteroidal AIs, the steroidal AI exemestane may exert beneficial effects on bone through its primary metabolite 17-hydroexemestane. We investigated 17-hydroexemestane and observed it bound estrogen receptor α (ERα) very weakly and androgen receptor (AR) strongly. Next, we evaluated 17-hydroexemestane in MCF-7 and T47D breast cancer cells and attributed dependency of its effects on ER or AR using the antiestrogen fulvestrant or the antiandrogen bicalutamide. 17-Hydroexemestane induced proliferation, stimulated cell cycle progression and regulated transcription at high sub-micromolar and micromolar concentrations through ER in both cell lines, but through AR at low nanomolar concentrations selectively in T47D cells. Responses of each cell type to high and low concentrations of the non-aromatizable synthetic androgen R1881 paralleled those of 17-hydroexemestane. 17-Hydroexemestane down-regulated ERα protein levels at high concentrations in a cell type–specific manner similarly as 17β-estradiol, and increased AR protein accumulation at low concentrations in both cell types similarly as R1881. Computer docking indicated that the 17β-OH group of 17-hydroexemestane relative to the 17-keto group of exemestane contributed significantly more intermolecular interaction energy toward binding AR than ERα. Molecular modeling also indicated that 17-hydroexemestane interacted with ERα and AR through selective recognition motifs employed by 17β-estradiol and R1881, respectively. We conclude that 17-hydroexemestane exerts biological effects as an androgen. These results may have important implications for long-term maintenance of patients with AIs. [Mol Cancer Ther 2007;6(11):2817–27]

Introduction

The third-generation aromatase inhibitors (AI) anastrozole (Arimidex; refs. 1, 2), letrozole (Femara; refs. 3, 4), and exemestane (Aromasin; refs. 5, 6), by virtue of blocking extragonadal conversion of androgens to estrogens and giving rise to an estrogen-depleted environment, exhibit improved efficacy over tamoxifen in the adjuvant therapy of estrogen receptor (ER)–positive breast cancer in postmenopausal women (7). Clinical trials evaluating these AIs showed a reduced incidence of contralateral primary breast cancer in the AI groups compared with tamoxifen (1–6); hence, AIs are currently being evaluated as chemopreventives in ongoing studies (8). AIs also exhibit reduced overall toxicity compared with tamoxifen (1–6, 9), but the toxicity profiles are different: tamoxifen is associated with increased incidences of thromboembolic events and endometrial cancer, whereas AIs are associated with decreased bone mineral density (BMD), coupled with an increased risk of bone fractures (10–12) and severe musculoskeletal pain that limits patient compliance (13, 14). Because the available third-generation AIs all exhibit similar efficacies, the selection of a specific AI for long-term adjuvant therapy of breast cancer and as a chemopreventive in healthy women at high risk for breast cancer will likely be determined by safety and tolerability profiles.

AIs fall into two classes, steroidal as represented by exemestane, which acts as a suicide inhibitor of aromatase, and nonsteroidal including anastrozole and letrozole, which reversibly block aromatase activity (7). Possibly due to its steroid structure, exemestane may exhibit a unique pharmacology distinct from the nonsteroidal AIs. In two preclinical studies by Goss et al. (15, 16), exemestane was given to female ovariectomized rats, an animal model

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of osteoporosis, and found to reduce bone resorption markers and increase BMD and bone strength, whereas lowering serum cholesterol and low-density lipoprotein levels compared with ovariectomized controls. One of these preclinical studies also evaluated the nonsteroidal AI letrozole, but in contrast, found no benefit of letrozole on bone or lipid profiles (16). In a clinical study investigating the effects of 2 years of exemestane on bone compared with placebo without prior tamoxifen therapy in patients with surgically resected breast cancer at low risk for recurrence, exemestane did not enhance BMD loss in lumbar spine and only modestly enhanced BMD loss in the femoral neck compared with the placebo group (17). Interestingly, in this study, exemestane promoted bone metabolism by increasing levels of both bone resorption and formation markers (17). However, a clear-cut advantage of exemestane versus the nonsteroidal AIs on bone safety has not been shown in humans, possibly because all other clinical studies compared the AI to tamoxifen (9, 12, 18) or the AI to placebo with prior tamoxifen therapy (10, 11). Drawing conclusions from these studies is difficult because tamoxifen preserves BMD, thereby protecting against fractures, and withdrawal of tamoxifen may have lasting effects on BMD (19).

Maintenance of BMD in women is a known estrogenic effect (20). However, androgen receptors (AR) are also expressed in multiple bone cell types (21, 22), and studies show that androgens maintain BMD in ovariectomized rats (23, 24) and in women (21, 25–27). In ovariectomized rats, physiologic concentrations of androstenedione, a weak androgen and a substrate of aromatase, reduced loss of bone, and the antiandrogen bicalutamide abrogated this effect (23), but anastrozole did not (23). Therefore, the protective effect of androstenedione on maintenance of BMD was androgen mediated and not due to aromatization of androstenedione to estrogen. Furthermore, the non-aromatizable androgen 5α-dihydrotestosterone has been shown to stimulate bone growth in osteopenic ovariectomized rats (24). In pre- and postmenopausal women, endogenous androgen levels correlate with BMD (25, 26). Furthermore, a study comparing estrogen to a synthetic androgen in postmenopausal osteoporotic women showed that both steroids were equally effective in reducing bone resorption (27). Also, a 2-year double-blind trial showed that estrogen plus a non-aromatizable androgen significantly improved BMD over estrogen alone in surgically menopausal women (28). Therefore, exogenous androgens promote BMD maintenance in women when used alone (27) and in conjunction with estrogen (28).

Although exemestane does not bind ER, it is structurally related to androstenedione and has weak affinity for AR (29, 30). At high doses, exemestane exerts possible androgenic activity *in vivo* by inducing an increase in ventral prostate weight in immature castrated rats (29). Recently, Miki et al. (22) showed in human osteoblast hFOB and osteosarcoma Saos-2 cells that exemestane promoted proliferation, which was partially blocked by the antiandrogen hydroxyflutamide, and increased alkaline phosphatase activity. However, metabolites of exemestane may be mediating these effects. Exemestane is given p.o. at 25 mg/day and rapidly absorbed, showing peak plasma levels within 2 to 4 h and a direct relationship between dosage and peak plasma levels after single (10–200 mg) or repeated doses (0.5–50 mg; refs. 30, 31). Single-dose studies suggested that exemestane has a short elimination half-life, but multiple-dose studies show its terminal half-life to be about 24 h. Exemestane undergoes complex metabolism, and the primary metabolite in plasma has been identified as 17-hydroexemestane, which accumulates to a concentration of about 10% of its parent compound (30). Taking the possible action of metabolites into consideration, Goss et al. (16) administered 17-hydroexemestane to ovariectomized rats and found that it produced the same bone-sparing effects and favorable changes in circulating lipid levels as exemestane. Also, Miki et al. (22) stated that 17-hydroexemestane promoted proliferation of the osteoblast and osteosarcoma cells similar to exemestane, but the data were not shown, and the authors did not further explore 17-hydroexemestane activities. Additionally, Miki et al. (22) showed that the osteoblasts efficiently metabolized androstenedione to testosterone, which involves the reduction of the 17-keto group of androstenedione to a hydroxyl group. Similar metabolism would convert exemestane to 17-hydroexemestane, and thus, activities of exemestane in the osteoblasts may have been mediated by a metabolite of exemestane. Hence, a thorough investigation of exemestane and 17-hydroexemestane activities through ER and AR is warranted to provide evidence regarding whether exemestane could display a more favorable safety and toxicity profile than nonsteroidal AIs for long-term adjuvant use and as a chemopreventive of breast cancer in postmenopausal women. Therefore, we evaluated the pharmacologic actions of exemestane and its primary metabolite 17-hydroexemestane on ER- and AR-regulated activities in a range of cellular and molecular assays. First, we determined the relative binding affinity (RBA) of 17-hydroexemestane to ERα and AR. Next, using MCF-7 and T47D breast cancer cells, we examined the ability of 17-hydroexemestane to stimulate cell proliferation and cell cycle progression (Supplementary Material)[4] via ER and AR, to regulate ER- and AR-dependent transcription, and to modulate ERα and AR protein levels. Lastly, we investigated intermolecular interactions between 17-hydroexemestane and ERα and AR using molecular modeling.

Materials and Methods

Compounds and Cell Lines

Exemestane and 17-hydroexemestane were provided by Pfizer. Fulvestrant (ICI 182,780, Faslodex) and bicalutamide (Casodex) were provided by Dr. Alan E. Wakeling and Dr. Barrington J.A. Furr (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom), respectively. All other

[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

compounds were obtained from Sigma-Aldrich, and cell culture reagents were from Invitrogen. All test agents were dissolved in ethanol and added to the medium at 1:1,000 (v/v). MCF-7/WS8 and T47D:A18 human mammary carcinoma cells, clonally selected from their parental counterparts for sensitivity to growth stimulation by E_2 (32), were used in all experiments indicating MCF-7 and T47D cells. Cells were maintained in steroid-replete RPMI 1640, but 3 days before all experiments, were cultured in steroid-free media as previously described (32, 33).

Competitive Hormone-Binding Assays

Competitive hormone-binding assays were conducted using fluorescence polarization–based ERα and AR Competitor Assay kits (Invitrogen) as previously described (34).

Cellular Proliferation Assays

Cellular proliferation following 7 days in culture was determined by DNA mass per well in 12-well plates using the fluorescent DNA dye Hoechst 33258 as previously described (32).

Reporter Gene Assays

Reporter gene assays were conducted by transfecting cells with either an ERE(5x)-regulated (pERE(5x)TA-ffLuc; ref. 33) or ARE(5x)-regulated (pAR-Luc; Panomics) firefly luciferase expression plasmid and co-transfected with a basal TATA promoter-regulated (pTA-srLuc) *Renilla* luciferase expression plasmid as previously described (33).

Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was used to determine AR and ribosomal large phosphoprotein subunit P0 (RLP0; 36B4) mRNA levels as previously described (35).

Immunoblot Analyses

Immunoblots, prepared as previously described (33), were probed with primary antibodies against AR (AR 441; Lab Vision), ERα (AER 611; Lab Vision), and β-actin (AC-15; Sigma-Aldrich).

Molecular Modeling and Virtual Docking Calculations

The three-dimensional conformations for E_2, 17-hydroexemestane, exemestane, R1881, and dexamethasone were generated with Omega version 2.1 software (OpenEye Scientific Software). These compounds were docked using the following X-ray crystallographic structures: 1GWR (ERα co-complexed with E_2, 2.4-Å resolution; ref. 36) and 1XQ3 (AR co-complexed with R1881, 2.25-Å resolution; ref. 37). ERα and AR ligand-binding pockets were built using a ligand-centered box and the receptor-bound conformation of the respective ligand: E_2 (for 1GWR) and R1881 (for 1XQ3). The volume of the cavity differs for the two receptors: 648 $Å^3$ for 1GWR and 532 $Å^3$ for 1XQ3. All receptor and ligand bonds were kept rigid. The receptor structures were filled with water because ERα (38) and AR crystal structures (39) indicate that specific stable hydrogen bond (H-bond) networks form among particular water molecules, ligands, and amino acid side chains. Docking was done with FRED version 2.2 software (OpenEye) using a short refinement step for the ligands within the receptor and using the MMFF94 force field. The best 30 conformations for each compound were compared and ranked by FRED's Chemscore function. For each ligand-docked receptor evaluated, the docked conformation with the lowest total intermolecular interaction energy (kJ/mol) was selected. To address whether water could be displaced by a compound during the process of binding, docking calculations were also done using receptors modeled with water removed as presented in Supplementary Table S1[4] and the differences between the methods in Supplementary Table S2.[4]

Curve Fitting and Statistical Analyses

All statistical tests, curve fitting, and determination of half-maximal inhibitory concentrations (IC_{50}) and half-maximal effective concentrations (EC_{50}) were done using GraphPad Prism 4.03 (GraphPad Software). Significant differences were determined using one-way ANOVA with Bonferroni multiple comparison post-test.

Results

Experimentally Determined Binding of 17-Hydroexemestane and Exemestane to ERα and AR

Structures of the compounds relevant to these studies, the steroidal AI parent compound exemestane, its primary metabolite 17-hydroexemestane, E_2, and the synthetic non-aromatizable androgen R1881, are shown in Fig. 1A. Importantly, the only difference between parental exemestane and its metabolite 17-hydroexemestane is a hydroxyl group in the metabolite in place of a ketone in the parent compound at the 17β position, whereas both compounds share a 3-keto group. For steroidal estrogens, elimination or modification of the 17β-OH group reduces binding to ERα, but that of the 3-OH group is much more dramatic (40). For steroidal androgens, the trend is reversed; elimination or modification of the 17β-OH group is more significant for AR binding than that of the 3-keto group (41). The 3-keto group found in both exemestane and 17-hydroexemestane also favors binding to AR (41).

We tested the binding of exemestane and 17-hydroexemestane to ERα and AR using fluorescence polarization–based competitive hormone-binding assays (Fig. 1B and C; Table 1). For purposes of comparison, compound affinities were arbitrarily categorized with respect to their RBAs as strong (100 to ≥1), moderate (<1 to ≥0.1), weak (<0.1 to ≥0.01), very weak (<0.01 to detectable binding defined as 50% competition), and inactive (compound did not compete for at least 50% binding). E_2 competitively bound ERα with an IC_{50} of 1.33×10^{-9} mol/L (RBA = 100; Fig. 1B), and R1881 competitively bound AR with an IC_{50} of 1.34×10^{-8} mol/L (RBA = 100; Fig. 1C). Considering ERα (Fig. 1B), both R1881 and 17-hydroexemestane competed for binding to ERα with IC_{50}s of 1.02×10^{-6} mol/L (RBA = 0.130) and 2.12×10^{-5} mol/L (RBA = 0.006), respectively, which categorized R1881 as a moderate and 17-hydroexemestane as a very weak ERα ligand. Neither exemestane nor dexamethasone significantly competed for binding to ERα. Regarding AR (Fig. 1C), 17-hydroexemestane and exemestane competed for binding to AR with IC_{50}s of 3.96×10^{-8} mol/L (RBA = 33.8) and 2.03×10^{-6} mol/L (RBA = 0.658), respectively, which classified

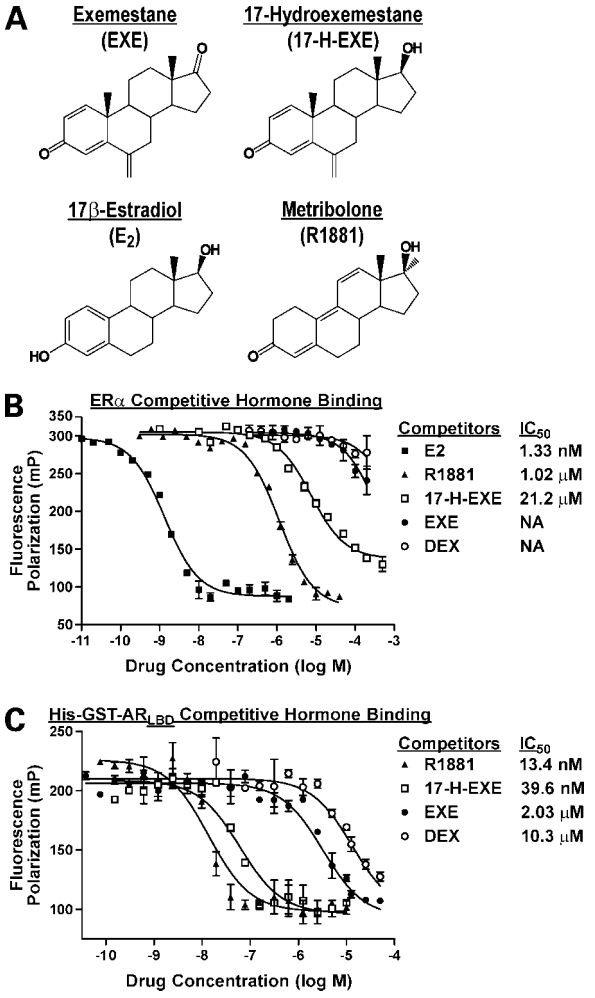

Figure 1. Compounds examined in this study and their RBAs for ERα and AR. **A,** structures of exemestane, its primary metabolite 17-hydroexemestane E_2, and R1881. ERα (**B**) and AR (**C**) fluorescence polarization-based competitive hormone-binding assays. Baculovirus-produced human ERα and rat AR ligand-binding domain tagged with a His-glutathione *S*-transferase epitope (His-GST-AR_{LBD}) were used at final concentrations of 15 and 25 nmol/L, respectively. The fluorescently labeled ERα and AR ligands, Fluormone ES2 and Fluormone AL Green, respectively, were both used at a final concentration of 1 nmol/L. The competing test compounds were E_2, R1881, 17-hydroexemestane, exemestane, and dexamethasone (DEX) as indicated. *Point,* mean of triplicate determinations; *bars,* 95% confidence intervals. Curve fitting was done using GraphPad Prism software (version 4.03). IC_{50}s corresponding to a half-maximal shift in polarization values of the test compounds were determined using the maximum and minimum polarization values of the E_2-competitive binding curve for ERα or of the R1881-competitive binding curve for AR as appropriate.

17-hydroexemestane as a strong and exemestane as a weak AR ligand. However, dexamethasone would also be categorized as a weak AR ligand. Hence, the observed very weak ERα binding and strong AR binding of 17-hydroexemestane was consistent with what previously reported structure-activity relationships (40, 41) would have predicted due to reduction of the 17-keto group in exemestane to a 17β-OH in the metabolite.

Proliferation Responses to 17-Hydroexemestane and Exemestane

We examined the effects of exemestane and 17-hydroexemestane on 7 days of proliferation in ERα- and AR-positive MCF-7 and T47D mammary carcinoma cells (Fig. 2). As expected, both cell lines were growth stimulated by E_2, with growth EC_{50}s of 1.7×10^{-12} mol/L E_2 for MCF-7 cells (Fig. 2A) and 7.1×10^{-12} mol/L E_2 for T47D cells (Fig. 2B). These growth responses to E_2 were completely blocked by fulvestrant (all P values <0.001), validating the E_2 responsiveness via ER in these cell lines.

Both cell lines were also growth stimulated by R1881 (Fig. 2A and B) and 17-hydroexemestane (Fig. 2C and D), whereas exemestane did not exert any significant effect on proliferation (Fig. 2C and D). Considering MCF-7 cells, R1881 exhibited a growth EC_{50} of 2.4×10^{-8} mol/L (Fig. 2A), or approximately 4 orders of magnitude higher than that of E_2. Similarly, 17-hydroexemestane exhibited a growth EC_{50} of 2.7×10^{-6} mol/L in MCF-7 cells (Fig. 2C) or approximately 6 orders of magnitude higher than that of E_2. These growth responses to R1881 and 17-hydroexemestane in MCF-7 cells were completely blocked by cotreatment with fulvestrant (Fig. 2A and B; both P values <0.001). Therefore, whereas R1881, a non-aromatizable synthetic androgen, stimulated growth of MCF-7 cells, it did so by acting through ER. Hence, at high concentrations, R1881 exerted estrogenic activity. Similarly, at high concentrations, 17-hydroexemestane also exerted estrogenic activity and stimulated growth of MCF-7 cells by acting through ER.

Interestingly, in T47D cells, the growth response to R1881 and 17-hydroexemestane followed an apparent bimodal pattern, which was different than in MCF-7 cells. In T47D cells, proliferative effects of high concentrations of R1881 (5×10^{-6} mol/L; Fig. 2B) and 17-hydroexemestane (5×10^{-6} mol/L; Fig. 2D) were only partially blocked by fulvestrant (both P values <0.001), down to the level of growth observed at nanomolar concentrations of these compounds. However, proliferative effects of lower concentrations of R1881 (10^{-9} mol/L) and 17-hydroexemestane (10^{-8} mol/L) were completely blocked by the antiandrogen bicalutamide (both P values <0.001). Based on these observed levels of inhibition by bicalutamide and fulvestrant, maximal concentrations at which R1881 and 17-hydroexemestane stimulated growth through AR-dependent activities were 10^{-7} and 10^{-6} mol/L, respectively, and above these concentrations, R1881 and 17-hydroexemestane stimulated growth through ER-dependent activities. Using this information to define concentration ranges in which these compounds exert AR-mediated or ER-mediated effects in T47D cells, the growth EC_{50}s via AR of R1881 and 17-hydroexemestane were 1.0×10^{-10} mol/L (Fig. 2B) and 4.3×10^{-10} mol/L (Fig. 2D), respectively. Similarly, the growth EC_{50}s via ER of R1881 and 17-hydroexemestane in T47D cells were 3.1×10^{-7} mol/L (Fig. 2B) and 1.5×10^{-6} mol/L (Fig. 2D), respectively. Hence, in T47D cells, both R1881 and 17-hydroexemestane stimulated growth via AR at lower

concentrations and via ER at higher concentrations. These results were consistent with the observed binding affinities of these compounds to ERα (Fig. 1B) and AR (Fig. 1C).

Cell Cycle Progression Responses to 17-Hydroexemestane

As shown in Supplementary Fig. S1,[4] 17-hydroexemestane at 10^{-8} mol/L acted through AR to stimulate S-phase entry in T47D cells by 1.9-fold ($P < 0.001$) but, at 5×10^{-6} mol/L, acted through ER to stimulate S-phase entry in MCF-7 cells by 2.2-fold ($P < 0.001$). Hence, 17-hydroexemestane effects on cell cycle progression were consistent with its effects on proliferation (Fig. 2).

Regulation of ERα and AR Transcriptional Activities by 17-Hydroexemestane

Next, we investigated the ability of 17-hydroexemestane to regulate ER and AR transcriptional activity by transfecting cells with an ERE(5x)-regulated or ARE(5x)-regulated dual-luciferase plasmid set, treating cells with test compounds, and measuring dual-luciferase activity 44 h after treatment (Fig. 3A–C). E_2 at 10^{-10} mol/L induced ERE(5x)-regulated transcription by 19.4-fold in MCF-7 cells (Fig. 3A; $P < 0.001$), and 11.3-fold in T47D cells (Fig. 3B; $P < 0.001$) compared with control-treated cells; this E_2-induced transcriptional activity was blocked by fulvestrant (both P values <0.001), validating dependence on ER for ERE(5x)-regulated transcription. At high sub-micromolar and micromolar concentrations, R1881 stimulated ERE(5x)-regulated transcription in both cell lines, with maximal inductions of 22.7-fold at 5×10^{-6} mol/L in MCF-7 cells (Fig. 3A; $P < 0.001$), and 7.9-fold at 5×10^{-6} mol/L in T47D cells (Fig. 3B; $P < 0.001$) compared with control-treated cells. The ability of R1881 at 5×10^{-6} mol/L to induce ERE(5x)-regulated transcription was blocked by fulvestrant (Fig. 3A and B; both P values <0.001), indicating that at high concentrations, R1881 acted as an estrogen. In a similar manner as R1881, 17-hydroexemestane stimulated ERE(5x)-regulated transcription in a concentration-dependent manner at sub-micromolar and micromolar concentrations (Fig. 3A and B). At 5×10^{-6} mol/L, 17-hydroexemestane maximally induced ERE(5x)-regulated transcription by 7.7-fold in MCF-7 cells (Fig. 3A; $P < 0.001$) and 3.3-fold in T47D cells (Fig. 3B; $P < 0.001$) compared with control-treated cells; this transcriptional activation was blocked by fulvestrant (both P values <0.001). Therefore, at high concentrations, 17-hydroexemestane acted as an estrogen and induced ER transcriptional activity.

In a similar manner, AR-dependent transcriptional activity was investigated. T47D cells showed a concentration-dependent induction of ARE(5x)-regulated transcription in response to R1881, with 10^{-9} mol/L R1881 inducing transcription by 8.5-fold and 10^{-6} mol/L R1881 maximally inducing transcription by 12.7-fold relative to control-treated cells (Fig. 3C; both P values <0.001). Bicalutamide blocked 10^{-9} mol/L R1881-mediated induction of ARE(5x)-regulated transcription (Fig. 3C; $P < 0.001$), confirming dependence on AR. MCF-7 cells failed to respond to 10^{-6} mol/L R1881 with induction of ARE(5x)-regulated transcription (data not shown), although these cells express AR protein. This supports our prior results that T47D cells were growth stimulated by R1881 through an AR-dependent mechanism (Fig. 2B), but that MCF-7 cells were not (Fig. 2A). As expected, 10^{-6} mol/L E_2 failed to induce ARE(5x)-regulated transcription (Fig. 3C). Next, 17-hydroexemestane was evaluated in T47D cells and, in a concentration-dependent manner, induced ARE(5x)-regulated transcription with maximal induction of 4.7-fold occurring at 5×10^{-6} mol/L relative to control treatment (Fig. 3C; $P < 0.001$). However, because high concentrations of 17-hydroexemestane were needed to induce this synthetic ARE(5x)-regulated promoter, we tested whether lower concentrations of 17-hydroexemestane could modulate endogenous AR mRNA expression, which is known to be negatively feedback regulated by its gene product (42). Using real-time PCR, AR mRNA levels were determined in T47D cells following 24 h of treatment with test compounds (Fig. 3D). R1881 at 10^{-9} mol/L significantly down-regulated

Table 1. Compound affinity for ERα and AR determined experimentally using a competitive hormone-binding assay (Fig. 1B and C), and by computer docking in which receptors were modeled as filled with water

Compound	Receptor	Competitive hormone binding			Intermolecular interaction energy (kJ/mol)				
		IC_{50} (mol/L)	95% CI (mol/L)	RBA (%)	Total score	Lipophilic	H-bond	Steric clash	RTB penalty
E_2	ERα	1.33×10^{-9}	$1.18–1.49 \times 10^{-9}$	100	−31.90	−25.96	−6.00	0.06	0
R1881	ERα	1.02×10^{-6}	$0.90–1.15 \times 10^{-6}$	0.130	−29.96	−26.01	−4.32	0.37	0
17-Hydroexemestane	ERα	2.12×10^{-5}	$1.73–2.61 \times 10^{-5}$	0.006	−29.14	−27.73	−3.34	1.93	0
Exemestane	ERα	NA			−27.33	−25.98	−3.34	1.99	0
Dexamethasone	ERα	NA			−23.71	−29.70	−4.18	9.07	1.10
R1881	AR	1.34×10^{-8}	$1.00–1.79 \times 10^{-8}$	100	−32.75	−28.47	−4.56	0.28	0
17-Hydroexemestane	AR	3.96×10^{-8}	$2.74–5.71 \times 10^{-8}$	33.8	−31.95	−30.54	−4.76	3.35	0
Exemestane	AR	2.03×10^{-6}	$1.39–2.97 \times 10^{-6}$	0.658	−26.48	−28.80	−2.11	4.43	0
Dexamethasone	AR	1.03×10^{-5}	$0.75–1.43 \times 10^{-5}$	0.130	−24.53	−32.21	−2.49	9.07	1.10

Abbreviations: RTB Penalty, rotable bond penalty; NA, not applicable; test compound did not compete for at least 50% binding of ERα.

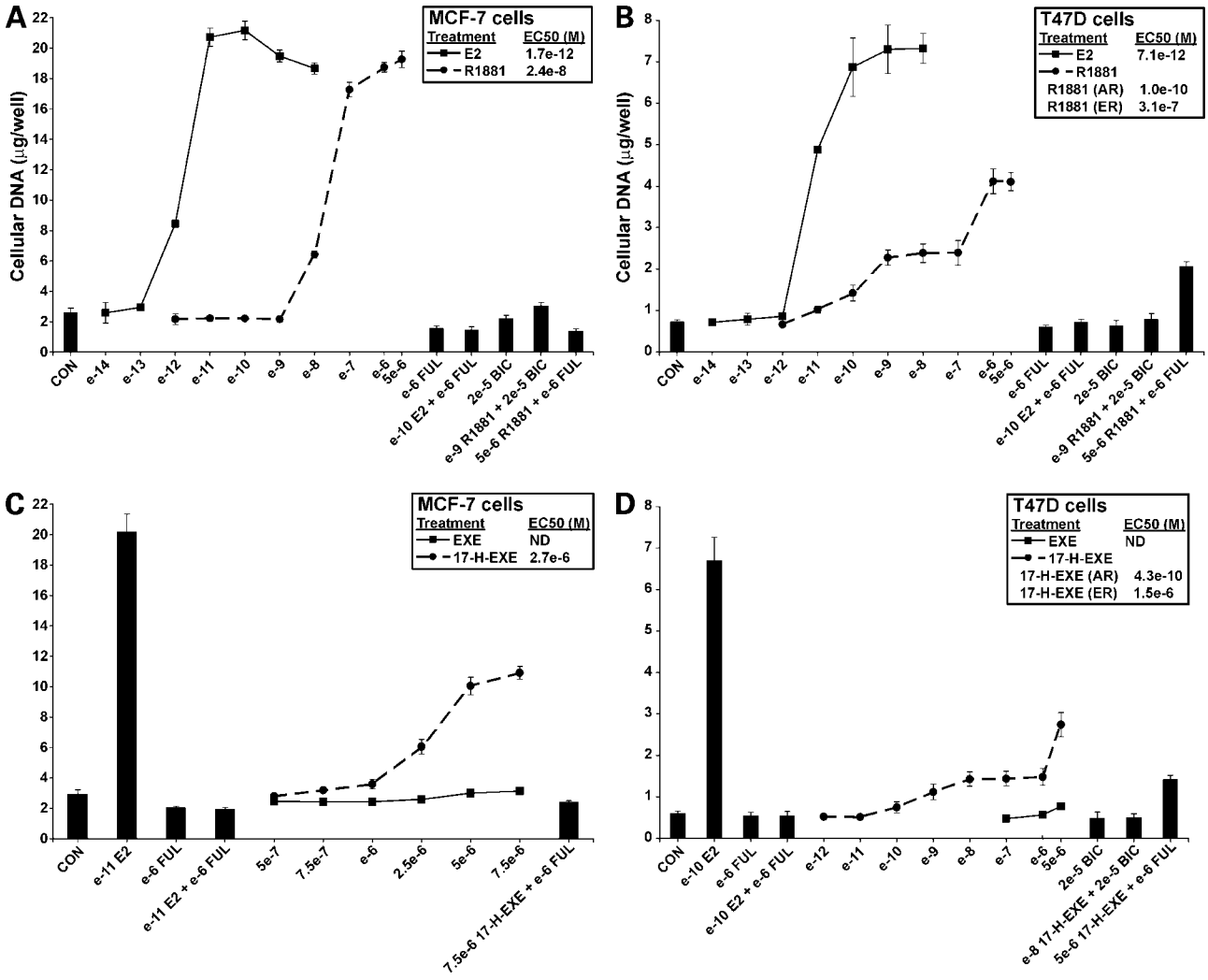

Figure 2. 17-Hydroexemestane and R1881 stimulate cellular proliferation. DNA-based cellular proliferation assays of (**A**) MCF-7 cells treated with E_2 and R1881, (**B**) T47D cells treated with E_2 and R1881, (**C**) MCF-7 cells treated with exemestane and 17-hydroexemestane, and (**D**) T47D cells treated with exemestane and 17-hydroexemestane. Cells were cultured in steroid-free medium for 3 d before the assays. MCF-7 cells were seeded at 15,000 cells per well and T47D cells at 20,000 cells per well in 12-well plates. Cells were treated on days 0 (the day after seeding), 3, and 6, and then collected on day 7. Cellular DNA quantities were determined using the fluorescent DNA-binding dye Hoechst 33258 and compared against a standard curve. Data shown represent the mean of four replicates and SDs. DNA values were fitted to a sigmoidal dose-response curve and growth EC_{50}s calculated using GraphPad Prism 4.03 software. At high concentrations, 17-hydroexemestane and R1881 increased growth via ER in both cell lines but, at low concentrations, stimulated growth via AR selectively in T47D cells. Abbreviations: CON, control; FUL, fulvetsrant; BIC, bicalutamide.

AR mRNA expression by 48% ($P < 0.001$), whereas 10^{-9} mol/L E_2 did not (Fig. 3D). Bicalutamide prevented R1881-mediated decrease in AR mRNA expression (Fig. 3D), validating that AR mRNA levels were negatively feedback regulated. Similarly, a low 10^{-8} mol/L concentration of 17-hydroexemestane led to a 41% decrease in AR mRNA levels ($P < 0.01$), with increased 17-hydroexemestane concentrations further decreasing AR mRNA expression (Fig. 3D). Bicalutamide blocked 17-hydroexemestane–mediated down-regulation of AR mRNA expression ($P < 0.01$), whereas fulvestrant did not (Fig. 3D). Therefore, 17-hydroexemestane acted as an androgen via AR to feedback-regulate the expression of endogenous AR mRNA in T47D cells.

Modulation of AR and ERα Protein Levels by 17-Hydroexemestane

Androgens and estrogens modulate protein expression levels of their cognate receptors. R1881 stabilizes AR protein allowing its accumulation (43), whereas E_2 promotes ERα degradation in a cell type–dependent manner (32). Therefore, we investigated the effects of 17-hydroexemestane on AR and ERα protein levels by treating cells with test compounds for 24 h and analyzing receptor levels by immunoblotting. E_2 decreased ERα protein levels in MCF-7 (Fig. 4A), but not T47D cells (Fig. 4B), as we have previously shown (32). As expected, fulvestrant promoted ERα protein degradation in both cell lines. E_2 did not significantly affect AR protein accumulation in MCF-7 cells

(Fig. 4A), but did down-regulate AR protein levels in T47D cells (Fig. 4B). Also, fulvestrant and E_2 plus fulvestrant treatments did not significantly affect AR protein levels in MCF-7 cells (Fig. 4A), but did modestly up-regulate AR protein levels in T47D cells (Fig. 4B). As expected, R1881 caused an increase in accumulation of AR protein in both cell lines (Fig. 4A and B), likely by stabilizing the protein (43). Next, we characterized the effects of low 10^{-8} mol/L and high 5×10^{-6} mol/L concentrations of 17-hydroxexemestane on ERα and AR expression. The high 5×10^{-6} mol/L concentration of 17-hydroxexemestane led to decreased ERα protein levels in MCF-7 (Fig. 4A), but not in T47D cells (Fig. 4B); this pattern indicates that 5×10^{-6} mol/L 17-hydroxexemestane acted as an estrogen to regulate ERα protein in a cell type–dependent manner. Similar to R1881, treatment with low 10^{-8} mol/L or high 5×10^{-6} mol/L concentrations of 17-hydroxexemestane led to increased AR protein accumulation in both cell lines (Fig. 4A and B), indicating that 17-hydroxexemestane acted as an androgen likely by stabilizing AR protein. Therefore, 17-hydroxexemestane modulated ERα and AR protein accumulation as would an estrogen and an androgen, respectively.

Molecular Docking of 17-Hydroxexemestane and Exemestane to ERα and AR

To investigate the mechanism by which 17-hydroxexemestane binds ERα as a very weak ligand and AR as a

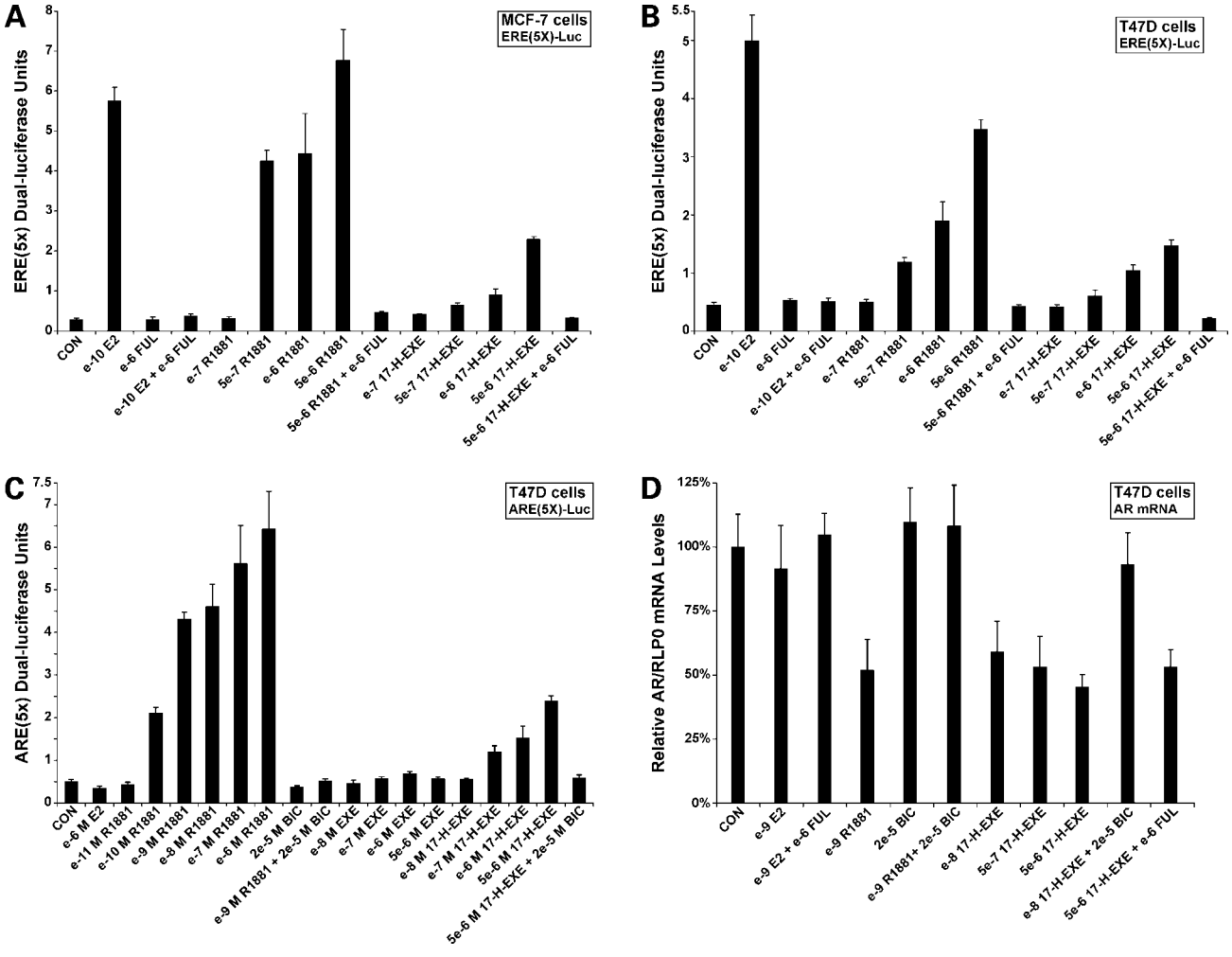

Figure 3. 17-Hydroxexemestane and R1881 regulate ER transcriptional activity at high concentrations and AR transcriptional activity at low concentrations. ERE(5x)-regulated dual-luciferase activity in (**A**) MCF-7 cells and (**B**) T47D cells. (**C**) ARE(5x)-regulated reporter gene activity in T47D cells. *A–C*, Under steroid-free conditions, cells were transiently transfected with pERE(5x)TA-ffLuc or pARE(5x)-Luc (firefly luciferase reporter plasmids) and the internal normalization control pTA-srLuc (*Renilla* luciferase reporter plasmid). Four hours after transfection, cells were treated as indicated and then again the following day. Cells were assayed 44 h after transfection for dual-luciferase activity. Data shown are the mean of triplicate determinations and associated SDs. 17-Hydroxexemestane and R881 stimulated ERE(5x)-regulated transcription in MCF-7 and T47D cells and ARE(5x)-regulated transcriptional activity in T47D cells. **D,** AR mRNA levels in T47D cells as determined by real-time PCR. T47D cells were treated as indicated for 24 h. RNA was isolated and converted to cDNA. Continuous accumulation of PCR products was monitored using the double strand-specific DNA dye SYBR Green. Quantitative measurements of AR mRNA and the endogenous normalization control RLP0 mRNA were determined by comparison to a standard curve of known quantities of serially diluted AR or RLP0 PCR product. The data represent the mean and SDs of three independent samples, each of which was measured in triplicate. 17-Hydroxexemestane and R881 down-regulated AR mRNA levels at nanomolar concentrations in an AR-dependent manner.

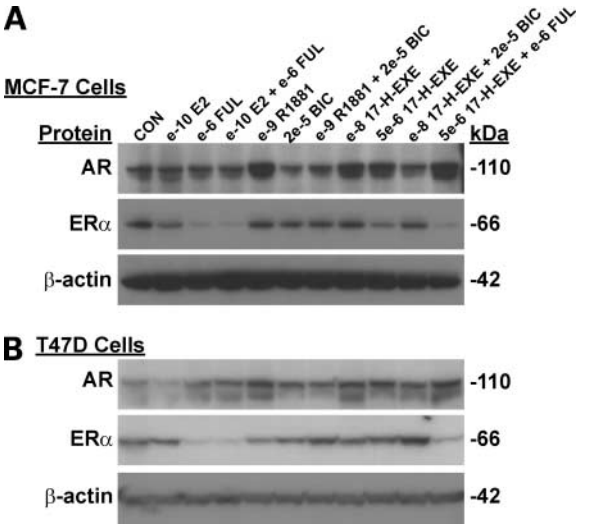

Figure 4. 17-Hydroexemestane modulates AR and ERα protein levels. Immunoblot analysis of AR and ERα in (**A**) MCF-7 cells and (**B**) T47D cells. Cells were treated as indicated for 24 h, and 20 μg of cellular protein were resolved by 4% to 12% SDS-PAGE and then transferred to a nylon membrane. Membranes were probed for AR, ERα, and β-actin, and immunoreactive bands were visualized by chemiluminescence and autoradiography. Cropped blots are shown. 17-hydroexemestane up-regulated AR protein levels at 10^{-8} mol/L in both cell lines and down-regulated ERα in MCF-7 cells at 5×10^{-6} mol/L.

strong ligand, molecular models were constructed *in silico*. The trends in the computed intermolecular interaction energies matched the experimentally determined RBAs (Table 1). Superimposition of the docked and crystallographic structures of E_2 complexed with ERα (Fig. 5A) and of R1881 complexed with AR (Fig. 5B) showed that the docking models recapitulated the molecular recognition patterns of the crystal structures.

Considering ERα, the intermolecular interaction energies of R1881 and 17-hydroexemestane were less favorable than E_2 by 1.94 and 2.76 kJ/mol, respectively, due to decreased H-bond interactions and increased steric clash (Table 1). Exemestane was much less favorable than E_2 by 4.57 kJ/mol (Table 1). Hence, the 17β-OH group of 17-hydroexemestane compared with the 17-keto group of exemestane contributed −1.81 kJ/mol toward increased affinity for ERα. Interestingly, the docking calculations suggested that the higher affinity of 17-hydroexemestane over exemestane for ERα was not due to increased H-bonding mediated by the 17β-OH group, but rather increased lipophilic interactions (Table 1) due to a slight repositioning of the compound as a consequence of 17β-OH group. In the E_2 docked to ERα model, H-bonds between E_2 and Glu^{353}, Arg^{394}, and His^{524} side chains were observed (Fig. 5A). In the docked 17-hydroexemestane to ERα model (Fig. 5C), the same Arg^{394} and His^{524} interactions were maintained, except that there was a loss of the Glu^{353} interaction. The R1881 docked to ERα model is shown in Supplementary Fig. S2A.[4]

Considering AR, the intermolecular interaction energy of 17-hydroexemestane was only 0.8 kJ/mol less favorable than R1881, whereas exemestane was significantly less favorable than R1881 by 6.27 kJ/mol (Table 1). Docking of 17-hydroexemestane to AR, compared with the parent drug exemestane, indicated that 17-hydroexemestane exhibited improved lipophilic interactions by −2.11 kJ/mol, more favorable H-bonding interactions by −2.65 kJ/mol, and decreased steric clash by −1.08 kJ/mol. Hence, the 17β-OH group in 17-hydroexemestane compared with the 17-keto group in exemestane contributed −5.47 kJ/mol toward higher affinity for binding AR (Table 1). In the R1881 docked to AR model, H-bonds between R1881 and Asn^{705}, Gln^{711} and Arg^{752} were observed (Fig. 5B). The OH side chain of Thr^{877} was in close proximity to both docked R1881 (Fig. 5B) and 17-hydroexemestane (Fig. 5D), but the angle was not favorable for H-bonding. Docking of 17-hydroexemestane to AR (Fig. 5D) indicated a short 2.78-Å H-bond between the 17β-OH group of the ligand and Asn^{705}, but not between the 3-keto group of the ligand and Gln^{711} and Arg^{752}. Hence, the short 2.78-Å H-bond observed in the 17-hydroexemestane docked to AR model was important in mediating high affinity binding. The exemestane docked to AR model is shown in Supplementary Fig. S2B.[4]

Discussion

We observed that 17-hydroexemestane, the primary metabolite of exemestane, bound to ERα as a very weak ligand and acted through ER at high sub-micromolar and micromolar concentrations to stimulate growth, promote cell cycle progression, induce ERE-regulated reporter gene expression, and down-modulate ERα protein levels in breast cancer cells. However, we also observed that 17-hydroexemestane bound to AR as a strong ligand and found in T47D cells that 17-hydroexemestane stimulated growth, induced cell cycle progression, down-modulated AR mRNA expression, and stabilized AR protein levels, with all of these effects occurring at low nanomolar concentrations and blocked by bicalutamide. Moreover, computer docking indicated that the 17β-OH group of 17-hydroexemestane versus the 17-keto group of exemestane contributed significantly more toward increasing affinity to AR than to ERα. Molecular modeling also indicated that 17β-OH group of 17-hydroexemestane interacted with AR through an important H-bond of Asn^{705}, a conserved recognition motif employed by R1881. Therefore, we propose that the primary mechanism of action of exemestane *in vivo* is mediated by 17-hydroexemestane regulating AR activities.

The Food and Drug Administration label for exemestane (Aromasin; Pfizer) reports that in postmenopausal women with advanced breast cancer, the mean AUC (area under the curve) values of exemestane following repeated doses was 75.4 ng·h/mL (254 nmol·h/L), which was almost twice that in healthy postmenopausal women (41.4 ng·h/mL; 140 nmol·h/L; ref. 31). Because circulating levels of 17-hydroexemestane can reach about 1/10 the level of the parent compound (30), we hypothesize that circulating levels of 17-hydroexemestane are sufficient to bind AR and

regulate AR-dependent activities. Furthermore, a subpopulation of patients may exist who metabolize exemestane at higher rates, leading to correspondingly higher circulating 17-hydroexemestane levels. For instance, one of three patients administered 800 mg of exemestane, the highest dose evaluated, achieved 17-hydroexemestane plasma levels approximately one-half the level of the parent compound (30). Based on our results, we would predict that higher circulating levels of 17-hydroexemestane would associate with decreased rates of BMD loss and risk of bone fractures in postmenopausal women. We suggest that circulating levels of 17-hydroexemestane and exemestane should be determined in clinical trials and correlated to disease outcome and toxicity profiles such as BMD loss.

Although the clinical studies reported thus far were not designed to directly compare one AI versus another, comparisons in the rate of BMD loss from baseline to year 1, and from year 1 to 2 can be made. In the bone safety subprotocol of the IES (Intergroup Exemestane Study) trial, the rate of BMD loss was greatest within 6 months of switching from tamoxifen to exemestane at −2.7% in the lumbar spine and −1.4% in the hip, but thereafter, BMD loss progressively slowed in months 6 to 12 and again in months 12 to 24 to only −1.0% and −0.8% in the lumbar spine and hip, respectively (10), which is in the same range as would be expected for postmenopausal women in general. However, in the bone safety substudy of the MA.17 trial, patients administered letrozole experienced a relatively constant rate of BMD loss for 2 years: at 12 months, the rate of BMD loss from baseline was −3.3% and −1.43% in lumbar spine and hip, respectively, and from year 1 to year 2, −2.05% and −2.17% in lumbar spine and hip, respectively (11). In the bone substudy of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial, the rate of BMD loss from baseline to year 1 was −2.2% in lumbar spine and −1.5% in hip and from year 1 to year 2, −1.8% in lumbar spine and −1.9% in hip (18). Collectively, these results suggest that after the initial

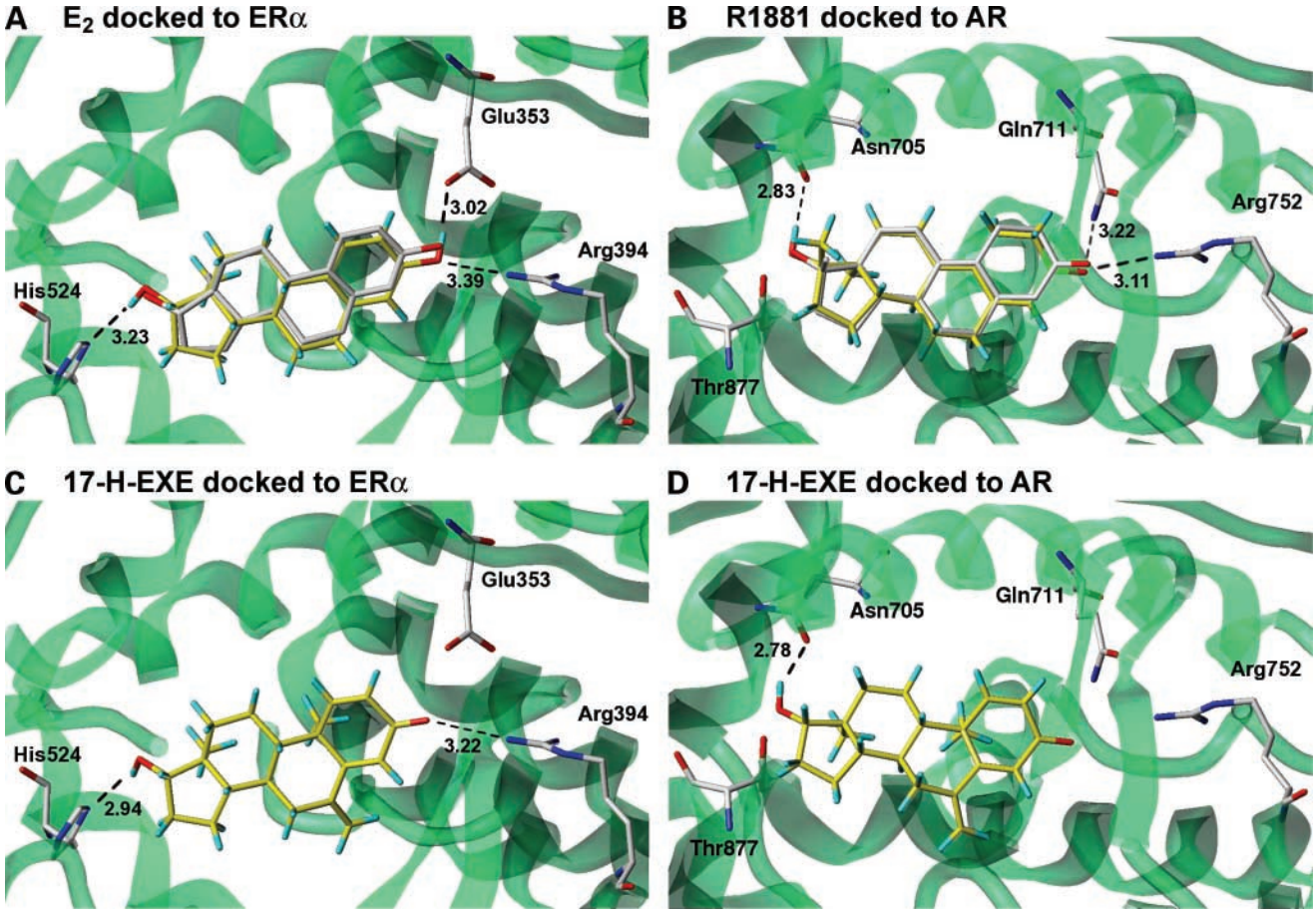

Figure 5. Intermolecular interactions of ligands complexed with ERα and AR by computer docking. **A,** superposition of E_2 from the X-ray crystal structure (*gray*) and modeled E_2 (*yellow*) docked to ERα. **B,** superposition of R1881 from the crystal structure (*gray*) and modeled R1881 (*yellow*) docked to AR. **C,** modeled 17-hydroexemestane docked to ERα. **D,** modeled 17-hydroexemestane docked to AR. *Cyan, red*, and *blue,* hydrogen, oxygen, and nitrogen atoms, respectively. *Green,* carbon backbone of the protein. Hydrogens from the X-ray crystal conformations of E_2 (**A**) and R1881 (**C**) were omitted. H-bonds were shown to the modeled compound conformations only. *Dashed lines,* intermolecular H-bonds up to 3.5 Å; their length in angstroms is indicated.

12 months of AI therapy, exemestane may be associated with slower rates of BMD loss compared with nonsteroidal AIs. Furthermore, although not directly comparable, the fracture rate per 1,000 woman-years in the ATAC trial was 22.6 for anastrozole and 15.6 for tamoxifen (1), whereas in the IES trial, the incidence rate per 1,000 woman-years for multiple fractures was 19.2 for exemestane and 15.1 for tamoxifen (10). These results show that although both anastrozole and exemestane were associated with higher fracture rates than tamoxifen, they also suggest that exemestane may be associated with a lower fracture rate than anastrozole. Clinical trials now under way to directly compare the different AIs will hopefully provide clear results.

Androgens regulate growth of normal and neoplastic mammary cells in a cell type-specific manner, either by inhibiting or stimulating growth (44). However, the mechanisms by which androgens via AR regulate breast cancer growth remain elusive. Female AR knock-out mice exhibit decreased ductal branching and terminal end buds in prepubertal animals and retarded lobuloalveolar development in adult animals (45). Likewise, targeted disruption of AR in MCF-7 cells also leads to severe inhibition of proliferation (45). Epidemiologic analyses indicate a positive correlation between androgen levels and the incidence of breast cancer; meta-analysis from nine prospective studies showed that a doubling in testosterone concentrations in postmenopausal women translated into an increased relative risk of 1.42 unadjusted and 1.32 adjusted for E_2 (46). AR status in breast cancer associates with both positive and negative indicators and clinical outcome. AR expression has been found in 84% (47) to 91% (48) of clinical breast cancers, and associated with ER status, but has also been found in 49% of ER-negative tumors (49). Patients with tumors that coexpress AR with ER and progesterone receptor have shown longer disease-free survival (DFS) than patients whose tumors were negative for all three receptors (48), but AR protein levels have also served as an independent predictor of axillary metastases in multivariate analysis (47) Furthermore, AR expression has correlated with decreased histopathologic grade, greater age, and postmenopausal status, but also lymph node–positive status (50). In AR-positive/ER-negative tumors, AR expression again associated with positive and negative indicators/outcome such as increased age, postmenopausal status, and longer DFS but also tumor grade, tumor size, and HER-2/neu overexpression (49).

Patients who fail AI therapy, whether the AI was steroidal or nonsteroidal, likely harbor tumor cells that have been selected for growth in an estrogen-depleted environment and, hence, are not dependent on ER activity for survival. Not all androgens are metabolized by aromatase to estrogens; for instance, dihydrotestosterone cannot be converted to an estrogen by aromatase (44). Thus, a possible mechanism for failure of AI therapy in the clinic is androgen-stimulated breast cancer growth, a largely unrecognized alternative mechanism. We observed cellular proliferation of T47D cells in response to R1881 and 17-hydroexemestane, and these effects were blocked by bicalutamide. Therefore, T47D cells contain a functional AR signaling pathway that promoted growth in the absence of estrogen. Because functional AR signaling could be etiologically involved in a subpopulation of clinical breast cancers, those patients who have AR-positive tumors and achieve high circulating levels of 17-hydroexemestane, yet whose disease progresses while on exemestane therapy, may respond to AR-based therapy such as the antiandrogen bicalutamide.

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References

1. Howell A, Cuzick J, Baum M, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 2005;365:60 – 2.

2. Jonat W, Gnant M, Boccardo F, et al. Effectiveness of switching from adjuvant tamoxifen to anastrozole in postmenopausal women with hormone-sensitive early-stage breast cancer: a meta-analysis. Lancet Oncol 2006;7:991 – 6.

3. Coates AS, Keshaviah A, Thurlimann B, et al. Five years of letrozole compared with tamoxifen as initial adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer: update of study BIG 1 – 98. J Clin Oncol 2007;25:486 – 92.

4. Goss PE, Ingle JN, Martino S, et al. Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17. J Natl Cancer Inst 2005;97:1262 – 71.

5. Coombes RC, Hall E, Gibson LJ, et al. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. N Engl J Med 2004;350:1081 – 92.

6. Coombes RC, Kilburn LS, Snowdon CF, et al. Survival and safety of exemestane versus tamoxifen after 2 – 3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial. Lancet 2007;369:559 – 70.

7. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007;72:7 – 25.

8. Howell A, Clarke RB, Evans G, et al. Estrogen deprivation for breast cancer prevention. Recent Results Cancer Res 2007;174:151 – 67.

9. Buzdar A, Howell A, Cuzick J, et al. Comprehensive side-effect profile of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: long-term safety analysis of the ATAC trial. Lancet Oncol 2006; 7:633 – 43.

10. Coleman RE, Banks LM, Girgis SI, et al. Skeletal effects of exemestane on bone-mineral density, bone biomarkers, and fracture incidence in postmenopausal women with early breast cancer participating in the Intergroup Exemestane Study (IES): a randomised controlled study. Lancet Oncol 2007;8:119 – 27.

11. Perez EA, Josse RG, Pritchard KI, et al. Effect of letrozole versus placebo on bone mineral density in women with primary breast cancer completing 5 or more years of adjuvant tamoxifen: a companion study to NCIC CTG MA.17. J Clin Oncol 2006;24:3629 – 35.

12. Eastell R, Hannon RA, Cuzick J, Dowsett M, Clack G, Adams JE. Effect of an aromatase inhibitor on bmd and bone turnover markers: 2-year results of the Anastrozole, Tamoxifen, Alone or in Combination (ATAC) trial (18233230). J Bone Miner Res 2006;21:1215 – 23.

13. Morales L, Pans S, Paridaens R, et al. Debilitating musculoskeletal pain and stiffness with letrozole and exemestane: associated tenosynovial changes on magnetic resonance imaging. Breast Cancer Res Treat 2007;104:87 – 91.

14. Mackey J, Gelmon K. Adjuvant aromatase inhibitors in breast cancer therapy: significance of musculoskeletal complications. Curr Opin Oncol 2007;19 Suppl 1:S9 – 18.

15. Goss PE, Qi S, Josse RG, et al. The steroidal aromatase inhibitor exemestane prevents bone loss in ovariectomized rats. Bone 2004;34: 384 – 92.

16. Goss PE, Qi S, Cheung AM, Hu H, Mendes M, Pritzker KP. Effects of the steroidal aromatase inhibitor exemestane and the nonsteroidal aromatase inhibitor letrozole on bone and lipid metabolism in ovariectomized rats. Clin Cancer Res 2004;10:5717 – 23.

17. Lonning PE, Geisler J, Krag LE, et al. Effects of exemestane administered for 2 years versus placebo on bone mineral density, bone biomarkers, and plasma lipids in patients with surgically resected early breast cancer. J Clin Oncol 2005;23:5126 – 37.

18. Coleman RE, Group AT. Effect of anastrozole on bone mineral density: 5-year results from the 'Arimidex,' Tamoxifen, Alone or in Combination (ATAC) trial. J Clin Oncol (Meeting Abstracts) 2006;24:511.

19. Love RR, Mazess RB, Barden HS, et al. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 1992;326:852 – 6.

20. Cummings SR, Browner WS, Bauer D, et al. Endogenous hormones and the risk of hip and vertebral fractures among older women. N Engl J Med 1998;339:733 – 8.

21. Hofbauer LC, Khosla S. Androgen effects on bone metabolism: recent progress and controversies. Eur J Endocrinol 1999;140:271 – 86.

22. Miki Y, Suzuki T, Hatori M, et al. Effects of aromatase inhibitors on human osteoblast and osteoblast-like cells: a possible androgenic bone protective effects induced by exemestane. Bone 2007;40:876 – 87.

23. Lea CK, Flanagan AM. Physiological plasma levels of androgens reduce bone loss in the ovariectomized rat. Am J Physiol 1998;274: E328 – 35.

24. Tobias JH, Gallagher A, Chambers TJ. 5 α-Dihydrotestosterone partially restores cancellous bone volume in osteopenic ovariectomized rats. Am J Physiol 1994;267:E853 – 9.

25. Buchanan JR, Hospodar P, Myers C, Leuenberger P, Demers LM. Effect of excess endogenous androgens on bone density in young women. J Clin Endocrinol Metab 1988;67:937 – 43.

26. Dixon JE, Rodin A, Murby B, Chapman MG, Fogelman I. Bone mass in hirsute women with androgen excess. Clin Endocrinol (Oxf) 1989;30: 271 – 7.

27. Riggs BL, Jowsey J, Goldsmith RS, Kelly PJ, Hoffman DL, Arnaud CD. Short- and long-term effects of estrogen and synthetic anabolic hormone in postmenopausal osteoporosis. J Clin Invest 1972;51:1659 – 63.

28. Barrett-Connor E, Young R, Notelovitz M, et al. A two-year, double-blind comparison of estrogen-androgen and conjugated estrogens in surgically menopausal women. Effects on bone mineral density, symptoms and lipid profiles. J Reprod Med 1999;44:1012 – 20.

29. di Salle E, Giudici D, Ornati G, et al. 4-Aminoandrostenedione derivatives: a novel class of irreversible aromatase inhibitors. Comparison with FCE 24304 and 4-hydroxyandrostenedione. J Steroid Biochem Mol Biol 1990;37:369 – 74.

30. Evans TR, Di Salle E, Ornati G, et al. Phase I and endocrine study of exemestane (FCE 24304), a new aromatase inhibitor, in postmenopausal women. Cancer Res 1992;52:5933 – 9.

31. Pfizer Inc. Product information: Aromasin, exemestane tablets. New York, NY 10017; 2007.

32. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. Cancer Res 1996; 56:2321 – 30.

33. Ariazi EA, Kraus RJ, Farrell ML, Jordan VC, Mertz JE. Estrogen-related receptor α1 transcriptional activities are regulated in part via the ErbB2/HER2 signaling pathway. Mol Cancer Res 2007;5:71 – 85.

34. Abdelrahim M, Ariazi E, Kim K, et al. 3-Methylcholanthrene and other aryl hydrocarbon receptor agonists directly activate estrogen receptor α. Cancer Res 2006;66:2459 – 67.

35. Bauer JA, Thompson TA, Church DR, Ariazi EA, Wilding G. Growth inhibition and differentiation in human prostate carcinoma cells induced by the vitamin D analog 1α,24-dihydroxyvitamin D2. Prostate 2003;55: 159 – 67.

36. Warnmark A, Treuter E, Gustafsson JA, Hubbard RE, Brzozowski AM, Pike AC. Interaction of transcriptional intermediary factor 2 nuclear receptor box peptides with the coactivator binding site of estrogen receptor α. J Biol Chem 2002;277:21862 – 8.

37. He B, Gampe RT, Jr., Kole AJ, et al. Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance. Mol Cell 2004;16: 425 – 38.

38. Brzozowski AM, Pike AC, Dauter Z, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997;389:753 – 8.

39. Matias PM, Donner P, Coelho R, et al. Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. J Biol Chem 2000;275: 26164 – 71.

40. Fang H, Tong W, Shi LM, et al. Structure-activity relationships for a large diverse set of natural, synthetic, and environmental estrogens. Chem Res Toxicol 2001;14:280 – 94.

41. Fang H, Tong W, Branham WS, et al. Study of 202 natural, synthetic, and environmental chemicals for binding to the androgen receptor. Chem Res Toxicol 2003;16:1338 – 58.

42. Wolf DA, Herzinger T, Hermeking H, Blaschke D, Horz W. Transcriptional and posttranscriptional regulation of human androgen receptor expression by androgen. Mol Endocrinol 1993;7:924 – 36.

43. Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM. Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. Mol Endocrinol 1995;9:208 – 18.

44. Somboonporn W, Davis SR. Testosterone effects on the breast: implications for testosterone therapy for women. Endocr Rev 2004;25: 374 – 88.

45. Yeh S, Hu Y-C, Wang P-H, et al. Abnormal mammary gland development and growth retardation in female mice and MCF7 breast cancer cells lacking androgen receptor. J Exp Med 2003;198:1899 – 908.

46. Group EHaBCC. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst 2002;94:606 – 16.

47. Soreide JA, Lea OA, Varhaug JE, Skarstein A, Kvinnsland S. Androgen receptors in operable breast cancer: relation to other steroid hormone receptors, correlations to prognostic factors and predictive value for effect of adjuvant tamoxifen treatment. Eur J Surg Oncol 1992;18: 112 – 8.

48. Kuenen-Boumeester V, Van der Kwast TH, Claassen CC, et al. The clinical significance of androgen receptors in breast cancer and their relation to histological and cell biological parameters. Eur J Cancer 1996; 32A:1560 – 5.

49. Agoff SN, Swanson PE, Linden H, Hawes SE, Lawton TJ. Androgen receptor expression in estrogen receptor-negative breast cancer. Immunohistochemical, clinical, and prognostic associations. Am J Clin Pathol 2003;120:725 – 31.

50. Bieche I, Parfait B, Tozlu S, Lidereau R, Vidaud M. Quantitation of androgen receptor gene expression in sporadic breast tumors by real-time RT-PCR: evidence that MYC is an AR-regulated gene. Carcinogenesis 2001;22:1521 – 6.

Aspirin Sensitizes Cancer Cells to TRAIL–Induced Apoptosis by Reducing Survivin Levels

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Abstract

Purpose: Although tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) and agonistic antibodies targeting its receptors are promising cancer therapies because of their tumor selectivity, many tumors are resistant to TRAIL-based therapies. We examined whether the nonsteroidal anti-inflammatory drug aspirin sensitized cancer cells to TRAIL agonists *in vitro* and *in vivo* and investigated the underlying mechanism.

Experimental Design: The effects of aspirin on sensitivity to TRAIL agonists and expression of apoptosis regulators was determined in human breast cancer cell lines and xenograft tumors. The specific role of survivin depletion in the TRAIL-sensitizing effects of aspirin was determined by silencing survivin.

Results: Aspirin sensitized human breast cancer cells, but not untransformed human mammary epithelial cells, to TRAIL-induced caspase activation and apoptosis by a cyclooxygenase-2–independent mechanism. Aspirin also sensitized breast cancer cells to apoptosis induced by a human agonistic TRAIL receptor-2 monoclonal antibody (lexatumumab). Aspirin treatment led to G_1 cell cycle arrest and a robust reduction in the levels of the antiapoptotic protein survivin by inducing its proteasomal degradation, but did not affect the levels of many other apoptosis regulators. Silencing survivin with small interfering RNAs sensitized breast cancer cells to TRAIL-induced apoptosis, underscoring the functional role of survivin depletion in the TRAIL-sensitizing actions of aspirin. Moreover, aspirin acted synergistically with TRAIL to promote apoptosis and reduce tumor burden in an orthotopic breast cancer xenograft model.

Conclusions: Aspirin sensitizes transformed breast epithelial cells to TRAIL-based therapies *in vitro* and *in vivo* by a novel mechanism involving survivin depletion. These findings provide the first *in vivo* evidence for the therapeutic utility of this combination.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL/Apo2L) is a proapoptotic cytokine that preferentially induces apoptosis in transformed cells (1). TRAIL activates the extrinsic apoptotic pathway by binding to its cell surface death receptors TRAIL receptor-1 (TRAIL-R1)/DR4 or TRAIL receptor-2 (TRAIL-R2)/DR5, which each contain a cytoplasmic death domain that is required for apoptosis induction. Upon ligand binding, the death domain–containing protein FADD is recruited to the death-inducing signaling complex (DISC), which in turn, leads to the recruitment and activation of apical procaspase-8 and procaspase-10 at the DISC via a homophilic interaction involving their respective death effector domains. In contrast, two decoy receptors DcR1/TRAIL-R3 and DcR2/TRAIL-R4, which lack functional death domains, act to neutralize the cytotoxicity of TRAIL by sequestering the ligand from its death receptors. In cells with robust activation of caspase-8 and caspase-10 (type I cells), these initiator caspases directly activate the executioner caspases (caspase-3, caspase-6, and caspase-7) and trigger apoptosis (2). In type II cells, the initiator caspases cleave the BH3-only protein Bid, which then translocates to the mitochondria to activate the intrinsic apoptotic pathway by inducing cytochrome *c* release by a Bax/Bak-dependent mechanism (3, 4). Cytosolic cytochrome *c* activates caspase-3 by inducing the Apaf-1–dependent activation of the apical procaspase-9 in the apoptosome (5). Importantly, the mitochondria play an essential role in TRAIL-induced apoptosis in type II cells: mutation or deletion of Bax results in resistance to TRAIL (6, 7).

Given the potential tumor selectivity of its proapoptotic actions, recombinant TRAIL has garnered a great deal of interest as a cancer therapy and is currently in early stage clinical trials. In preclinical models, TRAIL induces apoptosis in diverse cancer cells *in vitro* and suppresses primary tumor growth and metastases *in vivo* (8–10). In addition, the safety

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of recombinant TRAIL at doses up to 10 mg/kg/d for 7 days has been documented in non–human primates (8). Similarly, agonistic monoclonal antibodies (mAb) targeting TRAIL-R1 or TRAIL-R2 have been shown to induce apoptosis in cancer cells *in vitro* and in xenograft carcinomas *in vivo*, and these antibodies are well tolerated in rodents (11–15). Indeed, fully human agonistic mAbs specifically targeting TRAIL-R1 (mapatumumab) or TRAIL-R2 (lexatumumab) are currently in clinical trials. Collectively, these results suggest that recombinant TRAIL and/or agonistic mAbs targeting its death receptors may prove to be an efficacious and minimally toxic strategy to treat cancer.

One of the major obstacles confronting TRAIL-based cancer therapies is the intrinsic or acquired resistance of many human tumors to TRAIL-induced apoptosis (7, 8, 16). TRAIL-resistance is likely to be mediated by multiple defects in the TRAIL signaling pathway, including inactivating mutations in TRAIL-R1 and TRAIL-R2, loss of the initiator caspase-8 and the proapoptotic Bcl-2 family member Bax, and overexpression of antiapoptotic Bcl-2 family members such as Bcl-2, Bcl-x_L, and Mcl-1 (7, 17–22). Hence, optimal TRAIL-based therapies for many tumors will need to incorporate agents which sensitize cancer cells to TRAIL-induced apoptosis. For example, chemotherapy and radiation sensitize cancer cells and xenograft tumors to TRAIL- or TRAIL receptor antibody–induced apoptosis at least partly by increasing the expression of TRAIL-R2 and Bax (7, 8, 12, 15, 16). In addition, several potential chemopreventive agents and/or emerging cancer therapies, including PPARγ ligands (thiazolidinediones and triterpenoids), resveratrol and inhibitors of histone deacetylase, the proteasome and cyclin-dependent kinases have been shown to promote TRAIL-induced apoptosis in cancer cells (13, 23–27). These latter agents are particularly attractive because they do not abrogate the tumor selectivity of TRAIL-based therapies, and they are likely to have less systemic toxicity than conventional cytotoxic agents or radiation.

To identify additional TRAIL-sensitizing agents, we examined the hypothesis that aspirin (acetylsalicylic acid, ASA) might act synergistically with TRAIL and/or TRAIL receptor agonist antibodies to induce apoptosis. Aspirin is a nonsteroidal anti-inflammatory drug which has been reported to reduce the risk of colorectal adenomas and carcinomas, breast cancer, and other malignancies, particularly long-term and higher dose use of aspirin (28–31). Aspirin inhibits both cyclooxygenase (COX)-1 and COX-2 enzymes that catalyze the rate-limiting step in prostaglandin synthesis (32). In addition, 5 mmol/L of aspirin induces G_1 cell cycle arrest and triggers COX-independent apoptosis in cancer cells at least in part by activating Bax and releasing cytochrome *c* from mitochondria (33, 34). We postulated that these latter mitochondrial apoptotic effects of aspirin would sensitize cancer cells to death receptor–mediated apoptosis initiated by TRAIL by amplifying caspase activation. Although aspirin has recently been shown to promote TRAIL-induced apoptosis *in vitro* (35), we report here that aspirin sensitizes breast cancer cells to TRAIL-induced caspase activation and apoptosis at least in part by a novel mechanism involving the proteasomal degradation of the antiapoptotic protein survivin. Survivin is a member of the IAP (inhibitor of apoptosis) family that is preferentially expressed in transformed cells and confers resistance to diverse apoptotic stimuli, including TRAIL (23, 25, 27, 36). We also show for the first time that aspirin acts synergistically with TRAIL to promote apoptosis and suppress human breast xenograft tumor growth *in vivo*, thereby underscoring the potential therapeutic utility of this combination.

Materials and Methods

Cell lines and reagents. Human MDA-MB-435 and T47D breast carcinoma cells (American Type Culture Collection) were grown in DMEM (Mediatech) supplemented with 4.5 g/L of glucose and 4 mmol/L of L-glutamine, 100 units/mL of penicillin/streptomycin and 10% FCS (Invitrogen), whereas HCT116 colon cancer cells were grown in McCoy's 5A medium (Invitrogen) supplemented with 100 units/mL of penicillin/streptomycin and 10% FCS. Although the origin of MDA-MB-435 cells has been debated, recent studies suggest that they are breast cancer cells that have undergone lineage infidelity (37). Human mammary epithelial cells (HMEC; Cambrex) were grown as described in the manufacturer's instructions. All cells were grown in 5% CO_2 atmosphere at 37°C. Aspirin (ASA) was purchased from Sigma-Aldrich and dissolved in 100% ethanol for *in vitro* studies. Recombinant TRAIL (amino acids 95-281) was expressed in *Escherichia coli* and purified as described (23, 38). Human TRAIL-R1 (mapatumumab) and TRAIL-R2 (lexatumumab) agonistic antibodies of IgG_1 isotype were kindly provided by Dr. Robin Humphreys (Human Genome Sciences, Rockville, MD; refs. 13–15).

Induction and scoring of apoptosis. Cells were preincubated with ASA (0-5 mmol/L) for 48 h and then treated with TRAIL (0-2.5 μg/mL), mapatumumab, or lexatumumab (0 or 2.5 μg/mL) for 16 h. Apoptosis was measured by two independent methods. First, the percentage of apoptotic (condensed/fragmented) nuclei were scored by Hoechst (Sigma-Aldrich) staining and fluorescence microscopy as described (39). Three independent experiments were done, counting at least 200 nuclei in each experiment. Apoptotic cells were also identified by flow cytometry–based Annexin V–labeling using the Annexin-PE Apoptosis Detection Kit I (BD Bioscience) according to the manufacturer's instructions.

Cell cycle analyses. Cells were treated with vehicle or ASA (1 mmol/L or 5 mmol/L) for 48 h. Cells were washed twice in PBS and then incubated overnight in 70% ethanol/30% PBS at -20°C. The fixed cells were then washed twice and incubated with a propidium iodide solution (50 μg/mL propidium iodide, 0.2 mg/mL RNase A, and 0.1% Triton X-100 in PBS) for 20 min at 37°C. The cell cycle distribution of cells was determined by flow cytometry–based analysis of DNA content using ModFit software.

Crystal violet cell survival assays. Cells were plated on six-well plates (3×10^5 cells/well), allowed to adhere overnight, preincubated with vehicle or 5 mmol/L of ASA for 48 h, and then treated with vehicle or 2.5 μg/mL of TRAIL for 16 h. Cells were then washed with PBS, and fresh growth medium was added. The medium was changed every other day for 5 additional days. Surviving cells were fixed and stained with crystal violet solution (40% ethanol, 60% PBS, and 0.5% crystal violet).

Immunoblotting. Cells were lysed in a modified radioimmunoprecipitation assay buffer (50 mmol/L Tris, 0.1% SDS, 150 mmol/L NaCl, 0.5% sodium deoxycholate, and 1% NP40). Proteins were detected by immunoblotting as described (40) with the following antibodies: TRAIL-R1, TRAIL-R2, DcR2 (Stressgen), tubulin (Sigma-Aldrich), caspase-3, RIP, Bcl-X_L, FADD, TRADD, DcR1, FLIP, Mcl-1, caspase-8, and Bak (BD Biosciences), XIAP and survivin (R & D Systems), Bcl-2 and Bax (Santa Cruz Biotechnology). For proteasome inhibitor experiments, cells were preincubated with vehicle or 5 mmol/L of ASA for 48 h and then treated with the proteasomal inhibitor epoxomicin (0-200 nmol/L; EMD Biosciences; ref. 41) for 16 h (longer treatments with epoxomicin resulted in cell death).

Real-time reverse transcription-PCR. Total RNA was prepared from MDA-MB-435 cells treated with vehicle or 5 mmol/L of ASA for 64 h using the RNeasy mini kit (Qiagen). RNA was used to synthesize cDNA using the Superscript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen). Survivin mRNA and glyceraldehyde-3-phosphate dehydrogenase mRNA (for normalization) were amplified by real-time PCR using TaqMan universal PCR master mix and an ABI PRISM 7000 sequence detection system instrument (Applied Biosystems) according to the manufacturer's instructions. A predesigned 20× mix of primers and TaqMan MGB probes for survivin and glyceraldehyde-3-phosphate dehydrogenase were purchased from ABI. cDNA (100 ng), primers (900 nmol/L), and probes (250 nmol/L) were used in PCR reactions (50°C for 2 min, 95°C for 1 min, and 40 cycles of 95°C for 15 s, and 60°C for 1 min).

Survivin silencing using small interfering RNAs. MDA-MB-435 cells (2.5×10^5 cells/well) were plated on six-well plates and transiently transfected with 50 nmol/L of Smartpool small interfering RNAs (siRNA) targeting human survivin (Dharmacon) or a nonsilencing siRNA targeting luciferase (Dharmacon NS2) using OligofectAMINE reagent (Invitrogen) according to the manufacturer's instructions. Survivin levels were determined by immunoblotting at various time points after transfection. For apoptosis experiments, the medium was changed 72 h after siRNA transfection, and cells were treated with vehicle, 2.5 μg/mL of TRAIL or 2.5 μg/mL of lexatumumab for 16 h. Apoptotic nuclei were scored as described earlier (Induction and scoring of apoptosis).

Orthotopic breast cancer xenograft experiments. Pieces of human MDA-MB-435 breast carcinoma xenografts (1 mm^3) were implanted subcutaneously into both mammary fat pads of 4- to 5-week old female athymic *nu/nu* mice (Harlan Sprague-Dawley) as described (23). Three weeks later, mice (10 per group) were randomly assigned to one of six treatment groups: vehicle (0.75% methylcellulose; Sigma-Aldrich), ASA 100 mg/kg/d by oral gavage, ASA 400 mg/kg/d by oral gavage, TRAIL 10 mg/kg/d i.p., ASA 100 mg/kg/d orally + TRAIL 10 mg/kg/d i.p., or ASA 400 mg/kg/d orally + TRAIL 10 mg/kg/d i.p. For these experiments, ASA was ground into a fine powder in a mortar and pestle, suspended in 0.75% methylcellulose, and administered to mice by oral gavage. Mice were treated for 3 weeks, and tumor volume was measured weekly as described (23). The induction of apoptosis in formalin-fixed, paraffin-embedded breast cancer xenografts was measured by terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) assay (In Situ Cell Death Detection kit, TMR Red; Roche). These experiments were approved by the Animal Care and Use Committee of Northwestern University.

Statistical analysis. Statistical significance was determined by ANOVAs with Bonferroni posttests or two-tailed unpaired *t* tests with Welch's correction (xenograft experiments) using Prism 4 (GraphPad Software). $P < 0.05$ was considered statistically significant.

Results

***Aspirin promotes TRAIL-induced apoptosis of human breast carcinoma cells* in vitro.** To determine whether ASA sensitizes breast cancer cells to TRAIL, estrogen receptor (ER)–negative human MDA-MB-435 breast cancer cells were preincubated with ASA (0-5 mmol/L) for 48 hours and then treated with TRAIL (0-2.5 μg/mL) for 16 hours. Consistent with our previous findings (23), MDA-MB-435 cells were resistant to TRAIL-induced apoptosis when this cytokine was used as a single agent at concentrations as high as 2.5 μg/mL (Fig. 1A, *left*). MDA-MB-435 cells were also highly resistant to ASA alone at concentrations up to 5 mmol/L. Strikingly, ASA (at 5 mmol/L but not at 1 mmol/L) robustly sensitized MDA-MB-435 cells to TRAIL-induced apoptosis. Synergistic effects of 5 mmol/L ASA were observed at concentrations of TRAIL as low as 0.1 μg/mL, and the combination of 5 mmol/L of ASA and 2.5 μg/mL of TRAIL resulted in >70% of cells undergoing apoptosis. Preincubation with ASA was essential and shorter preincubation times were associated with less robust sensitization to TRAIL-induced apoptosis (data not shown). Flow cytometry–based Annexin V labeling of apoptotic cells revealed comparable findings: MDA-MB-435 cells were resistant to TRAIL or ASA alone, but were sensitive to the combined treatment (Fig. 1A, *right*). ASA also dramatically promoted TRAIL-induced apoptosis of ER-positive T47D breast cancer cells, which similar to MDA-MB-435 cells, were resistant to either agent alone (Fig. 1B, *left*). In contrast, the combination of 5 mmol/L of ASA and 2.5 μg/mL of TRAIL induced minimal apoptosis in untransformed HMECs (Fig. 1B, *right*), suggesting that ASA may not abrogate the potential tumor selectivity of TRAIL. Collectively, these results indicate that high-dose ASA sensitizes ER-negative and ER-positive breast cancer cells, but not untransformed HMECs, to TRAIL-induced apoptosis *in vitro*.

We next examined whether ASA sensitized breast cancer cells to apoptosis induced by human agonistic mAbs targeting TRAIL-R1 (mapatumumab) or TRAIL-R2 (lexatumumab; refs. 13–15). To this end, human T47D breast cancer cells were preincubated with vehicle or 5 mmol/L of ASA for 48 hours and then treated with mapatumumab (0 or 2.5 μg/mL), lexatumumab (0 or 2.5 μg/mL), or both mAbs (0 or 2.5 μg/mL each). T47D cells were resistant to mapatumumab (abbreviated "Mapa") or lexatumumab (abbreviated "Lexa") alone or in combination and to ASA alone (Fig. 1C). However, ASA preincubation resulted in a striking sensitization of these cells to lexatumumab-induced apoptosis, inducing levels of cell death comparable to the combination of ASA and TRAIL (Fig. 1B, *left*). In contrast, ASA did not sensitize T47D cells to mapatumumab-induced apoptosis (Fig. 1C), even though these cells express TRAIL-R1 (data not shown). Similar results were obtained in MDA-MB-435 cells (data not shown). Moreover, the addition of mapatumumab did not significantly increase apoptosis induction by ASA and lexatumumab. Taken together, these results indicate that ASA sensitizes breast cancer cells to apoptosis induced by an agonistic mAb targeting TRAIL-R2.

To determine whether the TRAIL-sensitizing effects of ASA were mediated by COX-2 inhibition, we preincubated COX-2–deficient HCT116 colon carcinoma cells with vehicle or 5 mmol/L of ASA for 48 hours and then treated cells with TRAIL (0-0.5 μg/mL) for 16 hours. Although HCT116 cells were partially sensitive to TRAIL alone, ASA dramatically enhanced TRAIL-induced apoptosis in these COX-2–deficient cells (Fig. 1D). These findings show that the TRAIL-sensitizing actions of ASA are COX-2–independent.

Aspirin induces G_1 cell cycle arrest and cooperates with TRAIL to reduce long-term cell survival. Because several drugs which induce G_1 cell cycle arrest have been shown to promote TRAIL-induced apoptosis, and aspirin has been reported to trigger G_1 arrest (23, 25, 33), we postulated that ASA might sensitize breast cancer cells to TRAIL by this mechanism. Treatment of MDA-MB-435 cells with 5 mmol/L of ASA for 48 hours resulted in a robust G_1 arrest, whereas 1 mmol/L of ASA did not affect cell cycle distribution (Fig. 2A) or promote TRAIL-induced apoptosis (Fig. 1A). Similar results were observed in T47D cells (data not shown). To determine whether the enhanced apoptosis observed by the combination of ASA and TRAIL resulted in greater long-term reductions in cell survival than treatment with either agent alone, we

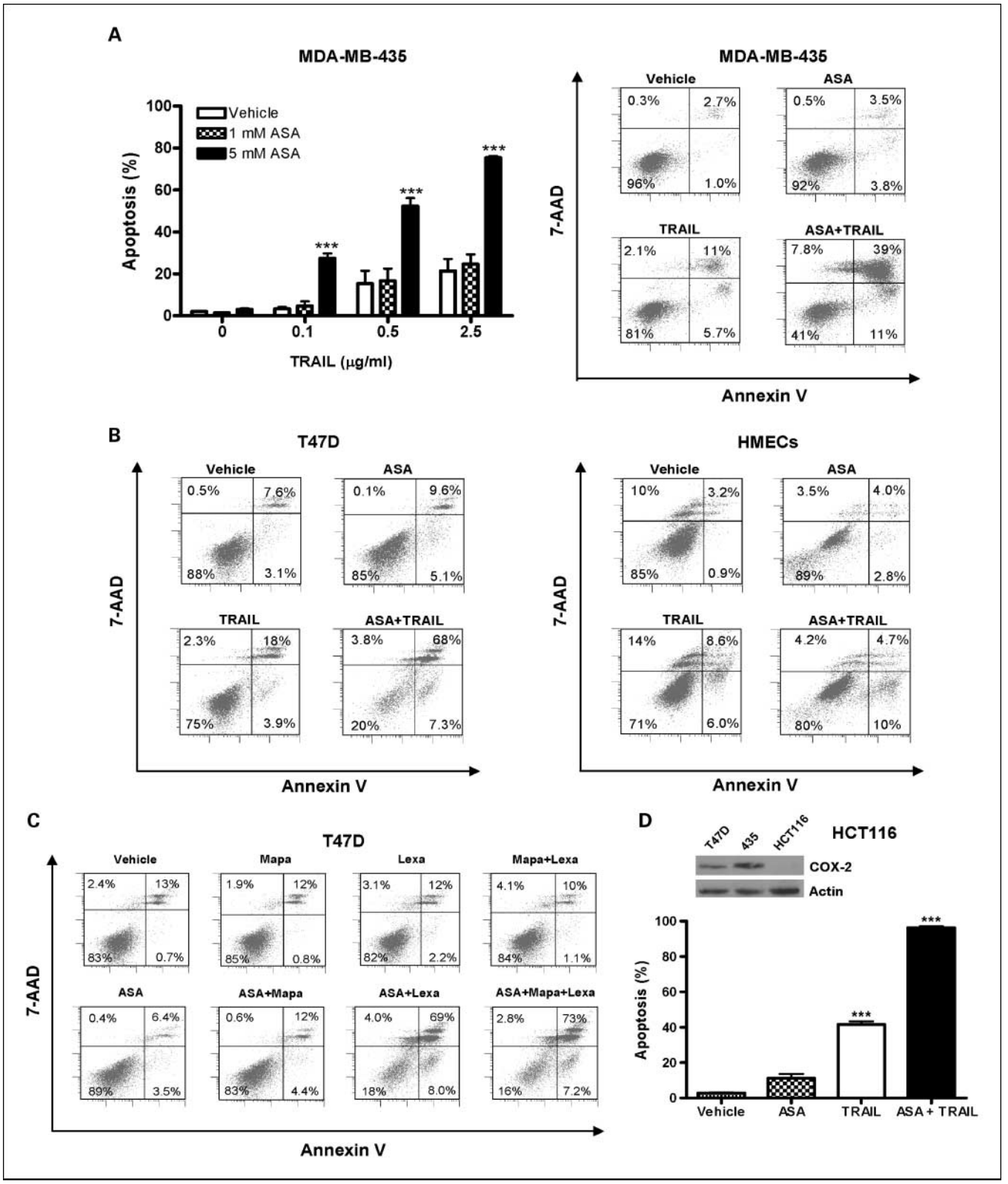

Fig. 1. Aspirin promotes TRAIL-induced apoptosis of human breast cancer cells, but not HMECs, *in vitro. A,* MDA-MB-435 breast carcinoma cells were preincubated with vehicle or ASA (1 or 5 mmol/L) for 48 h and then treated with TRAIL (0-2.5 μg/mL) for 16 h (*left*). Apoptotic nuclei were scored (*columns,* mean; *bars,* SE; $n = 3$). ***, $P < 0.001$ vs. vehicle-treated cells. *A,* MDA-MB-435 cells (*right*); *B,* T47D breast cancer cells (*left*) or HMECs (*right*) were preincubated with vehicle or 5 mmol/L of ASA for 48 h and then treated with TRAIL (0 or 2.5 μg/mL) for 16 h. Apoptosis was measured by Annexin V labeling. *C,* T47D cells were pretreated with vehicle or 5 mmol/L of ASA for 48 h and then treated with the TRAIL-R1 agonistic mAb mapatumumab (*Mapa*, 0 or 2.5 μg/mL), the TRAIL-R2 agonistic mAb lexatumumab (*Lexa*, 0 or 2.5 μg/mL), or both mAbs (0 or 2.5 μg/mL each) for 16 h. Apoptosis was measured by Annexin V labeling. *D,* ASA sensitizes COX-2–deficient HCT116 colon cancer cells to TRAIL-induced apoptosis. HCT116 cells were pretreated with ASA (0-5 mmol/L) for 48 h and then treated with TRAIL (0-0.5 μg/mL) for 16 h. Apoptotic nuclei were scored (*columns,* mean; *bars,* SE; $n = 3$). ***, $P < 0.001$ vs. vehicle-treated cells.

preincubated T47D cells with vehicle or 5 mmol/L of ASA for 48 hours, treated them with vehicle or 2.5 μg/mL of TRAIL for 16 hours, washed the cells, and grew them for 5 additional days in the absence of drugs. Under these conditions, cells treated with vehicle, ASA alone, or TRAIL alone remained viable and continued to grow following the removal of these agents (Fig. 2B). In contrast, the combination of ASA and TRAIL led to the virtual elimination of all viable cells, indicating that the TRAIL-sensitizing actions of ASA are accompanied by robust long-term reductions in cell survival.

Aspirin promotes TRAIL-induced caspase activation and reduces survivin protein levels. To examine whether ASA promotes TRAIL-induced caspase activation, we incubated MDA-MB-435 breast cancer cells with vehicle control (C) or 5 mmol/L of ASA for 64 hours, 2.5 μg/mL of TRAIL for 16 hours, or 5 mmol/L of ASA for 48 hours followed by 2.5 μg/mL of TRAIL for 16 hours. Combined treatment with ASA and TRAIL induced proteolytic cleavage of procaspase-8 and procaspase-3 and the caspase substrates BID and PARP (detected by diminished intensity of each full-length protein; Fig. 3A). In contrast, little caspase activation or caspase substrate cleavage was observed in cells treated with either ASA or TRAIL alone. To elucidate the mechanisms by which ASA promotes TRAIL-induced caspase activation, we examined the effects of treating MDA-MB-435 cells with 5 mmol/L of ASA for 64 hours on the expression of DISC proteins (Fig. 3B) or apoptosis regulators (Fig. 3C) by immunoblotting. Although ASA had minimal to no effect on the expression levels of the vast majority of these proteins, ASA treatment profoundly reduced the levels of survivin, an antiapoptotic protein previously implicated as a mediator of TRAIL-resistance in diverse cancers (23, 25, 27, 36). Importantly, 1 mmol/L of ASA did not reduce survivin levels (Fig. 3D), induce G_1 arrest (Fig. 2A), or promote TRAIL-induced apoptosis (Fig. 1A). Moreover, the time course of survivin depletion by 5 mmol/L of ASA (Fig. 3D) coincided closely with its TRAIL-sensitizing effects: maximal survivin depletion and TRAIL-sensitization were observed after prolonged exposure (64 hours) to ASA. These results suggest that the TRAIL-sensitizing effects of ASA might be due, at least in part, to the observed reduction in survivin protein levels.

Aspirin does not affect survivin mRNA levels but induces its proteasomal degradation. To determine the mechanisms by which ASA reduces survivin protein levels, we first examined the effect of ASA on survivin gene expression by real-time reverse transcription-PCR. Treatment of MDA-MB-435 cells with 5 mmol/L of ASA for 64 hours did not significantly affect survivin mRNA levels compared with vehicle-treated cells (Fig. 4A). However, the reduction in survivin protein levels induced by ASA was suppressed by the proteasome inhibitor epoxomicin (41) in a dose-dependent manner, with maximal suppression by 200 nmol/L of epoxomicin (Fig. 4B). These results indicate that ASA regulates survivin protein levels by a posttranscriptional mechanism that requires the proteasome.

***Silencing survivin promotes TRAIL-induced apoptosis* in vitro.** To examine the specific contribution of survivin to the TRAIL-sensitizing actions of ASA, we selectively inhibited the expression of survivin by RNA interference using siRNAs. MDA-MB-435 cells were transiently transfected with siRNAs targeting human survivin or a nonsilencing siRNA. Survivin siRNAs markedly reduced the expression of survivin, whereas the nonsilencing siRNA had no effect on survivin levels (Fig. 5A). Moreover, silencing survivin sensitized MDA-MB-435 cells to TRAIL- or lexatumumab-induced apoptosis, but did not induce apoptosis in the absence of TRAIL agonists (Fig. 5B). Stable silencing of survivin using short hairpin RNAs also sensitized breast cancer cells to TRAIL-induced apoptosis (data not shown). These results indicate that survivin negatively regulates TRAIL agonist–induced apoptosis and that the TRAIL-sensitizing effects of ASA are partly mediated by the observed reduction of survivin levels.

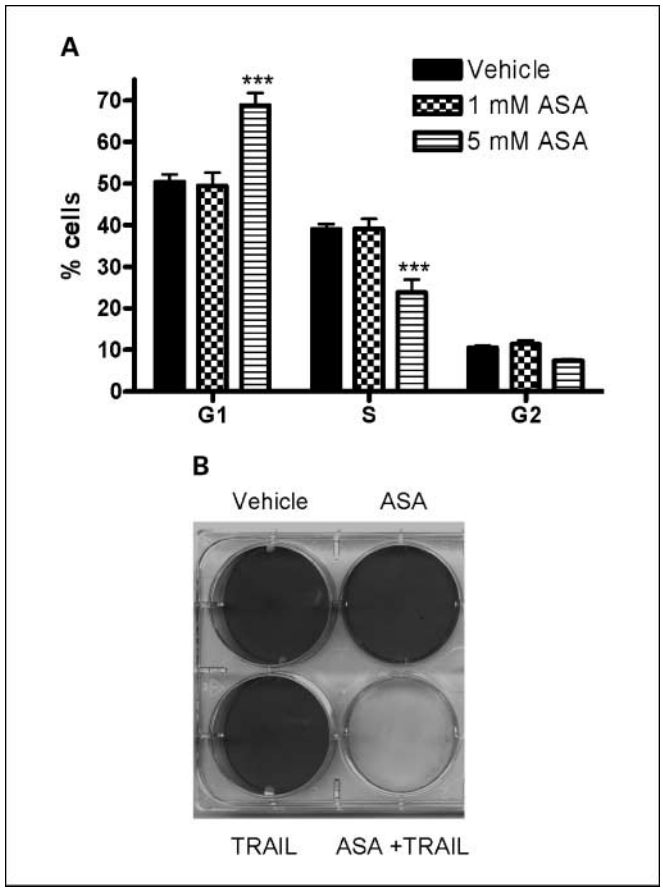

Fig. 2. Aspirin induces G_1 cell cycle arrest and cooperates with TRAIL to reduce long-term cell survival. *A,* MDA-MB-435 cells were treated with vehicle or ASA (1 or 5 mmol/L) for 48 h. The cell cycle distribution of treated cells was determined by flow cytometry–based analysis of DNA content (*columns,* mean; *bars,* SE; $n = 3$). ***, $P < 0.001$ vs. vehicle-treated cells. *B,* T47D cells were preincubated with vehicle or 5 mmol/L of ASA for 48 h, and then treated with vehicle or 2.5 μg/mL of TRAIL for 16 h. Cells were washed and cultured for 5 additional days in the absence of drugs. Viable cells were stained with crystal violet. Representative of three independent experiments.

***Aspirin sensitizes human breast xenograft tumors to TRAIL* in vivo.** To determine the antitumor efficacy of ASA and TRAIL *in vivo*, we treated female athymic nude mice with orthotopic MDA-MB-435 xenograft tumors with vehicle, ASA alone (100 or 400 mg/kg/d by oral gavage), TRAIL alone (10 mg/kg/d i.p.), or ASA (100 or 400 mg/kg/d) and TRAIL (10 mg/kg/d) for 3 weeks. Neither ASA nor TRAIL alone suppressed tumor growth compared with vehicle-treated mice (Fig. 6A). In contrast, the combination of high-dose ASA (400 mg/kg/d) and TRAIL robustly inhibited tumor growth,

whereas low-dose ASA (100 mg/kg/d) and TRAIL did not significantly affect tumor growth. High-dose ASA also sensitized xenograft tumors to TRAIL-induced apoptosis as determined by TUNEL staining (Fig. 6B). Moreover, high-dose ASA and TRAIL treatment reduced survivin levels in the breast xenograft tumors compared with the levels observed in vehicle-treated mice (Fig. 6C), consistent with the observed *in vitro* effects of ASA. Taken together, these findings indicate that ASA promotes TRAIL-induced apoptosis *in vivo*, thereby suggesting that this combination may be an effective therapy for breast cancer.

Discussion

Most human breast carcinoma cell lines are highly resistant to TRAIL-induced apoptosis even though they express its death receptors, TRAIL-R1 and TRAIL-R2 (16, 38), indicating that TRAIL-resistance in breast cancer is likely mediated by defects in the TRAIL signaling pathway downstream of death receptor activation. From a therapeutic standpoint, the high prevalence of TRAIL-resistance points to the need to identify agents which sensitize breast tumors to TRAIL-induced apoptosis without compromising the tumor selectivity and limited systemic toxicity of TRAIL. We have shown that ASA is a TRAIL-sensitizing agent *in vitro* and *in vivo* that fulfils many of these criteria. Specifically, we have shown that ASA (5 mmol/L) sensitizes ER-positive T47D and ER-negative MDA-MB-435 breast cancer cells, but not normal HMECs, to TRAIL-induced apoptosis. Both T47D and MDA-MB-435 breast cancer cells harbor a mutation in *TP53* (42, 43), indicating that the combination of ASA and TRAIL is effective against cancer cells with defects in p53-dependent apoptosis. Moreover, we show that ASA sensitizes breast cancer cells to lexatumumab, a fully human agonistic mAb targeting TRAIL-R2 (13, 15). Intriguingly, we observed that ASA does not promote apoptosis induced by an agonistic antibody targeting TRAIL-R1 (mapatumumab) even though the breast cancer cell lines used in this study express TRAIL-R1. These findings suggest that TRAIL-R2 is the principal death receptor used by TRAIL agonists in breast cancer cells sensitized to these agents by ASA. The molecular mechanisms underlying the observed differential effect of ASA on TRAIL-R1– and TRAIL-R2–dependent apoptosis are

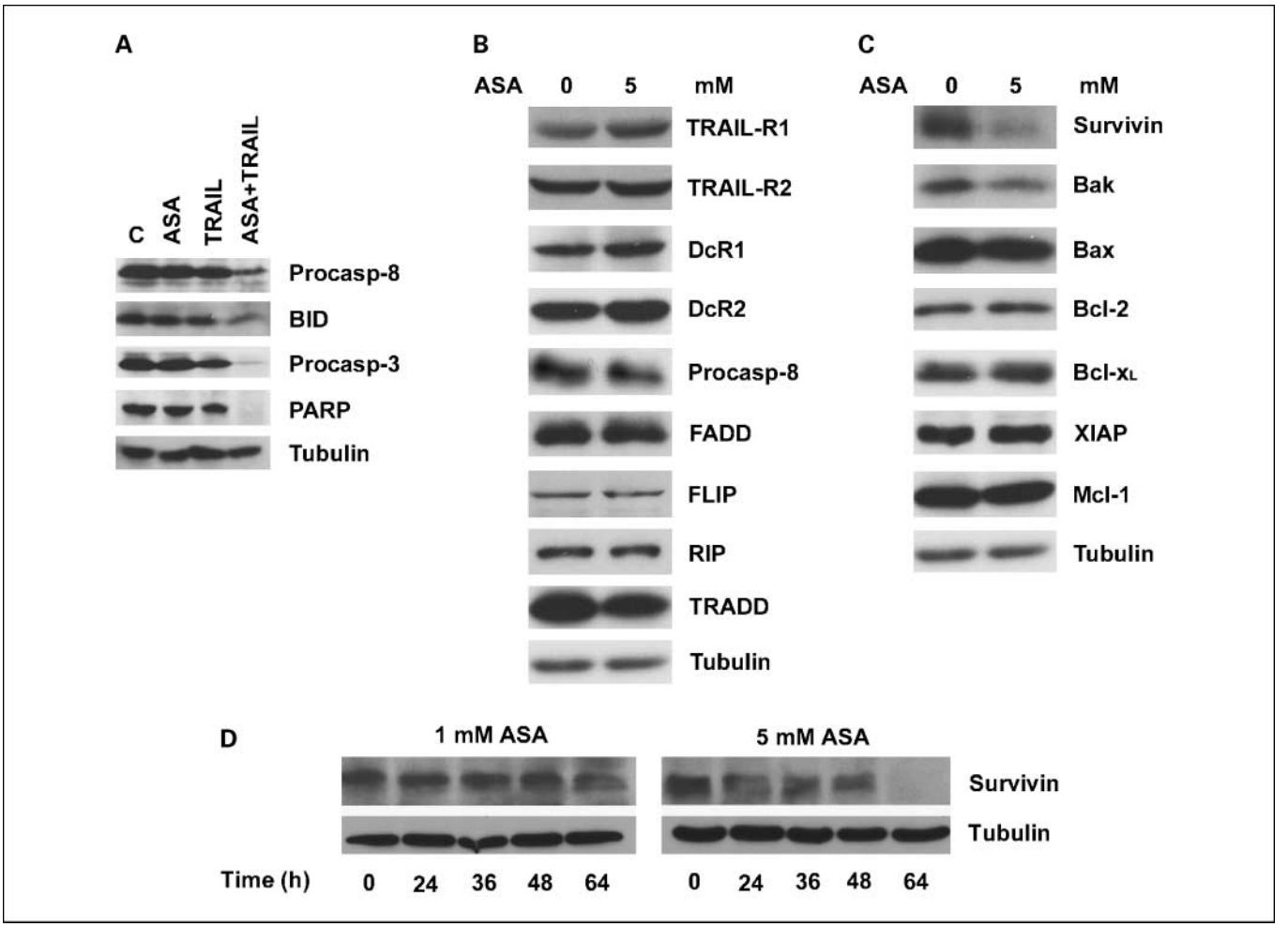

Fig. 3. Aspirin promotes TRAIL-induced caspase activation and selectively reduces survivin protein levels. *A,* MDA-MB-435 cells were treated with vehicle control (*C*), 5 mmol/L of ASA alone for 64 h, 2.5 μg/mL of TRAIL alone for 16 h, or 5 mmol/L of ASA for 48 h followed by 2.5 μg/mL of TRAIL for 16 h. Procaspase-8, BID, procaspase-3, and PARP were detected by immunoblotting. Tubulin was used as a control for protein loading. *B* and *C,* MDA-MB-435 cells were treated with ASA (0 or 5 mmol/L) for 64 h and the expression levels of DISC proteins (*B*) or other apoptosis regulators (*C*) were measured by immunoblotting. *D,* MDA-MB-435 cells were treated with ASA (1 or 5 mmol/L) for the indicated number of hours, and survivin levels were determined by immunoblotting.

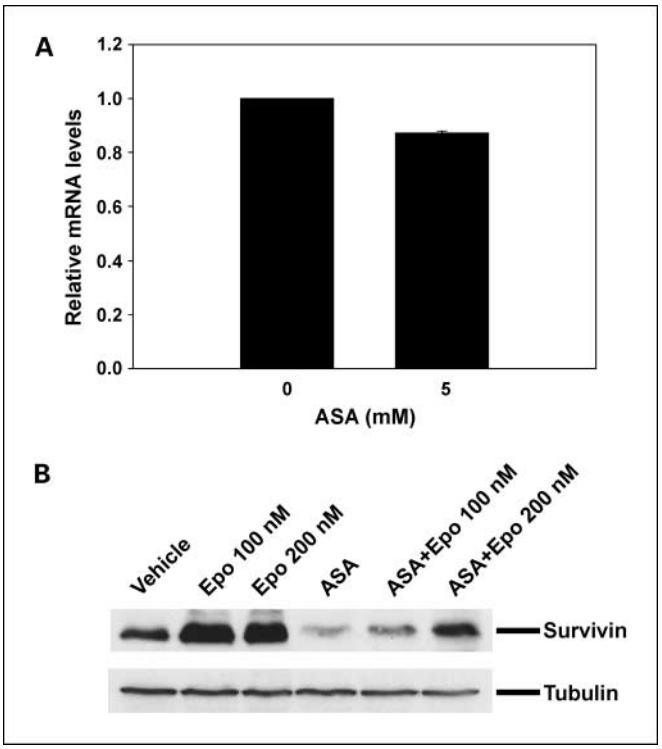

Fig. 4. Aspirin reduces survivin protein levels by inducing its proteasomal degradation. *A,* MDA-MB-435 cells were treated with vehicle or 5 mmol/L of ASA for 64 h. Survivin mRNA levels were measured by quantitative real-time reverse transcription-PCR. Columns, mean of survivin mRNA levels relative to vehicle-treated cells; bars, SE ($n = 3$). *B,* MDA-MB-435 cells were preincubated with vehicle or 5 mmol/L of ASA for 48 h, and then treated with the proteasome inhibitor epoxomicin (0-200 nmol/L) for 16 h. Survivin expression was examined by immunoblotting.

unclear. ASA does not affect the expression level of TRAIL-R2, but may affect its localization, posttranslational modification, or other specific components of the TRAIL-R2 signaling complex. Although ASA was recently reported to sensitize prostate and colon cancer cells to TRAIL *in vitro* (35), we have shown for the first time that ASA promotes TRAIL-induced apoptosis and inhibits xenograft tumor growth in mice, thereby providing critical *in vivo* proof of concept data supporting this novel combination cancer therapy. Importantly, the doses of ASA used in our xenograft studies (up to 400 mg/kg/d) suppress intestinal and mammary tumors when given to mice for their entire life span (44), confirming the feasibility of this dosing even for prolonged periods. Collectively, our results indicate that ASA selectively sensitizes transformed cells to TRAIL-based therapies *in vitro* and *in vivo* by a TRAIL-R2–dependent mechanism, and they suggest that this combination may be an effective cancer therapy that warrants additional study.

We have also shown that ASA sensitizes breast cancer cells to TRAIL-induced caspase activation and apoptosis at least in part by a mechanism involving G_1 cell cycle arrest and survivin depletion. Specifically, we have shown that ASA concentrations (5 mmol/L but not 1 mmol/L) which sensitize cancer cells to TRAIL also induce G_1 cell cycle arrest and robustly reduce the protein levels of survivin, but do not significantly affect the expression levels of many other apoptosis regulators or DISC components. Importantly, the TRAIL-sensitizing effects of ASA against breast xenograft tumors *in vivo* were also accompanied by a reduction in survivin levels. Survivin expression is cell cycle–dependent, with the highest levels at G_2-M, followed by a rapid reduction in survivin levels at G_1 mediated in part by proteasomal degradation of survivin (45). We observed that ASA does not affect survivin mRNA levels but acts by a posttranscriptional proteasome-dependent mechanism to reduce survivin protein levels. Hence, our results suggest that ASA reduces survivin levels by inducing G_1 arrest, which in turn, triggers the cell cycle–dependent proteasomal degradation of survivin (33, 45). The functional relevance of ASA-induced survivin degradation for its TRAIL-sensitizing actions is underscored by our observation that silencing survivin promotes TRAIL- or lexatumumab-induced apoptosis, albeit less dramatically than ASA treatment. This latter discrepancy may reflect the more modest reduction of survivin levels by RNA interference than by ASA treatment and/or the potential effects of ASA on other yet to be identified apoptosis regulators. Nevertheless, our findings provide unequivocal evidence that survivin is a negative regulator of TRAIL agonist–induced apoptosis and a functionally important target of ASA's TRAIL-sensitizing actions. These results are consistent with prior published reports from our group and others demonstrating that survivin confers protection against TRAIL and that suppressing survivin expression by a variety of strategies promotes TRAIL-induced apoptosis (23, 25, 27). Although the precise mechanism(s) by which survivin inhibits apoptosis remains controversial, recent work suggests that survivin sequesters Smac and/or procaspase-9, thereby suppressing caspase-3 activation (46, 47). In addition, survivin binds and stabilizes XIAP, which inhibits caspase-9 activation (48). Because

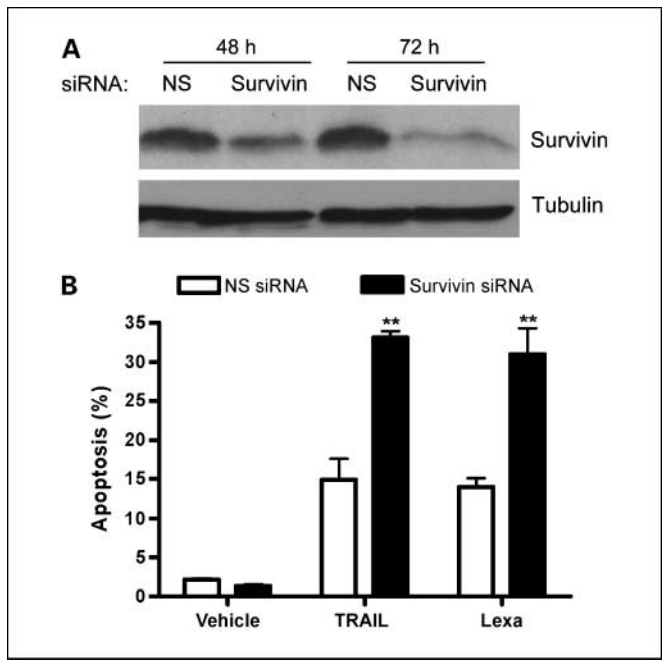

Fig. 5. Silencing survivin promotes TRAIL-induced apoptosis *in vitro. A,* MDA-MB-435 cells were transiently transfected with siRNAs targeting human survivin or a nonsilencing (*NS*) siRNA. Survivin levels were determined by immunoblotting at 48 and 72 h after transfection. *B,* MDA-MB-435 cells were transfected as in (*A*). Seventy-two hours later, cells were treated with vehicle, 2.5 μg/mL of TRAIL, or 2.5 μg/mL of lexatumumab (*Lexa*) for 16 h, and apoptotic nuclei were scored (*columns,* mean; *bars,* SE; $n = 3$). **, $P < 0.01$ vs. vehicle-treated cells.

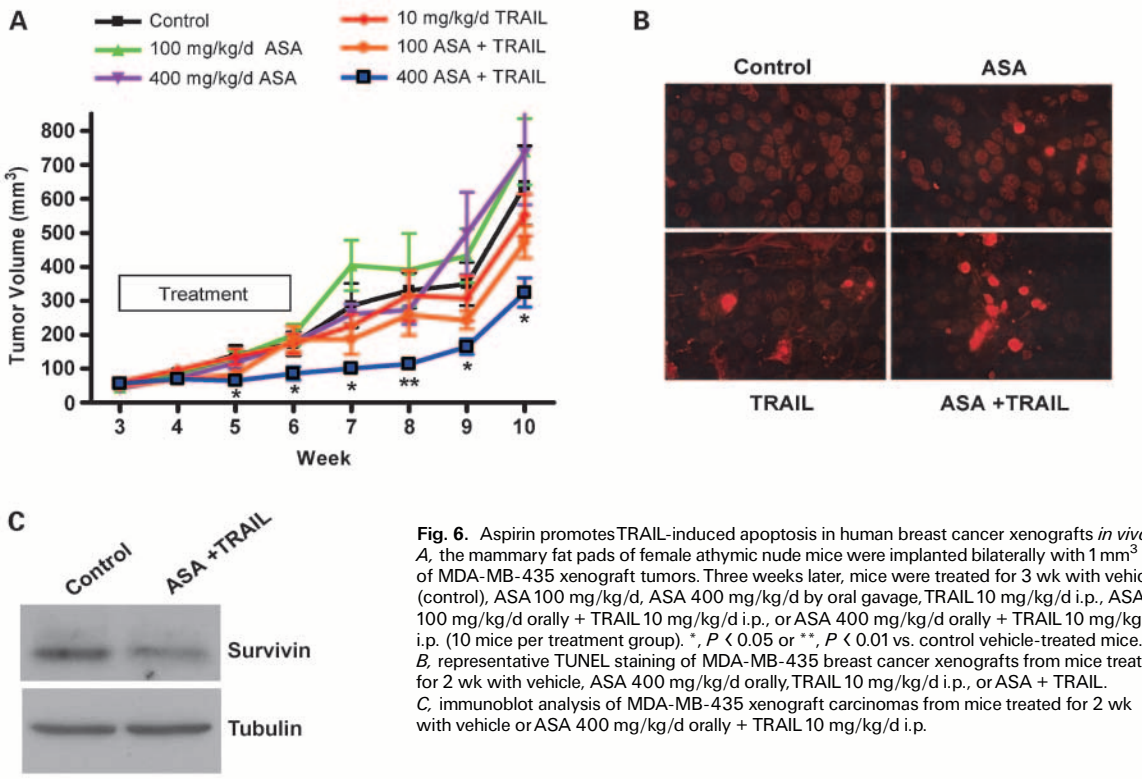

Fig. 6. Aspirin promotes TRAIL-induced apoptosis in human breast cancer xenografts *in vivo*. *A,* the mammary fat pads of female athymic nude mice were implanted bilaterally with 1 mm^3 pieces of MDA-MB-435 xenograft tumors. Three weeks later, mice were treated for 3 wk with vehicle (control), ASA 100 mg/kg/d, ASA 400 mg/kg/d by oral gavage, TRAIL 10 mg/kg/d i.p., ASA 100 mg/kg/d orally + TRAIL 10 mg/kg/d i.p., or ASA 400 mg/kg/d orally + TRAIL 10 mg/kg/d i.p. (10 mice per treatment group). *, $P < 0.05$ or **, $P < 0.01$ vs. control vehicle-treated mice. *B,* representative TUNEL staining of MDA-MB-435 breast cancer xenografts from mice treated for 2 wk with vehicle, ASA 400 mg/kg/d orally, TRAIL 10 mg/kg/d i.p., or ASA + TRAIL. *C,* immunoblot analysis of MDA-MB-435 xenograft carcinomas from mice treated for 2 wk with vehicle or ASA 400 mg/kg/d orally + TRAIL 10 mg/kg/d i.p.

the intrinsic apoptotic pathway plays an essential role in TRAIL-induced apoptosis in some cell types (6, 7), the inhibition of this pathway by survivin likely renders these cells resistant to TRAIL. Taken together, our findings indicate that the TRAIL-sensitizing effects of ASA are mediated at least in part by the proteasomal degradation of survivin.

As noted, Kim et al. have shown that ASA (0.1-10 mmol/L) promotes TRAIL-induced apoptosis in prostate and colon cancer cells *in vitro* by repressing *Bcl-2* gene expression by a COX-2–independent mechanism (35). However, we did not observe significant changes in the expression of any Bcl-2 family member, including Bcl-2, after treating breast cancer cells with 5 mmol/L of ASA for 64 hours, whereas Kim et al. did not examine survivin levels. This disparity may reflect differences in the duration of ASA treatment (64 hours versus 20-24 hours in the Kim et al. study) or in the cell types examined. Intriguingly, a very recent publication from the same group also implicated survivin depletion in TRAIL-sensitization by ASA in prostate cancer cells, although ASA repressed survivin expression by a transcriptional mechanism (49). It is entirely plausible that the TRAIL-sensitizing actions of ASA may reflect the effects of ASA on the expression of multiple apoptosis regulators, including survivin, Bcl-2, and potentially others. In agreement with Kim et al., our findings suggest that the TRAIL-sensitizing actions of ASA are independent of COX-2 inhibition because ASA promotes TRAIL-induced apoptosis in COX-2–deficient HCT116 colon cancer cells. Indeed, Kim et al. showed that silencing COX-2 had no effect on TRAIL-sensitization by ASA (35). Consistent with these findings, the TRAIL-sensitizing actions of COX-2 inhibitors have been reported to be an off-target effect of these drugs that is independent of COX-2 inhibition (50).

In summary, this report provides the first *in vivo* proof of concept data demonstrating the efficacy of the combination of ASA and TRAIL to reduce tumor burden in mice that reflects ASA's ability to promote TRAIL-induced apoptosis *in vivo*. We also show that the TRAIL-sensitizing effects of ASA are tumor-specific, p53-independent, and mediated by the proteasome-dependent degradation of survivin, a key negative regulator of TRAIL-induced apoptosis. Indeed, several other TRAIL-sensitizing agents with different molecular targets, including cyclin-dependent kinase inhibitors, PPARγ ligands, and resveratrol promote TRAIL-induced apoptosis at least in part by reducing survivin levels (23, 25, 27), thereby underscoring the importance of overcoming the antiapoptotic blockade imposed by survivin. Finally, we have shown that ASA treatment or survivin silencing also sensitizes breast cancer cells to apoptosis induced by an agonistic antibody targeting TRAIL-R2 but not TRAIL-R1, suggesting that this fully human TRAIL-R2 mAb (lexatumumab) may be a viable therapeutic alternative to TRAIL in treating cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Kelley SK, Ashkenazi A. Targeting death receptors in cancer with Apo2L/TRAIL. Curr Opin Pharmacol 2004;4:333–9.
2. Scaffidi C, Fulda S, Srinivasan A, et al. Two CD95 (APO-1/Fas) signaling pathways. EMBO J 1998;17: 1675–87.
3. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998;94:491–501.
4. Wei MC, Zong WX, Cheng EH, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 2001;292:727–30.
5. Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES. Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell 1998;1:949–57.
6. Deng Y, Lin Y, Wu X. TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. Genes Dev 2002;16:33–45.
7. LeBlanc H, Lawrence D, Varfolomeev E, et al. Tumor-cell resistance to death receptor-induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. Nat Med 2002;8:274–81.
8. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 1999;104:155–62.
9. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. Nat Med 1999;5:157–63.
10. Thai le M, Labrinidis A, Hay S, et al. Apo2l/Tumor necrosis factor-related apoptosis-inducing ligand prevents breast cancer-induced bone destruction in a mouse model. Cancer Res 2006;66:5363–70.
11. Ichikawa K, Liu W, Zhao L, et al. Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. Nat Med 2001;7: 954–60.
12. Buchsbaum DJ, Zhou T, Grizzle WE, et al. Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or a radiation therapy in a human breast cancer model. Clin Cancer Res 2003;9:3731–41.
13. Georgakis GV, Li Y, Humphreys R, et al. Activity of selective fully human agonistic antibodies to the TRAIL death receptors TRAIL-R1 and TRAIL-R2 in primary and cultured lymphoma cells: induction of apoptosis and enhancement of doxorubicin- and bortezomib-induced cell death. Br J Haematol 2005; 130:501–10.
14. Pukac L, Kanakaraj P, Humphreys R, et al. HGS-ETR1, a fully human TRAIL-receptor 1 monoclonal antibody, induces cell death in multiple tumour types *in vitro* and *in vivo*. Br J Cancer 2005;92:1430–41.
15. Marini P, Denzinger S, Schiller D, et al. Combined treatment of colorectal tumours with agonistic TRAIL receptor antibodies HGS-ETR1 and HGS-ETR2 and radiotherapy: enhanced effects *in vitro* and dose-dependent growth delay *in vivo*. Oncogene 2006;25: 5145–54.
16. Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Res 1999;59: 734–41.
17. Pai SI, Wu GS, Ozoren N, et al. Rare loss-of-function mutation of a death receptor gene in head and neck cancer. Cancer Res 1998;58:3513–18.
18. Fisher MJ, Virmani AK, Wu L, et al. Nucleotide substitution in the ectodomain of trail receptor DR4 is associated with lung cancer and head and neck cancer. Clin Cancer Res 2001;7:1688–97.
19. Teitz T, Wei T, Valentine MB, et al. Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. Nat Med 2000;6:529–35.
20. Burns TF, El-Deiry WS. Identification of inhibitors of TRAIL-induced death (ITIDs) in the TRAIL-sensitive colon carcinoma cell line SW480 using a genetic approach. J Biol Chem 2001;276:37879–86.
21. Fulda S, Meyer E, Debatin KM. Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression. Oncogene 2002;21:2283–94.
22. Clohessy JG. Zhuang J, de Boer J, Gil-Gomez G, Brady HJ. Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c release and its role in receptor-mediated apoptosis. J Biol Chem 2006;281:5750–9.
23. Lu M, Kwan T, Yu C, et al. Peroxisome proliferator-activated receptor γ agonists promote TRAIL-induced apoptosis by reducing survivin levels via cyclin D3 repression and cell cycle arrest. J Biol Chem 2005;280: 6742–51.
24. Hyer ML, Croxton R, Krajewska M, et al. Synthetic triterpenoids cooperate with tumor necrosis factor-related apoptosis-inducing ligand to induce apoptosis of breast cancer cells. Cancer Res 2005;65:4799–808.
25. Fulda S, Debatin KM. Sensitization for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by the chemopreventive agent resveratrol. Cancer Res 2004;64:337–46.
26. Nebbioso A, Clarke N, Voltz E, et al. Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells. Nat Med 2005;11:77–84.
27. Kim EH, Kim SU, Shin DY, Choi KS. Roscovitine sensitizes glioma cells to TRAIL-mediated apoptosis by downregulation of survivin and XIAP. Oncogene 2004;23:446–56.
28. Baron JA, Cole BF, Sandler RS, et al. A randomized trial of aspirin to prevent colorectal adenomas. N Engl J Med 2003;348:891–99.
29. Chan AT, Giovannucci EL, Meyerhardt JA, Schernhammer ES, Curhan GC, Fuchs CS. Long-term use of aspirin and nonsteroidal anti-inflammatory drugs and risk of colorectal cancer. JAMA 2005;294:914–23.
30. Harris RE, Chlebowski RT, Jackson RD, et al. Breast cancer and nonsteroidal anti-inflammatory drugs: prospective results from the Women's Health Initiative. Cancer Res 2003;63:6096–101.
31. Terry MB, Gammon MD, Zhang FF, et al. Association of frequency and duration of aspirin use and hormone receptor status with breast cancer risk. JAMA 2004; 291:2433–40.
32. Markowitz SD. Aspirin and colon cancer-targeting prevention? N Engl J Med 2007;356:2195–8.
33. Elder D J, Hague A, Hicks DJ, Paraskeva C. Differential growth inhibition by the aspirin metabolite salicylate in human colorectal tumor cell lines: enhanced apoptosis in carcinoma and *in vitro*-transformed adenoma relative to adenoma relative to adenoma cell lines. Cancer Res 1996;56:2273–6.
34. Zimmermann KC, Waterhouse NJ, Goldstein JC, Schuler M, Green DR. Aspirin induces apoptosis through release of cytochrome c from mitochondria. Neoplasia 2006;2:505–13.
35. Kim KM, Song JJ, An JY, Kwon YT, Lee YJ. Pretreatment of acetylsalicylic acid promotes tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by down-regulating BCL-2 gene expression. J Biol Chem 2005;280:41047–56.
36. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nature Med 1997;3:917–21.
37. Sellappan S, Grijalva R, Zhou X, et al. Lineage infidelity of MDA-MB-435 cells: expression of melanocyte proteins in a breast cancer cell line. Cancer Res 2004;64:3479–85.
38. Kamradt MC, Lu M, Werner ME, et al. The small heat shock protein αβ-crystallin is a novel inhibitor of TRAIL-induced apoptosis that suppresses the activation of caspase-3. J Biol Chem 2005;280:11059–66.
39. Kamradt MC, Chen F, Cryns VL. The small heat shock protein αβ-crystallin negatively regulates cytochrome c- and caspase-8-dependent activation of caspase-3 by inhibiting its autoproteolytic maturation. J Biol Chem 2001;276:16059–63.
40. Moyano JV, Evans JR, Chen F, et al. αβ-Crystallin is a novel oncoprotein that predicts poor clinical outcome in breast cancer. J Clin Invest 2006;116: 261–70.
41. Meng L, Mohan R, Kwok BH, Elofsson M, Sin N, Crews CM. Epoxomicin, a potent and selective proteasome inhibitor, exhibits *in vivo* antiinflammatory activity. Proc Natl Acad Sci U S A 1999;96:10403–8.
42. Runnebaum IB, Nagarajan M, Bowman M, Soto D, Sukumar S. Mutations in p53 as potential molecular markers for human breast cancer. Proc Natl Acad Sci U S A 1991;88:10657–61.
43. Carvajal D, Tovar C, Yang H, Vu BT, Heimbrook DC, Vassilev LT. Activation of p53 by MDM2 antagonists can protect proliferating cells from mitotic inhibitors. Cancer Res 2005;65:1918–24.
44. Sansom OJ, Stark LA, Dunlop MG, Clarke AR. Suppression of intestinal and mammary neoplasia by lifetime administration of aspirin in Apc(Min/+) and Apc(Min/+), Msh2(-/-) mice. Cancer Res 2001;61: 7060–64.
45. Zhao J, Tenev T, Martins LM, Downward J, Lemoine NR. The ubiquitin-proteasome pathway regulates survivin degradation in a cell cycle-dependent manner. J Cell Sci 2000;113:4363–71.
46. Song Z, Yao X, Wu M. Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis. J Biol Chem 2003;278:23130–40.
47. Marusawa H, Matsuzawa S, Welsh K, et al. C. HBXIP functions as a cofactor of survivin in apoptosis suppression. EMBO J 2003;22:2729–40.
48. Dohi T, Okada K, Xia F, et al. An IAP-IAP complex inhibits apoptosis. J Biol Chem 2004;279: 34087–90.
49. Yoo J, Lee YJ. Aspirin enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in hormone-refractory prostate cancer cells through survivin down-regulation. Mol Pharmacol 2007;72:1586–92.
50. Totzke G, Schulze-Osthoff K, Janicke RU. Cyclooxygenase-2 (COX-2) inhibitors sensitize tumor cells specifically to death receptor-induced apoptosis independently of COX-2 inhibition. Oncogene 2003;22: 8021–30.

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Overexpression of CEACAM6 promotes migration and invasion of oestrogen-deprived breast cancer cells

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ABSTRACT

Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is an intercellular adhesion molecule that is overexpressed in a wide variety of human cancers, including colon, breast and lung and is associated with tumourigenesis, tumour cell adhesion, invasion and metastasis. In this study, we showed that CEACAM6 was overexpressed in a panel of oestrogen receptor (ERα)-positive human breast cancer cell lines (MCF-7:5C and MCF-7:2A) that have acquired resistance to oestrogen deprivation, and this overexpression was associated with a more aggressive invasive phenotype *in vitro*. Expression array analysis revealed that MCF-7:5C and MCF-7:2A cells overexpressed CEACAM6 mRNA by 27-fold and 12-fold, respectively, and were 6–15-times more invasive compared to non-invasive wild-type MCF-7 cells which expressed low levels of CEACAM6. Suppression of CEACAM6 expression using small interfering RNA (siRNA) completely reversed migration and invasion of MCF-7:5C and MCF-7:2A cells and it significantly reduced phosphorylated Akt and c-Src expression in these cells. In conclusion, our findings establish CEACAM6 as a unique mediator of migration and invasion of drug resistant oestrogen-deprived breast cancer cells and suggest that this protein could be an important biomarker of metastasis.

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1. Introduction

Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is a glycosylphosphatidylinositol-anchored cell surface protein that functions as a homotypic intercellular adhesion molecule.[1] It is overexpressed in a number of human malignancies including pancreatic cancer, gastrointestinal cancer and breast cancers[2,3], and increased levels of CEACAM6 are inversely correlated to the differentiation state of cancer cells. Previous studies have shown that CEACAM6 is overexpressed in pancreatic adenocarcinoma cells, and its overexpression is associated with greater *in vivo* metastatic ability and increased invasiveness and migration.[4,5] More recently, Poola and co-workers[6] reported that the expression of CEACAM6 in atypical ductal hyperplasia was associated with the development of invasive breast cancer (IBC). Currently, however, the role of CEACAM6 overexpression in breast cancer migration and invasion is not known.

Invasion and metastasis are the hallmarks of cancer malignancy, and they are the primary cause of patient mortality during breast cancer progression.[7] Invasion refers to the ability of cancer cells to penetrate through the membranes that separate them from healthy tissues and blood vessels, and metastasis refers to the spreading of cancer cells to other parts of the body.[8] In order for a transformed cell to metastasize, it must first lose adhesion, penetrate and invade the surrounding extracellular matrix (ECM), enter the vascular system and adhere to distant organs.[8] These processes require extensive alterations in gene expression profiles,

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including the down-regulation of genes involved in cell anchorage and the up-regulation of genes involved in cell motility and matrix degradation.[7,9,10]

Aromatase inhibitors (AIs) are anti-oestrogen agents that suppress oestrogen production in peripheral tissues and breast tumours by inhibiting or inactivating aromatase, the enzyme which catalyses the conversion of androgens to oestrogens in post-menopausal women.[11] Several randomized trials[12–15] have shown that third generation AIs are superior to adjuvant tamoxifen in terms of improved disease-free survival and less side-effects. Unfortunately, one of the consequences of prolonged oestrogen deprivation/suppression is the development of drug resistance.[16,17] Previous studies have shown that acquisition of tamoxifen resistance in breast cancer cells is associated with a significant increase in motility and invasion[18,19] along with increased CEACAM6 expression[20]; however, it is unknown whether acquired resistance to oestrogen deprivation affects tumour cell migration and invasion and whether CEACAM6 plays a role in this process.

In this study, we investigated the role of CEACAM6 in cellular migration and invasion of breast cancer cells that have acquired resistance to oestrogen deprivation. We found that CEACAM6 was significantly overexpressed in oestrogen-deprived MCF-7:5C and MCF-7:2A breast cancer cells and that these cells were markedly more migratory and invasive than parental MCF-7 cells. Suppression of CEACAM6 expression by small interfering RNA (siRNA) completely reversed the invasive phenotype of MCF-7:5C and MCF-7:2A cells. E-cadherin and β-catenin were also significantly reduced in these cells. The mechanism of action of CEACAM6 appears to involve, in part, the c-Src and Akt signalling pathways.

2. Materials and methods

2.1. Reagents

17 Beta-oestradiol was purchased from Sigma Chemical Co. (St Louis, MO); PP2 was purchased from EMD Biosciences Inc. (La Jolla, CA); LY294002 was purchased from Promega (Madison, WI); fulvestrant was obtained as a generous gift from AstraZeneca (Macclesfield, United Kingdom); Affymetrix Human Genome U133 Plus 2.0 Arrays were purchased from Affymetrix (Santa Clara, CA); foetal bovine serum (FBS), cell culture medium and other reagents were purchased from Invitrogen (Carlsbad, CA).

2.2. Cell lines and culture conditions

Wild-type MCF-7 human breast cancer cells[21] were obtained from Dr. Dean Edwards (University of Texas, San Antonio, TX) and were maintained in fully oestrogenized medium (RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1× non-essential amino acids and bovine insulin at 6 ng/mL (Sigma–Aldrich, St. Louis, MO). MCF-7:5C[21–23] and MCF-7:2A[24] cells were clonally selected from parental MCF-7 cells following long-term culture (>1 year) in phenol red-free RPMI 1640 media containing 10% dextran-coated charcoal stripped FBS (SFS).

2.3. RNA preparation and microarray hybridisation

Total RNA was prepared using the Qiagen RNeasy Mini kit. A DNase I digestion step was included to eliminate DNA contamination. cRNA was generated, labelled, and hybridised to the Affymetrix Human Genome U133 Plus 2.0 Arrays by the Northwestern University Genomics Core (Chicago, IL). Arrays were washed, stained and scanned according to the directions detailed in the Affymetrix GeneChip® Expression Analysis Technical Manual.

2.4. Microarray data analysis

Assessment of data quality was conducted following default guidelines in the Affymetrixs GeneChip® Expression Analysis Data Analysis Fundamentals Training Manual. Data were extracted and normalised using *Affymetrix Microarray Suite* (MAS5.0) following recommended protocols for background and chip-correction. Global scaling for average signal intensity for all arrays was set to 500. Four biological replicates from each of the three cell lines were arrayed to determine consistent and reproducible patterns of gene expression. All but one array showed a high degree of reproducibility within a set of replicate hybridisations, leaving at least three array replicates per cell line for further analysis. Genes across all arrays with an expression intensity <70 were removed. To eliminate genes with variable expression within a group of replicates, normalised gene intensity ratios (signal intensities divided by the median gene intensity all hybridisations) were derived, then the standard deviation of the log-transformed normalised intensity ratios were calculated for each group of replicates. Genes with a standard deviation >0.15 were excluded. Lastly, to filter for genes with variable expression between cell lines, genes were retained that showed a standard deviation of >0.3. A total of 904 genes met the filtering criteria described and were examined by hierarchical clustering using resources available at TGen.[c] Uncentred Pearson's correlation with average linkage was used on $\log_2$-transformed data, with induced genes indicated in red and repressed genes in green. Random permutation analysis was performed as previously described [25] using 10,000 permutations. Genes with a p-value <0.01 and an alpha value <0.01 were used for gene ontology analysis.

2.5. Cell proliferation assay

Cell proliferation assay was performed as previously described.[22] The DNA content of the cells was determined using a Fluorescent DNA Quantitation kit (Bio-Rad Laboratories, Hercules, CA). For each analysis, three replicate wells were used, and at least three independent experiments were performed.

2.6. Western blot analysis

Western blot analyses were performed as previously described.[22] Separated proteins were transferred onto nitrocel-

[c] Internet address: http://biodiscovery.tgen.org/microarray/.

lulose membranes (Milllipore) and incubated overnight at 4 °C with the respective primary antibodies; CEACAM6 and CEACAM5 (Signet Laboratories, Dedham, MA); ERα, N-cadherin, β-catenin, CXCR4, MMP9, E-cadherin and CD44 (Santa Cruz Biotechnology, Santa Cruz, CA); fibronectin (Chemicon International, Temecula, CA); c-Src and p-Src^{Tyr529} (Biosource International, Carmarillo, CA); AKT and p-AKT^{Ser473} (Cell Signaling Technology, Beverly, MA); and β-actin (Sigma Chemical Co., St Louis, MO). Secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) were used with an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL) to visualise the resolved proteins.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR) for ERα and CEACAM6

Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Ten micrograms of total RNA for each sample were converted to first-strand cDNA using SuperScript III with a combination of random hexamers and oligo(dT) as primers (Invitrogen). Quantitative real-time PCR assays were done as previously described[22] with the Taqman Universal or SYBR Green PCR Master Mixes and an ABI 7700 sequence detection system (PE Applied Biosystems, Foster City, CA). The ERα forward and reverse primers were 5′-AAGAGGGTGCCAGGCTTTGT-3′ and 5′-CAGGATCTCTAGCCAGGCAC AT-3′, respectively. The *ERα* probe was 5′-[FAM]-ATTTGACCCTCCATGATCAGGTCC ACC-[TAMRA]-3′. The forward and reverse primers for *CEACAM6* were synthesised by Sigma Genosys (Sigma–Aldrich). The sequences for *CEACAM6* forward and reverse primers were 5′-GACGTTTGTGTGGATTGCTGGAACGC-3′ and 5′-TGCCACGCAGCCTCTAACC-3′, respectively. The reporter dye at the 5′-end of each probe was FAM and the quencher dye at the 3′-end was TAMRA. The 18S ribosomal RNA (*18S rRNA*) gene was used as an endogenous control to normalise for differences in the amount of total RNA in each sample, *18S rRNA* primers and probes were purchased from Applied Biosystems. Relative expression of the target gene was calculated using the 2 delta CT method described previously[26] (Relative expression = $2^{-\Delta CT}$; where $\Delta CT = C_{T\ (\text{Target gene})} - C_{T\ (\text{endogenous control gene})}$), where *18S rRNA* is the endogenous control gene. To determine relative RNA levels within the samples, standard curves for the PCR were prepared by using cDNA from one sample and making twofold serial dilutions covering the range equivalent to 20–0.625 ng RNA (for 18S rRNA analyses, the range was 4–0.125 ng).

2.8. Cell migration and invasion assays

Cell migration was measured in a Boyden chamber using Transwell filters obtained from Corning (Cambridge, MA). Cells (1×10^5) in 0.5 mL serum-free medium were placed in the upper chamber, and the lower chamber was loaded with 0.8 mL medium containing 10% SFS. Cells that migrated to the lower surface of filters were stained with Wright Giemsa solution, and five fields of each well were counted after 24 or 48 h of incubation at 37 °C with 5% CO_2. Three wells were examined for each condition and cell type, and the experiments were repeated in triplicate. Cell invasion assay was performed using the Chemicon cell invasion kit (Chemicon International, Temecula, CA) in accordance with the manufacturer's protocol. Cells (1×10^5/ml) were seeded onto 12-well cell culture chamber using inserts with 8 μM pore size polycarbonate membrane over a thin layer of extracellular matrix. Following incubation of the plates for 48 h at 37 °C, cells that had invaded through the ECM layer and migrated to the lower surface of the membrane were stained and counted under the microscope in at least 10 different fields and photographed.

2.9. CEACAM6 siRNA-mediated gene knockdown

CEACAM6-specific siRNA (Silencer™ Predesigned siRNA; sense: GCCCUGGUGUAUUU UCAUtt, antisense: AUCGAAAAUACAC CAGGGCtg) (AM16704) and scramble sequence control siRNA (Silencer™ Negative Control siRNA) were purchased from Ambion (Austin, TX). Transfection complexes were prepared in Opti-MEM serum-free medium (Invitrogen) by mixing 0.3 μL of siPORT NeoFX transfection reagent (Ambion) and 10 nM CEACAM6 siRNA or negative control siRNA (Ambion). Cells (9×10^4 cells per well) were reverse-transfected in 12-well plates simultaneously with addition of transfection complexes. The medium was replaced with phenol red-free RPMI supplemented with 10% SFS 24 h after transfection and cultures were harvested for CEACAM6 protein and mRNA analyses.

2.10. Statistical analyses

Statistical analyses were performed using Microsoft Excel (Seattle, WA). Differences between groups were evaluated using Student's *t*-test. Data were considered significant if $p < 0.05$.

3. Results

3.1. Characterisation of long-term oestrogen-deprived breast cancer cells

The growth of oestrogen-deprived MCF-7:5C and MCF-7:2A cells is compared to parental MCF-7 cells in Fig. 1A. Both MCF-7:5C and MCF-7:2A cells grew robustly in the absence of oestrogen whereas MCF-7 cells grew minimally without oestrogen. The doubling times were 2.7, 3.4, and 6 d for MCF-7:5C, MCF-7:2A and MCF-7 cells, respectively. We also examined cell morphology changes associated with resistance to long term oestrogen deprivation using phase-contrast microscopy. Fig. 1B shows that MCF-7 cells grew as a uniform monolayer of tightly associated cells with limited cell spreading but distinct cellular boundaries, whereas oestrogen-deprived MCF-7:5C and MCF-7:2A cells grew in a less uniform monolayer with cellular boundaries that were obscured. ERα mRNA and protein expression were also significantly increased in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells and treatment with oestradiol or the pure anti-oestrogen fulvestrant significantly down-regulated its expression (Fig. 1C and D) in all three cell lines. Overall, these results show that oestrogen deprivation increases ERα expression and alters the morphology of MCF-7:5C and MCF-7:2A cells.

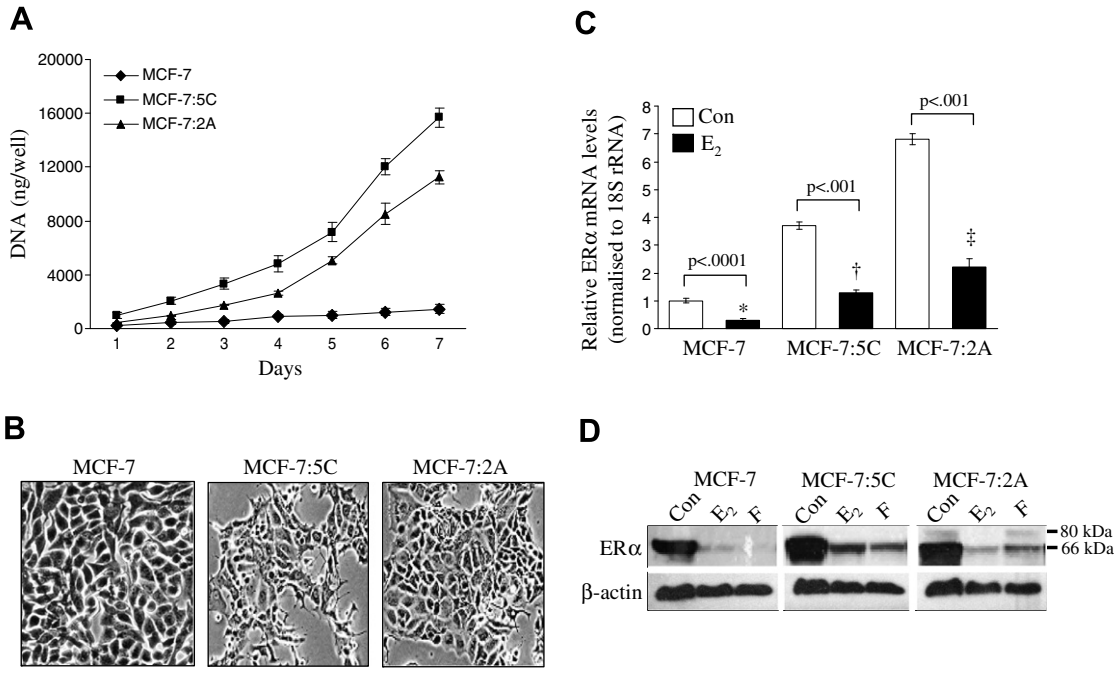

Fig. 1 – Characterisation of long-term oestrogen-deprived breast cancer cells. (A) For proliferation assays, cells were seeded in 24-well dishes (30,000 per well) in oestrogen-free RPMI media and total DNA was quantitated at the indicated time points. (B) Phase-contrast microscopy pictures of MCF-7, MCF-7:5C and MCF-7:2A cells. Images were produced by the Olympus DP-3030 camera and Olympus IX-70 software. Magnification, ×10. (C) ERα mRNA level was determined by quantitative RT-PCR. Relative expression of the target gene was calculated using the 2 delta CT method, where 18S rRNA was used as the endogenous control gene. All reactions were performed in triplicates and the error bar represents the standard deviation. (D) ERα protein levels were determined by immunoblotting with a specific ERα antibody. Cells were treated with 1 nM oestradiol or 1 μM fulvestrant for 48 h and 50 μg of protein lysates was analysed. β-Actin was used as a loading control.

3.2. *Global gene expression profiles of oestrogen-deprived breast cancer cells*

Transcriptional profiling of parental MCF-7 cells and oestrogen-deprived MCF-7:5C and MCF-7:2A cells was performed using Affymetrix Human Genome U133 Plus 2.0 Array. Two-dimensional hierarchical clustering was performed to analyse differences in gene expression patterns between MCF-7 cells and MCF-7:5C and MCF-7:2A cells. Data filtering identified 904 genes that were significantly altered between MCF-7:5C and MCF-7:2A cells and parental MCF-7 cells (Fig. 2A and Supplementary Fig. S1). The sample dendogram showed that MCF-7:2A cells and MCF-7 cells clustered more closely, whereas MCF-7:5C cells clustered on a more distant branch, suggesting that MCF-7:2A cells are more similar to parental MCF-7 cells than MCF-7:5C cells (Fig. 2A). In order to define cell signalling mechanisms that differed significantly between parental MCF-7 and MCF-7:5C and MCF-7:2A cells, random permutation weighted gene analysis was performed as described in Section 2. A comparison of MCF-7 expression data with that of MCF-7:5C and MCF-7:2A revealed that 4068 genes were highly differentially expressed (Supplementary Table 1). Gene Ontology analysis showed a significant number of genes associated with cell cycle control, proliferation, growth factor signalling, cell adhesion and motility and invasion. In particular, we found that CEACAM6 was overexpressed by 27-fold in MCF-7:5C cells and 12-fold in MCF-7:2A cells (Fig. 2B), and it was highly weighted in our random permutation analysis (p-value < .0001) (Supplementary Table 1).

3.3. *Oestrogen deprivation increases CEACAM6 expression and enhances migration and invasion of oestrogen-deprived breast cancer cells*

To confirm our microarray data, CEACAM6 mRNA expression was determined by quantitative RT-PCR. Fig. 3A shows that CEACAM6 mRNA was significantly upregulated in oestrogen-deprived MCF-7:5C and MCF-7:2A cells compared with parental MCF-7 cells. Similarly, by Western blotting, CEACAM6 protein was undetectable in MCF-7 cells but was strongly expressed in MCF-7:5C and MCF-7:2A cells (Fig. 3B). Other invasion proteins such as CEACAM5, MMP9, CXCR4 and CD44 were also markedly elevated in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells (Fig. 3B). This finding is consistent with a recent study by Mackay and coworkers[27] which revealed that many genes associated with extracellular matrix remodelling were significantly upregulated following aromatase inhibitor treatment of primary breast tumours.

3.4. *Oestrogen deprivation increases migration and invasion of breast cancer cells*

Since MCF-7:5C and MCF-7:2A cells overexpressed several invasion genes, we next assessed the migratory and invasive

potential of these cells *in vitro*. Cell migration was measured using a modified Boyden chamber assay with 10% SFS as a chemoattractant. As shown in Fig. 3C, MCF-7:5C and MCF-7:2A cells had the highest numbers of migrating cells compared to MCF-7 cells; a phenotype that correlated with CEACAM6 expression. Similar results were obtained when the different cell lines were tested for their ability to invade through membranes coated with Matrigel. Fig. 3D shows that MCF-7:5C and MCF-7:2A cells had the highest number of invading cells, while MCF-7 cells were non-invasive. The invasive ability of the cell lines was as follows: MCF-7:5C > MCF-7:2A > MCF-7.

3.5. *CEACAM6 suppression inhibits invasion and migration of MCF-7:5C cells*

To test the hypothesis that CEACAM6 is required for cell migration and invasion, we used siRNA to suppress CEACAM6 expression. MCF-7:5C cells were transfected with CEACAM6-specific or control (scrambled sequence) siRNA, and Western blot analysis was performed 72 h post-transfection. Fig. 4A (top) shows that CEACAM6 protein was significantly suppressed (75–85%) in MCF-7:5C cells transfected with the CEACAM6-specific siRNA but not the control siRNA. siRNA suppression of CEACAM6 expression was also confirmed at the transcript level using qRT-PCR at 48 h following transfection (Fig. 4A, bottom). To clarify the role of CEACAM6 in cell invasion, MCF-7:5C cells were pretreated with CEACAM6 siRNA or control siRNA for 48 h and invasion was measured over the subsequent 48 h. Fig. 4B shows that CEACAM6 siRNA almost completely reversed the invasiveness of MCF-7:5C cells, whereas control siRNA did not affect cell invasion. The invasiveness of MCF-7:5C cells was inhibited by nearly 80% when CEACAM6 expression was suppressed. A similar trend was observed for cell migration (data not shown). Suppression of CEACAM6 also significantly reduced phosphorylated Akt and phosphorylated c-Src in MCF-7:5C cells (Fig. 4C). E-cadherin and β-catenin were also significantly reduced in MCF-7:5C and MCF-7:2A cells, whereas pAkt and N-cadherin were significantly upregulated in these cells compared to parental MCF-7 cells (Fig. 4D). Similar experiments performed in MCF-7:2A cells also showed a dramatic reduction (60%) in invasion following CEACAM6 suppression (data not shown).

3.6. *Oestradiol down-regulates CEACAM6 expression and blocks migration and invasion of MCF-7:5C cells*

We also examined whether CEACAM6 expression is hormonally regulated in MCF-7:5C and MCF-7:2A cells. As shown in Fig. 5A and B, oestradiol completely down-regulated CEACAM6 mRNA and protein expression in MCF-7:5C and MCF-7:2A cells. This down-regulation was an ERα-mediated event since pretreatment with the anti-oestrogen fulvestrant, which is known to degrade ERα[28,29], was able to reverse the inhibitory effect of oestradiol on CEACAM6 protein in both cell lines (Fig. 5B). Fulvestrant also completely counteracted the anti-invasive effects of oestradiol in MCF-7:5C cells (Fig. 5C). Interestingly, oestradiol enhanced the invasiveness of parental MCF-7 cells (Fig. 5D) without significantly changing CEACAM6 protein level in these cells (Fig. 5B).

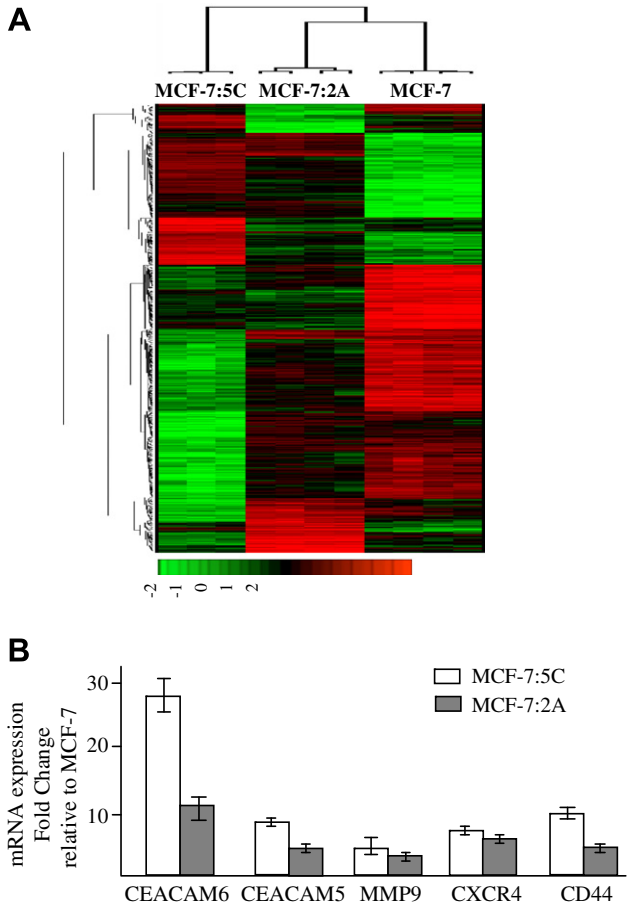

Fig. 2 – Overview of global gene expression patterns in wild-type MCF-7 cells and oestrogen-deprived MCF-7:5C and MCF-7:2A variant clones. (A) Unsupervised hierarchical clustering dendogram of 904 genes most differentially expressed across the three cell lines. Each row represents a single gene. Red, genes with high expression levels and green, genes with low expression levels. The similarities in the expression pattern amongst the three cell lines are presented as a "condition tree" on the top of the matrix. (B) Expression levels of invasion genes in MCF-7:5C and MCF-7:2A cells compared to parental MCF-7 cells, as identified by microarray analysis.

3.7. *Inhibition of c-Src reduces the invasiveness of MCF-7:5C and MCF-7:2A cells*

Previous studies have reported that CEACAM6 cross-linking initiates c-Src-dependent cross-talk between CEACAM6 and αvβ3 integrin, leading to increased ECM-adhesion and invasion.[30] We therefore determined c-Src kinase activity in oestrogen-deprived MCF-7:2A and MCF-7:5C cells by measuring phosphorylation of c-Src at Tyr^{529}. Both MCF-7:5C and MCF-7:2A cells showed significantly elevated levels of phosphorylated c-Src^{Y529} compared to parental MCF-7 cells, and treatment with the c-Src kinase inhibitor PP2 significantly reduced the invasiveness of MCF-7:5C and MCF-7:2A cells (Supplementary Fig. S2). Inhibition of Akt phosphorylation using the PI3K inhibitor LY294002 also significantly reduced cell growth and invasion of these cells (Supplementary

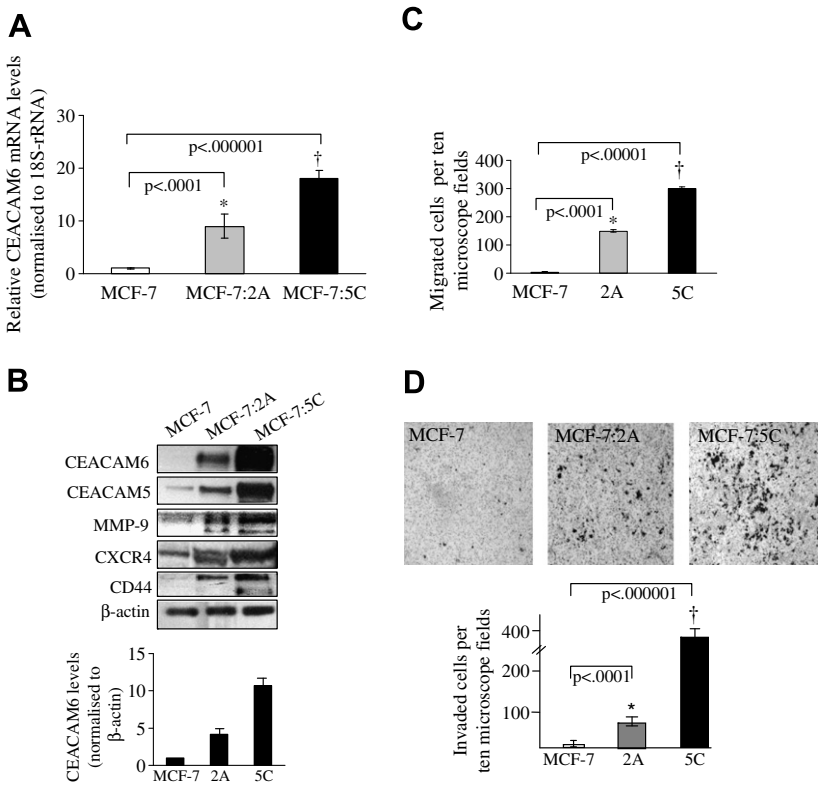

Fig. 3 – CEACAM6 promotes cell migration and invasion of oestrogen-deprived breast cancer cells. (A) CEACAM6 mRNA levels in parental MCF-7 cells and oestrogen-deprived MCF-7:5C and MCF-7:2A cells were measured by qRT-PCR. Relative expression of the target gene was calculated using the 2 delta CT method, where 18S rRNA was used as the endogenous control gene. All reactions were performed in triplicates, and the error bar represents the standard deviation. (B) Western blot analysis of CEACAM6 and other invasion proteins in MCF-7, MCF-7:5C and MCF-7:2A cells. The relative ratio of CEACAM6 was calculated by densitometry (bottom). The *bar graph* (bottom) depicts the averages of the data obtained from three individual experiments, and data are expressed as means ± SE. (C) Quantification of cells migrating across Transwell filters. (D) Cells that invaded through the Matrigel-coated transwells were fixed, stained, visualised at 20× magnification by light microscopy and photographed. Each panel represents an example of three replicates. Ten random fields were counted per insert at 20×.

Fig. S2), thus suggesting an important role for the c-Src and Akt signalling pathways in invasion.

4. Discussion

Despite advances in detection and treatment of metastatic breast cancer, mortality from this disease remains high because current therapies are limited by the emergence of therapy-resistant cancer cells. In this study, we showed that oestrogen deprivation significantly increased the motility and invasiveness of two ERα-positive human breast cancer cell lines that have acquired resistance to oestrogen deprivation, and that these cells overexpressed the invasive gene CEACAM6. Furthermore, knockdown of CEACAM6 expression completely inhibited the invasiveness of MCF-7:5C and MCF-7:2A cells and caused a reduction in phosphorylated c-Src and pAkt expression. A significant reduction in E-cadherin and β-catenin was also observed in MCF-7:5C and MCF-7:2A cells compared to parental MCF-7 cells. To our knowledge, this study is the first to demonstrate a critical role for CEACAM6 in migration and invasion of breast cancer cells that have acquired resistance to oestrogen deprivation.

Previous studies have reported that overexpression of CEACAM6 in pancreatic adenocarcinoma cells is associated with enhanced cellular invasiveness and increased metastatic potential *in vivo*, and that this effect is completely attenuated by suppression of CEACAM6 expression.[4] Recently, Scott and coworkers[20] reported that CEACAM6 was upregulated by 20-fold in tamoxifen-resistant MCF-7 cells compared to tamoxifen-sensitive cells, and that hormone sensitivity could be partially restored in the tamoxifen-resistant cells by siRNA silencing of CEACAM6. This *in vitro* data were substantiated in clinical breast cancer where it was demonstrated that CEACAM6 was overexpressed in primary breast tumours that subsequently relapsed following adjuvant tamoxifen and in a multivariate analysis, only CEACAM6 remained a significant predictor of recurrence.[31] These findings are consistent with our present study which shows that CEACAM6 is significantly upregulated in oestrogen-deprived breast cancer cells that have acquired resistance to oestrogen suppression, and knockdown of CEACAM6 expression reverses the invasive phenotype of these cells. The fact that CEACAM6 is identified independently in two model systems using endocrine agents with distinct modes of action suggests that it may play an important role in endocrine

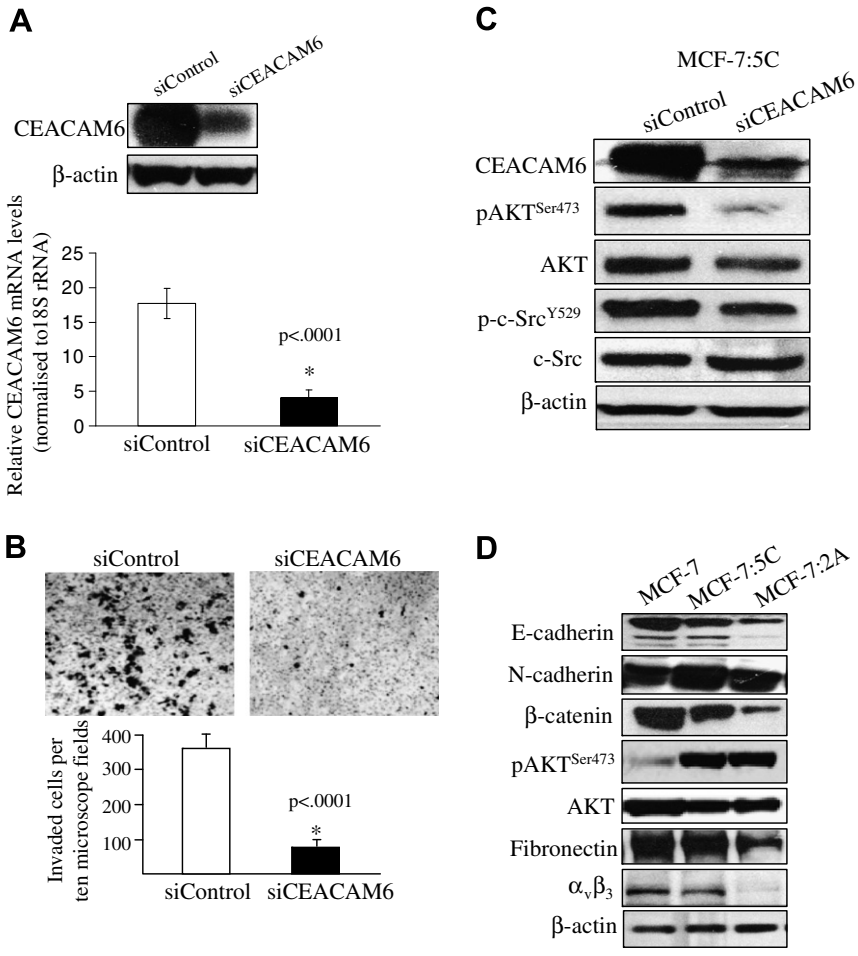

Fig. 4 – CEACAM6 suppression completely blocks invasion of MCF-7:5C breast cancer cells. (A) siRNA-mediated gene knockdown of CEACAM6 was verified by Western blot (top panel) and qRT-PCR (bottom panel). For qRT-PCR experiments, relative expression of CEACAM6 gene was calculated using the 2 delta CT method, where 18S rRNA was used as the endogenous control gene. All reactions were performed in triplicates and the error bar represents the standard deviation. (B) Matrigel invasion assay of siControl and siCEACAM6-transfected MCF-7:5C cells. (C) Immunoblot analysis of MCF-7:5C cells transfected with CEACAM6 siRNA or control siRNA for 72 h. β-Actin was used as a loading control. (D) Western blot analyses of E-cadherin, β-catenin, N-cadherin, Akt and pAKT protein expression in MCF-7, MCF-7:5C and MCF-7:2A cells.

resistance. Currently, the mechanism by which CEACAM6 facilitates invasion is not fully understood. However, there is evidence that CEACAM6, along with other GPI-anchored proteins, is capable of modulating the activity of intracellular tyrosine kinases such as c-Src.[32,33] In particular, studies by Duxbury and coworkers[30,34] showed that c-Src activity was increased in CEACAM6-overexpressing BxPC3 human pancreatic cancer cells and decreased following suppression of CEACAM6 expression, and that inhibition of c-Src activity significantly suppressed CEACAM6-mediated cellular invasiveness. We found that phosphorylated c-Src was significantly elevated in MCF-7:5C and MCF-7:2A cells, and that suppression of CEACAM6 expression reduced its level in these cells. Pharmacological blockade of c-Src using the Src tyrosine kinase inhibitor pyrazolopyrimidine (PP2) also inhibited the invasiveness of MCF-7:5C and MCF-7:2A cells. In addition, we found markedly elevated levels of phosphorylated Akt^{ser473} in MCF-7:5C and MCF-7:2A cells, which were dramatically reduced following CEACAM6 suppression. Akt is a serine/threonine protein kinase that mediates cell survival, proliferation[35,36], tumour cell migration and invasion and metastasis,[37] and previous studies have shown that c-Src activates the PI3K/Akt signalling pathway.[38] Thus, it is possible that activation of both c-Src and Akt might play a role in mediating CEACAM6-induced migration and invasion.

The epithelial-to-mesenchymal transition (EMT) plays a key role in metastasis and is characterised by the conversion of epithelial cancer cells to a more motile phenotype that facilitates invasion. A critical molecular feature of EMT is the down-regulation of E-cadherin,[39] a cell adhesion molecule present in the plasma membrane of most normal epithelial cells. E-cadherin acts *de facto* as a tumour suppressor inhibiting invasion and metastasis and is frequently repressed or degraded during transformation. In our study, E-cadherin and β-catenin were significantly decreased, whereas N-cadherin was markedly increased in invasive MCF-7:5C and MCF-7:2A cells compared to non-invasive MCF-7 cells. In addition, our cell morphology studies showed EMT-like changes in

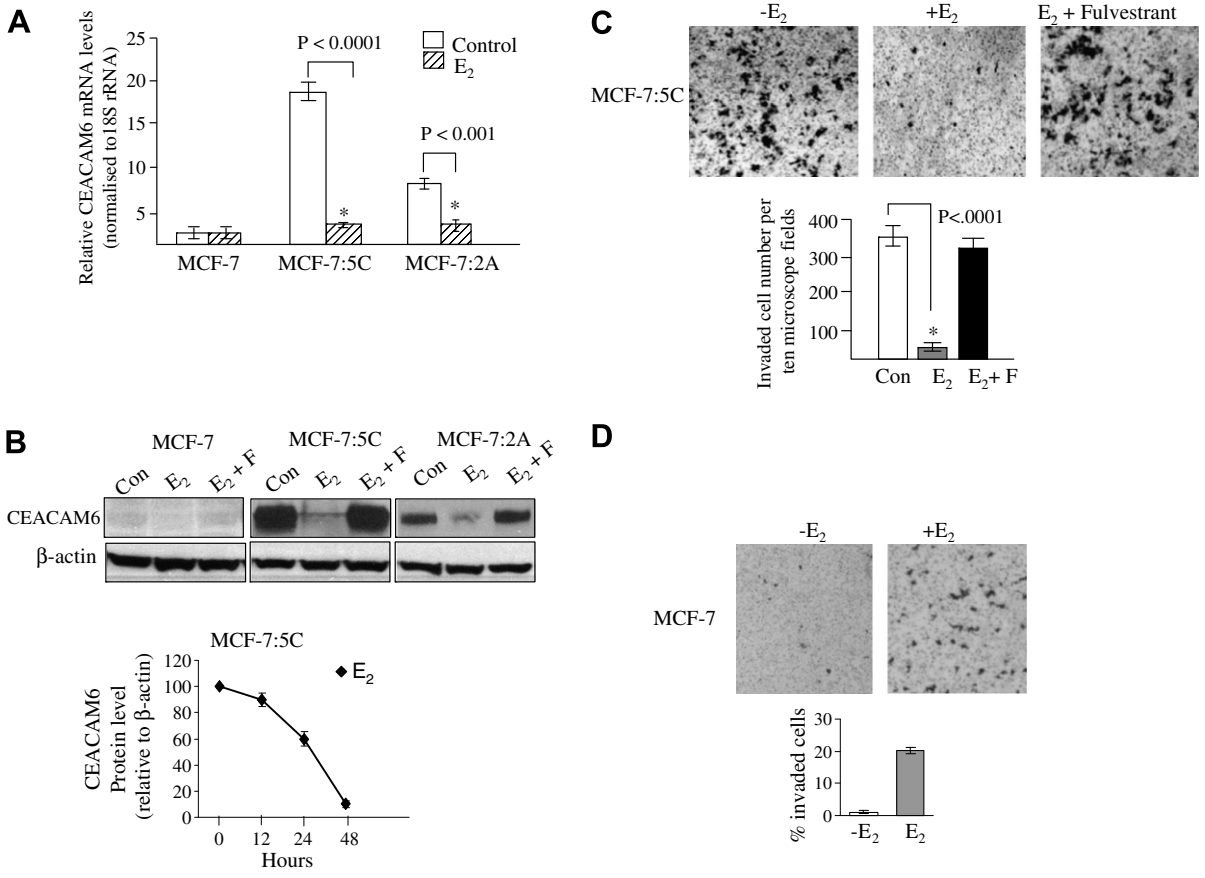

Fig. 5 – 17β-Oestradiol suppresses CEACAM6 expression and blocks invasion of oestrogen-deprived breast cancer cells. (A) Quantitative RT-PCR analyses of CEACAM6 mRNA expression in MCF-7:5C and MCF-7:2A cells following treatment with 1 nM oestradiol (E_2) for 48 h. Expression levels were internally normalised to the housekeeping gene 18S rRNA (error bars, SE) (B) Western blot analysis of CEACAM6 protein expression in MCF-7, MCF-7:2A and MCF-7:5C cells. *Line graph* shows the time-dependent effect of E_2 on CEACAM6 protein level in MCF-7:5C cells. (C) Invasion of MCF-7:5C cells is blocked by E_2 but not the pure anti-oestrogen fulvestrant. Invasion assay was performed as previously described in Fig. 3. (D) Effect of oestradiol on the invasiveness of wild-type MCF-7 cells. Each panel represents an example of three replicates.

MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells. A variety of signal transduction pathways impinge on the regulation of E-cadherin levels or subcellular distribution. In particular, Akt/PKB has been shown to repress transcription of the E-cadherin gene, which leads to conversion of epithelial cells into invasive mesenchymal cells.[40] We have found that MCF-7:5C and MCF-7:2A both cells overexpress phosphorylated Akt, and gene ontology analysis of expression data obtained for MCF-7:5C and MCF-7:2A cells reveals that the P13K/Akt signalling pathway is significantly ($p = 0.002$) altered compared to parental MCF-7 cells.

In conclusion, we have identified CEACAM6 as a critical gene in the regulation of migration and invasion of breast cancer cells that have acquired resistance to oestrogen deprivation. Since aromatase inhibitors are now considered the standard of care for the hormonal treatment of early breast cancer in postmenopausal women, this finding has important clinical implications for these patients because it suggests that extended use of aromatase inhibitors may potentially lead to the development of metastatic disease. CEACAM6 can thus serve as a powerful predictor of future recurrence and may also represent a promising new therapeutic target for breast cancer.

Conflict of interest statement

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2008.05.016.

REFERENCES

1. Kuroki M, Matsuo Y, Kinugasa T, Matsuoka Y. Three different NCA species, CGM6/CD67, NCA-95, and NCA-90, are comprised in the major 90 to 100-kDa band of granulocyte NCA detectable upon SDS–polyacrylamide gel electrophoresis. *Biochem Biophys Res Commun* 1992;**182**:501–6.
2. Scholzel S, Zimmermann W, Schwarzkopf G, Grunert F, Rogaczewski B, Thompson J. Carcinoembryonic antigen family members CEACAM6 and CEACAM7 are differentially expressed in normal tissues and oppositely deregulated in hyperplastic colorectal polyps and early adenomas. *Am J Pathol* 2000;**156**:595–605.
3. Blumenthal RD, Leon E, Hansen HJ, Goldenberg DM. Expression patterns of CEACAM5 and CEACAM6 in primary and metastatic cancers. *BMC Cancer* 2007;**7**:2.
4. Duxbury MS, Ito H, Benoit E, Ashley SW, Whang EE. CEACAM6 is a determinant of pancreatic adenocarcinoma cellular invasiveness. *Br J Cancer* 2004;**91**:1384–90.
5. Duxbury MS, Matros E, Clancy T, et al. CEACAM6 is a novel biomarker in pancreatic adenocarcinoma and PanIN lesions. *Ann Surg* 2005;**241**:491–6.
6. Poola I, Shokrani B, Bhatnagar R, DeWitty RL, Yue Q, Bonney G. Expression of carcinoembryonic antigen cell adhesion molecule 6 oncoprotein in atypical ductal hyperplastic tissues is associated with the development of invasive breast cancer. *Clin Cancer Res* 2006;**12**:4773–83.
7. Chau NM, Ashcroft M. Akt2: a role in breast cancer metastasis. *Breast Cancer Res* 2004;**6**:55–7.
8. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* 2003;**3**:453–8.
9. Vleminckx K, Vakaet Jr L, Mareel M, Fiers W, van Roy F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* 1991;**66**:107–19.
10. van den Brule FA, Engel J, Stetler-Stevenson WG, Liu FT, Sobel V, Castronovo V. Genes involved in tumor invasion and metastasis are differentially modulated by estradiol and progestin in human breast-cancer cells. *Int J Cancer* 1992;**52**:653–7.
11. Brodie AM, Coombes RC, Dowsett M. Aromatase inhibitors: basic and clinical studies. *J Steroid Biochem* 1987;**27**:899–903.
12. Baum M, Budzar AU, Cuzick J, et al. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. *Lancet* 2002;**359**:2131–9.
13. Thurlimann B, Keshaviah A, Coates AS, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *New Engl J Med* 2005;**353**:2747–57.
14. Conte P, Frassoldati A. Aromatase inhibitors in the adjuvant treatment of postmenopausal women with early breast cancer: putting safety issues into perspective. *Breast J* 2007;**13**:28–35.
15. Coombes RC, Kilburn LS, Snowdon CF, et al. Survival and safety of exemestane versus tamoxifen after 2–3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial. *Lancet* 2007;**369**:559–70.
16. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;**5**:207–13.
17. Osipo C, Liu H, Meeke K, Jordan VC. The consequences of exhaustive antiestrogen therapy in breast cancer: estrogen-induced tumor cell death. *Exp Biol Med (Maywood)* 2004;**229**:722–31.
18. Hiscox S, Morgan L, Barrow D, Dutkowskil C, Wakeling A, Nicholson RI. Tamoxifen resistance in breast cancer cells is accompanied by an enhanced motile and invasive phenotype: inhibition by gefitinib ('Iressa', ZD1839). *Clin Exp Metastasis* 2004;**21**:201–12.
19. Hiscox S, Jiang WG, Obermeier K, et al. Tamoxifen resistance in MCF7 cells promotes EMT-like behaviour and involves modulation of beta-catenin phosphorylation. *Int J Cancer* 2006;**118**:290–301.
20. Scott DJ, Parkes AT, Ponchel F, Cummings M, Poola I, Speirs V. Changes in expression of steroid receptors, their downstream target genes and their associated co-regulators during the sequential acquisition of tamoxifen resistance in vitro. *Int J Oncol* 2007;**31**:557–65.
21. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* 1992;**90**:77–86.
22. Lewis JS, Meeke K, Osipo C, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 2005;**97**:1746–59.
23. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 2005;**94**:131–41.
24. Pink JJ, Jiang SY, Fritsch M, Jordan VC. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* 1995;**55**:2583–90.
25. Bittner M, Meltzer P, Chen Y, et al. Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature* 2000;**406**:536–40.
26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;**25**:402–8.
27. Mackay A, Urruticoechea A, Dixon JM, et al. Molecular response to aromatase inhibitor treatment in primary breast cancer. *Breast Cancer Res* 2007;**9**:R37.
28. Gibson MK, Nemmers LA, Beckman Jr WC, Davis VL, Curtis KS, Korach KS. The mechanism of ICI 164,384 antiestrogenicity involves rapid loss of estrogen receptor in uterine tissue. *Endocrinology* 1991;**129**:2000–10.
29. Dauvois S, Danielian PS, White R, Parker MG. Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover. *Proc Natl Acad Sci USA* 1992;**89**:4037–41.
30. Duxbury MS, Ito H, Ashley SW, Whang EE. c-Src-dependent cross-talk between CEACAM6 and alphavbeta3 integrin enhances pancreatic adenocarcinoma cell adhesion to extracellular matrix components. *Biochem Biophys Res Commun* 2004;**317**:133–41.
31. Maraqa L, Cummings M, Peter MB, et al. Carcinoembryonic antigen cell adhesion molecule 6 predicts breast cancer recurrence following adjuvant tamoxifen. *Clin Cancer Res* 2008;**14**:405–11.
32. Stefanova I, Horejsi V, Ansotegui IJ, Knapp W, Stockinger H. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* 1991;**254**:1016–9.
33. Skubitz KM, Campbell KD, Ahmed K, Skubitz AP. CD66 family members are associated with tyrosine kinase activity in human neutrophils. *J Immunol* 1995;**155**:5382–90.
34. Duxbury MS, Ito H, Ashley SW, Whang EE. CEACAM6 cross-linking induces caveolin-1-dependent, Src-mediated focal adhesion kinase phosphorylation in BxPC3 pancreatic adenocarcinoma cells. *J Biol Chem* 2004;**279**:23176–82.

35. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev* 1999;**13**:2905–27.
36. Bellacosa A, Kumar CC, Di Cristofano A, Testa JR. Activation of AKT kinases in cancer: implications for therapeutic targeting. *Adv Cancer Res* 2005;**94**:29–86.
37. Sekharam M, Zhao H, Sun M, et al. Insulin-like growth factor 1 receptor enhances invasion and induces resistance to apoptosis of colon cancer cells through the Akt/Bcl-x(L) pathway. *Cancer Res* 2003;**63**:7708–16.
38. Jones RJ, Brunton VG, Frame MC. Adhesion-linked kinases in cancer; emphasis on src, focal adhesion kinase and PI 3-kinase. *Eur J Cancer* 2000;**36**:1595–606.
39. Thiery JP. Epithelial–mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 2003;**15**:740–6.
40. Cheng GZ, Chan J, Wang Q, Zhang W, Sun CD, Wang LH. Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel. *Cancer Res* 2007;**67**:1979–87.

Research article

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Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis

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Abstract

Introduction Estrogen deprivation using aromatase inhibitors is one of the standard treatments for postmenopausal women with estrogen receptor (ER)-positive breast cancer. However, one of the consequences of prolonged estrogen suppression is acquired drug resistance. Our group is interested in studying antihormone resistance and has previously reported the development of an estrogen deprived human breast cancer cell line, MCF-7:5C, which undergoes apoptosis in the presence of estradiol. In contrast, another estrogen deprived cell line, MCF-7:2A, appears to have elevated levels of glutathione (GSH) and is resistant to estradiol-induced apoptosis. In the present study, we evaluated whether buthionine sulfoximine (BSO), a potent inhibitor of glutathione (GSH) synthesis, is capable of sensitizing antihormone resistant MCF-7:2A cells to estradiol-induced apoptosis.

Methods Estrogen deprived MCF-7:2A cells were treated with 1 nM 17β-estradiol (E_2), 100 μM BSO, or 1 nM E_2 + 100 μM BSO combination *in vitro*, and the effects of these agents on cell growth and apoptosis were evaluated by DNA quantitation assay and annexin V and terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining. The in vitro results of the MCF-7:2A cell line were further confirmed *in vivo* in a mouse xenograft model.

Results Exposure of MCF-7:2A cells to 1 nM E_2 plus 100 μM BSO combination for 48 to 96 h produced a sevenfold increase in apoptosis whereas the individual treatments had no significant effect on growth. Induction of apoptosis by the combination treatment of E_2 plus BSO was evidenced by changes in Bcl-2 and Bax expression. The combination treatment also markedly increased phosphorylated c-Jun N-terminal kinase (JNK) levels in MCF-7:2A cells and blockade of the JNK pathway attenuated the apoptotic effect of E_2 plus BSO. Our *in vitro* findings corroborated *in vivo* data from a mouse xenograft model in which daily administration of BSO either as a single agent or in combination with E_2 significantly reduced tumor growth of MCF-7:2A cells.

Conclusions Our data indicates that GSH participates in retarding apoptosis in antihormone-resistant human breast cancer cells and that depletion of this molecule by BSO may be critical in predisposing resistant cells to E_2-induced apoptotic cell death. We suggest that these data may form the basis of improving therapeutic strategies for the treatment of antihormone resistant ER-positive breast cancer.

Introduction

Currently, estrogen deprivation using aromatase inhibitors is one of the standard treatments for postmenopausal women with estrogen receptor (ER)-positive breast cancer [1]. Unfortunately, a major clinical problem with the use of prolonged estrogen deprivation is the development of drug resistance (that is, hormone-independent growth) [2,3]. Our laboratory as well as other investigators, have instigated a major effort in

BSO: L-buthionine sulfoximine; E_2: 17β-estradiol; ER: estrogen receptor; FBS: fetal bovine serum; GCS: glutamylcysteine; GPx2: glutathione peroxidase; GS: glutathione synthetase; GSH: glutathione; H&E: hematoxylin and eosin; JNK: c-Jun N-terminal kinase; Rh123: rhodamine 123; SFS: dextran coated charcoal-treated FBS; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

studying antihormone resistance in breast cancer and have developed model systems of estrogen deprivation that are sensitive [4-6] or resistant to the apoptotic actions of estrogen [7]. In particular, we have previously reported the development of an estrogen deprived breast cancer cell line, MCF-7:5C, which undergoes estradiol-induced apoptosis after 2 days of treatment via the mitochondrial pathway [8]. In contrast, we have another estrogen deprived breast cancer cell line, MCF-7:2A, which appears to be resistant to estradiol-induced apoptosis [7]. We are studying resistance to estrogen induced apoptosis because clinical experience shows us that only 30% of patients respond to estrogen induced apoptosis once exhaustive antihormonal therapy occurs [9]. An important goal would be to see whether the apoptotic effect of estrogen can be enhanced in antihormone resistant cells. This new, targeted approach to the treatment of metastatic breast cancer could open the door to novel approaches to treatment with drug combinations.

L-Buthionine sulfoximine (BSO) is a specific γ-glutamylcysteine synthetase inhibitor that blocks the rate-limiting step of glutathionine (GSH) biosynthesis and in doing so depletes the intracellular GSH pool in both cultured cells and in whole animals [10]. GSH is a water-soluble tripeptide composed of glutamine, cysteine, and glycine. Reduced glutathione is the most abundant intracellular small molecule thiol present in mammalian cells and it serves as a potent intracellular antioxidant protecting cells from toxins such free radicals [11,12]. Changes in GSH homeostasis have been implicated in the etiology and progression of a variety of human diseases, including breast cancer [13]. In particular, studies have shown that elevated levels of GSH prevent apoptotic cell death whereas depletion of GSH facilitates apoptosis [10,14]. BSO depletes cellular GSH [10] and sensitizes tumor cells to apoptosis induced by standard chemotherapeutic agents [15,16].

Apoptosis (programmed cell death) is required for normal development and tissue homeostasis in multicellular organisms. Deregulation of apoptosis is fundamental to many diseases, such as cancer, stroke, heart disease, neurodegenerative disorders, and autoimmune disorders [17]. There are two main pathways for apoptosis, namely the extrinsic receptor mediated pathway and the intrinsic mitochondria-mediated pathway [18,19]. Components of the extrinsic pathway include the death receptors FasR/FasL, DR4/DR5, and tumor necrosis factor (TNF) [20], whereas the intrinsic pathway centers on the Bcl-2 family of proteins which comprises both proapoptotic proteins, such as Bax, Bak, and Bid and antiapoptotic proteins, such as Bcl-2 and Bcl-xL [18,19]. The Bcl-2 family proteins regulate apoptosis by altering mitochondrial membrane permeabilization which leads to the release of apoptogenic factors such as cytochrome *c*, procaspases, and apoptosis inducing factor (AIF). In particular, Bcl-2 and Bcl-xL inhibit apoptosis by maintaining mitochondrial membrane integrity whereas Bax and Bak facilitate apoptosis by initiating the loss of outer mitochondrial integrity [21]. Apart from its action on the mitochondria, there is also evidence that Bcl-2 possesses antioxidant property. Bcl-2 overexpression increases cellular GSH level which is associated with increased resistance to chemotherapy-induced apoptosis [22,23] whereas GSH depletion restores apoptosis in Bcl-2 expressing cells [16].

Based on microarray studies we found that the antihormone resistant MCF-7:2A cells express markedly elevated levels of glutathione synthetase (GS) and glutathione peroxidase 2 (GPx2); two enzymes that are involved in glutathione synthesis, which suggests that resistance to estrogen-induced apoptosis might be due to elevated levels of GSH present in the cells. If MCF-7:2A cells do indeed possess high levels of GSH, then it is possible that the use of BSO – as a single agent – might be able to sensitize these cells to estrogen-induced apoptosis. As mentioned before, there is current clinical interest in using low dose estradiol therapy to treat antihormone resistant breast cancer [24] however only a minimal 30% of patients respond to this therapeutic strategy. A combination of BSO and estradiol could possibly be used to improve the efficacy of estradiol as an apoptotic agent if glutathione depletion is fundamental to tumor cell survival. We have addressed the hypothesis that by altering glutathione levels we may be able to enhance apoptosis to estrogen and have employed BSO as our agent of choice because of earlier work clinically, which may provide a foundation for subsequent clinical trials.

In the present study, we show that depletion of cellular GSH by BSO sensitizes antihormone-resistant MCF-7:2A cells to estradiol-induced apoptosis that is mediated, in part, by the mitochondrial pathway and also activation of the c-Jun N-terminal kinase (JNK) signaling pathway. We further show that BSO, either alone or in combination with estradiol, causes tumor regression of MCF-7:2A cells *in vivo*.

Materials and methods

Cell lines and reagents

The MCF-7 human breast cancer cell line was obtained from Dr Dean Edwards (University of Texas, San Antonio, TX, USA) and was maintained in phenol red RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 × non-essential amino acids and bovine insulin at 6 ng/mL. The clonal cell line, MCF-7:2A, was derived by growing MCF-7 cells in estrogen-free media for more than 1 year, followed by two rounds of limiting dilution cloning [7]. These cells were grown in phenol red-free RPMI 1640 medium supplemented with 10% 4 × dextran-coated, charcoal-treated FBS (SFS). All reagents for cell culture were obtained from Invitrogen (Life Technologies, Carlsbad, CA, USA). DL-Buthionine sulfoximine (BSO) and 17β-estradiol (E_2) were from Sigma (St Louis, MO, USA), rhodamine 123 (Rh123) was from Invitrogen (Life Technoli-

gies, Carlsbad, CA, USA). LY294002 and SP600125 were from EMD (Gibbstown, NJ, USA)

Western blot analysis

The antibodies used for western blotting included those against stress-activated protein kinase (SAPK)/JNK, phospho-SAPK/JNK (Thr183/Tyr185), caspase-7, caspase-9, phospho-Bcl-2 (Ser70), and poly(ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Danvers, MA, USA), cytochrome *c* and β-actin (Sigma, St Louis, MO, USA), cytochrome oxidase subunit IV (Cox IV; Invitrogen, Carlsbad, CA, USA), Bax, Bcl-2, and Bcl-xL (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Western blotting analysis was performed as previously described [8].

Cell proliferation assays

Proliferation assay was performed as previously described [8]. Briefly, MCF-7 and MCF-7:2A cells were seeded in estrogen-free RPMI media containing 10% SFS at a density of 2×10^4 cells per well in 24-well plates. After 24 h, cells were treated with the respective drugs for 2, 5, and 7 days with retreatment on alternate days. The DNA content of the cells was determined as previously described [25] using a Fluorescent DNA Quantitation kit (Bio-Rad, Hercules, CA, USA). For each analysis, six replicate wells were used, and at least three independent experiments were performed.

Cell proliferation was also determined by cell counting using a hemocytometer. MCF-7 and MCF-7:2A cells were seeded at a density of 0.5×10^6 cells in 100 mm dishes and after 24 h cells were treated with 1 nM E_2, 100 μM BSO, or 1 nM E_2 plus 100 μM BSO for 7 days with re-treatment on alternate days. For each analysis, three replicate dishes were used, and at least three independent experiments were performed.

Detection of apoptosis by annexin V staining

The annexin V-fluorescein isothiocyanate (FITC) labeled Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) was used to detect and quantify apoptosis by flow cytometry, according to the manufacturer's instructions.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining for apoptosis

Apoptosis was also determined by the TUNEL assay using an *in situ* cell death detection kit conjugated with horse-radish peroxidase (POD) (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer's instructions. Briefly, fixed cells were washed, permeabilized, and then incubated with 50 μL of terminal deoxynucleotidyl transferase end-labeling cocktail for 60 min at 37°C in a humidified atmosphere in the dark. For signal conversion, slides were incubated with 50 μL of converter-POD (anti-fluorescein antibody conjugated with horseradish peroxidase) for 30 min at 37°C, rinsed with PBS, and then incubated with 50 μL of 3,3'-diaminobenzidine (DAB) substrate solution for 10 min at 25°C. The slides were then rinsed with phosphate-buffered saline (PBS), mounted under glass coverslips, and analyzed under a light microscope using an inverted Nikon TE300 (Nikon, Melville, NY, USA).

GSH assay

Total cellular GSH was measured using the Total Glutathione Colorimetric microplate assay Kit (Oxford Biomedical Research), according to the manufacturer's protocol. Cells were plated at 0.5×10^6/well of a six-well plate and allowed to recover overnight. After appropriate treatments, cells were washed in PBS and then lysed in 100 to 150 μl of buffer (100 mM $NaPO_4$, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5) containing 0.1% Triton X-100 and frozen at -80°C until analysis. To measure total glutathione, proteins were precipitated with sulfosalicylic acid at a final concentration of 1%. Samples were then spun for 10 min in a microcentrifuge to pellet proteins, and supernatant was diluted 1:20 in buffer before being measured. For all measurements, 50-μl triplicates of each sample were used for glutathione determination. The GSH level was obtained by comparing with the GSH standards and represented as nmol/mg of protein.

Mitochondrial transmembrane potential ($\Delta\Psi_m$) and cytochrome c release

Changes in the mitochondrial membrane potential ($\Delta\Psi m$) were examined by monitoring the cells after staining with rhodamine 123. Briefly, estradiol plus BSO-treated MCF-7:2A cells were washed twice with PBS and incubated with 1 μg/mL rhodamine 123 at 37°C for 30 min. Cells were then washed twice with PBS, and Rh123 intensity was determined by flow cytometry. Cells with reduced fluorescence were counted as having lost some of their mitochondrial membrane potential.

For cytochrome *c* release assays, cells were lysed in lysis buffer (10 mmol/L *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES; pH 7.5), 10 mmol/L KCl, and 1 mmol/L EDTA) with protease inhibitor cocktail (Sigma), frozen and thawed three times, and centrifuged at 2,000 *g* for 5 min. The supernatants were centrifuged at 10,000 *g* for 15 min at 4°C, and the mitochondrial pellets were dissolved in sodium dodecyl sulfate (SDS) sample buffer, subjected to 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by immunoblotting with monoclonal antibodies against cytochrome *c* and Cox IV.

RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated using TRI reagent (Invitrogen) according to the manufacturer's protocol. RNA (2 μg) was reverse transcribed to cDNA using the SuperScript II RNase H-reverse transcriptase system (Invitrogen, Carlsbad, CA, USA). Aliquots of the cDNA were combined with the SYBR green kit and primers, and assayed in triplicate by real-time quantitative PCR using a GeneAmp® 5700 Sequence detection system

(Applied Biosystems Inc, Foster City, CA, USA). Quantitation was performed using the comparative threshold cycle (Ct) method with 18S rRNA as the normalization gene, as previously described [8]. GS and GPx2 primers were designed using Primer Express™ software following the manufacturer's guidelines. Primers were synthesized by Applied Biosystems. Quantitative PCR was performed using the following conditions: 40 cycles; denaturation at 95 C for 15 s, annealing at 63 C for 1 min, and polymerization at 72 C for 1 min. Primer sequences were: GS forward: CACCAGCT GGGGAAGCATCT; reverse: GGTGAGGGGAAGAGCGT GAA, GPx2 forward: TTG ATT AAG GCT TTC TTT GGT AGG; reverse: TTT CAA TAA ATC AGG TCC CAG G.

Small interfering RNA (siRNA) transfection

Bcl-2-specific siRNA was chemically synthesized by Dharmacon Inc (Chicago, IL, USA). A non-targeting siRNA duplex was used as negative control. For transfection, MCF-7:2A cells were seeded in complete medium without antibiotics the day before the experiment in 12-well plates at a density of 70,000 cells per well. After 24 h, cells were transfected with 100 nM of Bcl-2 siRNA or control siRNA, using DharmaFect 1 transfection reagent (Dharmacon Inc, Chicago, IL, USA), according to the manufacturer's protocol. The cells were harvested 48 h post transfection and analyzed by western blot. Transfected cells were also treated with estradiol for an additional 72 h and apoptotic cells were measured using annexin V staining.

Inhibition of MCF-7:2A cell tumorigenesis by BSO in nude mice

Female CrTac:NCr-Foxn1nu athymic mice (4 to 5 weeks old) were purchased from Taconic (Germantown, NY, USA). Animal experiments were conducted at the Fox Chase Cancer Center (Philadelphia, PA, USA). The research protocol was approved, and mice were maintained in accordance with institutional guidelines of the Fox Chase Cancer Center Animal Care and Use Committee. Mice were acclimatized to the animal facility for 1 week before they received injections of MCF-7:2A human breast cancer cells: 2×10^7 cells were resuspended in 100 μL PBS (Collaborative Biomedical Products, Bedford, MA, USA) and were bilaterally injected into the mammary fat pads of 20 ovariectomized mice. Tumors were allowed to develop for 20 days until they reached a mean cross-sectional area of 0.32 cm^2, when treatment was initiated with placebo (saline), E_2 (0.3 cm capsule), BSO (4 mmol/kg weight), or BSO (4 mmol/kg weight) plus E_2 (0.3 cm capsule) for an additional 7 days. For the estradiol treatment, 0.3 cm silastic estradiol capsules (Baxter HealthCare, Mundeleine, IL, USA) were implanted subcutaneously in the mice. These capsules produced a mean serum estradiol level of 83.8 pg/mL [26], to achieve postmenopausal serum levels of estradiol. BSO was dissolved in saline and was administered intraperitoneally daily for 7 days. The cross-sectional tumor area was calculated by multiplying the length (l) by the width (w) by π and dividing the product by 4 ($lw\pi/4$). Animals were given food and water *ad libitum*. Mice from each group ($n = 5$) were killed at the conclusion of the experiment and immunohistochemical analysis was performed.

Tissue preparation and immunohistochemistry

Tumors from mice treated with placebo, E_2, BSO, or BSO plus E_2 were excised and fixed in 10% formalin, embedded in paraffin wax blocks and sectioned. Subsequently, sections of the tumor blocks were stained with hematoxylin and eosin (H&E), Ki67, or PARP antibody (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) by the pathology core facility at Fox Chase Cancer Center.

Statistical analysis

Statistical analysis was performed using the Student t test, and a p value of < 0.05 was considered significant. Data are expressed as the mean ± standard error of the mean (SEM). The mean value was obtained from at least three independent experiments.

Results

Estrogen deprivation increases glutathione levels in MCF-7:2A breast cancer cells

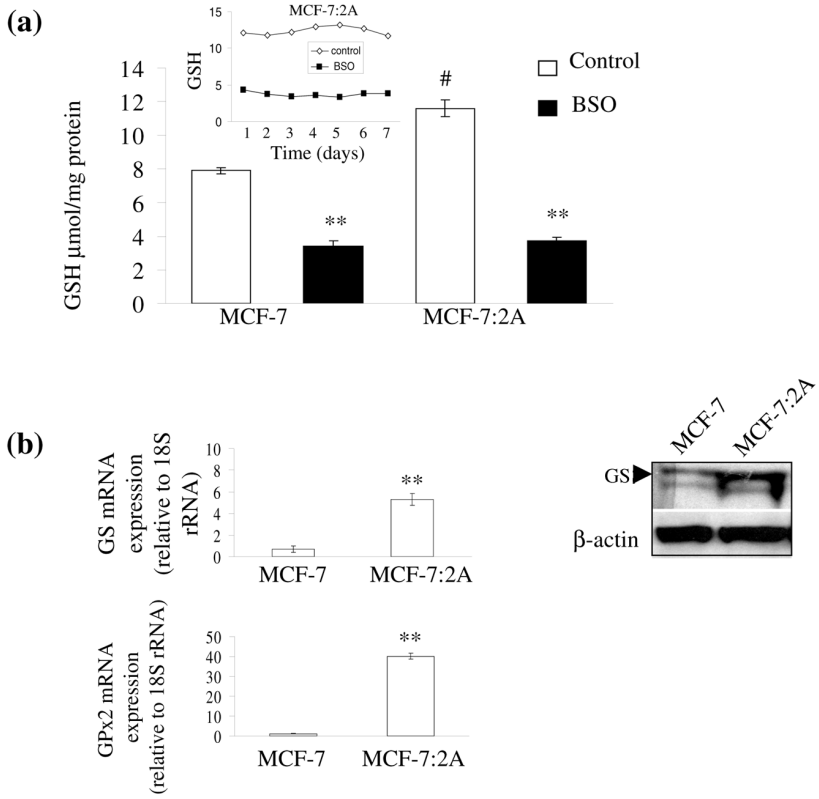
Elevated glutathione levels and the activity of its related enzymes have been characterized as one of the factors which could render breast cancer cells resistant to apoptosis. We have previously shown that MCF-7:2A breast cancer cells are resistant to estrogen-induced apoptosis [7], therefore we measured glutathione levels in these cells along with parental MCF-7 cells. Figure 1a showed that glutathione levels were significantly higher in MCF-7:2A cells (11.9 μM/mg protein) compared to MCF-7 cells (7.8 μM/mg protein) and treatment with BSO (100 μM), an inhibitor of glutathione synthesis, for 24 h depleted glutathione content by approximately 55% and 68% in MCF-7 and MCF-7:2A cells, respectively. It is worth noting that glutathione levels were consistently elevated in MCF-7:2A cells up to 7 days and the inhibitory effect of BSO persisted throughout that incubation period (Figure 1a, insert).

We next examined whether the expression of glutathione-related enzymes was altered in these cells. Using quantitative real-time PCR, we found a 6-fold increase in glutathione synthetase (GS) expression and a 40-fold increase in glutathione peroxidase 2 (GPx2) expressions in MCF-7:2A cells compared to parental MCF-7 cells (Figure 1b). Western blot analysis also showed a marked increase in GS protein level in MCF-7:2A cells compared to parental MCF-7 cells (Figure 1b, right panel).

BSO enhances the apoptotic effect of E_2 in MCF-7:2A cells

We next examined whether depletion of glutathione levels by BSO sensitizes MCF-7:2A cells to estrogen-induced apoptosis. For proliferation assays, MCF-7 and MCF-7:2A cells were

Figure 1

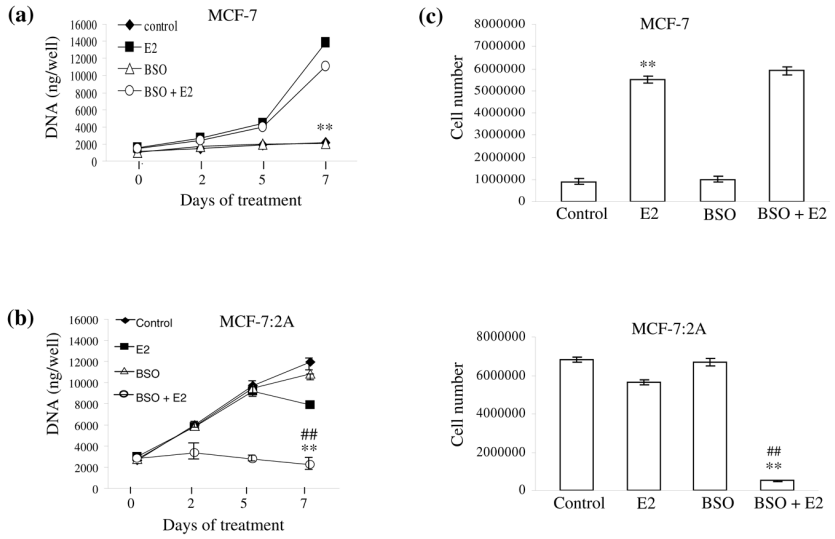


Intracellular glutathione (GSH) levels in wild-type MCF-7 cells and antihormone-resistant MCF-7:2A breast cancer cells. (a) MCF-7 and MCF-7:2A cells were seeded at 2 × 10^6 cells per 100 mm culture plates in phenol red RPMI media containing 10% fetal bovine serum (FBS) and phenol red-free RPMI media containing 10% 4× dextran coated charcoal-treated FBS (SFS), respectively, and after 24 h were treated with nothing (control) (white columns) or 100 μM buthionine sulfoximine (BSO) (black columns) for 24 h. Total cellular glutathione was measured using a Glutathione Colorimetric microplate assay kit, as described in Materials and methods. Columns, mean from three separate experiments; bars, ± standard error of the mean (SEM). **, $p < 0.001$ compared with control cells; #, $p < 0.05$ compared with MCF-7 control cells. Insert graph shows glutathione levels in MCF-7:2A cells over a 7-day period. (b) Quantitative real-time polymerase chain reaction (PCR) of glutathione sythetase (GS) (top left) and glutathione peroxidase 2 (GPx2) (bottom left) mRNA expression in MCF-7 and MCF-7:2A cells. **, $p < 0.001$ compared with MCF-7 control cells. Western blot analysis of GS protein expression in MCF-7:2A cells is also shown (top right).

seeded in estrogen-free media, and after 24 h, were treated with 100 μM BSO, 1 nM E_2, or 100 μM BSO plus 1 nM estradiol for 2, 5, and 7 days. Figure 2a shows that the growth of parental MCF-7 cells was stimulated sevenfold over the control cells by 1 nM estradiol during the course of the 7-day assay and that treatment with BSO, either alone or in combination with estradiol, did not significantly alter the growth of these cells. In contrast, MCF-7:2A cells treated with the combination of 100 μM BSO and 1 nM estradiol showed a significant time-dependent decrease in cell growth relative to cells treated with either estradiol or BSO alone. The growth inhibitory effect of BSO and estradiol was observed as early as 48 h after treatment and persisted over the time course of the experiment with maximum cell death at the 7-day time point. The combination of estradiol plus BSO also significantly reduced the proliferation of MCF-7:2A cells (Fig. 2c, bottom) but it did not affect the growth of wild type MCF-7 cells (Figure 2c, top). Furthermore, we found that treatment with the antiestrogen 4-hydroxytamoxifen (4-OHT) almost completely reversed the growth inhibitory effect of estradiol and BSO in MCF-7:2A cells (see Additional data file 1) which suggests the involvement of the ER in this process.

Based on the above finding, we next determined whether MCF-7:2A cells underwent apoptotic cell death upon BSO and estradiol treatment. We performed a TUNEL assay, which detects the fragmentation of DNA, which is characteristic of cells undergoing apoptotic cell death. As shown in Figure 3a, the percentage of TUNEL-positive cells significantly increased with the combination of BSO and estradiol but not with estradiol or BSO alone. After treatment with BSO and estradiol (96 h), as many as 53% of cells displayed TUNEL-positive staining, whereas, only 1% of the control cells and 5% of the estradiol-treated cells were TUNEL-positive. BSO-treated cells

Figure 2



Effect of buthionine sulfoximine (BSO) plus estradiol on the growth of wild-type MCF-7 cells and antihormone-resistant MCF-7:2A cells. (a) MCF-7 cells were grown in estrogen-free media for 3 days prior to the start of the growth assay. On the day of the experiment, 30,000 cells were seeded in 24-well plates and after 24 h were treated with < 0.1% ethanol vehicle (control), 1 nM 17β-estradiol (E_2), 100 μM BSO, or 100 μM BSO plus 1 nM E_2 for 7 days. At the indicated time points, cells were harvested and total DNA (ng/well) was quantitated as described in Materials and methods. The data represent the mean of three independent experiments; bars, ± standard error of the mean (SEM). **, $p < 0.001$ compared with control cells. (b) MCF-7:2A cells were seeded at the same density as MCF-7 cells and were treated similarly. The data represent the mean of three independent experiments; bars, ± SEM. **, $p < 0.001$ compared with control cells; ##, $p < 0.001$ compared with estradiol-treated cells. (c) The effect of BSO plus estradiol on cell proliferation was also determined by cell counting using a hemocytometer. For experiment, 0.5×10^6 MCF-7 (top) and MCF-7:2A (bottom) cells were seeded in 15-cm dishes and after 24 h were treated with 1 nM estradiol, 100 μM BSO, or E_2 plus BSO combination for 7 days. Data shown represents the mean of three independent experiments; bars, ± SEM. **, $p < 0.001$ compared with control cells; ##, $p < 0.001$ compared with estradiol-treated cells.

looked similar to control cells. As expected, parental MCF-7 cells showed very little TUNEL-positive staining in the presence of estradiol alone or BSO plus estradiol combined (Figure 2b, top panel), thus indicating a lack of apoptosis in these cells.

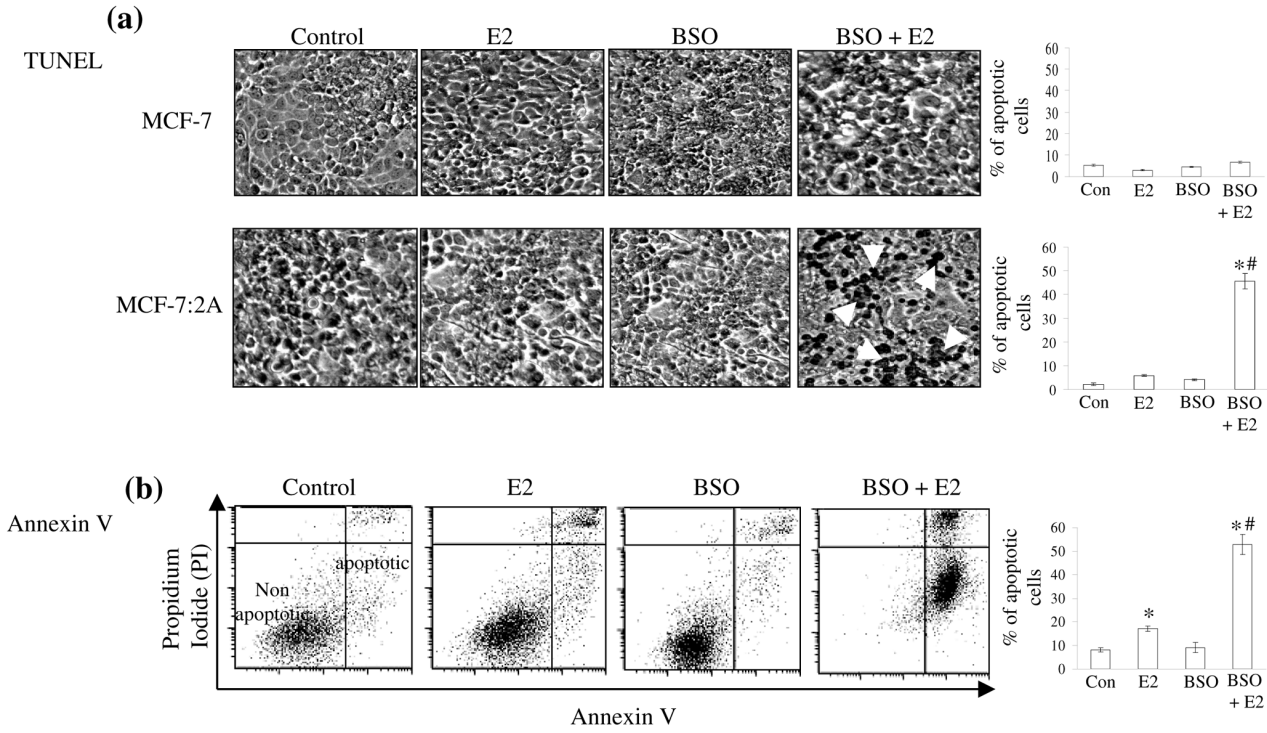
To further substantiate the apoptotic effect of BSO and estradiol in MCF-7:2A cells, annexin V-PI immunostaining was performed by flow cytometry. Figure 3b shows that in the BSO plus estradiol-treated group, approximately 55.6% of cells stained positive for annexin V whereas in the control group and estradiol-treated group, approximately 7.4% and approximately 15.6%, respectively, of cells stained positive for annexin V. For the BSO-treated group, only 8.7% of cells stained positive for annexin.

Role of the mitochondrial pathway in BSO plus estradiol-induced apoptosis in MCF-7:2A cells

To examine the role of the mitochondrial pathway in BSO plus estradiol-induced apoptosis, western blot analyses was used to measure Bax, Bcl-2, phosphorylated Bcl-2, and Bcl-xL protein levels in MCF-7:2A cells following treatment with 1 nM estradiol alone, 100 μM BSO, or BSO plus estradiol for 48 h. We found that Bcl-2, phospho-Bcl-2, and Bcl-xL protein levels were almost completely reduced in MCF-7:2A cells treated with BSO plus estradiol compared to control, BSO, or estradiol alone. In addition, a marked increase in Bax expression was also observed in MCF-7:2A cells following BSO plus estradiol combined treatment (Figure 4a). In contrast, similar experiments performed with parental MCF-7 cells showed that BSO plus estradiol slightly increased Bcl-2 and phospho-Bcl-2 protein levels in these cells with a more dramatic effect observed with estradiol alone (Figure 4a). It is worth noting that in MCF-7:2A cells endogenous levels of Bcl-2 and phosphorylated Bcl-2 were markedly elevated compared to parental MCF-7 cells. This finding is consistent with previous reports which show that overexpression of Bcl-2 increases glutathione levels and inhibits mitochondrial dysfunction and cell death elicited by glutathione-depleting reagents [27].

Although estradiol, as an individual treatment, did not significantly induce apoptosis in MCF-7:2A cells, it did decrease Bcl-2 protein level in these cells. We therefore tested whether siRNA knockdown of Bcl-2 expression would sensitize MCF-7:2A cells to estradiol-induced apoptosis. Expression of Bcl-2 following knockdown was analyzed by western blotting. As expected, Bcl-2 protein levels were significantly reduced following transfection of MCF-7:2A cells with Bcl-2 siRNA com-

Figure 3



Buthionine sulfoximine (BSO) plus estradiol induce apoptosis in MCF-7:2A cells. (a) Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining for apoptosis in MCF-7:2A cells following BSO plus 17β-estradiol (E_2) treatment for 96 h were performed as described in Materials and methods. Slides were photographed through a brightfield microscope under 100 × magnification. TUNEL-positive cells were stained black (white arrows). Columns (right), mean percentage of apoptotic cells (annexin V-positive cells) from three independent experiments performed in triplicate; bars, ± standard error of the mean (SEM). *, $p < 0.001$ compared with control cells; #, $p < 0.001$ compared with estradiol-treated cells. (b) Annexin V staining for apoptosis. Cells were seeded in 100 mm plates at a density of 1×10^6 per plate and after 24 h were treated with ethanol vehicle (control), 1 nM E_2, or BSO plus E_2 for 72 h and then stained with fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (PI) and analyzed by flow cytometry. PI was used as a cell viability marker. Representative cytograms are shown for each group. Quantitation of apoptosis (percentage of control) in the different treatment groups is shown on the right. bars, ± SEM. *, $p < 0.05$ compared with control cells; #, $p < 0.01$ compared with estradiol-treated cells.

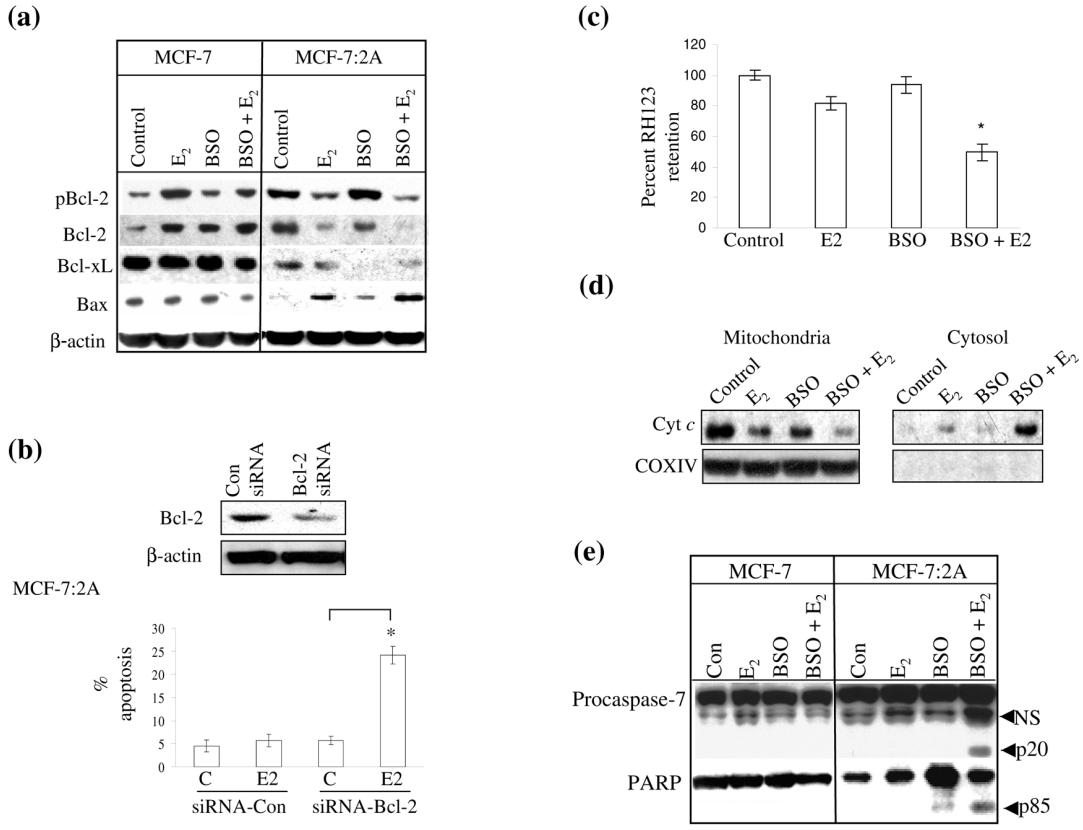
pared to control siRNA (Figure 4b, top panel). Using annexin V staining, we found that apoptosis was increased by 20% in Bcl-2 siRNA transfected cells compared with cells transfected with the control siRNA (Figure 4b, bottom panel), thus suggesting that suppression of antiapoptotic factors such as Bcl-2 has the ability to partially sensitize hormone-independent MCF-7:2A cells to apoptosis.

We next examined mitochondrial membrane integrity using the Rh123 retention assay. Cells were treated with nothing (control), estradiol, BSO, or BSO plus estradiol for 48 h. Figure 4c shows that BSO plus estradiol treatment reduced Rh123 fluorescence in MCF-7:2A cells by approximately 50% compared to control, whereas, estradiol or BSO, as individual treatments, did not significantly alter Rh123 retention levels in these cells. BSO plus estradiol also enhanced cytochrome *c* release in MCF-7:2A cells. Figure 4d shows that in the control cells, cytochrome *c* was detected primarily in the mitochondria and was undetectable in the cytosol; however, in the presence of BSO plus estradiol (48 h), all of cytochrome *c* was observed in the cytosol. BSO or estradiol, as individual treatments, did not significantly alter mitochondrial release of cytochrome *c*. The translocation of cytochrome *c* from the mitochondria to the cytosol following BSO plus estradiol treatment coincided with cleavage of caspase 7 and PARP (Figure 4e), which is a molecular signature of apoptosis. Cleavage of PARP and caspase 7 was blocked by the pan-caspase inhibitor z-VAD (data not shown).

The apoptotic effect of BSO and estradiol in MCF-7:2A cells is regulated, in part, by JNK signaling

Emerging evidence supports a role for JNK in stress-induced mitochondrial apoptotic pathways in a variety of cell systems [28]. Therefore, we examined the possible involvement of c-Jun/JNK pathway in BSO plus estradiol-induced apoptosis in MCF-7:2A cells. JNK activation was determined by western

Figure 4



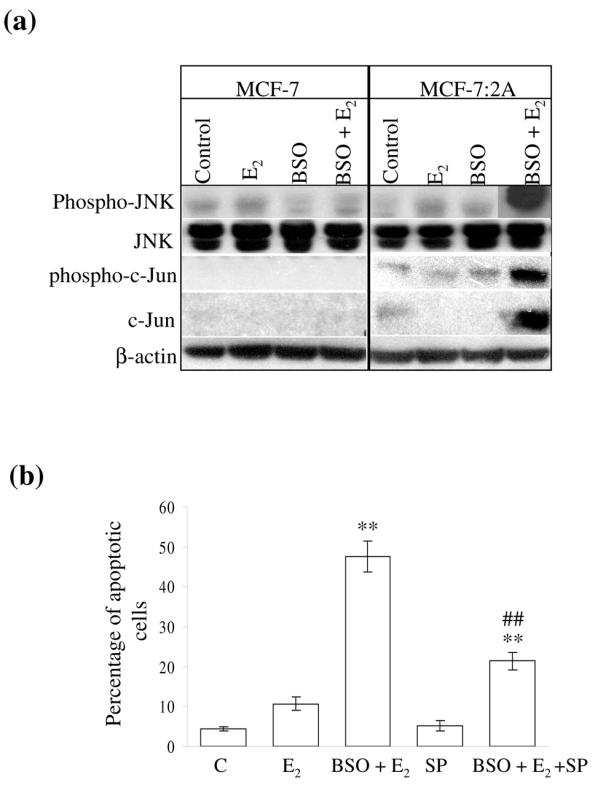
Effect of buthionine sulfoximine (BSO) and 17β-estradiol (E_2) on Bcl-2 family protein expression and mitochondrial function in MCF-7 and MCF-7:2A cells. (a) Western blot analysis for pBcl-2, Bcl-2, Bcl-x_L, and Bax protein expression in parental MCF-7 cells and MCF-7:2A cells following 48 h of treatment with ethanol vehicle (Control), 1 nM E_2, 100 μM BSO, or E_2 + BSO. Equal loading was confirmed by reprobing with an antibody against β-actin. (b) Small interfering RNA (siRNA) knockdown of Bcl-2 partially sensitizes MCF-7:2A cells to E_2-induced apoptosis. Cells were transfected with 100 nM siRNA-Bcl-2 or siRNA-Con (control) and expression levels of Bcl-2 was determined by immunoblot analysis (top). Annexin V staining (bottom) showing the effects of siRNA-con and siRNA-Bcl-2 on apoptosis induced by estradiol treatment in MCF-7:2A cells. *, $p < 0.001$. (c) Loss of mitochondrial potential in MCF-7:2A cells was determined by rhodamine 123 (Rh123) retention assay. The percentage of cells retaining Rh123 in each treatment group was compared with untreated control. (d) Cytochrome *c* release from the mitochondria to the cytosol after treatment with E_2 alone or BSO and E_2 for 48 h was determined as described in Materials and methods. Anti-Cox IV antibody was used as a control to demonstrate that mitochondrial protein fractionation was successfully achieved. (e) Cleavage of caspase 7 and poly(ADP-ribose) polymerase (PARP) (72 h) was assessed by western blot using specific antibodies. The upper band of caspase 7 represents the full-length protein and the lower band (p20, arrow) represents the cleaved activated product; NS, nonspecific. Full length PARP is approximately 116 kDa; cleaved (active) PARP is 85 kDa (arrow). The results are representative of three independent experiments.

blot analysis after 48-h treatment of cells with BSO plus estradiol. A profound induction of the p54 and p46 isoforms of phosphorylated JNK as well as a significant increase in phospho-c-Jun and c-Jun were observed in MCF-7:2A cells treated with BSO plus estradiol compared to BSO alone or control (Figure 5a). Interestingly, treatment with estradiol alone also significantly increased phosphorylated JNK in MCF-7:2A cells. We also found that pretreatment of MCF-7:2A cells with the JNK inhibitor, SP600125 (20 μM) markedly reduced the apoptotic effect of BSO plus estradiol in these cells (Figure 5b). Overall, these results suggest a possible involvement of the c-Jun/JNK signaling pathway in BSO plus estradiol-induced apoptosis in MCF-7:2A cells.

BSO inhibits the growth of MCF-7:2A cells *in vivo*

To determine whether the effect of BSO plus estradiol was relevant *in vivo*, we used a xenograft model in which MCF-7:2A cells were injected into CrTac:NCr-Foxn1nu athymic mice (n = 20). At 20 days post injection, tumors grew to a mean cross-sectional area of 0.30 cm^2 and mice were randomized to four groups; placebo (saline), estradiol, BSO, or the combination of BSO plus estradiol, as described in materials and methods. After 7 days of treatment, tumor growth was reduced by 25% in mice treated with estradiol alone whereas in the BSO and BSO plus estradiol group tumor growth was reduced by 40% and 60%, respectively, compared to the placebo group which showed a 7% increase in growth (Figure 6a). Interestingly, we

Figure 5



Activation of c-Jun N-terminal kinase (JNK) signaling pathway in MCF-7:2A cells in response to buthionine sulfoximine (BSO) and 17β-estradiol (E_2) treatment. (a) MCF-7 and MCF-7:2A cells were treated with ethanol vehicle (control), 1 nM E_2 or 100 μM BSO plus E_2 for 48 h and protein levels of phosphorylated JNK, JNK, phosphorylated c-Jun, and c-Jun were analyzed by western blotting. β-Actin was used as a control. (b) Inhibition of JNK activation by SP600125 (SP) partially reverses the apoptotic effect of BSO and estradiol in MCF-7:2A cells. Cells were pretreated with 20 μM SP600125 or vehicle for 24 h, then further incubated for 48 h with 1 nM E_2, E_2 + 100 μM BSO, 20 μM SP, or E_2 + BSO + SP and apoptosis was determined by annexin V-propidium iodide (PI) staining as described in Materials and methods. Columns, mean percentage of apoptotic cells from three independent experiments performed in triplicate; bars, ± standard error of the mean (SEM). **, $p < 0.001$ compared with control (C) cells; ##, $p < 0.01$ compared with E_2 plus BSO-treated cells.

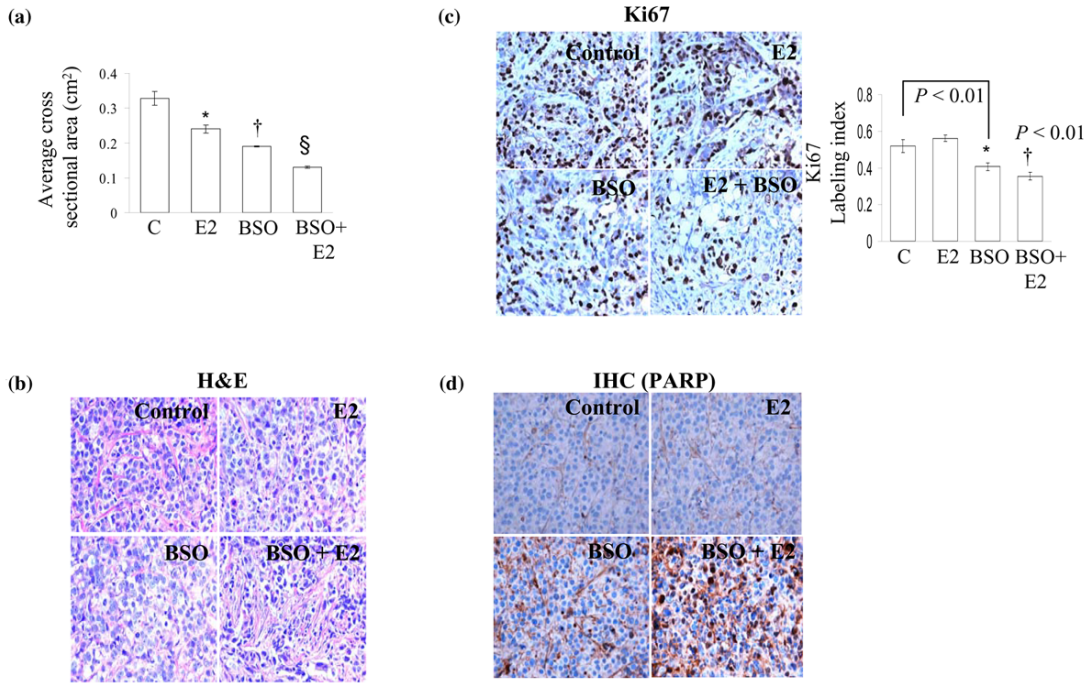
found that BSO *in vitro* had a relatively small effect on growth, however, *in vivo* its effect was very pronounced, thus suggesting the possibility of altered glutathione metabolism *in vivo*. We performed histology on tumors taken from placebo, estradiol, BSO, or BSO plus estradiol groups at day 27. H&E staining of the BSO plus estradiol-treated tumors revealed less tumor cells and more intercellular matrix, significantly less mitoses, chromatin clumping and dark staining which are associated with apoptosis, and enhanced abnormalities in shape and size, compared to tumors from placebo or BSO or estradiol-treated groups (Figure 6b). We also characterized the proliferative status of these cells by staining tumors for the expression of Ki67, a marker of cell proliferation. We observed a 32% decrease ($p < 0.001$) in the number of Ki67 stained tumors from the BSO plus estradiol-treated group and a 21% decrease in the BSO-treated group compared to the placebo group whereas estradiol treatment caused an 8% increase in Ki67 staining (Figure 6c). Immunohistochemistry of paraffin-embedded tumor sections of mice treated with the combination of BSO and estradiol showed increased immunostaining for proteolytically cleaved PARP (marker for apoptosis) compared to control, estradiol, or BSO-treated groups (Figure 6d). Overall, these data show that BSO either alone or in combination with estradiol, reduces tumor growth by inhibiting proliferation and increasing apoptosis.

Discussion

In the current study, we investigated whether suppression of the antioxidant glutathione by BSO has the ability to sensitize antihormone resistant MCF-7:2A breast cancer cells to estradiol-induced apoptosis. Our results showed that glutathione levels and the enzymes involved in its synthesis, glutathione synthetase and glutathione peroxidase, were significantly elevated in MCF-7:2A cells compared to parental MCF-7 cells and that suppression of glutathione by BSO sensitized these cells to estrogen-induced apoptosis *in vitro* and *in vivo*. The BSO-mediated estradiol-induced apoptosis was associated with a marked decrease in the expression of antiapoptotic Bcl-2 and Bcl-xL proteins and a significant increase in proapoptotic Bax protein. It is worth noting that high-dose estrogen was generally considered the endocrine therapy of choice for postmenopausal women with breast cancer prior to the introduction of tamoxifen, however, due to undesirable side effects, the use of high-dose estrogen was largely abandoned [29]. Here, we show that the killing effect of estradiol in antihormone resistant cells can be achieved at physiological concentrations when it is combined with non-toxic concentrations of BSO. Our present findings are consistent with previous studies which have shown that the cytotoxicity of a number of chemotherapeutic drugs, including melphalan [30], doxorubicin [31], and bleomycin [32], are significantly enhanced when glutathione is depleted by BSO.

An important target of BSO plus estradiol-induced apoptosis appears to be Bcl-2, whose protein expression was dramatically decreased in MCF-7:2A cells following glutathione depletion. Previous studies have shown that Bcl-2 functions as an antioxidant to block apoptosis and that Bcl-2 protein levels and glutathione intracellular concentration is coordinately regulated with a decrease in either favoring cell death [23,33]. It is believed that one mechanism by which Bcl-2 may function as an antioxidant is through upregulation of glutathione, leading to rapid detoxification of reactive oxygen species and inhibition of free radical-mediated mitochondrial damage. Bcl-2 also has the ability to shift the entire cellular redox potential to a more reduced state, which is independent of its effect on glutathione levels [33]. It is worth noting that glutathione levels

Figure 6



Buthionine sulfoximine (BSO) inhibits the growth of MCF-7:2A tumors in vivo. Athymic nude mice (4 to 5 weeks old, n = 20) were injected with MCF-7:2A breast cancer cells and after 20 days when tumors had reached a mean cross-sectional area of 0.3 cm^2, animals were randomized into 4 groups and were treated with placebo (saline), 17β-estradiol (E_2), BSO, or BSO plus E_2 for 7 days as described in Materials and methods. BSO (4 mmol/kg weight) was diluted in saline and was injected intraperitoneally daily. (a) Tumor size was measured everyday and cross-sectional area was calculated by multiplying the length (*l*) by the width (*w*) by π and dividing the product by 4 ($lw\pi/4$). Data is shown as mean ± standard error of the mean (SEM). *, $p < 0.05$, control group compared with the E_2 group; †, $p < 0.002$ control group compared with BSO group; § $p < 0.001$ control group compared with BSO + E2 group. (b) Microscopy of hematoxylin and eosin (H&E)-stained histological sections of MCF-7:2A tumors treated with placebo, E_2, BSO, or BSO plus E_2. (c) Immunohistochemical analysis of the proliferation marker Ki-67 in MCF-7:2A tumors treated with placebo, E_2, BSO, or BSO plus E_2. (d) Paraffin-embedded tumor sections of mice treated with E_2, BSO, or BSO plus E_2 were immunostained for proteolytically cleaved poly(ADP-ribose) polymerase (PARP), which exists only when cells undergo apoptosis. Three to four tumors per treatment group were analyzed.

and Bcl-2 protein expression were significantly elevated in MCF-7:2A cells compared to parental MCF-7 cells. In phase I trials [34,35], the concentration of BSO in blood has been shown to reach 0.5 to 1 mM, whereas, in mice [36,37] the concentration has been estimated to be 5 to 6 mM following an *in vivo* treatment of 4 mmol/kg. In our study, we showed that 100 μM BSO decreased glutathione concentrations by approximately 60% after 24 h and that BSO enhanced the apoptotic effect of estradiol in MCF-7:2A breast cancer cells as early as 48 h after treatment. Interestingly, treatment with BSO alone did not cause apoptosis in MCF-7:2A cells, indicating that glutathione depletion alone may not trigger apoptosis in these cells. This finding is consistent with previous studies by Mirkovic *et al*. [38] which showed that inhibition of glutathione by BSO did not increase susceptibility of mouse lymphoma cells to radiation-induced apoptosis even under conditions where glutathione levels were lowered by 50%. Other groups have made similar observations using BSO [39]. One possible explanation for this apparent contradiction might be the fact that BSO does not lower glutathione levels in mitochondria as effectively as it does in the cytoplasm [40]. Mitochondrial glutathione concentrations are regulated and have been implicated in apoptotic cell death [41], hence, it would be of interest to evaluate relative glutathione concentrations in the mitochondrial matrix of MCF-7:2A cells following treatment with BSO either alone or in combination with estradiol. Another possibility could be that cellular thiols other than glutathione may play important roles in regulating apoptosis [39]. The flavoprotein thioredoxin has been shown to be upregulated in several human tumors and is implicated in both cancer cell growth and apoptotic resistance [42]. However, it is not known whether Bcl-2 or other apoptotic regulators can influence the levels of thioredoxin or whether such modulation may contribute to resistance in human tumor cells.

Apart from Bcl-2, we also found that proapoptotic Bax protein was markedly increased in MCF-7:2A cells by the combination of BSO plus estradiol and this induction coincided with a loss of mitochondrial membrane integrity and cytochrome *c* release. Bax is normally found as a monomer in the cytosol of

non-apoptotic cells and it oligomerizes and translocates to the outer mitochondrial membrane in response to apoptotic stimuli and induces mitochondrial membrane permeabilization and cytochrome *c* release [19]. In MCF-7:2A cells, Bax protein was induced as early as 24 h after BSO plus estradiol treatment (Figure 4) and suppression of Bax expression using siRNA was able to partially reverse the apoptotic effect of the combination treatment (data not shown). The induction of Bax coincided with cytochrome *c* release from the mitochondria into the cytosol, which was followed by activation of caspase 7, and PARP cleavage. It is worth noting that pretreatment of cells with the universal caspase inhibitor z-VAD almost completely blocked the apoptotic effect of BSO plus estradiol. It is also worth noting that antiapoptotic Bcl-2 and Bcl-xL proteins were also markedly decreased in MCF-7:2A cells following the combination treatment of BSO plus estradiol (Figure 4) and overexpression of Bcl-xL partially blocked the apoptotic effect of BSO plus estradiol (data not shown). This finding is important because there is evidence that suggests that the ratio rather than the amount of antiapoptotic vs proapoptotic proteins determines whether apoptosis will proceed [43]. Thus, it is reasonable to suggest that the apoptotic effect of BSO plus estradiol is mediated, in part, by the mitochondrial pathway through their ability to alter the ratio between proapoptotic and antiapoptotic proteins in target cells.

In addition to the mitochondrial pathway, BSO plus estradiol appears to induce apoptosis, in part, through activation of the JNK signaling pathway. JNKs are a group of mitogen-activated protein kinases (MAPKs) that bind the N-terminal activation domain of the transcription factor c-Jun and phosphorylate c-Jun on amino acid residues Ser63 and Ser73 [44]. JNKs are stimulated by multiple factors including cytokines, DNA-damaging agents, and environmental stresses and are important in controlling programmed cell death or apoptosis. The inhibition of JNKs has been shown to enhance chemotherapy-induced inhibition of tumor cell growth, suggesting that JNKs may provide a molecular target for the treatment of cancer [44]. We found that JNK activation (as measured by the increased levels of phospho-JNK1/2 and the JNK substrate phospho-c-Jun) correlated well with BSO plus estradiol-induced apoptosis in MCF-7:2A cells and pharmacologic disruption of this pathway using the JNK inhibitor SP600125 significantly attenuated this effect. Previously, Chen and coworkers [45] reported that BSO enhanced the apoptotic effect of arsenic (As_2O_3) in leukemia and lymphoma cells through activation of JNK and upregulation of death receptor (DR)5 and that inhibition of JNK by SP600125 decreased DR5 upregulation and apoptotic induction in U937 leukemia cells treated with arsenic plus BSO. While the exact mechanism by which JNK promotes apoptosis is not currently known, the phosphorylation of transcription factors such as c-Jun and p53, as well as pro- and antiapoptotic Bcl-2 family members [46] has been suggested to be of importance. It is worth noting that treatment with BSO plus estradiol markedly increased phosphorylated c-Jun in MCF-7:2A cells and decreased phosphorylated Bcl-2 in these cells. These findings thus suggest that BSO plus estradiol might mediate their apoptotic effect, in part, through activation of JNK.

Conclusion

We have demonstrated that glutathione depletion by BSO sensitizes hormone-resistant MCF-7:2A human breast cancer cells to estradiol-induced apoptosis *in vitro* and *in vivo*. This finding has important clinical implications; particularly for the use of estrogen deprivation as long-term therapy, and it suggest that, if and when resistance develops, a strategy of treatment with estrogen combined with BSO may be effective in sensitizing resistant cells to apoptosis. It is worth noting that recently, Lonning and coworkers [9] reported a 33% complete response (that is, stable disease) with high dose diethylstilbestrol (DES) in postmenopausal patients with advanced breast cancer who were heavily pretreated with endocrine agents. However, 67% of the patients showed partial or no response [9] so the key to future clinical progress in the treatment of antihormone resistant breast cancer is to improve current treatment strategies. We are currently evaluating the optimal dose of daily estradiol therapy to reverse antihormonal resistance [4] but the goal is to enhance the estradiol-induced apoptotic response. The present findings suggest that BSO is indeed capable of enhancing the apoptotic effect of estradiol in antihormone resistant breast cancer cells. It is worth noting that a phase I study of BSO administered with the anticancer drug melphalan showed that continuous-infusion of BSO was relatively nontoxic and resulted in depletion of tumor glutathione [35,47]. Thus it is possible that future clinical studies of BSO infusions combined with low dose estrogen hold the promise of improving disease control for patients with antihormone resistant ER-positive metastatic breast cancer.

Competing interests

The authors declare that they have no competing interests. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

Authors' contributions

JSLW designed and coordinated the studies, analyzed the data and interpreted the results, generated the figures, and wrote and revised the manuscript. HK performed the cell proliferation assays and the western blots. CW performed the glutathione assay. RP and JP performed the animal experiments. AJK performed the immunohistochemistry. VCJ is the Principal Investigator (PI) of the laboratory in which all experiments were conducted and is the recipient of the grant that partially funded the project. VCJ was instrumental in revising the manuscript. All authors read, assisted in revision and approved the final manuscript.

Additional files

The following Additional files are available online:

Additional file 1

Powerpoint file showing the growth inhibitory effect of buthionine sulfoximine (BSO) and 17β-estradiol (E_2) in MCF-7:2A cells is reversed by the antiestrogen 4-hydroxytamoxifen (4-OHT). MCF-7:2A cells (30,000/well) were seeded in 24-well plates and after 24 h were treated with < 0.1% ethanol vehicle (control), 1 nM E_2, 100 μM BSO, 100 μM BSO plus 1 nM E_2, 1 μM 4-OHT, 4-OHT + E_2, 4-OHT + BSO, 4-OHT + E_2 + BSO for 7 days. At the indicated time points, cells were harvested and total DNA (μg/well) was quantitated as described in Materials and methods. The data represent the mean of three independent experiments; bars, ± standard error of the mean (SEM). *, $p < 0.01$ compared with control cells; #, $p < 0.01$ compared to E_2-treated cells.
See http://www.biomedcentral.com/content/supplementary/bcr2208-S1.ppt

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References

1. Herold CI, Blackwell KL: **Aromatase inhibitors for breast cancer: proven efficacy across the spectrum of disease.** *Clin Breast Cancer* 2008, **8:**50-64.
2. Santen RJ, Song RX, Masamura S, Yue W, Fan P, Sogon T, Hayashi S, Nakachi K, Eguchi H: **Adaptation to estradiol deprivation causes up-regulation of growth factor pathways and hypersensitivity to estradiol in breast cancer cells.** *Adv Exp Med Biol* 2008, **630:**19-34.
3. Jordan VC, O'Malley BW: **Selective estrogen-receptor modulators and antihormonal resistance in breast cancer.** *J Clin Oncol* 2007, **25:**5815-5824.
4. Jordan VC: **The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer – survival or death?** *J Clin Oncol* 2008, **26:**3073-3082.
5. Santen RJ, Allred DC: **The estrogen paradox.** *Nat Clin Pract Endocrinol Metab* 2007, **3:**496-497.
6. Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, Yue W, Wang J, Santen RJ: **Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol.** *J Natl Cancer Inst* 2001, **93:**1714-1723.
7. Pink JJ, Jiang SY, Fritsch M, Jordan VC: **An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein.** *Cancer Res* 1995, **55:**2583-2590.
8. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, Jordan VC: **Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation.** *J Natl Cancer Inst* 2005, **97:**1746-1759.
9. Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jorgensen LM, Mella O, Howell A: **High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy.** *Breast Cancer Res Treat* 2001, **67:**111-116.
10. Anderson CP, Tsai JM, Meek WE, Liu RM, Tang Y, Forman HJ, Reynolds CP: **Depletion of glutathione by buthionine sulfoxine is cytotoxic for human neuroblastoma cell lines via apoptosis.** *Exp Cell Res* 1999, **246:**183-192.
11. Schroder CP, Godwin AK, O'Dwyer PJ, Tew KD, Hamilton TC, Ozols RF: **Glutathione and drug resistance.** *Cancer Invest* 1996, **14:**158-168.
12. Anderson ME: **Glutathione: an overview of biosynthesis and modulation.** *Chem Biol Interact* 1998, **111–112:**1-14.
13. Townsend DM, Tew KD, Tapiero H: **The importance of glutathione in human disease.** *Biomed Pharmacother* 2003, **57:**145-155.
14. Hammond CL, Lee TK, Ballatori N: **Novel roles for glutathione in gene expression, cell death, and membrane transport of organic solutes.** *J Hepatol* 2001, **34:**946-954.
15. Schnelldorfer T, Gansauge S, Gansauge F, Schlosser S, Beger HG, Nussler AK: **Glutathione depletion causes cell growth inhibition and enhanced apoptosis in pancreatic cancer cells.** *Cancer* 2000, **89:**1440-1447.
16. Rudin CM, Yang Z, Schumaker LM, VanderWeele DJ, Newkirk K, Egorin MJ, Zuhowski EG, Cullen KJ: **Inhibition of glutathione synthesis reverses Bcl-2-mediated cisplatin resistance.** *Cancer Res* 2003, **63:**312-318.
17. Fulda S, Debatin KM: **Targeting inhibitor of apoptosis proteins (IAPs) for diagnosis and treatment of human diseases.** *Recent Patents Anticancer Drug Discov* 2006, **1:**81-89.
18. Fulda S, Debatin KM: **Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy.** *Oncogene* 2006, **25:**4798-4811.
19. Youle RJ, Strasser A: **The BCL-2 protein family: opposing activities that mediate cell death.** *Nat Rev Mol Cell Biol* 2008, **9:**47-59.
20. Peter ME, Krammer PH: **The CD95(APO-1/Fas) DISC and beyond.** *Cell Death Differ* 2003, **10:**26-35.
21. Newmeyer DD, Ferguson-Miller S: **Mitochondria: releasing power for life and unleashing the machineries of death.** *Cell* 2003, **112:**481-490.
22. Meredith MJ, Cusick CL, Soltaninassab S, Sekhar KS, Lu S, Freeman ML: **Expression of Bcl-2 increases intracellular glutathione by inhibiting methionine-dependent GSH efflux.** *Biochem Biophys Res Commun* 1998, **248:**458-463.
23. Voehringer DW: **BCL-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity.** *Free Radic Biol Med* 1999, **27:**945-950.
24. Swaby RF, Jordan VC: **Low-dose estrogen therapy to reverse acquired antihormonal resistance in the treatment of breast cancer.** *Clin Breast Cancer* 2008, **8:**124-133.
25. Labarca C, Paigen K: **A simple, rapid, and sensitive DNA assay procedure.** *Anal Biochem* 1980, **102:**344-352.
26. O'Regan RM, Cisneros A, England GM, MacGregor JI, Muenzner HD, Assikis VJ, Bilimoria MM, Piette M, Dragan YP, Pitot HC, Chatterton R, Jordan VC: **Effects of the antiestrogens tamoxifen, toremifene, and ICI 182,780 on endometrial cancer growth.** *J Natl Cancer Inst* 1998, **90:**1552-1558.
27. Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T, Bredesen DE: **Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species.** *Science* 1993, **262:**1274-1277.
28. Bossy-Wetzel E, Bakiri L, Yaniv M: **Induction of apoptosis by the transcription factor c-Jun.** *EMBO J* 1997, **16:**1695-1709.
29. Haddow AW, Paterson E: **Influence of synthetic oestrogens upon advanced malignant disease.** *BMJ* 1944, **2:**393-398.
30. Kramer RA, Greene K, Ahmad S, Vistica DT: **Chemosensitization of L-phenylalanine mustard by the thiol-modulating agent buthionine sulfoximine.** *Cancer Res* 1987, **47:**1593-1597.
31. Dusre L, Mimnaugh EG, Myers CE, Sinha BK: **Potentiation of doxorubicin cytotoxicity by buthionine sulfoximine in multidrug-resistant human breast tumor cells.** *Cancer Res* 1989, **49:**511-515.
32. Russo A, DeGraff W, Friedman N, Mitchell JB: **Selective modulation of glutathione levels in human normal versus tumor cells and subsequent differential response to chemotherapy drugs.** *Cancer Res* 1986, **46:**2845-2848.

33. Ellerby LM, Ellerby HM, Park SM, Holleran AL, Murphy AN, Fiskum G, Kane DJ, Testa MP, Kayalar C, Bredesen DE: **Shift of the cellular oxidation-reduction potential in neural cells expressing Bcl-2.** *J Neurochem* 1996, **67:**1259-1267.
34. O'Dwyer PJ, Hamilton TC, LaCreta FP, Gallo JM, Kilpatrick D, Halbherr T, Brennan J, Bookman MA, Hoffman J, Young RC, Comis RL, Ozols RF: **Phase I trial of buthionine sulfoximine in combination with melphalan in patients with cancer.** *J Clin Oncol* 1996, **14:**249-256.
35. Bailey HH, Ripple G, Tutsch KD, Arzoomanian RZ, Alberti D, Feierabend C, Mahvi D, Schink J, Pomplun M, Mulcahy RT, Wilding G: **Phase I study of continuous-infusion L-S, R-buthionine sulfoximine with intravenous melphalan.** *J Natl Cancer Inst* 1997, **89:**1789-1796.
36. Kang YJ, Uthus EO: **Suppression of plasma estradiol and progesterone concentrations by buthionine sulfoximine in female rats.** *Biochem Pharmacol* 1996, **51:**567-570.
37. Morrison JP, Coleman MC, Aunan ES, Walsh SA, Spitz DR, Kregel KC: **Aging reduces responsiveness to BSO- and heat stress-induced perturbations of glutathione and antioxidant enzymes.** *Am J Physiol Regul Integr Comp Physiol* 2005, **289:**R1035-1041.
38. Mirkovic N, Voehringer DW, Story MD, McConkey DJ, McDonnell TJ, Meyn RE: **Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is reversed by depleting cellular thiols.** *Oncogene* 1997, **15:**1461-1470.
39. Sato N, Iwata S, Nakamura K, Hori T, Mori K, Yodoi J: **Thiol-mediated redox regulation of apoptosis. Possible roles of cellular thiols other than glutathione in T cell apoptosis.** *J Immunol* 1995, **154:**3194-3203.
40. Griffith OW, Meister A: **Origin and turnover of mitochondrial glutathione.** *Proc Natl Acad Sci USA* 1985, **82:**4668-4672.
41. Martensson J, Meister A: **Mitochondrial damage in muscle occurs after marked depletion of glutathione and is prevented by giving glutathione monoester.** *Proc Natl Acad Sci USA* 1989, **86:**471-475.
42. Baker A, Payne CM, Briehl MM, Powis G: **Thioredoxin, a gene found overexpressed in human cancer, inhibits apoptosis *in vitro* and *in vivo*.** *Cancer Res* 1997, **57:**5162-5167.
43. Jacobson MD, Raff MC: **Programmed cell death and Bcl-2 protection in very low oxygen.** *Nature* 1995, **374:**814-816.
44. Vasilevskaya I, O'Dwyer PJ: **Role of Jun and Jun kinase in resistance of cancer cells to therapy.** *Drug Resist Updat* 2003, **6:**147-156.
45. Chen D, Chan R, Waxman S, Jing Y: **Buthionine sulfoximine enhancement of arsenic trioxide-induced apoptosis in leukemia and lymphoma cells is mediated via activation of c-Jun NH_2-terminal kinase and up-regulation of death receptors.** *Cancer Res* 2006, **66:**11416-11423.
46. Park J, Kim I, Oh YJ, Lee K, Han PL, Choi EJ: **Activation of c-Jun N-terminal kinase antagonizes an anti-apoptotic action of Bcl-2.** *J Biol Chem* 1997, **272:**16725-16728.
47. Bailey HH, Mulcahy RT, Tutsch KD, Arzoomanian RZ, Alberti D, Tombes MB, Wilding G, Pomplun M, Spriggs DR: **Phase I clinical trial of intravenous L-buthionine sulfoximine and melphalan: an attempt at modulation of glutathione.** *J Clin Oncol* 1994, **12:**194-205.

Estrogen promotes the survival and pulmonary metastasis of tuberin-null cells

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Lymphangioleiomyomatosis (LAM) is an often fatal disease primarily affecting young women in which tuberin (TSC2)-null cells metastasize to the lungs. The mechanisms underlying the striking female predominance of LAM are unknown. We report here that 17-β-estradiol (E_2) causes a 3- to 5-fold increase in pulmonary metastases in male and female mice, respectively, and a striking increase in circulating tumor cells in mice bearing tuberin-null xenograft tumors. E_2-induced metastasis is associated with activation of p42/44 MAPK and is completely inhibited by treatment with the MEK1/2 inhibitor, CI-1040. In vitro, E_2 inhibits anoikis of tuberin-null cells. Finally, using a bioluminescence approach, we found that E_2 enhances the survival and lung colonization of intravenously injected tuberin-null cells by 3-fold, which is blocked by treatment with CI-1040. Taken together these results reveal a new model for LAM pathogenesis in which activation of MEK-dependent pathways by E_2 leads to pulmonary metastasis via enhanced survival of detached tuberin-null cells.

anoikis | MAPK | lymphangioleiomyomatosis | Bim | Rheb

LAM, the pulmonary manifestation of tuberous sclerosis complex (TSC), affects women almost exclusively (1). LAM affects 30−40% of women with TSC (2, 3). In a Mayo Clinic series, LAM was the third most frequent cause of TSC-related death, after renal disease and brain tumors (4). LAM can also occur in women who do not have germline mutations in *TSC1* or *TSC2* (sporadic LAM). LAM cells from both TSC-LAM and sporadic LAM carry inactivating mutations in both alleles of the *TSC1* or *TSC2* genes (5). The protein products of *TSC1* and *TSC2*, hamartin and tuberin, respectively, form heterodimers (6, 7) that inhibit the small GTPase *R*as *h*omologue *e*nriched in *b*rain (Rheb), via tuberin's highly conserved GTPase activating domain. In its active form, Rheb activates the mammalian target of rapamycin (mTOR) complex 1 (TORC1), which is a key regulator of protein translation, cell size, and cell proliferation (8). Evidence of TORC1 activation, including hyperphosphorylation of ribosomal protein S6, has been observed in tumor specimens from TSC patients and LAM patients (9–11). Independent of its activation of mTOR, Rheb inhibits the activity of B-Raf and C-Raf/Raf-1 kinase, resulting in reduced phosphorylation of p42/44 MAPK (12–14), but the impact of the Raf/MEK/MAPK pathway on disease pathogenesis is undefined.

LAM is characterized pathologically by widespread proliferation of abnormal smooth muscle cells and by cystic changes within the lung parenchyma (1). About 60% of women with the sporadic form of LAM also have renal angiomyolipomas. The presence of *TSC2* mutations in LAM cells and renal angiomyolipoma cells from women with sporadic LAM, but not in normal tissues, has led to the hypothesis that LAM cells spread to the lungs via a metastatic mechanism, despite the fact that LAM cells have a histologically benign appearance (15, 16). Genetic and fluorescent in situ hybridization analyses of recurrent LAM after lung transplantation support this benign metastatic model (16).

The female predominance of LAM, coupled with the genetic data indicating that LAM cells are metastatic, suggests that estrogen may promote the metastasis of tuberin-null cells. Both LAM cells and angiomyolipoma cells express estrogen receptor alpha (17), and there are reports of symptom mitigation in LAM patients after oophorectomy and worsening of symptoms during pregnancy (1). However, the molecular and cellular mechanisms that may underlie an impact of estrogen on the metastasis of LAM cells are not well defined, in part because of the lack of in vivo models that recapitulate the metastatic behavior of LAM cells.

We report here that estrogen promotes the pulmonary metastasis of Tsc2-null ELT3 cells. This enhanced metastasis is associated with elevated levels of circulating tumor cells and with activation of p42/44 MAPK. When Tsc2-null cells are injected intravenously, E_2 enhances their survival and lung colonization, and in vitro, E_2 inhibits anoikis of Tsc2-null cells. In vivo, the MEK inhibitor CI-1040 blocks E_2-induced lung metastasis, decreases circulating tumor cells, and reduces lung colonization. Taken together, these data reveal that the MEK pathway is a critical component of the estrogen-dependent metastatic potential of Tsc2-null cells and lead to a unique model of LAM pathogenesis with therapeutic implications in which E_2 promotes the survival of disseminated LAM cells, thereby facilitating lung colonization and metastasis.

Results

Estrogen Promotes the Pulmonary Metastasis of Tuberin-Deficient ELT3 Cells in Ovariectomized Female Mice and in Male Mice. To study the role of E_2 in the metastasis of Tsc2-null cells, we used ELT3 cells, which were originally derived from a uterine leiomyoma in the Eker rat model of Tsc2 and, similar to LAM cells, express smooth muscle cell markers and estrogen receptor alpha (18, 19). To confirm that ELT3 cells proliferate in response to estrogen stimulation in vitro, cell growth was measured using ^{3}H-thymidine incorporation. E_2 treatment resulted in a significant increase in ^{3}H-thymidine incorporation by 2.8-fold on day 5 ($P = 0.03$, Fig. 1*A*), similar to the findings of Howe *et al.* (19).

ELT3 cells were inoculated s.c. into the flanks of ovariectomized CB17-*SCID* mice, which were supplemented 1 week before with either placebo or E_2 pellets (2.5 mg, 90-day release). Tumors arose in 100% of both estrogen and placebo-treated mice. At postinoculation week 8, estrogen-treated mice had a mean tumor area

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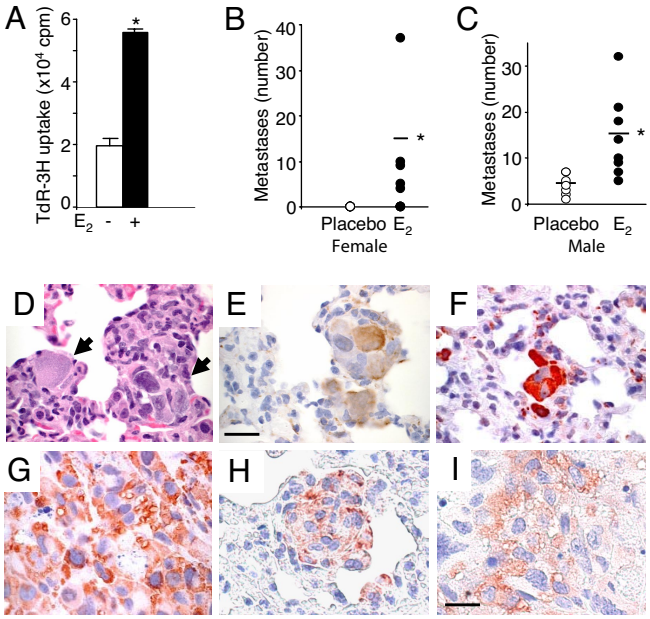

Fig. 1. Estrogen promotes the lung metastasis of tuberin-deficient ELT3 cells in female and male mice. (*A*) The proliferation of ELT3 cells in response to E_2 was measured by ^{3}H-thymidine incorporation after 5 days of growth. (*B–J*) ELT3 cells were injected s.c. into the flanks of female ovariectomized and male *SCID* mice implanted with E_2 ($n = 9$) or placebo ($n = 10$) pellets. (*B*) Lung metastases were scored from E_2 ($n = 9$) or placebo-treated ($n = 10$) mice. (*C*) The number of lung metastases in male mice was scored from placebo ($n = 10$) and E_2-treated ($n = 9$) mice. (*D–I*) Consecutive lung sections containing metastases (arrows) from an E_2-treated female mouse were stained with H&E (*D*), anti-smooth muscle actin (*E*), and anti-phospho-S6 (*F*). (Scale bar, 50 μM.) (*G*) Anti-phospho-S6 immunostain of the primary xenograft tumor of an estrogen-treated female mouse. (*H* and *I*) Phospho-S6 immunoreactivity of a metastasis (*H*) and xenograft tumor (*I*) of an estrogen-treated male mouse. (Scale bar, 20 μM.) *, $P < 0.05$, Student's *t* test.

of 287 ± 43 mm^2, whereas placebo-treated mice had a mean tumor area of 130 ± 20 mm^2 ($P = 0.0035$), consistent with previous findings (19). The proliferative potential of ELT3 cells in vivo was examined using Ki-67 immunoreactivity. The number of Ki-67 positive cells in estrogen-treated tumors was 17% higher than the number in placebo-treated tumors ($P = 0.03$).

Pulmonary metastases were identified in 5 of 9 E_2-treated mice (56%), with an average of 15 metastases/mouse (range 4–37) (Fig. 1*B*). In contrast, only 1 of 9 placebo-treated mice (10%) developed a single metastasis ($P = 0.039$). To determine whether the enhanced metastasis was directly related to tumor size, a subset of placebo-treated mice ($n = 4$) and estrogen-treated mice ($n = 4$) that developed primary tumors at similar size (209 ± 16 and 198 ± 20 mm^2, respectively) was analyzed separately. Three of the estrogen-treated mice developed pulmonary metastases with an average of 6 metastases/mouse, while none of the placebo-treated mice developed metastases.

Next, we inoculated ELT3 cells into male mice. At 8 weeks post-cell inoculation, E_2-treated animals developed tumors that were 2.9-fold larger than those in the placebo-treated animals. As in the female mice, E_2 significantly enhanced the frequency and the number of pulmonary metastases. At 8 weeks post-inoculation, 10 of 10 (100%) of the E_2-treated mice developed metastases, with an average of 14 metastases/mouse (range 5–32). In contrast, 7 of 10 (70%) of the placebo-treated mice developed metastases, with an average of 4 metastases/mouse (range 1–7, $P = 0.013$) (Fig. 1*C*). As expected, the metastatic and primary tumor cells were immunoreactive for smooth muscle actin and phospho-ribosomal protein S6 (Fig. 1 *D–I*).

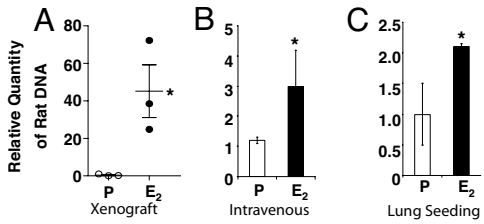

Fig. 2. Estrogen increases circulating tumor cells in mice bearing xenograft tumors and enhances the survival and lung seeding of intravenously injected Tsc2-null cells. (*A*) DNA prepared from the blood of placebo ($n = 3$) and E_2-treated ($n = 3$) mice bearing xenograft tumors of similar size (≈1,000 mm^3) was analyzed by real-time PCR using rat-specific primers to quantitate circulating tumor cells. (*B*) Levels of circulating tumor cell DNA 6 h after i.v. injection of ELT3 cells into placebo ($n = 3$) and E_2-treated ($n = 3$) mice. (*C*) Levels of tumor cell DNA in the lungs 24 h after i.v. injection of ELT3 cells into placebo ($n = 3$) and E_2-treated ($n = 3$) mice. *, $P < 0.05$, Student's *t* test.

Estrogen Increases Circulating Tumor Cell DNA. To determine whether the mechanism of E_2-driven metastasis of ELT3 cells is associated with an increase in survival of ELT3 cells in the circulation, we analyzed blood collected from xenograft mice at 7 weeks post-cell inoculation. Real-time PCR with rat-specific primers was used to measure the relative quantity of tumor cells circulating in the blood. We selected 6 animals (3 placebo, 3 E_2-treated) bearing tumors of similar size (≈1,000 mm^3) for this analysis. The E_2-treated animals had a striking increase in the amount of circulating tumor cell DNA as compared to that in the placebo-treated animals ($P = 0.034$, Fig. 2*A*).

This increased level of circulating tumor cell DNA suggested that E_2 may promote the survival of Tsc2-null cells upon dissemination from the primary tumor site. To test this, we injected 2×10^5 ELT3 cells intravenously and again measured the amount of tumor cell DNA using real-time PCR. E_2 treatment resulted in a 2.5-fold increase in circulating cells 6 h post-injection ($P = 0.047$, Fig. 2*B*). To determine whether this enhanced survival of circulating cells was associated with increased colonization of the lungs, the mice were killed 24 h after injection, and the lungs were analyzed by real-time PCR. E_2 treated mice had a 2-fold increase in the lung seeding of ELT3 cells ($P = 0.039$, Fig. 2*C*).

Estrogen Promotes the Lung Colonization of ELT3 Cells in Vivo. To identify the earliest time points at which estrogen exerts an effect on the survival of intravenously injected Tsc2-null cells, ELT3 cells that stably express luciferase (ELT3-Luc) were intravenously injected. The level of bioluminescence was evaluated using the Xenogen IVIS System. At 1 h post-cell injection, similar levels of bioluminescence were observed in the chest regions of E_2 and placebo-treated mice. By 3 h, the bioluminescence in the chest regions was 2-fold higher in the E_2-treated animals than in the placebo-treated animals, and at 24 h post-cell injection it was 5-fold higher in the E_2-treated animals ($P = 0.043$, Fig. 3 *A* and *B*). After sacrifice, the lungs were dissected and imaged in Petri dishes to confirm that the bioluminescent signals in the chest regions of the living mice were a result of lung colonization (Fig. 3*C*).

Estrogen Activates p42/44 MAPK in ELT3 Cells in Vitro and in Vivo. These results suggested that E_2 promotes the survival of disseminated ELT3 cells. To determine the mechanism of this, we focused on the Raf/MEK/MAPK signaling cascade. This pathway is inhibited in cells lacking TSC2 via Rheb's inhibition of B-Raf and C-Raf/Raf-1 kinase (13, 14). E_2 has been shown to activate p42/44 MAPK in ELT3 cells and in LAM patient-derived cells (11, 20, 21). To confirm that E_2 activates MAPK in ELT3 cells, we treated the cells with 10 nM E_2 and examined the phosphorylation status of p42/44 MAPK by immunoblotting. Within 15 min, E_2 induced the phosphorylation of p42/44 MAPK (Fig. 4*A*). We also found that

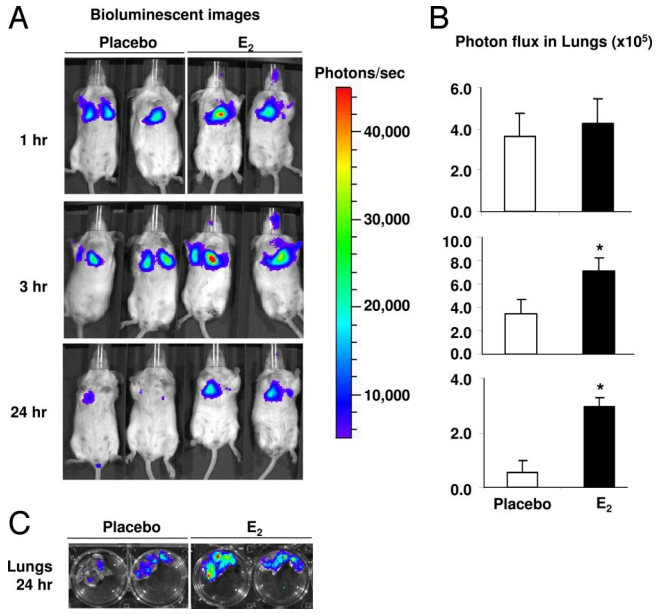

Fig. 3. Estrogen promotes the lung colonization of Tsc2-null ELT3 cells. (*A*) ELT3-luciferase cells were injected intravenously into ovariectomized female placebo ($n = 3$) and E_2-treated ($n = 3$) mice. Lung colonization was measured using bioluminescence at 1, 3, and 24 h after injection. Representative images are shown. (*B*) Total photon flux/second present in the chest regions in placebo ($n = 3$) and E_2-treated ($n = 3$) animals. *, $P < 0.05$, Student's *t* test. (*C*) Lungs were dissected 24 h postcell injection and bioluminescence was imaged in Petri dishes.

E_2-induced phosphorylation of p42/44 MAPK was blocked by the MEK1/2 inhibitor PD98059 (Fig. 4*A*), which is in contrast to the prior work of Finlay *et al.* (20). E_2 is known to rapidly activate C-Raf (22). We hypothesized that E_2 reactivates MAPK via a Rheb-independent pathway in cells lacking tuberin. In a separate experiment, we found that E_2 rapidly (within 2 min) increased the phosphorylation of C-Raf at Ser-338, a site which is closely linked with C-Raf activity (Fig. 4*B*). However, E_2 does not affect mTOR activation as measured by ribosomal protein S6 phosphorylation (Fig. 4*C*). These results suggest that E_2 does not regulate Rheb activity and that the potential of E_2 to impact the Raf/MEK/ERK kinase cascade is Rheb independent. Nuclear translocation of phospho-MAPK was observed within 5 min of E_2 exposure (Fig. 4*D*).

These in vitro findings led us to examine whether E_2 activates p42/44 MAPK in ELT3 cells in vivo. In lungs from E_2-treated animals, nuclear phospho-p42/44 MAPK staining was observed in metastases but not in adjacent normal tissues (Fig. 4 *E* and *F*). In the primary xenograft tumors, the percentage of cells with primarily nuclear phospho-MAPK was significantly higher in the tumors from the E_2-treated animals, compared to the tumors from placebo-treated animals (65% vs. 28%, $P = 0.001$, Fig. 4 *G–I*).

Estrogen Increases the Resistance of ELT3 Cells to Anoikis in Vitro. These in vivo findings suggest that estrogen enhances the survival of circulating tumor cells in a MAPK-dependent manner. Because detached cells normally undergo apoptosis (23–25), a critical first step in cancer progression is the development of resistance to matrix deprivation-induced apoptosis (anoikis) (26, 27). Therefore, to investigate the mechanism of E_2-prolonged survival of ELT3 cells in the circulation, we examined the effect of estrogen on anoikis. ELT3 cells were treated for 24 h with either 10 nM E_2 or control and then plated onto PolyHEMA, which prevents attachment and therefore induces anoikis. Cell lysates were immunoblotted for cleaved caspase-3, which is a measure of apoptosis. E_2 treatment reduced caspase-3 cleavage at 6, 16, and 24 h (Fig. 5*A*). E_2 treatment also significantly reduced DNA fragmentation at 1 and 24 h ($P =$

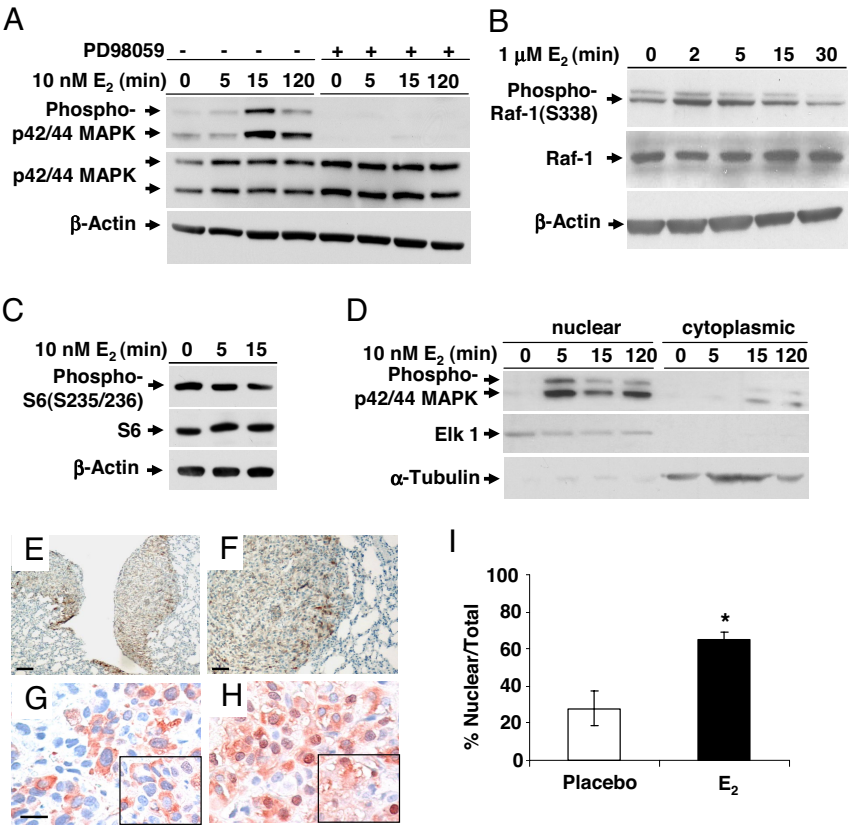

Fig. 4. Estrogen activates p42/44 MAPK in ELT3 cells in vitro and in vivo. (*A*) ELT3 cells were grown in phenol red-free and serum-free media for 24 h and then stimulated with 10 nM E_2 for 0, 5, 15, or 120 min. Levels of phosphorylated p42/44 MAPK and total MAPK were determined by immunoblot analysis. Pretreatment with PD98059 blocked E_2-induced MAPK activation. β-Actin immunoblotting was included as a loading control. (*B*) Levels of phosphorylated C-Raf/Raf-1 and total Raf-1 after E_2 stimulation. (*C*) Levels of phosphorylated S6 after E_2 stimulation. (*D*) The nuclear and cytoplasmic fractions were separated, and levels of phospho-p42/44 MAPK were examined by immunoblot analysis. Anti-ELK1 and anti-α-tubulin were included as loading controls for the nuclear and cytosolic fractions, respectively. (*E* and *F*) Pulmonary metastases from an E_2-treated mouse showed hyperphosphorylation of p42/44 MAPK. (Scale bar, 50 μM and 125 μM.) (*G* and *H*) Phospho-p42/44 MAPK (T202/Y204) immunostaining of primary tumor sections from placebo-treated (*G*) and E_2-treated (*H*) mice. (Scale bar, 20 μM.) (*I*) Percentage of cells with nuclear immunoreactivity of phospho-p42/44 MAPK was scored from 4 random fields per section. *, $P < 0.05$, Student's *t* test.

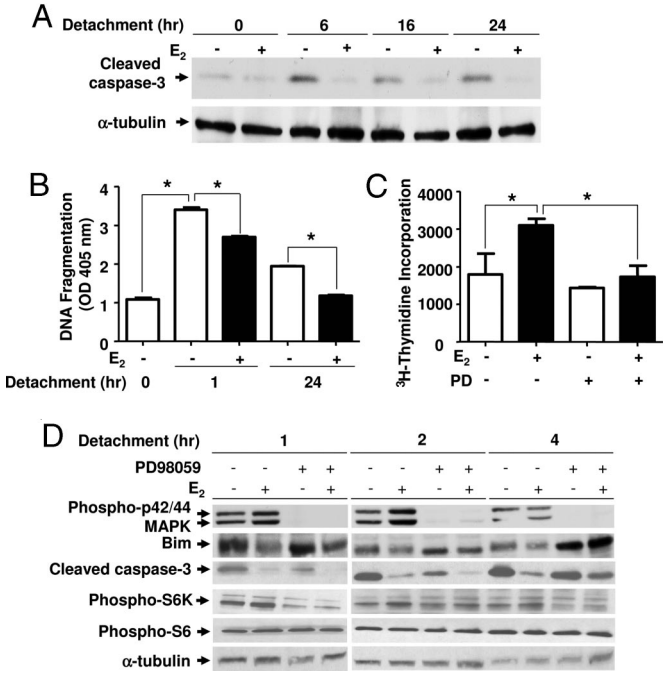

Fig. 5. Estrogen increases the resistance of ELT3 cells to anoikis. ELT3 cells were grown in phenol red-free and serum-free media for 24 h and then treated with 10 nM E_2 for 24 h before culturing on PolyHEMA plates. The MEK1/2 inhibitor PD98059 was begun 15 min before detachment. (*A*) The level of cleaved caspase-3 was determined by immunoblot analysis. α-Tubulin is included as a loading control. (*B*) DNA fragmentation was assessed by ELISA. (*C*) Cell growth was measured by ^{3}H-thymidine incorporation after 24 h of growth on PolyHEMA plates in the presence or absence of E_2, followed by 24 h of growth on adherent plates in the absence of E_2. (*D*) Levels of phospho-p42/44 MAPK, MAPK, Bim, cleaved caspase-3, phospho-S6K, and phospho-S6 were determined by immunoblot analysis. α-Tubulin is included as a loading control. *, $P < 0.05$, Student's *t* test.

0.001 and $P = 0.015$, Fig. 5*B*), which indicates that E_2 inhibits anoikis of Tsc2-null cells.

To confirm further that E_2 promotes the survival of detached cells, ELT3 cells were plated onto PolyHEMA plates for 24 h and replated onto normal tissue culture dishes. Cell growth was measured using ^{3}H-thymidine incorporation. E_2 treatment resulted in a significant increase in ^{3}H-thymidine incorporation 24 h after replating ($P = 0.008$, Figure 5*C*). This E_2-enhanced survival was blocked by treatment with the MEK1/2 inhibitor PD98059 ($P = 0.035$, Fig. 5*C*).

To determine the components that mediate estrogen-enhanced resistance of ELT3 cells to anoikis, we analyzed the proapoptotic protein, Bcl-2 interacting mediator of cell death (Bim), which is known to be a critical activator of anoikis (23). Bim is phosphorylated by protein kinases, including p42/44 MAPK, which leads to rapid proteasomal-mediated degradation and increased cell survival (28). Bim protein level was examined by immunoblotting. We found that estrogen decreased the accumulation of Bim after 1 h in detachment conditions (Fig. 5*D*). Preincubation with the MEK inhibitor PD98059 partially blocked estrogen's inhibition of Bim accumulation and caspase-3 cleavage after 4 h in detachment conditions (Fig. 5*D*). We also examined the phosphorylation of S6K and S6 in detachment conditions and found that the phosphorylation of S6K and S6 did not change with E_2 stimulation. Interestingly, treatment with PD98059 decreased the phosphorylation of S6K 1 h after detachment (Fig. 5*D*).

The MEK1/2 Inhibitor CI-1040 Blocks the Estrogen-Driven Metastasis of ELT3 Cells in Vivo. These in vitro and in vivo results suggest that E_2-induced activation of the MEK/MAPK pathway contributes to the metastatic potential of circulating Tsc2-null ELT3 cells. To determine the effect of inhibiting the MEK/MAPK pathway on the pulmonary metastasis of Tsc2-null cells in vivo, we used the MEK1/2 inhibitor, CI-1040. Beginning 1 day post-subcutaneous inoculation of ELT3 cells, animals, implanted with either placebo or estrogen pellets, were treated with CI-1040 (150 mg/kg day by gavage, twice a day) (29). CI-1040 delayed tumor formation (Fig. 6*A*) and reduced the size of primary tumors by 25% in E_2 animals (Fig. 6*B*), although these data did not reach statistical significance. CI-1040, however, significantly reduced the levels of circulating ELT3 cells in the blood of E_2-treated animals by 84% ($P = 0.042$, Fig. 6*C*). Most strikingly, no lung metastases were detected in mice treated with E_2 plus CI-1040 ($P = 0.046$, Fig. 6 *D* and *E*).

To investigate further the role of MEK/ERK on the survival of ELT3 cells in the circulation, ELT3-luciferase cells were intravenously injected into mice treated with E_2 alone or E_2 plus CI-1040. At 2 h post-cell injection, similar levels of bioluminescence were observed in the chest regions of all mice. At 5 h, the bioluminescence in the chest regions of the E_2 plus CI-1040 treated mice was decreased by 55%, as compared to that in the E_2-treated mice ($P = 0.02$, Fig. 6*F*). After sacrifice at 60 h postcell injection, the bioluminescent signals in the ex vivo lungs of the E_2 plus CI-1040-treated mice were significantly reduced by 96%, as compared to the signals in the E_2-treated animals ($P = 0.0045$, Fig. 6*F*).

Inhibition of mTOR Blocks Estrogen-Induced Pulmonary Metastasis of Tsc2-Null Cells. To determine the role of mTOR signaling pathway in the estrogen-induced metastasis of tuberin-deficient ELT3 cells, the mTORC1 inhibitor RAD001 (4 mg/kg/day by gavage) was administered 5 days per week beginning 1 day post-cell inoculation. RAD001 completely blocked both primary tumor development (Fig. 7*A*) and lung metastasis (Fig. 7*B*) in the presence of estrogen or placebo.

Discussion

LAM is associated with a very unusual disease mechanism: the metastasis of histologically benign TSC1 or TSC2-null cells. LAM has one of the strongest gender predispositions of any extragenital human disease, with a higher female-to-male ratio than even breast cancer. Estrogen receptor alpha is expressed in LAM cells and in angiomyolipoma cells from LAM patients (17), and estrogen has been shown to activate p42/44 MAPK and stimulate the proliferation of Tsc2-null ELT3 cells and TSC2-null angiomyolipoma cells (11). Estrogen has also been shown to enhance liver hemangioma development in Tsc2± mice (30). Despite these findings, the role of estrogen in LAM pathogenesis is not well defined.

We report here that estrogen treatment of both female and male mice bearing Tsc2-null ELT3 xenograft tumors results in an increase in pulmonary metastases. The estrogen-driven metastasis of ELT3 cells was associated with activation of p42/44 MAPK both in vitro and in vivo. Treatment of the mice with the MEK1/2 inhibitor CI-1040 completely blocked the lung metastases in estrogen-treated animals, while causing only a 25% reduction in the size of the primary xenograft tumors, indicating that activation of MEK by E_2 is a critical factor in the metastasis of Tsc2-null cells. In contrast to CI-1040, the mTOR inhibitor RAD001 completely blocked formation of the primary tumor.

Estrogen is known to activate the MAPK pathway (31–34). We speculate that tuberin-null cells may be particularly sensitive to activation of the Raf/MEK/MAPK signaling cascade by estrogen, because at baseline this signaling pathway is inhibited by Rheb, the target of tuberin's GTPase activating protein domain (12–14). Metastasis is a complex process, and there are numerous mechanisms through which estrogen's activation of MEK may enhance the metastasis of Tsc2-null cells. Our in vitro studies revealed that estrogen induces resistance to anoikis in Tsc2-null cells, which suggests that one of these mechanisms involves the survival of detached cells. Consistent with this, we found markedly elevated

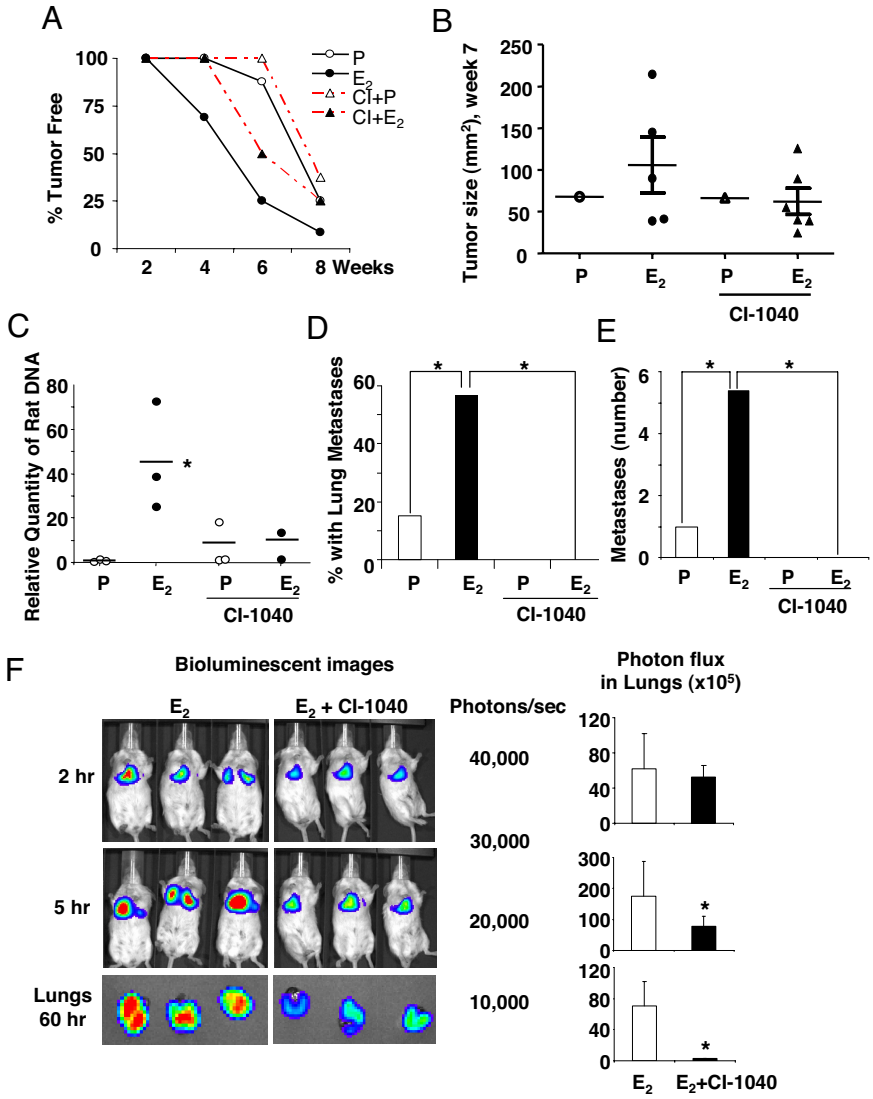

Fig. 6. The MEK1/2 inhibitor CI-1040 blocks the estrogen-driven metastasis of ELT3 cells in vivo. ELT3 cells were injected into female ovariectomized nude mice implanted with estrogen or placebo pellets. Animals were treated with CI-1040 (150 mg/kg/day by gavage, twice a day) starting 1 day post-ELT3 cell inoculation for the xenograft experiments (*A–E*), or 2 days before cell inoculation for i.v. injection (*F*). (*A*) Tumor development was recorded as the percentage of tumor-free animals post-cell inoculation. (*B*) The primary tumor area was calculated at 7 weeks post-cell inoculation. (*C*) The level of circulating ELT3 cells was measured from blood samples of xenograft animals using rat-specific qPCR amplification. (*D*) The percentage of mice with lung metastases in the placebo and estrogen-treated groups was compared. (*E*) The number of lung metastases was scored. (*F*) ELT3-luciferase cells were injected intravenously into ovariectomized female E_2-treated ($n = 5$) and CI-1040 plus E_2-treated ($n = 5$) mice. Lung colonization was measured using bioluminescence 2 and 5 h after injection. Total photon flux/second present in the chest regions were quantified and compared between E_2 ($n = 5$) and CI-1040 plus E_2-treated ($n = 5$) animals. Lungs were dissected and imaged 60 h post-cell injection. Total photon flux/second present in ex vivo lungs were quantified and compared between E_2 ($n = 5$) and CI-1040 plus E_2-treated ($n = 5$) animals. *, $P < 0.05$, Student's *t* test.

levels of circulating tumor cells in estrogen-treated mice bearing xenograft tumors. We also found that estrogen treatment enhances the survival of intravenously injected cells in the peripheral blood. These data are of particular interest because circulating LAM cells can be detected in the blood and pleural fluid of women with LAM (35). Our data provide a rationale for the potential use of circulating cells as a quantitative and rapid biomarker of response to targeted therapy in women with LAM.

In addition to promoting the levels of ELT3 cells in the peripheral blood, as measured by real-time RT-PCR using rat-specific primers, estrogen also enhanced the survival of intravenously injected luciferase-expressing ELT3 cells within the lungs. Three hours after injection, there was significantly more bioluminescence in the chest regions of the E_2-treated animals, and by 24 h this difference was even more marked. Importantly, however, 1 h after the i.v. injection of ELT3-luciferase cells, similar levels of bioluminescence were present in the lungs of estrogen-treated and placebo-treated animals, which demonstrates that similar numbers of injected cells reach the lungs. These data suggest that E_2 promotes the survival of Tsc2-null cells within the lungs.

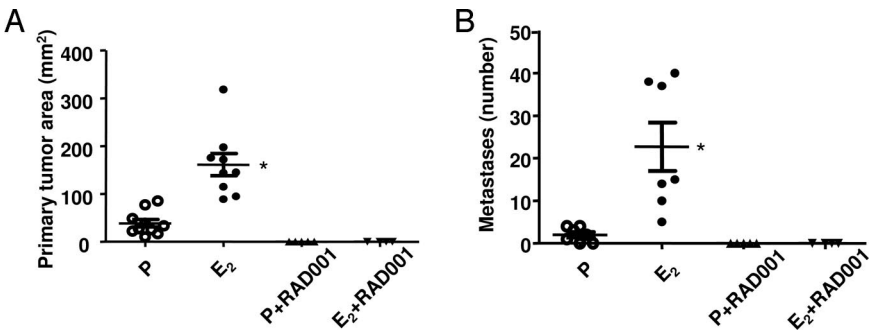

Fig. 7. The mTOR inhibitor RAD001 blocks primary tumor development and estrogen-driven metastasis of ELT3 cells in vivo. ELT3 cells were injected into female ovariectomized nude mice implanted with estrogen or placebo pellets. Animals were treated with RAD001 (4 mg/kg/day by gavage) starting 1 day post-ELT3 cell inoculation. (*A*) The primary tumor area was calculated at 8 weeks post-cell inoculation. (*B*) The number of lung metastases was scored at 8 weeks post-cell inoculation. *, $P < 0.05$, Student's *t* test.

The lack of an in vivo model of LAM has been a significant barrier in LAM research. While not a perfect surrogate, ELT3 cells have important features in common with LAM cells, including loss of Tsc2, activation of mTOR, and expression of estrogen receptor alpha and smooth muscle markers (18, 19). We are optimistic that this model of estrogen-induced metastasis will allow agents to be tested preclinically, thereby facilitating the development of therapies for LAM. Currently the only effective therapy for end-stage LAM is lung transplantation, and many women die while awaiting a donor lung or as a complication of the transplantation. There are multiple nodes that one can target in the estrogen/MEK/MAPK pathway, including inhibition of estrogen production, inhibition of the estrogen receptor, and inhibition of Raf/MEK.

Taken together, our data highlight a unique model for LAM pathogenesis in which activation of MEK by estrogen promotes the survival of detached tuberin-null cells. It will be important to confirm these findings using patient-derived cells, although this will be challenging because of the difficulties in establishing cultures of LAM cells. An alternative would be to measure levels of circulating LAM cells in women receiving hormonal therapy in the context of a clinical trial. If our model is correct, then important effects of estrogen on LAM pathogenesis may occur before the LAM cells reach the lungs and/or within the first hours of their reaching the lungs. Therefore, targeting estrogen signaling may have a major role in the treatment of early-stage LAM and/or in the prevention of LAM in women with TSC.

Methods

ELT-3 cells are Eker rat uterine leiomyoma-derived smooth muscle cells and were used in all in vitro and in vivo studies. For in vivo studies, female ovariectomized CB17-*SCID* mice were implanted with 17-beta estradiol or placebo pellets (2.5 mg, 90-day release) 1 week prior to cell inoculation. For xenograft tumor establishment, 2×10^6 ELT3 cells were bilaterally injected into the rear flanks of the mice. For intravenous injections, 2×10^5 ELT3 or ELT3-Luc cells were injected into the lateral tail vein. Lung metastases were scored from 5-micron H&E-stained sections of each lobe. CI-1040 (150 mg/kg day by gavage, twice per day) or RAD001 (4 mg/kg per day by gavage) was initiated 1 day after cell inoculation. To detect circulating ELT3 cells, 0.5 mL of mouse blood was collected by intraocular bleed, red blood cells were lysed, and genomic DNA was extracted. At death, lungs were dissected for DNA extraction. The assay for rat DNA was adapted from the method described by Walker *et al*. (36). Bioluminescent reporter imaging was performed to monitor the lung seeding of ELT3-Luciferase cells. Ten minutes prior to imaging, animals were injected with luciferin (Xenogen) (120 mg/kg, i.p.). Bioluminescent signals were recorded at indicated times post-cell injection using the Xenogen IVIS System. Total photon flux at the chest regions and from the dissected lungs was analyzed. For anoikis studies, ELT3 cells with or without 10 nM E_2 pretreatment were plated onto poly-hydroxyethyl methacrylate (PolyHEMA) culture dishes. Cell death as a function of DNA fragmentation was detected using Cell Death Detection ELISA kit (Roche Diagnostics). Full methods are available in *SI Text*.

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1. Sullivan EJ (1998) Lymphangioleiomyomatosis: review. *Chest* 114:1689–1703.
2. Costello LC, Hartman TE, Ryu JH (2000) High frequency of pulmonary lymphangioleiomyomatosis in women with tuberous sclerosis complex. *Mayo Clin Proc* 75:591–594.
3. Franz DN, *et al.* (2001) Mutational and radiographic analysis of pulmonary disease consistent with lymphangioleiomyomatosis and micronodular pneumocyte hyperplasia in women with tuberous sclerosis. *Am J Respir Crit Care Med* 164:661–668.
4. Shepherd CW, Gomez MR, Lie JT, Crowson CS (1991) Causes of death in patients with tuberous sclerosis. *Mayo Clin Proc* 66:792–796.
5. Yu J, Astrinidis A, Henske EP (2001) Chromosome 16 loss of heterozygosity in tuberous sclerosis and sporadic lymphangiomyomatosis. *Am J Respir Crit Care Med* 164:1537–1540.
6. Plank TL, Yeung RS, Henske EP (1998) Hamartin, the product of the tuberous sclerosis 1 (TSC1) gene, interacts with tuberin and appears to be localized to cytoplasmic vesicles. *Cancer Res* 58:4766–4770.
7. van Slegtenhorst M, *et al.* (1998) Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products. *Hum Mol Genet* 7:1053–1057.
8. Crino PB, Nathanson KL, Henske EP (2006) The tuberous sclerosis complex. *N Engl J Med* 355:1345–1356.
9. El-Hashemite N, Zhang H, Henske EP, Kwiatkowski DJ (2003) Mutation in TSC2 and activation of mammalian target of rapamycin signalling pathway in renal angiomyolipoma. *Lancet* 361:1348–1349.
10. Karbowniczek M, Yu J, Henske EP (2003) Renal angiomyolipomas from patients with sporadic lymphangiomyomatosis contain both neoplastic and non-neoplastic vascular structures. *Am J Pathol* 162:491–500.
11. Yu J, Astrinidis A, Howard S, Henske EP (2004) Estradiol and tamoxifen stimulate LAM-associated angiomyolipoma cell growth and activate both genomic and non-genomic signaling pathways. *Am J Physiol Lung Cell Mol Physiol* 286:L694–700.
12. Im E, *et al.* (2002) Rheb is in a high activation state and inhibits B-Raf kinase in mammalian cells. *Oncogene* 21:6356–6365.
13. Karbowniczek M, *et al.* (2004) Regulation of B-Raf kinase activity by tuberin and Rheb is mammalian target of rapamycin (mTOR)-independent. *J Biol Chem* 279:29930–29937.
14. Karbowniczek M, Robertson GP, Henske EP (2006) Rheb inhibits C-raf activity and B-raf/C-raf heterodimerization. *J Biol Chem* 281:25447–25456.
15. Carsillo T, Astrinidis A, Henske EP (2000) Mutations in the tuberous sclerosis complex gene TSC2 are a cause of sporadic pulmonary lymphangioleiomyomatosis. *Proc Natl Acad Sci USA* 97:6085–6090.
16. Karbowniczek M, *et al.* (2003) Recurrent lymphangiomyomatosis after transplantation: genetic analyses reveal a metastatic mechanism. *Am J Respir Crit Care Med* 167:976–982.
17. Logginidou H, Ao X, Russo I, Henske EP (2000) Frequent estrogen and progesterone receptor immunoreactivity in renal angiomyolipomas from women with pulmonary lymphangioleiomyomatosis. *Chest* 117:25–30.
18. Howe SR, *et al.* (1995) Rodent model of reproductive tract leiomyomata. Establishment and characterization of tumor-derived cell lines. *Am J Pathol* 146:1568–1579.
19. Howe SR, Gottardis MM, Everitt JI, Walker C (1995) Estrogen stimulation and tamoxifen inhibition of leiomyoma cell growth in vitro and in vivo. *Endocrinology* 136:4996–5003.
20. Finlay GA, Hunter DS, Walker CL, Paulson KE, Fanburg BL (2003) Regulation of PDGF production and ERK activation by estrogen is associated with TSC2 gene expression. *Am J Physiol Cell Physiol* 285:C409–418.
21. Finlay GA, *et al.* (2004) Estrogen-induced smooth muscle cell growth is regulated by tuberin and associated with altered activation of platelet-derived growth factor receptor-beta and ERK-1/2. *J Biol Chem* 279:23114–23122.
22. Pratt MA, Satkunaratnam A, Novosad DM (1998) Estrogen activates raf-1 kinase and induces expression of egr-1 in MCF-7 breast cancer cells. *Mol Cell Biochem* 189:119–125.
23. Reginato MJ, *et al.* (2003) Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. *Nat Cell Biol* 5:733–740.
24. Rytomaa M, Martins LM, Downward J (1999) Involvement of FADD and caspase-8 signalling in detachment-induced apoptosis. *Curr Biol* 9:1043–1046.
25. Schulze A, Lehmann K, Jefferies HB, McMahon M, Downward J (2001) Analysis of the transcriptional program induced by Raf in epithelial cells. *Genes Dev* 15:981–994.
26. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100:57–70.
27. Eckert LB, *et al.* (2004) Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoikis. *Cancer Res* 64:4585–4592.
28. Tan TT, *et al.* (2005) Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. *Cancer Cell* 7:227–238.
29. Sebolt-Leopold JS, *et al.* (1999) Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nat Med* 5:810–816.
30. El-Hashemite N, Walker V, Kwiatkowski DJ (2005) Estrogen enhances whereas tamoxifen retards development of Tsc mouse liver hemangioma: a tumor related to renal angiomyolipoma and pulmonary lymphangioleiomyomatosis. *Cancer Res* 65:2474–2481.
31. Migliaccio A, *et al.* (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J* 15:1292–1300.
32. Razandi M, Pedram A, Park ST, Levin ER (2003) Proximal events in signaling by plasma membrane estrogen receptors. *J Biol Chem* 278:2701–2712.
33. Song RX, *et al.* (2002) Linkage of rapid estrogen action to MAPK activation by ERalpha-Shc association and Shc pathway activation. *Mol Endocrinol* 16:116–127.
34. Song RX, Zhang Z, Chen Y, Bao Y, Santen RJ (2007) Estrogen signaling via a linear pathway involving insulin-like growth factor I receptor, matrix metalloproteinases, and epidermal growth factor receptor to activate mitogen-activated protein kinase in MCF-7 breast cancer cells. *Endocrinology* 148:4091–4101.
35. Crooks DM, *et al.* (2004) Molecular and genetic analysis of disseminated neoplastic cells in lymphangioleiomyomatosis. *Proc Natl Acad Sci USA* 101:17462–17467.
36. Walker JA, *et al.* (2004) Quantitative PCR for DNA identification based on genome-specific interspersed repetitive elements. *Genomics* 83:518–527.

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Potential of L-buthionine sulfoximine to enhance the apoptotic action of estradiol to reverse acquired antihormonal resistance in metastatic breast cancer☆

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ABSTRACT

L-Buthionine sulfoximine (BSO) is a potent inhibitor of glutathione biosynthesis and studies have shown that it is capable of enhancing the apoptotic effects of several chemotherapeutic agents. Previous studies have shown that long-term antihormonal therapy leads to acquired drug resistance and that estrogen, which is normally a survival signal, is a potent apoptotic agent in these resistant cells. Interestingly, we have developed an antihormone-resistant breast cancer cell line, MCF-7:2A, which is resistant to estrogen-induced apoptosis but has elevated levels of glutathione. In the present study, we examined whether BSO is capable of sensitizing antihormone-resistant MCF-7:2A cells to estrogen-induced apoptosis. Our results showed that treatment of MCF-7:2A cells with 1 nM E2 plus 100 μM BSO combination for 1 week reduced the growth of these cells by almost 80–90% whereas the individual treatments had no significant effect on growth. TUNEL and 4′,6-diamidino-2-phenylindole (DAPI) staining showed that the inhibitory effect of the combination treatment was due to apoptosis. Our data indicates that glutathione participates in retarding apoptosis in antihormone-resistant human breast cancer cells and that depletion of this molecule by BSO may be critical in predisposing resistant cells to estrogen-induced apoptosis.

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1. Introduction

Breast cancer continues to be the most common malignancy affecting women. Although great strides have been made in the treatment and cure of early stage breast cancer, metastatic breast cancer remains incurable resulting in 40,000 deaths per year in the United States alone [1]. Approximately two-thirds of all breast cancers contain the estrogen receptor (ER) and/or progesterone receptor (PgR) and are termed hormonally sensitive disease. A significant proportion of these hormonally sensitive breast cancers are dependent upon estrogenic stimulation for survival and growth [2].

Historically, various techniques employing estrogen deprivation have been utilized to exploit this feature in the treatment of hormonally sensitive breast cancers. Until recently, tamoxifen has been considered to be the hormonal therapy of choice for the treatment of ER-positive breast cancers [3]. Now, survival benefits have been demonstrated for the third generation aromatase inhibitors [4] and the pure anti-estrogen, fulvestrant, that causes degradation of the ER [5].

The use of exhaustive anti-estrogen therapies has consequences for the tumor [6]. With continued long-term estrogen deprivation, these initially hormonally sensitive breast cancer cells become sequentially resistant to further anti-estrogen therapy [7–9], indicating that they develop sophisticated survival mechanisms to sustain growth in estrogen deprived environments (Fig. 1). Jordan and colleagues have demonstrated that when estrogen receptor positive breast cancer cells are grown and maintained in long-term estrogen deprived (LTED) environments, they can ultimately develop enhanced responsiveness to greatly diminished levels of estrogen [6,7,10]. These pre-clinical animal models show that initially, ER expressing tumors are stimulated by estrogen and respond appropriately to tamoxifen with tumor regression. However, with continued exposure to tamoxifen, the tumors become resistant and re-grow [9]. Additionally, treatment of these LTED tumors with post-menopausal levels of estrogen inhibits tumor growth as well as causes regression of established tamoxifen resistant tumors [7,8,11,12] (Fig. 1).

Clinical data supports the use of estrogen to treat hormonally sensitive breast cancers. In the past, pharmacologic doses of estrogen were a commonly employed therapy that resulted in durable responses with regression of disease [13] with as high as 40% response rate as first-line treatment in patients with hormonally sensitive breast cancer with metastatic disease [3] and approximately 31% (44% clinical benefit rate) in patients heavily pre-treated with previous endocrine therapies [14]. Long-term survival data for

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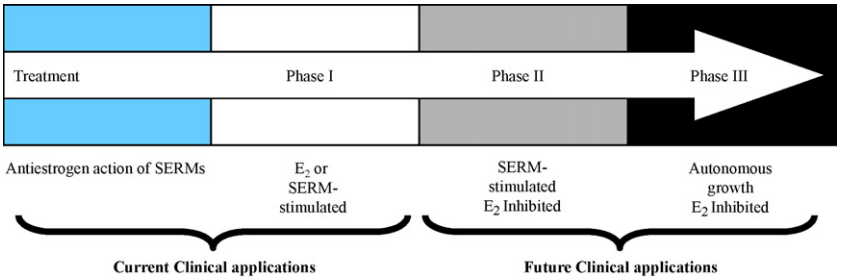
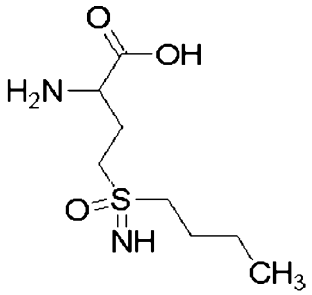


Fig. 1. Evolution of drug resistance to selective estrogen receptor modulations (SERMs). Acquired resistance occurs during long-term treatment with a SERM and is evidenced by SERM-stimulated breast tumor growth. Tumors also continue to exploit estrogen for growth when the SERM is stopped, so a dual signal transduction process develops. The aromatase inhibitors prevent tumor growth in SERM-resistant disease and fulvestrant that destroys the ER is also effective. This phase of drug resistance is referred to as phase I resistance. Continued exposure to a SERM results in continued SERM-stimulated growth, but eventually autonomous growth occurs that is unresponsive to fulvestrant or aromatase inhibitors. The event that distinguishes phase I from phase II acquired resistance is a remarkable switching mechanism that now causes apoptosis, rather than growth, with physiologic levels of estrogen.

pharmacologic estrogen treatment in the patients treated as first-line therapy for hormonally sensitive metastatic breast cancer has yielded a statistically significant 5-year survival benefit in favor of estrogen when compared to tamoxifen, 35% and 16% respectively. This clinical data is consistent with the pre-clinical models of Jordan and colleagues that show that after exhaustive anti-hormonal treatment, estrogen treatment produces tumor apoptosis and rapid tumor regression [8,9].

Therefore, we have hypothesized that treatment with a defined course of estrogen in post-menopausal women with ER-positive metastatic breast cancer whose disease has progressed after initial response to sequential anti-estrogen therapies, will result in clinical responses and may potentially reverse hormonally refractory disease, resulting in additional clinical benefit with further endocrine treatment such as an aromatase inhibitor, in this heavily endocrine pre-treated population. We are currently evaluating the optimal dose of daily estradiol therapy to reverse antihormonal resistance [6] but the goal is to enhance the estradiol-induced apoptotic response.

Increased intracellular glutathione has long been associated with tumor cell resistance to various cytotoxic agents. Studies have shown that L-buthionine sulfoximine (BSO) (Fig. 2), a potent inhibitor of glutathione biosynthesis [15], sensitizes tumor cells to apoptosis induced by standard chemotherapeutic drugs *in vitro* and *in vivo* [16,17]. We previously reported the development of a long-term estrogen deprived breast cancer cell line, MCF-7:2A [18], which appeared to be resistant to estradiol-induced apoptosis but expressed elevated levels of glutathione. We believe that the combination of BSO and estradiol could possibly be used to improve the efficacy of estradiol as an apoptotic agent if glutathione depletion is fundamental to tumor cell survival. Our goal is to address the hypothesis that by altering glutathione levels, we may be able to enhance estrogen-induced apoptosis and have employed BSO as our agent of choice.



Chemical structure of L-buthionine sulfoximine (BSO)

Fig. 2. Chemical structure of L-buthionine sulfoximine.

In the current study, we investigated the *in vitro* effect of the combination of BSO and estradiol (E2) on MCF-7:2A cell viability in relation to apoptosis. We found that BSO or E2, as individual treatments, did not significantly alter the viability of MCF-7:2A cells nor induced apoptosis. However, the combined treatment of BSO and E2 depleted glutathione content and induced significant apoptosis in MCF-7:2A cells. In contrast, similar experiments performed in wild-type hormone responsive MCF-7 cells showed no apoptosis or growth inhibition following the combination treatment of BSO and E2. Our data indicates that glutathione participates in retarding apoptosis in antihormone-resistant human breast cancer cells and that depletion of this molecule by BSO may be critical in predisposing resistant cells to E2-induced apoptotic cell death. We suggest that these data may form the basis of improving therapeutic strategies for the treatment of antihormone-resistant ER-positive breast cancer.

2. Materials and methods

2.1. Cell culture and reagents

The MCF-7 human breast cancer cell line was obtained from Dr. Dean Edwards (University of Texas, San Antonio, TX) and was maintained in phenol red RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1× non-essential amino acids and bovine insulin at 6 ng/mL. The clonal cell line, MCF-7:2A [18], was derived by growing MCF-7 cells in estrogen-free media for more than 1 year, followed by two rounds of limiting dilution cloning. These cells were grown in phenol red-free RPMI 1640 medium supplemented with 10% 4× dextran-coated, charcoal-treated FBS (SFS). All reagents for cell culture were obtained from Invitrogen. BSO and 17beta-estradiol (E2) were from Sigma.

2.2. Cell proliferation

Prior to the start of the cell growth assay, parental MCF-7 cells were grown in estrogen-free RPMI media containing 10% SFS for 3 days. This procedure was performed in order to remove any endogenous estrogen from the serum. On the day of the experiment, MCF-7 and MCF-7:2A cells were seeded in estrogen-free RPMI media containing 10% SFS at a density of 5×10^5 cells per 15-cm dish. After 24 h, cells were treated with nothing (control), 10^{-9} M E2, increasing concentrations of BSO (10 μM to 2.5 mM) either alone or combined with 10^{-9} M E2 for 1 week with retreatment on alternate days. At the indicated time point, the DNA content of the cells was determined as previously described [8] using a Fluorescent DNA Quantitation kit (Bio-Rad). For each analysis, six replicate wells were used, and at least three independent experiments were performed.

2.3. TUNEL staining for apoptosis

Apoptosis was determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay using an *in situ* cell death detection kit, POD (Roche Molecular Biochemicals), according to the manufacturer's instructions. Briefly, fixed cells were washed, permeabilized, and then incubated with 50 μL of terminal deoxynucleotidyl transferase end-labeling cocktail for 60 min at 37 °C in a humidified atmosphere in the dark. For signal conversion, slides were incubated with 50 μL of converter-POD (anti-fluorescein antibody conjugated with horse-radish peroxidase) for 30 min at 37 °C, rinsed with PBS, and then incubated with 50 μL of DAB substrate solution for 10 min at 25 °C. The slides were then rinsed with PBS, mounted under glass coverslips, and analyzed under a light microscope (Inverted Nikon TE300).

2.4. 4′,6-Diamidino-2-phenylindole (DAPI) staining for apoptosis

MCF-7:2A cells were grown (overnight) in RPMI medium containing 10% dextran-coated charcoal stripped fetal bovine serum (SFS) and then treated with ethanol vehicle (i.e., control), 1 nM estradiol, 100 μM BSO, or BSO + E2 for 72 h. The cells were then washed in PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and washed again in PBS. Cells were then treated with 1 μg/mL of DAPI (Sigma Chemical Co.) for 30 min, washed again with PBS for 5 min, and treated with 50 μL of VectaShield (Vector Laboratories, Burlingame, CA). Stained nuclei were visualized and photographed using a Zeiss fluorescence microscope (Provis AX70; Olympus Optical Co., Japan). Apoptotic cells were morphologically defined by cytoplasmic and nuclear shrinkage and by chromatin condensation or fragmentation.

2.5. Glutathione assay

Total cellular glutathione was measured using the Total Glutathione Colorimetric microplate assay Kit (Oxford Biomedical Research), according to the manufacture's protocol. Cells were plated at 0.5×10^6/well of a six-well plate and allowed to recover overnight. After appropriate treatments, cells were washed in PBS and then lysed in 100–150 μL of buffer (100 mM $NaPO_4$, 1 mM EDTA, pH 7.5) containing 0.1% Triton X-100 and frozen at −80 °C until analysis. To measure total glutathione, proteins were precipitated with sulfosalicylic acid at a final concentration of 1%. Samples were then spun for 10 min in a microcentrifuge to pellet proteins, and supernatant was diluted 1:20 in buffer before being measured. For all measurements, 50-μL triplicates of each sample were used for glutathione determination. The GSH level was obtained by comparing with the GSH standards and represented as nmol/mg of protein.

2.6. Statistical analysis

Statistical analysis was performed using Student's *t*-test, and a *P* value of <0.05 was considered significant. Data are expressed as the mean ± S.E. The mean value was obtained from at least three independent experiments.

3. Results

3.1. Glutathione levels are elevated in estrogen deprived MCF-7:2A breast cancer cells

Previous studies have shown that GSH levels in primary breast tumors are more than twice the levels found in normal breast tissue, and levels in lymph node metastases are more than four times the levels in normal breast tissue [19]. Recently, we reported the development of an estrogen deprived breast cancer cell line MCF-7:2A that is resistant to estrogen-induced apoptosis and expresses high levels of the glutathione synthetase gene GSS. To determine whether GSH levels were elevated in our apoptosis-resistant MCF-7:2A breast cancer cell line glutathione assays were performed on these cells. Fig. 3A shows that MCF-7:2A cells had significantly higher levels of GSH at 24, 48, and 72 h (11.9–15.8 nmol/mg protein) compared to wild-type MCF-7 cells (7.8–7.6 nmol/mg protein) and this trend continued up to day 7 (data not show). We next examined whether the GSH synthesis inhibitor BSO was capable of suppressing GSH levels in these cells. Fig. 3B shows that treatment with 100 μM of BSO for 48 h suppressed GSH levels by ∼55% in MCF-7 cells and by ∼75% in MCF-7:2A cells. Longer treatment with BSO (>48 h) yielded similar levels of inhibition (data not shown). These results indicate a possible link between elevated GSH levels and resistance to estrogen-induced apoptosis and they suggest that suppression of GSH by BSO has the ability to reverse the resistant phenotype of the MCF-7:2A cells.

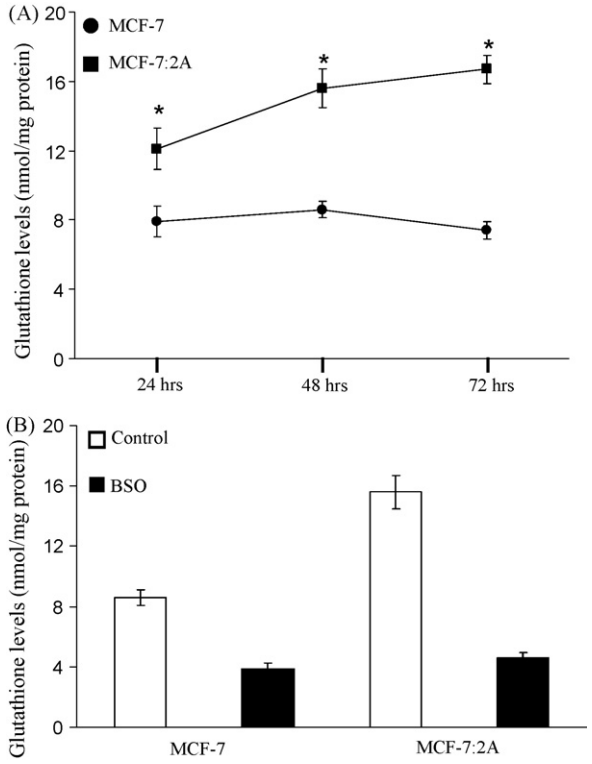


Fig. 3. Intracellular glutathione levels in wild-type MCF-7 cells and antihormone-resistant MCF-7:2A breast cancer cells. (A) Cells were seeded at 2×10^6 cells per 100 mm culture plates in estrogen-free media and total cellular glutathione was measured over a 72-h time period using a glutathione colorimetric assay kit, as described in Section 2. *$P < .0001$, with respect to MCF-7 cells. (B) BSO reduces glutathione levels in MCF-7 and MCF-7:2A cells. For experiment, cells were treated with 100 μM BSO for 48 h and levels of glutathione were measured as described in Section 2. Bars ± S.E.

3.2. Glutathione suppression by BSO sensitizes antihormone-resistant MCF-7:2A cells to estrogen-induced apoptosis

We next examined whether depletion of glutathione by BSO has the ability to sensitize MCF-7:2A cells to estrogen-induced apoptosis. Wild-type MCF-7 cells and estrogen deprived MCF-7:2A cells were seeded in estrogen-free media, and after 24 h, were treated with nothing (control), 1 nM estradiol, or 10 μM to 10 mM BSO in the presence or absence of 1 nM estradiol for 7 days. Fig. 4A shows that the growth of MCF-7 cells was stimulated 5-fold over the con-

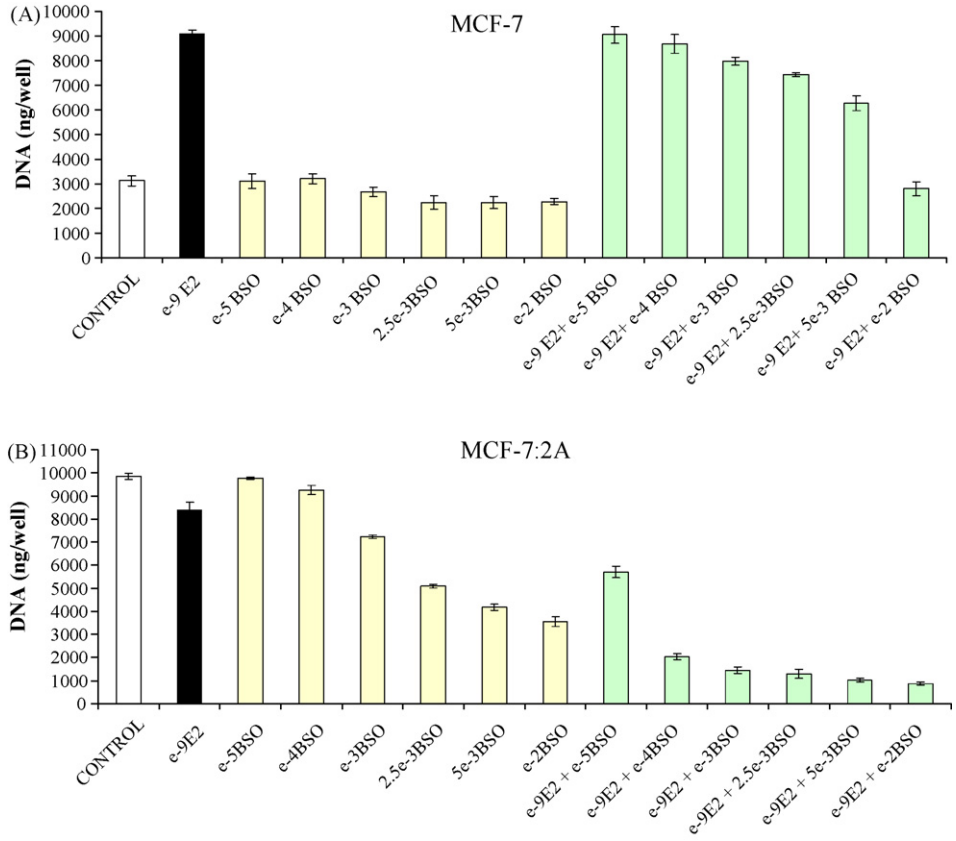


Fig. 4. BSO enhances the growth inhibitory effect of estradiol in antihormone-resistant MCF-7:2A cells. (A) MCF-7 cells were grown in estrogen-free media for 3 days prior to the start of the growth assay. On the day of the experiment, cells were seeded in 24-well plates and after 24 h were treated with various concentrations (10 μM to 10 mM) of BSO in the presence or absence of 1 nM (10^{-9} M) E2 for 7 days. At the indicated time points, cells were harvested and total DNA (ng/well) was quantitated as described in Section 2. (B) MCF-7:2A cells were treated similarly as described above. The data represents the mean of three independent experiments.

trol cells by 1 nM estradiol during the course of the 7-day assay and that treatment with BSO, either alone or in combination with estradiol, did not significantly alter the growth of these cells except at very high concentrations (>1 mM). In contrast, MCF-7:2A cells treated with the combination of BSO and estradiol showed a significant concentration dependent decrease in cell growth relative to cells treated with estradiol or BSO alone (Fig. 4B). It is noteworthy that 100 μM BSO, as a single agent, did not cause growth inhibition of MCF-7:2A cells. However, when combined with 1 nM estradiol the combination caused an 80–90% decrease in growth (Fig. 4B). The cell killing effect of BSO and estradiol was observed as early as 48 h after treatment and persisted over the time course of the experiment with maximum cell death at the 7-day time point. The concentration of BSO used in this study is already known to be clinically achievable without significant side effects [20,21].

Based on the above finding, we next determined whether MCF-7:2A cells underwent apoptotic cell death following BSO plus estradiol treatment. TUNEL assay was performed on cells treated with 100 μM BSO, 1 nM estradiol, or 100 μM BSO plus 1 nM estradiol for 72 h to detect fragmentation of DNA, a characteristic of apoptotic cell death. Fig. 5A shows that the percentage of TUNEL-positive cells significantly increased with the combination of BSO and estradiol but not with estradiol or BSO alone. After treatment with BSO and estradiol (72 h), as many as 53% of cells displayed TUNEL-positive staining, whereas, only 1% of the control cells and 5% of the estradiol treated cells were TUNEL-positive (Fig. 5A). BSO-treated cells looked similar to control cells. As expected, wild-type MCF-7 cells showed very little TUNEL-positive staining in the presence of estradiol alone or BSO plus estradiol combined (data not shown), thus indicating a lack of apoptosis in these cells. DAPI staining of MCF-7:2A cells treated with BSO and estradiol further confirmed that the cells were undergoing apoptosis (Fig. 5B). In addition, phase contrast microscopy of MCF-7:2A cells showed morphological changes associated with apoptosis following BSO and estradiol treatment (Fig. 5C). Overall, these results indicate that BSO, as a single agent, causes neither growth inhibition nor cell death, but is capable of sensitizing antihormone-resistant MCF-7:2A cells to estradiol-induced apoptosis at clinically achievable concentrations.

4. Discussion

In the current study, we investigated whether suppression of the antioxidant glutathione by BSO has the ability to sensitize antihormone-resistant MCF-7:2A breast cancer cells to estradiol-induced apoptosis. Our results showed that glutathione levels were significantly elevated in antihormone-resistant MCF-7:2A breast cancer cells compared to wild-type MCF-7 cells and that the combination treatment of BSO and estradiol caused a dramatic increase in apoptosis whereas the individual treatments had no effect on growth. Noteworthy, the killing effect of BSO and estradiol occurred at clinically achievable concentrations and was observed as early as 48 h. These findings are consistent with previous studies which have shown that the cytotoxicity of a number of chemotherapeutic drugs, including melphalan [22], doxorubicin [23], and bleomycin [24], are significantly enhanced when glutathione is depleted by BSO.

Our laboratory has previously demonstrated that when estrogen receptor positive breast cancer cells are grown and maintained in LTED environments, they can ultimately develop enhanced

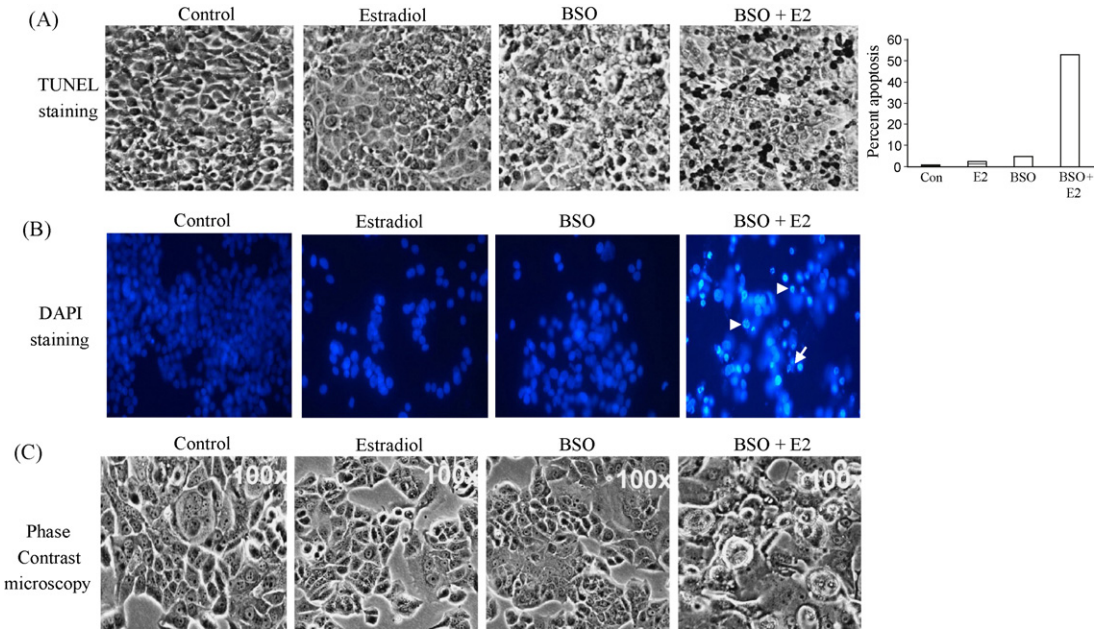


Fig. 5. BSO enhances the apoptotic effect of estradiol in MCF-7:2A breast cancer cells. (A) Cells were treated with 1 nM E2, 100 μM BSO, or 1 nM E2 + 100 μM BSO for 72 h and TUNEL staining for apoptosis was performed as described in Section 2. Slides were photographed through brightfield microscope under 100× magnification. TUNEL-positive cells were stained black (white arrows). Columns (right), mean percentage of apoptotic cells (annexin V-positive cells) from three independent experiments done in triplicate; bars, SEs. (B) Fluorescent microscopic analysis of apoptotic cells stained with 4′,6-diamidino-2-phenylindole (DAPI). MCF-7:2A cells were treated with 1 nM E2, 100 μM BSO, or 1 nM E2 + 100 μM BSO as described above for 72 h. To assess the number of cells undergoing apoptosis, round and/or shrunken nuclei of DAPI-stained cells were counted (white arrows). At least 200 cells per slide were counted by two individuals to control for subjective variability. Experiments were repeated three times with similar results. Representative slides are shown. Scale bars = 50 μM. (C) Phase contrast microscopy of MCF-7:2A cells treated with 1 nM E2, 100 μM BSO, or 1 nM E2 + 100 μM BSO for 72 h.

responsiveness to greatly diminished levels of estrogen [7,9]. These pre-clinical animal models show that initially, estrogen receptor expressing tumors are stimulated by estrogen and respond appropriately to tamoxifen with tumor regression. However, with continued exposure to tamoxifen, the tumors become resistant and re-grow [9]. Additionally, treatment of these LTED tumors with post-menopausal levels of estrogen inhibits tumor growth as well as causes regression of established tamoxifen resistant tumors [7,9,11,12] (Fig. 1). Mechanistic studies indicate that the apoptotic action of estrogen is due to its ability to either activate the fasR/FasL death receptor pathway [11,25] or to disrupt mitochondrial function through activation of the bcl-2 family proteins [7]. The paradoxical action of estrogen in these resistant cells is hypothesized to be due to increased sensitivity to estrogen due to adaptation to estrogen deprivation caused either by tamoxifen or an aromatase inhibitor [26]. It is believed that this "estrogen hypersensitivity" helps to explain the effectiveness of high-dose estrogen in patients with extensive prior endocrine therapy [14].

Interestingly, our present findings indicate that the ability of estradiol to induce apoptosis in antihormone-resistant cells is influenced by the level of glutathione present in the cells. Glutathione levels were elevated ∼1.4- to 1.6-fold in antihormone-resistant MCF-7:2A cells compared to wild-type MCF-7 cells and these cells failed to undergo apoptosis following 1 week of treatment with physiological concentrations of estradiol alone. In the presence of BSO, however, which depleted intracellular glutathione by ∼60–70%, the combination treatment of BSO and estradiol caused a dramatic increase in apoptosis which was observed as early as 48 h with maximum induction observed at day 7. Previous studies have shown that glutathione is an important component of tumor drug resistance [21] and that depletion of intracellular glutathione by BSO significantly enhances the cytotoxicity of many cytotoxic agents, principally alkylating agents [15,20,27] and platinating compounds [16] but also irradiation [28] and anthracyclines [29]. The concentration of BSO used in our study was within the range of 10 μM to 1 mM, which is similar to what has previously been reported in the literature. However, we did observe some toxicity at higher concentrations of BSO (>1 mM) in wild-type MCF-7 and antihormone-resistant MCF-7:2A cells (Fig. 4). It should be noted that BSO, at a clinically achievable concentration of 100 μM, was used for all of our combination experiments with estradiol since this concentration, as an individual treatment, did not significantly alter the growth of MCF-7:2A cells.

Glutathione, a sulfhydryl containing tripeptide, is involved in detoxifying cells from various toxins including chemotherapeutic agents [30,31]. Previous studies have demonstrated a strong correlation between elevated glutathione levels and increased resistance to chemotherapy in cancer cells [32]. This resistance was not limited to the particular chemotherapy agent used to induce resistance, but was also evident when other chemotherapeutic agents were tested for cross-resistance [32]. Additionally, translational studies of *in vitro* cell lines derived from patients with chemorefractory disease were found to have elevated glutathione levels [33]. BSO inhibits γ-glutamylcysteine synthetase (γ-GCS), the rate limiting enzyme in the production of glutathione, thus depleting glutathione levels within the cell [34]. Both, GSH as well as resultant increase in γ-GCS levels as a result of BSO treatment can be monitored peripherally in patients by analysis of peripheral mononuclear cells (PMNs) [35]. BSO also exhibits selectivity in that *in vitro* studies have demonstrated greater depletion of glutathione levels in tumor tissues than sampled normal tissues [30]. Based on its ability to target intracellular glutathione and reverse therapeutic resistance in refractory cancers, BSO is thought to be a potential antineoplastic agent and/or "therapeutic sensitizer" worthy of clinical evaluation.

Early phase clinical trials of BSO at doses resulting in both peripheral and tumor GSH depletion show that BSO can be safely administered to patients with refractory disease. BSO was administered intravenously twice daily either alone or together with chemotherapy to cancer patients whose disease who disease had progressed despite multiple lines of previous chemotherapy

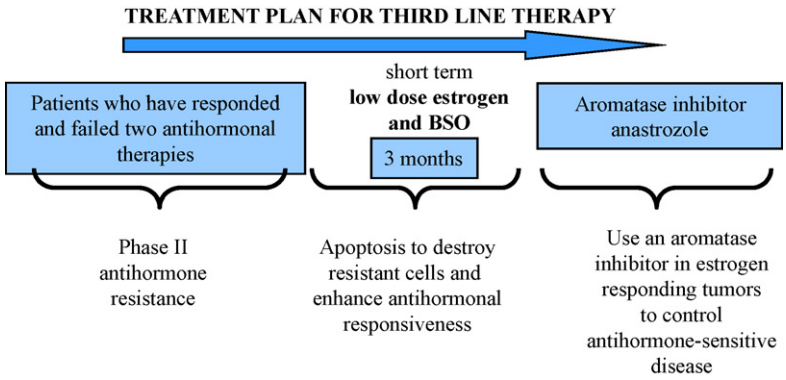


Fig. 6. Clinical protocol to investigate the efficacy of estradiol plus BSO combination treatment to induce apoptosis in long-term endocrine refractory breast cancer. An anticipated treatment plan for third-line endocrine therapy. Patients must have responded and experience treatment failure with two successive antihormone therapies to be eligible for a course of low-dose estradiol combined with BSO therapy for 3 months. The anticipated response rate is 30% and responding patients will be treated with anastrozole until relapse. The overall goal is to increase response rates and maintain patients for longer on antihormone strategies before chemotherapy is required.

[35,36]. In these patients treated with escalating doses of BSO, nausea and vomiting amenable to anti-emetic therapy were the main toxicities. Bone marrow suppression correlating with extent of previous chemotherapy exposure was found to be the rate limiting toxicity in the combination studies. No other significant toxicities were noted. Intracellular glutathione levels measured in PMNs decreased in a linear manner with repeated doses of BSO to a maximum of approximately 10–40% of baseline values [35,36]. When tested in sequential tumor biopsies, glutathione was also found to be depleted to a variable extent in a similarly predictable pattern [36]. Additionally, BSO administration resulted in an initial rapid inhibition of γ-GCS activity followed by γ-GCS recovery during the intervening time between dosings. In fact, γ-GCS levels mirrored peripheral BSO concentrations in patients thus demonstrating targeted delivery of BSO. Clinically, responses to treatment, including complete responses, have been achieved [27,35,36].

In this present study, we demonstrated that glutathione depletion by BSO sensitized antihormone-resistant MCF-7:2A human breast cancer cells to estradiol-induced apoptosis *in vitro*. Taken together, it would be reasonable to incorporate this data into our working translational model for clinical evaluation (Fig. 6). We therefore propose utilizing BSO together with estrogen in patients for a defined therapeutic course in patients with hormonally sensitive metastatic breast cancer whose disease has progressed on prior antihormonal therapies to significantly reduce their disease burden, while potentially reversing resistance to antihormonal therapies. This would then be followed by continuing treatment with an aromatase inhibitor for maintenance of additional clinical benefit for these patients (Fig. 6). Our future goal will be to address this hypothesis in the context of a clinical trial based on these new pre-clinical findings.

Acknowledgments

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References

[1] Cancer Facts and Figures, www.cancer.org, 2005.
[2] M. Lippman, G. Bolan, K. Huff, The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture, Cancer Research 36 (12) (1976) 4595–4601.
[3] J.N. Ingle, D.L. Ahmann, S.J. Green, J.H. Edmonson, H.F. Bisel, L.K. Kvols, W.C. Nichols, E.T. Creagan, R.G. Hahn, J. Rubin, S. Frytak, Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer, The New England Journal of Medicine 304 (1) (1981) 16–21.
[4] P.E. Goss, K. Strasser, Aromatase inhibitors in the treatment and prevention of breast cancer, Journal of Clinical Oncology 19 (3) (2001) 881–894.
[5] A. Howell, J.F. Robertson, J. Quaresma Albano, A. Aschermannova, L. Mauriac, U.R. Kleeberg, I. Vergote, B. Erikstein, A. Webster, C. Morris, Fulvestrant, formerly ICI, 182,780 is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment, Journal of Clinical Oncology 20 (16) (2002) 3396–3403.
[6] V.C. Jordan, The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer—survival or death? Journal of Clinical Oncology 26 (18) (2008) 3073–3082.
[7] J.S. Lewis, K. Meeke, C. Osipo, E.A. Ross, N. Kidawi, T. Li, E. Bell, N.S. Chandel, V.C. Jordan, Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation, Journal of the National Cancer Institute 97 (23) (2005) 1746–1759.
[8] J.S. Lewis, C. Osipo, K. Meeke, V.C. Jordan, Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal, The Journal of Steroid Biochemistry and Molecular Biology 94 (1–3) (2005) 131–141.
[9] K. Yao, E.S. Lee, D.J. Bentrem, G. England, J.I. Schafer, R.M. O'Regan, V.C. Jordan, Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice, Clinical Cancer Research 6 (5) (2000) 2028–2036.
[10] J.S. Lewis, D. Cheng, V.C. Jordan, Targeting oestrogen to kill the cancer but not the patient, British Journal of Cancer 90 (5) (2004) 944–949.
[11] C. Osipo, C. Gajdos, H. Liu, B. Chen, V.C. Jordan, Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer, Journal of the National Cancer Institute 95 (21) (2003) 1597–1608.
[12] R.X. Song, G. Mor, F. Naftolin, R.A. McPherson, J. Song, Z. Zhang, W. Yue, J. Wang, R.J. Santen, Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol, Journal of the National Cancer Institute 93 (22) (2001) 1714–1723.
[13] W.J. Haddow, A.E. Paterson, Influence of synthetic oestrogens upon advanced malignant disease, BMJ 2 (1944) 393–398.
[14] P.E. Lonning, P.D. Taylor, G. Anker, J. Iddon, L. Wie, L.M. Jorgensen, O. Mella, A. Howell, High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy, Breast Cancer Research and Treatment 67 (2) (2001) 111–116.
[15] M.E. Anderson, Glutathione: an overview of biosynthesis and modulation, Chemico-Biological Interactions 111–112 (1998) 1–14.
[16] C.M. Rudin, Z. Yang, L.M. Schumaker, D.J. VanderWeele, K. Newkirk, M.J. Egorin, E.G. Zuhowski, K.J. Cullen, Inhibition of glutathione synthesis reverses Bcl-2-mediated cisplatin resistance, Cancer Research 63 (2) (2003) 312–318.
[17] T. Schnelldorfer, S. Gansauge, F. Gansauge, S. Schlosser, H.G. Beger, A.K. Nussler, Glutathione depletion causes cell growth inhibition and enhanced apoptosis in pancreatic cancer cells, Cancer 89 (7) (2000) 1440–1447.
[18] J.J. Pink, S.Y. Jiang, M. Fritsch, V.C. Jordan, An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein, Cancer Research 55 (12) (1995) 2583–2590.
[19] R.R. Perry, J.A. Mazetta, M. Levin, S.C. Barranco, Glutathione levels and variability in breast tumors and normal tissue, Cancer 72 (3) (1993) 783–787.
[20] C.P. Anderson, R.C. Seeger, N. Satake, H.L. Monforte-Munoz, N. Keshelava, H.H. Bailey, C.P. Reynolds, Buthionine sulfoximine and myeloablative concentrations of melphalan overcome resistance in a melphalan-resistant neuroblastoma cell line, Journal of Pediatric Hematology Oncology 23 (8) (2001) 500–505.
[21] H.H. Bailey, L-S,R-buthionine sulfoximine: historical development and clinical issues, Chemico-Biological Interactions 111–112 (1998) 239–254.
[22] R.A. Kramer, K. Greene, S. Ahmad, D.T. Vistica, Chemosensitization of L-phenylalanine mustard by the thiol-modulating agent buthionine sulfoximine, Cancer Research 47 (6) (1987) 1593–1597.

[23] L. Dusre, E.G. Mimnaugh, C.E. Myers, B.K. Sinha, Potentiation of doxorubicin cytotoxicity by buthionine sulfoximine in multidrug-resistant human breast tumor cells, Cancer Research 49 (3) (1989) 511–515.
[24] A. Russo, W. DeGraff, N. Friedman, J.B. Mitchell, Selective modulation of glutathione levels in human normal versus tumor cells and subsequent differential response to chemotherapy drugs, Cancer Research 46 (6) (1986) 2845–2848.
[25] H. Liu, E.S. Lee, C. Gajdos, S.T. Pearce, B. Chen, C. Osipo, J. Loweth, K. McKian, A. De Los Reyes, L. Wing, V.C. Jordan, Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo, Journal of the National Cancer Institute 95 (21) (2003) 1586–1597.
[26] S. Masamura, S.J. Santner, D.F. Heitjan, R.J. Santen, Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells, The Journal of Clinical Endocrinology and Metabolism 80 (10) (1995) 2918–2925.
[27] H.H. Bailey, G. Ripple, K.D. Tutsch, R.Z. Arzoomanian, D. Alberti, C. Feierabend, D. Mahvi, J. Schink, M. Pomplun, R.T. Mulcahy, G. Wilding, Phase I study of continuous-infusion L-S,R-buthionine sulfoximine with intravenous melphalan, Journal of the National Cancer Institute 89 (23) (1997) 1789–1796.
[28] A. Rosi, S. Grande, A.M. Luciani, A. Palma, C. Giovannini, L. Guidoni, O. Sapora, V. Viti, Role of glutathione in apoptosis induced by radiation as determined by 1H MR spectra of cultured tumor cells, Radiation Research 167 (3) (2007) 268–282.
[29] Z. Benderra, A. Trussardi, H. Morjani, A.M. Villa, S.M. Doglia, M. Manfait, Regulation of cellular glutathione modulates nuclear accumulation of daunorubicin in human MCF7 cells overexpressing multidrug resistance associated protein, European Journal of Cancer 36 (3) (2000) 428–434.
[30] P. Calvert, K.S. Yao, T.C. Hamilton, P.J. O'Dwyer, Clinical studies of reversal of drug resistance based on glutathione, Chemico-Biological Interactions 111–112 (1998) 213–224.
[31] M.F. Renschler, The emerging role of reactive oxygen species in cancer therapy, European Journal of Cancer 40 (13) (2004) 1934–1940.
[32] T.C. Hamilton, M.A. Winker, K.G. Louie, G. Batist, B.C. Behrens, T. Tsuruo, K.R. Grotzinger, W.M. McKoy, R.C. Young, R.F. Ozols, Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion, Biochemical Pharmacology 34 (14) (1985) 2583–2586.
[33] R.F. Ozols, K.G. Louie, J. Plowman, B.C. Behrens, R.L. Fine, D. Dykes, T.C. Hamilton, Enhanced melphalan cytotoxicity in human ovarian cancer in vitro and in tumor-bearing nude mice by buthionine sulfoximine depletion of glutathione, Biochemical Pharmacology 36 (1) (1987) 147–153.
[34] O.W. Griffith, A. Meister, Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine), The Journal of Biological Chemistry 254 (16) (1979) 7558–7560.
[35] H.H. Bailey, R.T. Mulcahy, K.D. Tutsch, R.Z. Arzoomanian, D. Alberti, M.B. Tombes, G. Wilding, M. Pomplun, D.R. Spriggs, Phase I clinical trial of intravenous L-buthionine sulfoximine and melphalan: an attempt at modulation of glutathione, Journal of Clinical Oncology 12 (1) (1994) 194–205.
[36] P.J. O'Dwyer, T.C. Hamilton, F.P. LaCreta, J.M. Gallo, D. Kilpatrick, T. Halbherr, J. Brennan, M.A. Bookman, J. Hoffman, R.C. Young, R.L. Comis, R.F. Ozols, Phase I trial of buthionine sulfoximine in combination with melphalan in patients with cancer, Journal of Clinical Oncology 14 (1) (1996) 249–256.

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PRECLINICAL STUDY

A prospective study of variability in mammographic density during the menstrual cycle

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Abstract Mammographic breast density has been proposed as a surrogate endpoint in breast cancer prevention studies, but little is known about its variability over time, particularly in relation to menstrual cycle phase. The purpose of this study was to assess variation in breast density on digital mammograms using quantitative and qualitative density measures. Menstrual cycle phase was determined by salivary estradiol and progesterone assays. 73 healthy subjects with regular menses had 1–3 mammograms with paired saliva collection during a 12-month period. The mean difference in density as a percentage of the mean density was calculated for follicular–luteal ($n = 50$), luteal–luteal ($n = 26$) and follicular–follicular ($n = 23$) pairs in the same woman using the same breast. Two density measures (measurement of dense area and BIRADS) were used. The mean luteal density exceeded the mean follicular density by 7.1–9.2%, but density differences between luteal pairs and follicular pairs did not exceed 5%. The intraclass correlation for measurement of dense area was greater than 85% in all phases of the menstrual cycle, but was below 50% for BIRADS for luteal–follicular and follicular–follicular pairs. Our study provides estimates of the amount of variation in mammographic density during the menstrual cycle, and that inherent in repeated density measurement in premenopausal women, and suggests that menstrual phase of mammographic evaluation should be controlled for in intervention studies where density is being used as a surrogate measure.

Keywords Menstrual cycle · Mammographic density · Breast density · Mammogram

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Mammographic breast density is both an independent breast cancer risk factor [1–3] and a contributing factor to false negative mammograms [4–6]. Studies using self-reported dates of menstrual bleeding have suggested that mammographic density is greater in the luteal than the follicular phase of the menstrual cycle [7, 8]. A small prospective study of 11 women also indicated that density was increased in the luteal phase, although significant variation in the relationship between density and menstrual cycle phase among subjects was observed [9]. If it is true that breasts are less dense mammographically in the follicular than in the luteal phase, the simple, low-cost measure of performing screening mammography within the

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follicular phase may enhance screening performance. This would be attractive since even in modern studies of mammographic screening, approximately 15% of cancers in premenopausal women are not visualized [10–12]. In addition, mammographic density is increasingly being proposed as a useful surrogate endpoint in early phase studies of breast cancer prevention, but the measurement variability of this parameter over time within the same woman in the same menstrual phase has never been prospectively evaluated. The existing literature is largely retrospective and does not adequately address variations in the technical aspects of film-screen mammography, in radiologist estimation of density [13, 14], and in regularity of menstrual cycling in individual women [15]. These sources of variation need prospective evaluation in order to recommend that mammography should be scheduled during the follicular phase of the menstrual cycle, and for appropriate design of trials where mammographic density is used as a surrogate endpoint.

We have performed a prospective study of mammographic density in the follicular and luteal phase of the menstrual cycle to determine if significant variation in breast density occurs during the menstrual cycle, and to assess variability of density over time. We used digital mammography and documented menstrual cycle phase by salivary measurement of estradiol (E2) and progesterone (P) throughout the cycle in healthy, regularly cycling premenopausal women over a period of 12 months.

Materials and methods

Subjects

Healthy premenopausal women ages 29–49 years undergoing annual screening mammography were recruited as a convenience sample from the Lynn Sage Comprehensive Breast Center of Northwestern Memorial Hospital after Institutional Review Board approval of the study. A medical history was obtained prior to entry by face-to-face interview by a trained interviewer. Women were eligible if they reported regular menses for the 6 months preceding study entry. Women were ineligible if they were pregnant, lactating, planning pregnancy within the next year, had been diagnosed with ovarian dysfunction, or were taking oral contraceptives, tamoxifen, or other estrogen- or progesterone-containing drugs. Use of oral contraceptives more than 6 months prior to study entry was permissible. Subjects who had a history of tamoxifen use for more than 1-month at any time were ineligible. Mammography was scheduled after eligible subjects consented to participate in order to allow saliva collection during the month of the baseline mammogram. Subjects were queried prior to follow-up mammograms to confirm that they had continued to avoid the use of medications which would render them ineligible for participation. Demographic data were collected only at study entry and included age, weight, height, age at menarche, gravidity, parity, history of hormone use, family history of breast cancer, and prior breast biopsy. A total of three mammograms were obtained. Subjects underwent their initial mammogram at an unassigned phase of the menstrual cycle, i.e., in either the follicular or the luteal phase. Subjects were randomly assigned to have a second mammogram 6 months later during either the follicular or luteal phase of the cycle. The final mammogram was obtained approximately 12 months after study entry during the opposite phase of the menstrual cycle from the 6-month randomized mammogram. Subjects who became amenorrheic after a single mammogram were excluded from the study. The timing of the mammographic exams is summarized in Fig. 1.

A total of 73 women were recruited to the study. Thirty-five had three mammograms with saliva determinations during the same menstrual cycle, and 12 had two mammograms with matching saliva. Three subjects with multiple saliva samples were excluded due to collection during a different menstrual cycle than the one in which the mammogram was obtained. Eighteen consented subjects provided only a single mammogram with paired saliva and then dropped out of the study due to relocation, pregnancy, desire to use hormonal medication, or unspecified reasons.

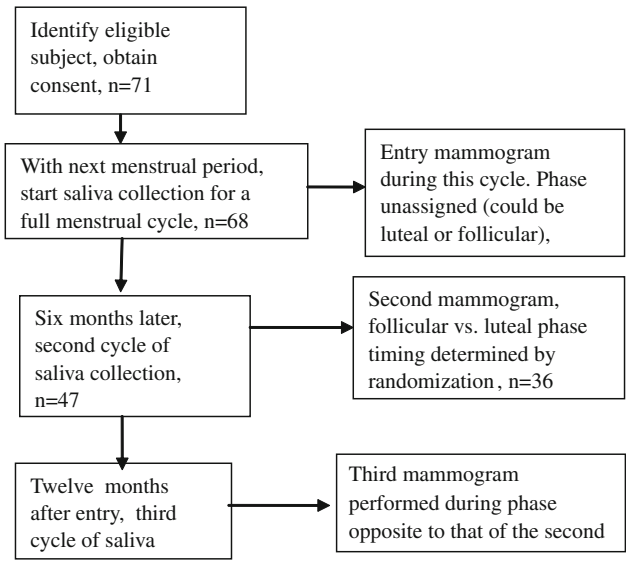

Fig. 1 Flow diagram of the study schema saliva was collected during each of the three menstrual cycles in which a mammogram was performed. The entry mammogram occurred during an unassigned phase of the menstrual cycle. The 6-month mammogram was randomized to the luteal or follicular phase, and the 12-month mammogram was performed during the opposite phase

Salivary collection and analysis

Saliva was collected for three full menstrual cycles (a cycle was defined as starting at the onset of menstruation and ending at the onset of the next menstrual period, or 30 days, whichever came earlier). The entry cycle began with the first period following recruitment and consent; the second was 6 months later, and the third was 12 months following the entry cycle. The purpose in collecting saliva was to determine the occurrence of ovulation and the relative level of estradiol and progesterone in ovulatory cycles. Consenting subjects were given a box containing 32 vials with 0.5 mg of sodium azide dried in the bottom as a preservative and sugarless chewing gum for saliva collection, at the beginning of each collection cycle. Boxes with saliva samples were returned to the clinic by the subjects at the end of each monthly collection period. Collections were scheduled to be repeated every 6 months for a total of three menstrual cycles. Beginning on the first day of menstrual bleeding, subjects began saliva collection. In the morning prior to food intake or teeth brushing, subjects chewed one-half of a stick of sugarless gum to increase salivation and deposited 7–10 ml of saliva into a vial, mixed the saliva with the preservative, and recorded both the date of saliva collection on the vial and whether menstrual bleeding was present. This procedure was repeated daily for 30 days or until the beginning of the next menstrual period. Saliva was stored in a light-tight box in the subject's home without refrigeration and was brought to the Breast Center at the end of the month. Progesterone (P) concentrations in samples prepared in this way are completely stable for at least 2 months [16, 17]. The stability of E2 was tested again in this study by repeating assays of four samples that were stored at room temperature. No deterioration was observed in E2 concentrations for 4 months.

Upon receipt in the laboratory, samples were stored at −20°C. For analysis, the samples were thawed and centrifuged for 30 min at 5,000*g* to remove cellular debris. Batches of samples collected from different subjects over a 2-month period were prepared for assay. Each assay included two quality control preparations for estimating assay precision. The quality control preparation was a pool of saliva that had been stripped of steroids by incubation with agarose-coated charcoal and to which 44 pmol/l of E2 and 636 pmol/l of P had been added. E2 and P were assayed by direct radioimmunoassay as described in detail previously [16, 17]. Briefly, the P assay utilizes [1,2,6,7-3H] P and an antiserum prepared by one of the authors (R T C) that has cross-reactivities of 0.5% with 17-hydroxy P, 2.0% with pregnanedione, 0.95% with corticosterone, 11.4% with 5α-pregnanedione, 1.9% with 5β-pregnanolone, and <0.1% with seven other steroids tested. Standards were prepared in 0.1 M PBS, pH 7.0, containing 0.015 M NaN3 and 0.1% gelatin. The volume of sample used was 0.1 ml. Intraassay and interassay coefficients of variance (CV) were 7.9 and 19.9%, respectively. Salivary E2 was measured with a double antibody RIA, also described previously [17]. Antiserum and 125I-labeled E2 tracer were obtained from Diagnostic Systems Laboratories (Webster, TX, USA). The antiserum has cross-reactivities of 2.4% with estrone, 0.01% with estrone sulfate, 0.21% with 16-ketooestradiol, 2.6% with estradiol-3-glucuride, 0.64% with estriol, and <0.1% with nonphenolic steroids tested. The antiserum was diluted to give 40% binding. Standards were prepared by diluting a methanolic stock solution of E2 with the same gelatin buffer used for P. A precipitating antibody solution was prepared by titrating the amount of sheep antirabbit gamma globulin required for precipitation of 0.1 ml rabbit serum, and adding this to propylene glycol (4.8 g/dl). The total volume of sample required was 0.4 ml. Intra- and interassay CV were 9.9 and 20.2%, respectively. Any samples with extremely high values (more than 3 SD above mean values from previous studies: >1,500 pmol/L for P and >100 pmol/L forE2) or with a pink or red color were tested for the presence of oxyhemoglobin with the Hemoccult procedure (Beckman-Coulter, Fullerton, CA, USA) to rule out blood from bleeding gums. [18] No positive tests were found.

Analyses

The last day of the menstrual cycle (the day before menstrual bleeding) was designated cycle-day zero. The luteal phase of the cycle was defined as the 14-day period after ovulation in an idealized 28-day menstrual cycle; the follicular phase as the time prior to ovulation. Saliva samples from day-28 through the end of the cycle were assayed for E2. Those from day-17 through day-zero were assayed for progesterone. The criteria used to determine the day of ovulation were: (1) progesterone values of >190 pmol/l on 3 or more days in a 4-day period occurring within days -4 to -10 from the end of the cycle. This was an arbitrary value based on a detectable increase over follicular phase levels based on a previous study of similar, ovulatory subjects in whom conception had occurred [19]; (2) cycle-length of >24 days; and (3) E2 values of <44 pmol/l during the first 10 days of the cycle. With these criteria met, the day of ovulation was determined by counting back 13 days from the last day of the cycle. Frequently, there was a peak of E2 within ±2 days from day-13 followed by a drop in estradiol concentration of >50% on the next day (62% of ovulatory cycles). If so, we choose the day after the peak as the day of ovulation [20]. Hormone levels were expressed either as a mean or a sum over all days in the cycle.

Density measurements

All mammograms in this study were obtained using full field digital mammography performed on a single General Electric Senographe 2000D Digital Mammography System (GE Medical Systems, Inc., Milwaukee, WI). A semiautomated analysis based on methods developed by Byng et al. [13, 21] for quantification of breast density in screen-film mammography was used for each measurement. Measurements were made using "processed" or thickness-equalized digital mammograms that displayed all breast tissue out to the skin line; no additional image processing was performed. The analysis consisted of four steps for each digital mammogram: (1) display of the digital mammogram; (2) determination of the area of the entire breast, excluding lesion markers and pectoralis major muscle; (3) determination of a threshold signal value, which defined the edges of the fibroglandular regions; and (4) determination of the area of fibroglandular tissue within the breast by summing the number of pixels with signal values above the threshold value. Two digital density measures were obtained using computer analysis to estimate the fraction of the image consisting of fibroglandular tissue. A 2-dimensional (2D) density measured the dense area of the breast (i.e., the ratio of fibroglandular area to total breast area) and was defined as (2D) density = Area of fibroglandular tissue/Total breast area excluding pectoral muscle.

The 2D densities obtained from the mediolateral oblique and the craniocaudal views of each subject were averaged to obtain a single 2D density value for each breast of each subject. The 3-dimensional (3D) density included an additional factor representing the degree of density, i.e., the density difference between the mean fibroglandular tissue signal ($S_{fibroglandular}$) and the threshold signal ($S_{threshold}$). The formula for 3D breast density is:

$$(3D)\ \text{density} = \frac{\text{Area of fibroglandular tissue} \times \log(S_{fibroglandular} - S_{threshold})}{\text{Total breast area excluding pectoral muscle}}$$

The digital density measurements were obtained by a single observer (EH).

In addition, to determine if measurable differences in density determined from digital mammography were perceptible to the human reader, the BIRADS lexicon [22] of mammographic density [(1) almost entirely fat, (2) scattered fibroglandular densities, (3) heterogeneously dense, (4) extremely dense] was rated for each subject based on all four views by an experienced, MQSA-qualified mammographer who was unaware of the results of the digital density measures.

Statistical analysis

Mean density measures were compared across BIRADS categories using one-way analysis of variance. To determine the degree of similarity between menstrual phases, mean differences were expressed as a percent of the overall mean (combined mean of the two phases being compared). Differences between pair types were compared using the independent sample *t*-test with adjustment for multiple pairs per woman by calculating a design effect based on the intrawoman correlation. In comparing the mean density between luteal to follicular phases, the sample size of 50 in this analysis had 80% power to detect an effect size of 0.57 where effect size is defined as a mean difference between phases divided by the standard deviation of the difference. Actual effect sizes for this analysis ranged from 0.11 to 0.29.

Menstrual cycles were designated ovulatory if the progesterone value exceeded 100 pmol/l for a minimum of three consecutive days of the cycle. The standard deviation of all midluteal phase values was 87. If a normal distribution with mean zero and standard deviation 87, truncated at zero, is assumed for the anovulatory values, the probability of achieving three or more values greater than 100 in an anovulatory cycle in which the actual response is zero is no greater than 0.004. This indicates a low chance of a false positive result from anovulatory cycles.

Reliability analyses were done on pairs of density measures within the same woman. For luteal–follicular pairs, different cycle phases were observed at different visits, but the paired measures had to be from the same breast. A similar procedure was done to pair observations in the same cycle phase. To determine the degree of reliability of repeated measures within women where the measures may have been in different phases or in the same phase, Spearman correlation coefficients were calculated. Also, the intrawoman correlation coefficient and the concordance coefficient [23] with its 95% confidence interval were also calculated. Numbers near 1.00 for all these statistics indicate a high degree of reliability.

Results

The baseline characteristics of the study population are summarized in Table 1. One-third of the participants were

Table 1 Characteristics of study participants

Sample size	71
Age	
n	71
Mean (SD)	41.8 (3.8)
Median (range)	42.0 (29–50)
Age at menarche	
n	71
Mean (SD)	12.6 (1.7)
Median (range)	12.0 (9–19)
Race	
White	51 (72%)
Black	9 (13%)
Hispanic	8 (11%)
Other	3 (4%)
Total	71 (100%)
Gravidity/parity	
G0/P0	23 (33%)
G > 0/P0	10 (14%)
G > 0/P1	9 (13%)
G > 0/P2	19 (27%)
G > 0/P3	9 (13%)
Total	70 (100%)
Hx hormone use	
Yes	40 (56%)
No	31 (44%)
Total	71 (100%)
Family Hx breast Ca	
Yes	32 (46%)
No	38 (54%)
Total	70 (100%)
Previous biopsy	
Yes	17 (25%)
No	52 (75%)
Total	69 (100%)
Body mass index (BMI)	
<25	39 (55%)
≥25	32 (45%)
Total	71 (10%)

nulliparous, 46% reported a family history of breast cancer, 25% had a prior benign breast biopsy, and 56% had exposure to hormonal medications in the past. The baseline value for each of the breast density measures across BIRADS category is shown in Table 2. A higher BIRADS classification by the radiologist was associated with a significant increase in numeric digital density reading for the 2D and 3D density measures. The relationship between age, race, age at menarche, gravidity, parity, age at first birth, a history of hormone use, family history of breast

Table 2 Variability in density measures across BIRADS categories

BIRADS	*N*	2D		3D	
		Mean	SEM	Mean	SEM
1	4	0.097	0.026	0.078	0.018
2	22	0.289	0.032	0.364	0.051
3	43	0.479	0.023	0.717	0.042
4	4	0.691	0.072	1.046	0.115
P-value		<0.0001		<0.0001	

cancer, prior breast biopsy, and body mass index (BMI) and each of the density measures was examined. Increased BMI was significantly associated with decreased breast density when 2D, 3D, or BIRADS density measures were used ($P < 0.001$). These associations were observed when BMI was examined as a continuous variable, as well as when it was dichotomized. Significant differences in BIRADS ratings were also noted on the basis of ethnicity, with Caucasian women having higher mean density ratings than those of other ethnicities ($P = 0.027$). BIRADS density ratings also differed significantly on the basis of prior breast biopsy. The 17 subjects with prior breast biopsy had a mean BIRADS density of 2.94 (SEM 0.16) versus 2.58 (SEM 0.09) for the 52 subjects who had no prior breast biopsy ($P = 0.03$). Because the 3D density measure did not provide additional information beyond that obtained from the universally used 2D density (or percent dense area) measure, the remainder of the results are reported only for the 2D and BIRADS measures.

Hormonal levels for salivary E2 and P were calculated as the mean value over a menstrual cycle. The incidence of anovulatory cycles was 25 of 130 menstrual cycles during the period of observation. However, none of the subjects were anovulatory in all three cycles examined. Over all cycles, the mean E2 level was 7.8 pmol/l (range, 0.03–59.7) and the mean P level was 106 pmol/l (range, 3.10–294). No correlation between breast density and mean levels of E2 and P within the same month for either measure of breast density was observed. Density measurements for the right and left breast in all subjects were compared in the initial menstrual cycle after study entry to determine the within-person variation between breasts. The absolute difference in the 2D breast density between the right and left breasts was 0.033 (SD 0.003), which is 8.1% of the mean breast density. Based on these findings, all subsequent density comparisons for phase of the menstrual cycle were made using the same breast.

There were 36 women with density data at two or three time points for whom two or three ovulatory cycles could be classified by phase. To compare different (luteal vs. follicular) or similar (luteal vs. luteal, follicular vs. follicular) cycles, the breast was taken as the unit of analysis. For each

woman and each analysis, any possible comparative pair was selected, the only stipulation being that the pair had to be from the same breast. This process resulted in different numbers of women and breast pairs in the individual analyses. There were 50 follicular and luteal paired samples in 23 women available for the analysis of density change by menstrual cycle phase. Eleven women contributed data from one breast for a total of 15 pairs, and 12 women contributed data from both breasts for a total of 35 pairs. Density measurements taken during the luteal phase of different menstrual cycles ($n = 26$ pairs in 17 women) included 12 women contributing data from 1 breast for a total of 12 pairs, and 5 women contributing data from both breasts for a total of 14 pairs. In the follicular phase of different menstrual cycles ($n = 23$ pairs in 13 women), seven women contributed data from one breast for a total of seven pairs, and six had data from both breasts for a total of 14 pairs. Density measures taken during the luteal or follicular phases of different menstrual cycles were compared to determine the amount of variation inherent in the density measurement. The mean density values for each type of density measurement according to menstrual cycle phase are shown in Table 3.

The mean difference in density as a percentage of the mean density was calculated for each follicular–luteal, luteal–luteal, and follicular–follicular pair (Table 3). Figure 2 demonstrates the changes in 2D breast density between the follicular and the luteal phases of the menstrual cycle, as well as between two luteal phase measurements and two follicular phase measurements. The density was higher in the luteal phase compared with the follicular phase, using both 2D and BIRADS measures of breast density, but these differences did not reach statistical significance. The mean luteal density exceeded the mean follicular density by 7.1% for 2D, and 9.2% for BIRADS density measures. Differences in mean densities between two different luteal phases, and two different follicular phases, were not found to be statistically significant for either of the measures of density. The variation between measures in the same woman ranged from 0.9 to 3.2% for 2D measures, and 1.5–5.1% for BIRADS (Table 3). The extent of the correlation between the pairs within the same woman was analyzed by several correlation methods. Table 4 summarizes Spearman correlation coefficients, intraclass (intrapair) correlation coefficients (ICC), and concordance coefficients. Since a high Spearman correlation does not necessarily indicate reproducibility, the concordance coefficient was used to determine how close the data pairs lie on a 45-degree line through the origin, which would give a concordance of 1.00. The 2D pairs were highly correlated in all phases of the menstrual cycle (Table 4), with correlation coefficients above 84%. Similarly, BIRADS reliability for luteal–luteal pairs was high,

Table 3 Density measurements by menstrual cycle phase

F–L differences ($n = 50$) mean (SEM)					
Density measure	Mean (SEM) F	Mean (SEM) L	Mean (SEM) L–F	L–F as % of mean of L and F	*P*-value comparing % to 0
2D	0.433 (0.037)	0.466 (0.043)	0.032 (0.020)	7.1% (3.3%)	0.13
BIRADS	2.50 (0.13)	2.74 (0.12)	0.24 (0.12)	9.2% (3.4%)	0.053
L–L differences ($n = 28$) mean (SEM)					
Density measure	Mean (SEM) L1	Mean (SEM) L2	Mean (SEM) L2–L1	L2–L1 as % of mean of L1 and L2	*P*-value comparing % to 0
2D	0.394 (0.046)	0.407 (0.038)	0.016 (0.018)	3.2% (5.0%)	0.52
BIRADS	2.62 (0.11)	2.65 (0.11)	0.04 (0.05)	1.5% (1.9%)	0.43
F–F differences ($n = 23$) mean (SEM)					
Density measure	Mean (SEM) F1	Mean (SEM) F2	Mean (SEM) F2–F1	F2–F1 as % of mean of F1 and F2	*P*-value comparing % to 0
2D	0.468 (0.064)	0.472 (0.061)	0.004 (0.032)	0.9% (6.0%)	0.90
BIRADS	2.61 (0.19)	2.48 (0.20)	0.13 (0.19)	5.1% (6.3%)	0.50

F, follicular; L, luteal; F1, first follicular cycle measurement; L1, first luteal cycle measurement; F2, second follicular cycle measurement; L2, second luteal cycle measurement

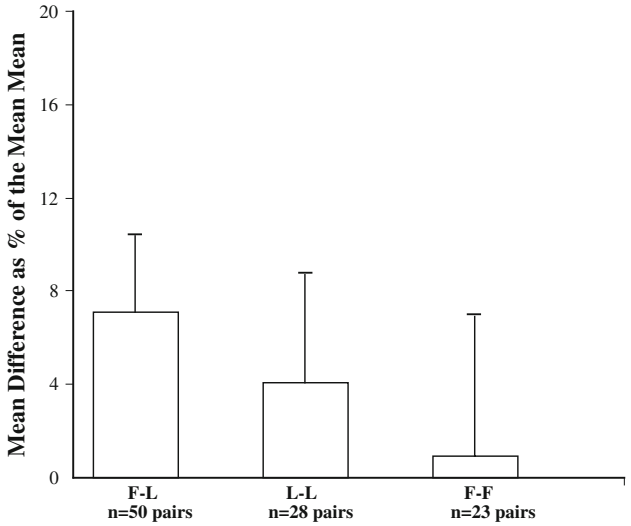

Fig. 2 Changes in breast density by menstrual cycle phase. Differences between 2D density measures for mammograms obtained in the follicular (F) and luteal (L) phases of the menstrual cycle (F–L), in two different luteal phases of the menstrual cycle (L–L), and in two different follicular phases of the menstrual cycle (F–F). Values reported are mean difference (SEM) between phases, as a percent of the mean of the phases

Table 4 Reproducibility measures

Density measure	Correlation *P*-value	ICC	Concordance (95% CI)
Follicular–Luteal pairs ($n = 50$)			
2D	0.75	0.85	0.84
	$P < 0.0001$		(0.74, 0.90)
BIRADS	0.45	0.48	0.49
	$P = 0.001$		(0.27, 0.66)
Luteal–Luteal pairs ($n = 26$)			
2D	0.84	0.88	0.88
	$P < 0.0001$		(0.75, 0.94)
BIRADS	0.99	0.93	0.93
	$P < 0.0001$		(0.13, 0.30)
Follicular–Follicular pairs ($n = 23$)			
2D	0.89	0.85	0.84
	$P < 0.0001$		(0.69, 0.92)
BIRADS	0.41	0.46	0.46
	$P < 0.0001$		(0.09, 0.71)

F, follicular; L, luteal; Correlation, Spearman correlation; ICC, intra-pair correlation; Concordance, Lin's concordance coefficient; SEM, mean difference

with all coefficients above 0.93. BIRADS reliability for luteal–follicular pairs and for follicular–follicular pairs was lower than for the 2D density measures, with coefficients below 50%.

Discussion

Variations in mammographic breast density reflect differences in the X-ray attenuation of the epithelial, stromal, and fatty components of the breast [24]. Studies examining the histologic correlates of breast density have found that both epithelial and stromal proliferation are associated with increased breast density [25, 26], while studies examining histologic changes in the breast during different phases of the menstrual cycle demonstrate morphologic changes in the epithelial and stromal components based on menstrual cycle phase [27, 28]. These observations provide a biologic rationale for examining variations in mammographic breast density on the basis of menstrual cycle phase. Although we found no significant correlation between levels of salivary estradiol and progesterone and breast density, a finding consistent with a number of studies which have failed to find an association between blood levels of estrone, estradiol, or progesterone, and breast density in premenopausal women [29, 30], our study documents a decrease in breast density that ranges from 7 to 9% of the mean density in the follicular, compared to the luteal, phase of the menstrual cycle. This was observed when density was measured as the percent dense area or 2D density, or using the BIRADS lexicon, although these differences did not reach statistical significance. In comparison, differences in density measurements made in the follicular phase of two different menstrual cycles in the same woman, or in the luteal phase of two different menstrual cycles, were smaller and ranged from 0.9 to 5% of the mean density. In addition, density variations of 8–11% between the right and left breasts of the same women were noted. The density measurements were performed in different phases of different menstrual cycles by design to allow the comparison of F–F and L–L as well as F–L differences in the same individual. All three of these comparisons would not have been possible in the same cycle. Additionally, when mammographic density is used as a surrogate endpoint in prevention studies, the measurements are performed in different cycles, often 6 months apart. The same is true for diagnostic mammography, which is rarely repeated within the same cycle. Our design was an attempt to reflect the real-world conditions under which these density measurements may be relevant, and in this sense it is strengthened by the fact that measurements were performed over the span of a year. We acknowledge that comparing density in the F–L phases of the same cycle may have been cleaner, but we feel our data are more pertinent to practical application.

A statistically significant difference in breast density between follicular and luteal phase may have been observable with a larger sample size; however, the clinical importance of the observed size of the density difference is uncertain. Although breast density is known to decrease the

sensitivity and specificity of mammography, the threshold increase in density which results in a decreased specificity and sensitivity is unknown. Carney et al. [31] used the mammograms of 329,495 women to examine the relationship between age, breast density, and the sensitivity and specificity of mammography. Although an increase in density was significantly associated with a decrease in sensitivity, for women under age 50, the difference in mammographic sensitivity between those with entirely fatty breasts and those with extremely dense breasts was about 6%. The difference in 2D mammographic density observed in this study between the follicular and luteal phases of the cycle was substantially smaller than that seen between completely fatty breasts (BIRADS 1) and very dense breasts (BIRADS 4, Table 1), and it is unlikely that this small difference would have a major impact upon the cancer detection rate.

Our findings regarding the magnitude of density changes associated with menstrual cycle phase are comparable with some previous work on this subject. In a cross-sectional study of 2,591 women ages 40–49 undergoing screening mammography, White et al. [8], observed a decrease in breast density during the follicular phase. In that study, each subject had a single mammogram, and density was classified as predominantly fat, heterogeneously dense, or extremely dense. Twenty-eight percent of women having a luteal phase mammogram were categorized as having extremely dense breasts compared with 24% of those who had a mammogram in the follicular phase, a difference which remained significant after adjustment for body mass index. However, adjustment for other variables, such as age, parity, and ethnicity, which are known to influence mammographic density, was not carried out [2]. In a similar analysis of women in the Canadian National Breast Screening Study, Baines et al. [7] found higher false negative and false positive rates for mammography during the luteal phase, an observation potentially attributable to changes in density. Ursin et al. [9] performed a prospective study in which 11 premenopausal women had a mammogram in both the follicular and the luteal phases of the same menstrual cycle. An average absolute density increase of 1.2% was seen in the luteal phase. The larger 3.2% absolute difference seen in our study may be attributable to the more careful hormone-based menstrual cycle analyses. There are a number of important differences in methodology between our study and these earlier reports [7–9]. We documented menstrual cycle phase using daily saliva collection to measure E2 and P, and precisely determined the day of ovulation. This is particularly important for assessment of menstrual cycle phase in a population of premenopausal women in their forties, where anovulatory cycles occurred in 19% of the cycles collected for our study. Failure to account for these anovulatory cycles introduces a source of error in previous studies, which relied on patient recall of the last menstrual period to determine the date of ovulation. In addition, since the follicular phase duration can vary substantially between (and within) women, it is not possible to accurately classify menstrual phase without knowledge of the date of the next menstrual period. It is therefore likely that there was random misclassification of the menstrual phase in studies that relied on recall of LMP for menstrual dating.

We also measured density on mammograms that were acquired digitally, eliminating variations in film optical density due to different batches of film, differences in exposure conditions, or differences in film processing conditions that can occur when density is measured directly from X-ray films or from films which have been digitized. In addition, we used computer-aided methods of density determination which allowed quantification of the percent dense area, independent of breast size. These computer-aided measurements of density have been shown by others to be highly reproducible [32, 33], and allowed a more exact quantification of density changes than was possible in other studies examining this question where only a BIRADS-type classification was assigned [8]. Our study documents that the radiologists' assessment of breast density, as measured by BIRADS category, is less reproducible than the measurement of percent dense area. We have documented a high degree of reproducibility of the 2D density measure over the 12-month time period of the study, regardless of whether measurements were made in the same phase of the menstrual cycle or between the luteal and follicular phases. Repeat BIRADS measurements were less well correlated, suggesting that this method of evaluating density change should be avoided in studies where density is used as a surrogate marker, as discussed below. However, the size of our study did not allow us to determine whether breast cancer risk factors, such as a family history or history of prior breast biopsy, or patient characteristics, such as body mass index, altered the relationship between density and menstrual cycle phase.

Our findings have implications for intervention studies that use breast density as an intermediate endpoint. Decreases in breast density have been suggested to be a surrogate biomarker for a reduction in breast cancer risk [2, 34–36]. Treatment with tamoxifen, an agent known to reduce breast cancer risk [37], has been shown to reduce breast density by 4–13% [35, 36, 38]. These studies were conducted in mixed populations of pre- and postmenopausal women, and did not control for the phase of the menstrual cycle in which the mammogram was obtained or the amount of variation inherent in density measurement. The observed 4–13% decrease in density with tamoxifen treatment reported by Chow et al. [35], Brisson et al. [36], and Cuzick et al. [38] is consistent with the difference in

density we observed between the luteal and follicular phases of the menstrual cycle and the right and left breasts. These studies did demonstrate a reduction in density compared with control populations, but two of the three (36, 38) suggested that density reductions were greatest in premenopausal women. Recently, Cuzick et al. [39] reported that in the IBIS 1 tamoxifen prevention trial, the 46% of women receiving tamoxifen who had a density reduction of 10% or more experienced a 52% reduction in breast cancer incidence compared to controls ($P = 0.01$), while the 54% who had no or lesser density reductions had a nonsignificant 8% decrease in breast cancer incidence. Although the follicular–luteal differences we observed were not statistically significant, studies examining breast density as a surrogate biomarker in premenopausal women can minimize the variability of density measurement by controlling for the phase of the menstrual cycle in which the mammogram is obtained, and using the same breast for all measurements. Our data on differences in measurements obtained between two luteal or two follicular phases suggesting that changes in density of less than 5% are unlikely to represent therapeutic effects is supported by the findings of Cuzick et al. In summary, our study, using salivary hormone assays for accurate menstrual cycle phase determination, documents a high degree of reproducibility of quantitative density measurements (but not of BIRADS measurements) over a 12-month time period, with nonsignificant changes in density between the luteal and follicular phases of the menstrual cycle when quantitative density measures are used.

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References

1. Vacek PM, Geller BM (2004) A prospective study of breast cancer risk using routine mammographic breast density measurements. Cancer Epidemiol Biomarkers Prev 13:715–722
2. Boyd NF, Jensen HM, Cooke G, Han HL, Lockwood GA, Miller AB (2000) Mammographic densities and the prevalence and incidence of histological types of benign breast disease. Reference pathologists of the Canadian national breast screening study. Eur J Cancer Prev 9:15–24
3. Byrne C, Schairer C, Wolfe J et al (1995) Mammographic features and breast cancer risk: effects with time, age, and menopause status. J Natl Cancer Inst 87:1622–1629
4. Ciatto S, Visioli C, Paci E, Zappa M (2004) Breast density as a determinant of interval cancer at mammographic screening. Br J Cancer 90:393–396
5. Kerlikowske K, Grady D, Barclay J, Sickles EA, Ernster V (1996) Effect of age, breast density, and family history on the sensitivity of first screening mammography. JAMA 276:33–38
6. Mandelson MT, Oestreicher N, Porter PL et al (2000) Breast density as a predictor of mammographic detection: comparison of interval- and screen-detected cancers. J Natl Cancer Inst 92: 1081–1087
7. Baines CJ, Vidmar M, McKeown-Eyssen G, Tibshirani R (1997) Impact of menstrual phase on false-negative mammograms in the Canadian national breast screening study. Cancer 80:720–724
8. White E, Velentgas P, Mandelson MT et al (1998) Variation in mammographic breast density by time in menstrual cycle among women aged 40–49 years. J Natl Cancer Inst 90:906–910
9. Ursin G, Parisky YR, Pike MC, Spicer DV (2001) Mammographic density changes during the menstrual cycle. Cancer Epidemiol Biomarkers Prev 10:141–142
10. Warren RM, Young JR, McLean L et al (2003) Radiology review of the UKCCCR Breast Screening Frequency Trial: potential improvements in sensitivity, lead time of radiological signs. Clin Radiol 58:128–132
11. Rosenberg RD, Hunt WC, Williamson MR et al (1998) Effects of age, breast density, ethnicity, estrogen replacement therapy on screening mammographic sensitivity, cancer stage at diagnosis: review of 183, 134 screening mammograms in Albuquerque, New Mexico. Radiology 209:511–518
12. Destounis SV, DiNitto P, Logan-Young W, Bonaccio E, Zuley ML, Willison KM (2004) Can computer-aided detection with double reading of screening mammograms help decrease the false-negative rate? Initial experience. Radiology 232:578–584
13. Byng JW, Boyd NF, Fishell E, Jong RA, Yaffe MJ (1994) The quantitative analysis of mammographic densities. Phys Med Biol 39:1629–1638
14. Jong R, Fishell E, Little L, Lockwood G, Boyd NF (1996) Mammographic signs of potential relevance to breast cancer risk: the agreement of radiologists' classification. Eur J Cancer Prev 5:281–286
15. Chatterton RT Jr, Mateo ET, Hou N et al (2005) Characteristics of salivary profiles of oestradiol and progesterone in premenopausal women. J Endocrinol 186:77–84
16. Lu YC, Chatterton RT Jr, Vogelsong KM, May LK (1997) Direct radioimmunoassay of progesterone in saliva. J Immunoassay 18:149–163
17. Lu Y, Bentley GR, Gann PH, Hodges KR, Chatterton RT (1999) Salivary estradiol and progesterone levels in conception and nonconception cycles in women: evaluation of a new assay for salivary estradiol. Fertil Steril 71:863–868
18. Kivlighan KT, Granger DA, Schwartz EB (2005) Blood contamination and the measurement of salivary progesterone and estradiol. Horm Behav 47:367–370
19. Vitzthum VJ, Bentley GR, Spielvogel H et al (2002) Salivary progesterone levels and rate of ovulation are significantly lower in poorer than in better-off urban-dwelling Bolivian women. Hum Reprod 17:1906–1913
20. Lipson SF, Ellison PT (1996) Comparison of salivary steroid profiles in naturally occurring conception and non-conception cycles. Hum Reprod 11:2090–2096
21. Byng JW, Yaffe MJ, Jong RA et al (1998) Analysis of mammographic density and breast cancer risk from digitized mammograms. Radiographics 18:1587–1598
22. The ACR Breast Imaging Reporting and Data System (BIRADS) 4th Ed. (www.ACR.org) (2003)
23. Lin LI (1989) A concordance correlation coefficient to evaluate reproducibility. Biometrics 45:255–268
24. Johns PC, Yaffe MJ (1987) X-ray characterisation of normal and neoplastic breast tissues. Phys Med Biol 32:675–695
25. Li T, Sun L, Miller N et al (2005) The association of measured breast tissue characteristics with mammographic density and other risk factors for breast cancer. Cancer Epidemiol Biomarkers Prev 14:343–349
26. Guo YP, Martin LJ, Hanna W et al (2001) Growth factors and stromal matrix proteins associated with mammographic densities. Cancer Epidemiol Biomarkers Prev 10:243–248

27. Vogel PM, Georgiade NG, Fetter BF, Vogel FS, McCarty KS Jr (1981) The correlation of histologic changes in the human breast with the menstrual cycle. Am J Pathol 104:23–34
28. Longacre TA, Bartow SA (1986) A correlative morphologic study of human breast and endometrium in the menstrual cycle. Am J Surg Pathol 10:382–393
29. Boyd NF, Stone J, Martin LJ et al (2002) The association of breast mitogens with mammographic densities. Br J Cancer 87: 876–882
30. Noh JJ, Maskarinec G, Pagano I, Cheung LW, Stanczyk FZ (2006) Mammographic densities and circulating hormones: a cross-sectional study in premenopausal women. Breast 15:20–28
31. Carney PA, Miglioretti DL, Yankaskas BC et al (2003) Individual and combined effects of age, breast density, and hormone replacement therapy use on the accuracy of screening mammography. Ann Intern Med 138:168–175
32. Boyd NF, Dite GS, Stone J et al (2002) Heritability of mammographic density, a risk factor for breast cancer. N Engl J Med 347:886–894
33. Ursin G, Ma H, Wu AH et al (2003) Mammographic density and breast cancer in three ethnic groups. Cancer Epidemiol Biomarkers Prev 12:332–338
34. Atkinson C, Warren R, Bingham SA, Day NE (1999) Mammographic patterns as a predictive biomarker of breast cancer risk: effect of tamoxifen. Cancer Epidemiol Biomarkers Prev 8:863–866
35. Chow CK, Venzon D, Jones EC, Premkumar A, O'Shaughnessy J, Zujewski J (2000) Effect of tamoxifen on mammographic density. Cancer Epidemiol Biomarkers Prev 9:917–921
36. Brisson J, Brisson B, Cote G, Maunsell E, Berube S, Robert J (2000) Tamoxifen and mammographic breast densities. Cancer Epidemiol Biomarkers Prev 9:911–915
37. Fisher B, Costantino JP, Wickerham DL et al (2005) Tamoxifen for the prevention of breast cancer: current status of the national surgical adjuvant breast and bowel project P-1 study. J Natl Cancer Inst 97:1652–1662
38. Cuzick J, Warwick J, Pinney E, Warren RM, Duffy SW (2004) Tamoxifen and breast density in women at increased risk of breast cancer. J Natl Cancer Inst 96:621–628
39. Cuzick J, Warwick J, Pinney L et al (2009) Change in breast density as a biomarker of breast cancer risk reduction; results from IBIS-1. Cancer Res 69(Supplement 2):61

Expression of estrogen receptor alpha with a Tet-off adenoviral system induces G0/G1 cell cycle arrest in SKBr3 breast cancer cells

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Abstract. Endocrine therapies targeting estrogen action are pivotal for the prevention and treatment of ER-positive breast cancers. Previous studies sought to recreate hormone responsiveness by the stable expression of ERα in the ER-negative MDA-MB-231 breast cancer cells. Paradoxically, estrogen inhibits breast cancer cell growth when an exogenous ERα is expressed. In this study, we have built on previous studies by developing a Tet-off adenoviral system to express ERα in the ER-negative SKBr3 breast cancer cells that over-express both EGFR and HER2. This system efficiently delivers ERα and the expression level of ERα is controlled by doxycycline in a concentration-dependent manner. The growth of SKBr3 was inhibited by ERα expression and further inhibited in the presence of 1 nM 17ß-estradiol. SKBr3 cells were arrested at G0/G1 cell cycle upon ERα expression, which corresponded to an increase of $p21^{Cip1\ Waf1}$, hypo-phosphorylation of pRb and decrease of E2F1. Estrogen also reduced EGFR and HER2 expression in SKBr3 cells after ERα was expressed. Given that estrogen-induced increase of $p21^{Cip1\ Waf1}$ and decrease of E2F1 was also observed in MDA-MB-231 cells stably transfected with ERα, our results suggest that a common pathway might be shared by different breast cancer cell lines whose growth is suppressed by ectopic ERα and estrogen.

Introduction

Antihormone agents such as tamoxifen and aromatase inhibitors have been widely used to treat estrogen receptor-positive (ER-positive) breast tumors whose growth depends on estrogen (1). However, acquired drug resistance develops as a consequence of long-term antihormone treatment. Interestingly, estrogen exerts apoptotic actions on long-term (>5 years) tamoxifen-resistant breast tumors (2) or long-term (>1 year) estrogen-deprived breast cancer cells (aromatase inhibitor-resistant) (3-5). In addition, the long-term tamoxifen-resistant MCF-7 breast cancer xenografts on ovariectomized athymic mice regrow and become tamoxifen-responsive again after short exposure to physiological estrogen (6). These discoveries suggest a novel strategy to kill antihormone-resistant breast cancer cells with low dose estrogen for short period and re-sensitize the tumors for further antihormone therapy. Phase II clinical trial is now ongoing to treat patients with 12-week course of low-dose estrogen after exhaustive antihormone therapy (7). It seems that estrogen induces apoptosis through different mechanisms in different breast cancer cell models. In one model, estrogen kills LTED breast cancer cells by activating the Fas/FasL signaling pathway (3). However, in another model, estrogen induces apoptosis in MCF-7:5C cells predominantly through a mitochondrial mechanism (5).

Although the development of antihormone therapies is improving cancer care for ER-positive patients, these endocrine therapies are ineffective for the treatment of ER-negative tumors that comprise about 30% of breast cancers. Therefore, it is of value to understand whether the re-introducing of ER expression into ER-negative breast cancer cells that are absolutely antihormone-resistant can modulate responsiveness to endocrine therapies. Multiple approaches are being tested in the laboratory on cultured cell lines and animal models to examine if ER-positive phenotypes can be re-created. Epigenetic methods using DNA methyltransferase (DNMT) inhibitors and/or histone deacetylase (HDAC) inhibitors have been shown to restore ERα expression in ER-negative breast cancer cells, whose growth is then inhibited by antiestrogens (reviewed in ref. 8). Estrogen blocks the growth inhibitory effects of antiestrogen on these cells when ER is restored using the epigenetic methods (9). Additionally, the study of estrogen and antiestrogen action has been described when ectopic ER is expressed in ER-negative cells. One way is to stably transfect ER-negative cells with plasmids encoding ERα. Surprisingly, estrogen treatment leads to growth inhibition rather than stimulation in ER-negative Chinese hamster ovary (CHO) cells and MDA-MB-231 breast cancer cells transfected with a wild-type ERα cDNA (10,11). The estrogen-mediated

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growth inhibition of MDA-MB-231 cells stably transfected with ERα seems to require regulation of E2F1 (12). Stable transfection normally takes months for a colony to be selected and expanded, and a more efficient adenoviral system was developed to express ERα (13). The growth of MDA-MB-231 cells that express ERα delivered by the adenoviral system is also suppressed by estradiol (14).

Antihormone-resistance is often linked with excessive growth factor signaling that has elevated ErbB family cell membrane receptor tyrosin kinases such as EGFR (ErbB-1) and HER2/c-neu (ErbB-2) (15). Most studies to express ectopic ERα have used MDA-MB-231 cells that over-express EGFR. It is important to examine how ER-negative breast cancers cells with high HER2 react to estrogen when an exogenous ERα is expressed. Potential new drug targets could be identified in ER-negative cancers if estrogen triggers apoptosis or growth inhibition through a common mechanism shared by different types of ER-negative cancer cells when an exogenous ERα is introduced. In this study, a Tet-off adenoviral system was developed to deliver ERα to ER-negative breast cancer SKBr3 cells that over-express both EGFR and HER2. The Tet-off adenoviral system is highly efficient and the expression level of ERα is controlled by addition of doxycycline in a concentration-dependent manner. Using this system, we examined the function of ERα and estradiol on cell proliferation. The results suggest that estrogen suppresses the proliferation of SKBr3 cells through a similar mechanism as estrogen does in MDA-MB-231 cells when an ectopic ERα is expressed. The mechanism involves upregulation of $p21^{Cip1\ Waf1}$ and down-regulation of E2F1. The effect of estrogen on growth receptor expression was also examined in SKBr3 cells when exogenous ERα was expressed.

Materials and methods

Cells and culture conditions. SKBr3 and MDA-MB-231 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). MCF-7 cells were from Dr Dean Edwards (University of Texas, San Antonio). MCF-7/F cells were derived from MCF-7 as described (16). SKBr3, MCF-7, and MCF-7/F cells were maintained in full serum RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ ml streptomycin, 1X essential amino acid (all from Invitrogen, Carlsbad, CA) and 6 ng/ml bovine insulin (Sigma-Aldrich, St. Louis, MO). MDA-MB-231 cells were maintained in minimal essential medium supplemented with 5% calf serum and other supplements as the RPMI-1640 complete medium. T47D:C42 cells were cloned from T47D (from ATCC) (17,18) and maintained in estrogen-free RPMI medium which is phenol red-free RPMI-1640 supplemented with 10% dextran-coated charcoal-stripped fetal bovine serum (SFS) and other supplements as the full serum RPMI-1640 medium. All cells were grown at 37°C with 5% CO_2.

Adenoviruses and viral infection. Ad-TRE-ERα adenovirus was custom-generated by Vector Biolabs (Philadelphia, PA) using human type 5 adenoviral backbone with E1 and E3 regions deleted. Adeno-X Tet-off adenovirus stock was purchased from Clontech (Mountain View, CA). It was subsequently amplified with Adeno-X Maxi Purification Kit (Clontech) and the titer was measured with Adeno-X Rapid Titer Kit (Clontech), following the instructions from the manufacturer. Ad-CMV-GFP was purchased from Vector Biolabs. For viral infection, SKBr3 cells were cultured in estrogen-free RPMI medium 3 days before the infection and throughout the experiments. Each adenovirus was added to resuspended cells at 30 MOI (multiplicity of infection), then the cells were divided equally and 1 μg/ml doxycycline was added to half of the cells. Subsequently, $3x10^4$ cells/well were seeded in 24-well plates for cell proliferation assay and $1.5x10^5$ cells/well were seeded in 6-well plates for protein or RNA preparation. After 24 h, the medium was replaced with fresh medium with or without 1 μg/ml doxycycline containing ethanol (EtOH), fulvestrant or 17ß-estradiol at concentrations indicated in the figures. The compound-containing medium was replaced every other day until the cells were harvested.

Cell proliferation assay. Cell DNA content was determined as a measure of cell proliferation using the Fluorescent DNA Quantitation Kit (Bio-Rad, Hercules, CA), which includes 10X TEN buffer, Hoechst dye and calf thymus DNA. Briefly, the cells were washed with 1X phosphate-buffered saline (PBS, Invitrogen), incubated in 0.5 ml 0.1X TEN buffer (diluted from the 10X TEN buffer) for 1 h at 4°C then sonicated for 10 sec. Hoechst dye was diluted in 10X TEN buffer to a final concentration of 25 μg/ml, and 20 μl of the diluted dye was incubated with 0.2 ml of the cell lysate for 1 h at room temperature. The fluorescence was measured with a Mithras LB 940 fluorometer (Oak Ridge, TN) and the total DNA amount was calculated based on a standard curve prepared from calf thymus DNA.

Western blot analysis. Cells were washed twice with 1X PBS and lysed in RIPA buffer (Sigma-Aldrich) supplemented with Complete Protease Inhibitor Cocktail Tablets at 1 tablet/10 ml (Roche, Indianapolis, IN). The protein concentration was determined using the BCA Protein Assay Reagent (Thermo, Rockford, IL) following instructions from the manufacturer. Total protein were separated by 4-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Invitrogen) and electro-blotted to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature in TBST buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat milk then incubated overnight at 4°C with primary antibodies. After being washed 3 times with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature, washed again with TBST and visualized using ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ). The antibodies against EGFR (Cat# 2232), Rb (Cat# 9309), Rb-P(s807/811) (Cat# 9308) and mTOR (Cat# 2983) were purchased from Cell Signaling (Danvers, MA). Antibodies against HER2 (Ab-20) and ERα (Ab-15) were from Thermo Lab Vision/NeoMarkers (Fremont, CA). Antibody against ß-actin (AC-15) was from Sigma-Aldrich. Antibodies against p21 (Cat# sc-469) and E2F1 (Cat# sc-193) were from Santa Cruz Biotechnology (Santa Cruz, CA). The HRP-conjugated

anti-mouse or anti-rabbit secondary antibodies were from Cell Signaling.

ERE-Luciferase reporter assay. SKBr3 cells were infected and seeded in 24-well plates as described above. Twenty-four hours after infection, 0.3 μg 5X ERE-firefly-luciferase reporter plasmid and 0.1 μg control TA-Renilla-luciferase plasmid (19) were used to transfect each well of cells using 15 μl FuGENE® HD transfection reagent (Roche) following instructions from the manufacturer. After 24 h, the medium was replaced with fresh medium containing different compounds as indicated in the figure. Cells were harvested 48 h after treatment and the activities of firefly and Renilla luciferases were analyzed with Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) following instructions from the manufacturer.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) assay. Total RNA was isolated with RNeasy Mini Kit (Qiagen, Valencia, CA) and quantitated with spectrometer. The cDNA was prepared from 1 μg RNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a 20-μl reaction mix assembled according to instructions from the manufacturer. The reaction mix was incubated at 25°C for 10 min and 85°C for 90 min then diluted with 200 μl water. Two microliters of the diluted products were used for subsequent real-time PCR amplification using either Power SYBR® Green PCR Master Mix or Taqman® Universal PCR Master Mix, both from Applied Biosystems. The reactions were performed with 7900 HT Fast Real-Time PCR System (Applied Biosystems) in 384-well plates using the standard settings. The sequences of the primers are as follows: PS2-F, 5'-CATCGACGTCCCT CCAGAAGAG; PS2-R, 5'-CTCTGGGACTAATCACCGTG CTG; PR-F, 5'-CGCGCTCTACCCTGCACTC; PR-R, 5'-TGA ATCCGGCCTCAGGTAGTT; E2F1-F, 5'-CCCAACTCCCT CTACCCTTGA; E2F1-R, 5'-TCTGTCTCCCTCCCTCACT TTC; p21-F, 5'-CTGGAGACTCTCAGGGTCGAA; p21-P, 5'-6ACGGCGGCAGACCAGCATGA[BHQ1]; p21-R, 5'-GGCGTTTGGAGTGGTAGAAATCT; Rb-F, 5'-CTTGCAT GGCTCTCAGATTCAC; Rb-R, 5'-AGAGGACAAGCAGA TTCAAGGTG; 36B4-F, 5'-GTGTTCGACAATGGCAGCAT; 36B4-R, 5'-GACACCCTCCAGGAAGCGA.

Cell cycle analysis. The adenovirus-infected SKBr3 cells were plated at $1x10^6$ per 10-cm culture dish and treated with different compounds for 48 h. All the cells were harvested and fixed in 70% ethanol in 1X PBS overnight at 4°C. The fixed cells were washed twice with 1X PBS and incubated with propidium iodide (PI) staining buffer (1X PBS, 0.1% Triton X-100, 200 μg/ml RNase A, and 50 μg/ml PI) for 30 min at 37°C. The stained cells were analyzed using FACScan flow cytometer (Becton Dickinson, San Jose, CA) and the data were analyzed using FlowJo program (Tree Star Inc., Ashland, OR).

Statistical analysis. Data were expressed as means ± standard deviation (SD) for at least three independent repeated experiments. Statistical significance ($p<0.05$) between two groups was assessed by unpaired, one-tailed t-test.

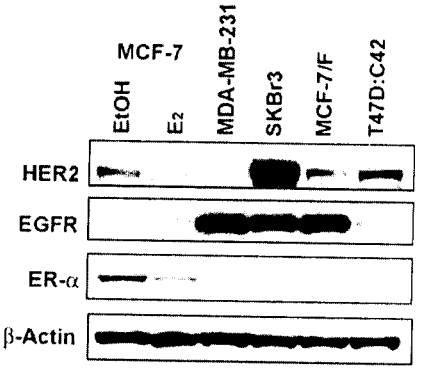

Figure 1. Comparison of HER2, EGFR and ERα expression between SKBr3 and other breast cancer cells. MCF-7 cells were grown in estrogen-free RPMI medium for 4 days then treated with either EtOH control or 1 nM E_2 for 2 more days before harvest. Other cells were grown in medium as described in Materials and methods. Fifty micrograms of total proteins were used for Western blot analysis for HER2, EGFR and ERα. The ß-actin was also examined as a loading control.

Results

Expression of ERα in SKBr3 breast cancer cells with Tet-off adenoviral system. Most studies expressing ectopic ERα in ER-negative breast cancer cells have used MDA-MB-231 cells which have high levels of EGFR, but low levels of HER2. Since about 20% breast cancers are HER2-positive, it is important to examine if hormone-responsiveness could be restored in ER-negative breast cancer cells that over-express HER2. Therefore, we chose SKBr3 cells which over-express both HER2 and EGFR for this study. The expression of HER2, EGFR and ERα were compared between SKBr3 and several other breast cancer cell lines as shown in Fig. 1. The ER-positive MCF-7 cells expressed low levels of EGFR and HER2, and estrogen treatment decreased HER2 expression. MDA-MB-231 cells had high levels of EGFR but little HER2. The ER-negative MCF-7/F cells derived from MCF-7 (16) highly expressed EGFR and moderately expressed HER2. Another ER-negative T47D:C42 cells cloned from ER-positive T47D cells (17,18) had moderate expression of HER2 and little expression of EGFR. Only SKBr3 cell had high levels of both HER2 and EGFR.

A Tet-off adenoviral delivery system was developed to express ERα in SKBr3 cells. The infection efficiency of adenoviruses in SKBr3 cells was analyzed using a green fluorescent protein (GFP) reporter adenovirus (Ad-CMV-GFP). As shown in Fig. 2A, >95% cells were infected and expressing GFP. The adenoviral system is more efficient than plasmid transfection which normally has <50% efficiency, thus a lengthy selection for stable-transfected cell colonies can be avoided using the adenoviral system since almost all the cells were infected and expressed the delivered gene of interest. The expression of ERα can be turned off by doxycycline when cells are co-infected with Adeno-X Tet-Off and Ad-TRE-ERα adenovirus simultaneously. As shown in Fig. 2B, the expression level of ERα decreased as the concentration of doxycycline increased from 0 to 0.8 ng/ml, and ERα expression was almost undetectable as doxycycline concentration was

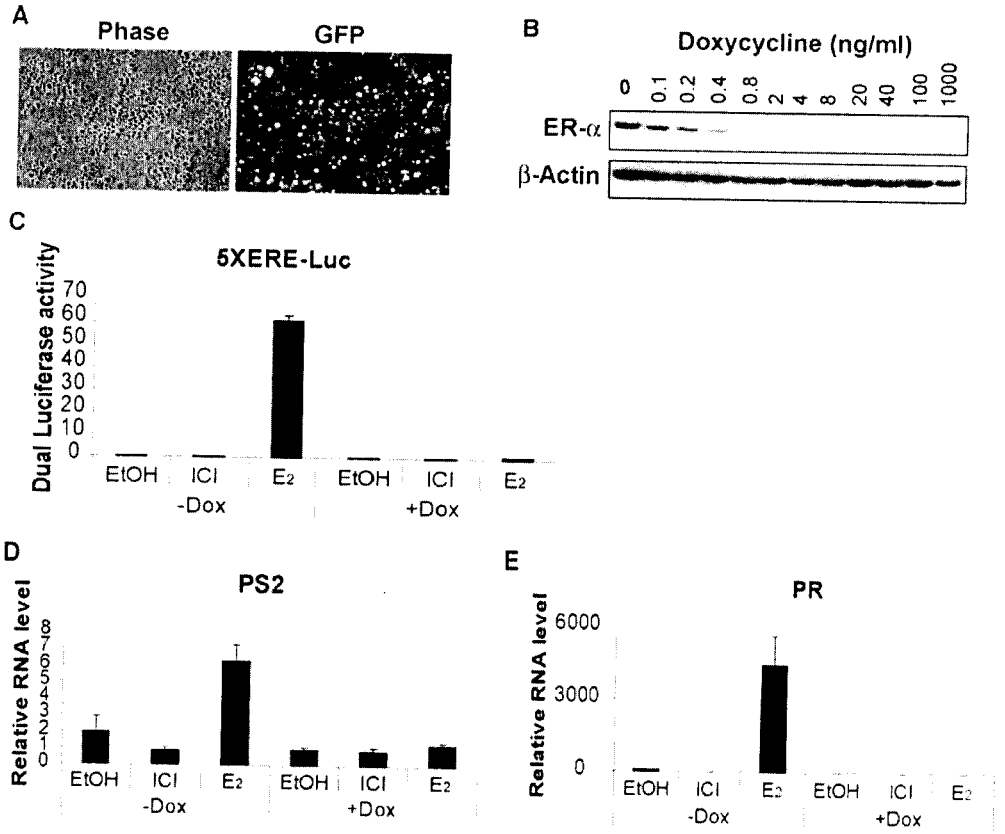

Figure 2. The Tet-off adenoviral system to express ERα in SKBr3 cells. (A) SKBr3 cells were infected with Ad-CMV-GFP and observed 24 h after infection with a TE300 fluorescence microscope (Nikon Instruments, Melville, NY). (B) SKBr3 cells were co-infected by Adeno-X Tet-off and Ad-TRE-ERα in the presence of doxycycline at various concentrations. The cells were harvested 48 h after infection and total protein was extracted for Western blot analysis (C) SKBr3 cells infected by Adeno-X Tet-off and Ad-TRE-ERα in the presence (+Dox) or absence (-Dox) of 1 μg/ml doxycycline were transfected with 5xERE-firefly-luciferase and TA-Renilla-luciferase plasmids. The cells were harvested for dual luciferase activity assay after 48-h treatment with the compounds as indicated. The ratio of firefly luciferase vs Renilla luciferase activities were plotted and the number of the +Dox/EtOH sample was arbitrarily set to be 1 for easy comparison. (D) SKBr3 cells infected by Adeno-X Tet-off and Ad-TRE-ERα in the presence (+Dox) or absence (-Dox) of 1 μg/ml doxycycline were treated with 0.1% EtOH, 1 μM fulvestrant (ICI) or 1 nM 17ß-estradiol (E_2) for 48 h. The total RNA was extracted for real-time RT-PCR analysis of PS2 or PR (E) against endogenous control 36B4 using a relative standard curve generated by 10-fold serial dilution of MCF-7 cDNA. The value of the +Dox/EtOH sample was arbitrarily set to be 1 for easy comparison.

above 2 ng/ml. The ERα expressed in SKBr3 cells by the adenovirus is fully functional. It activated luciferase reporter containing 5 estrogen receptor elements (5X ERE) in the presence of 1 nM E_2 while the luciferase reporter was not detected either when ERα was not expressed (+Dox) or when EtOH control or pure antiestrogen fulvestrant (ICI) was added (Fig. 2C). Real-time RT-PCR assay also indicated that the exogenous ERα induced the endogenous estrogen-responsive genes PS2 and progesterone receptor (PR) in response to E_2. The RNA level of PS2 was doubled by expression of ERα itself (compare -Dox/EtOH and +Dox/EtOH), and addition of 1 nM E_2 further increased PS2 RNA to 6-fold (compare -Dox/E_2 and +Dox/EtOH), but addition of fulvestrant did not change PS2 RNA expression (Fig. 2D). The induction of PR RNA was more dramatic, as PR RNA was barely detectable without ERα expression (+Dox) or with ERα but in the presence of EtOH control or antiestrogen fulvestrant. However, E_2 addition increased PR RNA level by thousands of folds when ERα was expressed (compare -Dox/E_2 and +Dox/EtOH, Fig. 2E).

Cell proliferation of SKBr3 cells after ERα expression. We next examined the effects of ERα on SKBr3 cell proliferation by measuring the total cellular DNA content. As shown in Fig. 3A, growth of SKBr3 cells was irresponsive to fulvestrant, 4-hydoxytamoxifen or E_2 if no ERα was expressed. However, expression of ERα itself reduced cell proliferation to about 70% (compare -Dox/EtOH and +Dox/EtOH), although the reduction was not statistically significant, similar inhibition was repeatedly observed in independent experiments. The ERα-mediated growth suppression was abolished by fulvestrant, and addition of 1 nM E_2 or 1 μM 4-hydroxytamoxifen inhibited SKBr3 cell proliferation to about 40 and 50% respectively, which was statistically significant (compare with the +Dox/EtOH control). With the ectopic expression of ERα, E_2 inhibited the growth of SKBr3 cells in a dose-dependent manner, as shown in Fig. 3B. Statistical difference was reached when E_2 concentration was $\geq 10^{-10}$ M (0.1 nM), comparing with the +Dox/EtOH control. Similar results were obtained in the time-dependent growth curve shown in Fig. 3C.

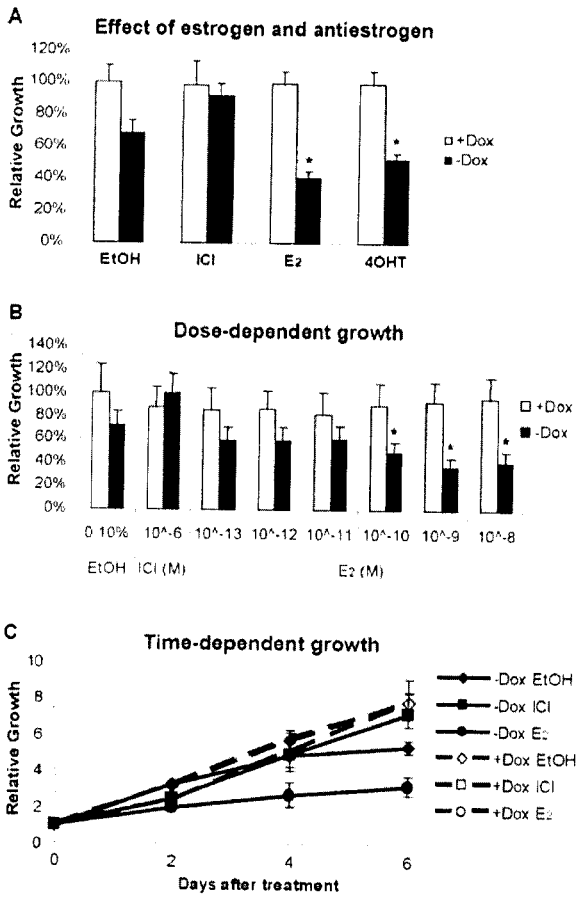

Figure 3. The effects of ERα expression and estrogen/antiestrogen treatment on the proliferation of SKBr3 cells. SKBr3 cells were infected by Adeno-X Tet-off and Ad-TRE-ERα in the presence (+Dox) or absence (-Dox) of 1 μg/ml doxycycline, treated by the 0.1% EtOH (v/v), 1 μM fulvestrant (ICI), 1 μM 4-hydroxytamoxifen (4OHT) or E_2 (at final concentration of 1 nM or as indicated in the graph) and harvested for DNA quantification. (A) Growth with different ER ligands treated for 6 days. (B) Dose-dependent growth with various E_2 concentrations treated for 6 days. (C) Time-dependent growth with cells harvested every 2 days after treatment. *Samples with a statistically significant difference ($p<0.05$ by t-test) from the +Dox/EtOH control.

ERα expression arrests SKBr3 cells at G0/G1 cycle. Next, flow cytometry analysis was performed to examine cell cycle progression of SKBr3 cells when ERα was expressed. As shown in Fig. 4, about 50% cells were at G0/G1 cell cycle without ERα (+Dox) or with ERα but in the presence of fulvestrant (-Dox/ICI). However, the population of cells at G0/G1 cell cycle increased to about 80% when ERα was expressed in the presence of EtOH control or 1 nM E_2. Apoptosis was not observed in SKBr3 cells as there was no significant cell accumulation at sub-G1 phase (cell debris) when ERα was expressed. Annexin V/PI staining, caspase activity assay or PARP-cleavage assay all confirmed that apoptosis did not occur (data not shown).

Modulation of E2F1 cell cycle checkpoint proteins by E_2 and ERα. The transcription factor E2F1 plays an important role in G1 to S cell cycle progression. Before cells enter S phase, hypo-phosphorylated pRb protein binds to E2F1 and prevents it from activating downstream genes essential for DNA replication and cell proliferation. Activation of cyclin-dependent kinases (CDKs) phosphorylates pRb and releases E2F1 for action. CDK inhibitory proteins such as $p21^{Cip1\,Waf1}$, $p27^{Kip1}$ and $p16^{INK4A}$ inhibit CDKs activity thus lead to hypo-phosphorylation of pRb and inactivation of E2F1, which in turn causes cell cycle arrest at G0/G1 phase. Stender *et al* (12) found that E2F1 and p21 were differentially regulated by estrogen in ER-positive MCF-7 cells and ER-stably-transfected MDA-MB-231 cells. Therefore, we also examined modification of $p21^{Cip1\,Waf1}$/pRb/E2F1 pathway proteins by E_2 and ERα in SKBr3 cells. As shown in Fig. 5A, $p21^{Cip1\,Waf1}$ was undetectable without ERα expression (+Dox) or with ERα expression but in the presence of fulvestrant. The $p21^{Cip1\,Waf1}$ protein level was increased by ERα expression and further increased by the addition of E_2, which coordinated with the phosphorylation status of pRb. Opposite regulation of E2F1 was observed by ERα expression and E_2 treatment. The RNA levels of $p21^{Cip1\,Waf1}$ and E2F1 were regulated in a similar patten as the protein levels (Fig. 5B). A moderate down-regulation of pRb at protein level was also observed in ERα-expressing samples but not at the RNA level. This might be resulted from the up-regulation of $p21^{Cip1\,Waf1}$ because $p21^{Cip1\,Waf1}$ mediates pRb protein degradation (20).

The effects of estrogen on HER2 and EGFR expression. Intimate crosstalk between hormone receptor signaling and growth factor receptor signaling is a major contributor to breast cancer progression and endocrine resistance (15). However, an inverse correlation is often found between ER and HER2 (21,22), and estrogen down-regulates HER2 expression in ER-positive MCF-7 cells (23) (Fig. 1). Growth factor signaling is essential for SKBr3 cell proliferation, therefore we examined the effects of estrogen and exogenous ERα on the expression of HER2 and EGFR. As shown in Fig. 6, ERα expression itself had little effect on HER2 and EGFR expression (compare -Dox/EtOH and +Dox/EtOH), however, 2-day treatment with E_2 decreased EGFR protein level and 6-day treatment of E_2 also reduced HER2 protein level. These results suggest that ectopic expression of ERα and E_2 treatment might be a way to switch the more aggressive growth-factor receptor-positive tumors to the prognostically more favorable hormone-sensitive type.

Discussion

Tet-off adenoviral system is a valuable approach to deliver ectopic genes. In this study, we developed a Tet-off adenovirus to express ERα in ER-negative SKBr3 cells. Adenoviruses infect the cells and deliver the gene of interest with over 95% efficiency, thus can be used to study cellular effects of the interested gene in a 'transient expression' experiment. This is not always possible using the traditional plasmid transfection with <50% delivery efficiency because the background is high when most cells are not expressing the gene of interest. Instead, a stably-transfected clone has to be selected and expanded, which is a time-consuming process. In addition, the phenotype of a stably-transfected clone may not be the

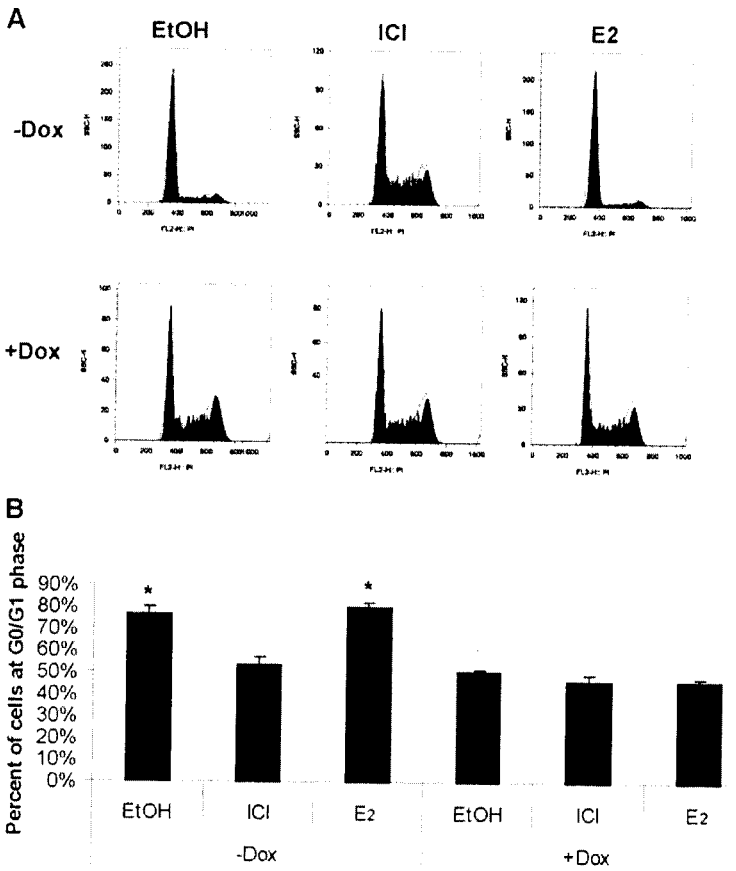

Figure 4. Cell cycle analysis of SKBr3 cells expressing ERα. SKBr3 cells were infected by Adeno-X Tet-off and Ad-TRE-ERα in the presence (+Dox) or absence (-Dox) of 1 μg/ml doxycycline, treated by 0.1% EtOH, 1 μM fulvestrant (ICI) or 1 nM E_2 for 2 days and harvested for cell cycle analysis. (A) Flow cytometry analysis of cell cycle distribution. (B) Percentage of cells at G0/G1 cell cycle from three independent experiments. *Samples with a statistically significant difference ($p<0.05$ by t-test) from the +Dox/EtOH control.

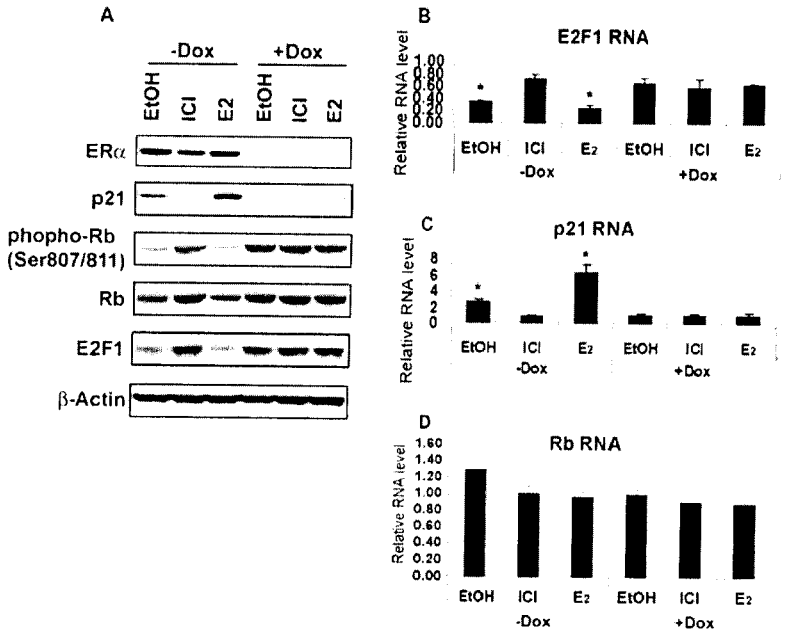

Figure 5. Modification of $p21^{Cip1\ Waf1}$, pRb and E2F1 by ERα/E_2 in SKBr3 cells. SKBr3 cells were infected, treated and harvested as in Fig. 4. Protein was extracted for Western blot analysis (A) and RNA was prepared for real-time RT-PCR analysis to detect E2F1 (B), $p21^{Cip1\ Waf1}$ (C) or pRb (D) as described in Fig. 2. *Samples with a statistically significant difference ($p<0.05$ by t-test) from the +Dox/EtOH control.

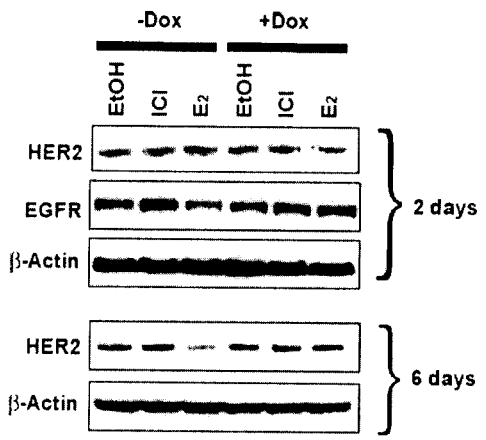

Figure 6. The effects of ERα expression and estrogen treatment on the expression of HER2 and EGFR in SKBr3 cells. SKBr3 cells were infected, treated for 2 days or 6 days then harvested for protein extraction and Western blot analysis.

direct result of the interested gene expression but the result of the random gene insertion at the host genome. The inability of adenoviruses to integrate into the host genome minimizes the complications of destroying or activating other host genes, thus adenoviral vector is a valuable tool to express exogenous genes for gene therapy. Adenovirus-based therapy to express p53 tumor suppressor, Advxin™ (Introgen Therapeutics, Austin, TX), has demonstrated safety profile and clinic efficacy in several tumor types and approval is being sought in Europe and the United States to treat recurrent, refractory head and neck cancer (24). Another similar adenoviral p53 transfer therapy, Gendicine® (Benda Pharmaceutical, China), has been approved to treat head and neck cancer in China (25). Therefore, adenovirus-based vectors could potentially be developed in the future to express ERα in ER-negative breast cancers to restore hormone responsiveness.

The Tet-off system adds another advantage to the expression method by controlling the expression level of interested gene. As shown in Fig. 2B, the amount of ERα expressed is regulated by doxycycline. This provides a valuable approach to study gene function in a dose-dependent manner, which is not achievable using a constitutively-expressing vector. Moreover, expression of the interested gene can be turned on or off by removal or addition of doxycycline at any time, thus studying the gene function in a timely fashion is possible.

Ectopic ERα expression and E_2 treatment arrest SKBr3 cells at G0/G1 cell cycle. In MDA-MB-231 cells, ectopic ERα expression by adenovirus itself had no effect on cell proliferation, but treatment of E_2 suppressed cell proliferation (14). However, in SKBr3 cells, the expression of ERα itself inhibits cell proliferation and E_2 treatment amplifies the growth inhibitory effects, while pure antiestrogen fulvestrant abolished growth inhibitory effects of ERα (Fig. 3). It is possible that ERα has more ligand-independent activity in SKBr3 cells which over-express both HER2 and EGFR than in MDA-MB-231 cells that only over express EGFR. Estrogen exerts similar growth inhibitory effects on MDA-MB-231 and SKBr3 cells when ERα is expressed, but ERα-expressing MDA-MB-231 and SKBr3 cells respond differently to tamoxifen which is ineffective in MDA-MB-231 cells (14) but inhibitory in SKBr3 cells (Fig. 3A). The mechanisms remain to be elucidated and could be that these two cell types have various cellular profile of transcription factors and different levels of nuclear receptor coregulators.

The proliferation inhibition mediated by ERα and E_2 in SKBr3 cells is likely due to cell cycle arrest at G0/G1 phase (Fig. 4), since significant apoptosis was not observed. Similar to MDA-MB-231 cells, E_2 and ERα modify the expression of G1 to S phase checkpoint proteins $p21^{Cip1\ Waf1}$ and E2F1 in SKBr3 cells (Fig. 5), suggesting an important role of E2F1 in hormone-mediated regulation of cell proliferation. E2F1 is critical to control cell cycle progression and apoptosis (26), and its overexpression is often linked to poor prognosis of breast cancer (27-30). Therefore, E2F1 is a potential drug target for breast cancer. In addition, E_2 treatment down-regulates expression of HER2 and EGFR in ERα-expressing SKBr3 cells (Fig. 6), suggesting that growth factor signalling could be diminished by E_2/ERα and that a less aggressive hormone-responsive cancer type can be re-created.

Strategically, it is important to note that the ectopic E_2/ERα complex is able to block cell cycle progression at G0/G1 phase. A similar effect occurs with endogenous E_2/ERα complex in the MCF-7:5C cell line that is resistant to estrogen withdrawal (5). However, in contrast to the MCF-7:5C cells that progress to apoptosis, SKBr3 cells with ectopic ERα do not. It will be important to discover the reason for the failure to trigger apoptosis because the ectopic ERα could be used to define and identify a common pathway for future drug discovery. In other words, a proportion of cancers that never had the ER may have a vestigial pathway that could be activated to provoke apoptosis. The Tet-off adenoviral ERα system may be an approach to discover the veracity of this drug discovery strategy.

Acknowledgements

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References

1. Jordan VC: A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 69: 1243-1254, 2009.
2. Wolf DM and Jordan VC: A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127: 23-33, 1993.
3. Song RX, Mor G, Naftolin F, *et al*: Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 93: 1714-1723, 2001.

4. Lewis JS, Osipo C, Meeke K and Jordan VC: Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 94: 131-141, 2005.
5. Lewis JS, Meeke K, Osipo C, *et al*: Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97: 1746-1759, 2005.
6. Yao K, Lee ES, Bentrem DJ, *et al*: Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6: 2028-2036, 2000.
7. Jordan VC: The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer - urvival or death? J Clin Oncol 26: 3073-3082, 2008.
8. Brinkman JA and El-Ashry D: ER re-expression and re-sensitization to endocrine therapies in ER-negative breast cancers. J Mammary Gland Biol Neoplasia 14: 67-78, 2009.
9. Bayliss J, Hilger A, Vishnu P, Diehl K and El-Ashry D: Reversal of the estrogen receptor negative phenotype in breast cancer and restoration of antiestrogen response. Clin Cancer Res 13: 7029-7036, 2007.
10. Jiang SY and Jordan VC: Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. J Natl Cancer Inst 84: 580-591, 1992.
11. Levenson AS and Jordan VC: Transfection of human estrogen receptor (ER) cDNA into ER-negative mammalian cell lines. J Steroid Biochem Mol Biol 51: 229-239, 1994.
12. Stender JD, Frasor J, Komm B, Chang KCN, Kraus WL and Katzenellenbogen BS: Estrogen-regulated gene networks in human breast cancer cells: involvement of E2F1 in the regulation of cell proliferation. Mol Endocrinol 21: 2112-2123, 2007.
13. Lazennec G, Alcorn JL and Katzenellenbogen BS: Adenovirus-mediated delivery of a dominant negative estrogen receptor gene abrogates estrogen-stimulated gene expression and breast cancer cell proliferation. Mol Endocrinol 13: 969-980, 1999.
14. Lazennec G and Katzenellenbogen BS: Expression of human estrogen receptor using an efficient adenoviral gene delivery system is able to restore hormone-dependent features to estrogen receptor-negative breast carcinoma cells. Mol Cell Endocrinol 149: 93-105, 1999.
15. Arpino G, Wiechmann L, Osborne CK and Schiff R: Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. Endocr Rev 29: 217-233, 2008.
16. Liu H, Cheng D, Weichel AK, *et al*: Cooperative effect of gefitinib and fumitremorgin c on cell growth and chemosensitivity in estrogen receptor alpha negative fulvestrant-resistant MCF-7 cells. Int J Oncol 29: 1237-1246, 2006.
17. Murphy CS, Pink JJ and Jordan VC: Characterization of a receptor-negative, hormone-nonresponsive clone derived from a T47D human breast cancer cell line kept under estrogen-free conditions. Cancer Res 50: 7285-7292, 1990.
18. Pink JJ, Bilimoria MM, Assikis J and Jordan VC: Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. Br J Cancer 74: 1227-1236, 1996.
19. Ariazi EA, Kraus RJ, Farrell ML, Jordan VC and Mertz JE: Estrogen-related receptor alpha1 transcriptional activities are regulated in part via the ErbB2/HER2 signaling pathway. Mol Cancer Res 5: 71-85, 2007.
20. Broude EV, Swift ME, Vivo C, *et al*: p21(Waf1/Cip1/Sdi1) mediates retinoblastoma protein degradation. Oncogene 26: 6954-6958, 2007.
21. Schiff R, Massarweh SA, Shou J, *et al*: Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators. Cancer Chemother Pharmacol 56 (Suppl. 1): 10-20, 2005.
22. Huang HJ, Neven P, Drijkoningen M, *et al*: Hormone receptors do not predict the HER2/neu status in all age groups of women with an operable breast cancer. Ann Oncol 16: 1755-1761, 2005.
23. Read LD, Keith D Jr, Slamon DJ and Katzenellenbogen BS: Hormonal modulation of HER-2/neu protooncogene messenger ribonucleic acid and p185 protein expression in human breast cancer cell lines. Cancer Res 50: 3947-3951, 1990.
24. Senzer N and Nemunaitis J: A review of contusugene ladenovec (Advexin) p53 therapy. Curr Opin Mol Ther 11: 54-61, 2009.
25. Patil SD, Rhodes DG and Burgess DJ: DNA-based therapeutics and DNA delivery systems: a comprehensive review. AAPS J 7: E61-E77, 2005.
26. De Gregori J and Johnson DG: Distinct and overlapping roles for E2F family members in transcription, proliferation and apoptosis. Curr Mol Med 6: 739-748, 2006.
27. Vuaroqueaux V, Urban P, Labuhn M, *et al*: Low E2F1 transcript levels are a strong determinant of favorable breast cancer outcome. Breast Cancer Res 9: R33, 2007.
28. Baldini E, Camerini A, Sgambato A, *et al*: Cyclin A and E2F1 overexpression correlate with reduced disease-free survival in node-negative breast cancer patients. Anticancer Res 26: 4415-4421, 2006.
29. Han S, Park K, Bae BN, *et al*: E2F1 expression is related with the poor survival of lymph node-positive breast cancer patients treated with fluorouracil, doxorubicin and cyclophosphamide. Breast Cancer Res Treat 82: 11-16, 2003.
30. Zhang SY, Liu SC, Al-Saleem LF, *et al*: E2F-1: a proliferative marker of breast neoplasia. Cancer Epidemiol Biomarkers Prev 9: 395-401, 2000.

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Tumor and Stem Cell Biology

Cancer Research

The G Protein–Coupled Receptor GPR30 Inhibits Proliferation of Estrogen Receptor–Positive Breast Cancer Cells

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Abstract

The G protein–coupled receptor GPR30 binds 17β-estradiol (E_2) yet differs from classic estrogen receptors (ERα and ERβ). GPR30 can mediate E_2-induced nongenomic signaling, but its role in ERα-positive breast cancer remains unclear. Gene expression microarray data from five cohorts comprising 1,250 breast carcinomas showed an association between increased GPR30 expression and ERα-positive status. We therefore examined GPR30 in estrogenic activities in ER-positive MCF-7 breast cancer cells using G-1 and diethylstilbestrol (DES), ligands that selectively activate GPR30 and ER, respectively, and small interfering RNAs. In expression studies, E_2 and DES, but not G-1, transiently downregulated both ER and GPR30, indicating that this was ER mediated. In Ca^{2+} mobilization studies, GPR30, but not ERα, mediated E_2-induced Ca^{2+} responses because E_2, 4-hydroxytamoxifen (activates GPR30), and G-1, but not DES, elicited cytosolic Ca^{2+} increases not only in MCF-7 cells but also in ER-negative SKBr3 cells. Additionally, in MCF-7 cells, GPR30 depletion blocked E_2-induced and G-1–induced Ca^{2+} mobilization, but ERα depletion did not. Interestingly, GPR30-coupled Ca^{2+} responses were sustained and inositol triphosphate receptor mediated in ER-positive MCF-7 cells but transitory and ryanodine receptor mediated in ER-negative SKBr3 cells. Proliferation studies involving GPR30 depletion indicated that the role of GPR30 was to promote SKBr3 cell growth but reduce MCF-7 cell growth. Supporting this, G-1 profoundly inhibited MCF-7 cell growth, potentially via p53 and p21 induction. Further, flow cytometry showed that G-1 blocked MCF-7 cell cycle progression at the G_1 phase. Thus, GPR30 antagonizes growth of ERα-positive breast cancer and may represent a new target to combat this disease. *Cancer Res; 70(3); 1184–94. ©2010 AACR.*

Introduction

The G protein–coupled receptor GPR30 is a seven-transmembrane domain protein identified as a novel 17β-estradiol (E_2)–binding protein structurally distinct from the classic estrogen receptors α and β (ERα and ERβ). GPR30 can mediate rapid E_2-induced nongenomic signaling events, including stimulation of adenylyl cyclase, and, via transactivation of epidermal growth factor receptors, induces mobilization of intracellular calcium (Ca^{2+}) stores and activation of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways (1, 2). GPR30 also exhibits prognostic utility in endometrial (3), ovarian (4), and breast cancer (5, 6) and can modulate growth of hormonally responsive cancer cells (7–11). Therefore, GPR30 likely plays important roles in modulating estrogen responsiveness and in the development and/or progression of hormonally responsive cancers. Moreover, GPR30 represents a promising new target for drug discovery in hormonally responsive disease.

In addition to E_2, the selective ER modulator (SERM) tamoxifen, one of its active metabolites 4-hydroxytamoxifen (4OHT), and the complete antiestrogen fulvestrant all activate GPR30 (12–14). GPR30 does not significantly bind the nonsteroidal full ER agonist diethylstilbestrol (DES; ref. 12). Drug discovery efforts have yielded two GPR30-selective high-affinity ligands: an agonist termed G-1 (15) and, recently, an antagonist termed G-15 (16). G-1 and G-15 do not bind ERs at concentrations up to 10^{-6} mol/L (15, 16). Moreover, G-1 specificity for GPR30 was illustrated by showing it does not significantly bind 25 other G protein–coupled receptors (17).

GPR30 has been shown to mediate the proliferative effects of E_2 in thyroid (7), endometrial (8, 18), ovarian (9), and

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ER-negative SKBr3 breast cancer cell lines (9, 10) because GPR30 depletion, using antisense oligonucleotides or RNA interference (RNAi) methodologies, abrogated E_2-stimulated growth in these cells. However, GPR30-mediated growth effects differ in ER-positive MCF-7 breast cancer cells. Ahola and colleagues (11) reported that transient overexpression of GPR30 in MCF-7 cells inhibited bromodeoxyuridine incorporation, an indicator of proliferation.

We investigated GPR30 largely in ER-positive MCF-7 with some comparisons to ER-negative SKBr3 breast cancer cells. First, a statistical association was sought between GPR30 and ERα-positive status in publicly available breast carcinoma microarray data sets. Next, the contribution of ERα and GPR30 in several E_2-responsive activities, including regulation of GPR30 expression, intracellular Ca^{2+} mobilization, cellular growth, and cell cycle progression, was studied using receptor-specific ligands and small interfering RNA (siRNA) methodology.

Materials and Methods

Breast cancer microarray data mining. GPR30 mRNA levels and ER status were extracted from gene expression microarrays comprising 1,250 breast carcinomas across five distinct cohorts. The first cohort or the NKI cohort (n = 295; Netherlands Cancer Institute, Amsterdam, the Netherlands) was derived from van de Vijver and colleagues (19).[8] NKI data were obtained using two-color 60-polymer oligonucleotide arrays. cRNA from one tumor was competitively hybridized against a pooled reference cRNA from all tumors. Expression values corresponded to normalized $\log_2$ ratio intensity units. ER-positive status was supplied with the microarray data (19). Pearson's correlation coefficients were computed between GPR30 and all other genes using the R software package.[9] Cohorts 2 to 5 were obtained from Gene Expression Omnibus (GEO; ref. 20). These cohorts are termed the Uppsala cohort (GSE3494/GSE4922/GSE6532; samples collected in Uppsala County, Sweden), the Stockholm cohort (GSE1456; samples collected at the Karolinska Hospital in Stockholm, Sweden), the EMC cohort (GSE2034/GSE5327; samples collected at the Erasmus Medical Center, Rotterdam, the Netherlands), and the TRANSBIG cohort (GSE7390; samples collected by the translational research network managed by the Breast International Group). The Uppsala and EMC cohorts contained samples processed at the same institution that span multiple GEO accession numbers. These four cohorts used Affymetrix microarray technology. Where available, raw data (in the form of CEL files) were downloaded; otherwise, MAS5.0 normalized data were downloaded (CEL files were available for all studies except GSE2034 and GSE5327). All data preprocessing and MAS5.0 normalization were performed using R software and the justMAS function in the simpleAffy library from Bioconductor (no background correction, target intensity of 600; ref. 21). After normalization, gene expression data were extracted for the GPR30 probe 210640_s_at. ER status was provided via Supplementary Data in GEO.

Compounds and cell lines. E_2, DES, and 2-aminoethyldiphenylborinate (2APB) were from Sigma-Aldrich. G-1 and fulvestrant (ICI 182,780, Faslodex) were from Tocris. Xestospongin C (XeC) and ryanodine (Ry) were from Calbiochem, EMD Biosciences. All agents were added to culture medium at 1:10,000 to 1:1,000 (v/v). Fura-2 AM and all cell culture reagents were from Invitrogen. MCF-7:WS8 human mammary carcinoma cells were used in all experiments indicating MCF-7 cells; they were clonally selected for sensitivity to E_2-stimulated growth (22). SKBr3 cells were purchased from the American Type Culture Collection. Both cell lines were maintained in estrogenized medium [RPMI-1640 plus 10% fetal bovine serum (FBS)]. MCF-7 cells were switched to estrogen-free medium (phenol red–free RPMI-1640 plus 10% charcoal-stripped FBS) for 2 d before all experiments, except where noted.

Real-time quantitative PCR assays. Quantitative PCR (qPCR) was conducted as previously described (23). Target mRNA levels were normalized to PUM1 [pumilio homologue 1 (*Drosophila*)] mRNA levels (24). See Supplementary Materials and Methods for primer sequences. Data were analyzed by comparison with a serial dilution series of MCF-7 cell cDNA. Values in each group were averaged from four biological replicates (unless otherwise indicated), and each biological replicate was averaged from four technical replicates.

siRNA transfection. MCF-7 cells that had been maintained in estrogenized medium were transfected with siRNAs for 6 h in serum-free Opti-MEM (Invitrogen) using Dharmafect 1 (Dharmacon RNAi Technologies) followed by overnight recovery in estrogenized medium. The transfection was carried out a second time, and then the cells were immediately switched to estrogen-free medium and again allowed overnight recovery. siRNAs were transfected at 200 nmol/L final concentration, except in Ca^{2+} experiments in which siRNAs were transfected at 100 nmol/L and cotransfected with siGLO Green, a 6-carboxyfluorescein–labeled inactive double-stranded RNA. See Supplementary Materials and Methods for ERα and GPR30 siRNA sequences (Dharmacon RNAi Technologies).

Ca^{2+} imaging. Cytoplasmic Ca^{2+}concentrations were measured using Fura-2 AM and microscopy as previously described (25). Flat SKBr3 cells were imaged because they were the major morphologic cell type, whereas rounded SKBr3 cells were not imaged. All compounds were administered at 1 min.

Cellular proliferation. Cell growth was assessed using Hoechst 33258 (Invitrogen) and compared with a standard curve of serial-diluted calf thymus DNA as previously described (26, 27).

Cell cycle analyses. Cell cycle distribution was determined by propidium iodide staining and using a fluorescence-activated cell sorter (Becton Dickinson) as previously described (27). Data were analyzed using FlowJo 7.2.5 for Windows (Tree Star).

[8] http://www.rii.com/publications/2002/nejm.html
[9] http://www.R-project.org

Immunoblot analyses. Immunoblotting was carried out using 40 μg protein per lane as previously described (23). Membranes were probed using antibodies against ERα (AER 611; Lab Vision), p53 (DO-1; Calbiochem), p21 (F-5; Santa Cruz Biotechnology), cyclin D1 (DCS-6; Santa Cruz Biotechnology), cyclin B1 (D-11; Santa Cruz Biotechnology), and β-actin (AC-15; Sigma-Aldrich). Membranes were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Statistical analyses. Mann-Whitney tests were performed using Prism 4.03 for Windows (GraphPad Software). All other statistical analyses were performed using Excel 2003 for Windows (Microsoft). Error is represented by SEMs in Ca^{2+} mobilization experiments and by SDs in all other experiments. Also in the Ca^{2+} experiments, *P* values reflect one-way ANOVA comparing the maximum Ca^{2+} concentration versus the concentration at the treatment start time, unless otherwise stated.

Results

Increased GPR30 mRNA expression associates with ERα-positive status in 1,250 breast carcinomas. Evidence of a relationship between GPR30 and ERα expression was sought by mining publicly available and well-annotated gene expression microarray data sets across five independent cohorts comprising 1,250 breast carcinomas. In the NKI cohort (n = 254), data were collected using two-color oligonucleotide microarrays (Fig. 1A). According to the nonparametric Mann-Whitney rank sum test, GPR30 mRNA levels were significantly higher in ERα-positive versus ERα-negative tumors ($P < 0.0001$). The upper range of GPR30 expression was 7.7-fold higher on a linear scale in the ERα-positive compared with ERα-negative carcinomas. In addition, GPR30 and ERα mRNA levels correlated as continuous variables (Pearson's coefficient $\rho = 0.30$, adjusted $P < 0.0001$). The other four cohorts used one-color Affymetrix oligonucleotide microarrays (Fig. 1B). In each of these four cohorts, GPR30 mRNA levels were significantly higher in the ERα-positive compared with the ERα-negative breast cancers (Uppsala cohort, n = 244, P = 0.040; Stockholm cohort, n = 159, P = 0.0091; EMC cohort, n = 344, P = 0.0050; TRANSBIG cohort, n = 198, P = 0.0024).

E_2 downregulates GPR30 mRNA expression via ER and not GPR30. GPR30 regulation in response to E_2 was investigated. MCF-7 cells were treated with E_2 or without E_2 (control, vehicle only) over a 96-hour time course followed by determination of ERα and GPR30 mRNA levels by qPCR. As expected, E_2 steadily downregulated ERα mRNA levels by 59% over 96 hours (Fig. 2A). E_2 also downregulated GPR30 but with faster kinetics than with ERα (Fig. 2B); GPR30 mRNA levels were decreased by 37% at 2 hours (P = 0.0013) and by 79% at 24 hours ($P < 0.0001$). Afterwards, GPR30 mRNA levels rebounded. Additionally, GPR30 mRNA expression decreased in a concentration-dependent manner from 10^{-12} mol/L E_2 to 10^{-10} mol/L E_2 (Fig. 2C). The GPR30-specific agonist G-1 did not alter GPR30 mRNA expression, but the ER-specific agonist DES did repress GPR30 expression relative to control treatment by 54% P = 0.0009), which was very similar to the effect of E_2 (Fig. 2D). Fulvestrant completely blocked E_2 and DES effects. Therefore, E_2 likely acted via ER and not GPR30 to transiently downregulate GPR30 mRNA expression.

GPR30 and not ERα mediates E_2-induced Ca^{2+} mobilization responses. To begin to delineate whether endogenous ERα and/or GPR30 mediates E_2-induced Ca^{2+} responses in breast cancer cells, changes in intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ were measured in ER-positive MCF-7 and ER-negative SKBr3 breast cancer cells at the single-cell level using Fura-2 AM (Fig. 3). In ER-positive MCF-7 cells (Fig. 3A), E_2 induced $[Ca^{2+}]_i$ by 112 ± 1.6 nmol/L (n = 47 cells, P = 0.0063), G-1 by 511 ± 3.4 nmol/L (n = 58 cells, P = 0.0007), and 4OHT by 234 ± 3.4 nmol/L (n = 31 cells, P = 0.0017), whereas DES did not significantly increase the $[Ca^{2+}]_i$ (change = 41 ± 0.8 nmol/L, n = 23 cells, P = 0.66). In ER-negative SKBr3 cells (Fig. 3B), the rank order of ligand-induced cytosolic Ca^{2+} increases was the same as in MCF-7 cells, but the magnitude of the increases was much greater and the responses were transitory instead of sustained. In ER-negative SKBr3 cells, E_2 induced oscillating increases in $[Ca^{2+}]_i$ with an average maximum of 294 ± 1.6 nmol/L (n = 36 cells, P = 0.0037). G-1 and 4OHT induced transitory increases in $[Ca^{2+}]_i$ of 1,517 ± 10.3 nmol/L (n = 79 cells, P = 0.0001) and of 558 ± 2.7 nmol/L (n = 37 cells, P = 0.0013), respectively, whereas DES did not ($[Ca^{2+}]_i$ change = 51 ± 1.3 nmol/L, n = 21 cells, P = 0.26). Therefore, because G-1 and two ER ligands that also bind GPR30, E_2 and 4OHT, but not ER-selective DES, elicited Ca^{2+} responses in both ER-positive MCF-7 cells and ER-negative SKBr3 cells, they likely did so via GPR30.

Two of the major Ca^{2+} channels, inositol triphosphate receptors (IP_3R) and Ry receptors (RyR; ref. 28), were tested for whether they mediated G-1–induced Ca^{2+} mobilization. The pharmacologic probes 2APB and XeC, both of which inhibit IP_3Rs, and Ry, which at high concentrations blocks RyRs, were used. Cells were pretreated for 30 min before inducing Ca^{2+} responses with G-1. In MCF-7 cells, both 2APB and XeC blocked G-1–induced $[Ca^{2+}]_i$ increases by 78% (both 2APB + G-1 versus G-1 alone and XeC + G-1 versus G-1 alone, P = 0.0018) but Ry did not. In contrast, in SKBr3 cells, Ry blocked G-1–induced $[Ca^{2+}]_i$ increases by 80% (Ry + G-1 versus G-1 alone, P = 0.0006), whereas XeC did not. In addition, 2APB allowed G-1 to almost fully induce $[Ca^{2+}]_i$ increases, although the response was significantly lower by 17% versus G-1 alone (P = 0.0094), but this was likely due to blockade of store-operated Ca^{2+} entry, another activity of 2APB. Therefore, GPR30 was coupled to IP_3Rs in ER-positive MCF-7 cells but to RyRs in ER-negative SKBr3 cells.

To confirm that GPR30 and not ERα mediates Ca^{2+} mobilization in response to E_2 in MCF-7 cells, cells were transfected with siRNAs targeting these receptors (characterization of siRNAs in Supplementary Materials and Methods) and a nontargeting siRNA pool as a control. First, the GPR30 siRNA was validated by showing that it led to an almost complete blockade of G-1–induced Ca^{2+} responses ($[Ca^{2+}]_i$ increase = 58 ± 1.3 nmol/L, n = 19 cells, P = 0.62; Fig. 4A). Next, E_2-induced Ca^{2+} mobilization responses were investigated

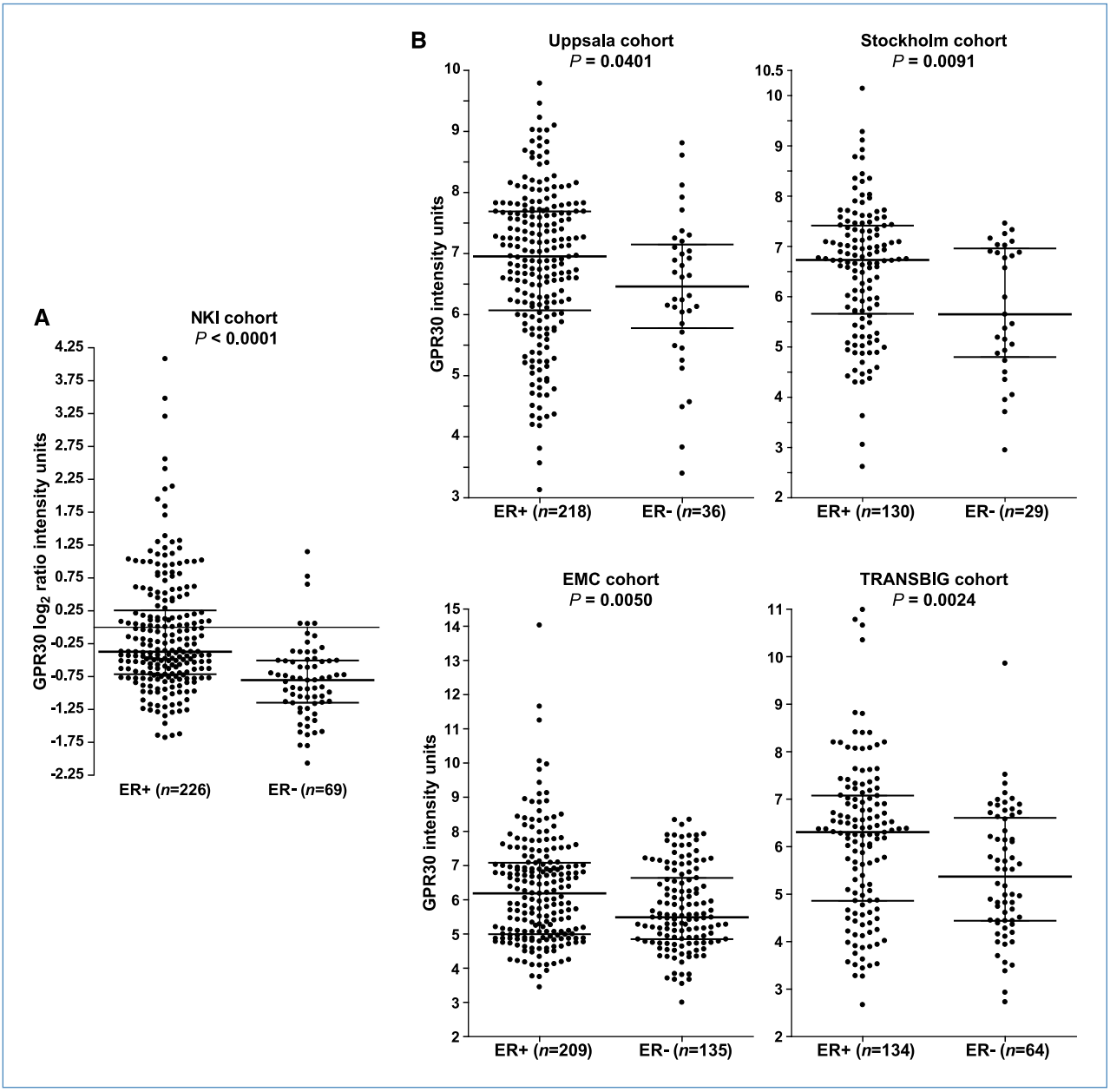

Figure 1. GPR30 mRNA expression shows an association with ERα-positive status in human breast carcinomas. A, GPR30 mRNA levels in the NKI cohort derived from two-color arrays. Expression values are normalized $\log_2$ ratio intensity units corresponding to a single tumor cRNA hybridized against a pooled reference cRNA from all tumors. B, GPR30 mRNA levels in the Uppsala, Stockholm, EMC, and TRANSBIG cohorts all derived from one-color arrays. Expression values are MAS5.0 normalized intensity units. A and B, sample sizes of ERα-positive (*ER+*) and ER-negative (*ER–*) cancers are shown, and bars indicate the 75th, 50th (median), and 25th percentiles. Significance was assessed using the nonparametric Mann-Whitney rank test.

(Fig. 4B). In nontargeting siRNA–transfected cells, E_2 induced an $[Ca^{2+}]_i$ increase of 159 ± 1.6 nmol/L (n = 14 cells, P = 0.0075). However, in ERα siRNA–transfected cells, E_2 caused almost a 2-fold further rise in $[Ca^{2+}]_i$ (314 ± 3.2 nmol/L, n = 17 cells; P = 0.0006). In GPR30 siRNA–transfected cells, the E_2-induced Ca^{2+} response was blocked ($[Ca^{2+}]_i$ increase = 52 ± 0.8 nmol/L, n = 16 cells, P = 0.35). ERα and GPR30 expression were depleted in the appropriate siRNA-transfected cells (Fig. 4C). However, GPR30 mRNA levels were increased by 73% in ERα-depleted cells, a finding consistent with the prior observation that E_2 and DES repressed GPR30 expression (Fig. 2B–D). Hence, the 2-fold potentiation of E_2-induced $[Ca^{2+}]_i$ in ERα-depleted cells likely reflected, at least in part, the increased GPR30 expression.

GPR30 functions to promote growth of ER-negative SKBr3 cells but to inhibit growth of ER-positive MCF-7 cells. The role of GPR30 in cellular proliferation was examined

by transfecting cells with nontargeting and GPR30 siRNAs and then measuring cellular DNA mass after 5 days of growth. First, SKBr3 cells were evaluated (Fig. 5A). The number of cells seeded in each group was similar as indicated by a lack of difference in DNA masses at day 0. After 5 days of growth, DNA mass was 45% lower in GPR30 siRNA compared with nontargeting siRNA–transfected cells ($P < 0.0001$). Thus, the function of GPR30 was to promote growth of SKBr3 cells, in accordance with other reports (9, 10). Next, MCF-7 cells were similarly evaluated (Fig. 5B). Again, equivalent numbers of nontargeting and GPR30 siRNA–transfected cells were seeded as indicated by DNA masses at day 0. After 5 days, GPR30 depletion did not affect basal growth (control treatment). However, GPR30 depletion did potentiate E_2-stimulated growth by 2.1-fold (nontargeting versus GPR30 siRNA–transfected cells, $P < 0.0001$). Analysis of progesterone receptor (PgR) and TFF1 (pS2) mRNA levels by qPCR indicated no significant differences in their induction by E_2 between nontargeting and GPR30 siRNA–transfected cells (data not shown). Therefore, in contrast to SKBr3 cells, the function of GPR30 in MCF-7 cells was to inhibit growth.

The role of GPR30 in MCF-7 cell proliferation was further evaluated by examining effects of G-1 on E_2-stimulated (Fig. 5C) and DES-stimulated (Fig. 5D) growth over 6 days. G-1 blocked the concentration-dependent growth stimulatory response of E_2 (all E_2 treatment groups versus paired E_2 + G-1

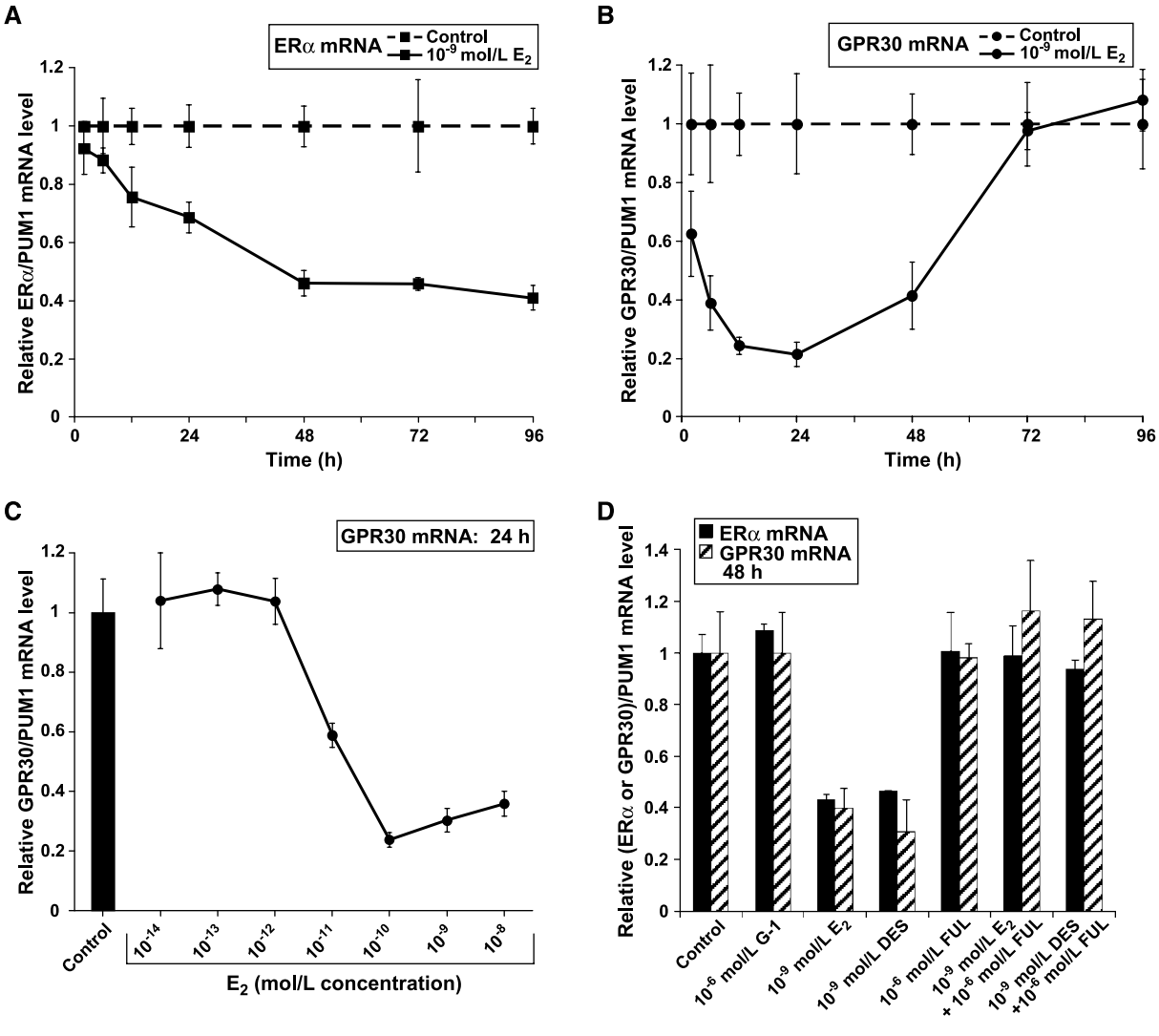

Figure 2. E_2 represses ERα and GPR30 mRNA levels via ER and not GPR30 in MCF-7 cells. E_2 regulation of ERα (A) and GPR30 mRNA (B) levels across a time course. MCF-7 cells were treated with 10^{-9} mol/L E_2 or with the vehicle ethanol alone for 2, 6, 12, 24, 48, 72, and 96 h. C, GPR30 mRNA levels in response to 24-h treatment with a serial dilution series of E_2. D, ERα and GPR30 mRNA levels in response to 48-h treatment with ER and GPR30 ligands as determined by qPCR. Each data point represents the average of six (A and B) or four (C and D) biological replicates. *FUL,* fulvestrant.

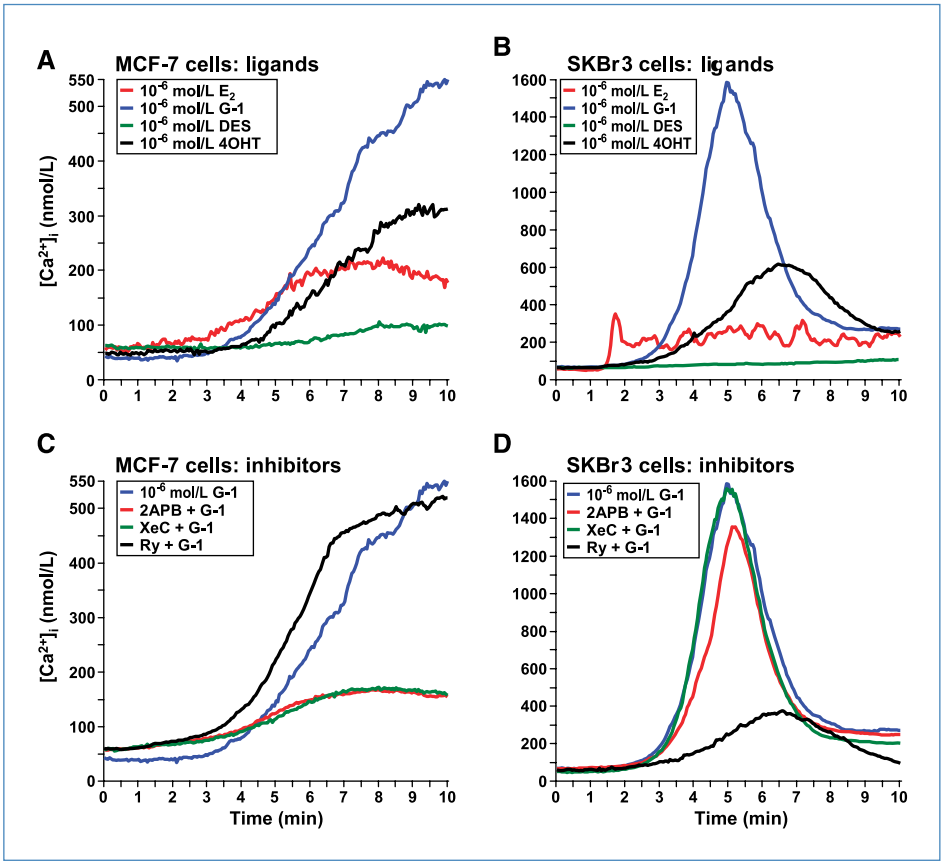

Figure 3. ER ligands that also activate GPR30 induce Ca^{2+} mobilization responses in both ER-positive MCF-7 and ER-negative SKBr3 cells. Ligand-induced Ca^{2+} responses (A and B) and blockade of G-1–induced responses using Ca^{2+} channel inhibitors (C and D) in MCF-7 and SKBr3 cells. Cells were loaded with Fura-2 AM, and intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ were determined in individual cells versus time using fluorescence microscopy. Cells were perfused with all ligands at 10^{-6} mol/L starting at 1 min. 2APB was used at 10^{-4} mol/L, XeC at 10^{-5} mol/L, and Ry at 10^{-5} mol/L. SKBr3 cells with flat, not rounded, morphology were imaged. G-1–induced Ca^{2+} traces in A and B were redrawn in C and D, respectively.

treatment groups, $P = 0.0001$, one-way ANOVA); in particular, G-1 inhibited 10^{-10} mol/L E_2-stimulated growth by 77% relative to E_2 alone ($P < 0.0001$, t test). G-1 also blocked DES-stimulated growth by 72% (DES versus DES + G-1, $P < 0.0001$). Additionally, in both the E_2 and DES experiments, G-1 inhibited basal (control treatment) growth by 32% ($P < 0.0001$) and 47% ($P < 0.0001$), respectively. Therefore, G-1–activated GPR30 blocked growth of ER-positive breast cancer cells but did so independently of ligand-activated ER.

G-1–activated GPR30 blocks cell cycle progression at G_1 phase. The effect of G-1 on cell cycle progression was investigated. MCF-7 cells were synchronized by estrogen withdrawal and then treated with E_2 and G-1 for 24 hours followed by propidium iodide staining and flow cytometric analysis (Fig. 6A). Treatment with G-1 alone significantly decreased the proportion of S-phase cells from 19.8% (control) to 14.7% (G-1; $P < 0.0001$). Importantly, the addition of G-1 to E_2 led to retention of an additional 11.6% of the cells in G_1 phase (42.7% in E_2-treated cells versus 54.4% in E_2 + G-1–treated cells, $P < 0.0001$) and prevented 13.2% of cells from entering S phase (37.7% in E_2-treated cells versus 24.5% in E_2 + G-1–treated cells, $P < 0.0001$). Therefore, G-1 blocked E_2-stimulated cells from cell cycle progression at the G_1 phase.

The G-1–induced cell cycle block was further investigated by measuring protein expression of the tumor suppressor p53, the cyclin-dependent kinase inhibitor (CDK-I) p21 (Fig. 6B), the G_1-phase–specific cyclin D1, and the G_2/M-phase–specific cyclin B1 (Fig. 6C). MCF-7 cells were treated with E_2 and G-1 and then collected at 24, 48, and 72 hours for immunoblot analysis. Both p53 and p21 proteins were upregulated in G-1 and E_2 + G-1–treated cells across all time points compared with control-treated cells (Fig. 6B). As expected, E_2 upregulated both cyclins D1 and B1 across the time course compared with control treatment, whereas G-1 alone did not (Fig. 6C). However, the addition of G-1 to E_2 potentiated the upregulation of cyclin D1 while nearly completely preventing cyclin B1 accumulation compared with E_2 alone across the time points. Because cyclin D1 is induced during G_1 phase and degraded in S phase (29, 30), whereas cyclin B1 accumulates during G_2-phase and degrades on M-phase entry (31), these data are consistent with G-1 blocking cell cycle progression in G_1 phase before cyclin D1 degradation occurred and before cyclin B1 accumulated.

Discussion

Filardo and colleagues (5) and Kuo and colleagues (6) have previously shown in breast carcinomas a positive association between GPR30 and ERα expression by immunohistochemistry and qPCR, respectively. We confirmed and extended this finding by examining gene expression

microarray data sets of five independent patient cohorts comprising 1,250 breast cancers. In all cohorts, high GPR30 mRNA levels showed an association with ERα positivity (Fig. 1). It is unknown why high GPR30 levels would be selected for in ERα-positive breast carcinomas given GPR30 attenuates growth of ER-positive breast cancer, but some GPR30-dependent functions may be necessary for tumorigenesis and cell survival, such as activation of adenylyl cyclase, PI3K, and MAPK (1, 2). Additional roles of GPR30 may be needed for disease progression, such as in cell migration (15, 32), which may then promote metastasis (5).

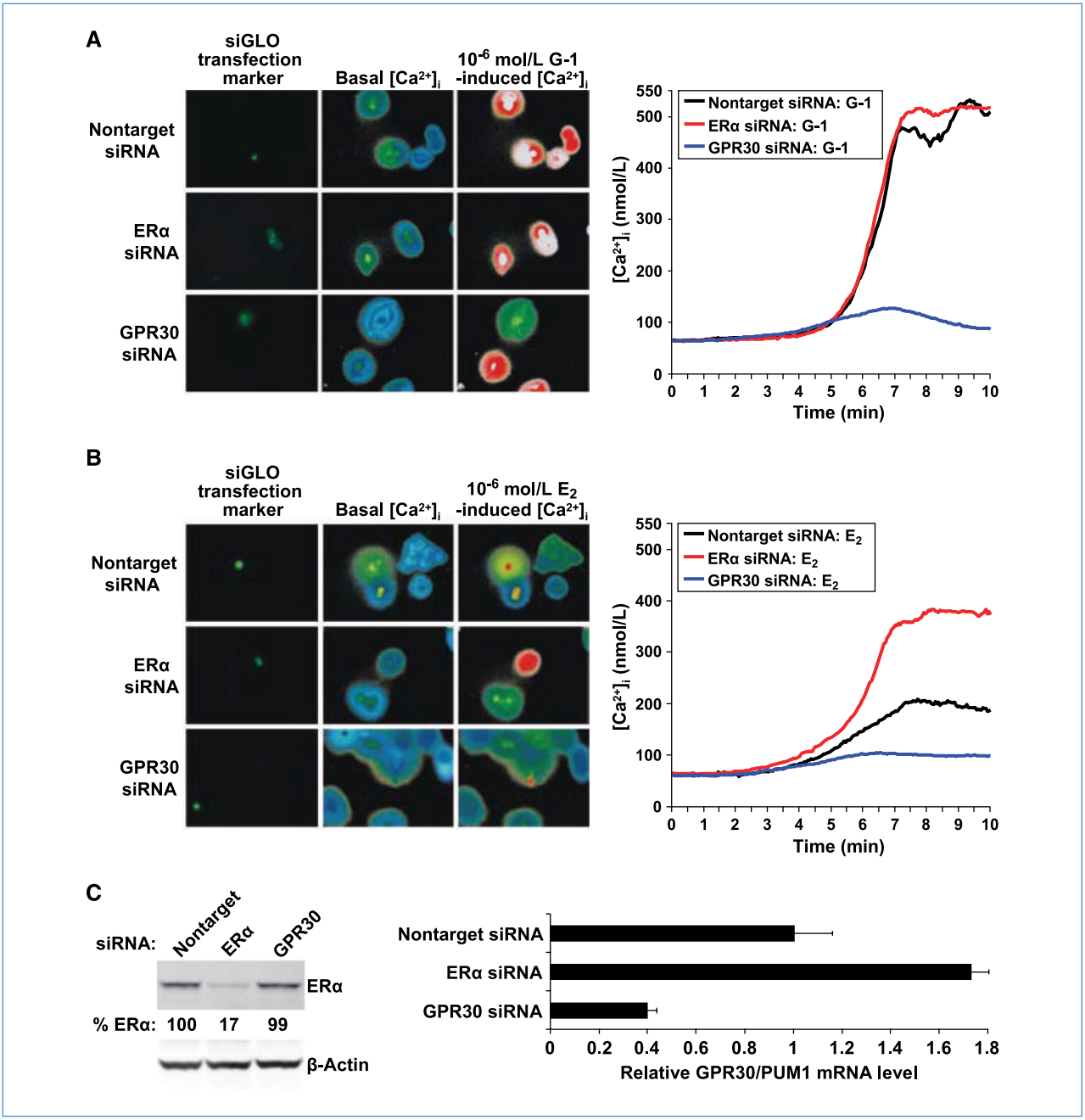

Figure 4. GPR30 and not ERα mediates E_2-induced Ca^{2+} mobilization in MCF-7 cells. G-1–induced (A) and E_2-induced (B) Ca^{2+} responses. Cells were transfected with nontargeting pool, ERα, and GPR30 siRNAs. Transfected cells were labeled using siGLO Green and appear green. Ca^{2+} imaging was performed 48 h following the transfection as in Fig. 3. Low levels of basal $[Ca^{2+}]_i$ are visualized as blue and then green, whereas higher levels of $[Ca^{2+}]_i$ are seen as red and then white. C, ERα protein levels were measured by immunoblotting and GPR30 mRNA levels by qPCR in siRNA-transfected cells 48 h following transfection.

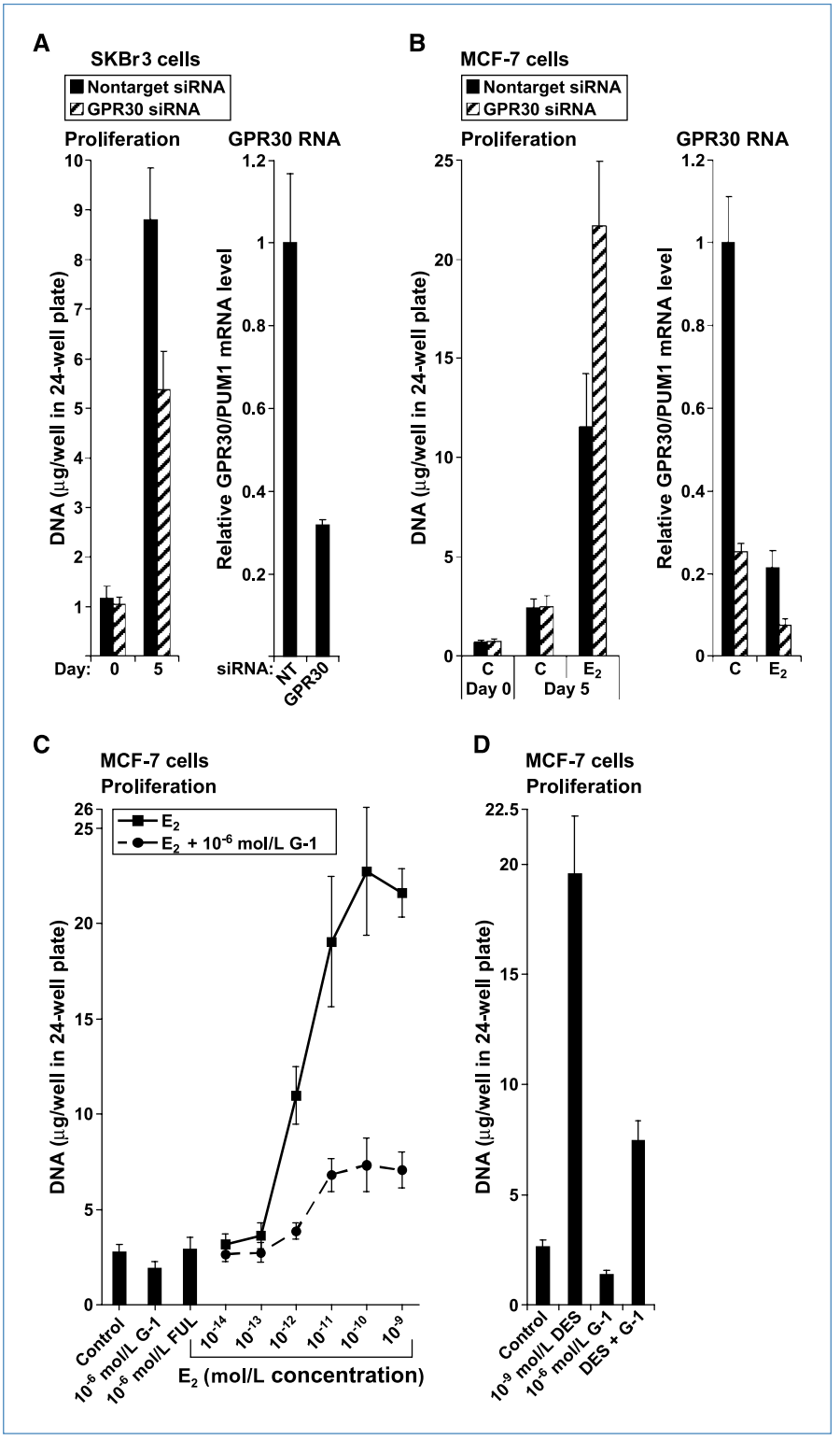

Figure 5. GPR30 promotes growth of ER-negative SKBr3 but inhibits growth of ER-positive MCF-7 cells. Proliferation of SKBr3 (A) and MCF-7 (B) cells transfected with the nontargeting pool and GPR30 siRNAs. Cells were transfected and then seeded at 15,000 per well in 24-well dishes. Medium was replenished the day after seeding on day 0 and every other day thereafter. Cells were collected on days 0 and 5. SKBr3 cells were cultivated in their passage medium, and MCF-7 cells in estrogen-free medium supplemented with 10^{-9} mol/L E_2 or without E_2 [control (*C*)]. Proliferation was assessed as cellular DNA mass (μg/well) using 24 replicate wells. GPR30 mRNA levels were determined by qPCR 48 h following the transfection in both cell lines, and in MCF-7 cells, after 24 h of 10^{-9} mol/L E_2 or control treatment. C and D, proliferation of MCF-7 cells over 6 d treated with a serial dilution series of E_2 (C) or with 10^{-9} mol/L DES (D) in the presence and absence of 10^{-6} mol/L G-1. Twelve replicate wells were used per group.

The interplay between GPR30 and ERα was further investigated using MCF-7 breast cancer cells. E_2 repressed GPR30 expression in a time- and concentration-dependent manner (Fig. 2B and C). In addition, DES but not G-1 downregulated GPR30; therefore, ER mediated this effect (Fig. 2D). The inverse functional relationship between ERα and GPR30 was also shown by depleting ERα, which led to derepression of GPR30 mRNA expression and consequently potentiated

E_2-induced Ca^{2+} mobilization responses (Fig. 4). E_2 is known to downregulate ERα expression in MCF-7 cells as a negative feedback regulatory loop to prevent overresponsiveness (26). Likewise, GPR30 may also be negatively regulated by E_2 via ER to prevent excessive GPR30-dependent activity, such as aberrantly high $[Ca^{2+}]_i$. Interestingly, the maximum increases in $[Ca^{2+}]_i$ were much larger in SKBr3 cells than in MCF-7 cells (Fig. 3B versus Fig. 3A). It is possible that this was due to the lack of ERs in SKBr3 cells, which translated into a lack of negative feedback regulation.

GPR30 depletion decreased growth of SKBr3 cells (Fig. 5A) but potentiated E_2-stimulated growth in MCF-7 cells (Fig. 5B), indicating that GPR30 functions to promote SKBr3 but to inhibit MCF-7 cellular proliferation. Also in MCF-7 cells, G-1 profoundly inhibited E_2-stimulated (Fig. 5C) and DES-stimulated growth (Fig. 5D) as well as decreased the percentage of cells entering S phase (Fig. 6A). However, these G-1 effects occurred in both the presence and the absence of E_2. These findings in MCF-7 cells complement those of Ahola and colleagues (11) who reported that transient

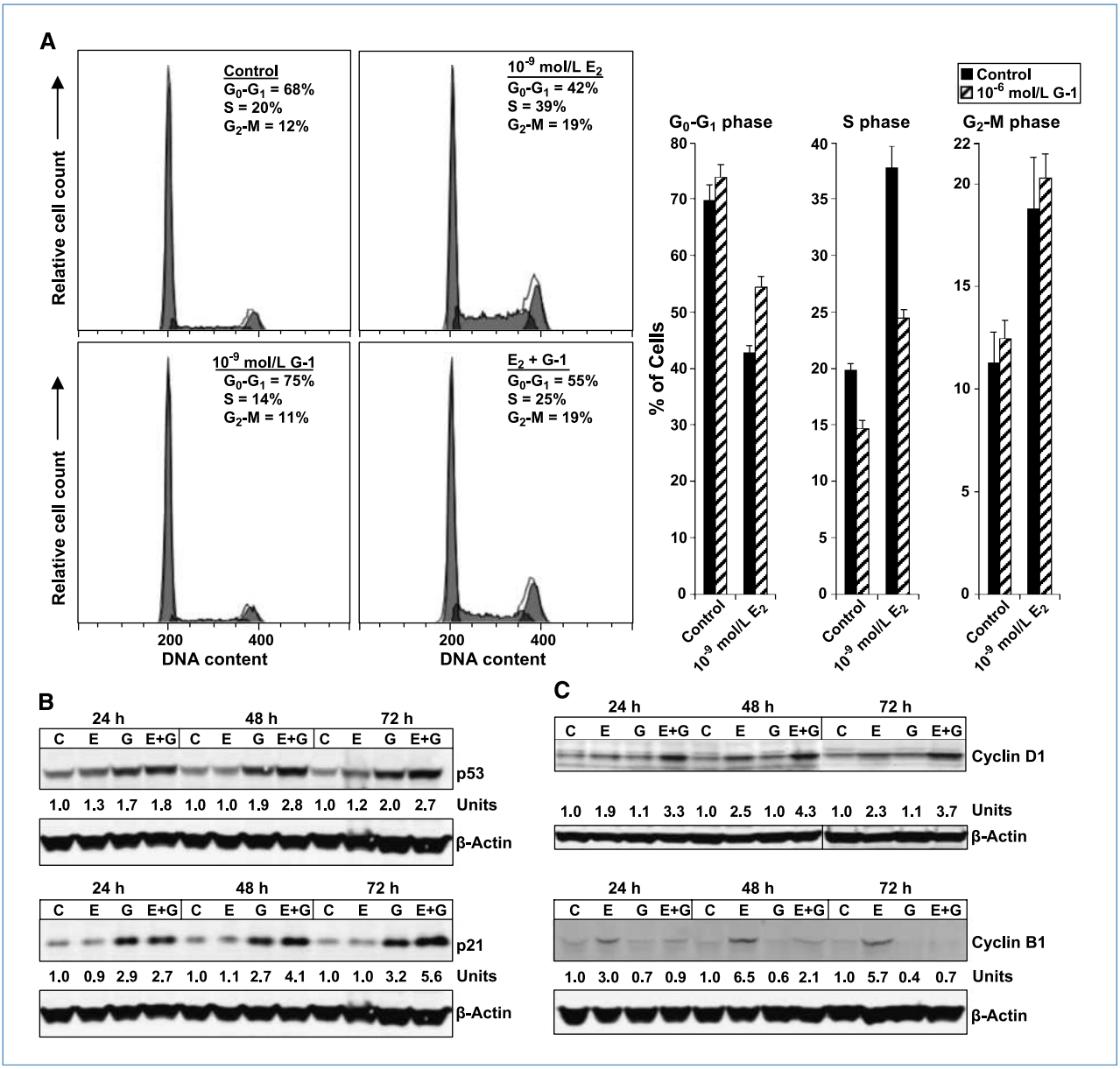

Figure 6. G-1 inhibits cell cycle progression in E_2-stimulated MCF-7 cells by producing a block at G_1 phase. A, cell cycle distribution as determined by propidium iodide staining of DNA content and flow cytometry. Cells were synchronized by 3-d cultivation in estrogen-free medium and then treated as indicated for 24 h. Thirty-thousand cells per sample and three replicates per group were collected. Representative histograms are shown. Immunoblot analyses of p53 and p21 (B) and of cyclin D1 and cyclin B1 (C) protein levels. MCF-7 cells were control (*C*)-, 10^{-9} mol/L E_2 (*E*)-, and 10^{-6} mol/L G-1 (*G*)–treated as indicated. Quantitated protein levels normalized to β-actin are indicated.

GPR30 overexpression decreased the percentage of proliferating MCF-7 cells independent of E_2. Indeed, GPR30 likely does not directly regulate ER transcriptional activity because there were no significant differences in E_2-induced mRNA expression of well-established ER target genes *PgR* and *TFF1* between GPR30 siRNA–transfected and nontargeting siRNA–transfected cells (data not shown).

Rather, we propose that GPR30 antagonizes growth of MCF-7 cells by inducing sustained increases in cytosolic Ca^{2+} concentrations (Figs. 3A and 4), in contrast to transitory increases in SKBr3 cells where GPR30 promotes growth. Aberrant sustained increases in intracellular Ca^{2+} levels can lead to inhibition of proliferation and induce apoptosis (33). For example, the plasma membrane Ca^{2+}-ATPase (PMCA) pumps Ca^{2+} across the plasma membrane out of the cell to lower cytosolic Ca^{2+} levels after Ca^{2+} increases. Partial inhibition of PMCA in MCF-7 cells causes a moderate increase in intracellular Ca^{2+} levels, which leads to inhibition of proliferation by altering cell cycle kinetics (34). Additionally, the mechanism of action of numerous antitumor agents involves increases in $[Ca^{2+}]_i$ (35).

It is possible that differences in GPR30-coupled Ca^{2+} signaling, which mediate sustained versus transitory responses, associate with ER status. In support of this hypothesis, GPR30 was coupled to differing Ca^{2+} channels: to IP_3Rs in ER-positive MCF-7 cells but to RyRs in ER-negative SKBr3 cells. Alternatively, sustained versus transitory Ca^{2+} responses could have been due to potential alterations in factors that participate in lowering cytosolic Ca^{2+}, such as plasma membrane or sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase pumps. We intend to explore these possibilities involving differing Ca^{2+} responses in future studies.

As shown by propidium iodide staining and flow cytometry, G-1 induced a cell cycle block at the G_1 phase (Fig. 6A). Consistent with a G_1-phase arrest, G-1 increased accumulation of the tumor suppressor p53, the CDK-I p21, and the G_1-phase–specific cyclin D1 but prevented E_2-induced accumulation of the G_2/M-phase–specific cyclin B1 (Fig. 6B and C). Ca^{2+} signaling has been shown to induce p53 via activation of cyclic AMP–responsive element binding protein (28). In MCF-7 cells, p53 induction by E_2 is Ca^{2+} and calmodulin kinase IV dependent (36). In addition, aberrant Ca^{2+} mobilization in response to anticancer/cytotoxic agents correlates with p53 induction (35). Thus, G-1 could lead to p53 induction via Ca^{2+} mobilization in MCF-7 cells. Then, p53 could induce p21 via a p53 response element to mediate arrest in G_1 phase of the cell cycle (28).

As a SERM, tamoxifen acts as an antiestrogen in ER-positive breast cancer but as an estrogen in the endometrium and bone (37). Similarly, G-1 inhibits growth of ER-positive MCF-7 breast cancer cells but promotes growth of the endometrium (16) and plays an important role in promoting bone growth *in vivo* (38, 39). Thus, the tissue-specific proliferative effects of G-1 may parallel those of tamoxifen. It is interesting to speculate that 4OHT-induced Ca^{2+} mobilization (Fig. 3A and B) may be involved in some of the tissue-specific effects of tamoxifen.

Taken together, GPR30 inhibits growth of ERα-positive breast cancer. Our studies also indicate that pharmacologic activation of GPR30 shows promise in combating ER-positive breast cancer. G-1 would also probably be well tolerated because it, like E_2, exerts beneficial effects against an animal model of multiple sclerosis but without E_2-associated side effects (17, 40). Thus, G-1 may represent the first in a new class of therapeutically relevant agents for use alone or in conjunction with conventional antihormonal therapeutics in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, Hathaway HJ. Estrogen signaling through the transmembrane G protein–coupled receptor GPR30. Annu Rev Physiol 2008;70:165–90.
2. Prossnitz ER, Barton M. Signaling, physiological functions and clinical relevance of the G protein–coupled estrogen receptor GPER. Prostaglandins Other Lipid Mediat 2009;89:89–97.
3. Smith HO, Leslie KK, Singh M, et al. GPR30: a novel indicator of poor survival for endometrial carcinoma. Am J Obstet Gynecol 2007;196:386.e1–9; discussion 386.e9–11.
4. Smith HO, Arias-Pulido H, Kuo DY, et al. GPR30 predicts poor survival for ovarian cancer. Gynecol Oncol 2009;114:465–71.
5. Filardo EJ, Graeber CT, Quinn JA, et al. Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression. Clin Cancer Res 2006;12:6359–66.
6. Kuo WH, Chang LY, Liu DL, et al. The interactions between GPR30 and the major biomarkers in infiltrating ductal carcinoma of the breast in an Asian population. Taiwan J Obstet Gynecol 2007;46:135–45.
7. Vivacqua A, Bonofiglio D, Albanito L, et al. 17β-Estradiol, genistein, and 4-hydroxytamoxifen induce the proliferation of thyroid cancer cells through the G protein–coupled receptor GPR30. Mol Pharmacol 2006;70:1414–23.
8. Vivacqua A, Bonofiglio D, Recchia AG, et al. The G protein–coupled

receptor GPR30 mediates the proliferative effects induced by 17β-estradiol and hydroxytamoxifen in endometrial cancer cells. Mol Endocrinol 2006;20:631–46.
9. Albanito L, Madeo A, Lappano R, et al. G protein–coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17β-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. Cancer Res 2007;67:1859–66.
10. Albanito L, Sisci D, Aquila S, et al. Epidermal growth factor induces G protein–coupled receptor 30 expression in estrogen receptor-negative breast cancer cells. Endocrinology 2008;149:3799–808.
11. Ahola TM, Manninen T, Alkio N, Ylikomi T. G protein–coupled receptor 30 is critical for a progestin-induced growth inhibition in MCF-7 breast cancer cells. Endocrinology 2002;143:3376–84.
12. Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology 2005;146:624–32.
13. Filardo EJ, Quinn JA, Bland KI, Frackelton AR, Jr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein–coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol 2000;14:1649–60.
14. Filardo EJ, Quinn JA, Frackelton AR, Jr., Bland KI. Estrogen action via the G protein–coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. Mol Endocrinol 2002;16:70–84.
15. Bologa CG, Revankar CM, Young SM, et al. Virtual and biomolecular screening converge on a selective agonist for GPR30. Nat Chem Biol 2006;2:207–12.
16. Dennis MK, Burai R, Ramesh C, et al. *In vivo* effects of a GPR30 antagonist. Nat Chem Biol 2009;5:421–7.
17. Blasko E, Haskell CA, Leung S, et al. Beneficial role of the GPR30 agonist G-1 in an animal model of multiple sclerosis. J Neuroimmunol 2009;214:67–77.
18. Lin BC, Suzawa M, Blind RD, et al. Stimulating the GPR30 estrogen receptor with a novel tamoxifen analogue activates SF-1 and promotes endometrial cell proliferation. Cancer Res 2009;69:5415–23.
19. van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002; 347:1999–2009.
20. Barrett T, Suzek TO, Troup DB, et al. NCBI GEO: mining millions of expression profiles-database and tools. Nucleic Acids Res 2005;33: D562–6.
21. Gentleman RC, Carey VJ, Bates DM, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 2004;5:R80.
22. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 1992;90:77–86.
23. Ariazi EA, Kraus RJ, Farrell ML, Jordan VC, Mertz JE. Estrogen-related receptor α1 transcriptional activities are regulated in part via the ErbB2/HER2 signaling pathway. Mol Cancer Res 2007;5:71–85.
24. Lyng MB, Laenkholm AV, Pallisgaard N, Ditzel HJ. Identification of genes for normalization of real-time RT-PCR data in breast carcinomas. BMC Cancer 2008;8:20.
25. Brailoiu E, Churamani D, Cai X, et al. Essential requirement for two-pore channel 1 in NAADP-mediated calcium signaling. J Cell Biol 2009;186:201–9.
26. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. Cancer Res 1996; 56:2321–30.
27. Liu H, Lee E-S, Gajdos C, et al. Apoptotic action of 17β-estradiol in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*. J Natl Cancer Inst 2003;95:1586–97.
28. Lipskaia L, Lompre AM. Alteration in temporal kinetics of Ca^{2+} signaling and control of growth and proliferation. Biol Cell 2004;96: 55–68.
29. Doisneau-Sixou SF, Sergio CM, Carroll JS, Hui R, Musgrove EA, Sutherland RL. Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. Endocr Relat Cancer 2003;10: 179–86.
30. Guo Y, Yang K, Harwalkar J, et al. Phosphorylation of cyclin D1 at Thr 286 during S phase leads to its proteasomal degradation and allows efficient DNA synthesis. Oncogene 2005;24:2599–612.
31. Pines J, Hunter T. Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. Cell 1989;58:833–46.
32. Pandey DP, Lappano R, Albanito L, Madeo A, Maggiolini M, Picard D. Estrogenic GPR30 signaling induces proliferation and migration of breast cancer cells through CTGF. EMBO J 2009;28:523–32.
33. Pinton P, Giorgi C, Siviero R, Zecchini E, Rizzuto R. Calcium and apoptosis: ER-mitochondria Ca^{2+} transfer in the control of apoptosis. Oncogene 2008;27:6407–18.
34. Lee WJ, Robinson JA, Holman NA, McCall MN, Roberts-Thomson SJ, Monteith GR. Antisense-mediated inhibition of the plasma membrane calcium-ATPase suppresses proliferation of MCF-7 cells. J Biol Chem 2005;280:27076–84.
35. Olofsson MH, Havelka AM, Brnjic S, Shoshan MC, Linder S. Charting calcium-regulated apoptosis pathways using chemical biology: role of calmodulin kinase II. BMC Chem Biol 2008;8:2.
36. Qin C, Nguyen T, Stewart J, Samudio I, Burghardt R, Safe S. Estrogen up-regulation of p53 gene expression in MCF-7 breast cancer cells is mediated by calmodulin kinase IV-dependent activation of a nuclear factor κB/CCAAT-binding transcription factor-1 complex. Mol Endocrinol 2002;16:1793–809.
37. Ariazi EA, Jordan VC. Estrogen receptors as therapeutic targets in breast cancer. In: Ottow E, Weinmann H, editors. Nuclear receptors as drug targets. Mörlenbach: Wiley-VCH; 2008, p. 127–99.
38. Martensson UE, Salehi SA, Windahl S, et al. Deletion of the G protein–coupled receptor 30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice. Endocrinology 2009; 150:687–98.
39. Chagin AS, Savendahl L. GPR30 estrogen receptor expression in the growth plate declines as puberty progresses. J Clin Endocrinol Metab 2007;92:4873–7.
40. Wang C, Dehghani B, Li Y, et al. Membrane estrogen receptor regulates experimental autoimmune encephalomyelitis through up-regulation of programmed death 1. J Immunol 2009;182:3294–303.

Supplementary Materials

Characterization of ERα and GPR30 siRNAs

Before examining effects of RNAi-mediated depletion of ERα and GPR30, an ERα siRNA pool, a GPR30 siRNA pool, and the four individual siRNAs in each pool were evaluated (Supplemental Fig. 1). Since ERα regulated expression of GPR30 in MCF-7 cells (Fig. 2B-C), siRNA–mediated depletion of ERα could potentially alter expression of GPR30. Therefore, evaluation of the ERα and GPR30 siRNAs was carried out in a non–breast cancer cell type. ERα–positive ECC-1 endometrial cancer cells were chosen since we had observed that E_2 does not significantly regulate GPR30 mRNA expression in this cell line (data not shown). ECC-1 cells were transfected with the siRNAs as described in the Methods, and 48 h later, ERα protein (Supplemental Fig. 1A) and GPR30 mRNA expression (Supplemental Fig. 1B) were determined by semi-quantitative immunoblot analysis and real-time qPCR, respectively. The ERα pool and individual siRNAs (#11 to #14) all effectively depleted ERα by greater than 90 %. Similarly, the GPR30 pool and individual siRNAs (#6 to #9) depleted GPR30 mRNA expression from 86 % to 71 %. However, the ERα pool siRNA, and ERα siRNAs #11 and #14 decreased GPR30 mRNA expression by 70 %, 44 %, and 62 % respectively, while ERα siRNA #13 did not. Likewise, the GPR30 siRNA pool and siRNAs #6 and #7 significantly decreased ERα protein expression by 92 %, 79 %, and 62 %, respectively, while GPR30 siRNA #8 did not. Since the ERα siRNAs led to varying decreases in GPR30 expression, and similarly since the GPR30 siRNAs led to varying decreases in ERα expression, it was concluded that these effects were off–target. Additionally, while the fourth GPR30 siRNA evaluated, #9, only showed a modest off-target effect of decreasing ERα protein levels by 22 %, it appeared to be toxic (data not shown). Therefore, for

all further siRNA–based experiments presented, the ERα siRNA #13 and GPR30 siRNA #8 were employed as these siRNAs exhibited the least off–target effects.

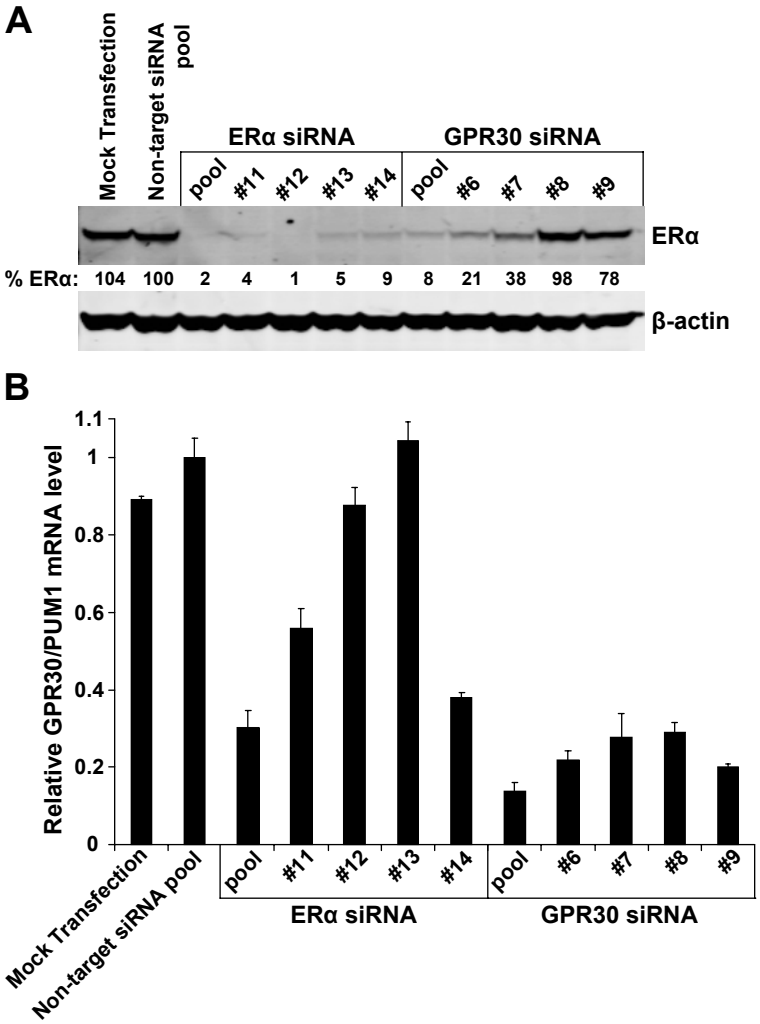
PCR primer sequences

PCR primer sequences used were as follows : ERα forward 5'–GGA GGG CAG GGG TGA A–3', ERα reverse 5'-GGC CAG GCT GTT CTT CTT AGA-3'; GPR30 forward 5'-TGG GGA AGA GGC CAC CA-3', GPR30 reverse 5'-CGT GGA GCT GCT CAC TCT CTG-3'; PUM1 forward 5'-AAT GCA GGC GCG AGA AAT-3', PUM1 reverse 5'-TTG TGC AGC TGA GGA ACT AAT GA-3'.

siRNA sense oligonucleotide sequences

The siRNA sense oligonucleotide sequences were as follows : GPR30 #6 GGG UGA AGC GCC UCA GUU Auu; GPR30 #7 GAC GAG GCC UGC UUC UGU Uuu, GPR30 #8 UAG GAA ACC UCA CGA CUG Guu; GPR30 #9 GGA UGA GCU UCG ACC GCU Auu; ESR1 #11 GAU CAA ACG CUC UAA GAA Guu; ESR1 #12 GAA UGU GCC UGG CUA GAG Auu; ESR1 #13 GAU GAA AGG UGG GAU ACG Auu; ESR1 #14 GCC AGC AGG UGC CCU ACU Auu.

Supplemental Figure 1. Characterization of ERα and GPR30 siRNA pools in ECC-1 endometrial cancer cells. (A) ERα protein levels by immnoblot analysis and **(B)** GPR30 mRNA levels by qPCR in ERα siRNA and GPR30 siRNA-transfected ECC-1 endometrial cancer cells. ECC-1 cells were transfected and assayed under estrogen-free conditions at 48 h following the transfection. The immunoblot was visualized using a Li-Cor Odyssey infrared scanner. Quantitation of ERα protein levels normalized to β-actin is indicated. GPR30 mRNA levels represent the average of 4 biological replicates and error bars their associated SDs. Testing of individual siRNAs indicated that only ERα siRNA #13 and GPR30 siRNA #8 exhibit on-target knockdown without off-target effects.


A
Mock Transfection
Non-target siRNA pool
ERα siRNA
GPR30 siRNA
pool
#11
#12
#13
#14
pool
#6
#7
#8
#9
ERα
% ERα:
104
100
2
4
1
5
9
8
21
38
98
78
β-actin
B
Relative GPR30/PUM1 mRNA level
1.1
1
0.8
0.6
0.4
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#11
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#13
#14
pool
#6
#7
#8
#9
ERα siRNA
GPR30 siRNA

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Experimental treatment of oestrogen receptor (ER) positive breast cancer with tamoxifen and brivanib alaninate, a VEGFR-2/FGFR-1 kinase inhibitor: A potential clinical application of angiogenesis inhibitors

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ABSTRACT

Purpose: Tamoxifen, a selective oestrogen receptor modulator (SERM), and brivanib alaninate, a vascular endothelial growth factor receptor 2 (VEGFR-2) inhibitor, are two target specific agents that result in a substantial decrease in tumour growth when given alone. Tamoxifen activates SERM stimulated breast and endometrial tumour growth. Tamoxifen and brivanib alaninate have side-effects that can affect therapeutic outcomes. The primary goal of the current study was to evaluate the therapeutic effects of lower doses of both agents when given in combination to mice with SERM sensitive, oestrogen stimulated tumour xenografts (MCF-7 E2 tumours). Experiments were conducted to evaluate the response of SERM stimulated breast (MCF-7 Tam, MCF-7 Ral) and endometrial tumours (EnCa 101) to demonstrate the activity of brivanib alaninate in SERM resistant models.

Experimental design: In the current study, tumour xenografts were minced and bi-transplanted into the mammary fat pads of athymic, ovariectomised mice. Preliminary experiments were conducted to determine an effective oral dose of tamoxifen and brivanib alaninate that had minimal effect on tumour growth. Doses of 125 μg of tamoxifen and 0.05 mg/g of brivanib alaninate were evaluated. An experiment was designed to evaluate the effect of the two agents together when started at the time of tumour implantation. An additional experiment was done in which tumours were already established and then treated, to obtain enough tumour tissue for molecular analysis.

Results: Brivanib alaninate was effective at inhibiting tumour growth in SERM sensitive (MCF-7 E2) and SERM stimulated (EnCa 101, MCF-7 Ral, MCF-7 Tam) models. The effect

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of the low dose drug combination as an anti-tumour strategy for SERM sensitive (MCF-7 E2) in early treatment was as effective as higher doses of either drug used alone. In established tumours, the combination is successful at decreasing tumour growth, while neither agent alone is effective. Molecular analysis revealed a decreased phosphorylation of VEGFR-2 in tumours that were treated with brivanib alaninate and an increase in VEGFA transcription to compensate for the blockade of VEGFR-2 by increasing the transcription of VEGFA. Tamoxifen increases the phosphorylation of VEGFR-2 and this effect is abrogated by brivanib alaninate. There was also increased necrosis in tumours treated with brivanib alaninate.

Conclusion: Historically, tamoxifen has a role in blocking angiogenesis as well as the blockade of the ER. Tamoxifen and a low dose of an angiogenesis inhibitor, brivanib alaninate, can potentially be combined not only to maximise therapeutic efficacy but also to retard SERM resistant tumour growth.

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1. Introduction

Angiogenesis is a major requirement for tumours to grow successfully and spread. Early work[1–3] characterised many of the factors involved in the regulation of angiogenesis and how these factors can become disregulated in tumour pathogenesis.[4–6] One of the most important factors in the positive modulation of angiogenesis is the vascular endothelial growth factor (VEGF) family of growth factors and their corresponding receptors. Angiogenesis in tumours is different from physiological angiogenesis seen with normal development and wound healing. In wound healing, angiogenesis is a carefully orchestrated process and occurs in a short time. By contrast, the blood vessels that form in the tumour bed are thin, disorganised and leaky. The growth of such vessels persists over years as long as viable tumour tissue is present.[3]

In oestrogen receptor (ER) positive breast cancer, it is clear that adjuvant anti-oestrogenical therapy must be extended to 5 years and beyond to prevent recurrence and improve survival.[7–9] However, toxicities, the development of resistance to anti-hormonal therapy, and side-effects from therapy such as clots and endometrial cancer with tamoxifen[10,11] and fractures and joint pain with aromatase inhibitors[11,12] often limit long-term treatment. Clearly new treatment strategies need to be developed to enhance the activity of anti-hormonal therapy by improving efficacy. Oestrogen enhances the angiogenic cascade critical for tumour growth, primarily through the release of VEGF.[13–16] Tamoxifen has a historical role in the prevention of tumour angiogenesis as it was one of the three drugs in the 'Navy Regimen' developed by Folkman.[17] Tamoxifen is also reported[18] to reduce angiogenesis for ER negative tumours. An anti-oestrogen for the treatment of ER positive breast cancer can potentially regulate VEGF production. However, with the development of acquired resistance[10] in breast and endometrial tumours it is axiomatic that selective oestrogen receptor modulator (SERM) (tamoxifen and raloxifene) stimulated tumours must induce angiogenesis to grow. We hypothesise that limiting angiogenesis with angiogenic drugs during anti-hormonal therapy could potentially improve adjuvant therapeutic regimens. However, there are significant toxicities with current antiangiogenic drugs that limit their usefulness for long-term therapy.

Several antiangiogenic agents are either used in clinical practice or are in clinical trials. Most notably, bevacizumab, a monoclonal antibody that binds to VEGFA and as a result, prevents phosphorylation and activation of its target receptors, vascular endothelial growth factor receptors 1 and 2 (VEGFR-1 and VEGFR-2), has shown promise in combination with chemotherapy for breast cancer.[19–21] In a phase 3 trial of 722 patients, the disease-free survival time in patients with metastatic breast cancer has been shown to double (5.9 versus 11.8 months) when treated with paclitaxel in conjunction with bevacizumab.[21] Unfortunately, the overall survival does not change when bevacizumab is included as a part of therapy. Toxicities such as infection (9.3% versus 2.9%), proteinuria (3.6% versus 0.0%), hypertension (14.8% versus 0.0%) and cerebrovascular ischaemia (1.9% versus 0.0%) also limit long-term therapy.[21] Part of the problem with the therapeutic use of monoclonal antibodies is that VEGFA is not the only ligand that can bind to these receptors. Other members of the VEGF family such as VEGFC and VEGFD can bind to VEGFR-2, while VEGFB has been shown to bind and activate VEGFR-1.[19,22] With this in mind, other agents, which target the tyrosine kinase domain of the receptor, have been developed and several pre-clinical and clinical trials are investigating the use of such agents.[2,23] Many of the newer agents that are being developed also target other growth factor receptor tyrosine kinases such as PDGF, FGFR, and c-Kit with the idea that blocking several receptors will prevent resistance to therapy that results from the activation of alternate pathways by co-regulatory proteins.[2]

One dual-targeting drug is brivanib alaninate (BMS 582664, Bristol Myers Squibb, Princeton, NJ), a VEGFR-2/FGFR-1 inhibitor. Pre-clinical studies *in vivo* have shown that brivanib alaninate is effective in reducing the growth of a lung tumour xenograft, L2987, a panel of human derived hepatocellular carcinomas,[24] and an ER negative breast tumour H3396.[25] Pharmacological studies in a phase I clinical trials have shown that doses of brivanib alaninate below 800 mg/d are tolerable, but have associated toxicities such as hypertension (>150/100), elevated transaminases, fatigue and dizziness as the dosage increases from a baseline of 180 mg/d. Several phase 1 clinical trials are underway in patients with a variety of solid tumours.[26,27]

We have addressed the hypothesis that combining tamoxifen, a SERM with a sub-therapeutic dose of brivanib alaninate would be a beneficial strategy for long-term therapy in the treatment of breast cancer. We report the first studies testing the efficacy of brivanib alaninate to control tumour growth of ER regulated SERM sensitive (MCF-7 E2) and SERM stimulated (MCF-7 Ral, MCF-7 Tam), and endometrial (EnCa 101) tumours. We find that the combination of tamoxifen and brivanib alaninate in a laboratory model provided a therapeutic advantage for the control of breast tumour growth over tamoxifen or brivanib alaninate alone.

2. Materials and methods

2.1. Tumour xenografts

SERM sensitive tumours were previously developed by injecting the mammary fat pads of ovariectomised, BALB/c athymic mice (Harlan Sprague Dawley, Madison, WI) with 1×10^7 WS8 human breast cancer cells.[28] Tumour growth was sustained with 0.3 cm silastic capsules containing estradiol (Sigma, St. Louis, MO) delivering 83.8 ± 34.6 pg/mL oestrogen over an eight-week period.[29] Over time, the tumours were serially passaged by bi-transplanting the established tumours into the mammary fat pads of estradiol treated mice. The development and characterisation of the SERM stimulated EnCa 101 endometrial cancer model,[30] MCF-7 Ral model,[31] and MCF-7 Tam model[32] have been reported previously.

For the experiments in the current study, athymic ovariectomised CrTac: NCr-Foxn1nu mice were obtained from Taconic (Hudson, NY). Mice were placed under anaesthesia, using a mixture of isoflurane and 100% oxygen delivered via inhalation. Healthy tumour tissue was sectioned into 1 mm^3 pieces and implanted bilaterally into the mammary fat pads. Estradiol capsules (0.3 cm silastic capsule) were placed subcutaneously on the dorsal surface of the mice to maintain tumour growth.

Tumours were measured with calipers once a week. Cross-sectional areas (CSAs) were calculated by measuring the length and width of the tumours and then using an Excel (Microsoft) spreadsheet to calculate the CSA (length (cm) × width (cm) × $\pi/4$). Growth curves were derived from the determining the average CSA per treatment group per week. In the case of EnCa 101 endometrial tumours, growth characteristics were atypical with a prolonged latent period of tumour spreading subcutaneously with an eventual rapid haemorrhagical growth phase reminiscent of the 'angiogenic trigger'. Tumour volumes were measured for EnCa 101 using the formula $4/3\pi r^3$.

Six sets of experiments were completed. The first experiment was specifically conducted to evaluate where VEGFR-2 and VEGFA are expressed and how expression changes in response to hormonal and anti-hormonal manipulation. Experiments 2–5 were conducted to determine dosing of brivanib alaninate to prevent the growth in MCF-7 E2, a SERM sensitive tumour, and MCF-7 Ral, MCF-7 Tam, and EnCa 101 SERM stimulated tumours. The fourth experiment determined the dosing of tamoxifen to block estradiol stimulated tumour growth in MCF-7 E2 tumours. The fifth and sixth experiments determined the effects of combined therapy when started 24 h after initial tumour implantation versus giving the drug to animals with the established tumours for a two-week time period.

2.2. Drug preparation

Bristol Myers Squibb (Princeton, NJ) provided brivanib alaninate in powder form. The drug was suspended in a citric acid buffer solution and the pH was gradually titrated to a pH of 3.5 after the drug dissolved. The final concentration was 10 mg/mL.

Tamoxifen (Sigma Chemical Co., St. Louis, MO) was weighed and suspended in 10% Tween 80/polyethylene glycol (PEG) 400 (99.5% PEG 400/0.5% Tween 80) and 90% carboxymethylcellulose (CMC, 1% CMC dissolved in double distilled water). The final concentration of the tamoxifen solution was 2.5 mg/mL and administered by gavage at the doses indicated. Administration of tamoxifen to animals bearing EnCa 101 tumours was at a dose of 500 μg/mouse by gavage.

Raloxifene (Evista, Eli Lilly, IN) was prepared by placing five raloxifene tablets in a conical tube and dissolving them via centrifugation in 27 mL double distilled water. Once the tablets were dissolved, 3 mL of 90% CMC and 10% PEG 400/Tween 80 was added to the raloxifene solution. The final concentration was 10 mg/mL. Raloxifene was administered at a daily dose of 1.5 mg/mouse by gavage.

Estradiol capsules were prepared by plugging one end 0.3 cm length of medical grade silastic tubing and filling it with 17β-estradiol (Sigma Chemical Company, St. Louis, MO) mixed 1:3 with elastomer. Capsules sealed by placing elastomer at the open ends and then sterilised with radiation (20,000 rad).[33]

Fulvestrant (Faslodex, AstraZeneca, Wilmington, DE) was purchased from the pharmacy at Fox Chase Cancer Center as a solution of fulvestrant suspended in EtOH and castor oil (50 mg/ml).

2.3. Drug administration

Brivanib alaninate was dosed orally 7 d a week, according to the weight of each mouse. Mice were weighed once weekly. For the high dose, a 20 g mouse was given 200 μL (2 mg) and for the low dose a 20 g mouse was given 100 μL (1 mg). Tamoxifen was also administered 7 d a week. Dosing of tamoxifen was as follows: 125 μg (50 μL), for 250 μg (100 μL), or 500 μg (200 μL). Fulvestrant was administered as 2 mg (40 μL) injections 5 d per week.

2.4. Experiment 1: the effect of hormonal manipulation on VEGFA and VEGFR-2 expression

Tumours were grown in the presence of estradiol (0.3 cm silastic capsule) until the tumours reached 0.4 cm^2. The mice were then treated with different drug regimens for 2 weeks. The treatments after tumours reached 0.4 cm^2 were as follows:

(1) continue estradiol (0.3 cm silastic capsule),
(2) withdraw estradiol,

(3) estradiol + 125 μg tamoxifen daily,
(4) withdraw estradiol (0.3 cm silastic capsule) and 2 mg/40 μL fulvestrant injections given subcutaneously daily.

2.5. *Experiment 2: effects of different doses of brivanib alaninate on SERM sensitive MCF-7 E2 tumours*

We evaluated the effects of a high dose and low dose of brivanib alaninate. The brivanib alaninate treatment was started 24 h after tumour implantation. Treatment groups (five animals) were as follows:

(1) estradiol (0.3 cm silastic capsule) + placebo given orally (citric acid buffer: pH 3.5),
(2) estradiol (0.3 cm silastic capsule) + low dose brivanib alaninate given orally (.05 mg/g),
(3) estradiol (0.3 cm silastic capsule) + high dose brivanib alaninate given orally (0.1 mg/g).

2.6. *Experiment 3: the effects of brivanib alaninate on SERM stimulated tumours*

2.6.1. *Experiment 3A: the effects of different doses of brivanib alaninate on SERM resistant MCF-7 Ral tumours*

We evaluated the effects of a high dose and low dose of brivanib alaninate. The brivanib alaninate treatment was started 24 h after tumour implantation. Treatment groups (five animals) were as follows:

placebo (citric acid buffer),
1.5 mg raloxifene,
2 mg fulvestrant – pure anti-oestrogen (subcutaneous),
1.5 mg raloxifene + high dose VEGFR antagonist (0.1 mg/g).

2.6.2. *Experiment 3B: the effect of brivanib alaninate on established SERM resistant MCF-7 Ral tumour models*

Tumours were grown up to an average 0.5 cm^2 CSA. The mice were randomised to receive 2 weeks of therapy with the high dose brivanib alaninate.

Groups:
10 mice each after randomisation:

1.5 mg raloxifene,
1.5 mg raloxifene + high dose brivanib alaninate (0.1 mg/g).

2.6.3. *Experiment 3C: the effect of brivanib alaninate on SERM resistant MCF-7 Tam tumours*

We examined the effects of the high dose brivanib alaninate on another SERM resistant model. There were two components to this experiment. The first was to determine whether brivanib alaninate inhibited tumour growth and the second was to determine whether brivanib alaninate was effective in established tumours

(1) 1.5 mg tamoxifen first 48 d of the experiment (8 mice, 16 tumours),
  – this group was used for the second part of the experiment,
(2) placebo: citric acid buffer (0.15 mL) (5 mice, 10 tumours),
(3) 1.5 mg tamoxifen + 0.1 mg/g brivanib alaninate (4 mice, 6 tumours).

Once the tumours in group one reached a CSA of 0.5 cm^2, 48 d after tumour implantation, group 1 was subdivided:

(1) continue 1.5 mg tamoxifen for two more weeks (4 mice, 8 tumours),
(2) 1.5 mg tamoxifen + high dose brivanib alaninate (0.1 mg/g) for 2 weeks (4 mice, 8 tumours).

2.6.4. *Experiment 3D: the effect of brivanib alaninate on EnCa Tam endometrial tumours*

This experiment determined whether brivanib alaninate inhibited the growth of endometrial tumours that normally grow with 500 μg of tamoxifen daily. Tumours initially are not evident until after one month after which they grow rapidly. Twenty mice were treated with tamoxifen for 40 d and then randomised into two groups. After randomisation, treatments were given for 3 weeks.

Control group: 500 μg tamoxifen daily(10 mice).
Experimental group: 500 μg tamoxifen daily + 0.1 mg/g brivanib alaninate (started on day 40) (10 mice).

2.7. *Experiment 4: determination of tamoxifen dosing in SERM sensitive MCF-7 E2 tumours*

We determined a dose response curve of various oral doses of tamoxifen to determine the lowest dose that was effective in decreasing the rate of tumour growth

(1) no estradiol,
(2) estradiol (0.3 cm silastic capsule),
(3) estradiol (0.3 cm silastic capsule) + 500 μg tamoxifen given orally,
(4) estradiol (0.3 cm silastic capsule) + 250 μg tamoxifen given orally,
(5) estradiol (0.3 cm silastic capsule) + 125 μg tamoxifen given orally.

2.8. *Experiment 5: the combined effect of a lower dose of tamoxifen and brivanib alaninate in SERM sensitive MCF-7 E2 tumours*

This experiment determined the combined effects of a sub-maximal dose of tamoxifen and a sub-maximal dose of brivanib alaninate on oestrogen stimulated tumour growth. Drug dosing was commenced 24 h after tumour implantation

(1) control with estradiol (0.3 cm silastic capsule),
(2) experimental group with estradiol (0.3 cm silastic capsule) + 125 μg tamoxifen given orally,
(3) experimental group with estradiol (0.3 cm silastic capsule) + low dose brivanib alaninate (.05 mg/g dose) given orally,

(4) experimental group with estradiol (0.3 cm silastic capsule) + 125 μg tamoxifen given orally + low dose brivanib alaninate (0.05 mg/g dose) given orally.

2.9. *Experiment 6: the combined effect of a lower dose of tamoxifen and brivanib alaninate in established SERM sensitive MCF-7 E2 tumours*

Experiment 8 was similar to Experiment 7 with one exception. The tumours were grown to an average CSA of 0.43 mm^3 and drug therapy was given for 2 weeks.

2.10. *Western immunoblotting*

Tumours were harvested and placed in foils and frozen immediately in liquid nitrogen. Tumours were kept at –80 °C until they were processed. For processing, tumours were placed in liquid nitrogen and homogenised using a mortar and pestle. The extract was suspended in RIPA buffer (Sigma, St. Louis, MO) with protease (Roche, Nutley, NJ) and phosphatase (Calbiochem, San Diego, CA) inhibitors. The mixture was briefly sonicated and centrifuged for 10 min at 5000*g*. The supernatant was removed and protein concentration was determined using the Bradford assay (BCA assay, Pierce, Rockford, IL) with a Spectramax machine (Molecular Devices, Sunnyvale, CA). Equal amounts (25 μg) and concentrations of protein were loaded into 4–12% Nupage Bis-tris (Invitrogen, Carlsabad, CA) gels, and transferred to nitrocellulose membranes. Immunoblotting was carried out with the following antibodies: total VEGFR-2 (1:1000, rabbit polyclonal, Cell Signaling Technologies, Beverly, MA), phospho-VEGFR-2 Tyr ^{951}(1:200, rabbit polyclonal, Santa Cruz, Biotechnology, Santa Cruz, CA), total FGFR-1 and total VEGFR-3 (1:200, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), total VEGFR-1 (rabbit polyclonal, 1:200, Labvision, Fremont, CA), total ER alpha (ERα) (rabbit polyclonal, 1:200, G20, Santa Cruz, Santa Cruz, CA), phospho-ERα (rabbit monoclonal, 1:2000, Ser 118, clone NL 44, Upstate, Billerica, MA), β-actin (mouse monoclonal, 1:30,000, Sigma–Aldrich, St. Louis, MO).

2.11. *Real time polymerase chain reaction (RT-PCR)*

Total RNA was extracted from frozen tumour tissues using RNA mini easy kit (Qiagen, Venlo, The Netherlands) as per the manufacturer's instructions. Two micrograms of total RNA were reverse transcribed using a cDNA high capacity reverse transcription kit (Applied Biosystem, Carlsbad, CA) in 20 μL of total volume, as per manufacturer's instruction. The resulting cDNA was diluted to a total volume of 200 μL using sterile water. The real time PCR was carried out on an ABI 7900 HT Fast Real Time PCR system using 1X SYBR green PCR master mix (Applied Biosystem, Carlsbad, CA) and 100 nM of forward and reverse primers. All the forward and reverse primers (Table 1) were designed using Primer Express 3 software (Applied Biosystem, Carlsbad, CA) except ERα[34] and mouse and human 36B4.[35,36] The fold change in the expression of each gene was calculated by the ΔΔCt[37] method using 36B4, a ribosomal phospho-protein as an internal control.

Table 1 – Primers used for RTPCR.

VEGFA	Fwd: 5′ GGGCAGAATCATCACGAAGTG 3′
	Rev: 5′ TCAGGGTACTCCTGGAAGATGTC 3′
VEGFB	Fwd: 5′ AGCCAGTGTGAATGCAGACCTA 3′
	Rev: 5′ AGTCCCAGCCCGGAACAG 3′
VEGFC	Fwd: 5′ CCTCAGCAAGACGTTATTTGAAATT 3′
	Rev: 5′ TGGCAAAACTGATTGTTACTGGTT 3′
VEGFD	Fwd: 5′ CGTACATTTCCAAACAGCTCTTTG 3′
	Rev: 5′ GGCAAGCACTTACAACCTGTATGA 3′
VEGFR-1	Fwd: 5′ TTCTCACAGGATCTAGTTCAGGTTCA 3′
	Rev: 5′ CTGCTTCCCCCCTGCAT 3′
VEGFR-2	Fwd: 5′ CAGAGTGGCAGTGAGCAAAGG 3′
	Rev: 5′ TTGTAGGCTCCAGTGTCATTTCC 3′
Mouse VEGFR-1	Fwd: 5′ TCCTATCGGCTGTCCATGAAA 3′
	Rev: 5′ CCAAATAGCGAGCAGACTTCAA 3′
Mouse VEGFR-2	Fwd: 5′ ACCAGCATGGCATCGTGTAC 3′
	Rev: 5′ CCTAGCGCAAAGAGACACATTG 3′
Mouse VEGFR-3	Fwd: 5′ GTATGAAATTGACCCGTACGAAAA 3′
	Rev: 5′ AGGAAATGAGGCTTGAGAGAAGATC 3′
VEGFA	Fwd: 5′ GGGCAGAATCATCACGAAGTG 3′
	Rev: 5′ TCAGGGTACTCCTGGAAGATGTC 3′
VEGFB	Fwd: 5′ AGCCAGTGTGAATGCAGACCTA 3′
	Rev: 5′ AGTCCCAGCCCGGAACAG 3′
VEGFC	Fwd: 5′ CCTCAGCAAGACGTTATTTGAAATT 3′
	Rev: 5′ TGGCAAAACTGATTGTTACTGGTT 3′
VEGFD	Fwd: 5′ CGTACATTTCCAAACAGCTCTTTG 3′
	Rev: 5′ GGCAAGCACTTACAACCTGTATGA 3′
VEGFR-1	Fwd: 5′ TTCTCACAGGATCTAGTTCAGGTTCA 3′
	Rev: 5′ CTGCTTCCCCCCTGCAT 3′
VEGFR-2	Fwd: 5′ CAGAGTGGCAGTGAGCAAAGG 3′
	Rev: 5′ TTGTAGGCTCCAGTGTCATTTCC 3′
Mouse VEGFR-1	Fwd: 5′ TCCTATCGGCTGTCCATGAAA 3′
	Rev: 5′ CCAAATAGCGAGCAGACTTCAA 3′
Mouse VEGFR-2	Fwd: 5′ ACCAGCATGGCATCGTGTAC 3′
	Rev: 5′ CCTAGCGCAAAGAGACACATTG 3′
Mouse VEGFR-3	Fwd: 5′ GTATGAAATTGACCCGTACGAAAA 3′
	Rev: 5′ AGGAAATGAGGCTTGAGAGAAGATC 3′

2.12. *Immunohistochemistry (IHC)/histology*

Staining (IHC) was done to determine VEGFR-2 and VEGFA expressions on tumour tissue from Experiments 2 and 6. Tumours were placed in formalin for 48 h and subsequently embedded in paraffin. Fixation was done with phosphate buffered formaldehyde 10% (F79-4, Fisher Scientific, Pittsburgh, PA). Xenografts were placed in the fixative for 48 h and subsequently embedded in paraffin. Paraffin sections were dewaxed using xylenes and hydrated using a series of ethanol. Antigen retrieval was performed with citrate buffer pH 6 for 10 min in a microwave oven (1500 W, 2 min at high and 8 min at the lowest power). Endogenous peroxidases were quenched with 0.3% hydrogen peroxide in methanol for 30 min. Sections were incubated overnight with the primary antibody raised against VEGFR-2 and VEGFA. Total VEGFR-2 (55B11) rabbit monoclonal antibody from Cell Signaling Technology (Beverly, MA) and anti-VEGF (A-20) purified rabbit polyclonal antibody from Santa Cruz (Santa Cruz, CA) were

diluted 1/100 (2 μg/mL) in phosphate buffered saline (PBS), washed the next day with PBS, incubated with biotinylated secondary antibodies (Vector Labs), incubated with Vecta Elite ABC kit (Vector Labs), developed with a DAB kit (Vector Labs) and lightly counterstained with Gill's haematoxylin. Negative controls were stained without primary antibody or with the corresponding concentration of rabbit IgG isotype. Specimens were documented photographically using a Nikon Optiphot microscope, equipped with an Optronics CCD camera. The stained sections were scored on the basis of staining intensity. The vast majority of tissues stained diffusely and all or more than 70% of the tumour tissue was stained in the positive specimens. The score was defined as weak (1+), positive (2+) or strong (3+).

For CD31 staining, in Experiment 3C, the sections were washed in PBS and then treated with 3% H_2O_2 for 10 min to block endogenous peroxidase activity and were blocked with normal rabbit serum. Then, the sections were incubated with rat anti-mouse CD31 (PECAM-1) monoclonal antibody (BD Pharmingen, San Diego, CA) at a 1:300 dilution overnight at 4 °C. Negative controls were incubated with the rat serum IgG at the same protein concentration. All sections were washed in PBS containing 0.05% Tween-20, and were then incubated with a second antibody, mouse anti-rat IgG (Vector laboratories, Burlingame, CA) at a 1:200 dilution for 30 min at room temperature again followed by washing with PBS containing 0.05% Tween-20. The sections were incubated in a 1:400 dilution of Extravadin Peroxidase (Sigma, St. Louis, MO) for 30 min. After washing in PBS containing 0.05% Tween-20, the sections were incubated in peroxidase substrate (Vector laboratories, Burlingame, CA) for 5 min. After washing we used a Biotinyl-Tyramide enhancement kit (TSA/Biotin Tyramide Reagent Pack, Perkin Elmer, Waltham, MA) according to the manufacturer's instructions. The sections were washed in PBS containing 0.05% Tween-20 and were counterstained with Gill's haematoxylin.

For general morphological evaluations, sections from each tumour were stained with haematoxylin and eosin (H and E).

2.13. Statistical analysis

Tumour growth data were analysed using random effects growth curve models, where tumour CSA was fit assuming a quadratic function of time. Let A_{ijt} be the CSA of tumour i on mouse j, in treatment group k, measured t days after treatment (or control) initiation. The growth curve model was of the following form:

$$A_{ijkt} = \beta_{0j} + t\beta_{1ij} + t^2\beta_{2ij} + \sum_{z=1,\ldots,K} \gamma_{0z} I(k = z) + \sum_{z=1,\ldots,K} t\gamma_{1z} I(k = z) + \sum_{z=1,\ldots,K} t^2\gamma_{2z} I(k = z) + \varepsilon_{ijkt}$$

where the βs were assumed random terms with mean zero, the γs were fixed effects and K is the number of treatment groups. Random effects were included to allow deviation of individual tumours from the mean growth of the group and to account for within-animal clustering. The estimated curves were plotted and the fit examined. Linear contrasts were used to estimate mean tumour size differences (and associated standard error) at a specified time t between any two pre-specified experimental groups. Wald tests were used to test the null hypothesis of equal tumour size between two experimental groups at time t. For experiments with randomisation and treatment initiated after day 0, only observations taken after randomisation were analysed. For example, in Experiment 8, only observations after initiation of brivanib alaninate or tamoxifen treatment ($\geqslant$35 d) were analysed. Bonferroni corrections were used to adjust for multiple testing within each experiment for these analyses. The experiment-wise type I error was 5%. The RNA expression data measured by RTPCR with high/low dose of VEGFR-2 inhibitor and combination treatment were analysed using Wilcoxon rank-sum tests. The RTPCR analyses were confirmatory and, therefore no adjustment of the type I error for multiple testing was used. All tests were two-sided. Statistical analyses were performed using STATA version 10.1.

For the CD31 counts that were done for the MCF-7 Tam model, the statistical analysis was done using a two tailed Student's t-test and a p-value that was less than 0.05 was considered significant.

3. Results

3.1. Immunohistochemistry

Immunohistochemistry was performed on representative MCF-7 E2 tumours to determine whether the VEGFR-2 receptor was expressed in response to estradiol and 2 weeks of tamoxifen. We also determined VEGFR-2 receptor expression in response to estradiol, estradiol withdrawal and the treatment with the pure anti-oestrogen, fulvestrant. This analysis demonstrated the presence of VEGFR-2 on both tumour cells and endothelial cells (Fig. 1A). In addition, VEGFR-2 and VEGFA expressions were increased on tumour cells in the presence of estradiol. It is interesting to note that the combination of estradiol and 2 weeks of 125 μg tamoxifen did not apparently change VEGFR-2 or VEGFA expression in comparison to estradiol treatment alone. However, as noted in Fig. 6A, tamoxifen was not effective at controlling established estradiol stimulated tumour growth during the two-week treatment period. With estradiol withdrawal alone, and the subsequent destruction of the ER with fulvestrant, there was very little expression of VEGFR-2 or VEGFA on the tumour cells (Fig. 1A and B).

3.2. Effects of different doses of brivanib alaninate in SERM sensitive MCF-7 E2 tumours

We evaluated the effects of a low dose (0.05 mg/g) and high dose (0.1 mg/g) of brivanib alaninate on estradiol stimulated tumour growth. The high dose was based on data demonstrating the highest effective dose with minimal toxicity and the low dose that was chosen was half of the high dose and the minimally effective dose as determined by Bristol Myers Squibb (Princeton, NJ).[38] Statistical comparisons were done to determine whether there was a difference in the average CSA of tumours treated with estradiol versus those that received the high dose (0.1 mg/g) or low dose (0.05 mg/g) of brivanib alaninate in the presence of estradiol. Estradiol caused tumour growth, while the high dose of brivanib alaninate pro-

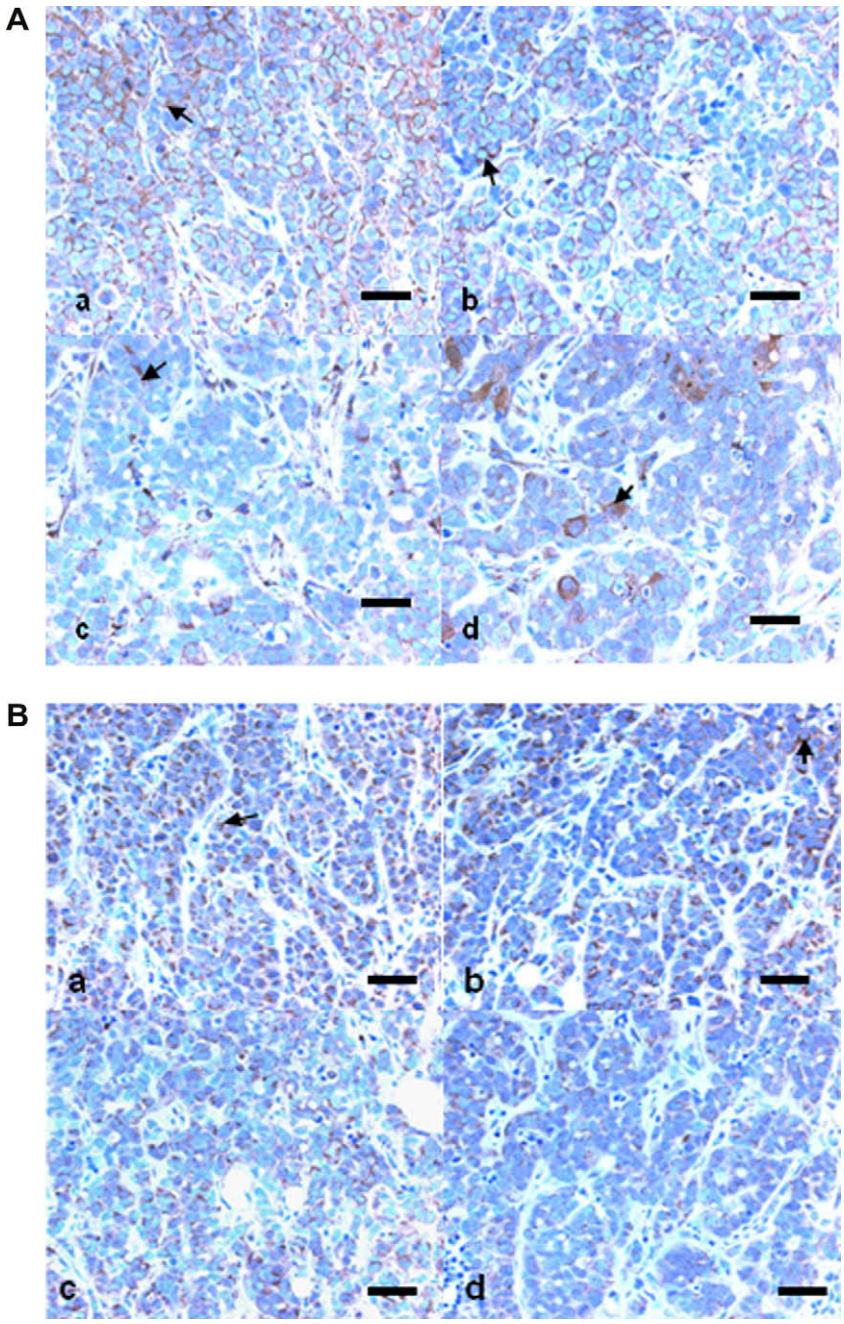

Fig. 1 – The distribution of the VEGFR-2 receptor (A) and VEGFA (B) in the MCF-7 E2 tumour model. Tumour bearing animals were treated with estradiol (a), estradiol and 2 weeks of 125 μg tamoxifen (b), estradiol and then 2 weeks of estradiol withdrawal (c), and estradiol followed by 2 weeks of estradiol withdrawal and fulvestrant (d). VEGFA and VEGFR-2 expressions decreased with estradiol withdrawal. The bars represent 50 μm.

duced a dramatic decrease in estradiol-stimulated growth (Fig. 2A). The average difference in tumour CSA at 6 weeks in the mice that received the high dose of the brivanib alaninate and estradiol versus estradiol was –0.37 cm^2 ($p = 0.001$, $\alpha = 0.025$). There was no significant difference (0.13 cm^2) in the average CSA of tumours treated with estradiol only and those treated with estradiol and the low dose of brivanib alaninate ($p = 0.202$, $\alpha = 0.025$).

The tumour tissue was further evaluated with H and E staining (Fig. 2B). The purpose of this analysis was to detect differences in the amount of necrotic tissue. In tumours in which angiogenesis and thus, oxygen and nutrient delivery is blocked, there would be a decrease in tumour cell viability and hence an increase in necrosis. In tumours that received brivanib alaninate, there was an increase in tissue necrosis as exemplified by the areas that stain pink only. The necrosis was most prominent in the tumours treated with the high dose of the brivanib alaninate. There was mild necrosis in the tumours that were treated with the low dose of the brivanib alaninate.

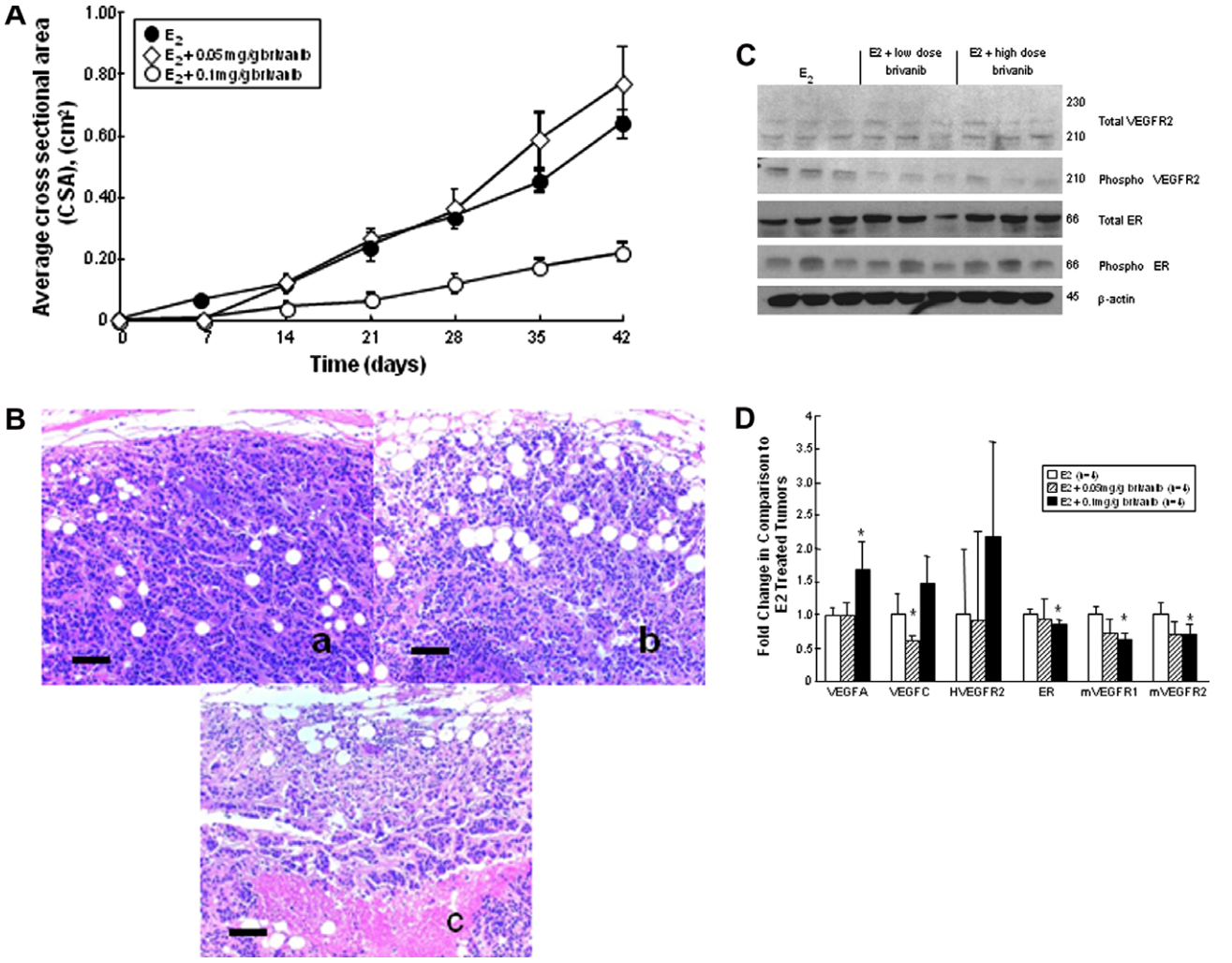

Fig. 2 – The growth characteristics of MCF-7 E2 tumours treated with estradiol alone or with estradiol and the lower (0.05 mg/g) and higher doses (0.1 mg/g) of brivanib alaninate. There were five ovariectomised, athymic mice and 10 tumours per group. The drug treatment resulted in a decreased average CSA of the tumours at the higher dose (0.1 mg/g) ($p = .001$, $\alpha = 0.025$), but there was no difference between the group treated with the low dose (0.05 mg/g) of brivanib alaninate and the oestrogen only group ($p = 0.2$, $\alpha = 0.025$). There were no significant differences in animal body weights between groups. H and E staining is shown in panel B and reveals that with increases in the dosing of the drug, there was an increase in the amount of necrotic tissue (*). The bar represents 100 μm. Panel C demonstrates that there was no significant change in the total amount of VEGFR-2 expressed by the tumours, but there was a decrease in the phosphorylation pattern of the tumours treated with brivanib alaninate, regardless of the dose given. The presence of ER and its phosphorylated form was indicative of active tumour tissue in all the samples. Panel D demonstrates analysis by RTPCR. There was a significant increase in VEGFA in the high dose group in comparison with tumours treated with oestrogen only ($p = 0.02$). There was a small, but significant decrease in ER mRNA in the high dose (0.1 mg/g) group ($p = 0.04$). VEGFC transcription decreased significantly in tumours treated with the low dose (0.05 mg/g) of brivanib alaninate. Mouse VEGFR-1 and mouse VEGFR-2 mRNA, which represented the endothelial component of the tumour, significantly decreased in the high dose (0.1 mg/g) group ($p = 0.02$, $p = .04$).

Western immunoblotting of tumour extracts did not reveal a difference in total VEGFR-2, but there was less phosphorylation at the tyrosine 951 residue of VEGFR-2 in brivanib alaninate treated animals (Fig. 2C). The presence of ER and phospho-ER demonstrated active tumour tissue and an activated ER. There was very little VEGFR-1, VEGFR-3 or FGFR-1 (data not shown) detected by immunoblotting. The use of RTPCR analysis confirmed a significant increase in VEGFA ($p = 0.02$) and a non-significant increase in human VEGFR-2 in tumours that were treated with the high dose of brivanib alaninate (Fig. 2D). There was a significant decrease in mouse VEGFR-1 and mouse VEGFR-2 in tumours that were treated with the higher ($p = 0.02$, $p = 0.04$) dose of brivanib alaninate. ER mRNA decreased slightly, but significantly (Fig. 2C) in those tumours that were treated with the higher dose of brivanib alaninate ($p = 0.04$), but there was no increase in ER protein by Western blotting analysis (Fig. 2C). There was a significant decrease in transcription of VEGFC mRNA ($p = 0.04$) in tumours treated with the lower dose of brivanib alaninate (Fig. 2D). There was very little or no VEGFB, VEGFD, mouse

VEGFR-3 or human VEGFR-1 present in the tumours as evidenced by high CT values (>35) detected by RTPCR analysis (data not shown).

3.3. *Effect of brivanib alaninate on SERM stimulated tumour growth*

To establish that an inhibitor of VEGFR-2 would block the growth of SERM stimulated tumours and as a consequence, would have the potential to retard the development of acquired SERM resistance in ER positive cancers, a series of models and designs was explored. The MCF-7 Ral tumour model[39] grows without raloxifene, and to a greater extent in the presence of raloxifene. Fulvestrant retards tumour growth.[39] This is illustrated in Fig. 3A. Statistical comparisons were done to determine whether there was a difference in the average CSA of tumours treated with raloxifene versus those treated with placebo, fulvestrant or high dose brivanib alaninate (0.1 mg/g). Raloxifene stimulated tumour growth was significantly decreased in the presence of high dose brivanib alaninate (0.1 mg/g) administered with raloxifene and the difference in average CSA was 0.391 cm^2 after 8 weeks ($p < 0.001$, $\alpha = 0.016$). A similar difference in average CSA (0.366 cm^2) was also observed with tumours treated with raloxifene versus tumours treated with fulvestrant ($p < 0.001$, $\alpha = 0.016$). There was no significant difference between the average CSAs of tumours (0.212 cm^2) in the presence or absence of raloxifene ($p = 0.024$, $\alpha = 0.016$). The addition of high dose brivanib alaninate (0.1 mg/g) to a daily regimen of 1.5 mg of raloxifene (0.1 mg/g) caused a rapid decrease in tumour growth (decrease in average CSA = –0.294 cm^2) ($p < 0.001$, $\alpha = 0.025$) in established raloxifene stimulated tumours (Fig. 3B) over a two-week period. At the time of randomisation, the group that was treated with raloxifene (1.5 mg) and brivanib alaninate (0.1 mg/g) demonstrated no difference in average CSA (–.0146 cm^2) than those that received raloxifene (1.5 mg) only ($p = 0.73$, $\alpha = 0.025$).

Our MCF-7 Tam SERM stimulated model showed similar effects with brivanib alaninate. Statistical comparisons were done to determine whether there was a difference in the average CSA of tumours treated with 1.5 mg tamoxifen daily versus vehicle or 1.5 mg tamoxifen + 0.1 mg/g brivanib alaninate. The difference in CSA between those tumours that received 1.5 mg tamoxifen daily versus 1.5 mg tamoxifen and the high dose brivanib alaninate (0.1 mg/g) daily ($p < 0.001$, $\alpha = 0.025$) was 0.395 cm^2. A similar difference in CSA (0.484 cm^2) was observed between tumours treated with tamoxifen alone versus control treated with vehicle only ($p < 0.001$, $\alpha = 0.025$). The tamoxifen (1.5 mg/daily) treated group was then randomised to continue 1.5 mg/d tamoxifen or 1.5 mg/d tamoxifen + high dose brivanib alaninate for 2 weeks. At the time of randomisation, the group that was treated with tamoxifen (1.5 mg) and brivanib alaninate (0.1 mg/g) demonstrated no difference in average CSA than those that received tamoxifen (1.5 mg/g) only ($p = 0.76$, $\alpha = 0.25$). The addition of brivanib alaninate (0.1 mg/g) caused a rapid tumour regression (difference in average CSA = –0.261 cm^2) of established tumours after 2 weeks of treatment ($p < 0.001$, $\alpha = 0.025$) (Fig. 3C). There was a significant decrease in blood vessel density (CD31 counts) in the group that received 0.1 mg/g brivanib alaninate and 1.5 mg tamoxifen for 2 weeks (average MVD/sq. mm = 76) in comparison with the group that continued receiving 1.5 mg tamoxifen (average MVD sq./mm = 156) ($p = 0.003$).

Finally, the tamoxifen-stimulated EnCa 101 endometrial tumour model[30] was also used to evaluate the efficacy of brivanib alaninate (0.1 mg/g). Animals with bi-transplanted tumours were treated with 500 μg of tamoxifen daily by oral gavage for 40 d and then randomised. One group received 500 μg of tamoxifen and 0.1 mg/g brivanib alaninate daily. The other group continued to receive 500 μg of tamoxifen. At the time of randomisation, the group that was treated with tamoxifen (500 μg) and brivanib alaninate (0.1 mg/g) had a larger average volume (difference = 40 mm^3) than those that received tamoxifen (500 μg) only ($p = 0.002$, $\alpha = 0.025$). Despite this initial difference, over a three-week period, animals treated with tamoxifen alone subsequently had an average tumour volume (difference = 0.168 mm^3) that was much greater than those animals treated with brivanib alaninate in combination with tamoxifen ($p < 0.001$, $\alpha = 0.025$) (Fig. 3D). All models demonstrated that a VEGFR-2 inhibitor, brivanib alaninate would prevent the growth of SERM stimulated tumours.

3.4. *Determination of tamoxifen dosing in SERM sensitive MCF-7 E2 tumours*

We determined an anti-oestrogenical dose of tamoxifen that would be approximately 50% effective in blocking estradiol stimulated tumour growth. Previously, 1.5 mg/d of tamoxifen has been used to almost completely block oestrogen stimulated tumour growth.[40] The differences in the CSAs of tumours treated with estradiol and 125 μg tamoxifen (–0.368 cm^2, $p = 0.01$, $\alpha = 0.016$), estradiol and 250 μg tamoxifen (–0.479 cm^2, $p = 0.001$, $\alpha = 0.016$) or estradiol and 500 μg tamoxifen (–0.479 cm^2, $p < 0.001$, $\alpha = 0.016$) versus estradiol alone were significant (Fig. 4). A dose of 125 μg was chosen for further testing in combination with brivanib alaninate to determine whether there would be an improvement in therapeutic efficacy.

3.5. *The combined effect of a lower dose of tamoxifen and brivanib alaninate in SERM sensitive MCF-7 E2 tumours*

We hypothesise that a sub-therapeutic dose of brivanib alaninate may enhance a sub-optimal effective daily dose of tamoxifen (125 μg) and thus improve tumour growth control. The strategy of limiting angiogenesis would optimise long-term anti-oestrogen therapy. Statistical comparisons were done to determine whether there was a difference in the average CSA of tumours treated with 125 μg tamoxifen + 0.05 mg/g brivanib alaninate versus 125 μg tamoxifen or 0.05 mg/g brivanib alaninate. The results illustrated in Fig. 5 demonstrated that the combination of 125 μg of tamoxifen and 0.05 mg/g of brivanib alaninate significantly improved the anti-tumour action tamoxifen or brivanib alaninate alone after 6 weeks. The difference in average CSAs of tumours treated with 125 μg tamoxifen and 0.05 mg/g brivanib alaninate versus those treated 125 μg tamoxifen (–0.128 cm^2, $p = 0.01$, $\alpha = 0.025$) was significant. Similarly, there was a significant difference in the CSA of those tumours treated with the combination therapy and those treated with brivanib alaninate (–0.449 cm^2, $p < 0.001$, $\alpha = 0.025$).

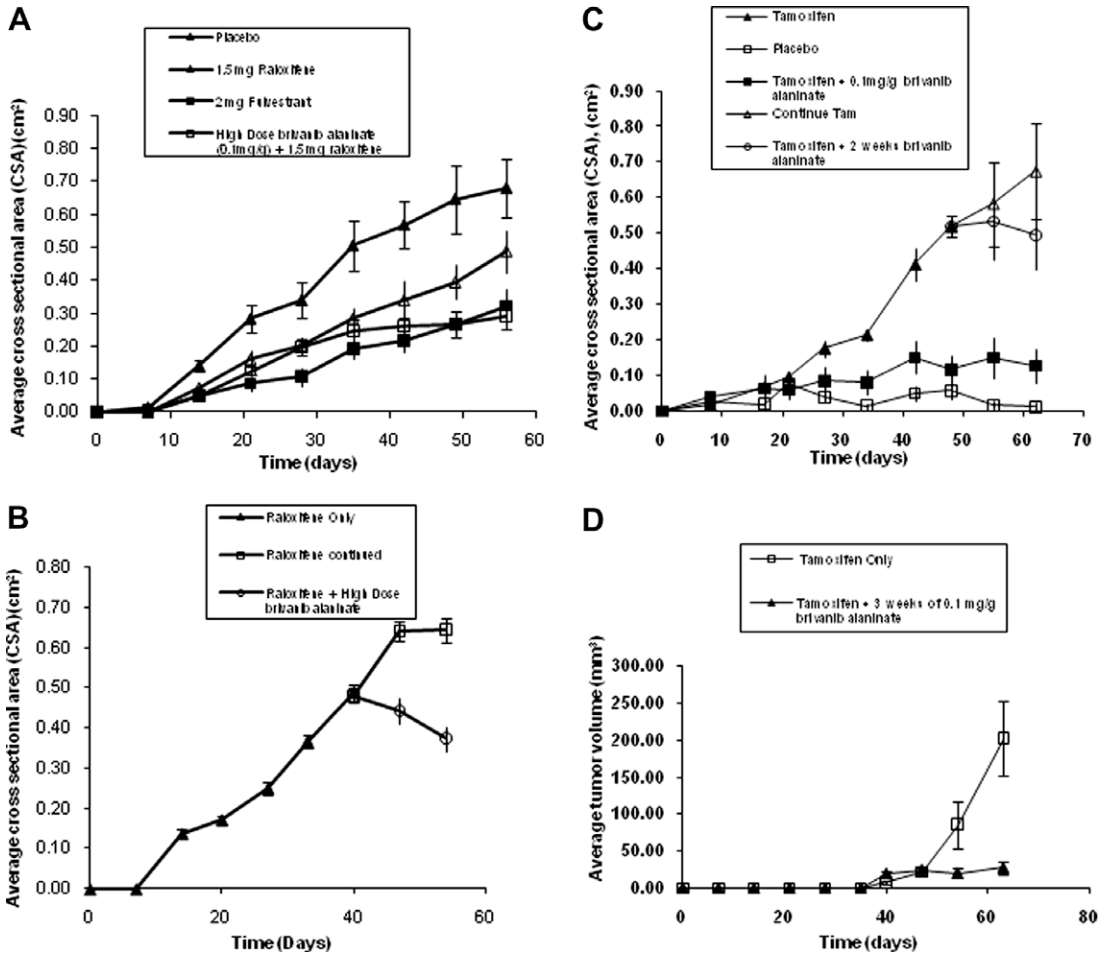

Fig. 3 – The anti-tumour effects of high dose (0.1 mg/g) brivanib alaninate on the growth of human tumours with acquired resistance to the SERMs raloxifene or tamoxifen. There were no significant differences in animal body weights between groups. Unless stated otherwise, all groups had 5 ovariectomised athymic mice with 10 tumours. (A) Raloxifene stimulated MCF-7 Ral. Groups were treated with raloxifene (1.5 mg daily by gavage), vehicle, fulvestrant (2 mg SQ 5 d per week), or raloxifene plus brivanib alaninate (0.1 mg/g by gavage). Brivanib alaninate (0.1 mg/g) significantly prevented the growth of raloxifene treated tumours ($p < 0.001$, $\alpha = 0.016$). (B) Raloxifene (1.5 mg daily by gavage) stimulated MCF-7 RAL. Twenty ovariectomised athymic mice were randomised into two groups of 10 mice each with continued raloxifene treatment (total of 17 tumours in the group) or raloxifene plus high dose brivanib alaninate (0.1 mg/g by gavage) (total of 19 tumours in the group). There was a significant decrease in tumour size with brivanib alaninate ($p < 0.001$, $\alpha = 0.025$). (C) Tamoxifen (1.5 mg daily by gavage) stimulated MCF-7 TAM tumours. Athymic, ovariectomised mice were initially placed into three groups to receive 1.5 mg tamoxifen (8 mice, 16 tumours), 1.5 mg tamoxifen plus 0.1 mg/g brivanib alaninate (4 mice, 6 tumours) or control vehicle (5 mice, 10 tumours). The group that received tamoxifen was randomised to continue tamoxifen (4 mice, 8 tumours) or receive tamoxifen with 0.1 mg/g brivanib alaninate (4 mice, 8 tumours) once the tumours reached an average CSA of 0.5 cm^2. The VEGFR inhibitor produced significant decreases in tamoxifen-stimulated growth rate in early implanted ($p < 0.001$, $\alpha = 0.025$) or established ($p < 0.001$, $\alpha = 0.025$) tumours. (D) Treatment of tamoxifen-stimulated (500 μg tamoxifen by gavage daily) EnCa 101 endometrial tumours was continued in two groups of 10 ovariectomised, athymic mice (20 tumours per group for 40 d). One group then received concomitant high dose brivanib alaninate (0.1 mg/g by gavage) for 3 weeks. Tumour volume was significantly decreased in animals treated with brivanib alaninate and tamoxifen compared to tamoxifen alone ($p < 0.001$, $\alpha = 0.025$).

3.6. *The combined effect of a lower dose of tamoxifen and brivanib alaninate in established SERM sensitive MCF-7 E2 tumours*

The goal of this experiment was to obtain sufficient tumour tissue for molecular analysis to evaluate the actions of tamoxifen and brivanib alaninate. The results are summarised in Figs. 6 and 7. The short-term combination of brivanib alaninate and tamoxifen decreased tumour size during the two-week period, whereas neither tamoxifen alone nor the brivanib alaninate alone prevented an increase in established tumour size (Fig. 6A). Statistical comparisons were done to determine whether there was a difference in the average CSA of tumours treated with 125 μg tamoxifen + 0.05 mg/g

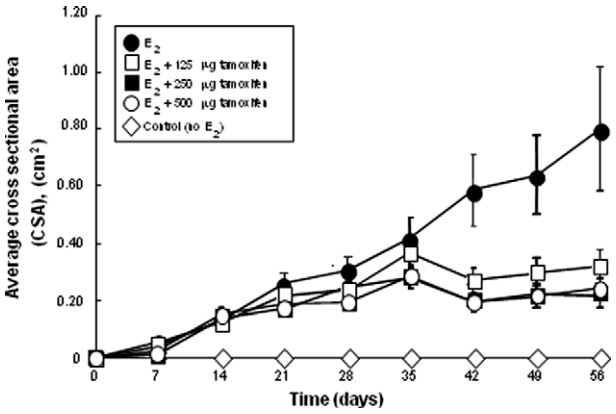

Fig. 4 – The effect of daily oral tamoxifen dosing on the estradiol-stimulated growth of MCF-7 E2 tumours delivered by an implanted 0.3 cm sustained release silastic capsule. There were five ovariectomised, athymic mice and 10 tumours per group. There was a dose-dependent decrease in estradiol stimulated tumour growth. The tumours did not grow without estradiol. The lowest dose of tamoxifen, 125 µg, suppressed tumour growth by 63%, whereas the higher doses (250 µg and 500 µg) suppressed tumour growth by 75%. There were no significant differences in animal body weights between groups.

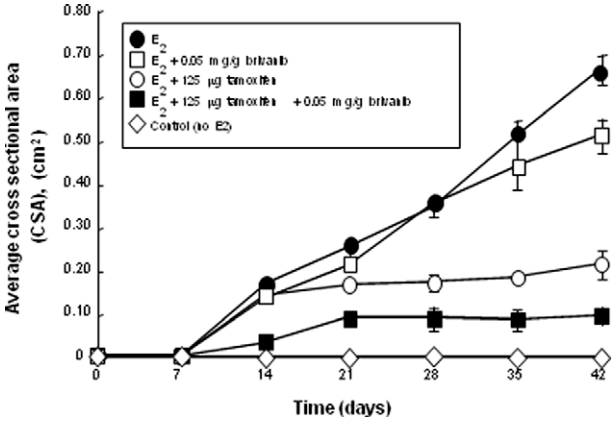

Fig. 5 – The effect of a combination of tamoxifen (125 µg daily oral dose) and 0.05 mg/g brivanib alaninate on the growth of established estradiol stimulated MCF-7 E2 tumours. There were five ovariectomised, athymic mice and 10 tumours per group. The combination of 125 µg tamoxifen with 0.05 mg/g brivanib alaninate improved the effects of 125 µg tamoxifen ($p < 0.01$, $\alpha = 0.025$) or 0.05 mg/g brivanib alaninate ($p < 0.001$, $\alpha = 0.025$). There were no significant differences in animal body weights between groups.

brivanib alaninate versus 125 µg tamoxifen or 0.05 mg/g brivanib alaninate. There was no difference in size at the time of randomisation (tamoxifen versus combination therapy ($p = 0.87$) and brivanib versus combination therapy ($p = 0.29$)). The average CSA was significantly different between tumours treated with 125 µg tamoxifen versus those treated with 125 µg tamoxifen and .05 mg/g brivanib alaninate (–0.292 cm^2, $p = 0.01$, $\alpha = 0.025$). The same observation was noted for those tumours treated with 0.05 mg/g brivanib alaninate versus those treated with 0.05 mg/g brivanib alaninate and 125 µg tamoxifen (–0.341 cm^2, $p = 0.007$, $\alpha = 0.025$).

Consistent with our findings, illustrated in Fig. 2B, representative histological analysis in this experiment confirmed (Fig. 6B) increased necrosis in tumours that received only brivanib alaninate or brivanib alaninate plus tamoxifen.

Western immunoblotting (Fig. 6C) demonstrated a decrease in phosphorylation of the VEGFR-2, but not total VEGFR-2 in the two groups that received brivanib alaninate. Total ER expression was reduced in the group receiving tamoxifen and the brivanib alaninate compared to tamoxifen alone.

RTPCR analysis (Fig. 6D) demonstrated an increase in mRNA for mouse VEGFR-1 and mouse VEGFR-2 in tumours that receive brivanib alaninate with ($p = 0.002$, $p = 0.002$) or without ($p = 0.001$, $p = 0.001$) tamoxifen. VEGFA mRNA is increased with tamoxifen ($p = 0.01$), brivanib alaninate ($p = 0.001$) or both drugs ($p = 0.002$) in combination. VEGFC increased with the tamoxifen treated group ($p = 0.001$), but decreased in the groups treated with the brivanib alaninate ($p = 0.004$). ER mRNA levels increased ($p = 0.04$) with the tamoxifen treated group, but decreased with the group that received both the VEGFR inhibitor and tamoxifen ($p = 0.04$).

We further validated our molecular studies with immunohistochemistry. There was little change in total VEGFR-2 (Fig. 7A), which was consistent with the findings in Western immunoblotting. VEGFA staining intensity increased in the tumours treated with tamoxifen and brivanib alaninate, which is consistent with the increased VEGFA mRNA seen in RTPCR analysis (Fig. 7B). The nuclear staining of the VEGF in the presence of brivanib (Fig. 7C) could be consistent with the report by Rosenbaum-Dekel et al.[41] with the nuclear localisation of L-VEGF, but no specific antibody was available to test the hypothesis.

4. Discussion

We report the first study to explore the potential of combining tamoxifen with low dose brivanib alaninate to block the growth of ER positive breast cancer. Previous studies have demonstrated the efficacy of brivanib in mouse models of human hepatocellular carcinoma[24] and to inhibit growth in ER negative H3396 xenografts in athymic mice.[25] Our strategy is to employ an anti-oestrogen (tamoxifen) to block oestrogen stimulated VEGF production and to use a combination with blockers of VEGFR-2 to reduce angiogenic survival mechanisms in both the tumour and endothelial cells to enhance tumour cell death. Our results demonstrate that the strategy is feasible. We have advanced the idea with the demonstration that a VEGFR-2 inhibitor, brivanib alaninate can not only inhibit the growth of small SERM stimulated implants derived from MCF-7 cells with acquired resistance to tamoxifen and raloxifene, but also can inhibit SERM stimulated growth of established tumours in athymic mice (Fig. 3A–C). Additionally, brivanib alaninate inhibits tamoxifen-stimulated endometrial cancer (EnCa 101) growth (Fig. 3D). Thus, the ability of a VEGFR-2 inhibitor to block the growth of tumours with acquired SERM resistance supports the idea that this strategy might improve adjuvant therapies.

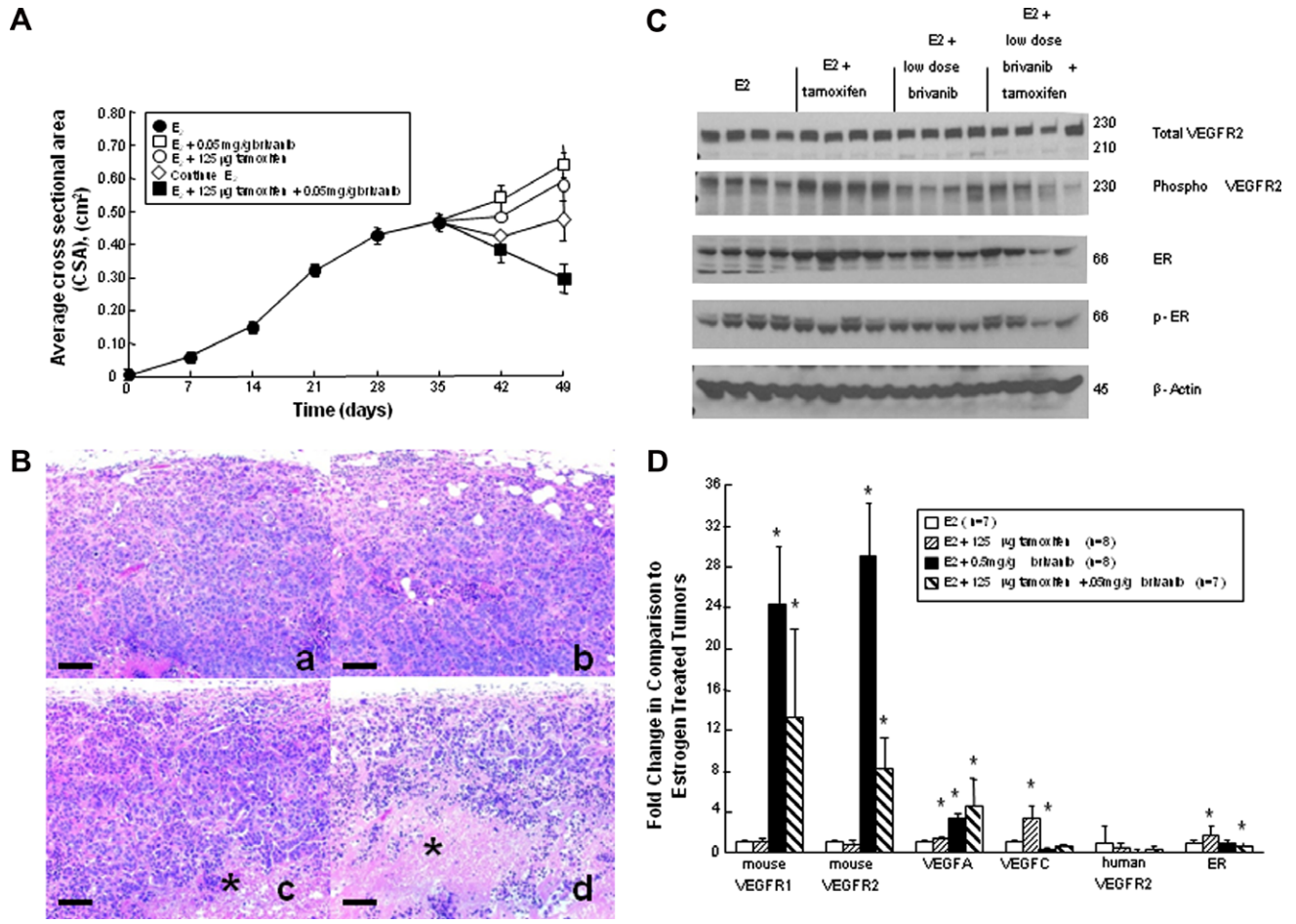

Fig. 6 – *Panel A:* the efficacy of a combination of 125 μg tamoxifen and 0.05 mg/g brivanib alaninate on the growth of established estradiol stimulated MCF-7 E2 tumours. There were five ovariectomised, athymic mice and 10 tumours per group. Tumours were grown to approximately 0.46 cm^2 and treated with the treatment regimens as indicated. There were no significant differences in animal body weights between groups. However, the decrease in average CSA was significant when comparing the combination treatment to tamoxifen (125 μg) treated tumours ($p = 0.01$, $\alpha = 0.025$) or those treated with 0.05 mg/g brivanib alaninate ($p = 0.007$, $\alpha = 0.025$). *Panel B:* H and E staining demonstrated an increase in necrotic tissue when brivanib alaninate was given alone or with tamoxifen. Once again, the bar represented a 100 μm distance. *Panel C:* Western blot analysis of tumour tissue did not illustrate a decrease in total VEGFR-2, regardless of the treatment group. The addition of brivanib alaninate, decreased the phosphorylation of VEGFR-2. Expression of ER and phosphorylated ER in all tumours, demonstrated the presence of active tumour tissue. *Panel D:* relative fold change in the mRNA levels of angiogenic factors in tumours relative to estradiol treatment alone. Mouse VEGFR-1 and mouse VEGFR-2 mRNA increased dramatically in tumours that received the inhibitor ($p = 0.001$, $p = 0.001$) or the inhibitor plus tamoxifen ($p = 0.002$, $p = 0.002$). VEGFA mRNA increased in tumours in response to tamoxifen treatment ($p = 0.01$) brivanib alaninate treatment ($p = 0.001$) and the combination of brivanib alaninate plus tamoxifen ($p = 0.002$). VEGFC increased in tamoxifen treated tumours ($p = 0.001$) and decreased in tumours treated with brivanib alaninate ($p < 0.004$). There was a significant, but small decrease in ER mRNA ($p = 0.04$) in tumours treated with the combination of tamoxifen plus brivanib alaninate and an increase in ER mRNA in tamoxifen treated tumours ($p = 0.04$).

Angiogenesis is important for tumour growth and metastasis. Stable transfection of MCF-7 cells with the VEGF gene results in hormone independent growth *in vivo* and tamoxifen resistance.[42] This is supported by the recent work by Aesoy and coworkers[43] using an anti-oestrogen resistant cell line (LCC2) *in vitro* that has constitutive VEGF secretion relative to wildtype MCF-7 cells. MCF-7 cells respond to 4-hydroxytamoxifen with a reduction in VEGF, but the anti-oestrogen resistant variant LCC2 does not. Oestrogen has been shown to increase the synthesis of VEGFA[13] and anti-oestrogens inhibit the process.[13,43] This observation was validated in our tumour models as the expression of VEGFA and VEGFR-2 is increased in the presence of oestrogen and decreased with oestrogen withdrawal (Fig. 1A and B). As there is strong evidence for the oestrogen mediated regulation of angiogenesis, combining an anti-oestrogen with an antiangiogenic inhibitor to diminish tumour growth is a reasonable therapeutic approach.

There are fewer side-effects such as malignant hypertension with angiogenesis inhibitors when used lower doses.[44]

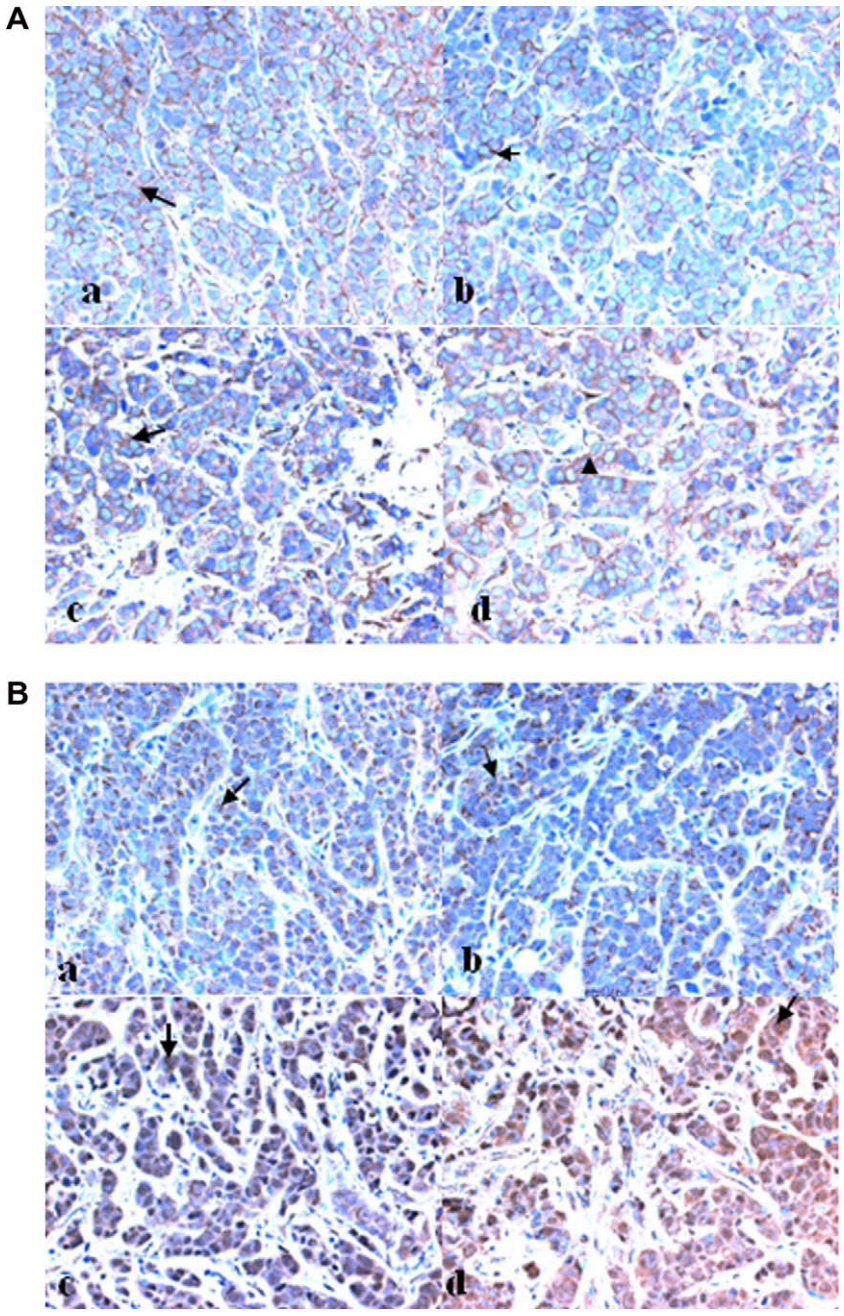

Fig. 7 – *Panel A*: there is no change in total VEGFR-2 expression by IHC in MCF-7 E2 tumours treated with estradiol (a), estradiol and 2 weeks of 125 μg tamoxifen (b), estradiol and 2 weeks of 0.05 mg/g brivanib alaninate (c), or estradiol and 2 weeks of the combination of 125 μg tamoxifen and 0.05 mg/g brivanib alaninate (d). *Panel B*: by IHC, the VEGFA staining intensity is greatest with 2 weeks of the combination of 125 μg tamoxifen and 0.05 mg/g brivanib alaninate (d). Staining intensity is the same with estradiol (a), estradiol and 2 weeks of 125 μg tamoxifen (b), estradiol, and 2 weeks of 0.05 mg/g brivanib alaninate (c). The bars represent 50 μm.

At higher doses, therapeutic efficacy may be diminished when drug dosing is reduced or abbreviated. Therefore, we advanced the concept of dual inhibition of angiogenesis further and tested a combination of sub-effective tamoxifen (125 μg) daily and the sub-therapeutic VEGFR-2 inhibitor brivanib alaninate (0.05 mg/g daily). The combination significantly decreased tumour growth compared with estradiol and either drug alone. This was true for the prevention of early tumour development following initial implantation (Fig. 5) or during the short-term treatment of established tumours (Figs. 6 and 7). Thus, we have shown that using a combination of lower, more tolerable doses of two drugs that are as efficacious as higher, less tolerable doses of either drug used alone, is a viable alternative for adjuvant therapy.

Drug treatments were evaluated in established tumours to provide tissue to investigate molecular mechanisms. Total

VEGFR-2 levels did not change in the tumours with treatment (Figs. 6C and 7A), but the phosphorylation patterns were different (Fig. 6C). Brivanib alaninate inhibits phosphorylation of the VEGFR-2 receptor. This confirmed the reported mechanism of action[24] of brivanib alaninate as an inhibitor of the VEGFR-2 tyrosine kinase. Treatment of established tumours with tamoxifen alone increased phosphorylation of VEGFR-2 and this increase in phosphorylation was inhibited when brivanib alaninate was combined with tamoxifen. Thus, it is possible to explain why a significant decrease in tumour size resulted from the use of a two-drug combination rather than a single drug that was individually ineffective in established tumours.

Similarly, transcription of VEGFC mRNA increased during tamoxifen treatment, but this was abrogated with brivanib alaninate. This is an important finding because VEGFC also activates VEGFR-2.[22] There was a compensatory rise in VEGFA with tamoxifen, brivanib alaninate, or the combination of the two drugs. However, with the combination of tamoxifen and brivanib alaninate, the compensatory mechanisms of the tumour to overcome blockade of the ER and VEGFR-2 failed as evidenced by increased tumour necrosis. The compensatory rise in VEGFA was validated by IHC in tumours treated with the combination of tamoxifen and brivanib alaninate. Overall, our findings confirm and extend the recent findings of Aesoy and co-workers[43] who demonstrate a breast cancer cell survival of VEGF/VEGFR-2/p38 feedback loop in cells resistant to anti-oestrogens.

Classically, the VEGF pathway in tumours has been thought to result from VEGF secretion from tumour cell activation of VEGF receptors on endothelial cells. However, accumulating evidence suggest that VEGFR-2 is most likely found on both cancer cells and endothelial cells.[43,45–47] By using IHC to localise VEGFR-2 in the MCF-7 tumour model, there is demonstrable expression of VEGFR-2 on the breast cancer cells (Fig. 1A). Moreover, there is evidence of oestrogen mediated regulation of VEGFR-2 expression on tumour cells as VEGFR-2 expression decreases with the withdrawal of 17β-estradiol. Ryden[48] also demonstrated that VEGFR-2 is expressed on tumour material from patients. These findings strengthen the argument to target VEGFR-2 in breast cancer.

By using RTPCR to differentiate between mouse and human VEGFR-2, we were able to evaluate the response to therapy in the endothelial (mouse) and the tumour cell (human) components. Interestingly, when the brivanib alaninate is started at the time of implantation there is a significant decrease in mouse VEGFR-1 and VEGFR-2. There was a trend towards an increase in human VEGFR-2 in mice treated with the higher dose of brivanib alaninate, with a significant decrease in mouse VEGFR-2 mRNA. When the angiogenesis inhibitor was given to mice with established tumours, there was a trend towards a decrease in human VEGFR-2 mRNA with a significant increase in mouse VEGFR-1 and VEGFR-2 mRNA. Thus, when the human VEGFR-2 is blocked, this then affects the endothelial component and the cells attempt to manufacture more receptor.

The ER is central to oestrogen-regulated events. As reported in previous studies, tamoxifen blocks the E2-mediated down-regulation of ER mRNA (Fig. 6D) and there is an increase in total ER expression[49] (Fig. 6C). Interestingly, the co-administration of brivanib alaninate prevented the tamoxifen induced increase in ER mRNA (Fig. 6C) and there was a decrease in total ER expression (Fig. 6D). It appears that the administration of an inhibitor of VEGFR-2 can modulate the ER during the anti-tumour process and this is an area worthy of further investigation. Conversely, the expression of VEGFR-2 on the cancer cells in response to oestrogen is clearly important to maintain control of tumour growth. These observations further validate the use of a combination of an anti-oestrogen and an angiogenesis inhibitor.

In addition to inhibiting VEGFR-2, the inhibitor has also shown activity against FGFR-1 in other tumour models, and is thus useful as a dual inhibitor for angiogenesis.[24] In the present study, however, we were unable to detect FGFR-1 in our specific model.

Despite the encouraging results obtained in the present study, several recent reports[50–52] of either the development of resistance to antiangiogenic drugs[50] or enhanced metastatic spread with low dose antiangiogenic drugs[51,52] deserve consideration. Clinical trials have shown that the majority of human tumour types do not respond to inhibitors of integrin as an antiangiogenic strategy. Laboratory models now show[52] that low concentrations of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ inhibitors increase tumour growth via VEGFR-2 trafficking. This promotes endothelial cell migration to VEGF. In related studies, inhibitors of VEGFR can either enhance tumour cell seeding in 'metastatic assays'[51] or cause adaptive–evasive responses by tumours with greater malignancy and increased invasiveness.[50] Clearly, the complexity of the angiogenic survival signalling pathways present a challenge to seek the clinical relevance of pre-clinical pharmacology. Nevertheless, in a recent review, Ebos and co-workers[53] contend that it remains unclear whether antiangiogenic therapy will lead to increased invasion or metastases after long- or short-term treatments. There are more than 40+ adjuvant clinical trials in progress, so the question of the premature tumour resistance caused by low dose antiangiogenesis inhibitors will probably be answered first in the clinical setting.[53]

With this concern in mind, we are currently considering an initial short-term testing platform in ER positive metastatic breast cancer that has failed exhaustive endocrine therapy.[54,55] It is known that apoptosis and tumour regression can be induced by both high or low dose oestrogen clinically,[56,57] but we propose to use low dose oestrogen to reduce thromboembolic events. The therapeutic application of low dose oestrogen treatment is a direct translation of laboratory studies over the past 15 years.[58,59] By combining a dose escalation schedule of brivanib alaninate, we will be able to monitor tumour response precisely for the 12-week treatment schedule. These preliminary clinical data will guide our future adjuvant applications.

In summary, antiangiogenic agents have been utilised clinically in patients who have breast cancer that is refractory to other agents.[44] In these instances, to see a partial clinical benefit, higher doses that are potentially toxic have to be used. The observations that elevations of VEGFA and VEGFR-2 are associated with poor prognosis and response to tamoxifen therapy[48,60] suggests that a strategy to combine

anti-hormone treatment with an antiangiogenic strategy may have merit to test in clinical trials. Based on an increasing laboratory database that implicates an elevation in angiogenic factors in endocrine resistant breast cancer in the presence of tamoxifen,[43] we have provided evidence that a combination of tamoxifen plus a low dose dual inhibitor of VEGFR-2 and FGFR-1, brivanib alaninate, effectively controlled tumour growth. The strategy of combining a tyrosine kinase inhibitor of VEGFR-2 has the advantage of reducing toxicity, permitting long-term therapy and therefore compliance to enhance efficacy for adjuvant tamoxifen therapy. Indeed, the strategy of inhibiting angiogenesis, might in fact, improve responsiveness of those ER positive tumours that are refractory to tamoxifen alone. We believe this issue should be addressed in clinical trial.

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Conflict of interest statement

A research grant was provided as partial funding of this project by Bristol Myers Squibb. Brivanib alaninate was also provided by Bristol Myers Squibb.

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REFERENCES

1. Folkman J. Angiogenesis. *Annu Rev Med* 2006;**57**:1–18.
2. Folkman J. Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov* 2007;**6**:273–86.
3. Naumov GN, Akslen LA, Folkman J. Role of angiogenesis in human tumor dormancy: animal models of the angiogenic switch. *Cell Cycle* 2006;**5**:1779–87.
4. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 2005;**307**:58–62.
5. Jain RK, Carmeliet PF. Vessels of death or life. *Sci Am* 2001;**285**:38–45.
6. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000;**407**:249–57.
7. Goss PE, Ingle JN, Pater JL, et al. Late extended adjuvant treatment with letrozole improves outcome in women with early-stage breast cancer who complete 5 years of tamoxifen. *J Clin Oncol* 2008;**26**:1948–55.
8. Muss HB, Tu D, Ingle JN, et al. Efficacy, toxicity, and quality of life in older women with early-stage breast cancer treated with letrozole or placebo after 5 years of tamoxifen: NCIC CTG intergroup trial MA.17. *J Clin Oncol* 2008;**26**:1956–64.
9. Mamounas EP, Jeong JH, Wickerham DL, et al. Benefit from exemestane as extended adjuvant therapy after 5 years of adjuvant tamoxifen: intention-to-treat analysis of the national surgical adjuvant breast and bowel project B-33 trial. *J Clin Oncol* 2008;**26**:1965–71.
10. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;**5**:207–13.
11. Buzdar A, Howell A, Cuzick J, et al. Comprehensive side-effect profile of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: long-term safety analysis of the ATAC trial. *Lancet Oncol* 2006;**7**:633–43.
12. Crivellari D, Sun Z, Coates AS, et al. Letrozole compared with tamoxifen for elderly patients with endocrine-responsive early breast cancer: the BIG 1-98 trial. *J Clin Oncol* 2008;**26**:1972–9.
13. Takei H, Lee ES, Jordan VC. In vitro regulation of vascular endothelial growth factor by estrogens and antiestrogens in estrogen-receptor positive breast cancer. *Breast Cancer* 2002;**9**:39–42.
14. Hyder SM, Nawaz Z, Chiappetta C, et al. Identification of functional estrogen response elements in the gene coding for the potent angiogenic factor vascular endothelial growth factor. *Cancer Res* 2000;**60**:3183–90.
15. Hyder SM. Sex-steroid regulation of vascular endothelial growth factor in breast cancer. *Endocr Relat Cancer* 2006;**13**:667–87.
16. Hyder SM, Stancel GM, Chiappetta C, et al. Uterine expression of vascular endothelial growth factor is increased by estradiol and tamoxifen. *Cancer Res* 1996;**56**:3954–60.
17. Kirk E. Dog's tale of survival opens door in cancer research. USA Today 2002. Available from: http://www.usatoday.com/news/health/2002-07-24-cover-cancer_x.htm.
18. Blackwell KL, Haroon ZA, Shan S, et al. Tamoxifen inhibits angiogenesis in estrogen receptor-negative animal models. *Clin Cancer Res* 2000;**6**:4359–64.
19. Ferrara N, Hillan KJ, Gerber HP, et al. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov* 2004;**3**:391–400.
20. Wedam SB, Low JA, Yang SX, et al. Antiangiogenic and antitumor effects of bevacizumab in patients with inflammatory and locally advanced breast cancer. *J Clin Oncol* 2006;**24**:769–77.
21. Miller K, Wang M, Gralow J, et al. Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med* 2007;**357**:2666–76.
22. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004;**25**:581–611.
23. Eskens FA, Verweij J. The clinical toxicity profile of vascular endothelial growth factor (VEGF) and vascular endothelial

growth factor receptor (VEGFR) targeting angiogenesis inhibitors; a review. *Eur J Cancer* 2006;**42**:3127–39.
24. Huynh H, Ngo VC, Fargnoli J, et al. Brivanib alaninate, a dual inhibitor of vascular endothelial growth factor receptor and fibroblast growth factor receptor tyrosine kinases, induces growth inhibition in mouse models of human hepatocellular carcinoma. *Clin Cancer Res* 2008;**14**:6146–53.
25. Bhide RS, Cai ZW, Zhang YZ, et al. Discovery and preclinical studies of (R)-1-(4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yloxy)propan-2-ol (BMS-540215), an in vivo active potent VEGFR-2 inhibitor. *J Med Chem* 2006;**49**:2143–6.
26. Rosen LS, Wilding G, Sweeney C, et al. Phase I dose escalation study to determine the safety, pharmacokinetics and pharmacodynamics of BMS-582664, a VEGFR/FGFR inhibitor in patients with advanced/metastatic solid tumors. *J Clin Oncol* 2006;**24**:3051.
27. Jonker DJ, Rosen LS, Sawyer M, et al. A phase I study of BMS-582664 (brivanib alaninate), an oral dual inhibitor of VEGFR and FGFR tyrosine kinases, in patients (pts) with advanced/metastatic solid tumors: safety, pharmacokinetic (PK), and pharmacodynamic (PD) findings. *J Clin Oncol* 2007;**25**:3559.
28. Gottardis MM, Robinson SP, Jordan VC. Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumoristatic action of tamoxifen. *J Steroid Biochem* 1988;**30**:311–4.
29. O'Regan RM, Cisneros A, England GM, et al. Effects of the antiestrogens tamoxifen, toremifene, and ICI 182, 780 on endometrial cancer growth. *J Natl Cancer Inst* 1998;**90**:1552–8.
30. Gottardis MM, Robinson SP, Satyaswaroop PG, et al. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 1988;**48**:812–5.
31. O'Regan RM, Gajdos C, Dardes RC, et al. Effects of raloxifene after tamoxifen on breast and endometrial tumor growth in athymic mice. *J Natl Cancer Inst* 2002;**94**:274–83.
32. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 1988;**48**:5183–8187.
33. Robinson SP, Jordan VC. Antiestrogenic action of toremifene on hormone-dependent, -independent, and heterogeneous breast tumor growth in the athymic mouse. *Cancer Res* 1989;**49**:1758–62.
34. Stossi F, Barnett DH, Frasor J, et al. Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER)α or ERβ in human osteosarcoma cells: distinct and common target genes for these receptors. *Endocrinology* 2004;**145**:3473–86.
35. Gowri PM, Sengupta S, Bertera S, et al. Lipin1 regulation by estrogen in uterus and liver: implications for diabetes and fertility. *Endocrinology* 2007;**148**:3685–93.
36. Frasor J, Danes JM, Komm B, et al. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. *Endocrinology* 2003;**144**:4562–74.
37. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(–$\Delta\Delta$C(T)) method. *Methods* 2001;**25**:402–8.
38. Cai ZW, Zhang Y, Borzilleri RM, et al. Discovery of brivanib alaninate ((S)-((R)-1-(4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yloxy)propan-2-yl)2-aminopropanoate), a novel prodrug of dual vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1 kinase inhibitor (BMS-540215). *J Med Chem* 2008;**51**:1976–80.
39. O'Regan RM, Osipo C, Ariazi E, et al. Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. *Clin Cancer Res* 2006;**12**:2255–63.
40. Dardes RC, O'Regan RM, Gajdos C, et al. Effects of a new clinically relevant antiestrogen (GW5638) related to tamoxifen on breast and endometrial cancer growth in vivo. *Clin Cancer Res* 2002;**8**:1995–2001.
41. Rosenbaum-Dekel Y, Fuchs A, Yakirevich E, et al. Nuclear localization of long-VEGF is associated with hypoxia and tumor angiogenesis. *Biochem Biophys Res Commun* 2005;**332**:271–8.
42. Qu Z, Van Ginkel S, Roy AM, et al. Vascular endothelial growth factor reduces tamoxifen efficacy and promotes metastatic colonization and desmoplasia in breast tumors. *Cancer Res* 2008;**68**:6232–40.
43. Aesoy R, Sanchez BC, Norum JH, et al. An autocrine VEGF/VEGFR2 and p38 signaling loop confers resistance to 4-hydroxytamoxifen in MCF-7 breast cancer cells. *Mol Cancer Res* 2008;**6**:1630–8.
44. Burstein HJ, Elias AD, Rugo HS, et al. Phase II study of sunitinib malate, an oral multitargeted tyrosine kinase inhibitor, in patients with metastatic breast cancer previously treated with an anthracycline and a taxane. *J Clin Oncol* 2008;**26**:1810–6.
45. Kranz A, Mattfeldt T, Waltenberger J. Molecular mediators of tumor angiogenesis: enhanced expression and activation of vascular endothelial growth factor receptor KDR in primary breast cancer. *Int J Cancer* 1999;**84**:293–8.
46. Scherbakov AM, Lobanova YS, Shatskaya VA, et al. Activation of mitogenic pathways and sensitization to estrogen-induced apoptosis: two independent characteristics of tamoxifen-resistant breast cancer cells? *Breast Cancer Res Treat* 2006;**100**:1–11.
47. Weigand M, Hantel P, Kreienberg R, et al. Autocrine vascular endothelial growth factor signalling in breast cancer. Evidence from cell lines and primary breast cancer cultures in vitro. *Angiogenesis* 2005;**8**:197–204.
48. Ryden L, Linderholm B, Nielsen NH, et al. Tumor specific VEGF-A and VEGFR2/KDR protein are co-expressed in breast cancer. *Breast Cancer Res Treat* 2003;**82**:147–54.
49. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 1996;**56**:2321–30.
50. Paez-Ribes M, Allen E, Hudock J, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 2009;**15**:220–31.
51. Ebos JM, Lee CR, Cruz-Munoz W, et al. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* 2009;**15**:232–9.
52. Reynolds AR, Hart IR, Watson AR, et al. Stimulation of tumor growth and angiogenesis by low concentrations of RGD-mimetic integrin inhibitors. *Nat Med* 2009;**1**:392–400.
53. Ebos JM, Lee CR, Kerbel RS. Tumor and host-mediated pathways of resistance and disease progression in response to antiangiogenic therapy. *Clin Cancer Res* 2009;**15**:5020–5.
54. Jordan VC, Lewis-Wambi J, Kim H, et al. Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients. *Breast* 2007;**16**:S105–13.
55. Jordan VC, Lewis-Wambi JS, Patel RR, et al. New hypotheses and opportunities in endocrine therapy: amplification of oestrogen-induced apoptosis. *Breast* 2009;**18**:S10–7.
56. Lonning PE, Taylor PD, Anker G, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 2001;**67**:111–6.

57. Ellis MJ, Gao F, Dehdashti F, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *JAMA* 2009;**302**:774–80.
58. Wolf DM, Jordan VC. *A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent results in cancer research*. Heidelberg: Springer-Verlag; 1993. p. 23–33.
59. Yao K, Lee ES, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;**6**:2028–36.
60. Ryden L, Stendahl M, Jonsson H, et al. Tumor-specific VEGF-A and VEGFR2 in postmenopausal breast cancer patients with long-term follow-up. Implication of a link between VEGF pathway and tamoxifen response. *Breast Cancer Res Treat* 2005;**89**:135–43.

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Structure−Function Relationships of Estrogenic Triphenylethylenes Related to Endoxifen and 4-Hydroxytamoxifen

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Estrogens can potentially be classified into planar (class I) or nonplanar (class II) categories, which might have biological consequences. 1,1,2-Triphenylethylene (TPE) derivatives were synthesized and evaluated against 17β-estradiol (E2) for their estrogenic activity in MCF-7 human breast cancer cells. All TPEs were estrogenic and, unlike 4-hydroxytamoxifen (4OHTAM) and Endoxifen, induced cell growth to a level comparable to that of E2. All the TPEs increased ERE activity in MCF-7:WS8 cells with the order of potency as followed: E2 > 1,1-bis(4,4′-hydroxyphenyl)-2-phenylbut-1-ene (**15**) > 1,1,2-tris(4-hydroxyphenyl)but-1-ene (**3**) > *Z* 4-(1-(4-hydroxyphenyl)-1-phenylbut-1-en-2-yl)phenol (**7**) > *E* 4-(1-(4-hydroxyphenyl)-1-phenylbut-1-en-2-yl)phenol (**6**) > *Z*(4-(1-(4-ethoxyphenyl)-1-(4-hydroxyphenyl)but-1-en-2-yl)phenol (**12**) > 4-OHTAM. Transient transfection of the ER-negative breast cancer cell line T47D:C4:2 with wild-type ER or D351G ER mutant revealed that all of the TPEs increased ERE activity in the cells expressing the wild-type ER but not the mutant, thus confirming the importance of Asp351 for ER activation by the TPEs. The findings confirm E2 as a class I estrogen and the TPEs as class II estrogens. Using available conformations of the ER liganded with 4OHTAM or diethylstilbestrol, the TPEs optimally occupy the 4OHTAM ER conformation that expresses Asp351.

Introduction

Breast cancer is one of the most frequently diagnosed cancers among women in the United States, with an estimated 192370 new cases of invasive disease and 40170 deaths in 2009.[1] Although the exact etiology of breast cancer is not known, there is strong evidence that estrogen plays a role in its development and progression.[2] The effects of estrogen are mediated via the estrogen receptors (ERs[a]), ER-alpha (ERα) and ER-beta (ERβ), which are present in more than 80% of breast tumors. With regard to the therapy of breast cancer, ERα remains the most important target and its presence in breast tumors is routinely used to predict response to selective ER modulators (SERMs), such as tamoxifen (TAM).[3,4] TAM (Figure 1) is also the first chemotherapeutic drug to target ER-positive breast cancer cells[5] and prevent tumorigenesis in high-risk women.[6] TAM is available worldwide to treat patients with ER-positive breast cancers.

TAM is a substituted derivative[7,8] of the long-acting estrogen triphenylethylene.[9] TAM efficacy depends on the formation of clinically active metabolites 4-hydroxytamoxifen (4OHTAM)[10] and Endoxifen[11] (Figure 1), which have a greater affinity to ERα and a much higher antiestrogenic potency in breast cancer cells compared to the parent drug.

We are unaware of the subtle molecular changes that occur when estrogen binds to the ER to produce the ER complex because the whole complex has not been crystallized. As a consequence of this gap in our knowledge, the modulation of ERα can only be deduced by exploring structure−function relationships. However, the ligand binding domain (LBD) of ERα has been crystallized[12,13] with the estrogens 17β-estradiol (E2), diethylstilbestrol (DES), and the SERMs, 4OHTAM and raloxifene (Figure 1). The resolution of the structure of the estrogen: LBD complex by X-ray crystallography demonstrates that the planar estrogens E2 and DES are sealed within the LBD by helix 12.[12,13] This activates activating function (AF)-2 at the upper surface of helix 12 by the interaction with coactivators to facilitate full estrogen action. In contrast, the bulky side chain of 4OHTAM and raloxifene prevents helix-12 from sealing the LBD and this produces antiestrogenic action.[12,13] However, although AF-2 is deactivated, the 4OHTAM:ERα complex has estrogen-like activity,[14] whereas raloxifene does not.[15] This is believed to be because the side chain of raloxifene shields and neutralizes asp351 to block estrogen action.[16] In contrast, the side chain of tamoxifen is too short. It appears that when helix 12 is not positioned correctly the exposed asp351 can interact with AF-1 to produce estrogen action. This estrogen-like activity can be

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[a] Abbreviations: ER, estrogen receptor; SERM, selective estrogen receptor modulators; TAM, tamoxifen; 4OHTAM, 4-hydroxytamoxifen; LBD, ligand binding domain; E2, 17β-estradiol; DES, diethylstilbestrol; AF, activating function; TPE, triphenylethylene; EC_{50}, effective concentration 50%; ERE, estrogen response element; rmsd, root-mean-square deviation; THF, tetrahydrofuran; RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum; SFS, stripped fetal bovine serum; PBS, phosphate buffered saline; OPTI-MEM, Optimum Eagle's Minimum Essential Media; RCSB, Research Collaboratory for Structural Bioinformatics; PDB, Protein Data Bank; OPLS, optimized potential for liquid simulations; IFD, induced fit.

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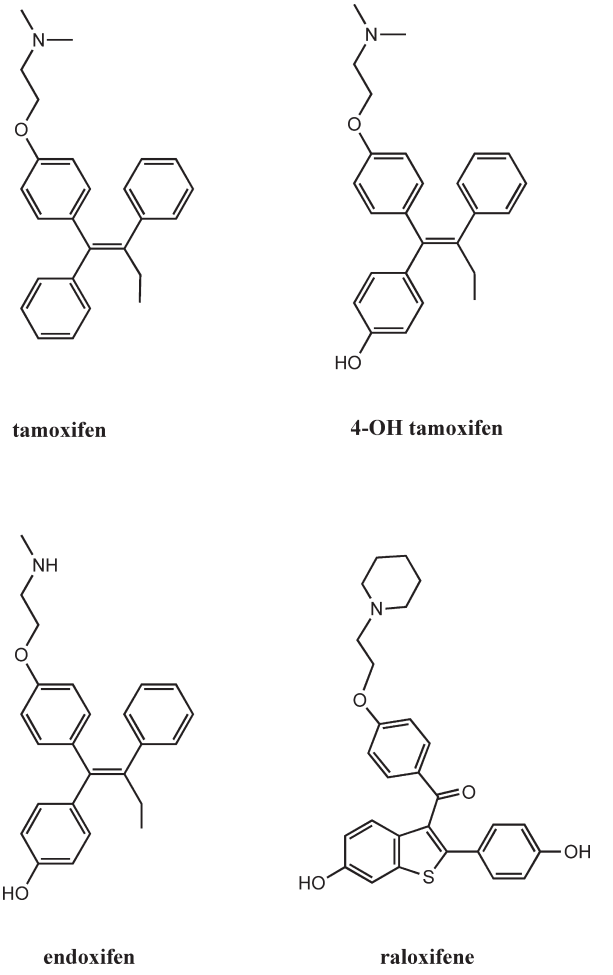


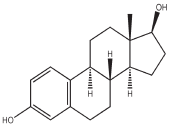
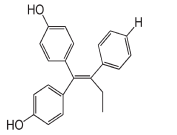
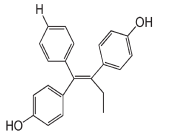
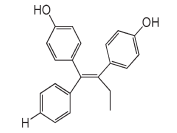
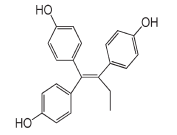
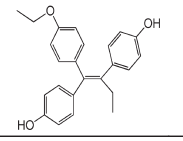
Figure 1. The formula of tamoxifen and its hydroxylated metabolites Endoxifen, and 4OHTAM. The related SERM raloxifene is shown for comparison.

inhibited by substituting asp351 for glycine an uncharged amino acid.[17]

Planar or nonplanar compounds are both classified as estrogens based on their actions to cause growth of the immature rodent uterus or provoke vaginal cornification in castrate animals. However, knowledge of the structure of the 4OHTAM:ER LBD complex[13] led to the idea that all estrogens may not be the same in their interactions with ER.[18] Previous studies suggest that nonplanar triphenylethylenes (TPEs) with a bulky phenyl substituent prevents helix-12 from completely sealing the LBD pocket.[18] This physical event creates a putative "antiestrogen like" configuration within the complex. However, the complex is not antiestrogenic because Asp351 is exposed to communicate with AF-1, thereby causing estrogen-like action. Thus, there are putative class I (planar) and class II (nonplanar) estrogens.[18] A similar classification and conclusion has been proposed,[19] but the biological consequences of this classification are unknown.

In this report, we further addressed the hypothesis that the shape of the ER complex can be controlled by the shape of an estrogen. We have synthesized a range of hydroxylated TPEs to establish new tools to investigate the relationship of shape with estrogenic activity through the exposure of Asp351. For convenience, the structure of nonsteroidal antiestrogens described in the text are illustrated in Figure 1 and the test compounds in Table 1.

Table 1. The EC_{50} Values for E2 and the Tested Triphenylethylenes in MCF-7:WS8 Cells Proliferation Assays

Compound	Structure	EC_{50}
E2		$1x10^{-3}$ nM
15		$5x10^{-2}$ nM
6		$1x10^{-1}$ nM
7		$1x10^{-1}$ nM
3		$1.5x10^{-1}$ nM
12		4.0 nM

Results

Chemistry. The general synthetic routes used to prepare substituted 1,1,2-tribenzyl-but-1-ene compounds are outlined in Scheme 1. Desoxyanisoin was treated with potassium *t*-butoxide followed by reflux with ethyl iodide to give **1** in 74% yield. Intermediate **1** was refluxed with the formed Grignard reagent of 4-bromoanisole and then treated with phosphoric acid to yield **2**. Removal of the methoxide groups was accomplished with boron tribromide to give **3**. Isomers **6**−**7** were synthesized from **1** by treatment with the formed Grignard reagent of bromobenzene followed by reflux in phosphoric acid to yield isomers **4**−**5**. Removal of the two methoxides was accomplished with boron tribromide resulting in isomers **6**−**7**. Compounds **11**−**12** were obtained by reaction of desoxyanisoin with glacial acetic acid and hydroiodic acid to give **8** in 90% yield. Dihydroxy **8** was protected using 3,4-dihydro-2*H*-pyran and *p*-toluene sulfonic acid to form **9**. Compound **9** was treated with potassium *t*-butoxide followed by reflux with ethyl iodide to yield **10** in 87%. Compound **10** was refluxed with the formed Grignard reagent of 4-bromophenetole, followed by acid hydrolysis using phosphoric acid to yield isomers **11**−**12**. Synthesis of 15 proceeded from reaction of anisole with 2-phenylbutyryl chloride to form monomethoxide **13** in 94% yield. Compound **13** was coupled with 4-methoxyphenyl magnesium bromide, followed by phosphoric acid to produce **14**. The methoxides of **14** were treated with boron tribromide to give dihydroxy **15**.

Pharmacology. We compared and contrasted the estrogen-like properties of the hydroxylated TPEs to promote proliferation in the ERα-positive human breast cancer cell

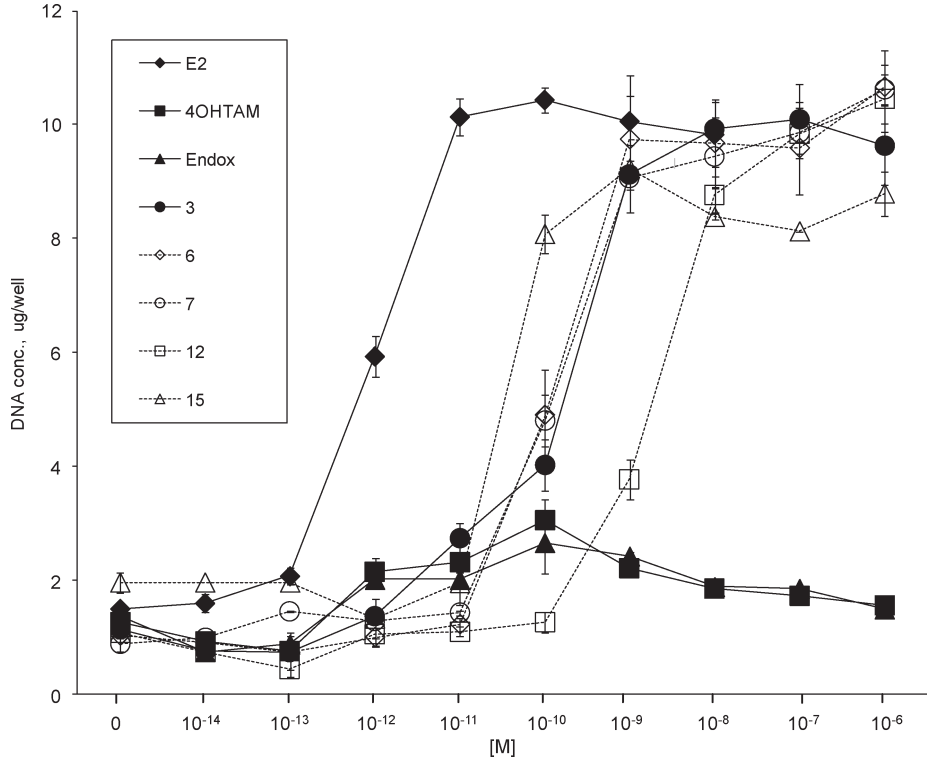
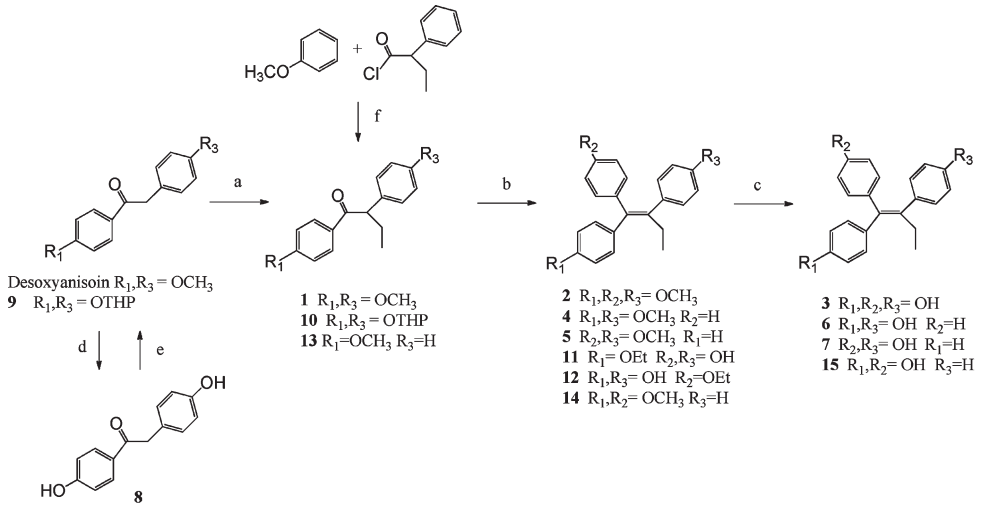


Figure 2. Effects of E2, test TPEs **3**, **6**, **7**, **12**, and **15** and antiestrogens 4OHTAM and Endoxifen on the proliferation of MCF-7:WS8 breast cancer cells. Cells were treated with the indicated compounds for 7 days.

Scheme 1. Synthesis of Substituted 1,1,2-Tribenzyl-but-1-ene Compounds[a]



[a] Reaction conditions: (a) KOtBu, ether, 1 h, then, EtI, reflux 12 h; (b) 4-$BrMgC_6H_4R_2$, THF, refluxed 12 h, then, H_3PO_4, refluxed 2 h; (c) BBr_3, CH_2Cl_2, 4 days; (d) HI, AcOH, 130−140 °C, 4 h; (e) C_5H_8O, *p*-$CH_3C_6H_4SO_3H$-H_2O, 0 °C 4.5 h; (f) $AlCl_3$, CS_2, 20 °C, 22 h.

line MCF-7:WS8. Compounds were compared with the tamoxifen metabolites 4-OHTAM and Endoxifen, which have a high affinity for the ER (because of the appropriately positioned phenolic hydroxyl) but are antiestrogenic because of the alkylaminoethoxy-side chain. To compare the biological activities of the tested TPEs, we employed DNA proliferation assays which are described in the Materials and Methods.

Figure 2 shows that our MCF-7:WS8 human breast cancer cells were exquisitely sensitive to E2, which produced a concentration-dependent increase in growth with maximal stimulation at 1×10^{-11} M. All of the TPE's were potent agonists with the ability to stimulate MCF-7:WS8 breast cancer cell growth, however, their agonist potency was less compared to E2, which had an effective concentration 50% (EC_{50}) of 1×10^{-12} M. The most potent of the phenolic TPEs was bisphenol (**15**), with an EC_{50} of approximately 5×10^{-11} M. The second potent were the *E* and *Z*-isomers of the diphenolic TPEs, compounds **6** and **7**, which both had an EC_{50} of approximately 1×10^{-10} M. The triphenolic TPE (**3**) was slightly less active, with an EC_{50} of approximately 1.5×10^{-10} M, whereas the ethoxy TPE (**12**) was the least potent, with an EC_{50} of approximately 4×10^{-9} M. The EC_{50} values for all the tested compounds are outlined

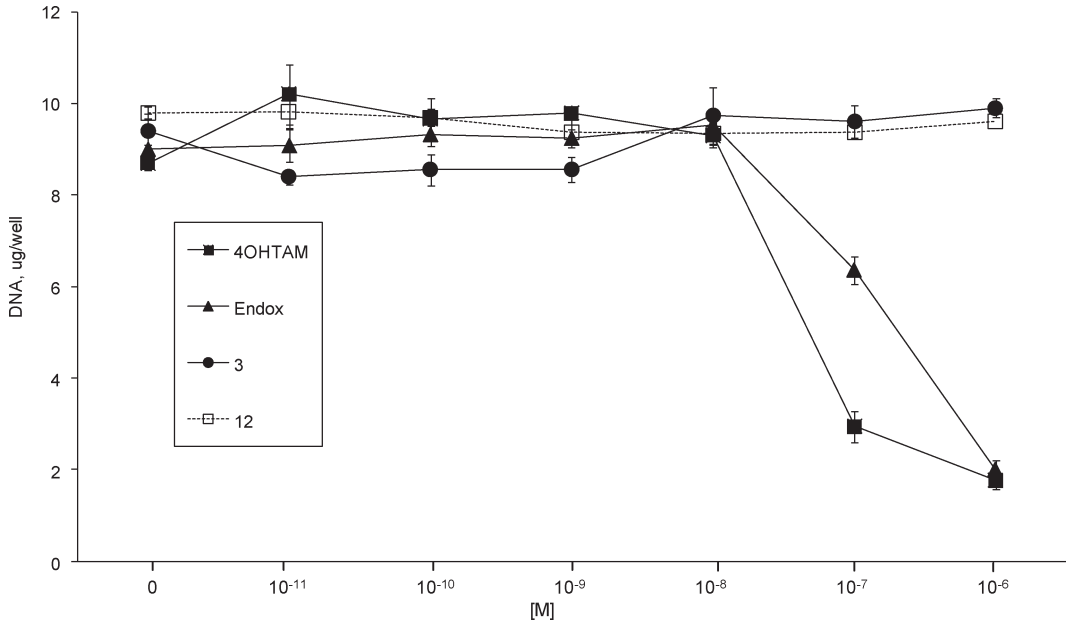


Figure 3. The ability of the tested TPEs **3** and **12** and 4OHTAM and Endoxifen to inhibit estradiol-stimulated MCF-7:WS8 breast cancer cell growth. Cells were treated with indicated compounds for 7 days.

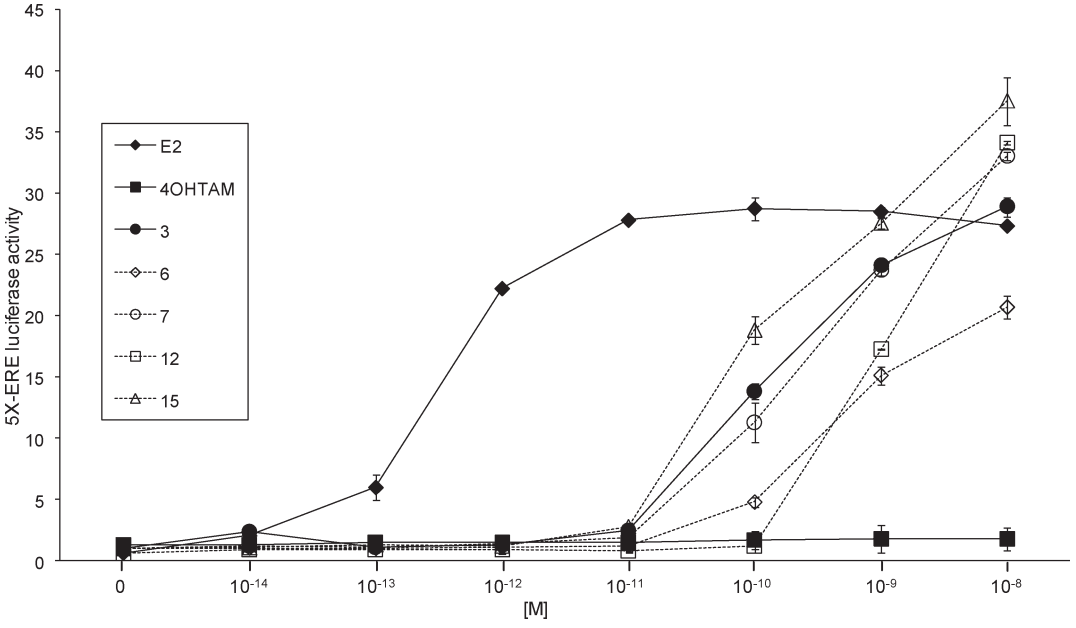


Figure 4. ERE luciferase assay in MCF-7:WS8 cells transiently transfected with an ERE luciferase construct and treated with E2, test TPEs **3**, **6**, **7**, **12**, and **15** and 4OHTAM.

in Table 1. The compound **12** was prepared to replicate a molecule without the alkyl nitrogen group of 4-OHTAM or Endoxifen, and this derivative had reduced estrogenic potency comparison to the other TPEs, however, the molecule remained a full estrogen agonist in our proliferation assays. The metabolites, 4-OHTAM and Endoxifen, had no significant agonist effect in MCF-7:WS8 cells, however, these compounds at 1 μM were able to completely inhibit estradiol-stimulated MCF-7:WS8 breast cancer cell growth (Figure 3), thus confirming their role as antagonists/antiestrogens. Similar experiments performed with compounds **3** and **12** showed an inability to block estradiol-stimulated growth in MCF-7:WS8 cells at concentrations up to 1 μM (Figure 3). On the basis of these findings, compounds **3** and **12** were classified as estrogens with a pharmacology, in this assay, indistinguishable from the natural planar estrogen E2.

It is interesting to note that compounds **6** and **7**, which are the *E*- and *Z*-isomers of the diphenolic TPEs, were equivalent in their agonistic potency, thus suggesting that isomerization occurs in vitro given an equilibrium mixture. This phenomenon has been noted previously with the *E*-isomer of 4-OHTAM,[20] but the true pharmacology of the separate isomers was eventually resolved by the synthesis of fixed ring analogues.[20,21] Both the *E*- and *Z*-isomers of 4-OHTAM are antiestrogenic because they block the proliferation of estradiol-stimulated growth in MCF-7 breast cancer cells and they inhibit estradiol-stimulated prolactin gene activation.

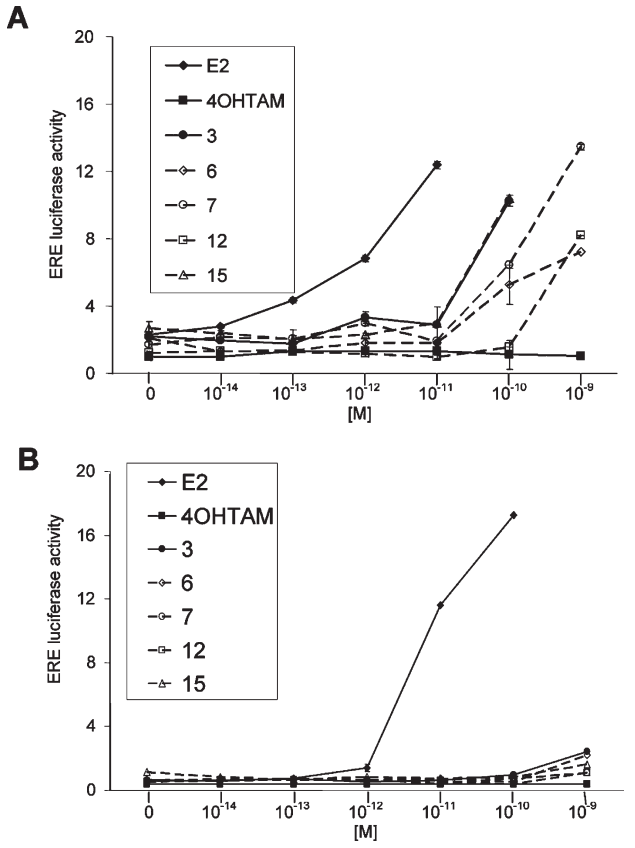


Figure 5. Luciferase assay in ER-negative T47D:C4:2 cells, transiently transfected with ERE luciferase and wild type (A) and D351G (B) mutant ER constructs, respectively, and treated with E2, tested TPEs **3**, **6**, **7**, **12**, and **15** and 4OHTAM. Results demonstrate that substitution of Asp351 to Gly in ER abrogates the agonistic activity on all tested TPEs (class II estrogens), except planar E2 (class I estrogen).

The *E*-isomer is however approximately 1/100 the potency of the *Z*-isomer.

To determine the ability of the test TPEs to activate the ER, MCF-7:WS8 cells were transiently transfected with an estrogen response element (ERE)-luciferase reporter gene encoding the firefly reporter gene with five consecutive EREs under the control of a TATA promoter. The binding of ligand-activated ER complex at the EREs in the promoter of the luciferase gene activates transcription. The measurement of the luciferase expression levels permits a determination of agonist activity of the TPE:ER complex. Figure 4 shows that all the phenolic TPEs were estrogenic, but E2 was 100 times more potent than the most potent TPE bisphenol (**15**). The order of potency was as follows: E2 > **15** > **3** > **7** > **6** > **12** > 4-OHTAM. None of the tested TPEs were antiestrogenic in this assay.

Our goal was to confirm and advance the hypothesis that the shape of the estrogen ER complex was different for planar and nonplanar (TPE -like) estrogens. This hypothesis has been advanced independently by ourselves[18,22] and Gust's group.[19] Through a series of studies using mutant ER expression in an ER negative breast cancer cell line, we found that the mutant D351G ER completely suppressed estrogen-like properties of 4-OHTAM at an endogenous TGFα target gene.[17] Use of this assay led us to classify planar estrogens (DES or E2) as class I and nonplanar estrogens (TPE-type) as class II. A broad group of compound structures were used in this study to establish whether a class II compound could become nonestrogenic with the D351G ER mutant.

Our series of phenolic TPEs were evaluated in the ER-negative breast cancer cell line T47D:C42,[23] which was transiently transfected with an ERE luciferase plasmid and either the wild-type ER or the D351G mutant ER. Figure 5A shows that in the presence of the wild-type ER all of the tested TPE compounds were potent agonists with the ability to significantly enhance ERE luciferase activity. In contrast, when the D351G mutant ER gene was transfected with the ERE luciferase reporter, only the planar E2 was estrogenic, whereas the TPEs did not activate the ERE reporter gene (Figure 5B). Overall, these results confirm the importance of Asp351 in ER activation by TPE ligands to trigger estrogen action.

Analysis of the Induced Fit Models for Tested TPEs. Data analysis was performed on top ranked poses for each of the tested TPEs and for comparison reasons on 4OHTAM (Figure 6A). The top ranked structure from induced fit for 4OHTAM has a ligand root-mean-square deviation (rmsd) of 0.55 Å compared with the experimental structure. In addition to the low ligand rmsd, there is a good similarity between the 3ert crystal structure and the top-ranked structures from docking (Figure 6B), the conformations of D351, E353, R394, T347, H524, and the rest of amino acids which line the binding site are nearly superimposable in both structures. Also, the well-known network of H-bonds is formed between 4OHTAM and E353, R394, and water molecules. The most significant difference is that in the top docked pose of 4OHTAM, the antiestrogenic chain is moved closer to D351 to form the interaction between the amino group of 4OHTAM and carboxylate of D351. Induced fit docking of the TPE derivatives: **3**, **6**, **7**, **12**, **15**, and Endoxifen in the ligand binding domain of ERα (3ert) has yielded ligand poses which display a binding mode (Figure 6B) very similar with that of 4OHTAM in the ER binding site (Figure 6A). Thus, the superimposition of the top ranked poses of each ligand onto the 4OHTAM cocrystallized with ERα (binding cavity filled with water) shows the ligands binding to the receptor in a similar mode with 4OHTAM, having the propensity to form the same hydrophobic contacts with the amino acids lining the binding cavity. Furthermore, the complex H-bond network is formed with E353, R394, H524, and a highly ordered water molecule positioned between E353 and R394 (Figure 6B). Interestingly, a H-bond has been noticed between the hydroxyl group of **15**, **3**, **7**, and the side chain of T347 is stabilized by an additional interaction with a water molecule from close proximity and precludes the interaction of the ligands with D351. The situation is different when water is removed from the binding site. In this case, the OH is shifted so that the H interacts with the carboxylate group of D351 and the HO group of T347 is shifted to form a H-bond with the oxygen. (data not shown). The molecular docking results have shown that most of the compounds form the H-bond network encountered in the case of agonists (E353, R394, H524, water) and display hydrophobic interactions with the amino acids lining the binding site. An interesting interaction is the hydrogen bond with T347 which seems to be stabilized by a water molecule and it was observed in different docking simulations (flexible and rigid). However, analysis of other ER crystal structures has not revealed additional data to confirm this interaction. Additional work has to be done to verify the hypothesis

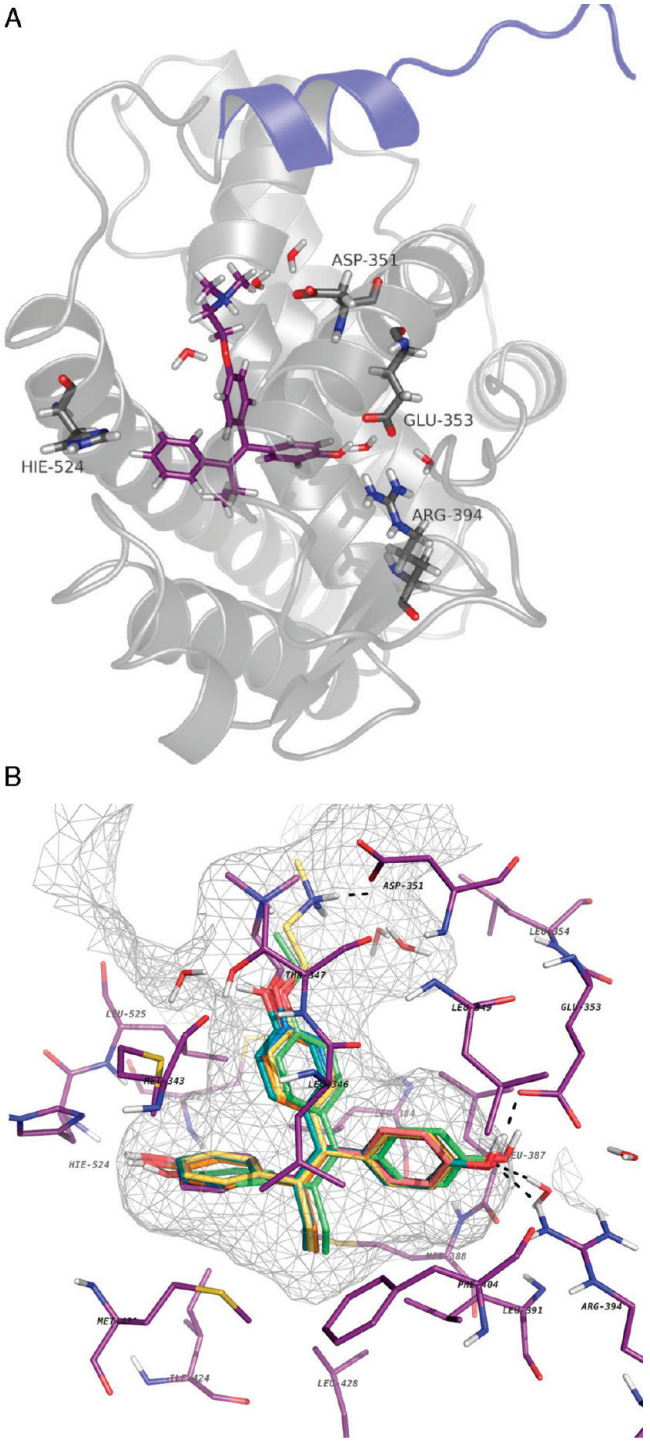


Figure 6. (A) Cartoon representation of the human ERα ligand binding domain complexed with 4-hydroxy tamoxifen, the antagonist conformation of the receptor. Helix 12 is depicted in blue, the amino acids involved in the H-bond network with the ligand are displayed as sticks. and the ligand is colored in purple. (B) Molecular docking of TPE derivatives into the binding site of ERα. For comparison reasons, the top ranked ligand−protein complex is superimposed on the crystal structure of the receptor cocrystallized with 4-OHT; the amino acids lining the biding sites of both complexes are shown and the complex H-bond network between ligand and the binding site is displayed. The induced fit docking poses of the ligands are colored as follow: **15** in cyan, **3** in blue, **6** in orange, **7** in pink, **12** in green, Endoxifen in yellow, while the crystal structure is depicted in purple.

(docking, binding energy calculations through semiempircal and/or ab initio methods, etc.). The interaction with D351 is weak (3.8−4 Å), and it was mostly noticed when the simulations were run with the receptor without water in the binding site. This would mean that D351 is exposed and not shielded so it could communicate intrinsic estrogenic properties of the complex to AF-1.

The best poses of the tested TPEs **3**, **6**, **7**, **12**, **15**, and Endoxifen, obtained from docking simulations ran against the antagonist conformation of the ER, were superimposed on the experimental agonist conformation of the ER (ER cocrystallized with estradiol, PDB code 1GWR) (Figure 7A). This has shown that these ligands are unlikely to be accommodated in the agonist conformation of the ER due to the sterical clashes between "Leu crown", mostly Leu525 and Leu540, helix 12, and ligands as depicted in Figure 7B, indicating, that these ligands most likely bind to ER's conformation more closely related with the antagonist form.

Discussion

The aim of this structure function relationship study was to evaluate the pharmacological properties of synthetic TPEs as estrogens in MCF-7 human breast cancer cells using the DNA proliferation assay and ERE luciferase assays. Our results show that all of the synthesized TPEs possess potent estrogen-like properties in our MCF-7 human breast cancer cells. These TPEs markedly increased cell growth and enhanced ERE luciferase activity. In contrast, the tamoxifen metabolites 4OHTAM and Endoxifen, which possess an alkylaminoethoxy side chain in their structure, failed to induce growth or increase ERE luciferase activity, thus confirming their role as antiestrogens.

X-ray crystallography of ER-4OHTAM and ER-Raloxifene complexes demonstrate that the presence of the alkyaminoethoxy side chain of 4OHTAM is crucial for the ER to gain an antagonistic conformation by displacing the H12 of the receptor by 4OHTAM's bulky side chain, thus preventing the binding of the coactivators.[13] On the basis of the results of our proliferation assays and the luciferase assays, it is clear that repositioning of the hydroxyl groups changed the biological potencies of the tested TPE compounds, which lowered their estrogenic potency compared to that of E2. However, the fact that these TPEs were able to significantly induce growth and ERE activation in MCF-7:WS8 cells demonstrated that they are still full agonists. The absence of the alkyaminoethoxy side chain on the tested TPEs does not allow these compounds to act as antiestrogens, like 4-OHTAM or Endoxifen, which possesses the alkyaminoethoxy side chain.[13] However, despite the changes in biological potencies of the tested TPEs, due to repositioning of the hydroxyl groups and addition of the ethoxy group, these compounds also maintained their ability to activate the ERE as was demonstrated in our ERE luciferase assays.

Another interesting aspect in our study is the importance of Asp351 in activation of the ER thereby acting as a molecular test for the presumed structure of the TPE:ER complex. On the basis of the X-ray crystallography of the ER in complex with 4OHTAM[13] and Raloxifene,[12] it was determined that the basic side chains of these antiestrogens are in proximity of Asp351 in the ER. It was hypothesized that this interaction with Raloxifene actually neutralizes and shields Asp351, preventing it from interacting with ligand-independent activating function 1 (AF-1). In contrast, 4OHTAM possesses some estrogenic activity because the side chain is too short.[13] Substitution of Asp351 with glycine leads to loss of estrogenic

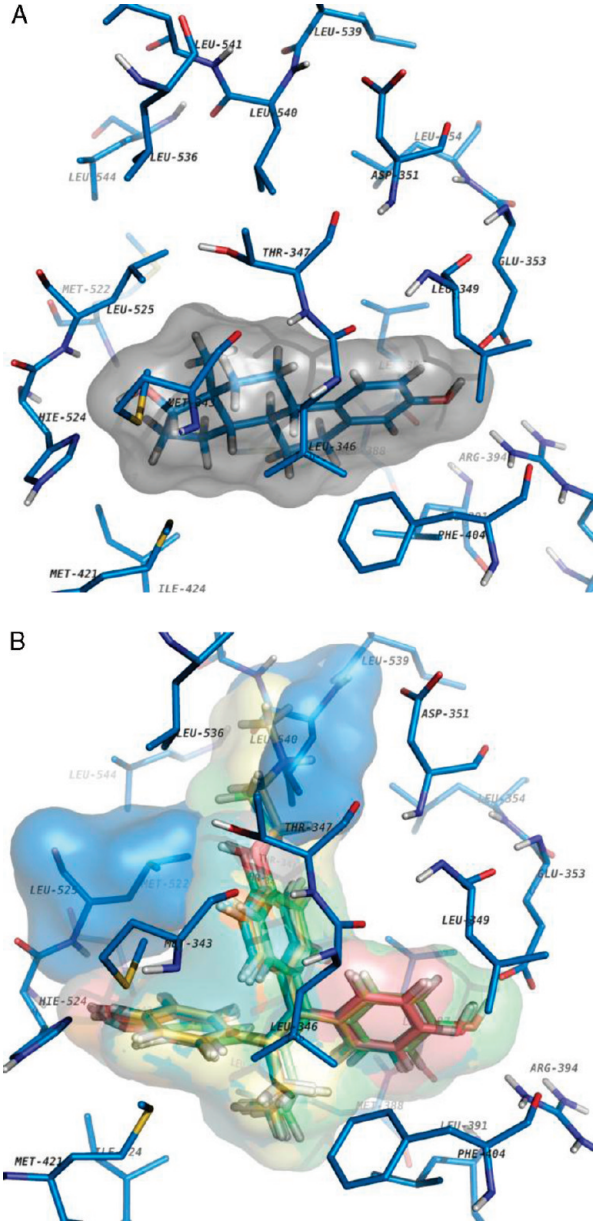


Figure 7. (A) View of ERα binding cavity. The X-ray crystal structure of ERα complexed with estradiol (PDB code 1GWR), the agonist conformation of the receptor. The amino acids lining the binding site are depicted as sticks colored by element. The color code is blue for carbon, red for oxygen, gray for nitrogen, and yellow for sulfur. The ligand is represented as sticks having the same colored code like the receptor and the ligand's surface is colored in gray. (B) View of ERα binding cavity. The best poses of BisPhen, TriOHTPE, EDiOHTPE, ZDiOHTPE, Z4EthoxDiOHTPE, Endox obtained from docking simulations ran against the antagonist conformation of the receptor are superimposed on the agonist conformation of the receptor, ERα cocrystallized with estradiol (PDB code 1GWR). The amino acids involved in steric clashes with the ligands, Leu525 and Leu540, are depicted as molecular surfaces colored in blue while the rest of amino acids lining the binding site are depicted as sticks colored by element, the color code is blue for carbon, red for oxygen, gray for nitrogen, and yellow for sulfur. The ligands are represented in sticks with the associated molecular surfaces. They respect the same coloring code with the exception of carbons which are colored as follow: BisPhen in cyan, TriOHTPE in blue, EDiOHTPE in orange, ZDiOHTPE in pink, ZEthoxDiOHTPE in green, Endoxifen in yellow. For clarity, waters and hydrogen atoms were omitted from the binding site.

activity of the ER bound with 4OHTAM.[17,24] Results from ERE luciferase assays in T47:C4:2 cells transiently transfected with wild type and D351G mutant ER expression plasmids demonstrated that wild type ER was activated by all of the tested TPEs, however substitution of Asp351 by Gly prevented the increase of ERE luciferase activity by all TPEs and only planar E2, which does not interact with Asp351 at all, or exposes it on the surface of the complex, was able to activate ERE in D351G ER transfected cells. This confirms and expands the classification of estrogens, where planar estrogens such as E2 are classified as class I and all TPE-related estrogens are classified as class II estrogens based on the mechanism of activation of the ER.[18]

It is important to note that all of the tested TPEs were agonists in our wild type ER assay systems, however, extensive studies of the structure−function relationship of phenolic TPEs by Gust and co-workers,[25,26] demonstrated that some of these compounds were potent antagonists in their MCF-7:2A cells stably transfected with an ERE luciferase plasmid. Specifically, these investigators found that compounds 1,1,2-tris(4-hydroxyphenyl)but-1-ene and 1,1-bis(4,4′-hydroxyphenyl)-2-phenylbut-1-ene, which correspond to compounds **3** and **15** in this study, were able to completely inhibit estradiol-stimulated ERE luciferase activity at 100 nM. The antagonistic potency of these compounds, however, did not correlate with results from the cytotoxicity assays performed in their wild-type MCF-7 cells.[25,26] Both compounds 1,1,2-tris(4-hydroxyphenyl)but-1-ene (designated as **3** in this study) and 1,1-bis(4,4′-hydroxyphenyl)-2-phenylbut-1-ene (designated as **15** in this study) produced weak cytotoxic effects only at concentrations above 5 uM, which were well beyond the concentration range used in our study. Thus it is possible that the variation in findings between our laboratory and that of Gust and co-workers[25,26] might be due to differences in our in vitro model systems and our experimental design.

Conclusions

We have confirmed and advanced the hypothesis[18,19,22] that estrogens can be classified into planar class I compounds (E2) and nonplanar class II compounds (TPEs). Armed with these new tools, we are now poised to examine the biological consequences of estrogen classification based on the shape of the resulting ER complex.

Materials and Methods

Chemistry. 1,1,2-Tris(4-hydroxyphenyl)but-1-ene (3). 1,1,2-Tris(4-hydroxyphenyl)but-1-ene (**3**) was synthesized according to the method of Lubczyk, Bachmann, and Gust.[26]

1,2-Bis(4-methoxyphenyl)butanone (1). Potassium *tert*-butoxide (1.35 g, 12 mmol) was added to a solution of desoxyanisoin (2.55 g, 10 mmol) in anhydrous ether under a nitrogen atmosphere, and the mixture was stirred for 1 h. At which time, iodoethane (0.8 mL, 10 mmol) was added dropwise and the mixture was refluxed for 12 h. Water (40 mL) was added, and the product was extracted with ether. The ether extracts were combined, dried over sodium sulfate, and evaporated under reduced pressure. The crude product was dissolved in carbon tetrachloride (10 mL), and petroleum ether was added to crystallize unreacted desoxyanisoin. Dexosyanisoin was filtered off, and the filtrate was evaporated in vacuo to yield **1** as colorless oil (2.11 g, 74%). ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 3H, J = 7.5 Hz), 1.81 (m, 1H), 2.15 (m, 1H), 3.75 (s, 3H, OCH_3), 3.82 (s, 3H, CH_3), 4.34 (t, 1H, J = 7.5 Hz), 6.84 (d, 2H, J = 8.7 Hz), 6.88

(d, 2H, J = 9.0 Hz), 7.26 (d, 2H, J = 9.0 Hz), 7.97 (d, 2H, J = 9.0 Hz).

***E*/*Z*-4,4′-(1-Phenylbut-1-ene-1,2-diyl)bis(methoxybenzene) (4) and (5).** Bromobenzene (1.12 mL, 1.664 g, 10.6 mmol) was added dropwise over 30 min to a stirred solution of magnesium turnings (0.26 g, 10.6 mmol) in dry tetrahydrofuran (THF) (10 mL) under a nitrogen atmosphere. Once the Grignard reagent formed and went into solution, **1** (2.03 g, 7.0 mmol) in THF (10 mL) was added dropwise over 60 min. The reaction was refluxed for 12 h then quenched with water (10 mL) and the THF removed under reduced pressure. The aqueous layer was extracted with ether (3 × 50 mL). The ether extracts were washed with saturated sodium bicarbonate and water and dried over sodium sulfate. The crude carbinol was refluxed with 85% phosphoric acid (10 mL) in dry THF (20 mL) for 2 h. The reaction mixture was diluted with water (30 mL) and extracted with of dichloromethane (3 × 50 mL). The dichloromethane layers were washed with sodium bicarbonate and water and dried over sodium sulfate. It was filtered and the solvent removed under reduced pressure, yielding a brown oil. Purification by flash chromatography over silica (3.0 × 30 cm) and elution with 200 mL of petroleum ether, 300 mL of 5% ether 95% pet ether, and 500 mL of 10% ether 90% petroleum ether yielded two isomers. Isomer *E* **4** (0.322 g; 15% yield) was collected in fractions 17 to 19 while *Z*-isomer **5** was collected in fractions 20 to 28 (0.746 g, 31% yield). ^{1}H NMR *E*-isomer ($CDCl_3$): δ = 0.96 (t, 3H, J = 7.5 Hz), 2.49 (q, 2H, J = 7.5 Hz), 3.76 (s, 3H), 3.84 (s, 3H), 6.71 (d, 2H, 8.7 Hz), 6.89 (dd, 4H, J = 8.7 and 2.1 Hz), 6.88−7.05 (m, 5H), 7.15 (d, 2H, J = 8.7). ^{1}H NMR *Z*-isomer ($CDCl_3$): δ = 0.93 (t, 3H, J = 7.5 Hz), 2.44 (q, 2H, J = 7.5 Hz), 3.71 (s, 3H), 3.78 (s, 3H), 6.57 (d, 2H, 8.7 Hz), 6.72 (d, 2H, J = 8.7 Hz), 6.89 (d, 2H, J = 8.7 Hz), 7.05 (d, 2H, J = 8.7), 7.22−7.37 (m, 5H).

***E*/*Z*-4-(1-(4-Hydroxyphenyl)-1-phenylbut-1-en-2-yl)phenol (6) and (7).** Boron tribromide (1.23 mL; 3.25 g; 0.0129 mols) in dichloromethane (5 mL) was added dropwise over 60 min to 4,4′-(1-phenylbut-1-ene-1,2-diyl)bis(methoxybenzene) **4** or **5** (0.746, 2.17 mmol) in dry dichloromethane (20 mL) cooled in a dry ice/ethanol bath while stirring under a nitrogen atmosphere. The solution turned dark immediately and was allowed to warm to room temperature after the addition was complete. The reaction mixture was stirred for a total of 4 days at room temperature. Excess boron tribromide was removed using a nitrogen stream then anhydrous methanol (3 × 25 mL) was added and it was evaporated in vacuo three times. It was recrystallized from benzene and purified further by preparative HPLC using 70% methanol 30% water. Fractions were collected as follows: *E*-isomer **6** (28−39 min, 1.814 abs; 40 mg); *Z*-isomer **7** (41−58 min, 2.007 Abs; 78 mg). ^{1}H NMR *E*-isomer **6** (MeOD): δ = 0.90 (t, 3H, J = 7.5 Hz), 2.47 (q, 2H, J = 7.5 Hz), 6.39 (d, 2H, J = 8.4 Hz), 6.65 (d, 2H, J = 8.7), 6.76 (d, 2H, J = 8.4 Hz), 7.02 (d, 2H, J = 8.7), 7.07−7.12 (m, 5H). ^{1}H NMR *Z*-isomer **7** (MeOD): δ = 0.90 (t, 3H, J = 7.5 Hz), 2.38 (q, 2H, J = 7.5 Hz), 6.43 (d, 2H, 8.4 Hz), 6.59 (d, 2H, J = 8.4 Hz), 6.66 (d, 2H, J = 8.4 Hz), 6.93 (d, 2H, J = 8.4 Hz), 7.16−7.32 (m, 5H). MS m/z calcd for $C_{22}H_{20}O_2$ 315.14 $(M - H)^-$; found 315 for both samples.

1,2-Bis(4-hydroxyphenyl)ethanone (8). Desoxyanisoin (1.0 g; 3.90 mmol) was dissolved in glacial acetic acid (1 mL) with stirring. Next, hydroiodic acid (5 mL, 36.5 mmol) was added and the solution was heated to 130−140 °C for 4 h. The reaction mixture was poured into water (50 mL) and the blue−gray colored solid was filtered and washed with water. It was dried in vacuo to yield **9** (0.80 g; 90%). Melting point 205−208 °C. ^{1}H NMR (MeOD): δ = 4.13 (s, 2H), 6.71 (d, 2H, J = 8.7 Hz), 6.83 (d, 2H, J = 8.7 Hz), 7.06 (d, 2H, J = 8.7 Hz), 7.93 (d, 2H, J = 8.7 Hz).

1,2-Bis(4-(tetrahydro-2*H*-pyran-2-yloxy)phenyl)ethanone (9). 1,2-Bis(4-hydroxyphenyl)ethanone (**8**) (700 mg, 3.07 mmol) was suspended in benzene (35 mL) with stirring under nitrogen atmosphere. Then, 3,4-dihydro-2*H*-pyran (6 mL, 65.6 mmol) was added, followed by *p*-toluenesulfonic acid monohydrate (53 mg, 0.28 mmol), and the solution was stirred at 0 °C for 4.5 h. The solution changed from purple to pink to clear. It was poured into saturated sodium bicarbonate solution and extracted with ethyl acetate. The combined ethyl acetate layers were washed with water, dried over magnesium sulfate, and evaporated in vacuo to a yellow solid. The residue was triturated with carbon tetrachloride to remove unreacted pyran. The white solid (548 mg) was collected by filtration, and the filtrate was purified by column chromatography over silica (2.7 × 4 on 2.7 × 22). The product was eluted with 100 mL of pet ether, 200 mL of 10% ether 90% pet ether, 200 mL of 20% ether 80% pet ether, 200 mL of 30% ether 70% pet ether, and 200 mL of 40% ether 60% pet ether. Fractions 33−40 were combined and evaporated in vacuo to give 243 mg of additional product (791 mg, 65% yield). ^{1}H NMR ($CDCl_3$): δ = 1.59−2.00 (m, 12H), 3.60 (m, 2H), 3.86 (m, 2H), 4.16 (s, 2H), 5.38 (t, 1H, J = 3.0 Hz), 5.50 (t, 1H, J = 3.0 Hz), 7.00 (d, 2H, J = 8.7 Hz), 7.07 (d, 2H, J = 8.7 Hz), 7.17 (d, 2H, J = 8.7 Hz), 7.97 (d, 2H, J = 9.0 Hz).

1,2-Bis(4-(tetrahydro-2*H*-pyran-2-yloxy)phenyl)butan-1-one (10). Potassium *tert*-butoxide **9** (229 mg, 2.04 mmol) was added to 1,2-bis(4-(tetrahydro-2*H*-pyran-2-yloxy)phenyl)ethanone (**9**) (672 mg, 1.69 mmol) dissolved in anhydrous THF (25 mL) under a nitrogen atmosphere with stirring. The mixture was stirred at room temperature for 1 h. Next, iodoethane (0.136 mL 1.70 mmol) was added dropwise, and the reaction mixture was refluxed for 6 h. After cooling, the THF was removed under reduced pressure. Water (30 mL) was added, and the product was extracted with ether. The ether extracts were combined, dried over sodium sulfate, and evaporated under reduced pressure to **10** (623 mg, 87% yield). ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 3H, J = 7.2 Hz), 1.60−1.97 (m, 12H), 2.13 (m, 2H, J = 7.2 Hz), 3.58 (m, 2H), 3.864 (m, 2H), 4.34 (t, 1H, J = 7.2 Hz), 5.34 (t, 1H, J = 3.0 Hz), 5.46 (t, 1H, J = 3.0 Hz), 6.95 (d, 2H, J = 8.4 Hz), 7.01 (d, 2H, J = 8.4 Hz), 7.20 (d, 2H, J = 8.7 Hz), 7.93 (d, 2H, J = 8.7 Hz).

***E*/*Z*(4-(1-(4-ethoxyphenyl)-1-(4-hydroxyphenyl)but-1-en-2-yl)-phenol (11) and (12).** 4-Bromophenetole (0.160 mL, 1.11 mmol) in dry THF (10 mL) was added dropwise over 30 min to magnesium turnings (27 mg, 1.11 mmol) with stirring under a nitrogen atmosphere. An iodine crystal was added to initiate the reaction, and it was refluxed until the magnesium turnings dissolved. Next, 1,2-bis(4-(tetrahydro-2*H*-pyran-2-yloxy)phenyl)-butan-1-one (**10**) (0.311 g; 0.735 mmol) was added and the reaction was refluxed for 12 h. After cooling, the reaction mixture was evaporated in vacuo to an orange residue. Water (20 mL), dichloromethane (20 mL), and acetic acid (1 drop) were added to the orange residue, and the aqueous layer was extracted with dichloromethane (3 × 25 mL). The organic extracts were washed with saturated sodium bicarbonate and water and dried over sodium sulfate. Filtration and evaporation provide the crude carbinol, which was hydrolyzed by refluxing for 2 h with 85% phosphoric acid (1 mL) in dry THF (10 mL). The reaction mixture was diluted with water (30 mL) and extracted with dichloromethane (3 × 50 mL). The dichloromethane was washed with sodium bicarbonate and water and dried over sodium sulfate. It was filtered, and the solvent was removed under reduced pressure to a yellow oil. It was purified by prep HPLC over C-18 Delta Pak column eluting with 40% H_2O and 60% MeOH. Flow rate was 100 mL/min. The elution was monitored by UV set to 254. Two fractions were collected: *E*-isomer **11** (54−64 m, 14 mg) and *Z*-isomer **12** (92−100 m, 16 mg). ^{1}H NMR **11** ($CDCl_3$): δ = 0.92 (t, 3H, J = 7.5 Hz), 1.34 (t, 3H, J = 6.9 Hz), 2.44 (q, 2H, J = 7.5 Hz), 3.90 (q, 2H, J = 6.9 Hz), 6.55 (d, 2H, J = 8.7 Hz), 6.63 (d, 2H, J = 8.4 Hz), 6.76 (d, 2H, J = 8.7 Hz), 6.80 (d, 2H, J = 8.7 Hz), 6.97 (d, 2H, J = 8.7 Hz), 7.12 (d, 2H, J = 8.4 Hz). ^{1}H NMR **12** ($CDCl_3$): δ = 0.92 (t, 3H, J = 7.5 Hz), 1.40 (t, 3H, J = 6.9 Hz), 2.45 (q, 2H, J = 7.5 Hz), 4.02 (q, 2H, J = 6.9 Hz), 6.49 (d, 2H, J = 8.7 Hz), 6.63

(d, 2H, J = 8.4 Hz), 6.73 (d, 2H, J = 7.8 Hz), 6.86 (d, 2H, J = 8.4 Hz), 6.97 (d, 2H, J = 8.4 Hz), 7.12 (d, 2H, J = 8.7 Hz). MS m/z calcd for $C_{24}H_{24}O_3$ 360; $(M - H)^-$ found 359; $(M + H)^+$ found 361.

1-(4-Methoxyphenyl)-2-phenylbutan-1-one (13). Anisole (5.861 g, 54.2 mmol) and 2-phenylbutyryl chloride (9.90 g, 54.2 mmol) were dissolved in 20 mL of carbon disulfide with stirring under a nitrogen atmosphere. The reaction mixture was cooled in an ice bath while $AlCl_3$ (7.6 g, 57.0 mmol) was added, keeping the temperature between 10 and 20 °C. It was stirred at room temperature for 20 h. The dark-red reaction mixture was poured into ice, and the aqueous layer was extracted with ether (3 × 75 mL). The combined ether layers were washed with 10% KOH, 10% HCl, and saturated sodium bicarbonate solutions. The ether layer was then dried over $MgSO_4$ and evaporated under reduced pressure to a yellow solid **18** (13.01 g; 94% yield); mp 41−42.5 °C. TLC: ($CHCl_3$) R_f = 0.57. NMR ($CDCl_3$): δ = 0.90 (t, 3H, J = 7.2 Hz), 1.84 (m, 1H, J = 7.2 Hz), 2.19 (m, 1H, J = 7.2 Hz), 3.82 (s, 3H), 4.40 (t, 1H, J = 7.2 Hz), 6.86 (d, 2H, J = 9 Hz), 7.16−7.41 (m, 5H), 7.96 (d, 2H, J = 9 Hz).

1,1-Bis(4,4′-methoxyphenyl)-2-phenylbut-1-ene (14). Compound **18** (1.0 g, 3.93 mmol) in 10 mL of THF was added dropwise over 15 min to a 1 M THF solution of 4-methoxyphenyl magnesium bromide (5.8 mL, 5.8 mmol) cooled in an ice bath under a nitrogen atmosphere with stirring. The solution was refluxed for 17.5 h. The reaction mixture was poured into 20 mL of water and 8 mL of 6N acetic acid. The aqueous layer was extracted with ether (3 × 50 mL). The ether extracts were washed with saturated sodium bicarbonate and brine, dried over sodium sulfate, and evaporated under reduced pressure to an oil. The crude carbinol was refluxed with 85% phosphoric acid (10 mL) in dry THF (20 mL) for 2 h. The reaction mixture was diluted with water (60 mL) and extracted with dichloromethane (3 × 50 mL). The dichloromethane layers were washed with sodium bicarbonate and water then dried over sodium sulfate. The solvent was removed under reduced pressure, yielding a cream solid. Purification by flash chromatography over silica (2.7 × 37 cm) and elution with 500 mL of hexanes gave pure **19** in fractions 7−18, which were combined and evaporated in vacuo; yield 1.30 g (96%); mp 116−118 °C. TLC: ($CHCl_3$) R_f = 0.68. NMR ($CDCl_3$): δ = 0.93 (t, 3H, J = 7.5 Hz), 2.48 (q, 2H, J = 7.5 Hz), 3.68 (s, 3H), 3.83 (s, 3H), 6.54 (d, 2H, J = 9 Hz), 6.77 (d, 2H, J = 9 Hz), 6.88 (d, 2H, J = 9 Hz), 7.07−7.19 (m, 7H).

1,1-Bis(4,4′-hydroxyphenyl)-2-phenylbut-1-ene (15). Compound **19** (1.9 g, 3.79 mmol) in 18 mL dry dichloromethane was cooled to −55 °C under a nitrogen atmosphere with stirring. Then, BBr_3 (2.17 mL, 22.95 mmol) in 10 mL of dichloromethane was added over 30 min while the temperature was kept at −55 °°C. The reaction mixture was stirred at room temperature for 90 h. The reaction was quenched by addition of methanol. The methanol was evaporated, and this was performed three more times, which resulted in a green residue. The crude product was purified by flash chromatography on a silica column (4 cm × 26 cm) equilibrated with hexane. It was eluted with chloroform:methanol (85:15), and 20 mL fractions were collected. Fractions 18−27 were combined and evaporated under reduced pressure. The resulting solid was recrystallized from chloroform but contained a small impurity by HPLC. It was purified by prep HPLC over C-18 Delta Pak column eluting with 20% H_2O and 80% MeOH. Flow rate was 30 mL/min. The UV detector was set to 254λ. The product was collect from 20 to 24 min, and the solvent was evaporated in vacuo to give **20** (75 mg, 5% yield); mp 206−206.5 °C. TLC: ($CHCl_3$ 9: methanol 1) R_f = 0.50. NMR ($CDCl_3$): δ = 0.92 (t, 3H, J = 7.5 Hz), 2.47 (q, 2H, J = 7.5 Hz), 4.48 (s, 1H), 4.71 (s. 1H), 6.46 (d, 2H, J = 8.4 Hz),; 6.73 (d, 2H, J = 8.4 Hz),; 6.81 (d, 2H, J = 8.4 Hz), 7.10−7.16 (m, 7H).

Cell Culture. The ER-positive human breast cancer cell line MCF-7:WS8 and the ER-negative breast cancer cell line T47D:C4:2 were used in our study. MCF-7:WS8 cells were cloned from wild type MCF-7 cells that were originally obtained from Dr. Dean Edwards (University of Texas, San Antonio, TX) and were maintained in phenol-red RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin at 100 units/mL, streptomycin at 100 μg/mL, 1× nonessential amino acids (all from Invitrogen, Carlsbad, CA), and bovine insulin at 6 ng/mL (Sigma-Aldrich, St. Louis, MO). The hormone-independent T47D:C4:2 cells were subcloned from T47D:C4 clones of T47D cells that were originally obtained from the ATCC (Rockville, MD). The T47D:C4:2 cells are ER-negative hormone-independent cells and they do not re-express ERα following growth in estrogen-containing media.[23] T47D:C4:2 cells were maintained in estrogen-free RPMI 1640 medium, containing 10% dextran charcoal-stripped fetal bovine serum (SFS). All cells were cultured in T185 culture flasks (Nalge Nunc International, Rochester, NY) and passaged twice a week in 1:4 ratio. All cultures were grown in 5%CO_2, 37 °C.

Cell Proliferation Assays. MCF-7:WS8 cells were cultured in estrogen-free medium (phenol red-free RPMI 1640 media supplemented with 10% charcoal-stripped FBS) for 4 days before beginning the proliferation assay. On day 0 of the experiment, MCF-7:WS8 cells were seeded in estrogen-free RPMI media containing 10% SFS at a density of 20000 cells per well respectively in NunclonΔ Surface 24-well plates (Nalge Nunc International, Rochester, NY). After 24 h, cells were treated with various concentrations of the tested compounds, prepared via serial dilutions. All concentration points were performed in triplicate. The compound-containing medium was changed on days 3 and 5, and the experiment was stopped on day 7. Cells were washed with cold PBS (Invitrogen, Carlsbad, CA) at least twice and analyzed with Fluorescent DNA quantification kit (Bio-Rad, Hercules, CA) according to manufacturers instructions, and samples were read on Mithras LB540 fluorometer/luminometer (Berthold Technologies, Oak Ridge, TN) in black wall 96-well plates (Nalge Nunc International, Rochester, NY) .

DNA Plasmids. Estrogen Response Element activity was determined via Luciferase assays with pERE(5X)TA-ffLuc and pTA-srLuc reporter plasmids. These plasmids contained the TATA-box basal promoter firefly and the *Renilla* luciferase reporter genes, respectively, and were constructed by insertion via *Hin*dIII linkers of the nucleotides −31 and +31 region of the herpes simplex virus thymidine kinase promoter into pGL3-Basic and phRG-B (Promega, Madison, WI).[27] For transient expression of wild-type ERα and 351 aspartate-to-glycine-substituted mutant ERα, pSG5HEGO and pSG5D351-GER plasmids were used, respectively. pSG5HEGO was originally provided by Professor Pierre Chambon, University of Strasbourg, France, and pSG5D351GER was generated using QuichChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and pSG5HEGO as a template.[17] All plasmids for this study were purified using HiSpeed Plasmid Maxi Kit (Qiagen, Valencia, CA) and were grown via OneShot TOP10 Chemically Competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA).

Transient Transfections and Luciferase Activity Assays. MCF-7:WS8 cells were cultured in estrogen-free RPMI media for 24 h prior to transfection. On the day of the experiment, cells were seeded in estrogen-free media at a density of 100000 cells per well in 24-well plates. After 24 h, MCF-7:WS8 cells were transfected with 28.8 μg of pERE(5X)TA-ffLuc and 9.6 μg of pTA-srLuc reporter plasmids, using 3 μL of TransIT-LT1 transfection reagent (Mirus Biolabs, Madison, WI) per 1 μg of plasmid DNA in 52.5 mL of OPTI-MEM serum-free media (Invitrogen, Carlsbad, CA). Transfection mix containing transfection complexes of the transfection reagent and plasmid DNA in OPTI-MEM media was added to cell in growth media to a final concentration of 0.3 μg pERE(5X)TA-ffLuc and 0.1 μg of pTA-srLuc reporter plasmids per well. After 18 h, transfection reagents were removed and fresh media was added. Cells were then treated with the various test compounds for 24 h. At the indicated time point, cells were washed once with cold PBS (Invitrogen, Carlsbad, CA), lysed, and ERE luciferase activity

was determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to manufacturers recommendations. Samples were then read on a Mithras MB540 fluorometer/luminometer (Berthold Technologies, Oak Ridge, TN).

T47D:C4:2 cells were seeded in estrogen-free RPMI 1640 media at a density of 200000 cells per well in 24-well plates. T47D:C4:2 cells are ER-negative cells, therefore these cells were transiently transfected with wild-type ERα (pSG5HEGO) or D351G mutant ERα (pSG5D351GER), along with pERE-(5X)TA-ffLuc and pTA-srLuc reporter plasmids. Transfection mix contained 7.2 μg of pSG5HEGO or 7.2 μg of pSG5D351GER, 7.2 μg of pERE(5X)TA-ffLuc, and 2.4 μg of pTA-srLuc reporter plasmids, and 3 μL of FuGene HD transfection reagent (Roche Diagnostics, Indianapolis, IN) per 1 μg of plasmid DNA, and 13 mL of OPTI-MEM serum-free media (Invitrogen, Carlsbad, CA) for 1 × 24-well plate. Transfection complexes of the reagent and plasmid DNA were added to cells in growth media to a final concentration of 0.3 μg of pSG5HEGO or 0.3 μg of pSG5D-351GER, 0.3 μg of pERE(5X)TA-ffLuc, and 0.1 μg of pTA-srLuc per well. After 18 h, transfection reagents were removed and fresh media was added. Cell were then treated with the various test compounds for 24 h, and ERE luciferase activity was determined as described above.

Reagents and Supplies. Estradiol (E2), 4-hydroxy-tamoxifen (4OHTAM), and bovine insulin, was obtained from Sigma, St. Louis, MO. Endoxifen (*Z*-isomer) was a kind gift from Dr. James Ingle (Mayo Clinic). Fetal bovine serum (FBS), 2 mM glutamine, penicillin at 100 units/mL, streptomycin at 100 μg/mL, 1× nonessential amino acids, RPMI 1640 with phenol-red media, PBS buffer, RPMI 1640 phenol-red-free media, and OPTI-MEM serum-free media were all obtained from Invitrogen, Carlsbad, CA. Fluorescent DNA quantification kit obtained from Bio-Rad, Hercules, CA. HiSpeed Plasmid Maxi Kit was obtained from Qiagen, Valencia, CA. TransIT-LT1 transfection reagent was obtained from Mirus Biolabs, Madison, WI. FuGene HD transfection reagent was obtained from Roche Diagnostics, Indianapolis, IN. Dual-Luciferase Reporter Assay System was obtained from Promega, Madison, WI. Anhydrous ether was purchased from Fisher. Potassium *tert*-butoxide, desoxyanisoin, magnesium turnings, bromobenzene, boron tribromide, anhydrous methylene chloride, 3,4-dihydro-2*H*-pyran, oxalyl chloride, *N*,*O*-dimethylhydroxylamine, aluminum chloride, and cyclohexene were purchased from Acros. Ethyl iodide, hydroiodic acid (55% ACS unstabilized), *p*-toluene sulfonic acid monohydrate, 2-fluoro-3-trifluoromethylbenzoic acid, 2,4-dimethoxymagnesium bromide (0.5 M solution in THF), anisole, 2-phenylbutyryl chloride, phenyl magnesium bromide (1 M solution in THF), sodium hydride (60% in mineral oil), and allylbromide was purchased from Aldrich. Carbon disulfide was purchased from Baker. THF was distilled and stored over calcium hydride. Benzene was distilled from calcium hydride and stored over molecular sieves. Flash chromatography was run using Whatman 230−400 mesh silica gel 60. Preparative chromatography was run on a Waters Delta Prep 3000 HPLC system using a prep pak C-18 delta-pak column (47 nn × 300 mm). UV detection was set at 254λ. Flow rate was 50 mL per min. ^{1}H NMR was performed on a Bruker WB Advance 300 MHz instrument. MS analysis performed by HT Laboratories (San Diego, CA) using electrospray ionization. LC-MS was performed using a Waters 2545 binary gradient module and a 2487 dual wavelength detector set to 254 and 365, a 2424 ELS detector, and a 3100 MS detector. The gradient was linear 5% MeOH 95% H_2O to 95% MeOH 5% H_2O over 20 min. The column was a Waters Delta Pak C-18 15μ 100A 3.9 mm × 300 mm (catalogue number 11797) run at a flow rate of 0.8 mL per min. The purity of all compounds was determined by LC-MS to be 95% or greater.

Molecular Modeling. The coordinates for the antagonist conformation of human ER ligand binding domain cocrystallized with 4OHTAM were extracted from the RCSB Protein Data Bank (PDB),[28] entry 3ert, was selected for further modeling with ER in antagonist conformation and 1GWR was selected for modeling of the ER in the agonist conformation. The protein was prepared for the docking experiments using the Protein Preparation Workflow (Protein Preparation Wizard, Schrödinger, LLC, Portland, OR) implemented in Schrödinger suite and accessible from within the Maestro program (Maestro 8.5, Schrödinger, LLC, Portland, OR). Briefly, the hydrogen atoms were added, water molecules beyond 5 Å from the ligand were deleted, and the orientation of hydroxyl groups, Asn, Gln, and the protonation state of His were optimized to maximize hydrogen bonding. All Asp, Glu, Arg, and Lys residues were left in their charged state. Finally, the ligand−protein complex was refined with a restrained minimization performed by Impref utility, which is based on the Impact molecular mechanics engine (Impact 4.5, Schrödinger, LLC, Portland, OR) and the OPLS2001 force field, setting a max rmsd of 0.30.

Ligands preparation for docking was performed with LigPrep (LigPrep 2.1, Schrödinger, LLC, Portland, OR) application which consists of a series of steps that perform conversions, apply corrections to the structure, generate ionization states and tautomers, and optimize the geometries.

Molecular docking was performed using Glide 4.5 (Glide 4.5, Schrödinger, LLC, Portland, OR) followed by the Induced Fit protocol (Induced Fit protocol, Schrödinger, LLC, Portland, OR), which is intended to circumvent the inflexible binding site and accounts for the side chain or backbone movements, or both, upon ligand binding.[29] In the first stage of the IFD protocol, softened-potential docking step, 20 poses per ligand were retained. In the second step, for each docking pose, a full cycle of protein refinement was performed, with Prime 1.6 (Prime 1.6, Schrödinger, LLC, Portland, OR) on all residues having at least one atom within 8 Å of an atom in any of the 20 ligand poses. The Prime refinement starts with a conformational search and minimization of the side chains of the selected residues and after convergence to a low-energy solution, an additional minimization of all selected residues (side chain and backbone) is performed with the truncated-Newton algorithm using the OPLS parameter set and a surface Generalized Born implicit solvent model. The obtained complexes are ranked according to Prime calculated energy (molecular mechanics and solvation), and those within 30 kcal/mol of the minimum energy structure are used in the last step of the process, redocking with Glide 4.5 (extended precision), and scoring. In the final round, the ligands used in the first docking step is redocked into each of the receptor structures retained from the refinement step. The final ranking of the complexes is done by a composite score which accounts for the receptor−ligand interaction energy (GlideScore) and receptor strain and solvation energies (Prime energy).[29]

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References

(1) *Cancer Facts and Figures*; www.cancer.org **2009**.
(2) Russo, I. H.; Russo, J. Role of hormones in mammary cancer initiation and progression. *J. Mammary Gland Biol. Neoplasia* **1998**, *3*, 49–61.

(3) EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. *Lancet* **1998**, *351*, 1451−1467.
(4) EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* **2005**, *365*, 1687−1717.
(5) Jordan, V. C Tamoxifen: catalyst for the change to targeted therapy. *Eur. J. Cancer* **2008**, *44*, 30–38.
(6) Fisher, B.; Costantino, J. P.; Wickerham, D. L.; Redmond, C. K.; Kavanah, M.; Cronin, W. M.; Vogel, V.; Robidoux, A.; Dimitrov, N.; Atkins, J.; Daly, M.; Wieand, S.; Tan-Chiu, E.; Ford, L.; Wolmark, N. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J. Natl. Cancer Inst.* **1998**, *90*, 1371–1388.
(7) Harper, M. J.; Walpole, A. L. Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. *Nature* **1966**, *212*, 87.
(8) Harper, M. J.; Walpole, A. L. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. *J. Reprod. Fertil.* **1967**, *13*, 101–119.
(9) Robson, J. M.; Schonberg, A. Estrous reactions, including mating, produced by triphenyl ethylene. *Nature* **1937**, *140*, 196.
(10) Jordan, V. C.; Collins, M. M.; Rowsby, L.; Prestwich, G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J. Endocrinol.* **1977**, *75*, 305–316.
(11) Johnson, M. D.; Zuo, H.; Lee, K. H.; Trebley, J. P.; Rae, J. M.; Weatherman, R. V.; Desta, Z.; Flockhart, D. A.; Skaar, T. C. Pharmacological characterization of 4-hydroxy-*N*-desmethyl tamoxifen, a novel active metabolite of tamoxifen. *Breast Cancer Res. Treat.* **2004**, *85*, 151–159.
(12) Brzozowski, A. M.; Pike, A. C.; Dauter, Z.; Hubbard, R. E.; Bonn, T.; Engstrom, O.; Ohman, L.; Greene, G. L.; Gustafsson, J. A.; Carlquist, M. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **1997**, *389*, 753–758.
(13) Shiau, A. K.; Barstad, D.; Loria, P. M.; Cheng, L.; Kushner, P. J.; Agard, D. A.; Greene, G. L. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **1998**, *95*, 927–937.
(14) Levenson, A. S.; Tonetti, D. A.; Jordan, V. C. The oestrogen-like effect of 4-hydroxytamoxifen on induction of transforming growth factor alpha mRNA in MDA-MB-231 breast cancer cells stably expressing the oestrogen receptor. *Br. J. Cancer* **1998**, *77*, 1812–1819.
(15) Levenson, A. S.; Catherino, W. H.; Jordan, V. C. Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. *J. Steroid Biochem. Mol. Biol.* **1997**, *60*, 261–268.
(16) Levenson, A. S.; Jordan, V. C. The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. *Cancer Res.* **1998**, *58*, 1872–1875.
(17) MacGregor Schafer, J.; Liu, H.; Bentrem, D. J.; Zapf, J. W.; Jordan, V. C. Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. *Cancer Res.* **2000**, *60*, 5097–5105.
(18) Jordan, V. C.; Schafer, J. M.; Levenson, A. S.; Liu, H.; Pease, K. M.; Simons, L. A.; Zapf, J. W. Molecular classification of estrogens. *Cancer Res.* **2001**, *61*, 6619–6623.
(19) Gust, R.; Keilitz, R.; Schmidt, K. Investigations of new lead structures for the design of selective estrogen receptor modulators. *J. Med. Chem.* **2001**, *44*, 1963–1970.
(20) Jordan, V. C.; Koch, R.; Langan, S.; McCague, R. Ligand interaction at the estrogen receptor to program antiestrogen action: a study with nonsteroidal compounds in vitro. *Endocrinology* **1988**, *122*, 1449–1454.
(21) Murphy, C. S.; Langan-Fahey, S. M.; McCague, R.; Jordan, V. C. Structure−function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. *Mol. Pharmacol.* **1990**, *38*, 737–743.
(22) Bentrem, D.; Fox, J. E.; Pearce, S. T.; Liu, H.; Pappas, S.; Kupfer, D.; Zapf, J. W.; Jordan, V. C. Distinct molecular conformations of the estrogen receptor alpha complex exploited by environmental estrogens. *Cancer Res.* **2003**, *63*, 7490–7496.
(23) Pink, J. J.; Bilimoria, M. M.; Assikis, J.; Jordan, V. C. Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. *Br. J. Cancer* **1996**, *74*, 1227–1236.
(24) Levenson, A. S.; MacGregor Schafer, J. I.; Bentrem, D. J.; Pease, K. M.; Jordan, V. C. Control of the estrogen-like actions of the tamoxifen−estrogen receptor complex by the surface amino acid at position 351. *J. Steroid Biochem. Mol. Biol.* **2001**, *76*, 61–70.
(25) Lubczyk, V.; Bachmann, H.; Gust, R. Investigations on estrogen receptor binding. The estrogenic, antiestrogenic, and cytotoxic properties of C2-alkyl-substituted 1,1-bis(4-hydroxyphenyl)-2-phenylethenes. *J. Med. Chem.* **2002**, *45*, 5358–5364.
(26) Lubczyk, V.; Bachmann, H.; Gust, R. Antiestrogenically active 1,1,2-tris(4-hydroxyphenyl)alkenes without basic side chain: synthesis and biological activity. *J. Med. Chem.* **2003**, *46*, 1484–1491.
(27) Ariazi, E. A.; Kraus, R. J.; Farrell, M. L.; Jordan, V. C.; Mertz, J. E. Estrogen-related receptor alpha1 transcriptional activities are regulated in part via the ErbB2/HER2 signaling pathway. *Mol. Cancer Res.* **2007**, *5*, 71–85.
(28) Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. The Protein Data Bank. *Nucleic Acids Res.* **2000**, *28*, 235–242.
(29) Sherman, W.; Day, T.; Jacobson, M. P.; Friesner, R. A.; Farid, R. Novel procedure for modeling ligand/receptor induced fit effects. *J. Med. Chem.* **2006**, *49*, 534–553.

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Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells

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Abstract

Background: X-box binding protein 1 (XBP1), a transcription factor involved in unfolded protein response, is also an estrogen-regulated gene and strongly correlates with estrogen receptor alpha (ERα) expression in breast cancers. We investigated the functional role of XBP1 in estrogen responsive breast and endometrial cancer cells as its functions are not fully understood.
Materials and methods: ERα positive breast (MCF7) and endometrial (ECC1) cancer cells were used to study XBP1 gene regulation by 17-β-estradiol (E2) and to investigate the role of XBP1 in E2-mediated growth using short interfering RNA. Quantitative real-time PCR and Western blot were used to assess RNA and protein levels. Recruitment of ERα and other cofactors at the promoter and enhancer region of the XBP1 gene was investigated by chromatin immunoprecipitation. Estrogen responsive element (ERE)-mediated transcriptional activity was evaluated by a luciferase reporter assay.
Results: E2 induced the transcription of XBP1 in both MCF7 and ECC1 cells. E2-dependent recruitment of ERα, steroid receptor coactivator (SRC)-1 and SRC-3, and RNA polymerase II were observed at the promoter and/or enhancer region of the XBP1 gene. Depletion of XBP1 markedly inhibited the E2-induced growth in MCF7 and ECC1 cells. However, ERE-mediated transcription was not altered in XBP1-overexpressing or XBP1-depleted MCF7 cells.
Conclusion: Our results confirm E2-induced transcription of XBP1 and demonstrate the crucial role of XBP1 in E2-induced growth of ERα positive breast and endometrial cancer cells without modulating the classical ERE-mediated transcription by ER. This knowledge creates new opportunities for therapeutic interventions.

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Introduction

Estrogen is the principal growth mediator of the estrogen receptor (ER) positive breast and endometrial cancers (1). Estrogen acts by binding to ERα or β and the resulting complex can activate transcription of estrogen responsive genes. Examples of estrogen responsive genes include the transcription factors which crucially regulate estrogen-dependent growth. X-box binding protein 1 (XBP1) is a transcription factor, identified as basic region leucine zipper belonging to the ATF/CREB family, strongly coexpressed in ERα positive luminal epithelial breast cancers (2, 3). Several DNA microarray studies have also found XBP1 as an estrogen-regulated gene in ER positive breast cancer cell lines as well as in breast cancers (4–9). In addition, recruitment of ERα on the XBP1 promoter as well as enhancer regions has been confirmed using chromatin immunoprecipitation (ChIP) followed by tiled microarray on human chromosomes 21 and 22 (10).

XBP1 is an important component of unfolded protein response (UPR) where it activates a distinct set of genes and regulates endoplasmic reticulum stress-mediated apoptosis (11). Studies have found that XBP1 is essential for survival of mouse embryonic fibroblasts and is also required for tumor growth of human fibrosarcoma cells under hypoxic conditions, as XBP1-deficient cells show impaired survival (12). Consistent with these findings, XBP1 knockout mice are found to be embryonic lethal as embryonic livers at 13.5 day from $XBP^{-/-}$ mouse exhibited increased apoptosis compared with wild type embryos (13). Further studies show that embryonic lethality of the $XBP^{-/-}$ can be rescued by selectively expressing XBP1 in the hepatocytes. However, these animals died in early postnatal period with pancreatic insufficiency (14). Likewise, XBP1 is essential for UPR and differentiation of plasma cells (15). In multiple myeloma, a plasma cell malignancy, XBP1 deficiency can induce apoptosis in response to endoplasmic reticulum stress (16). A recent study observed apoptosis in XBP1-depleted intestinal epithelial cells from mouse and a concurrent increase in their susceptibility to developing inflammatory bowel disease (17).

Interestingly, XBP1 is reported (18) to interact with ERα in a ligand-independent manner and can also induce transcription from estrogen responsive element (ERE) containing luciferase reporter gene even in the absence of estrogen. Fur-

ther studies found large-scale chromatin unfolding associated with XBP1-mediated increase in ERα transcriptional activity (19). Although these findings strongly suggest an interaction of XBP1 with ERα and its involvement in the ERα-mediated transcriptional process, the precise underlying mechanisms are unknown in ER positive breast and endometrial cancers.

Overexpression of XBP1 in ER positive breast cancer cells not only induces estrogen-independent growth of ER positive breast cancer cells but also confers resistance to the anti-estrogen tamoxifen (20). However, no data are available to explain the relevance of endogenous level of XBP1 and also how estrogen mediated upregulation of XBP1 could have a functional role in estrogen-induced growth of ERα positive breast and endometrial cancer cells.

We report the estrogen regulation of endogenous XBP1 and show that coactivators steroid receptor coactivator (SRC)-1 and SRC-3 along with ERα are recruited at the promoter and/or enhancer elements of the XBP1 gene. By depleting XBP1 levels using siRNA, we also show that XBP1 is required to mediate the estrogen-induced growth of MCF7 breast and ECC1 endometrial cancer cells.

Materials and methods

Cell culture and reagents

Cell culture media were purchased from Invitrogen Inc. (Grand Island, NY, USA) and fetal calf serum (FCS) was obtained from HyClone Laboratories (Logan, UT, USA). The ER positive breast cancer cells MCF7:WS8 used in this study were derived from MCF7 cells obtained from the American Type Culture Collection as reported previously (21). The ER positive endometrial cancer cells ECC1 cells were originally from Dr. Myles Brown's lab. MCF7 cells were routinely maintained in RPMI media and ECC1 cells were maintained in Dulbecco's Modified Eagle Medium media supplemented with 10% FCS, 6 ng/mL bovine insulin and penicillin and streptomycin. Three to four days prior to harvesting, cells were switched to phenol red-free media containing 10% charcoal dextran treated FCS. Media was changed every other day. The cells were treated with indicated reagents for the specified time and were subsequently harvested for total RNA isolation or protein lysate. Cycloheximide (CHX) and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were purchased from Sigma chemicals (St. Louis, MO, USA) and fulvestrant (FUL) was from Tocris Pharmaceuticals (Ellisville, MI, USA). Cells were pretreated for 30 min with CHX, DRB or FUL before treating with 17β-estradiol (E2) or vehicle for specified times as mentioned in Figure legends. All experiments were repeated at least three times, in triplicate to confirm the results.

Total RNA isolation and real-time polymerase chain reaction (PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and an RNeasy kit (Qiagen Inc.) according to the manufacturer's instructions. Real-time PCR was performed by reverse transcribing 1 μg of total RNA in a total volume of 20 μL using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) as per manufacturer's instructions and subsequently diluted to 200 μL with sterile water. The real-time PCR was performed in a 20 μL reaction which included 1× SYBR green PCR master mix (Applied Biosystems), 100 nM each of forward and reverse primers and 2 μL of diluted cDNA using an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) for 40 cycles (95°C for 15 s, 60°C for 1 min) following an initial 10 min incubation at 95°C. The fold change in expression of transcripts was calculated using the ΔΔCt method, with the ribosomal protein 36B4 mRNA as the internal control (22). The primer sequences used were the same as previously reported for XBP1 (10) and 36B4 (23).

Growth assay

Cells were grown in 10 cm plates in charcoal-stripped media for 4 days before plating for the growth assay. In total, 12,000 cells were plated in each well of 24-well plates and allowed to attach for at least 16 h before treating them with vehicle or 1 nM E2. One untreated plate was collected at the start day of treatment and this served as the baseline for the comparison of the growth of cells. Cells were then collected on the second, fourth and sixth days of treatment and frozen in –30°C. The growth was assessed by measuring the DNA content in each well using a fluorescent DNA quantitation kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Calf thymus DNA was used to plot the standard curve for the DNA assay with each set of quantitation. The experiments were repeated three times in quadruplicates to confirm the data.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed as described by Shang et al. (24) with minor modifications. Cells were grown in 15 cm plates in phenol red-free RPMI media containing 10% charcoal stripped fetal bovine serum for 3 days before treating with vehicle or 1 nM estradiol for 45 min. Cells were then washed with phosphate buffered saline (PBS) followed by crosslinking with 1% formaldehyde at room temperature for 15 min and then stopped it using 125 mM glycine. Cells were then rinsed with PBS and collected in PBS containing protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA) and 10 mM dithiothreitol (DTT), followed by centrifuging at 2000×*g* for 5 min at 4°C. Subsequently, cells were resuspended in nuclei isolation buffer (50 mM Tris Cl, 60 mM KCl, 0.5% NP40, protease inhibitors and 10 mM DTT) followed by centrifugation to isolate the nuclei. Isolated nuclei were resuspended in sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris Cl, 1% SDS, 10 mM EDTA, pH 8.1 with protease inhibitors) and sonicated (Microson ultrasonic dismembrator) three times at setting '10' followed by centrifugation at 14,000×*g* for 20 min at 4°C. Fixed chromatin supernatant was diluted using ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl with 1× protease inhibitors) followed by immunoclearing using normal rabbit serum and 60 μL of protein A agarose (Upstate Cell Signaling Solutions, Temecula, CA, USA). Immunoprecipitation was performed overnight with antibodies against ERα, SRC-1, SRC-3 and phospho-2-serine-RNA polymerase II (p-RNA polII). The immunocomplexes were precipitated using 60 μL of protein A agarose and incubated for an additional 2 h followed by centrifugation at 700×*g* for 5 min. The beads bound to immunocomplexes were sequentially washed 10 min each using buffer I (20 mM Tris Cl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100 and 150 mM NaCl), buffer II (20 mM Tris Cl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100 and 250 mM NaCl), buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Precipitates were then washed twice with TE buffer and extracted twice with freshly made 1% SDS and 0.1 M $NaHCO_3$. Pooled elutes were decrosslinked using 200 μM of NaCl and heated at 65°C overnight.

The DNA fragments were purified using a Qiaquick PCR purification kit (Qiagen Inc.). Then, 1–2 μL of eluted DNA was used for real-time PCR analysis. The primer sequences used are as follows: XBP1 promoter: 5′TCTGGAAAGCTCTCGGTTTG3′ (forward); 5′AATCCCTGGCCAAAGGTACT3′ (reverse); XBP1 enhancer: 5′ATACTTGGCAGCCTGTGACC3′ (forward); 5′GTGCCACAAAGCAGGAAAAA3′ (reverse). The data are expressed as percent input of 1/20th part of starting chromatin material and are representative of three separate experiments with similar results.

Western immunoblotting

MCF7 and ECC1 cells were grown in phenol red-free media containing charcoal stripped serum. After treatments with the compounds for the indicated time periods, cells were rinsed with cold PBS and then lysed by RIPA buffer (Sigma Chemicals, St. Louis, MO, USA) supplemented with protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA) and phosphatase inhibitor cocktails I and II (Calbiochem, La Jolla, CA, USA). Cell lysates were collected, sonicated (3× for 10 s, on ice) and centrifuged at 14,000 rpm for 20 min at 4°C. Cell supernatants were aliquoted and stored at −80°C. Protein concentration was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins (20–40 μg) were separated by electrophoresis using 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) and transferred onto PVDF transfer membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Primary antibodies used for Western blotting were raised against XBP1 (Santa Cruz, CA, USA) and β-actin (Sigma-Aldrich Corp., St. Louis, MO, USA). The bands were visualized using an ECL Western blotting detection system (GE Healthcare Bio-Sciences Corp) as per manufacturer's instructions. The experiments were repeated at least three times with similar results.

Short interfering RNA (siRNA) experiments

For siRNA experiments, MCF7 or ECC1 cells were grown in 10 cm plates, in antibiotic-free media. Cells were transfected with 100 nM XBP1 siRNA (on target plus, cat #009552; Dharmacon, Inc.) or control siRNA (on target plus, non-targeting pool, cat #D-001810; Dharmacon, Inc.) using 20 μL of Dharmafect transfection reagent as per manufacturer's instruction, for 48 h. Cells were allowed to recover in complete medium (without antibiotics) for 16 h, followed by reseeding in 24-well plates for growth assay or in 6-well plates for RNA and protein isolation. Cells were then treated with vehicle or E2 for indicated times. RNA and protein was extracted by methods as mentioned earlier. The experiments were repeated at least three times, in triplicate to confirm the results.

Luciferase reporter assays

Plasmid pERE(5X)TA-ffLuc (25) containing five tandem copies of the consensus palindromic ERE and firefly luciferase was transfected to assess the ERE-mediated transcriptional activity in the MCF7 cells. Plasmid pTA-srluc (25) expressing *renilla* luciferase reporter gene was cotransfected as an internal control. Then, 300 ng of pERE(5X)TA-ffLuc and 50 ng of pTA-srluc was cotransfected in each well containing 10^5 cells treated with control or XBP1 siRNA. Cells were further treated with vehicle or E2 for 48 h before harvesting for the assay.

In another set of experiments, cells were cotransfected with either 20 ng or 500 ng of XBP1 expressing plasmid along with 300 ng of pERE(5X)TA-ffLuc and 50 ng of pTA-srluc. The entire assay was performed using dual luciferase assay kits (Promega). All data are represented in terms of ratio of firefly/renilla RLU values. The experiments were repeated three times, in quadruplicates to confirm the results.

Statistics

Statistical significance of our data were assessed using the Student t-test. A p-value <0.05 was considered as statistically significant.

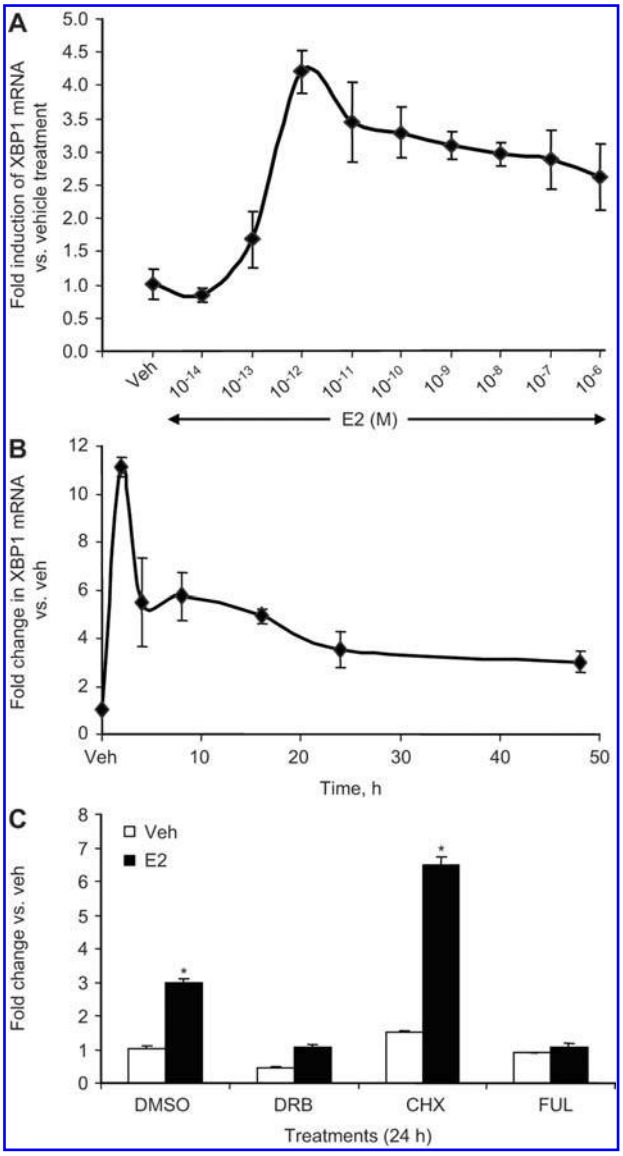

Figure 1 E2-mediated upregulation of XBP1 in MCF7 cells. MCF7 cells were treated with different concentrations of E2 for 8 h and expression of XBP1 was measured using quantitative real-time PCR and compared with vehicle-treated cells (A). MCF7 cells treated with E2 (1 nM) for 2, 4, 8, 16, 24 or 48 h and expression of XBP1 was measured using quantitative real-time PCR and compared with vehicle-treated cells (B). MCF7 cells were treated with CHX (10 μg/mL), DRB (75 μM) or FUL (1 μM) in absence or presence of E2 (1 nM) for 24 h and expression of XBP1 was assessed using real-time PCR (C). *$p<0.05$ compared with respective vehicle-treated group.

Results

Estrogen upregulates XBP1 in MCF7 and ECC1 cells and is a primary responsive gene

We first studied the dose-response of E2 on XBP1 mRNA regulation in MCF7 breast cancer cells after 8 h of E2 treatment using quantitative real-time PCR. Our data show that XBP1 mRNA was induced by E2 in a dose-dependent manner (Figure 1A). Low dose of E2 (10^{-14} M) was not able to induce any upregulation of XBP1 levels, whereas 10^{-12} M of E2 achieved the peak induction. Higher doses of E2 treatment induced XBP1 levels similar to 10^{-12} M of E2. We then studied the regulation of the XBP1 at various time points after 1 nM (10^{-9} M) E2 treatment and found that it was upregulated as early as 2 h after estrogen treatment and maintained an elevated level even after 48 h of estrogen treatment in breast cancer (MCF7) as well as in endometrial cancer (ECC1) cells (Figures 1B and 2A). This upregulation was completely abrogated in the presence of FUL, a complete anti-estrogen, indicating an ERα-mediated mechanism (Figures 1C and 2B). Pretreatment with CHX, an inhibitor of protein synthesis, in the presence of E2 did not alter the E2-mediated upregulation suggesting that de novo protein synthesis is not required for the estrogen-mediated upregulation of XBP1 (Figures 1C and 2B). Conversely, pretreatment with DRB, a transcriptional inhibitor, completely blocked the upregulation of XBP1 demonstrating involvement of transcriptional machinery in upregulation of XBP1 by estrogen (Figures 1C and 2B).

We also studied the regulation of XBP1 in ER negative breast cancer cells SKBR3 and MDA MB 231 cells. As expected, XBP1 was not regulated by E2 in these cells (Figure 2C). Furthermore, we investigated the levels of XBP1 in long-term estrogen-deprived MCF7 cells, known as MCF7:5C cells (26) which are estrogen-deprived resistant cells. Paradoxically, low levels of E2 induce apoptosis in these cells (27). Basal levels of XBP1 mRNA were found to be around 23-fold higher in MCF7:5C cells compared with MCF7 cells (Figure 2D). Interestingly, E2 treatment for 48 h

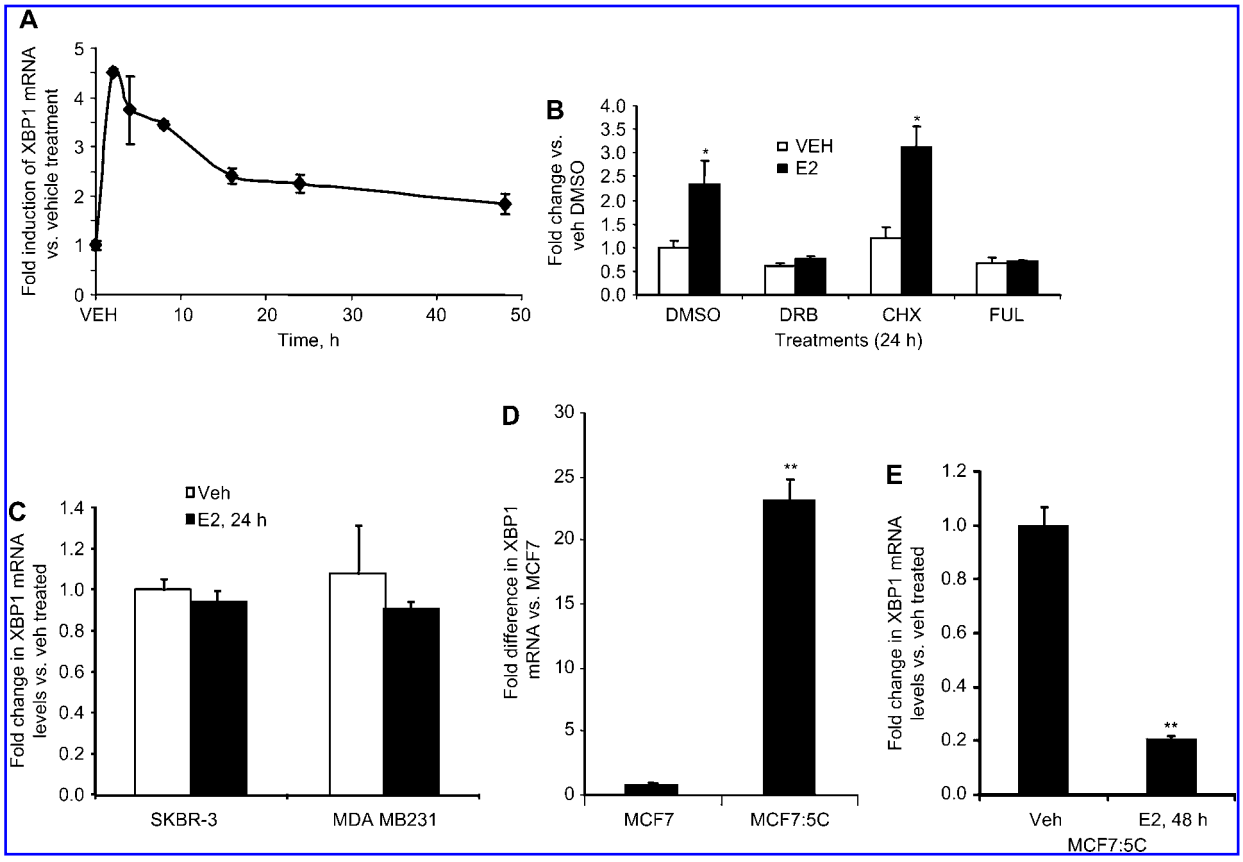

Figure 2 E2-mediated upregulation of XBP1 in ECC1 cells. ECC1 cells treated with E2 (1 nM) for 2, 4, 8, 16, 24 or 48 h and expression of XBP1 was measured using quantitative real-time PCR and compared with vehicle-treated cells (A). ECC1 cells were treated with CHX (10 μg/mL), DRB (75 μM) or FUL (1 μM) in absence or presence of E2 (1 nM) for 24 h and expression of XBP1 was assessed using real-time PCR (B). SKBR-3 and MDA-MB-231 cells were treated with E2 (1 nM) or vehicle (0.1% ethanol) for 24 h and expression of XBP1 was measured using quantitative real-time PCR and compared with vehicle-treated cells (C). Total RNA from MCF7 and MCF:5C was isolated and expression of XBP1 was measured using quantitative real-time PCR relative to MCF7 cells (D). MCF7:5C cells were treated with vehicle (0.1% ethanol) or E2 (1 nM) for 48 h and expression of XBP1 was measured using quantitative real-time PCR and compared with vehicle-treated cells (E). *$p < 0.05$ compared with vehicle-treated group (B). **$p < 0.05$ compared with MCF7 cells (D) or vehicle-treated group (E).

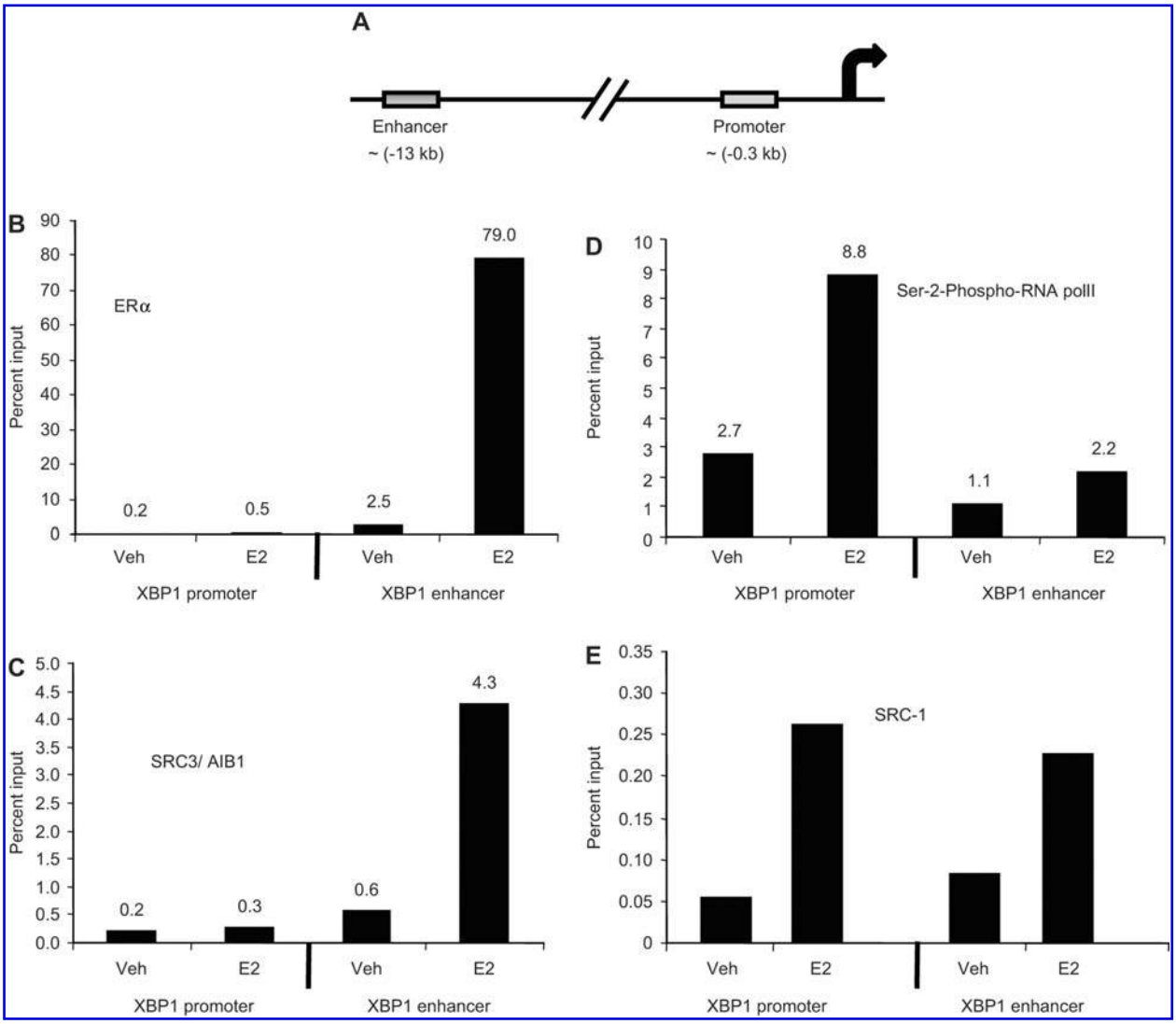

Figure 3 Recruitment of ERα, phospho-serine-2-RNA polII, SRC-1 and SRC-3, at the proximal promoter and distal enhancer region of the XBP1 gene assessed by chromatin immunoprecipitation (ChIP) assay. MCF7 cells were treated with vehicle or E2 (1 nM) for 45 min and ChIP assay was performed as mentioned in the materials and methods section. Schematic representation of the promoter and enhancer regions of the XBP1 gene (A). The extent of recruitment of the factors indicated is shown for promoter and enhancer region of the XBP1 gene. The data are expressed as percent input of 1/20th part of starting chromatin material in each case after subtracting non-specific binding. The data shown are representative of three separate experiments with similar results.

drastically downregulated the XBP1 levels in MCF7:5C cells (Figure 2E), which coincides with estrogen-induced apoptosis in these cells.

Recruitment of ERα and other factors at the promoter and enhancer regions of the XBP1 gene

To further confirm the direct involvement of ERα in transcriptional induction of the XBP1 gene, we performed ChIP assay to assess the recruitment of ERα, SRC-1, SRC-3 and serine-2-phosphorylated RNA polymerase II at ~0.3 kb (promoter) and ~13 kb (enhancer) upstream of the transcription start site of the XBP1 gene (Figure 3A) in the MCF7 cells treated with vehicle or 1 nM E2 for 45 min. We found higher occupancy of ERα and SRC-3 at the enhancer region but not at the promoter region. The occupancy of these factors at the enhancer region was further stimulated after 45 min of E2 treatment compared with vehicle treatment (Figure 3B and C). In contrast, serine-2-phosphorylated RNA polymerase II was found to be recruited 4-fold more at the promoter region than at the enhancer region after 45 min of E2 treatment (Figure 3D). Occupancy of SRC-1 was stimulated after E2 treatment in both the promoter and enhancer regions of the XBP1 gene (Figure 3E). These results indicate that the enhancer region of XBP1 is involved in the regulation of estrogen-induced transcriptional stimulation of the XBP1 gene.

XBP1 depletion inhibits estrogen-mediated growth

To investigate the functional importance of XBP1, we evaluated the effect through loss-of-function using a pool of

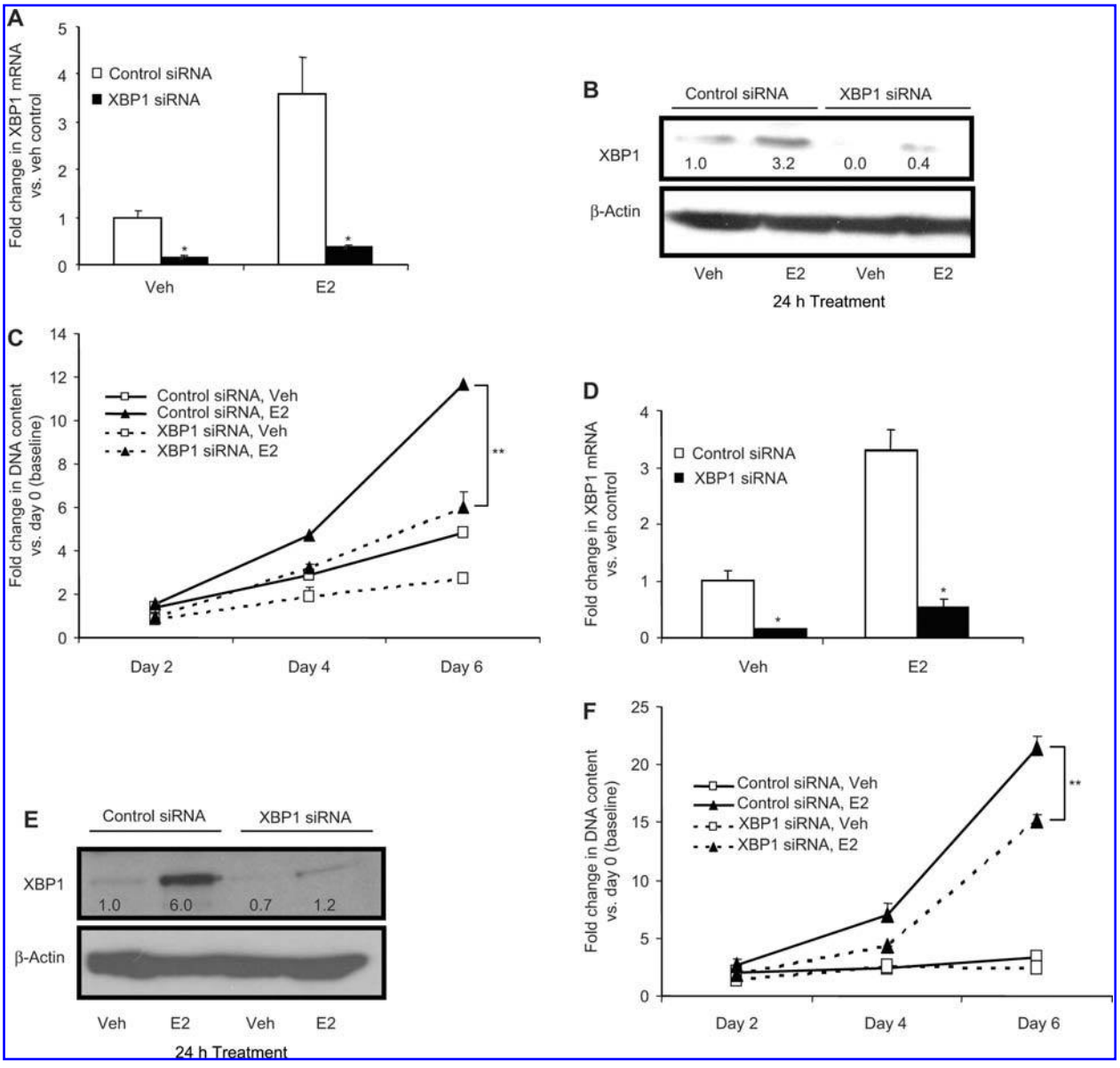

Figure 4 Short interfering RNA (siRNA)-mediated knockdown of XBP1 inhibits growth of MCF7 and ECC1 cells and its effect on estrogen-mediated growth. MCF7 and ECC1 cells, transfected with XBP1 siRNA or control siRNA, were treated with E2 (1 nM) or vehicle for 24 h and the extent of knockdown was assessed using quantitative real-time PCR compared with control siRNA, vehicle-treated cells (A and D) and Western blotting (B and E). Subsequently, cells were reseeded and the growth of the cells was monitored over a 6-day period. Total DNA content was measured as a marker of growth and the fold change in DNA content was calculated compared with the number of cells at the time of the start of the treatment (baseline) (C and F). *$p < 0.05$ compared with control siRNA group and **$p < 0.005$, using the unpaired Student t-test. The Western blots were scanned and quantified. Levels of XBP1 normalized for β-actin, relative to control siRNA-vehicle treated cells, are indicated below each band.

siRNA against XBP1 in MCF7 and ECC1 cells. The extent of XBP1 knockdown was confirmed by real-time PCR and Western blotting (Figure 4A and B; Figure 4D and E, respectively). A growth assay was performed after XBP1 knockdown by siRNA and total DNA content was used as a measure to determine the cell growth over a 6-day period. A parallel identical growth assay was performed using pool of non-targeting control siRNA for comparison. XBP1 knockdown attenuated the E2-induced growth of MCF7 and ECC1 cells by 49% and 30%, respectively, compared with cells treated with control siRNA (Figure 4C and F). These data indicated that the level of XBP1 expression is critical for inducing estradiol-mediated growth of breast and endometrial cancer cells.

We further investigated if the levels of ERα were altered in the XBP1-depleted MCF7 cells compared with control siRNA-treated MCF7 cells. No differences were detected in levels of ERα in XBP1-depleted cells compared with control siRNA-treated cells in presence of vehicle or E2 for 24 h (Figure 5). This rules out the possibility that growth

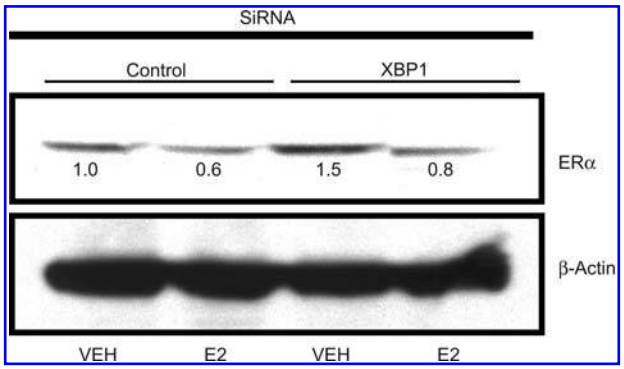

Figure 5 ERα levels in MCF7 cells treated with control or XBP1 siRNA. MCF7 cells were transfected with control or XBP1 siRNA and subsequently treated with vehicle or E2 for 24 h. ERα levels were assessed by Western blotting. Levels of β-actin are shown as loading control. The Western blots were scanned and quantified. Levels of ERα protein normalized for β-actin, relative to control siRNA-vehicle treated cells, are indicated below each band.

inhibition of XBP1-depleted cells was due to altered ERα levels.

XBP1 overexpression or XBP1 depletion does not affect ERE-mediated transcriptional activity

To understand the underlying mechanism by which XBP1 can influence estrogen-mediated growth, we examined the effect of XBP1 overexpression or depletion on the transcriptional activity of ER from a classical ERE. We performed an ERE-luciferase reporter assay in the MCF7 cells transiently transfected with XBP1 expression plasmid or XBP1 siRNA. No differences were observed (Figure 6A and B) in transcriptional activity (as measured by luciferase activity) of the ERE-luciferase reporter in the cells either overexpressing XBP1 or the cells depleted of XBP1 compared with their respective controls. This result suggests that levels of XBP1 in the cell might not affect the classical transcriptional activity of ERα mediated through the direct binding of ERE.

Discussion

Estrogen is the prime growth regulator of ER positive breast and endometrial cancer cells. To better understand the induction of estrogen-mediated growth in breast cancer, some studies (6, 28) have explored the estrogen-induced transcriptional network using DNA microarrays to identify downstream pathways. In these studies, many of the estrogen-regulated genes are identified as transcription factors which could be collectively responsible for the phenotypic manifestations of estrogen-induced growth of ER positive cancer cells. However, in the majority of cases, the precise role of the downstream events in growth is not understood. One solution which can be used to dissect the complexities of clinical tissues is to interrogate estrogen responsive cell lines. One such recent study (29) has noted that the genes are similarly regulated by estrogen in breast

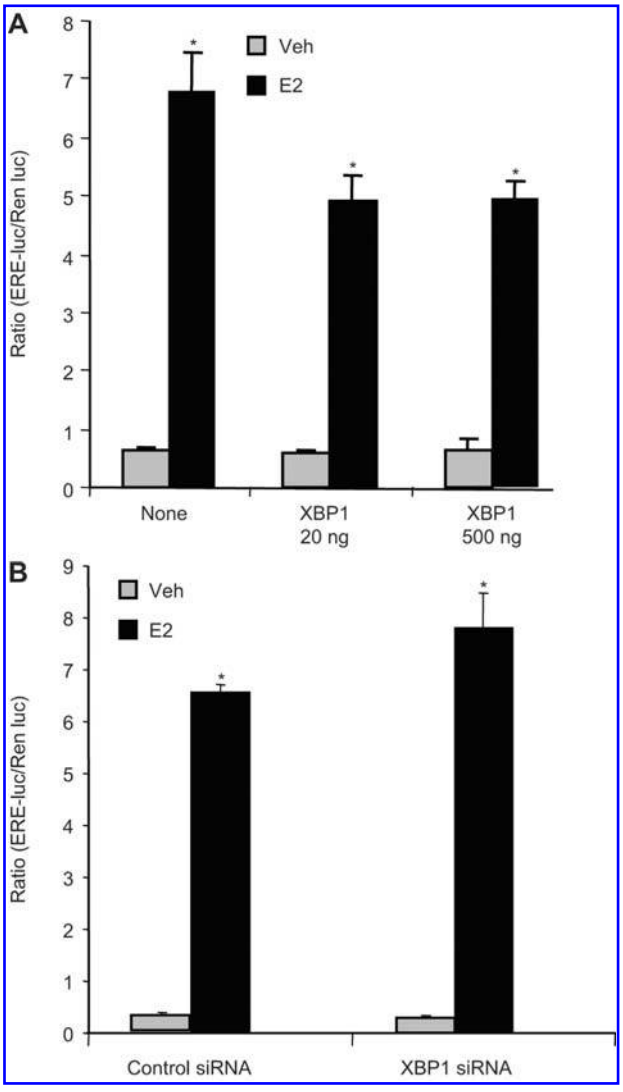

Figure 6 ERE-mediated luciferase activity in XBP1 overexpressing or XBP1 depleted cells. MCF7 cells were transfected with empty vector (none), 20 ng or 500 ng of XBP1-expressing plasmid and ERE-mediated luciferase activity was assessed in absence or presence of 1 nM E2 (A). MCF7 cells transfected with control or XBP1 siRNA were used to assess ERE-mediated luciferase activity in presence or absence of 1 nM E2 (B). Renilla luciferase activity was used as internal control and all values are represented as a ratio of ERE-luciferase and renilla luciferase activity. The values are average of at least four replicates ±SD. *$p < 0.05$ compared with respective vehicle-treated group.

cancer cells in vitro and human breast tumors. This provides a unique opportunity to study the underlying mechanism by manipulating specific genes in the cells, which can influence the progression of ER positive cancers and also provide potential targets for therapeutic intervention. In this context, some recent reports have identified the important roles played by the estrogen-regulated genes such as FOXA1 (10, 30, 31), GREB1 (32) and GATA-3 (30, 33) in regulating estrogen-induced growth in breast cancers.

In the present study, we evaluated the phenotypic effects of the E2-regulated gene XBP1, which has been consistently

shown to be highly coexpressed with ERα in breast cancer patients and is also known to be upregulated by estrogen in the ER positive breast cancer cells in vitro (4–9, 29). Although it is well established that XBP1 plays a key role in UPR and endoplasmic reticulum stress by acting as a transcription factor for the genes involved in UPR, its role in E2-dependent ER positive cancers is not fully understood.

Our results confirm that XBP1 is an E2-regulated gene which is in agreement with previous studies (6, 10). The E2-induction of XBP1 is mediated by ERα and does not need de novo protein synthesis for the upregulation, as CHX did not alter the regulation. Treatment with DRB, an inhibitor of transcription, completely blocked the upregulation of XBP1 by estrogen, indicating transcriptional regulation. The ChIP data further confirmed direct binding of ERα, SRC-1, SRC-3 and serine-2-phosphorylated RNA polymerase II at the promoter and/or enhancer region of the XBP1 gene. Interestingly, recruitment of ERα and SRC-3 (AIB1) was higher at the enhancer region of the XBP1 gene and very minimal at the promoter region. ERα recruitment was induced dramatically at the enhancer region after 45 min of E2 treatment. Recruitment of ERα was also accompanied by SRC-1 and SRC-3 at the enhancer region. However, as expected, recruitment of serine-2-phosphorylated RNA polymerase II was higher at the promoter region than the enhancer region. These data strongly suggest that the enhancer region of the XBP1 gene, which is approximately 13 kb upstream of the transcription start site, is involved in the transcriptional regulation of XBP1 by ER. Indeed, recent studies (34–36) have indicated that distal enhancers of E2-induced genes GREB1 and carbonic anhydrase 12 are involved in the transcriptional regulation by ER. It has been shown that the distal enhancer can interact with the proximal promoter region of these estrogen-regulated genes by intrachromosomal looping.

This study reports for the first time that the XBP1 level is critical for E2-induced growth of ER positive breast and endometrial cancer cells, as evidenced by marked inhibition of E2-induced growth of XBP1-deficient MCF7 and ECC1 cells. This specifically demonstrates that the endogenous level of XBP1 and its upregulation by estrogen is intimately involved in the growth regulation of estrogen responsive breast and endometrial cancer cells. A recent study demonstrated that overexpression of XBP1 in ER positive breast cancer cells can lead to anti-estrogen resistance, by regulating genes associated with apoptosis and cell cycle progression (20).

To further understand the mechanism by which XBP1 can influence E2-induced growth, we hypothesized that levels of XBP1 could affect the ERE-mediated transcriptional activity of ER. To test this we depleted or overexpressed XBP1 and performed an ERE-luciferase reporter assay. Our data show that the level of XBP1 in the MCF7 cells does not affect the transcriptional activity of ER mediated through classical ERE binding. This indicates that XBP1 can influence the growth of the cells by either regulating a subset of genes directly under the control of XBP1 or can also modulate the E2 regulation of the genes which are not exclusively regulated by classical ERE-mediated transcription. These data are, however, in contrast to a previous study (19) where XBP1 overexpression activated ER transcriptional activity in a ligand-independent manner. The differences in the results could be attributed to the exogenous overexpression of ERα in the previous study, whereas in the present study we relied on the intrinsic activity of ER in the MCF7 cells. Further investigations are required to address the associated mechanism of action.

In summary, our results demonstrate that XBP1 expression is estrogen regulated at the transcriptional level and the enhancer region of the XBP1 gene can play a critical role in regulating E2-mediated transcriptional activation. Our findings show that expression level of XBP1 is critical in achieving optimal E2-induced growth in breast and endometrial cancer cells without influencing the classical ERE-mediated transcriptional activity. Our findings also provide an explanation for the strong correlation observed between ERα and XBP1 expression in breast cancer patients. Taken together, we suggest that this novel mechanism for the regulation of cancer cell growth via XBP1 can be exploited as a novel drug target in future studies of anti-hormonal resistance in ER positive cancer cells.

Acknowledgements

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References

1. Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. Clin Cancer Res 2003;9:1980–9.
2. van't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415:530–6.
3. Finlin BS, Gau CL, Murphy GA, Shao H, Kimel T, Seitz RS, Chiu YF, Botstein D, Brown PO, Der CJ, Tamanoi F, Andres DA, Perou CM. RERG is a novel ras-related, estrogen-regulated and growth-inhibitory gene in breast cancer. J Biol Chem 2001; 276:42259–67.
4. Wang DY, Fulthorpe R, Liss SN, Edwards EA. Identification of estrogen-responsive genes by complementary deoxyribonucleic acid microarray and characterization of a novel early estrogen-induced gene: EEIG1. Mol Endocrinol 2004;18:402–11.
5. Tozlu S, Girault I, Vacher S, Vendrell J, Andrieu C, Spyratos F, Cohen P, Lidereau R, Bieche I. Identification of novel genes that co-cluster with estrogen receptor alpha in breast tumor biopsy specimens, using a large-scale real-time reverse transcription-PCR approach. Endocr Relat Cancer 2006;13:1109–20.
6. Cunliffe HE, Ringner M, Bilke S, Walker RL, Cheung JM, Chen Y, Meltzer PS. The gene expression response of breast cancer to growth regulators: patterns and correlation with tumor expression profiles. Cancer Res 2003;63:7158–66.

7. Bertucci F, Nasser V, Granjeaud S, Eisinger F, Adelaide J, Tagett R, Loriod B, Giaconia A, Benziane A, Devilard E, Jacquemier J, Viens P, Nguyen C, Birnbaum D, Houlgatte R. Gene expression profiles of poor-prognosis primary breast cancer correlate with survival. Hum Mol Genet 2002;11:863–72.
8. Bertucci F, Houlgatte R, Benziane A, Granjeaud S, Adelaide J, Tagett R, Loriod B, Jacquemier J, Viens P, Jordan B, Birnbaum D, Nguyen C. Gene expression profiling of primary breast carcinomas using arrays of candidate genes. Hum Mol Genet 2000;9:2981–91.
9. Fujimoto T, Onda M, Nagai H, Nagahata T, Ogawa K, Emi M. Upregulation and overexpression of human X-box binding protein 1 (hXBP-1) gene in primary breast cancers. Breast Cancer 2003;10:301–6.
10. Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoute J, Shao W, Hestermann EV, Geistlinger TR, Fox EA, Silver PA, Brown M. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. Cell 2005;122:33–43.
11. Koong AC, Chauhan V, Romero-Ramirez L. Targeting XBP-1 as a novel anti-cancer strategy. Cancer Biol Ther 2006;5: 756–9.
12. Romero-Ramirez L, Cao H, Nelson D, Hammond E, Lee AH, Yoshida H, Mori K, Glimcher LH, Denko NC, Giaccia AJ, Le QT, Koong AC. XBP1 is essential for survival under hypoxic conditions and is required for tumor growth. Cancer Res 2004;64:5943–7.
13. Reimold AM, Etkin A, Clauss I, Perkins A, Friend DS, Zhang J, Horton HF, Scott A, Orkin SH, Byrne MC, Grusby MJ, Glimcher LH. An essential role in liver development for transcription factor XBP-1. Genes Dev 2000;14:152–7.
14. Lee AH, Chu GC, Iwakoshi NN, Glimcher LH. XBP-1 is required for biogenesis of cellular secretory machinery of exocrine glands. EMBO J 2005;24:4368–80.
15. Iwakoshi NN, Lee AH, Glimcher LH. The X-box binding protein-1 transcription factor is required for plasma cell differentiation and the unfolded protein response. Immunol Rev 2003;194:29–38.
16. Lee AH, Iwakoshi NN, Anderson KC, Glimcher LH. Proteasome inhibitors disrupt the unfolded protein response in myeloma cells. Proc Natl Acad Sci USA 2003;100:9946–51.
17. Kaser A, Lee AH, Franke A, Glickman JN, Zeissig S, Tilg H, Nieuwenhuis EE, Higgins DE, Schreiber S, Glimcher LH, Blumberg RS. XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. Cell 2008;134:743–56.
18. Ding L, Yan J, Zhu J, Zhong H, Lu Q, Wang Z, Huang C, Ye Q. Ligand-independent activation of estrogen receptor alpha by XBP-1. Nucleic Acids Res 2003;31:5266–74.
19. Fang Y, Yan J, Ding L, Liu Y, Zhu J, Huang C, Zhao H, Lu Q, Zhang X, Yang X, Ye Q. XBP-1 increases ERα transcriptional activity through regulation of large-scale chromatin unfolding. Biochem Biophys Res Commun 2004;323:269–74.
20. Gomez BP, Riggins RB, Shajahan AN, Klimach U, Wang A, Crawford AC, Zhu Y, Zwart A, Wang M, Clarke R. Human X-box binding protein-1 confers both estrogen independence and antiestrogen resistance in breast cancer cell lines. FASEB J 2007;21:4013–27.
21. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. Cancer Res 1996;56:2321–30.
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–8.
23. Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 2003;144:4562–74.
24. Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 2000;103:843–52.
25. Ariazi EA, Kraus RJ, Farrell ML, Jordan VC, Mertz JE. Estrogen-related receptor α1 transcriptional activities are regulated in part via the ErbB2/HER2 signaling pathway. Mol Cancer Res 2007;5:71–85.
26. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 2005;94:131–41.
27. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, Jordan VC. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 2005;97:1746–59.
28. DeNardo DG, Kim HT, Hilsenbeck S, Cuba V, Tsimelzon A, Brown PH. Global gene expression analysis of estrogen receptor transcription factor cross talk in breast cancer: identification of estrogen-induced/activator protein-1-dependent genes. Mol Endocrinol 2005;19:362–78.
29. Creighton CJ, Cordero KE, Larios JM, Miller RS, Johnson MD, Chinnaiyan AM, Lippman ME, Rae JM. Genes regulated by estrogen in breast tumor cells in vitro are similarly regulated in vivo in tumor xenografts and human breast tumors. Genome Biol 2006;7:R28.
30. Lacroix M, Leclercq G. About GATA3, HNF3A, and XBP1, three genes co-expressed with the oestrogen receptor-alpha gene (ESR1) in breast cancer. Mol Cell Endocrinol 2004;219:1–7.
31. Laganiere J, Deblois G, Lefebvre C, Bataille AR, Robert F, Giguere V. From the cover: location analysis of estrogen receptor alpha target promoters reveals that FOXA1 defines a domain of the estrogen response. Proc Natl Acad Sci USA 2005; 102:11651–6.
32. Rae JM, Johnson MD, Scheys JO, Cordero KE, Larios JM, Lippman ME. GREB 1 is a critical regulator of hormone dependent breast cancer growth. Breast Cancer Res Treat 2005;92:141–9.
33. Eeckhoute J, Keeton EK, Lupien M, Krum SA, Carroll JS, Brown M. Positive cross-regulatory loop ties GATA-3 to estrogen receptor alpha expression in breast cancer. Cancer Res 2007;67:6477–83.
34. Barnett DH, Sheng S, Charn TH, Waheed A, Sly WS, Lin CY, Liu ET, Katzenellenbogen BS. Estrogen receptor regulation of carbonic anhydrase XII through a distal enhancer in breast cancer. Cancer Res 2008;68:3505–15.
35. Deschenes J, Bourdeau V, White JH, Mader S. Regulation of GREB1 transcription by estrogen receptor alpha through a multipartite enhancer spread over 20 kb of upstream flanking sequences. J Biol Chem 2007;282:17335–9.
36. Sun J, Nawaz Z, Slingerland JM. Long-range activation of GREB1 by estrogen receptor via three distal consensus estrogen-responsive elements in breast cancer cells. Mol Endocrinol 2007;21:2651–62.

Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: A unifying concept in anti-hormone resistance

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Abstract. We have previously demonstrated that prolonged treatments with raloxifene (RAL) *in vitro* will result in phase II RAL resistance and RAL-induced tumor growth. Clinical interest prompted us to re-examine RAL resistance *in vivo*, particularly the effects of long-term treatments (a decade or more) on the evolution of RAL resistance. In this study, we have addressed the question of this being a reproducible phenomenon in wild-type estrogen receptor (ER)-positive human breast cell line MCF-7. MCF-7 cells cultured under estrogen-deprived conditions in the presence of 1 μM RAL for more than a year develop RAL resistance resulting in an independent cell line, MCF7-RAL. The MCF7-RAL cells grow in response to both estradiol E_2 and RAL. Fulvestrant (FUL) blocks RAL and E_2-mediated growth. Transplantation of MCF7-RAL cells into athymic ovariectomized mice and treatment with physiologic doses of E_2 causes early E_2-stimulated tumor growth. In contrast, continuous treatment of implanted animals with daily oral RAL (1.5 mg daily) causes growth of small tumors within 15 weeks. Continuous re-transplantation of the tumors growing in RAL-treated mice indicated that RAL stimulated tumor growth. Tumors in the untreated mice did not grow. Bi-transplantation of MCF7-E_2 and MCF7-RAL tumors into the opposing mammary fat pads of the same ovariectomized animal demonstrated that MCF7-E_2 grew with E_2 stimulation and not with RAL. Conversely, MCF7-RAL tumors grew with RAL and not E_2, a characteristic of phase II resistance. Established phase II resistance of MCF7-RAL tumors was confirmed following up to 7 years of serial transplantation in RAL-treated athymic mice. The ERα was retained in these tumors. The cyclical nature of RAL resistance was confirmed and extended during a 2-year evolution of the resistant phases of the MCF7-RAL tumors. The MCF7-RAL tumors that initially were inhibited by E_2 grew in the presence of E_2 and subsequently grew with either RAL or E_2. RAL remained the major grow stimulus and RAL enhanced E_2-stimulated growth. Subsequent transplantation of E_2 stimulated tumors and evaluations of the actions of RAL, demonstrated robust E_2-stimulated growth that was blocked by RAL. These are the characteristics of the anti-estrogenic actions of RAL on E_2-stimulated breast cancer growth with a minor component of phase I RAL resistance. Continuous transplantation of the phase I RAL-stimulated tumors for >8 months causes reversion to phase II resistance. These data and literature reports of the cyclical nature of anti-androgen/androgen responsiveness of prostate cancer growth, illustrate the generality of the evolution of anti-hormonal resistance in sex steroid-sensitive target tissues.

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Introduction

Selective estrogen receptor (ER) modulators (SERMs) are compounds that bind to the ER and based on tissue specificity, act as agonists or antagonists (1). Tamoxifen (TAM), the first SERM, is a proven agent for treatment of breast cancer (2) and breast cancer chemoprevention (3,4). Laboratory studies during the 1980s demonstrated that long-term tamoxifen treatment stimulated the growth of ER-positive MCF-7 breast tumors *in vivo* (5,6). This unique form of acquired resistance to a cancer therapy raised clinical concerns

about extending adjuvant tamoxifen therapy. However, the risk of developing endometrial cancer during the use of tamoxifen for chemoprevention of breast cancer (4) prompted the examination of other compounds that would capitalize on the gains in breast cancer prevention made with tamoxifen but with a superior safety profile.

Raloxifene (also known as keoxifene or LY156,758) (7), a second generation SERM, inhibits the growth of 7,12-dimethylbenzanthracene (DMBA)-induced tumors in rats (8), prevents the development and growth of estrogen-dependent N-nitrosomethylurea (NMU)-induced mammary carcinoma in rats (9) and maintains bone density in ovariectomized rats (10). The recognition that non-steroidal anti-estrogens like tamoxifen and raloxifene selectively exhibited estrogen-like effects in bone and anti-estrogenic effects in breast and mammary tissue (9,10) suggested a new strategy to prevent breast cancer by treating post-menopausal women to prevent and treat osteoporosis and prevent breast cancer at the same time (11).

The clinical finding that patients treated with raloxifene to improve bone density (12) exhibited significant decrease in the rates of breast cancer (13), provided a clinical proof of the laboratory principle and demonstrated raloxifene's potential as a breast cancer chemopreventive agent. Data from the study of tamoxifen and raloxifene (STAR) trial (14), which directly compared raloxifene to tamoxifen for breast cancer chemoprevention, indicated that raloxifene has similar chemopreventive properties as tamoxifen but with a significantly better safety profile. A subsequent clinical trial (15) examining the effects of raloxifene on coronary heart disease (CHD) did not achieve its goals but confirmed the role of raloxifene as a breast cancer chemoprevention agent with no increase in endometrial cancer. The evaluation by Martino and coworkers (16) that long-term raloxifene treatment for the prevention of osteoporosis does not increase endometrial cancer but maintains an inhibiting effect on breast cancer incidence suggests that the clinical community may use raloxifene for indefinite periods. However, the discovery that acquired tamoxifen resistance evolves (17,18) raises new questions about acquired resistance to raloxifene treatments.

Acquired tamoxifen resistance is sub-divided into 3 phases: i) phase I, in which estrogen and the SERM stimulate tumor growth, ii) phase II, in which the SERM stimulates tumor growth and estrogen induces tumor regression; iii) phase III resistance or autonomous growth (1). Laboratory studies indicate that long-term SERM treatments result in hypersensitivity to low, physiological doses of estrogen resulting in breast tumor regression and possibly estrogen-induced apoptosis. It is important to note that these observations were initially made with an estrogen-supersensitive clone of MCF-7 breast cancer cells (WS8) using only tamoxifen treatment for 5-10 years *in vivo* (17,18) and raloxifene-resistant model (19,20) *in vitro* and few weeks (20) or a year or two (19,20) *in vivo*. These data are not confined to SERM-resistant models as similar observations were made in long-term estrogen-deprived breast cancer cells (21-24). The findings that physiological estrogen causes dramatic tumor repression in anti-hormone-resistant breast cancer (17,18) are reminiscent of the early clinical trials utilizing high doses of diethylstilbestrol (DES) (25,26) to treat breast cancer in post-menopausal patients many years after their menopause. Moreover, recent clinical trial (27,28) evaluating the role of estrogen treatments in women with advanced breast cancer following acquired resistance to anti-hormone therapy noted a 31% objective response and indicated a substantial role for high dose estrogen treatments in hormone-dependent breast cancer resistant to conventional endocrine therapies.

The current 10-year laboratory study has paralleled the translation of the new biology of apoptotic action (17,18,21,23) to clinical trials (27,28). Most importantly, the increasing clinical use of raloxifene for the prevention of osteoporosis in post-menopausal women implies that breast cancer that develops during a decade or more of raloxifene treatment will have developed raloxifene resistance. It is important to address this emerging clinical problem.

Our goal was to revisit this question by utilizing wild-type MCF-7 cells to recreate a raloxifene-resistant variant of MCF7 cells *in vitro*. The failure of wild-type MCF-7 cells to create acquired resistance *in vivo* would expose an inadequacy of laboratory models or imply that acquired raloxifene resistance would not occur in the clinic. This was not the case as the answer is yes to the first question and the answer to the second question requires clinical investigation. We subsequently used the new model *in vivo* to evaluate the actions of physiological estrogen and raloxifene on the growth responses of raloxifene-stimulated tumors passaged over a decade in ovariectomized athymic mice. This laboratory strategy mimics the clinical duration of raloxifene exposure.

Materials and methods

Cell lines and tissue culture. The MCF7 breast cells were a generous gift of Dr Myles Brown (Harvard) in 1995. The MCF7 cells were maintained in a DMEM red medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM non-essential amino acids (NEAA). Raloxifene-resistant MCF7 cells (MCF7-RAL) were derived by continuously culturing the MCF7 cells for up to 10 years in estrogen-free media: DMEM yellow media with 10% charcoal-stripped FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM NEAA, supplemented with 1 μM raloxifene-HCl. All cell lines were cultured at 37°C, 5% CO_2 and 95% humidity.

Verification of cell line identity by DNA fingerprinting. The identity of the cell lines was verified by DNA fingerprinting using the commercially available kit, PowerPlex® 1.2 System (Promega). This system allows the co-amplification and two-color detection of nine loci (eight STR loci and the Y-specific Amelogenin) and provides a powerful level of discrimination in excess of 1 in 10^8 (29). The following STR markers were tested: CSF1PO, TPOX, TH01, vWA, D16S539, D7S820, D13S317 and D5S818. The cells were harvested by trypsinization and DNA was isolated from the resultant cell pellets using standard methods (30). The PCR amplification was performed according to the manufacturer's recommended protocol. Fragment analysis of the PCR product was achieved using an ABI 3100 capillary sequencer (Applied Biosystems,

Foster City, CA). The GeneMapper® software (Applied Biosystems) was used to score the fragment sizes and generate an alphanumeric score for each locus. The data generated were then compared to allelic alphanumeric scores for MCF-7 and ECC-1 reported in the ATCC STR database generated using the same assay (ATCC, VA).

DNA growth assay. MCF7 and MCF7-RAL cells were seeded in estrogen-free media 4 days prior to start of the experiment. After 3 days of ligand starvation the appropriate numbers of cells were seeded in a 24-well plate. Twenty-four hours later, which was denoted as day 0, the cells were appropriately treated. The media containing treatments were changed every other day. All drugs were solubilized in ethanol and were added as 1:1000 dilutions. Following 15 days of treatment the DNA content of the cells was measured as previously described (31) with VersaFluor fluorometer (Bio-Rad Laboratories, Hercules, CA).

Animal procedures

MCF7 tumor models. The MCF7-E_2 breast tumor model was developed by bilaterally injecting $1x10^7$ MCF7 cells into the mammary fat pads of ovariectomized athymic CrTac: NCR-Foxn1<nu> mice (Taconic, Hudson, NY) (32), 4-6 weeks of age, implanted with silastic 17ß-estradiol capsules. The raloxifene-resistant MCF7-RAL model was similarly developed by injecting $1x10^7$ raloxifene-resistant MCF7-RAL cells into the mammary fat pads of ovariectomized female mice. RAL treatments were started 24 h post-implantation by administering 1.5 mg RAL or .005 mg TAM via oral gavage. The MCF7-RAL tumor xenograft model was maintained by excising the established MCF7-RAL tumors, removing all extraneous tissues and dissecting them into approximately 1-2 mm^3 pieces that were then implanted by trochar into the mammary fat pads of naïve mice subsequently treated with RAL. The RAL-resistant MCF7-RAL model was continuously passaged into RAL-treated athymic mice over a 10-year period. Established tumors were measured every week or as needed with Vernier calipers and cross sectional area of the tumor was calculated utilizing the formula: Length (l) x width (w) x $\pi/4$.

Drug administration. The raloxifene solution for oral gavage was prepared by grinding 10 commercially available Evista® tablets and dissolving them into 10% PEG 400/Tween-80 (Sigma, St. Louis, MO) solution to a final concentration of 15 mg/ml. Silastic 17ß-estradiol capsules were manufactured as previously described (33) and were subcutaneously implanted in the mice dorsal region. The 0.3-cm capsule delivered the equivalent of menopausal levels of estrogen while the 1.0-cm capsule delivered the equivalent of pre-menopausal levels of estrogen (34). Fulvestrant (Fasoldex/ICI 182,780, AstraZeneca) is commercially available and was purchased from the hospital pharmacy. Total fulvestrant (FUL) (10 mg) was injected bi-weekly, subcutaneously (35). All animal studies were approved by the Fox Chase institutional animal care and use committee.

RNA extractions, reverse transcriptase reactions and real-time qPCR. Total RNA was extracted with TRIzol reagent (Invitrogen) and further purified using RNeasy Mini and Midi kits (Qiagen, Valencia, CA). Total RNA (1 μg) was reversely transcribed with the High Capacity cDNA reverse transcriptase kit (Applied Biosystems) following manufacturer's instructions. The sequences of the primers utilized for real-time qPCR are as follows: *tff1* forward primer, 5'-CATC GACGTCCCTCCAGAAGAG-3'; *tff1* reverse primer, 5'-CTC TGGGACTAATCACCGTGCTG-3'; *36B4* forward primer, 5'-GTGTTCGACAATGGCAGGCAT-3'; *36B4* reverse primer, 5'-GACACCCTCCAGGAAGCGA-3'; c-myc forward primer, 5'-GCCACGTCTCCACACATCAG-3'; c-myc reverse primer, 5'-TCTTGGCAGCAGGAATAGTCCTT-3'; ebag9 forward primer, 5'-CTGGCAGAGGACGGAAATTA-3'; ebag9 reverse primer, 5'-TCATCCCAGGAAGTCCACTC-3'; the primer sets for *egfr* and *her2* were previously described (36,37). Real-time qPCR was performed using the 7900HT real-time PCR system (Applied Biosystems), the amplicons were detected with SYBR-Green and analysis was performed utilizing the $2^{-\Delta\Delta t}$ method (38).

Transient transfections and luciferase assays. MCF7 and MCF7-RAL cells were maintained in estrogen-free medium for 3 days and seeded at confluency of 150,000 cells per 6-well plate. The cells were co-transfected with 5ERE(5X)-TA ffluc and pTA-srluc utilizing TransIT LT1 transfection agent (Mirus, Madison, WI) (39). Luciferase activity was measured utilizing the Dual-luciferase reporter assay system (Promega) with Mithras LB 940 (Berhold Technologies, Bad Wildbad, Germany) microplate reader.

Protein isolation and Western blot. The MCF7 and MCF7-RAL cells were cultured in estrogen-free media for 3 days and seeded at 50-60% confluency. Twenty-four hours post-seeding the cells were treated with the appropriate drug or drug combination. Following 24-h treatment, the cells were washed with PBS and scraped off the plates. After brief centrifugation at 4°C the PBS was aspirated and the cells were resuspended in RIPA buffer (Sigma) supplemented with complete mini protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN), phosphatase inhibitor cocktail set (EMD Biosciences, La Jolla, CA) and benzonase (Call Biochem, La Jolla, CA). The cells were then incubated for additional 30 min at 4°C with rotation. The debris was removed with centrifugation at 12000 rpm for 30 min at 4°C.

Tumor protein lysates were generated by pulverizing flash-frozen tumors to a fine powder with a Bio-pulverizer (BioSpec Products Inc., Bartlesville, OK) and resuspending them in 400 μl of RIPA buffer supplemented with complete mini protease inhibitor cocktail tablets and phosphatase inhibitors cocktail set. The suspension was than sonicated 3 times at maximum power and centrifuged at 12000 rpm for 20 min at 4°C. Supernatants were collected and stored at -80°C.

Protein quantitation was performed with the Bicinchoninic acid (BCA) Protein Assay (Pierce, Rockford, IL) as per the manufacturer's protocol. Readings were obtained with a microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA). Protein (50 μg) was resolved by SDS-PAGE and Western blotting was performed as previously described (40). Antibodies used were as follows: ERα G-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phospho

A.

cell line:	D5S818 allele 1	D5S818 allele 2	D13S317 allele 1	D13S317 allele 2	D7S820 allele 1	D7S820 allele 2	D16S539 allele 1	D16S539 allele 2	vWA allele 1	vWA allele 2	TH01 allele 1	TH01 allele 2	Amelogenin allele 1	Amelogenin allele 2	TPOX allele 1	TPOX allele 2	CSF1PO allele 1	CSF1PO allele 2
MCF-7 ATCC	11	12	11	11	8	8	11	12	14	15	6	6	X	X	8	12	10	10
MCF7-WS8 p24		12	11	11	8	8	11	12		15	6	6	X	X	8	12	10	10
MCF7/5C p217		12	11	11	8	8	11	12	14	15	6	6	X	X	8	12	10	
MCF7/2A p549		12	11	11	8	8	11	12	14	15	6	6	X	X	8	12	10	10
MCF7/ICI p42		12	11	11	8	8	11	12		15	6	6	X	X	8	12	10	10
MCF7/RAL p83	12		11	11	8	8	11	12	14	15	6	6	X	X	8	12	10	10

B.

cell line	D5S818 allele 1	D5S818 allele 2	D13S317 allele 1	D13S317 allele 2	D7S820 allele 1	D7S820 allele 2	D16S539 allele 1	D16S539 allele 2	vWA allele 1	vWA allele 2	TH01 allele 1	TH01 allele 2	Amelogenin allele 1	Amelogenin allele 2	TPOX allele 1	TPOX allele 2	CSF1PO allele 1	CSF1PO allele 2
MCF-7 ATCC	11	12	11	11	8	8	11	12	14	15	6	6	X	X	8	12	10	10
MCF7 (GMB) p184	11	12	11	11	8	8	11	12	14	15	6	6	X	X	8	12	10	10
MCF7-RAL (GMB) p74	11	12	11	11	8	8	11	12	14	15	6	6	X	X	8	12	10	10
ECC1 ATCC		11			8				14	15			X	X				

Figure 1. Verification of cell line identity by DNA fingerprinting. See Materials and methods.

p42/44 MAPK (Thr202/Tyr204) (E10) antibody (Cell Signalling), p42/44 MAP kinase antibody (Cell Signalling). ß-actin antibody AC-15 (Sigma) was used as a loading control. Appropriate horseradish peroxidase-conjugated secondary antibody was used to visualize bands using an Amersham Western Blotting Detection kit (GE Healthcare).

Histology and immunohistochemistry. Tissues were fixed in 10% phosphate-buffered formaldehyde for 48 h, subsequently embedded in paraffin, sectioned and stained. Hematoxylin and eosin (H&E) staining was used to evaluate tumor tissue morphology and extent of necrosis. Immunohistochemistry for Ki-67 (dilution 1:6000) was performed using rabbit polyclonal antibodies from Vector Labs (Burlingame, CA) and Cell Signalling, respectively. Immunostaining was preceded by antigen retrieval in citrate buffer pH6 using a 750 W microwave oven, boiling the slides at maximum setting for 3 min and at low setting for another 7 min. A rabbit Vectastain kit (Vector) was used to develop the immunohistochemical reaction using diaminobenzidine as chromogen. Microphotographs were taken using a Nikon Optiphot research microscope with a x10 and x20 Plan/Apo objectives and a x10 ocular lens connected to a digital photographic camera (Optronics, Magnafire camera, Optronics, Goleta, CA).

Statistical analysis. The growth rates in Fig. 2A and B were estimated for each individual test by fitting the weight of DNA/well to the linear time term. The rates were compared using Wilcoxon rank-sum tests. The tumor growth data were analyzed using growth curve models, where tumor cross sectional area (CSA) was fit assuming a linear function of time. The intercepts and the slopes were used as random effects at the individual tumor level to allow deviation of individual tumor growth from the mean growth of the group. Random mouse effects were included to account for within-animal clustering. The estimated curves were plotted and the fit examined. The differences in rates were estimated by the interaction term between time and the treatment. The comparisons of either the DNA weight/well or CSA at each time point were also conducted by using Wilcoxon rank-sum tests. All tests were 2-sided with 0.05 type I error.

Results

Verification of cell line identity. DNA profiling of the cell lines was conducted using the PowerPlex 1.2 System resulting in the generation of allelic scores for 8 polymorphic STR loci and the amelogenin locus which are presented in Fig. 1A along with the scores for MCF-7 and ECC-1 cells reported in the ATCC STR database. Data from the amelogenin gene amplification were consistent with all samples being of female origin as expected. Allelic score data from the 8 polymorphic STR loci reveal a pattern almost identical among the 5 MCF7 lines that is very closely related to the scores reported for MCF-7 by the ATCC, and consistent with their presumptive identity. Scores for 5 of the 8 loci (D13S317, D7S820, D16S539, TH01 and TPOX) were identical among the study and ATCC MCF-7 cells (Fig. 1A, areas of identity

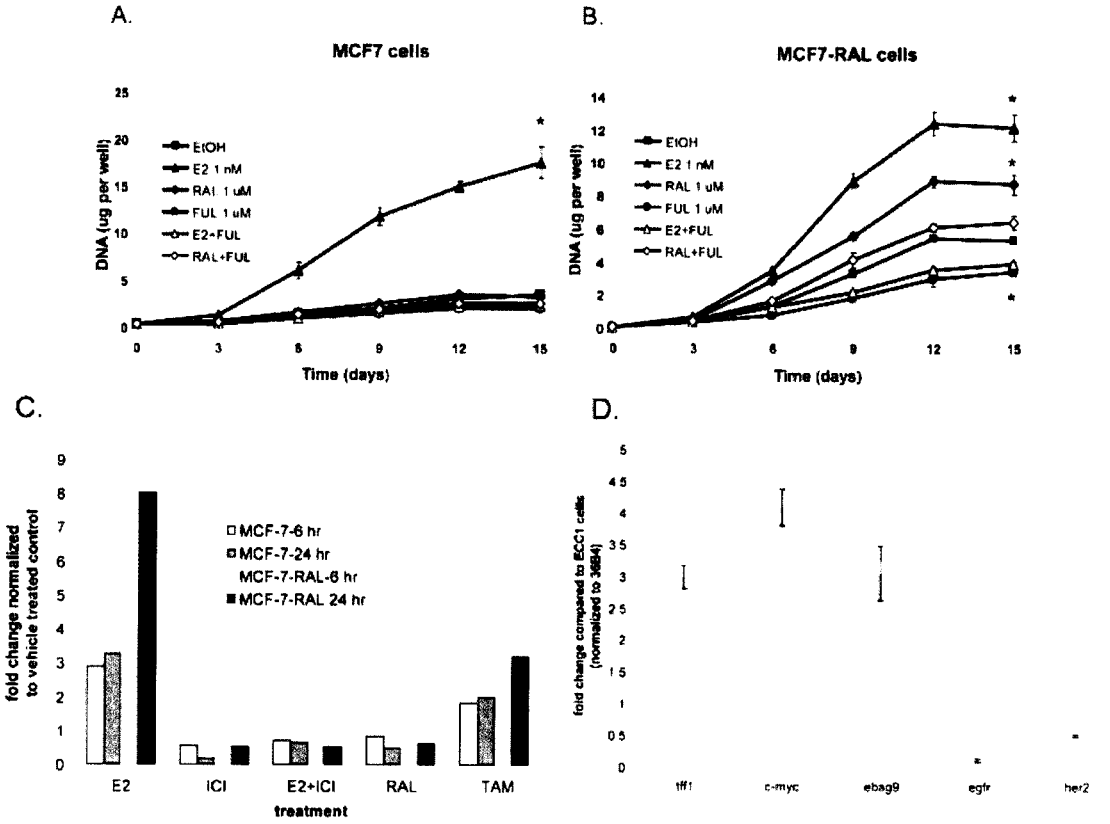

Figure 2. The MCF7-RAL cells are spontaneously growing cells that are stimulated by raloxifene (RAL) and 17β-estradiol (E_2). (A) Three days before seeding the MCF7 cells were cultured in E_2-free conditions, RPMI-yellow media with charcoal stripped FBS. The MCF-7 cells were than seeded in a 24-well plate and 24-h post seeding the cells were treated with vehicle, 1 nM E_2, 1 μM RAL, 1 μM fulvestrant (FUL) and combination of drugs as described in Materials and methods. (B) MCF7-RAL cells were seeded and treated in an identical manner as in (A). (C) MCF-7 and MCF7-RAL cells were either E_2 or RAL starved for 3 days before transfection with the appropriate reporters. Twenty-four h post transfection the cells were treated with vehicle control (EtOH), 1 nM E_2, 1 μM RAL, 1 μM TAM, 1 μM FUL and combination of 1 nM E_2 and1 μM FUL. Luciferase activity was measured 6 and 24 h after post treatment. (D) Expression of ERα-regulated genes in MCF7-RAL cells in steady state. Error bars = standard error of the mean (SEM); *$p<0.05$, statistically significant finding as compared to EtOH-treated cells.

highlighted in pink), but there was some evidence of genetic drift in some of the study lines. ATCC MCF-7 cells have D5S818 allelic scores of 11 and 12, whereas 4 of the study lines (WS8, 5C, 2A and ICI) only have one allele (12) (allelic loss highlighted in green), whereas the MCF7-RAL cells have two alleles at this locus: 12 and 13 (variant allele highlighted in blue). Similarly, for the vMA locus, the ATCC cells have alleles 14 and 15, as do the 5C, 2A and RAL cells, whereas the WS8 and ICI cells only have one allele; 15. Scores for the CSF1PO locus were identical among the lines showing a single allele (10), with the exception of the 5C cells that have an additional allele at this locus (11). The minor variations in the DNA profile exhibited by the MCF-7 cells are similar to the sort of genetic drift that has been seen previously among sub-lines of cells cultured independently (41), and overall these fingerprinting data confirm the presumptive identity of the lines as being of MCF-7 origin. Furthermore, the profiles from the study cell lines derived from MCF-7 (WS8) show that they are more closely related to each other than to the ATCC MCF-7 cells, again consistent with their having been derived from a common ancestor subline.

Development of a novel raloxifene-resistant tumor cell line, MCF7-RAL. To examine the effects of long-term raloxifene treatments on breast cancer cell growth we derived a novel breast raloxifene-resistant cell line, MCF7-RAL (GMB). The MCF7-RAL (GMB) cells were developed by continuously passaging cells in estrogen-free media supplemented with 1 μM raloxifene for at least 1 year. The fingerprinting data from the independently obtained MCF7 cells p184 and MCF7-RAL p74 (GMB) cells reveal a pattern of allelic scores that is identical to the scores reported for the ATCC MCF-7 cells, and highly divergent from the pattern reported for non-related cells such as the ATCC ECC-1 cells (Fig. 1B). These data suggest that the cell lines used in this study are in fact of ATCCMCF-7 origin and not a variant of the MCF-7WS8 clone. For clarity the MCF-7RAL (GMB) are referred to as MCF-7RAL throughout this paper.

Currently, the MCF7-RAL cells have been propagated in RAL containing medium for approximately 10 years. The growth characteristics *in vitro* were compared and contrasted with wild-type MCF-7. Within 3 days of treatment the MCF7 cells are significantly ($p=0.02$) stimulated by 1 nM E_2, 2.2-fold increase as compared to vehicle-treated controls (Fig. 2A).

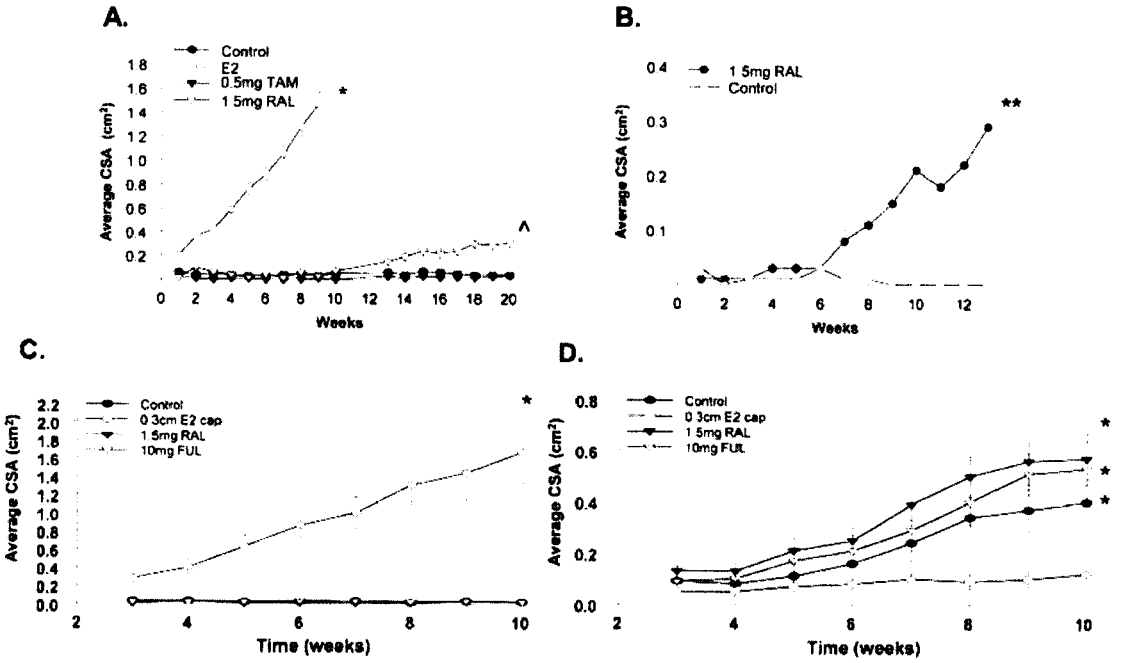

Figure 3. Establishment of MCF7-RAL tumor xenograft model. (A) MCF7-RAL-resistant cells ($1x10^7$) were injected into the axillary mammary fat pads of ovariectomized athymic mice. The mice were then divided into 4 groups and treated as follows: placebo, implanted with silastic 0.3-cm E_2 capsule, orally gavaged with RAL (1.5 mg daily) and TAM (1.5 mg daily). (B) A single tumor from the RAL-treated group was transplanted (passage 1) into 20 naïve ovariectomized athymic mice and divided into 2 groups: placebo and RAL treated. Error bars = SEM; *p<0.0001, E_2 vs. all other treatment groups; ^p=0.048 RAL vs. control. **p=0.05, RAL vs. control (C) MCF7-E_2 and MCF7-RAL tumor xenogratfs were bi-transplanted into each ovariectomized athymic mouse (total of 40). The MCF7-E_2 tumor was implanted in the left and the MCF7-RAL tumor was implanted in the right axillary mammary fat pad. The mice were randomized into groups of 10 and implanted with 0.3-cm E_2 capsule or treated with RAL (1.5 mg daily), FUL (5 mg s.c., twice a week) or no treatment (control). (C) MCF7-E_2 tumors; (D) MCF7-RAL tumors; error bars = SEM; *p<0.05, E_2 vs. all other treatment groups.

Maximum induction, 4.8-fold increase as compared to control was observed at day 15. The E_2-induced growth of the MCF7 cells was blocked by 1 μM FUL treatments. In contrast to E_2, 1 μM RAL did not stimulate the growth of the MCF7 cells. Similarly to the MCF7 cells, within 3 days of treatments, E_2 significantly (p=0.02) induced the growth of the MCF7-RAL cells (Fig. 2B). Maximum E_2 induction was observed at day 9, 2.67-fold increase as compared to control. At day 3 of treatment RAL also significantly (p=0.02) induced the growth of the MCF7-RAL cells. Maximum RAL induction was observed at day 6, 2.1-fold increase as compared to the controls. The E_2 and RAL-induced growth of the MCF7-RAL cells was significantly inhibited by 1 μM FUL treatments within 3 (p=0.04) and 6 days (p=0.02) of treatment, respectively. In addition, the MCF7-RAL cells were spontaneously growing.

To further characterize the RAL-resistant phenotype of the MCF7-RAL cells we determined the protein expression levels of ERα. To determine the protein levels of ERα in MCF7 and MCF7-RAL cells we treated the cells with EtOH, 1 μM RAL, 1 nM E_2 and 1 μM FUL for 48 h. The ERα protein levels in the MCF7-RAL cells are regulated in an identical manner as in the parental MCF7 cells (data not shown). Treatments with 1 nM E_2 and 1 μM FUL decreased the protein levels of ERα, while treatments with 1 μM RAL maintained the protein expression of ERα. The levels of total MAPK and total AKT in the MCF7-RAL cells appeared to remain unchanged, regardless of treatment, when compared to the parental, MCF7 cells. However, the levels of phosphorylated MAPK, increased in the EtOH-treated MCF7-RAL cells (data not shown). Luciferase reporter assays indicated that 1 nM E_2 treatments significantly induced transcriptional activation of the reporter in MCF7 and MCF7-RAL cells (Fig. 2C) consistent indicating similar activity of ERα in the parental and resistant cell line. Fulvestrant (FUL) and RAL treatments did not induce activation of the reporter. Furthermore, FUL treatments abolished the E_2-dependent reporter activity. TAM treatments significantly induced reporter activity in both MCF7 and MCF7-RAL cells at the 24-h time point.

The MCF7-RAL cells grew spontaneously and were inhibited by FUL treatment (Fig. 2B). To further characterize the RAL-resistant phenotype of the MCF7-RAL cells at steady state, we determined by quantitative real-time PCR, the basal mRNA expression of ERα-regulated genes in MCF7 and MCF7-RAL cells (Fig. 2D). In the basal state, the MCF7-RAL cells exhibited 3-fold up-regulation of *tff-1*, 4.1-fold up-regulation of the *c-myc* and 3.1-fold up-regulation of *ebag9*. In contrast, the levels of *egfr* and *her2* were down-regulated by 7.7- and 1.99-fold, respectively.

Development of an MCF7-RAL xenograft tumor model. To develop MCF7-RAL xenograft tumor model *in vivo*, $1x10^7$ MCF7-RAL cells were injected into the mammary fat pads of nude athymic mice as described in Materials and methods. The mice were treated with vehicle, implanted with 0.3-cm

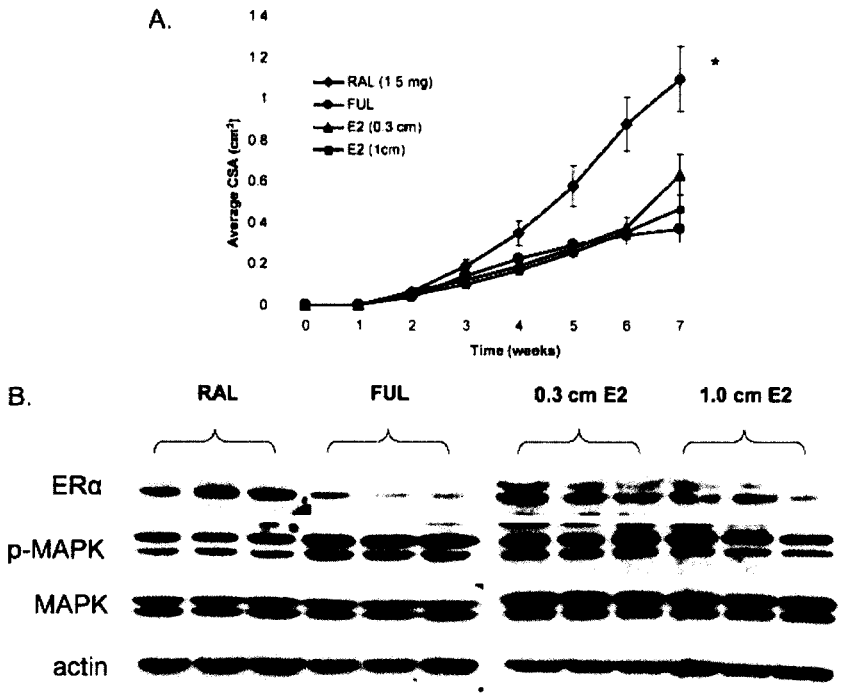

Figure 4. Pre- and post-menopausal concentrations of E_2 significantly impair the growth of long-term RAL-treated MCF7-RAL xenografts. (A) MCF7-RAL tumor xenografts serially transplanted for at least 8 years were implanted into 45 ovariectomized athymic mice. The animals were treated with RAL (1.5 mg daily), FUL (5 mg s.c twice weekly) or implanted with either 0.3-cm or 1.0-cm silastic E_2 capsules. (B) Western blot analysis of protein extracts collected from (A). *p=0.001 RAL vs. all other treatment groups.

silastic E_2 capsule or orally gavaged with 1.5 mg daily RAL or 0.5 mg daily TAM. At week 9, average cross sectional area (CSA) of the estradiol-treated group was 1.47 cm^2, significantly greater ($p<0.0001$) than the control and the other treatment groups (Fig. 3A). The E_2-treated mice grew large tumors and were sacrificed at week 10 because of ethical considerations. By week 15, palpable tumors were observed in the RAL-treated group (average CSA = 0.24 cm^2) which were significantly larger than the control group ($p=0.048$) (Fig. 3A). At week 20, a single tumor from the raloxifene-treated group was excised, resected and transplanted into 20 ovariectomized athymic mice (Fig. 3B). The mice were divided into control (no treatment) and a RAL- (1.5 mg daily) treated group. Starting at week 7, RAL promoted tumor growth which by the conclusion of the experiment at week 13 was statistically significant as compared to the control group ($p<0.05$) (Fig. 3B).

To further characterize the MCF7-RAL tumor xenograft model and to determine the effects of E_2 and RAL on estrogen and raloxifene-dependent breast tumor growth, we bi-transplanted MCF7-E_2 and MCF7-RAL tumors on opposite sides in the axillary mammary fat pads of the same animal. MCF7-E_2 xenografts were implanted into the left and the MCF7-RAL xenografts were implanted into the right mammary fat pad of 40 ovariectomized athymic mice. As anticipated the E_2-treated MCF7-E_2 tumors displayed robust tumor growth and at week 10 the mean tumor size was 1.67 cm^2 (Fig. 3C). No tumor growth was observed in the control, RAL- and FUL-treated groups (Fig. 3C). In contrast, at week 10, RAL and FUL stimulated MCF7-RAL tumor growth while the E_2-treated tumors exhibited minimal growth (Fig. 3D). At week 10, the mean size of the RAL- and FUL-treated tumors was 0.57 and 0.53 cm^2, respectively. Interestingly, spontaneous tumor growth was observed in the control MCF7-RAL (at this point considered passage 3) (mean tumor size = 0.4 cm^2, $p<0.05$ as compared to the E_2 group) (Fig. 3D).

Long-term RAL treatments of the MCF7-RAL tumor xenografts. To determine the effects of E_2 on long-term RAL-treated MCF7-RAL xenografts, we evaluated the effects of pre- and post-menopausal levels of E_2 (34) on the growth of MCF7-RAL tumors that were serially transplanted and continuously treated with RAL for at least 8 years. The MCF7-RAL tumor xenografts were transplanted into 45 ovariectomized athymic mice that were treated with RAL, FUL and 0.3- or 1.0-cm silastic E_2 capsules (Fig. 4A). At week 7, the RAL-treated xenografts exhibited a statistically significant ($p<0.001$) RAL-stimulated growth (mean CSA = 1.1 cm^2) as compared to the FUL, 0.3 and 1.0 cm E_2-treated tumors (mean CSA = 0.37, 0.63, 0.46 cm^2, respectively). There were no statistical differences between the FUL, 0.3 and 1.0 cm E_2-treated tumors. To further characterize the effects of E_2 on the long-term RAL-treated MCF7-RAL tumor xenografts we analyzed the ERα expression of the xenografts (Fig. 4B). The long-term RAL-treated MCF7-RAL xenografts continue to express ERα and RAL treatments increased the expression of ERα while FUL treatments down-regulated the expression of ERα. No differences in ERα protein expression was observed between the two different concentrations of E_2-treated tumors.

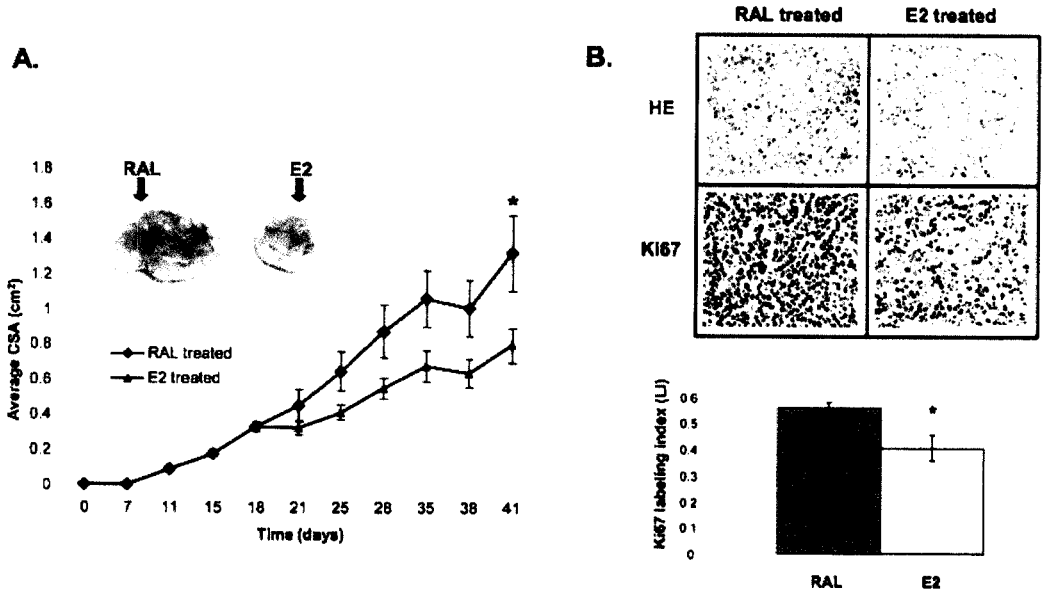

Figure 5. 17ß-estradiol treatments impair the growth of established MCF7-RAL xenografts. (A) Long-term RAL-treated MCF7-RAL xenografts were implanted into 30 ovariectomized athymic mice and the animals were treated with RAL until the cross sectional area (CSA) of the tumors reached 0.3 cm^2. The animals were than randomized into 2 groups: continued RAL treatments or implanted with 0.3-cm E_2 capsules. Estradiol treatments significantly impaired the growth of the MCF7-RAL xenografts by day 38 (20 days post-introduction of E_2). Insert: representative images of E_2- and RAL-treated tumors. (B) Histological analysis of tumors from (A). *p=0.02 RAL vs. E_2.

Estrogen treatments inhibit the growth of established MCF7-RAL tumors. To determine the effects of E_2 on established MCF7-RAL tumors, MCF7-RAL tumor xenografts were implanted into ovariectomized athymic nude mice and the animals were treated with RAL until the average CSA of the tumors reached 0.3 cm^2. At this point the animals were randomized into 2 groups: 1) continued RAL treatments and 2) implanted with 0.3-cm E_2 capsules (Fig. 5A). Within 3 days post-E_2 implantation, there were visible morphological and size differences between the RAL- and E_2-treated tumors (Fig. 5A insert). At day 7, the mean CSA was 0.64 cm^2 for the RAL-treated and 0.41 cm^2 for the E_2-treated tumors. At day 17 the CSA of the RAL-treated tumors was 1.00 cm^2 and the CSA of the E_2-treated tumors was 0.64 cm^2 (p=0.03). At the end-point of the experiment statistically significant differences (p=0.02) were observed between the RAL-treated tumors (average CSA = 1.32 cm^2) and the E_2-treated tumors (average CSA = 0.79 cm^2) (Fig. 5A).

Histological analysis of the RAL- and E_2-treated tumors at the conclusion of the experiment (Fig. 5B) by hematoxylin and eosin staining indicated that there are no significant morphological changes between the two treatment groups. However, significant differences in the expression of Ki-67, a known marker of proliferation, were observed between the two groups. There were significant statistical differences (p=0.02) between the average labeling index (LI) of the RAL-treated group and the E_2-treated group which were 0.56±0.04 and 0.40±0.09, respectively.

Effects of long-term estrogen treatments on the growth of MCF7-RAL tumors. To determine the effects of long-term E_2 treatments on the growth of MCF7-RAL tumor xenografts we transplanted long-term RAL-treated MCF7-RAL tumors into 45 ovariectomized athymic mice. The mice were divided into 3 groups: no treatment, RAL and 0.3 cm E_2 (Fig. 6A). Three weeks post-implantation the average CSA of the tumors were 0.17, 0.08 and 0.09 cm^2 for the RAL, placebo and the 0.3-cm E_2-treated tumors. At week 5, differences could be observed between the treatment groups; the average CSA of the RAL-treated tumors was 0.41 cm^2 and the average CSA of the 0.3-cm E_2-treated tumors was 0.11 cm^2. The average CSA of the untreated tumors was 0.2 cm^2 indicating spontaneously growing tumors. The 0.3-cm E_2 treatment was continued for additional 5 weeks and at week 10 the average CSA was 0.32 cm^2. At that point the E_2-treated tumors were excised, resected and bitransplanted into 25 ovariectomized athymic mice. The animals were divided into 5 groups: RAL, placebo, 0.3 cm E_2, FUL and combination of E_2- and RAL-treated (Fig. 6B). Treatment with RAL continued to induce the growth of the MCF7-RAL tumor and at week 8, the average CSA was 1.3 cm^2. At week 8, the average CSA of the placebo and the FUL-treated tumors was 0.36 and 0.29 cm^2. Unexpectedly, E_2 treatments either individually or in combination with RAL induced the growth of the MCF7-RAL xenografts. At week 8 the average CSA of the E_2-treated tumors was 0.64 cm^2 and combination of E_2 and RAL treatments resulted in tumor growth (average CSA = 1.15 cm^2). The placebo, E_2 and FUL treatments were continued and at week 10 the average CSA of the tumors was: 0.49, 1.16 and 0.52 cm^2. The growth rates of the RAL-treated tumors were significantly different compared to the placebo (p=0.003) and FUL-treated, tumors (p=0.005). However, the growth rate of the E_2- and E_2 + RAL-treated tumors was indistinguishable from the RAL-treated tumors. At week 10 the E_2-treated tumors were

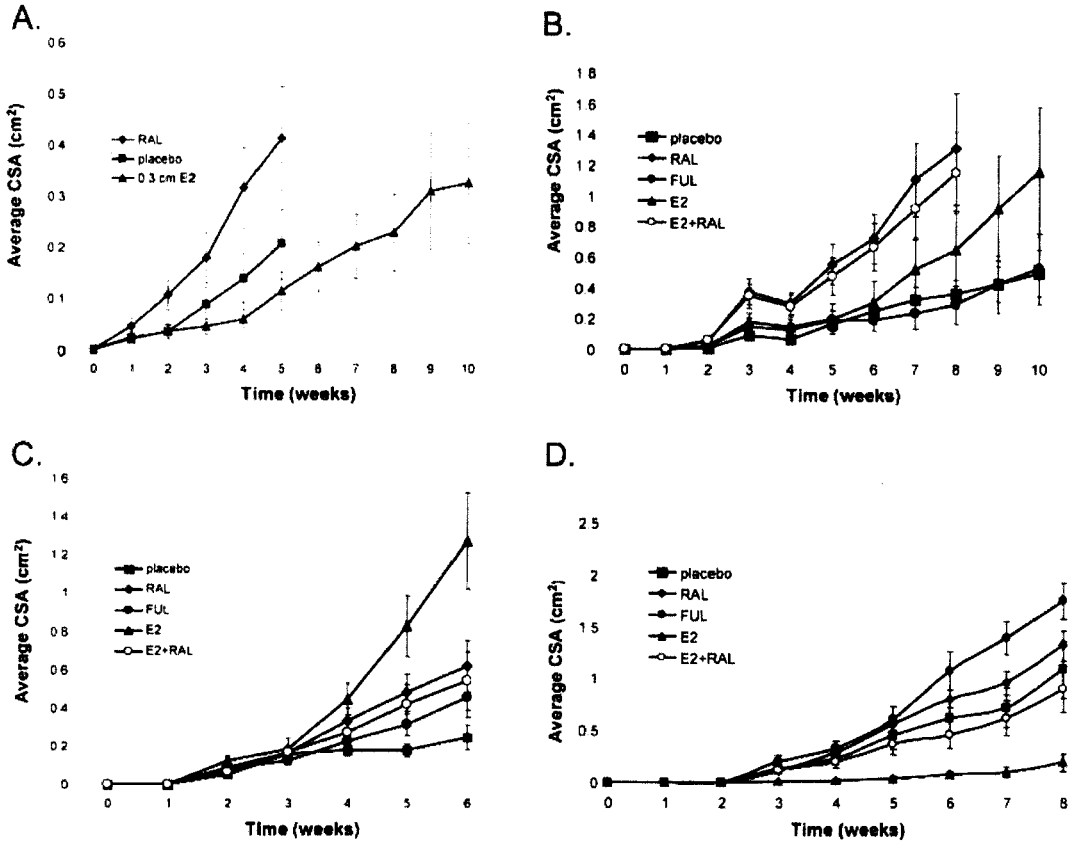

Figure 6. Long-term estrogen and raloxifene treatments result in changes in the phases of SERM resistance. (A) MCF7-RAL tumor xenografts were implanted into 45 ovariectomized athymic mice, the mice were divided into 3 groups and were either left untreated, treated with RAL (1.5 mg/daily) or implanted with 0.3-cm E_2 capsules. (B) E_2-treated tumors from (A) were resected and re-transplanted into 25 ovariectomized athymic mice that were either left untreated or treated with RAL (1.5 mg/daily), FUL (5 mg subcutaneously, twice weekly), implanted with 0.3-cm E_2 capsules and combination of RAL and E_2. (C) E_2-treated tumors from (B) were serially retransplanted into 25 ovariectomized athymic mice that were either left untreated or treated with RAL, FUL, implanted with 0.3-cm E_2 capsules and combination of RAL and E_2. (D) RAL-treated tumors from (C) were implanted into naïve animals and continuously treated with RAL for 28 weeks before being implanted into 25 naïve animals that were either left untreated or treated with raloxifene, FUL, implanted with 0.3-cm E_2 capsules and combination of RAL and E_2. See Results for a precise description of the evolution of raloxifene resistance and statistical significance of the findings in the individual experiments.

excised, resected and implanted into 25 naïve animals. The treatments were identical to the previous experiment and consisted of placebo, RAL, FUL, E_2 and combination of E_2 and RAL (Fig. 6C). At week 3 post-implantation there were no significant differences in the average CSA between the various treatments, and the average CSA was 0.05, 0.08, 0.08, 0.12 and 0.06 cm^2 for the placebo, RAL-, FUL-, E_2- and the E_2 + RAL-treated tumors. However, dramatic changes were observed at week 4 as E_2 treatments started to induce significant tumor growth (average CSA = 0.44 cm^2). In contrast RAL inhibited the estrogen-induced tumor growth as the combination of E_2 + RAL treatments average CSA was 0.27 cm^2. The average CSA of the RAL-treated tumors was 0.33 cm^2 and the placebo- and FUL-treated tumors were 0.18 and 0.22 cm^2, respectively. At conclusion of the experiment at week 6, the E_2-treated tumors reached average CSA of 1.27 cm^2. The average CSA of the RAL-treated tumors was 0.62 cm^2 and the E_2 + RAL group was 0.54 cm^2. The growth rate of the E_2-treated group was significantly different ($p<0.01$) from all other groups with the exception of the RAL-treated tumors, but approached significance ($p=0.06$). Upon conclusion of the experiment at week 6 the RAL-treated tumors were excised, resected and implanted into ovariectomized athymic animals that were continuously treated with RAL. Following 28 weeks of continuous RAL treatments the long-term treated MCF7-RAL tumor xenografts were implanted into 25 animals that were divided into 5 groups and treated as follows: placebo, RAL, E_2, FUL and E_2 + RAL (Fig. 6D). Within 3 weeks of treatments highly statistically significant differences ($p<0.01$) emerged between the E_2-treated tumors and all other treatment groups. At week 3 tumor growth was observed in the placebo, FUL, RAL and E_2 + RAL while negligible tumor growth was observed in the E_2-treated group (average CSA = 0.008 cm^2). These differences persisted throughout the duration of the experiment and at its conclusion at week 7, the average CSA of the E_2-treated tumors was 0.1 cm^2. In contrast, significant tumor growth was observed in all other treatment groups. Paradoxically, maximum tumor growth was observed in the FUL treatment groups (average CSA = 1.4 cm^2). Significant tumor growth was also observed in the RAL group (CSA = 0.96 cm^2) and in the placebo group (CSA

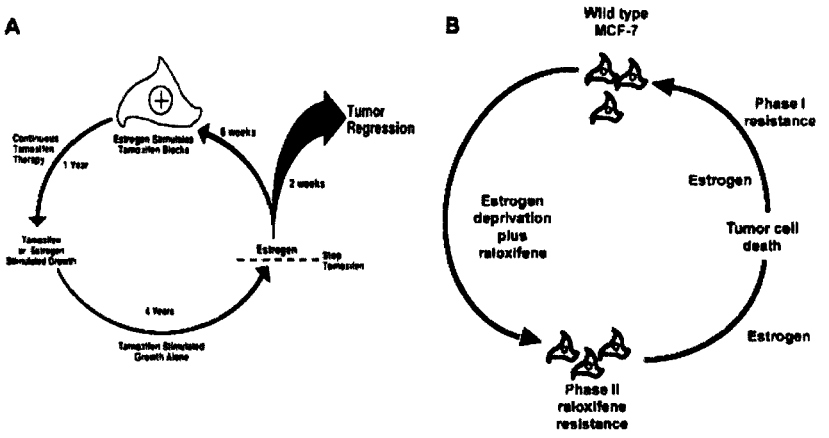

Figure 7. Proposed model of the evolution of acquired raloxifene resistance in ERα-positive MCF-7 breast cancer. On the left (Fig. 7A) is our original proposal from cyclical evolution of acquired resistance to tamoxifen in a clonal derivative (MCF-7 WS8) of wild-type MCF-7 cells originally acquired from Dr Dean Edwards (University of Texas, San Antonio, TX) in 1985. All steps in the cycle (17,18) were documented with experimental data in the peer reviewed literature. On the right (Fig. 7B) is a summary of our current results that illustrate the cyclical evolution of acquired resistance to raloxifene in wild-type MCF-7 cells (MCF-7 GMB) acquired from Dr Myles Brown (Dana Farber Cancer Center, Harvard University, Boston, MA) in 1995. The technique of employing an estrogen-deprived environment with raloxifene accelerates the evolution to phase II-acquired resistance where estradiol causes tumor regression. This process can be reversed through phase I-acquired resistance in a continuous estrogenic environment so tumor growth is again controlled by raloxifene treatment. Continuous raloxifene does again cause phase II-acquired resistance and exposes estrogen-induced tumor regression.

= 0.72 cm^2). The average CSA of the E_2 + RAL group was 0.62 cm^2, indicating that E_2 treatments significantly inhibited the RAL-stimulated tumor growth (p=0.03).

Discussion

In a previous study, we used a select clone of MCF7 cells (MCF7-WS8) (42) that is extremely sensitive to estrogen stimulation, to create an MCF7 raloxifene-resistant cell line *in vitro* (MCF7-RAL) (20). In a short-term growth experiment *in vivo* MCF7-RAL cells grew into tumors in response to raloxifene and tamoxifen but estradiol inhibited tumor growth (20). This biological response to SERMs and estradiol is classified as phase II SERM resistance (1). We have now addressed the question of the predictable creation and evolution of SERM resistance with raloxifene *in vivo* using a wild-type MCF7 cell line from a source that is external to our laboratory. The origins of the line (MCF7 GMB) were confirmed by genotyping (Fig. 1) and unlike the MCF7-WS8 cells were similar to the wild-type MCF7 from ATCC and the original MCF7 cells derived by Soule (43). We created a new MCF7-RAL cell line that is able not only to grow in response to raloxifene *in vitro* but eventually grow in response to raloxifene *in vivo* with phase II resistance, i.e. estradiol-inhibited tumor growth (Fig. 3). However, in this 10-year re-transplantation study *in vivo* we demonstrate the reversal of the biological characteristics of phase II anti-hormone-resistant tumor growth with long-term estradiol therapy to phase I resistance; i.e., estradiol- or raloxifene-stimulated growth, and then predominately estradiol-stimulated growth. Raloxifene now acts as an anti-estrogen, inhibiting estradiol-stimulated growth (Fig. 6C). Thus raloxifene has the potential to cause the classic evolution of SERM resistance in the clinical setting and reverse the process during long-term physiologic estrogen therapy. Nevertheless clinical studies need to be considered to evaluate the efficacy of estrogen on patients whose breast tumors develop during long-term raloxifene treatment to prevent osteoporosis (16). Current anti-hormonal therapies used for the treatment of breast cancer (tamoxifen or aromatase inhibitors) can develop acquired resistance in the clinical cells. The best clinical responses to estrogen are observed with high-dose (15 mg) DES therapy following exhaustive anti-hormonal therapy (27). Indeed, one patient had a complete response during the 5-year to DES therapy administered continuously and a further 5-year disease-free response following the cessation of therapy (44). In contrast, no complete responses were observed in the study of Ellis *et al* (28) probably because the patient population was not selected based on exhaustive anti-hormonal therapy but only failure of therapy following aromatase inhibitors. Experience in the laboratory demonstrates that long-term (>5 years) tamoxifen treatment is necessary to cause the evolution of tamoxifen resistance *in vivo* to expose the apoptotic actions of physiologic estrogen (18). Consistent with these observations, a profound antitumor effect was noted with physiologic estrogen after 10 years of alternating treatments with raloxifene and physiologic estrogen (Fig. 6D).

With regard to treatment strategies for SERM-resistant disease, it is important to note that the response to the injectable steroidal pure anti-estrogen fulvestrant is unpredictable (Fig. 6). At some stages of acquired resistance, fulvestrant acts as an antitumor agent but at other stimulates tumor growth (Fig. 6). This may in part explain the low reported efficacy of fulvestrant in clinical trials treating patients who already have acquired resistance to tamoxifen or aromatase inhibitors. However, it also appears that the recommended monthly doses of fulvestrant used clinically may be sub-optimal and in fact actually enhance tumor growth in tumors with phase II resistance with physiologic estrogen present (45). A recent clinical study on metastatic breast cancer demonstrates that doubling the monthly dose of fulvestrant enhances antitumor activity (46). In a laboratory study, an antitumor dose of fulvestrant in athymic animals implanted

with phase II-resistant tumors reversed the apoptotic actions of estrogen (45). In the present study, despite using repeated subcutaneous injections of fulvestrant weekly, tumor growth was enhanced in some tumor passages with long-term acquired resistance to raloxifene (Fig. 6D). It appears that the efficacy of fulvestrant may depend both upon bioavailability, pharmacokinetics and, as yet, unresolved pharmacodynamic factors of the steroidal antiestrogens at unknown targets within the tumor with acquired raloxifene resistance.

Two further conclusions emerged from the present study. The variant of MCF7 cells that is closely related to wild-type MCF-7 from ATCC could develop acquired resistance to raloxifene *in vitro* and the resulting cell line MCF7-RAL grew in response to either estradiol or raloxifene (Fig. 2). MCF7-RAL cells exhibited gene activation consistent with autonomous growth (Fig. 2C). The cells responded to estradiol both *in vitro* and *in vivo* as a growth stimulus but only developed raloxifene-stimulated tumors *in vivo* after 5 months of continuous treatment. This was confirmed by re-transplantation into raloxifene-treated ovariectomized athymic mice (Fig. 3B). In contrast to MCF7-RAL cells *in vitro*, estradiol is no longer a growth stimulus *in vivo* and completely inhibits tumor development (Fig. 3D). This new biology of estrogen action classifies the MCF7-RAL cells as phase II-resistant *in vivo*. Secondly, the observation that treatment with tamoxifen *in vivo* (Fig. 3A) did not result in tumor growth and that this MCF7 variant could not be used to develop acquired tamoxifen resistance *in vitro* (H. Liu, unpublished), was unusual and unanticipated based on previous studies over two decades. All cells died during incubation with 4-hydroxy-tamoxifen. This observation is currently under investigation as it may provide insight into the cytocidal actions of tamoxifen.

Based on this long-term study, and studies using prostate cancer cells, a general principle is emerging in cancer endocrinology. An androgen-independent cell line, LNCaP 104 R2 was derived from the androgen-dependent cell line, LNCaP 104 S (47). The LNCaP 104 R2 cells are androgen-independent, continue to express the androgen receptor (AR) and low concentrations of androgen in the media inhibited their growth. Implantation of the LNCap104-R2 cells in male athymic-castrated nude mice resulted in tumor growth, that was inhibited by implantation of testosterone capsules (48). In a subsequent study utilizing the LNCaP 104-R2 tumor model, Chuu *et al* (49) significantly impaired established tumor growth with androgen treatments; approximately 2 months post-cell injections. However, within 40 days of initiation of androgen treatments tumor growth resumed, which was a clear indication that the tumors adapted to the presence of the androgen and utilized it for growth. Subsequent androgen withdrawal inhibited tumor growth. These data are consistent with the assumption that androgen-dependent and androgen-independent tumor cells coexist in prostate cancer patients resulting in positive selection of androgen-independent tumor cells during androgen ablation therapies, resulting in androgen-independent growth. Therefore, intermittent androgen replacement therapy has been tested in recent years (50).

Nearly 20 years ago, we first described the antitumor potential of physiologic estrogen to destroy what is now known as phase II-acquired tamoxifen resistance (17) We noted that the interplay of apoptotic estrogen and tamoxifen would create a cyclical method for controlling the growth of ER-postitive breast cancer by purging with estrogen at the appropriate time and then continuing anti-hormone therapy (17). The cycles could be repeated. This original work is summarized in Fig. 7. Our current 10-year *in vitro* and *in vivo* study of the evolution of acquired raloxifene resistance was initiated to explore the potential of raloxifene to exhibit acquired resistance in breast cancer during the long-term treatment and prevention of osteoprosis (16). We conclude that the predictable evolution of acquired resistance to the SERM tamoxifen and estrogen deprivation (aromatase inhibitors) also occurs with raloxifene. The current conclusions are summarized in Fig. 7, following the creation of MCF7-RAL cells *in vitro* is a raloxifene/estrogen-free environment which was then transplanted into athymic mice. The development of phase II-acquired resistance i.e.: estrogen-induced apoptosis or estrogen-inhibited tumor growth (51) occurs with raloxifene and the principle is also true for the evolution of acquired androgen withdrawal in prostate cancer in the laboratory (47-49). Preliminary studies to translate these laboratory findings to aid patients have shown merit (27, 28,44). Further understanding of the mechanism of sex steroid-induced apoptosis (52) and the definition of vulnerable tumors following exhaustive anti-hormonal therapy have the potential to identify appropriate patient populations to amplify the effectiveness of a sex steroid apoptotic trigger in metastatic breast cancer and possibly prostate cancer (53).

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References

1. Jordan VC: Selective estrogen receptor modulation: Concept and consequences in cancer. Cancer Cell 5: 207-213, 2004.
2. Tamoxifen for early breast cancer: An overview of the randomised trials. Early breast cancer trialists' collaborative group. Lancet 351: 1451-1467, 1998.
3. Jordan VC: Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nat Rev Cancer 7: 46-53, 2007.
4. Fisher B, Costantino JP, Wickerham DL, *et al*: Tamoxifen for prevention of breast cancer: Report of the National Surgical Adjuvant Breast and Bowel project P-1 study. J Natl Cancer Inst 90: 1371-1388, 1998.
5. Gottardis MM and Jordan VC: Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 48: 5183-5187, 1988.
6. Gottardis MM, Wagner RJ, Borden EC and Jordan VC: Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro. Cancer Res 49: 4765-4769, 1989.
7. Black LJ, Jones CD and Falcone JF: Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 32: 1031-1036, 1983.

8. Clemens JA, Bennett DR, Black LJ and Jones CD: Effects of a new antiestrogen, keoxifene (ly156758), on growth of carcinogen-induced mammary tumors and on lH and prolactin levels. Life Sci 32: 2869-2875, 1983.
9. Gottardis MM and Jordan VC: Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea induced rat mammary carcinoma model. Cancer Res 47: 4020-4024, 1987.
10. Jordan VC, Phelps E and Lindgren JU: Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10: 31-35, 1987.
11. Jordan VC: Chemosuppression of breast cancer with tamoxifen: Laboratory evidence and future clinical investigations. Cancer Invest 6: 589-595, 1988.
12. Ettinger B, Black DM, Mitlak BH, *et al*: Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: Results from a 3-year randomized clinical trial. Multiple outcomes of raloxifene evaluation (more) investigators. JAMA 282: 637-645, 1999.
13. Cummings SR, Eckert S, Krueger KA, *et al*: The effect of raloxifene on risk of breast cancer in postmenopausal women: Results from the more randomized trial. Multiple outcomes of raloxifene evaluation. JAMA 281: 2189-2197, 1999.
14. Vogel VG, Costantino JP, Wickerham DL, *et al*: Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: The NSABP study of tamoxifen and raloxifene (STAR) P-2 trial. JAMA 295: 2727-2741, 2006.
15. Barrett-Connor E, Mosca L, Collins P, *et al*: Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 355: 125-137, 2006.
16. Martino S, Cauley JA, Barrett-Connor E, *et al*: Continuing outcomes relevant to evista: Breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 96: 1751-1761, 2004.
17. Wolf DM and Jordan VC: A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127: 23-33, 1993.
18. Yao K, Lee ES, Bentrem DJ, *et al*: Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6: 2028-2036, 2000.
19. O'Regan RM, Osipo C, Ariazi E, *et al*: Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. Clin Cancer Res 12: 2255-2263, 2006.
20. Liu H, Lee ES, Gajdos C, *et al*: Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 95: 1586-1597, 2003.
21. Song RX, Mor G, Naftolin F, *et al*: Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 93: 1714-1723, 2001.
22. Song RX, Zhang Z, Mor G and Santen RJ: Down-regulation of BCL-2 enhances estrogen apoptotic action in long-term estradiol-depleted ER(+) breast cancer cells. Apoptosis 10: 667-678, 2005.
23. Lewis JS, Meeke K, Osipo C, *et al*: Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97: 1746-1759, 2005.
24. Lewis JS, Osipo C, Meeke K and Jordan VC: Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 94: 131-141, 2005.
25. Kennedy BJ: Diethylstilbestrol versus testosterone propionate therapy in advanced breast cancer. Surg Gynecol Obstet 120: 1246-1250, 1965.
26. Haddow A, Watkinson JM, Paterson E and Koller PC: Influence of synthetic oestrogens on advanced malignant disease. Br Med J 2: 393-398, 1944.
27. Lonning PE, Taylor PD, Anker G, *et al*: High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat 67: 111-116, 2001.
28. Ellis MJ, Gao F, Dehdashti F, *et al*: Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: A phase 2 randomized study. JAMA 302: 774-780, 2009.
29. Lins AM, Micka KA, Sprecher CJ, *et al*: Development and population study of an eight-locus short tandem repeat (STR) multiplex system. J Forensic Sci 43: 1168-1180, 1998.
30. Jamerson MH, Johnson MD, Korsmeyer SJ, Furth PA and Dickson RB: Bax regulates c-myc-induced mammary tumour apoptosis but not proliferation in MMTV-c-myc transgenic mice. Br J Cancer 91: 1372-1379, 2004.
31. Labarca C and Paigen K: A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 102: 344-352, 1980.
32. Dardes RC, Schafer JM, Pearce ST, Osipo C, Chen B and Jordan VC: Regulation of estrogen target genes and growth by selective estrogen-receptor modulators in endometrial cancer cells. Gynecol Oncol 85: 498-506, 2002.
33. Robinson SP and Jordan VC: Antiestrogenic action of toremifene on hormone-dependent, -independent, and heterogeneous breast tumor growth in the athymic mouse. Cancer Res 49: 1758-1762, 1989.
34. O'Regan RM, Cisneros A, England GM, *et al*: Effects of the antiestrogens tamoxifen, toremifene, and ICI 182,780 on endometrial cancer growth. J Natl Cancer Inst 90: 1552-1558, 1998.
35. Ariazi EA, Lewis-Wambi JS, Gill SD, *et al*: Emerging principles for the development of resistance to antihormonal therapy: Implications for the clinical utility of fulvestrant. J Steroid Biochem Mol Biol 102: 128-138, 2006.
36. Ariazi EA, Kraus RJ, Farrell ML, Jordan VC and Mertz JE: Estrogen-related receptor alpha1 transcriptional activities are regulated in part via the erbb2/her2 signaling pathway. Mol Cancer Res 5: 71-85, 2007.
37. Rae JM, Johnson MD, Scheys JO, Cordero KE, Larios JM and Lippman ME: Greb 1 is a critical regulator of hormone dependent breast cancer growth. Breast Cancer Res Treat 92: 141-149, 2005.
38. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2-(delta)(delta)ct method. Methods 25: 402-408, 2001.
39. Ariazi EA, Clark GM and Mertz JE: Estrogen-related receptor alpha and estrogen-related receptor gamma associate with unfavorable and favorable biomarkers, respectively, in human breast cancer. Cancer Res 62: 6510-6518, 2002.
40. Pink JJ and Jordan VC: Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. Cancer Res 56: 2321-2330, 1996.
41. Rae JM, Creighton CJ, Meck JM, Haddad BR and Johnson MD: MDA-MB-435 cells are derived from M14 melanoma cells - loss for breast cancer, but a boon for melanoma research. Breast Cancer Res Treat 104: 13-19, 2007.
42. Jiang SY, Langan-Fahey SM, Stella AL, McCague R and Jordan VC: Point mutation of estrogen receptor (ER) in the ligand-binding domain changes the pharmacology of anti-estrogens in ER-negative breast cancer cells stably expressing complementary DNAs for ER. Mol Endocrinol 6: 2167-2174, 1992.
43. Brooks SC, Locke ER and Soule HD: Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J Biol Chem 248: 6251-6253, 1973.
44. Lonning PE: Additive endocrine therapy for advanced breast cancer - back to the future. Acta Oncol 48: 1092-1101, 2009.
45. Osipo C, Gajdos C, Liu H, Chen B and Jordan VC: Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 95: 1597-1608, 2003.
46. Robertson JF, Llombart-Cussac A, Rolski J, *et al*: Activity of fulvestrant 500 mg versus anastrozole 1 mg as first-line treatment for advanced breast cancer: Results from the first study. J Clin Oncol 27: 4530-4535, 2009.
47. Kokontis J, Takakura K, Hay N and Liao S: Increased androgen receptor activity and altered c-myc expression in prostate cancer cells after long-term androgen deprivation. Cancer Res 54: 1566-1573, 1994.
48. Umekita Y, Hiipakka RA, Kokontis JM and Liao S: Human prostate tumor growth in athymic mice: Inhibition by androgens and stimulation by finasteride. Proc Natl Acad Sci USA 93: 11802-11807, 1996.
49. Chuu CP, Hiipakka RA, Fukuchi J, Kokontis JM and Liao S: Androgen causes growth suppression and reversion of androgen-independent prostate cancer xenografts to an androgen-stimulated phenotype in athymic mice. Cancer Res 65: 2082-2084, 2005.
50. Abrahamsson PA: Potential benefits of intermittent androgen suppression therapy in the treatment of prostate cancer: A systematic review of the literature. Eur Urol (In press).
51. Jordan VC: The 38th David A. Karnofsky lecture: The paradoxical actions of estrogen in breast cancer - survival or death? J Clin Oncol 26: 3073-3082, 2008.
52. Maximov PY, Lewis-Wambi JS and Jordan VC: The paradox of oestradiol-induced breast cancer cell growth and apoptosis. Curr Signal Transduct Ther 4: 88-102, 2009.
53. Jordan VC: A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: The origins of targeted therapy and chemoprevention. Cancer Res 69: 1243-1254, 2009.

Research Article
See perspectives pp. 681, 686, and 689

Cancer Prevention Research

Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing Breast Cancer

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Abstract

The selective estrogen-receptor modulator (SERM) tamoxifen became the first U.S. Food and Drug Administration (FDA)–approved agent for reducing breast cancer risk but did not gain wide acceptance for prevention, largely because it increased endometrial cancer and thromboembolic events. The FDA approved the SERM raloxifene for breast cancer risk reduction following its demonstrated effectiveness in preventing invasive breast cancer in the Study of Tamoxifen and Raloxifene (STAR). Raloxifene caused less toxicity (versus tamoxifen), including reduced thromboembolic events and endometrial cancer. In this report, we present an updated analysis with an 81-month median follow-up. STAR women were randomly assigned to receive either tamoxifen (20 mg/d) or raloxifene (60 mg/d) for 5 years. The risk ratio (RR; raloxifene:tamoxifen) for invasive breast cancer was 1.24 (95% confidence interval [CI], 1.05–1.47) and for noninvasive disease, 1.22 (95% CI, 0.95–1.59). Compared with initial results, the RRs widened for invasive and narrowed for noninvasive breast cancer. Toxicity RRs (raloxifene:tamoxifen) were 0.55 (95% CI, 0.36–0.83; $P = 0.003$) for endometrial cancer (this difference was not significant in the initial results), 0.19 (95% CI, 0.12–0.29) for uterine hyperplasia, and 0.75 (95% CI, 0.60–0.93) for thromboembolic events. There were no significant mortality differences. Long-term raloxifene retained 76% of the effectiveness of tamoxifen in preventing invasive disease and grew closer over time to tamoxifen in preventing noninvasive disease, with far less toxicity (e.g., highly significantly less endometrial cancer). These results have important public health implications and clarify that both raloxifene and tamoxifen are good preventive choices for postmenopausal women with elevated risk for breast cancer. *Cancer Prev Res; 3(6); 696–706. ©2010 AACR.*

Introduction

Despite improvements in the detection and treatment of breast cancer, this disease still accounted for 192,000 new cases and 40,000 deaths in the United States in 2009 (1). Therefore, the concept of preventing the development of invasive breast cancer remains an attractive one. The selective estrogen-receptor modulator (SERM) tamoxifen has well-known benefits in the treatment of receptor-positive invasive breast cancer (2) and has been shown to be an effective chemoprevention therapy (3–6). However, in spite of its impressive efficacy in the prevention of breast cancer, tamoxifen has not been widely used for prevention because, in large part, of the increased risk of endometrial

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Note: Clinical Trial Registration for NSABP P-2: NCT00003906. The work described in this manuscript is original research and has not been previously published. The following recent related work has been published: ref. 9 and Land SR et al., JAMA 2006;295:2742–51. For earlier related publications, please see the references.

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cancer and thromboembolic events associated with its use. Another SERM, raloxifene, has been shown to reduce the incidence of breast cancer in a series of clinical trials designed primarily to evaluate it for treatment and prevention of osteoporosis in postmenopausal women (7, 8).

The National Surgical Adjuvant Breast and Bowel Project (NSABP) protocol P-2, the Study of Tamoxifen and Raloxifene (STAR), directly compared tamoxifen with raloxifene in 19,747 healthy postmenopausal women at an increased risk for development of breast cancer. With 47 months of follow-up, the initial STAR results demonstrated no significant difference between the two trial arms in the incidence of invasive breast cancer, both with an estimated decreased incidence of approximately 50% (vs untreated women; ref. 9). Raloxifene did not appear to be as effective as tamoxifen in reducing the incidence of noninvasive breast cancer (ductal carcinoma *in situ* [DCIS] and lobular carcinoma *in situ* [LCIS] combined). The toxicity and side-effect evaluations favored the raloxifene group, in which women had significantly fewer deep-vein thromboses and pulmonary emboli, cataracts, and hysterectomies for benign disease. The raloxifene group also had a nonsignificant reduction in endometrial cancer. This report provides updated STAR results.

Materials and Methods

STAR was a two-arm, randomized, double-blinded trial of tamoxifen versus raloxifene for the reduction of breast cancer incidence; participants and their physicians were unaware of the treatment that was being administered until the trial was unblinded in April 2006. All participants provided written informed consent that was reviewed and approved by the National Cancer Institute and the institutional review boards of all participating institutions. The details of the trial methodology, including the definition of endpoints and the methods used for randomization, schedule of patient follow-up, patient testing, and trial monitoring, are described in the initial report of 2006, for which the data were cut off as of December 31, 2005 (9). The update in the present report is based on a cut-off date of March 31, 2009, providing a median follow-up of 81 months. We focus here on updating findings for the primary endpoint (incidence of invasive breast cancer) and for all key secondary endpoints, including noninvasive breast cancer, endometrial and other cancers, and vascular-related events. In the original STAR report, no difference between treatment groups was noted for the secondary endpoints ischemic heart disease, stroke, and osteoporotic fractures. Because our new analyses confirmed that this lack of differences continued in the longer term, these endpoints are not included in this report.

Participant characteristics

Only women who were postmenopausal, at least 35 years of age, and who had a 5-year predicted breast cancer risk of at least 1.66% were eligible for STAR. The risk determination was based on the Gail model, as modified and applied in the Breast Cancer Prevention Trial (BCPT P-1; ref. 10). Participants were also required to meet the following criteria: not taking either tamoxifen or raloxifene, hormone therapy, oral contraceptives, or androgens for at least 3 months before randomization; not currently taking warfarin or cholestyramine; no history of stroke, transient ischemic attack, pulmonary embolism, or deep-vein thrombosis; no atrial fibrillation, uncontrolled diabetes, or uncontrolled hypertension; no psychiatric condition that would interfere with adherence; a performance status that would not restrict normal activity; and no history of previous malignancy except basal cell or squamous cell carcinoma of the skin, carcinoma *in situ* of the cervix, or LCIS of the breast. Eligible women were randomly assigned to receive either 20 mg/d of tamoxifen plus placebo, or 60 mg/d of raloxifene plus placebo for 5 years; the placebo tablets were necessary to maintain the double blinding of treatment assignment because the formulations of tamoxifen and raloxifene tablets were dissimilar.

A total 19,747 women were randomly assigned to one of the two groups between July 1, 1999, and November 4, 2004, and 19,471 of these women (9,726 tamoxifen; 9,745 raloxifene) were included in the analysis of the original report. Two hundred seventy-four women were not included because of a lack of follow-up information (146 tamoxifen; 128 raloxifene). Two other women (in the raloxifene group) were excluded because they had received a prophylactic bilateral mastectomy before randomization and were not at risk for the development of invasive breast cancer. Since the time of the initial report, follow-up information was collected on 20 of the women (10 tamoxifen; 10 raloxifene) who lacked follow-up information at the time of the original report. One woman (in the raloxifene group) in the original report has been excluded from the follow-up analyses because she was discovered to have been diagnosed with invasive breast cancer before randomization. Therefore, this update report includes the findings for 19,490 women—9,736 in the tamoxifen group and 9,754 in the raloxifene group.

The characteristics of the participants included in the current analysis are shown in Table 1. The mean age at entry to the trial was 58.5 years (SD, 7.4). Nine percent of the women were younger than 50 years, 49.8% were between ages 50 and 59, 32.4% were between ages 60 and 69, and 8.8% were aged 70 years or older. The percentages of racial/ethnic groups were as follows: White = 93.5%, African American = 2.4%, Hispanic = 2.0%, and "other" = 2.1%. More than half (51.5%) of the participants had undergone a hysterectomy before entry to the study; over 70% had a first-degree female relative with a history of breast cancer; and 23% had a history of atypical hyperplasia of the breast. The mean 5-year predicted breast cancer risk at entry was 4.03% (SD, 2.2), subdivided as follows: 30.2% with risks between 2.01% and 3.00%, 31.4% between 3.01% and 5.00%, and 27.3% greater than 5.00%. The mean lifetime risk was 14.73% (SD, 7.4).

Table 1. Characteristics at entry to the NSABP STAR Trial (P-2) for women included in the STAR-update analyses

Participant characteristics	Tamoxifen		Raloxifene	
	No.	%	No.	%
Age (years)				
≤49	884	9.1	878	9.0
50–59	4,856	49.9	4,855	49.8
60–69	3,137	32.2	3,174	32.5
≥70	859	8.8	847	8.7
Race/ethnicity				
White	9,105	93.5	9,115	93.4
African-American	233	2.4	243	2.5
Hispanic	192	2.0	193	2.0
Other	206	2.1	203	2.1
No. 1° relatives with breast cancer				
0	2,838	29.1	2,791	28.6
1	5,046	51.8	5,135	52.6
2	1,532	15.7	1,561	16.0
≥3	320	3.3	267	2.7
History of hysterectomy				
No	4,739	48.7	4,717	48.4
Yes	4,997	51.3	5,037	51.6
History of lobular carcinoma *in situ*				
No	8,844	90.8	8,865	90.9
Yes	892	9.2	889	9.1
History of breast atypical hyperplasia				
No	7,545	77.5	7,513	77.0
Yes	2,191	22.5	2,241	23.0
5-year predicted breast cancer risk (%)*				
≤2.00	1,055	10.8	1,102	11.3
2.01-3.00	2,993	30.7	2,893	29.7
3.01-5.00	3,042	31.2	3,086	31.6
≥5.01	2,646	27.2	2,673	27.4
Total	9,736	100.0	9,754	100.0

Abbreviation: NSABP STAR, National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene.
*Determined by the Gail model.

The mean duration of treatment was 43.5 months (SD, 20.7) for the tamoxifen group and 46.8 months (SD, 20.0) for the raloxifene group. Participant adherence to 5 years of therapy was within the limits anticipated when the trial was designed. Also, since the original report and unblinding of treatment assignment, any woman who had not completed her 5-year course of tamoxifen was offered the option to switch to raloxifene for the remaining portion of her treatment course. A total of 879 women chose this option.

Statistical analyses

Analyses included all randomly assigned at-risk women for whom follow-up information was available. All analyses were based on the intention-to-treat principle and used the treatment assignment determined at randomization, regardless of the treatment status at the time of analysis. Rates per 1,000 person-years for each of the study endpoints were determined for each treatment group by dividing the number of events within each treatment group by the total number of event-specific person-years of follow-up within the group. Comparisons of rates between treatment groups were based on the risk ratio (RR) and the 95% confidence interval (CI) for the RR. The RR was determined as the rate in the raloxifene group divided by the rate in the tamoxifen group. The 95% CI for each RR was determined assuming a Poisson distribution, conditioning on the total number of events and the person-years at risk. RRs for which the 95% CI did not include 1.00 were considered to be statistically significant. Plots of the cumulative incidence over time of follow-up were also developed. The cumulative incidence accounted for the competing risk of death (11). *P*-values to assess statistically significant differences between treatment group–specific cumulative incidence curves were determined by the log-rank test. All *P*-values are 2-sided using $P < 0.05$ to determine statistical significance. Analyses were performed using SAS version 9.1 software (SAS Institute, Inc.).

Results

Breast cancer

The updated findings for invasive breast cancer are shown in the left panel of Fig. 1. In contrast with the results documented in the original report, there is now a significant difference between the treatment groups, with 310 cases of invasive breast cancer in the raloxifene group and 247 in the tamoxifen group. The invasive breast cancer RR (raloxifene:tamoxifen) is 1.24 (95% CI, 1.05–1.47), indicating that the rate in the raloxifene group is about 24% higher than the rate in the tamoxifen group. As demonstrated in the BCPT, compared with placebo, tamoxifen reduces the risk of invasive breast cancer by about 50% (3). Therefore, if there were no breast cancer RR effect from raloxifene, the expected rate of breast cancer in the raloxifene group would be about twice the rate in the tamoxifen group, yielding an RR of 2.00. Based on this information and the actual 1.24 RR observed in this study, one can extrapolate that raloxifene is about 76% as effective as tamoxifen in reducing breast cancer risk [{(2.00–1.24)/(2.00–1.00)} × 100 = 76%]. Then, compared with placebo, raloxifene would reduce the risk of invasive breast cancer by about 38% (50% × 76% = 38%), versus the 50% reduction seen with tamoxifen.

The rate of invasive breast cancer by participant demographic characteristics is provided in Table 2. The number of events and the point estimates of the rate are higher in the raloxifene arm than in the tamoxifen arm for all categories of participant characteristics, and there is no indication of a quantitative interaction between treatment and any of the participant characteristics.

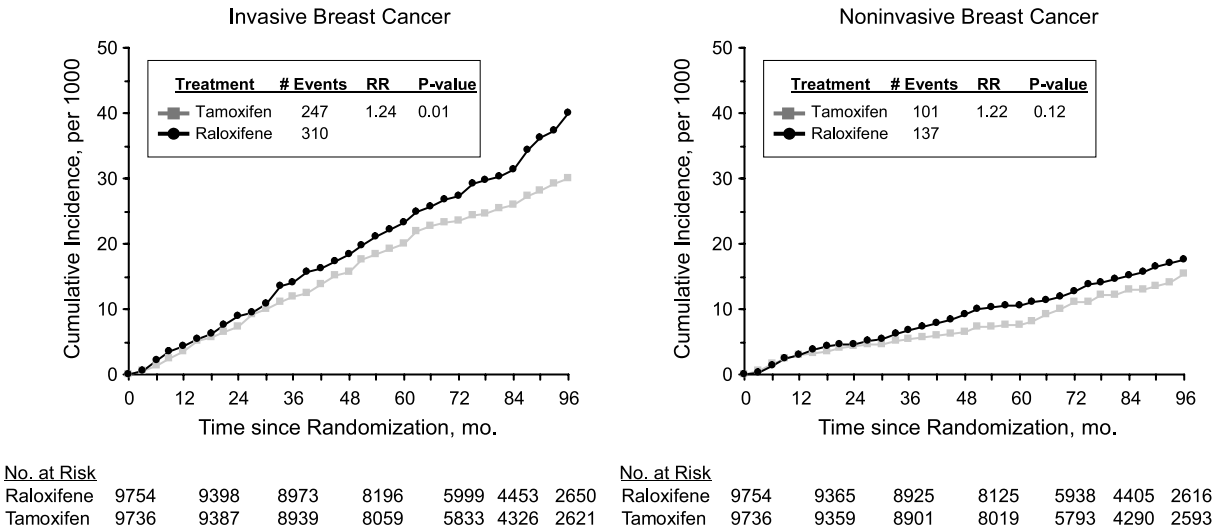

Fig. 1. Cumulative incidences of invasive and noninvasive breast cancer.

In our original report, the difference between treatment groups for the rate of noninvasive breast cancer was borderline for statistical significance (RR = 1.40; 95% CI, 0.98–2.00; P = 0.052). Currently, the difference between treatment groups for this event is less than originally seen (right panel of Fig. 1). There are 137 cases in the raloxifene group compared with 111 in the tamoxifen group, for an RR of 1.22 (95% CI, 0.95–1.59). The difference between

Table 2. Annual rates of invasive breast cancer—NSABP STAR Trial (P-2)

Participant characteristic at baseline	Number of events		Rate per 1000			RR*	RR (95% CI)
	Tamoxifen	Raloxifene	Tamoxifen	Raloxifene	Difference†		
Age at entry (years)							
≤49	10	15	1.84	2.80	−0.96	1.53	0.64–3.80
50-59	125	155	4.09	5.03	−0.94	1.23	0.97–1.57
≥60	112	140	4.47	5.48	−1.01	1.22	0.95–1.58
History of lobular carcinoma *in situ*							
No	197	253	3.54	4.50	−0.96	1.27	1.05–1.54
Yes	50	57	9.14	10.34	−1.20	1.13	0.76–1.69
History of atypical hyperplasia							
No	187	218	3.90	4.52	−0.62	1.16	0.95–1.42
Yes	60	92	4.58	6.79	−2.21	1.48	1.06–2.09
5-year predicted breast cancer risk (%)							
≤3.00	61	81	2.39	3.21	−0.82	1.34	0.95–1.90
3.01-5.00	84	91	4.43	4.63	−0.20	1.05	0.77–1.42
≥5.01	102	138	6.13	8.17	−2.04	1.33	1.02–1.74
No. 1^0 relatives with breast cancer							
0	82	105	4.77	6.17	−1.40	1.29	0.96–1.75
1	112	135	3.51	4.10	−0.59	1.17	0.90–1.51
≥2	53	70	4.44	5.96	−1.52	1.34	0.93–1.96
Total	247	310	4.04	5.02	−0.98	1.24	1.05–1.47

Abbreviations: CI, confidence interval; NSABP STAR, National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene; RR, risk ratio.

*Risk ratio for women in the raloxifene group compared to women in the tamoxifen group.

†Rate in the tamoxifen group minus rate in the raloxifene group.

Table 3. Annual rates of noninvasive breast cancer and uterine disease/hysterectomy—NSABP STAR Trial (P-2)

Disease/uterine event type	Events, *n*		Rate per 1,000			RR*	RR (95% CI)
	Tamoxifen	Raloxifene	Tamoxifen	Raloxifene	Difference†		
Noninvasive breast cancer							
DCIS	70	86	1.15	1.40	−0.25	1.22	0.88–1.69
LCIS	33	34	0.54	0.55	−0.01	1.02	0.61–1.70
Mixed	8	17	0.13	0.28	−0.15	2.11	0.86–5.64
Total	111	137	1.83	2.23	−0.40	1.22	0.95–1.59
Uterine disease and hysterectomy‡							
Invasive Cancer	65	37	2.25	1.23	1.02	0.55	0.36–0.83
Hyperplasia§	126	25	4.40	0.84	3.56	0.19	0.12–0.29
Without atypia§	104	21	3.63	0.70	2.93	0.19	0.11–0.31
With atypia§	22	4	0.77	0.13	0.64	0.17	0.04–0.51
Hysterectomy during follow-up	349	162	12.08	5.41	6.67	0.45	0.37–0.54

Abbreviations: CI, confidence interval; DCIS, ductal carcinoma *in situ*; LCIS, lobular carcinoma *in situ*; NSABP STAR, National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene; RR, risk ratio.

*Risk ratio for women in the raloxifene group compared with women in the tamoxifen group.

†Rate in the tamoxifen group minus rate in the raloxifene group.

‡Women at risk were those with an intact uterus at entry (see Table 1).

§Among women not diagnosed with uterine cancer.

treatment groups in noninvasive breast cancer appears to be limited to cases of pure DCIS or cases of mixed DCIS and LCIS (top portion of Table 3). There was no difference between the groups for pure LCIS cases; the numbers of women diagnosed with this condition were 33 (tamoxifen) and 34 (raloxifene; RR = 1.02; 95% CI, 0.61–1.70). In parallel with the analysis presented above for invasive breast cancer, tamoxifen was shown in the BCPT to reduce the risk of noninvasive breast cancer by about 50%. Therefore, if there were no noninvasive breast cancer risk reduction effect of raloxifene, the expected rate of noninvasive breast cancer in the raloxifene group would be about twice the rate in the tamoxifen group, yielding an RR (raloxifene:tamoxifen) of 2.00. Based on this information and the actual 1.22 RR observed in this study, one can extrapolate that raloxifene is about 78% as effective as tamoxifen in reducing noninvasive breast cancer risk [{(2.00-1.22)/(2.00-1.00)} × 100 = 78%]. Then, compared with placebo, raloxifene reduces the risk of noninvasive breast cancer by about 39% (50% × 78% = 39%).

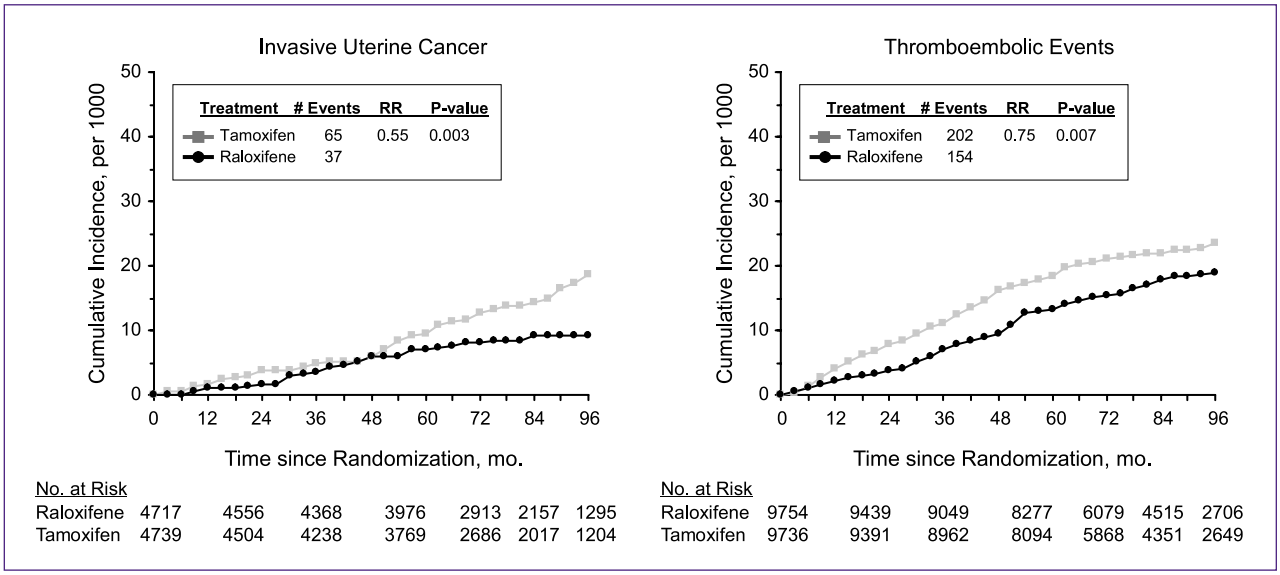

Fig. 2. Cumulative incidences of invasive uterine cancer and thromboembolic events.

Uterine disease

Invasive uterine cancer and uterine hyperplasia are well-established toxicities associated with tamoxifen treatment. When compared with tamoxifen, raloxifene does not have such a profile (bottom portion of Table 3). The incidence of invasive uterine cancer is significantly lower in the raloxifene group (P = 0.003; left panel of Fig. 2). The annual average rate per 1,000 was 2.25 in the tamoxifen group compared with 1.23 in the raloxifene group (RR = 0.55; 95% CI, 0.36–0.83). In our original report, the difference between treatment groups for the rate of invasive uterine cancer was not statistically significant. The average annual incidence rate of uterine hyperplasia, the majority of which was hyperplasia without atypia, was 5 times higher in the tamoxifen group (4.40 per 1,000) than in the raloxifene group (0.84 per 1,000; RR = 0.19; 95% CI, 0.12–0.29). The number of hysterectomies performed in the tamoxifen group (349), including those done for benign disease, was more than double that performed in the raloxifene group (162; RR = 0.45; 95% CI, 0.37–0.54).

Other cancers

Comparisons between treatment groups for the average annual rates of invasive cancer at sites other than the breast or uterus are presented in Table 4. These data are consistent with those in the original report, which also showed no significant differences for cancers other than in breast or uterus cancer.

Thromboembolic events

Pulmonary embolism and deep-vein thrombosis are other toxicities with a well-recognized association with tamoxifen treatment. The incidence of such events was

Table 4. Annual rates of site-specific invasive cancer cases other than breast and uterine cancer—NSABP STAR Trial (P-2)

Site of cancer	Events, *n*		Rate per 1000			RR*	RR (95% CI)
	Tamoxifen	Raloxifene	Tamoxifen	Raloxifene	Difference†		
Adrenal gland	0	1	0	0.02	–0.02	—	—
Bone/cartilage/connective tissue	3	4	0.05	0.06	–0.01	1.32	0.22–8.98
Buccal cavity and pharynx	4	6	0.06	0.10	–0.04	1.48	0.35–7.13
Cervix	3	0	0.05	0	0.05	—	—
Colorectal	48	45	0.78	0.72	0.06	0.93	0.60 to 1.42
Esophagus	2	0	0.03	0	0.03	—	—
Eye	1	1	0.02	0.02	0	0.99	0.01–77.48
Gallbladder	5	2	0.08	0.03	0.05	0.39	0.04–2.41
Kidney	14	21	0.23	0.34	–0.11	1.48	0.72–3.15
Larynx	0	1	0	0.02	–0.02	—	—
Leukemia/other lymph/hemato	60	53	0.97	0.85	0.12	0.87	0.59–1.28
Liver	7	2	0.11	0.03	0.08	0.28	0.03–1.48
Lung, trachea, bronchus	57	64	0.92	1.02	–0.10	1.11	0.76–1.61
Nasal/middle ear/sinuses	1	1	0.02	0.02	0	0.99	0.01–77.48
Nervous system	9	10	0.15	0.16	–0.01	1.10	0.40–3.05
Other gyn	2	2	0.03	0.03	0	0.99	0.07–13.62
Ovary	21	34	0.50	0.79	–0.29	1.58	0.89–2.86
Pancreas	12	11	0.19	0.18	0.01	0.90	0.36–2.24
Retroperitoneum	7	4	0.11	0.06	0.05	0.56	0.12–2.22
Skin	25	24	0.40	0.38	0.02	0.95	0.52–1.73
Small intestine	0	2	0	0.03	–0.03	—	—
Spleen	0	2	0	0.03	–0.03	—	—
Stomach	5	1	0.08	0.02	0.06	0.20	0.004–1.76
Thyroid gland	18	32	0.29	0.51	–0.22	1.76	0.96–3.32
Urinary bladder	15	12	0.24	0.19	0.05	0.79	0.34–1.81
Site unspecified/unspecified nature	15	19	0.24	0.30	–0.06	1.25	0.60–2.64
Secondary/uncertain	4	5	0.06	0.08	–0.02	1.23	0.27–6.22

Abbreviations: CI, confidence interval; gyn, gynecologic; hemato, hematopoietic; lymph, lymphatic; NSABP STAR, National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene; RR, risk ratio.

*Risk ratio for women in the raloxifene group compared with women in the tamoxifen group.

†Rate in the tamoxifen group minus rate in the raloxifene group.

Table 5. Rates of thromboembolic events, cataracts, and cataracts surgery—NSABP STAR Trial (P-2)

Type of event	Events, *n*		Rate per 1,000			RR*	RR (95% CI)
	Tamoxifen	Raloxifene	Tamoxifen	Raloxifene	Difference†		
Thromboembolic events	202	154	3.30	2.47	0.83	0.75	0.60–0.93
Pulmonary embolism	84	68	1.36	1.09	0.27	0.80	0.57–1.11
Deep-vein thrombosis	118	86	1.93	1.38	0.55	0.72	0.54–0.95
Cataracts and Cataract Surgery							
Developed cataracts during follow-up‡	739	603	14.58	11.69	2.89	0.80	0.72–0.89
Developed cataracts and had cataract surgery‡	575	462	11.18	8.85	2.33	0.79	0.70–0.90

Abbreviations: CI, confidence interval; NSABP STAR, National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene; RR, risk ratio.

*Risk ratio for women in the raloxifene group compared to women in the tamoxifen group.

†Rate in the tamoxifen group minus rate in the raloxifene group.

‡Women at risk were those with no prior history of cataracts at entry (8,341 and 8,336 tamoxifen and raloxifene participants, respectively).

significantly elevated in the tamoxifen group compared with the raloxifene group (P = 0.007; right panel of Fig. 2 and top of Table 5). The average annual rates of thromboembolic events were 3.30 per 1,000 (tamoxifen) and 2.47 per 1,000 (raloxifene; RR = 0.75; 95% CI, 0.60–0.93).

Cataracts

When compared with the results in the placebo group in the BCPT, tamoxifen increased the incidence of cataract development and cataract surgery (3). Raloxifene does not have this effect. In the original report of STAR, cataract events were significantly elevated in the tamoxifen group compared with the raloxifene group, and these differences persisted in the current analysis (bottom of Table 5). The rate of cataract development (RR = 0.80; 95% CI, 0.72–0.89) and the rate of cataract surgery (RR = 0.79; 95% CI, 0.70–0.90) are about 20% less in the raloxifene group than in the tamoxifen group.

Mortality

The number of deaths observed during follow-up is shown in Table 6. There is no statistically significant mortality difference between the treatment groups. Overall, 236 deaths occurred in the tamoxifen group and 202 deaths in the raloxifene group, for an RR of 0.84, which was not statistically significant (95% CI, 0.70–1.02). When the differences between treatment groups are compared by specific causes of death, the data are consistent with variation due to chance.

Discussion

Tamoxifen has been shown to reduce the risk of contralateral breast cancer in women with invasive breast cancer and DCIS (12, 13). The benefit appears to be very durable. After 2 to 5 years of adjuvant tamoxifen, the contralateral breast cancer reduction continued through at least 15 years of follow-up (2, 14). In primary prevention trials of tamoxifen in women at risk for the future development of breast cancer, 5 to 8 years of tamoxifen significantly reduced the incidence of invasive breast cancer, and this benefit persisted for at least 7 to 12 years (6, 15, 16).

Raloxifene has also been shown to reduce the incidence of primary invasive breast cancer (compared with placebo). The Multiple Outcomes of Raloxifene Evaluation (MORE) trial randomized 7,704 postmenopausal women with osteoporosis; with a median follow-up of 45 months, raloxifene (given for 4 years) reduced the incidence of breast cancer by 76% (RR = 0.24; 95% CI, 0.13–0.44; ref. 7). In the Raloxifene Use for the Heart (RUTH) trial, 10,101 postmenopausal women with coronary heart disease or multiple risk factors for this disease were assigned to either raloxifene (60 mg/d) or placebo. With 5.6 years median follow-up, raloxifene reduced the incidence of invasive breast cancer by a significant 44% (hazard ratio [HR] = 0.56; 95% CI, 0.38–0.83; ref. 17). As detailed in the initial report of STAR, after a median follow-up of 47 months, raloxifene was as effective as tamoxifen in reducing the risk of invasive breast cancer. The updated results reported here demonstrate that after a median follow-up of 81 months, which represents 60 months of treatment plus an additional 21 months of follow-up, raloxifene no longer appears to be as effective as tamoxifen in preventing primary invasive breast cancer. Raloxifene does appear, however, to retain approximately 76% of tamoxifen's effectiveness, which represents as much as a 38% reduction in invasive breast cancer (compared with an untreated group). The initial STAR report also suggested that raloxifene may not be as effective as tamoxifen in preventing the development of noninvasive breast cancers (LCIS and DCIS combined). The updated results show that the difference between the treatment groups has narrowed, and much like its effect against invasive breast cancer, raloxifene is about 78% as effective as tamoxifen in reducing the risk of noninvasive breast cancer. Patients with a history of LCIS or atypical hyperplasia of the breast

have a 4-fold to 10-fold increased risk of subsequent invasive disease, and tamoxifen and raloxifene were equally effective in reducing this risk in the initially reported STAR results. The current analyses indicate that this equality is no longer the case for STAR women with a history of atypical hyperplasia (RR = 1.48; 95% CI = 1.06-2.09), although results for the LCIS group remain similar to those reported originally (RR = 1.13; 95% CI, 0.76–1.69).

Only a slight difference was evident between treatment groups in the cumulative incidence of both invasive and noninvasive breast cancer (Fig. 1) through the first 20 months of the study. After 30 months, a clear separation of the treatment curves was observed, with a higher cumulative incidence of both invasive and noninvasive breast cancer in the raloxifene group. Why are we seeing this apparent diminution of raloxifene's benefits with longer follow-up? When the initial STAR results were published, all participants were notified of the results, and women who were still receiving tamoxifen were offered the option of crossing over to raloxifene therapy for the remainder of their 5 years of treatment. Only 879 women (9%) chose this option. The cross-over is unlikely to fully explain our updated findings.

Is nonadherence with the medication an issue? Only about 2% of orally administered raloxifene becomes bioavailable, and the biological half-life of raloxifene is much shorter than that of tamoxifen. Missing a day or 2 of raloxifene may result in a greater reduction of effectiveness than would similarly skipped doses of tamoxifen. However, overall adherence to protocol medication, as measured by pill counts, was similar in the two groups, and the protocol medication drop-off rates were higher in the tamoxifen group (38.9% versus 27.4%), indicating that nonadherence or drop-offs in the raloxifene group do not provide the answer. Raloxifene may simply be less potent than is tamoxifen. It was originally developed as a drug to treat breast cancer but was less effective than was tamoxifen in that setting as well (18).

The superiority of tamoxifen over raloxifene in reducing breast cancer risk comes with a cost: significantly more endometrial cancers, hysterectomies for benign disease, thromboembolic events, and cataracts. These toxicities may be acceptable for the treatment of breast cancer but have proved to be a barrier to the use of tamoxifen for preventing primary breast cancers. It is important to point out that, unlike raloxifene, tamoxifen is approved for use in premenopausal women, and the BCPT (NSABP P-1) showed no excessive risk of endometrial cancers or thromboembolic events in the tamoxifen-treated premenopausal group compared with the placebo group. For premenopausal women at increased risk, particularly those with biopsy-proven risk factors such as LCIS or atypical hyperplasia, tamoxifen has a positive risk/benefit ratio and should be presented as a treatment option. A similar risk/benefit ratio may exist in younger postmenopausal women with elevated Gail scores and a prior hysterectomy.

Our results demonstrate that raloxifene (compared with tamoxifen) retains substantial benefit in reducing the risk of invasive breast cancer and has fewer life-threatening side effects, including significantly fewer endometrial cancers, and these results are in keeping with those in the placebo-controlled raloxifene trials. We saw no significant increases in other primary cancers, although there were numerically more ovarian cancers and thyroid cancers. Neither of these tumors was noted to be of concern in the other raloxifene trials, but we plan to continue to follow STAR patients with particular attention to all potential long-term side effects.

The 5-year duration of therapy in STAR was a carryover from the P-1 trial of tamoxifen versus placebo, in which 5 years of tamoxifen was chosen based on the duration of treatment in adjuvant trials. In the combined results of MORE and the Continuing Outcomes Relevant to Evista (CORE) trial, which involved as much as 8 years of raloxifene therapy, a 66% reduction in the incidence of invasive breast cancer was seen in the raloxifene-treated group compared with the placebo group (HR = 0.34; 95% CI, 0.22–0.50). The women in the MORE/CORE studies were not selected based on breast cancer risk, and the majority had Gail scores below 1.66%, although some high-risk women were included.

Laboratory studies demonstrate that the antitumor actions of raloxifene and related hydroxylated SERMs depend on the duration of administration (19–21). In other words, longer administration periods are necessary to control tumorigenesis with short-acting SERMs with poor bioavailability (20). It may be that the long-term benefit of tamoxifen in controlling tumorigenesis occurs because of the development and evolution of a sophisticated SERM-resistant disease that becomes vulnerable to the apoptotic actions of physiologic estrogen (22) once tamoxifen is stopped. In contrast, the evolution of acquired SERM resistance may not advance as quickly with raloxifene as with tamoxifen, and raloxifene only remains therapeutically effective as long as it is given (8). It is unlikely that the optimal duration of raloxifene for chemoprevention will be evaluated in a breast cancer prevention setting; however, the use of raloxifene in treating and preventing osteoporosis is approved for an indefinite period of time. Therefore, continuing raloxifene therapy beyond 5 years might be an approach that would preserve its full chemopreventive activity.

Large randomized cancer-prevention trials with long-term clinical follow-up of a carefully characterized population of individuals provide a valuable resource beyond the primary aims of the study. In the NSABP STAR (P-2) and BCPT (P-1), baseline blood samples have been collected and stored from more than 30,000 women at an increased risk for breast cancer, as have tumor specimens from breast cancer events. Various studies have already been conducted using these resources, and others are underway, including a genome-wide-association study by NSABP in collaboration with the National Institutes of Health Pharmacogenetics Research Network (PGRN) and the RIKEN Yokohama Institute Center for Genomic Medicine; this study includes

Table 6. Distribution of Deaths - NSABP STAR Trial (P-2)

Cause of death	Deaths, *n*	
	Tamoxifen	Raloxifene
Cancer	101	86
Bladder	1	3
Bone, articular cartilage and connective tissue	1	1
Brain	6	4
Breast	11	4
Colon	4	3
Endocrine gland	0	1
Gallbladder	2	1
Kidney	1	1
Liver	7	1
Lung	25	28
Lymphatic/hematopoietic	12	11
Oral	2	1
Ovary	8	7
Pancreas	7	5
Peritoneum	2	0
Skin	2	0
Spleen	0	1
Stomach	2	1
Thyroid	1	0
Uterus	2	2
Other, uncertain, and unspecified sites	5	11
Circulatory/vascular disease	42	42
Aortic	1	2
Atherosclerosis	0	1
Cerebrovascular disease, unspecified	1	0
Hypertensive disease	1	4
Ischemic heart disease	13	8
Other heart disease	9	14
Peripheral vascular disease, unspecified	0	1
Polyarteritis nodosa	0	1
Pulmonary embolism	3	2
Primary pulmonary hypertension	1	0
Stroke	13	9
Other	93	74
Accident, auto	3	4
Accident, fire	1	0
Alcohol dependence syndrome	1	1
Asphyxiation and strangulation	1	0
Complications of surgery	0	1
Dementia	0	1
Diabetes	1	3
Disorders of metabolism	1	0
Emphysema	1	0
Injury, intracranial	2	2

(*Continued on the following page*)

Table 6. Distribution of Deaths - NSABP STAR Trial (P-2) (Cont'd)

Cause of death	Deaths, *n*	
	Tamoxifen	Raloxifene
Injury, other	1	0
Interferon toxicity	0	1
Intestinal infectious disease	0	1
Other conditions of the blood	0	2
Other conditions of the brain/neurological system	7	3
Other diseases of the digestive system	7	6
Other Diseases of the urinary system	2	1
Other respiratory disease	13	7
Pneumonia	2	4
Poisoning	2	0
Septicemia	4	3
Skin infections	0	1
Symptoms, signs, and ill-defined conditions	2	3
Unknown	42	30
Total deaths (rate per 1,000)	236 (3.81)	202 (3.22)
Risk ratio (95% CI)	0.70–1.02)	

Abbreviations: CI, confidence interval; NSABP STAR, National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene.

a detailed evaluation of cytochrome P450 2D6 (CYP2D6) status (refs. 23–27; access to these data and specimens is not restricted to NSABP members; the pathology section of the NSABP web site, ref. 28, describes the process by which one can submit applications for such projects).

In conclusion, with a median follow-up of 81 months, our long-term, updated results show that raloxifene retained 76% of the effectiveness of tamoxifen in preventing invasive disease, that its level of effectiveness grew closer over time to that of tamoxifen (78% as effective) in preventing noninvasive disease, and that raloxifene remained far less toxic (e.g., now with highly statistically significantly fewer endometrial cancers). These relative effects of the drugs in the longer term—including greater potency of tamoxifen in preventing invasive and noninvasive disease and significantly less endometrial toxicity with raloxifene—are more consistent with the profiles that were expected on the basis of findings from other published studies. With deep public-health implications, these results help to clarify that both raloxifene and tamoxifen are good preventive choices for higher-risk postmenopausal women, depending largely on a woman's personal risk factors for breast cancer. For postmenopausal women with elevated risk, these results should encourage widespread acceptance of raloxifene for breast cancer risk reduction, especially in women with an intact uterus who also face a risk of osteoporosis and fracture. The results should also promote greater acceptance of tamoxifen (given its greater efficacy) by premenopausal women who are at a very high risk for breast cancer. Such increased acceptances of both SERMs for breast cancer risk reduction ultimately would reduce the public health burden of the disease.

Disclosure of Potential Conflicts of Interest

V.G. Vogel: commercial research grant, Astra-Zeneca; honoraria from speakers bureau, Eli Lilly and Astra-Zeneca; consultant/advisory board, Eli Lilly and Astra-Zeneca. D.L. Wickerham: honoraria from speakers bureau, Astra-Zeneca; consultant/advisory board, Eli Lilly. The other authors disclosed no potential conflicts of interest.

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References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics 2009. CA Cancer J Clin 2009;59:225–49.
2. Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687–717.
3. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant

Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998;90: 1371–88.
4. Cuzick J, Forbes J, Edwards R, et al. First results from the International Breast Cancer Intervention Study (IBIS-1): a randomised prevention trial. Lancet 2002;360:817–24.
5. Cuzick J, Powles T, Veronesi U, et al. Overview of the main outcomes in breast cancer prevention trials. Lancet 2003;361:296–300.
6. Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. J Natl Cancer Inst 2007;99:283–90.
7. Cummings SR, Eckert S, Krueger KA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA 1999;281:2189–97.
8. Martino S, Cauley JA, Barrett-Conner E, et al. Continuing Outcomes Relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 2004;96:1751–61.
9. Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: The NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 2006;295:2727–41.
10. Costantino JP, Gail MH, Pee D, et al. Validation studies for models to project the risk of invasive and total breast cancer incidence. J Natl Cancer Inst 1999;91:1541–8.
11. Korn EL, Dorey FJ. Applications of crude incidence curves. Stat Med 1992;11:813–29.
12. Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: An overview of the randomised trials. Lancet 1998;351:1451–67.
13. Fisher B, Dignam J, Wolmark N, et al. Tamoxifen in treatment of intraductal breast cancer: National Surgical Adjuvant Breast and Bowel Project B-24 randomised controlled trial. Lancet 1999;353: 1993–2000.
14. Fisher B, Jeong JH, Bryant J, et al. Treatment of lymph-node-negative, oestrogen-receptor-positive breast cancer: long-term findings from National Surgical Adjuvant Breast and Bowel Project randomised clinical trials. Lancet 2004;364:858–68.
15. Fisher B, Costantino J, Wickerham DL, et al. Tamoxifen for the prevention of breast cancer: Current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 2005;97:1652–62.
16. Cuzick J, Forbes JF, Sestak I, et al. Long-term results of tamoxifen prophylaxis for breast cancer – 96-month follow-up of the randomized IBIS-1 trial. J Natl Cancer Inst 2007;99:272–82.
17. Barrett-Conner E, Mosca L, Collins P, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 2006;355:125–37.
18. Buzdar AU, Marcus C, Holmes F, Hug V, Hortobagyi G. Phase II evaluation of Ly156758 in metastatic breast cancer. Oncology 1998;45:344–5.
19. Gottardis MM, Jordan VC. The antitumor actions of keoxifene (raloxifene) and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987;47:4020–4.
20. Jordan VC, Allen KE. Evaluation of the antitumor activity of the nonsteroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 1980;16: 239–51.
21. Jordan VC, Gosden B. Inhibition of the uterotrophic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 1983;113:463–8.
22. Jordan VC. The 38th David A. Karnofsky lecture: The paradoxical actions of estrogen in breast cancer—survival or death? J Clin Oncol 2008;26:3073–82.
23. Abramson N, Costantino JP, Garber JE, Berliner N, Wickerham DL, Wolmark N. Effect of Factor V Leiden and prothrombin G20210→A mutations on thromboembolic risk in the National Surgical Adjuvant Breast and Bowel Project cancer prevention trial. J Natl Cancer Inst 2006;98:904–10.
24. Cushman M, Costantino JP, Tracy RP, et al. Tamoxifen and cardiac risk factors in healthy women: Suggestion of an anti-inflammatory effect. Arterioscler Thromb Vasc Biol 2001;21:255–61.
25. King MC, Wieand S, Hale K, et al. Tamoxifen and breast cancer incidence among women with inherited mutations in BRCA1 and BRCA2: National Surgical Adjuvant Breast and Bowel Project (NSABP-P-1) Breast Cancer Prevention Trial. JAMA 2001;286: 2251–6.
26. Cushman M, Costantino JP, Bovill EG, et al. Effect of tamoxifen on venous thrombosis risk factors in women without cancer: The breast cancer prevention trial. Br J Haematol 2003;120: 109–16.
27. Beattie MS, Costantino JP, Cummings SR, et al. Endogenous sex hormones, breast cancer risk, and tamoxifen response: an ancillary study in the NSABP breast cancer prevention trial (P-1). J Natl Cancer Inst 2006;98:110–5.
28. Available from: http://www.nsabp.pitt.edu.

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The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: a hypothesis

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Abstract

Background: Estrogens are classified as type I (planar) and type II (angular) based on their structures. In this study, we used triphenylethylenes (TPEs) compounds related to 4-hydroxytamoxifen 4OHT to address the hypothesis that the conformation of the liganded estrogen receptor (ERα) can dictate the E2-induced apoptosis of the ER+ breast cancer cells.

Materials and methods: ERα positive MCF7:5C cells were used to study apoptosis induced by E2, 4OHT and TPEs. Growth and apoptosis assays were used to evaluate apoptosis and the ability to reverse E2-induced apoptosis. ERα protein was measured by Western blotting to investigate the destruction of ERα by TPEs in MCF7 cells. Chromatin immunoprecipitation (ChIP) assays were performed to study the in vivo recruitment of ERα and SRC3 at classical E2-responsive promoter TFF1 (PS2) by TPEs. Molecular modeling was used to predict the binding mode of the TPE to the ERα.

Results: TPEs were not only unable to induce efficient apoptosis in MCF7:5C cells but also reversed the E2-induced apoptosis similar to 4OHT. Furthermore, the TPEs and 4OHT did not reduce the ERα protein levels unlike E2. ChIP assay confirmed very weak recruitment of SRC3 despite modest recruitment of ERα in the presence of TPEs. Molecular modeling suggests that TPE would bind in antagonistic mode with ERα.

Conclusion: Our results advances the hypothesis that the TPE liganded ERα complex structurally resembles the 4OHT bound ERα and cannot efficiently recruit co-activator SRC3. As a result, the TPE complex cannot induce apoptosis of ER+ breast cancer cells, although it can cause growth of the breast cancer cells. The conformation of the estrogen-ER complex differentially controls growth and apoptosis.

Keywords: breast cancer; estrogen; estrogen receptor; tamoxifen; triphenylethylenes.

Introduction

High dose estrogen therapy for the treatment of breast cancer is a pioneering application of translational research, as this was the first chemical therapy to be successful for the treatment of any type of cancer (1). High dose estrogen therapy for the treatment of breast cancer in postmenopausal women became an accepted standard of care for the treatment of breast cancer prior to the introduction of tamoxifen in the 1970s. Response rates to high dose estrogen therapy were dependent upon the duration of time from the menopause; patients treated in their seventies would have a response rate of 30%, whereas patients treated in their fifties had very few tumor responses. Although it was not realized at the time, the antitumor actions of estrogen were based upon estrogen deprivation. Sir Alexander Haddow FRS pioneered the development of high dose estrogen therapy, but in 1970, when he was selected as the inaugural Karnofsky lecturer at the American Society for Clinical Oncology, he remarked that little progress was being made in targeted therapeutics, and with regard to his own contribution of high dose estrogen treatment, he stated, ''…the extraordinary extent of tumor regression observed in perhaps 1% of post-menopausal cases (with estrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us…'' (2).

Now, some 40 years later, based upon decades of research on the impact of long-term adjuvant antihormone therapy on the evolution of drug resistance, a vulnerability of breast cancers has emerged, that was unanticipated. Selective estrogen receptor modulators (SERMs), e.g., tamoxifen and raloxifene, initially cause drug resistance in breast cancer cells that is identified by SERM stimulated growth and the tumor cells are also stimulated to grow with physiological estrogen (3, 4). However, this form of drug resistance occurs within approximately a year with SERM treatment; but, because successful 5 years of adjuvant tamoxifen therapy is given routinely to enhance survivorship (5), one would imagine other mechanisms of drug resistance occurring for micrometastatic disease during 5 years of adjuvant therapy. This would be a reasonable explanation for the lack of recurrences and increasing survivorship after tamoxifen is stopped (5). Studies in the laboratory demonstrate that drug resistance to

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SERMs evolves over approximately 5 years into a phase that is SERM-stimulated for growth, but physiological estrogen causes apoptosis and tumor regression (4, 6). The hypothesis has been offered that in fact long-term adjuvant tamoxifen therapy can reconfigure antihormone resistant breast cancer cells so that they are particularly sensitive to the apoptotic actions of physiological estrogen (from the patient's body) once 5 years of adjuvant tamoxifen has been stopped (7). Aromatase inhibitors are also administered to postmenopausal ER positive breast cancer patients for 5 years of adjuvant therapy (8, 9). Laboratory studies show that estrogen deprivation for prolonged periods sensitizes the resulting cells that are estrogen independent for growth to the apoptotic actions of estrogen (10, 11).

Using laboratory models for SERM and aromatase inhibitor resistant disease, mechanisms are emerging to define intrinsic and extrinsic pathways of estrogen-induced apoptosis (12). However, the question arises of how the estrogen receptor (ER) complex in one context can stimulate estrogen-stimulated growth, but the same complex will induce apoptosis in antihormone resistant cells. To address this paradox, we have drawn upon our previous contribution on the molecular classification of estrogens (13, 14) to interrogate the ER complex with structural derivatives of the antiestrogen, 4-hydroxytamoxifen (4OHT) and endoxifen (15). The molecular classification of estrogens is based upon the published X-ray crystallographic data for the planar estrogen diethylstilbestrol (DES) (incidentally, the synthetic estrogen earlier selected for high dose estrogen therapy for breast cancer) and 4OHT (the potent antiestrogenic metabolite of tamoxifen) (16). Simply stated, DES binds to the ligand binding domain (LBD) and is sealed within the cavity with helix 12 being the cap. In contrast, 4OHT, with its ''bulky side chain'' in the triphenylethylene (TPE) structure, pushes helix 12 back because of steric hindrance. The DES ER structure sealed with helix 12 allows the ER complex to be activated through activating function-2 (AF-2) that collaborates and cooperates with AF-1 at the opposing end of the ER. In this manner, the co-activators recruited to the complex initiate estrogen-stimulated growth and gene transcription. In contrast, the 4OHT ER complex cannot activate AF-2, but AF-1 is able to be activated through the exposed Asp351 that is inadequately neutralized and shielded by the dimethyl aminoethoxy side chain of 4OHT. This is classified as an antiestrogen complex, but it mechanistically explains the promiscuous estrogen-like activity of 4OHT (17, 18). Indeed, substitution of Asp351 for the non-ionic amino acid glycine completely abrogates the estrogen-like actions of the 4OHT ER complex (17).

In this paper, we offer the hypothesis that the shape of the ER complex with either planar estrogens (Class I) or angular estrogens (Class II) can modulate the apoptotic actions of estrogen through the shape of the resulting complex. We have previously synthesized a range of estrogenic TPEs, and all of these compounds will stimulate estrogen-stimulated growth of MCF-7 cells (15). Here, we investigate the actions of 4OHT and our model TPEs on estradiol-induced apoptosis in MCF-7:5C cells (19). We have discovered that the angular TPE estrogens do not cause rapid estrogen-induced apoptosis, even though they are potent stimulators of breast cancer cell growth. They do, in fact, block estradiol-induced apoptosis as effectively as 4OHT, a known antiestrogen. We propose the hypothesis that the shape of the ER complex and its ability to bind co-activators and transport them to the correct part of the cell is fundamentally important for the initiation of estrogen-induced apoptosis.

Materials and methods

Cell culture and reagents

Media for cell culture were purchased from Invitrogen Inc. (Grand Island, NY, USA) and fetal calf serum (FCS) was obtained from HyClone Laboratories (Logan, UT, USA). Compounds E2 and 4OHT were obtained from Sigma, St. Louis, MO, USA. The compounds trihydroxytriphenylethylene (3OHTPE) and ethoxytriphenylethylene (EtOX) were synthesized and the details of the synthesis have been reported previously (15). The ER positive breast cancer cells MCF-7:WS8 (hereafter mentioned as MCF7) and estrogen-deprived MCF7:5C were derived from MCF7 cells obtained from the Dr. Dean Edwards, San Antonio, TX, USA as reported previously (20). MCF7 cells were maintained in RPMI media supplemented with 10% FCS, 6 ng/mL bovine insulin and penicillin and streptomycin. MCF7:5C cells were maintained in phenol-red free RPMI media containing 10% charcoal dextran treated FCS, 6 ng/mL bovine insulin and penicillin and streptomycin. Three to four days prior to harvesting, the MCF7 cells were cultivated in phenol red-free media containing 10% charcoal dextran treated FCS. The cells were treated with indicated compounds (with media changes every 48 h) for the specified time and were subsequently harvested for protein lysate or growth assay. All the experiments were repeated at least three times, in triplicate to confirm the results.

Growth assay

For growth assay, 12,000 MCF7:5C cells were plated in each well of 24-well plates and the treatment of the cells with specific concentration of indicated compounds were started 24 h later (day 0). The media containing the compounds were changed on day 2 and day 4. On day 6, the cells were harvested for assessing the total DNA content in each well using a fluorescent DNA quantitation kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Calf thymus DNA was used to plot the standard curve for the DNA assay with each set of quantitation. The experiments were repeated three times in triplicate to confirm the data.

Western immunoblotting

The MCF7 cells were seeded on 10-cm Petri dishes at a density of 3 million cells per plate and were incubated overnight in phenol red-free RPMI 1640 media containing 10% charcoal dextran treated FCS, 6 ng/mL bovine insulin and penicillin and streptomycin. The cells were treated for 24 h with the indicated compounds and the cells were subsequently washed with cold phosphate buffered saline (PBS; Invitrogen, Carlsbad, CA, USA) twice and were lysed using 1× Lysis buffer (Cell Signaling Technology Inc., Denvers, MA, USA) that contained a 1× Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN, USA) and 1× phosphatase inhibitors (Calbiochem, Gibbstown, NJ, USA). The cells were lysed for 30 min on ice and subsequently centrifuged at 12,000 rpm

(15,000×*g*) for 20 min. Supernatants were transferred in fresh tubes and stored on ice, the concentration of proteins in the lysates were measured via a fluorescent Quant-iT Protein Assay Kit (Invitrogen, Carlsbad, IN, USA). Then, 20 μg of each protein sample diluted in a NuPAGE LDS loading dye was loaded and separated on NuPAGE 4%–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA). After electrophoresis, the samples were transferred onto Hybond-ECL Nitrocellulose Membranes (Amersham Biosciences, Piscataway, NJ, USA), which were subsequently blocked with blocking solution TBS-T (50 nM Tris-HCl pH 7.5, 150 nM NaCl, 0.1% Tween-20), containing 5% skim milk for 1 h at room temperature. The membranes were subsequently probed with primary antibodies anti-ERα (Santa Cruz, Biotechnology, Santa Cruz, CA, USA) and with anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA) diluted in blocking buffer at ratios recommended by the supplier at 4°C. The membranes were washed three times (10 min each) with the TBS-T buffer and subsequently incubated with the appropriate HRP-linked secondary antibodies (anti-mouse or anti-rabbit from Santa Cruz, Biotechnology, Santa Cruz, CA, USA) diluted in blocking buffer for 1 h at room temperature. The membranes were washed again as described above with TBS-T buffer and the signal was visualized using ECL Western Blotting Detection Reagents (GE Healthcare UK, Birminghamshire, UK).

Apoptosis assay

In total, 20,000 MCF7:5C cells were seeded in each well of a 96-well plate. Then, 24 h later cells were treated with indicated compounds in triplicate. Media containing the appropriate compounds were changed every 48 h. At the end of day 5, the cells were harvested using a colorimetric dye based apoptosis kit, APOPercentage™ (Biocolor Ltd., Carrickfergus, Antrim, UK) according to the manufacturer's instructions. Briefly, 30 min prior to the harvesting of the cells 5 μL of APOPercentage dye (which is selectively imported by the cells undergoing apoptosis) was added to each well and incubated for 30 min at 37°C, 5% CO_2 incubator. Subsequently, the cells were very carefully washed twice with PBS to wash off the unimported dye. The dye was thereafter released from the cells using a dye releasing reagent and the amount of dye imported was measured spectrophotometrically at 550 nm. The O.D. at 550 nm was directly proportional to the apoptosis of the cells.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed as described previously (21) with minor modifications. Briefly, cells were grown in phenol red-free RPMI media containing 10% charcoal stripped fetal bovine serum for 3 days before treating with vehicle, 1 nM E2, 4OHT (10^{-6} M) 3OHTPE (10^{-6} M) or EtOX (10^{-6} M) for 45 min. Cells were then washed with PBS and crosslinked with 1.25% formaldehyde. After stopping the crosslinking, cells were collected in PBS [containing protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA) and 10 mM dithiothreitol (DTT)], centrifuged and resuspended in nuclei isolation buffer (50 mM Tris-Cl, 60 mM KCl, 0.5% NP40, protease inhibitors and 10 mM DTT). Nuclei were isolated by centrifugation and resuspended in SDS lysis buffer (50 mM Tris-Cl, 1% SDS, 10 mM EDTA, pH 8.1 with protease inhibitors) followed by sonication and centrifugation at 14,000×*g* for 20 min at 4°C. The supernatant (fixed chromatin) were diluted using ChIP dilution buffer followed by immunoclearing using normal rabbit serum and 20 μL of Magna ChIP protein A agarose magnetic beads (Upstate Cell Signaling Solutions, Temecula, CA, USA). Immunoprecipitation was performed overnight with antibodies against ERα (1:1 mixture of cat # sc-543 and sc-7207; Santa Cruz Biotechnology, Inc.) and SRC-3 (cat # 13066; Santa Cruz Biotechnology, Inc.). The immune complexes were precipitated using 20 μL of Magna ChIP protein A agarose magnetic beads (Upstate Cell Signaling Solutions) and incubating for an additional 2 h followed by precipitating using a magnet. The beads bound to immunocomplexes were sequentially washed using buffer I (20 mM Tris-Cl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, and 150 mM NaCl), buffer II (20 mM Tris-Cl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, and 250 mM NaCl), buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Precipitates were then washed twice with TE buffer and extracted twice with freshly made 1% SDS and 0.1 M $NaHCO_3$. Pooled elutes were decrosslinked using 200 μM NaCl and heating at 65°C overnight. The DNA fragments were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA). Then, 2 μL of eluted DNA was used for real time PCR analysis. The primer sequences used are as follows: PS2 promoter: 5′-TGGGCTTCATGAGCTCCTTC-3′ (forward); 5′-TTCATAGTGAGAGATGGCCGG-3′ (reverse); the data are expressed as percent input of starting chromatin material after subtracting the percent input pulldown of the negative control (normal rabbit IgG).

Molecular modeling

The molecular modeling study was performed using the available X-ray crystallographic structures of ERα in the agonist and antagonist conformations. The 3D coordinates of ERα co-crystallized with E2 (1gwr) and 4OHT (3ert) were extracted from RCSB Protein Data Bank (PDB) (22) and these structures were prepared for docking using the Protein Preparation Workflow (Schrödinger, LLC, New York, NY, USA, 2008), accessible from within the Maestro 9.1 program (Schrödinger, LLC).

The ligand was prepared for docking with LigPrep 2.1 application (Schrödinger, LLC) and molecular docking was carried out with Glide 4.5 (Schrödinger, LLC) followed by the Induced Fit protocol (Schrödinger, LLC) using default parameters and 10 poses per ligand were retained for analysis.

Results

Reversal of E2-induced apoptosis in MCF7:5C cells by 4OHT, 3OHTPE and EtOX

17-β Estradiol induces apoptosis in ER+ MCF7:5C cells (19) which are long-term E2-deprived MCF7 breast cancer cells. Our aim was to evaluate the 4OHT and the TPEs, 3OHTPE and EtOX (Figure 1), for their ability to reverse the apoptosis induced by E2 in MCF7:5C cells in a concentration-dependent manner. Interestingly, the triphenylethylenes 3OHTPE and EtOX had previously been reported to be completely estrogenic as they can induce proliferation of MCF7 cells, unlike 4OHT (15). We found that 3OHTPE and EtOX were able to block the E2-induced apoptosis of MCF7:5C cells similar to the 4OHT in a concentration-dependent manner (Figure 2) as evident by DNA growth assay. The compounds alone at 10^{-6} M concentration were not able to induce significant apoptosis of MCF7:5C cells (Figure 2), whereas, as expected, drastic apoptosis was induced by E2 (1 nM) alone.

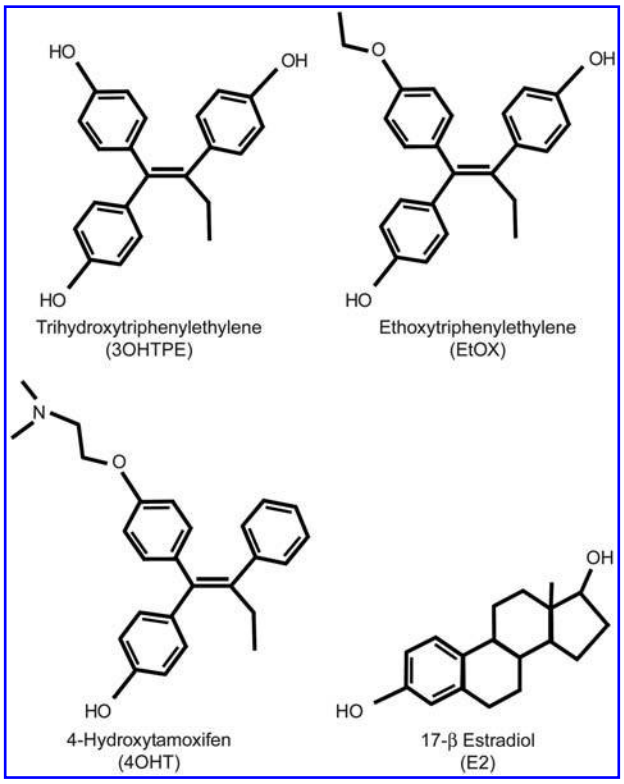

Figure 1 Structure of the compounds used in the study. Trihydoxytriphenylethylene (3OHTPE), ethoxytriphenylethylene (EtOX), 4-hydroxytamoxifen (4OHT) and 17-β estradiol (E2).

Estrogen receptor α levels are not decreased by 4OHT, 3OHTPE and EtOX

Treatment with estrogen in MCF7 cells causes a rapid destruction of ERα protein levels, whereas 4OHT impedes the destruction of ERα levels (23). Interestingly, despite acting as an estrogen agonist in MCF7 cells (15), the triphenylethylenes, 3OHTPE and EtOX, did not reduce the protein levels of ERα after 24 h of treatment at 10^{-6} M concentration, as evident by Western blot analysis of ERα protein levels which is similar to 4OHT treatment (Figure 3). As expected, ERα protein levels were drastically reduced after treatment with E2 (1 nM) for 24 h in MCF7 cells (Figure 3).

Induction of apoptosis by E2, 4OHT, 3OHTPE and EtOX

We further evaluated the apoptotic induction by 4OHT, 3OHTPE and EtOX and compared it with E2 in MCF7:5C cells using a dye-based kit which can measure the cells undergoing apoptosis as detailed in the Materials and methods section. We found that E2 (1 nM) produced a drastic increase in apoptotic cells after 5 days of treatment, whereas 4OHT (10^{-6} M) was completely ineffective (Figure 4). 3OHTPE (10^{-6} M) induced a modest level of apoptosis and a very slight apoptotic induction was observed after EtOX (10^{-6} M) treatment for 5 days (Figure 4).

Recruitment of ERα and SRC3/AIB1 at the promoter of PS2 (TFF1) gene by E2, 4OHT, 3OHTPE and EtOX

PS2 (TFF1) transcription is induced by E2 through a classical estrogen responsive element (ERE) at the promoter of the gene and its mechanism has been extensively studied (24, 25). We therefore evaluated the binding of the ERα and SRC3/AIB1 to the PS2 promoter after 45 min of treatment with 4OHT (10^{-6} M), 3OHTPE (10^{-6} M) or EtOX (10^{-6} M) in comparison with E2 (1 nM) in the MCF7:5C cells. Around a 17-fold increase in ERα recruitment was recorded with E2 treatment compared with vehicle treatment at the PS2 promoter (Figure 5A). In comparison, approximately 15-fold and approximately 11-fold increases in ERα recruitment was

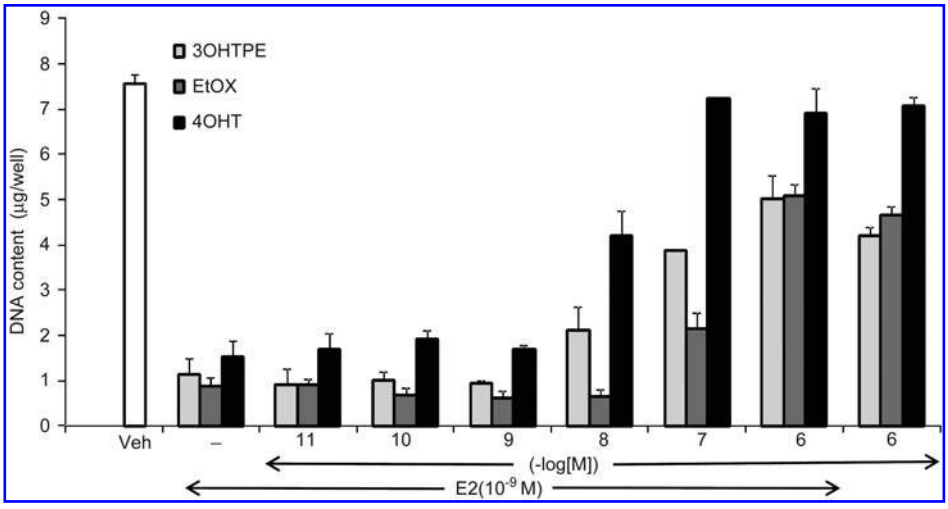

Figure 2 Reversal of E2-induced apoptosis of MCF7:5C cells by 4OHT, 3OHTPE and EtOX. MCF7:5C cells were treated with either vehicle (Veh), E2 (10^{-9} M) alone or E2 in combination with increasing concentration of the indicated compounds. Cells were also treated with compounds alone at 10^{-6} M concentration. After 7 days of treatment, the total DNA in the wells were estimated as a measure of cell survival.

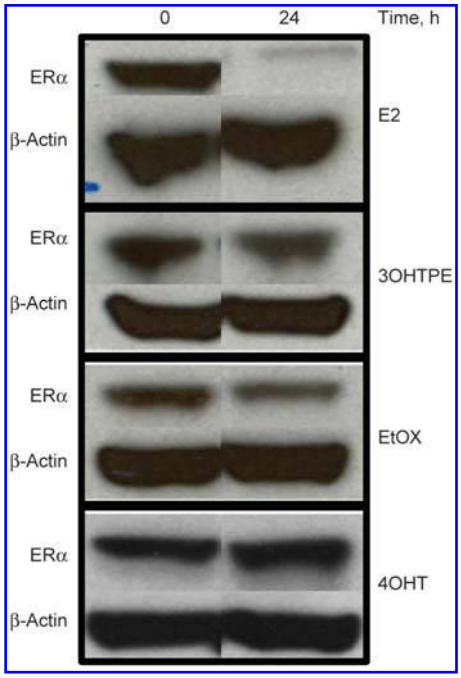

Figure 3 Levels of ERα protein after treatment with E2, 3OHTPE, EtOX or 4OHT. MCF7 cells were treated with E2 (10^{-9} M), 3OHTPE (10^{-6} M), EtOX (10^{-6} M) or 4OHT (10^{-6} M) for 24 h and total protein was isolated to estimate the ERα levels by Western blotting. Levels of β-actin were measured to ensure equal loading.

observed after treatment with 3OHTPE and EtOX, respectively, whereas only an approximately 5-fold increase in ERα recruitment was observed with 4OHT treatment (Figure 5A). Interestingly, in the case of SRC3/AIB1, very low levels of recruitment were observed after treatment with 3OHTPE and EtOX compared with E2 treatment (Figure 5B), whereas

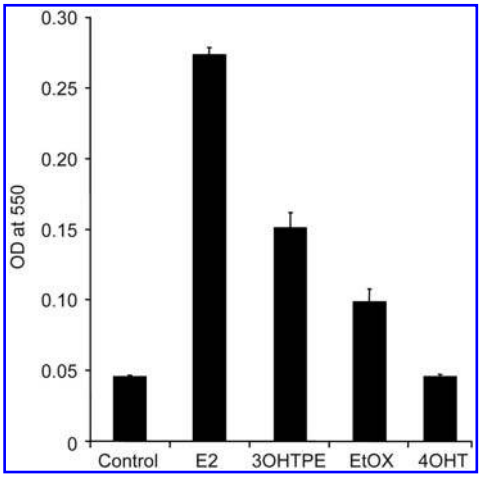

Figure 4 Induction of apoptosis by E2, 3OHTPE, EtOX or 4OHT. MCF7:5C cells were treated with E2 (10^{-9} M), 3OHTPE (10^{-6} M), EtOX (10^{-6} M) or 4OHT (10^{-6} M) and the induction of apoptosis was measured using a dye-based kit as detailed in Materials and methods section.

SRC3/AIB1 was not recruited at all after treatment with 4OHT (Figure 5B).

Binding of EtOX to the LBD of ERα

In an attempt to clarify the binding mode of EtOX to ERα, the flexible docking of this compound into the LBD of the receptor co-crystallized with 4OHT (Figure 6B) was performed, and also the best ranked ligand-receptor complex was superimposed onto the agonist conformation of ERα (Figure 6B). The results show that when EtOX is fitted in the binding site of the agonist conformation of ERα (1gwr), the ethoxy side chain of the ligand is bumping the side chains of L525 and L540 (Figure 6C) and it is unlikely for the ligand to bind in this conformation of the receptor. In contrast, when EtOX is docked into the binding site of ERα antagonist conformation (Figure 6D), the top ranked pose is fitted well in the binding cavity and it probably binds to an antagonist-related conformation of the receptor.

Discussion

Estrogen-induced apoptosis can be reversed in a concentration-related manner by the non-steroidal antiestrogen 4OHT. It is important to point out that in the ER+ MCF-7:5C cells

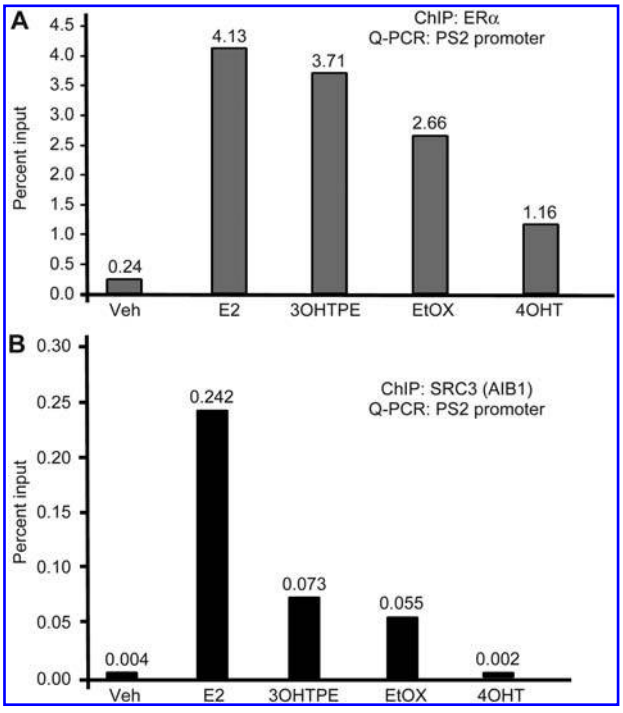

Figure 5 Recruitment of ERα (A) or SRC3 (AIB1) (B) at the promoter of PS2 (TFF1) gene. MCF7:5C cells were treated with E2 (10^{-9} M), 3OHTPE (10^{-6} M), EtOX (10^{-6} M) or 4OHT (10^{-6} M) for 45 min and cells were fixed with 1.25% formaldehyde before isolating the chromatin. ChIP was performed using ERα or SRC3 antibody and the immunoprecipitated DNA was quantified using specific primers for PS2 promoter by quantitative real time PCR. The values at the top of each bar represent the percent input after subtracting the negative control (rabbit IgG).

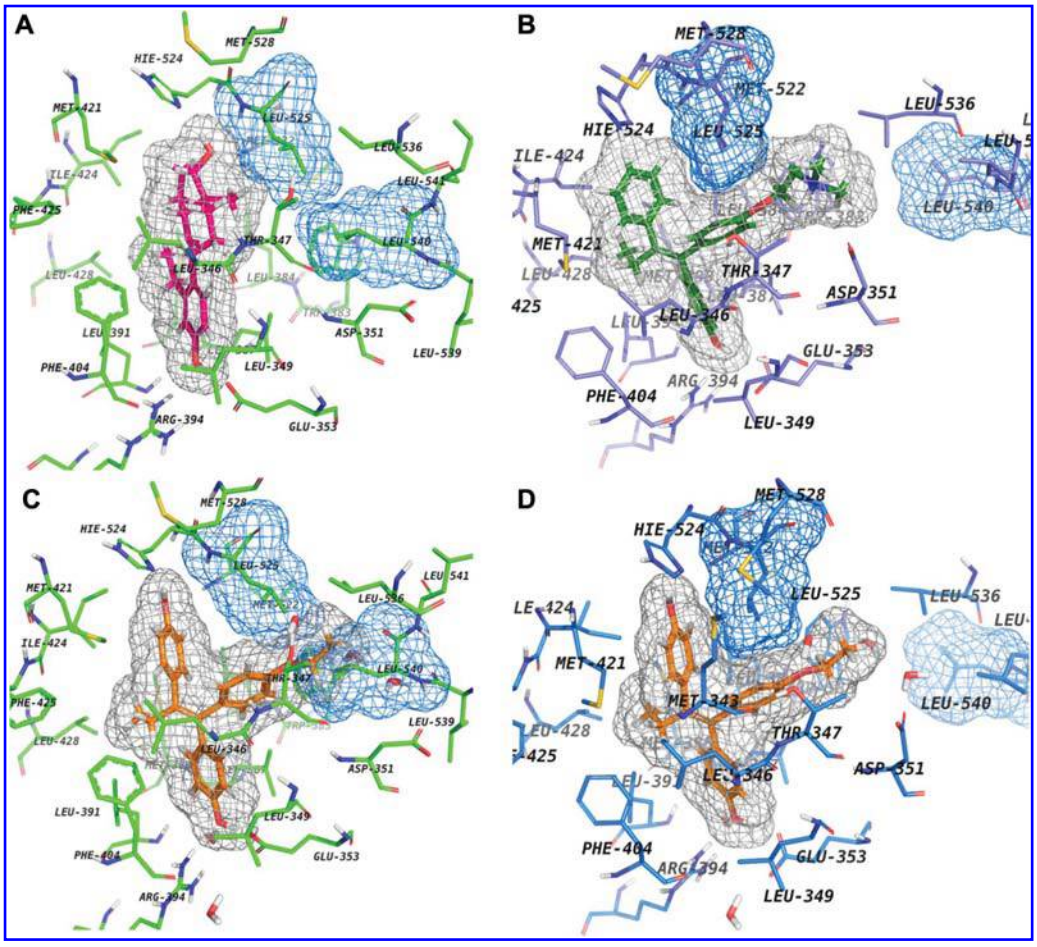

Figure 6 ERα binding site presented with different ligands. All ligands are shown with their corresponding molecular surfaces depicted as gray grids. Also, Leu525 and Leu540 are shown with their molecular surface depicted as blue grids. (A) The agonist conformation of ERα co-crystallized with E2 (colored in magenta) (PDB code: 1GWR); (B) 4OHT (depicted in green) co-crystallized with ERα – the antagonist conformation of the receptor (PDB code: 3ERT); (C) EtOX (colored in orange) is superimposed in the agonist conformation of the receptor (PDB code: 1GWR) and (D) same ligand is docked in the antagonist conformation of ERα (3ERT).

used in this study, 4OHT, although it binds to the ER, blocking apoptosis, does not produce any effect on cell growth when administered alone. These cells are completely resistant to the actions of nonsteroidal antiestrogens. The major finding in this study is that the test TPEs that are all fully estrogenic on cell replication in MCF-7 cells (15) also inhibit estrogen-induced apoptosis. Based on our previous study on the molecular classifications of estrogens (13), this leads to the suggestion that the angular TPEs are creating a shaped ER complex that is analogous to that observed in X-ray crystallography with 4OHT (26). Indeed, molecular modeling (Figure 6) demonstrates that the angular TPE would be unlikely to fit in the estradiol ER complex because steric hindrance would prevent helix 12 from sealing the LBD.

It seems that the TPEs can affect the ER complex in ways similar to 4OHT. 4OHT is known to impede the destruction of the 4OHT ER complex (23, 27). Similarly, the TPEs do not facilitate the rapid destruction of the TPE ER complex (Figure 3). Thus, Western blot analysis shows that the TPE ER levels are analogous to 4OHT ER levels rather than estradiol ER-like, i.e., rapidly destroyed. Indeed, the LeClercq group (28) have recently confirmed and extended our molecular classifications of estrogens, with a larger series of compounds and have also shown that an angular TPE does not cause the destruction of the ER complex in a manner analogous to estradiol when MCF-7 cells are examined by immunohistochemistry for the ER.

In a preliminary study, we have examined, using the ChIP assay, the binding of the ERα in the promoter region of the TFF1 (PS2) gene. The E2-ER complex has robust binding in the promoter region (Figure 5A) and SRC-3 is detected presumably bound to the ER complex (Figure 5B). In contrast, 4OHT ER complexes only have modest binding of ERα and virtually no SRC-3 in the promoter region. The TPEs permit some binding of the TPE ER complex in the promoter region but there are lower levels of SRC-3 and a reduced ability to stimulate PS2 synthesis (data not shown). A major conclusion of LeClerq's paper (28) is that the putative Class II estrogens (angular estrogens) that do not permit the appropriate sealing of the LBD with helix 12 do not efficiently bind co-activators. Our respective studies are therefore in agreement.

In summary, the proposed hypothesis is that the TPE-ER complex significantly changes the shape of the ER to adopt a conformation that mimics that adopted by 4OHT when it binds to the ER. A co-activator now has difficulty in binding to the TPE-ER complex appropriately, but whereas this does affect cell replication, it dramatically impairs the events that must be triggered to cause apoptosis. Future studies will confirm or refute our hypothesis based on the known intrinsic activity of mutant ERs and their capacity to investigate estrogen-target genes. Naturally, the absolute proof of our hypothesis would be the solution of the X-ray crystallography of a TPE-ER complex.

Acknowledgements

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References

1. Haddow A, Watkinson JM, Paterson E. Influence of synthetic oestrogens upon advanced malignant disease. Br Med J 1944; 2:393–8.
2. Haddow A. David A. Karnofsky memorial lecture. Thoughts on chemical therapy. Cancer 1970;26:737–54.
3. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 1988;48:5183–7.
4. Balaburski GM, Dardes RC, Johnson M, Haddad B, Zhu F, Ross EA, Sengupta S, Klein-Szanto A, Liu H, Lee ES, Kim H, Jordan VC. Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. Int J Oncol 2010;37:387–98.
5. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687–717.
6. Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, Jordan VC. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000;6:2028–36.
7. Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 1993;127:23–33.
8. Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, Forbes JF, Hoctin-Boes G, Houghton J, Locker GY, Tobias JS. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 2005;365:60–2.
9. Thurlimann B, Keshaviah A, Coates AS, Mouridsen H, Mauriac L, Forbes JF, Paridaens R, Castiglione-Gertsch M, Gelber RD, Rabaglio M, Smith I, Wardley A, Price KN, Goldhirsch A. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. N Engl J Med 2005;353: 2747–57.
10. Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, Yue W, Wang J, Santen RJ. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 2001;93:1714–23.
11. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, Jordan VC. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 2005;97:1746–59.
12. Maximov PY, Lewis-Wambi JS, Jordan VC. The paradox of oestradiol-induced breast cancer cell growth and apoptosis. Curr Signal Transduct Ther 2009;4:88–102.
13. Jordan VC, MacGregor Schafer JI, Levenson AS, Liu H, Pease KM, Simons LA, Zapf JW. Molecular classification of estrogens. Cancer Res 2001;61:6619–23.
14. Bentrem D, Fox JE, Pearce ST, Liu H, Pappas S, Kupfer D, Zapf JW, Jordan VC. Distinct molecular conformations of the estrogen receptor alpha complex exploited by environmental estrogens. Cancer Res 2003;63:7490–6.
15. Maximov PY, Myers CB, Curpan RF, Lewis-Wambi JS, Jordan VC. Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. J Med Chem 2010;53:3273–83.
16. Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 1977;75:305–16.
17. MacGregor Schafer J, Liu H, Bentrem DJ, Zapf JW, Jordan VC. Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. Cancer Res 2000;60: 5097–105.
18. Liu H, Lee ES, De Los Reyes A, Zapf JW, Jordan VC. Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor alpha complex. Cancer Res 2001;61: 3632–9.
19. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 2005;94:131–41.
20. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 1992;90:77–86.
21. Sengupta S, Sharma CGN, Jordan VC. Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells. Horm Mol Biol Clin Invest 2010;2:235–43.
22. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. The protein data bank. Nucleic Acids Res 2000;28:235–42.
23. Wijayaratne AL, McDonnell DP. The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. J Biol Chem 2001;276:35684–92.
24. Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, Gannon F. Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 2003;115:751–63.
25. Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoute J, Shao W, Hestermann EV, Geistlinger TR, Fox EA, Silver PA, Brown M. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. Cell 2005;122:33–43.

26. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;95:927–37.
27. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. Cancer Res 1996;56:2321–30.
28. Bourgoin-Voillard S, Gallo D, Laios I, Cleeren A, Bali LE, Jacquot Y, Nonclercq D, Laurent G, Tabet JC, Leclercq G. Capacity of type I and II ligands to confer to estrogen receptor alpha an appropriate conformation for the recruitment of coactivators containing a LxxLL motif – relationship with the regulation of receptor level and ERE-dependent transcription in MCF-7 cells. Biochem Pharmacol 2010;79:746–57.

Most Plastic Products Release Estrogenic Chemicals: A Potential Health Problem that Can Be Solved

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Background: Chemicals having estrogenic activity (EA) reportedly cause many adverse health effects, especially at low (picomolar to nanomolar) doses in fetal and juvenile mammals.

Objectives: We sought to determine whether commercially available plastic resins and products, including baby bottles and other products advertised as bisphenol A (BPA) free, release chemicals having EA.

Methods: We used a roboticized MCF-7 cell proliferation assay, which is very sensitive, accurate, and repeatable, to quantify the EA of chemicals leached into saline or ethanol extracts of many types of commercially available plastic materials, some exposed to common-use stresses (microwaving, ultraviolet radiation, and/or autoclaving).

Results: Almost all commercially available plastic products we sampled—independent of the type of resin, product, or retail source—leached chemicals having reliably detectable EA, including those advertised as BPA free. In some cases, BPA-free products released chemicals having more EA than did BPA-containing products.

Conclusions: Many plastic products are mischaracterized as being EA free if extracted with only one solvent and not exposed to common-use stresses. However, we can identify existing compounds, or have developed, monomers, additives, or processing agents that have no detectable EA and have similar costs. Hence, our data suggest that EA-free plastic products exposed to common-use stresses and extracted by saline and ethanol solvents could be cost-effectively made on a commercial scale and thereby eliminate a potential health risk posed by most currently available plastic products that leach chemicals having EA into food products.

Key words: bisphenol A, endocrine disruptor, endocrine-disrupting chemical, estrogen receptor binding, estrogenic activity, plastic. *Environ Health Perspect* 119:989–996 (2011). doi:10.1289/ehp.1003220 [Online 2 March 2011]

Chemicals that mimic or antagonize the actions of naturally occurring estrogens are defined as having estrogenic activity (EA), which is the most common form of endocrine disruptor activity [Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) 2003, 2006; National Research Council 1999]. Chemicals having EA typically interact with one or more of the classical nuclear estrogen receptor (ER) subtypes: ERα, ERβ, or nonclassical membrane or ER-related subtypes (Hewitt et al. 2005; Matsushima et al. 2008; National Research Council 1999). In mammals, chemicals having EA can produce many health-related problems, such as early puberty in females, reduced sperm counts, altered functions of reproductive organs, obesity, altered sex-specific behaviors, and increased rates of some breast, ovarian, testicular, and prostate cancers (Della Seta et al. 2006; Gray 2008; Kabuto et al. 2004; National Research Council 1999; Newbold et al. 2004; Patisaul et al. 2006, 2009). Fetal, newborn, and juvenile mammals are especially sensitive to very low (sometimes picomolar to nanomolar) doses of chemicals having EA (Gray 2008; vom Saal et al. 2005). Many of these effects observed in mammals are also expected to be produced in humans, because basic endocrine mechanisms have been highly conserved across all classes of vertebrates (Kavlock et al. 1996; National Research Council 1999).

Thermoplastics, which are used for many items that contain food, are made by polymerizing a specific monomer or monomers in the presence of catalysts into a high-molecular-weight chain known as a thermoplastic polymer [see Supplemental Material, Figure 1 (doi:10.1289/ehp.1003220)]. The resulting polymer is mixed with small quantities of various additives (antioxidants, plasticizers, clarifiers, etc.) and melted, mixed, extruded, and pelletized to form a base thermoplastic resin. Base resins are either used as is [e.g., bisphenol A (BPA)-based polycarbonate (PC), non-BPA-based polypropylene (PP) copolymer (PPCO), and non-BPA-based PP homopolymer (PPHO)] or, more commonly, mixed with other resins, additives, colorants, and/or extenders to form plastic compounds (e.g., polymer blends and precolored polymers). Plastic products are then made by using one or more plastic compounds or resins to form a finished plastic part that can be subjected to finishing processes that may use inks, adhesives, and so forth, to make a finished product.

As previously described (Begley et al. 1990, 2005; De Meulenaer and Huyghebaert 2004), plastic resins and manufacturing protocols [see Supplemental Material, Figure 1 (doi:10.1289/ehp.1003220)] collectively use many monomers and additives that may exhibit EA because they have physicochemical properties, often from an insufficiently hindered phenol (HP) group, that enable them to bind to ERs (see Supplemental Material, Table 1). Because polymerization of monomers is rarely complete and additives are not chemically part of the polymeric structure, chemicals having EA can leach from plastic products at very low (e.g., nanomolar to picomolar) concentrations that individually or in combination can produce adverse effects, especially in fetal to juvenile mammals. This leaching of monomers and additives from a plastic item into its contents is often accelerated if the product is exposed to common-use stresses such as ultraviolet (UV) radiation in sunlight, microwave radiation, and/or moist heat via boiling or dishwashing. The exact chemical composition of almost any commercially available plastic part is proprietary and not known. A single part may consist of 5–30 chemicals, and a plastic item containing many parts (e.g., a baby bottle) may consist of ≥ 100 chemicals, almost all of which can leach from the product, especially when stressed. Unless the selection of chemicals is carefully controlled, some of those chemicals will almost certainly have EA, and even when using all materials that initially test EA free, the stresses of manufacturing can change chemical structures or create chemical reactions to convert an EA-free chemical into one with EA.

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Very few studies (Soto et al. 1991; Till et al. 1982) have examined the extent to which plastics that presumably do not contain BPA nevertheless release other chemicals having detectable EA. For example, a recent comprehensive review [table on page 72 of Gray (2008)] described polyethylene (PE), PP, and PE terephthalate (PET) plastics as being "'OK' for use with respect to release of chemicals exhibiting EA."

Here, we report that most of the > 500 commercially available plastic products that we sampled—even those that are presumably BPA free—release chemicals having detectable EA, especially if they are assayed by more polar and less polar solvents and exposed to common-use stresses. That is, we show that, to reliably detect such leachable chemicals having EA, unstressed or stressed plastic resins or products should be extracted with more polar (e.g., saline) and less polar [e.g., ethanol (EtOH)] solutions and exposed to common-use stresses (boiling water, microwaving, and UV radiation).

Materials and Methods

We developed a sensitive and accurate roboticized version of the MCF-7 cell proliferation assay (E-SCREEN assay) that has been used for decades to reliably assess EA and anti-EA (Leusch et al. 2010; Soto et al. 1995) and is currently undergoing validation for international use by ICCVAM/NTP (National Toxicology Program) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). Chemicals with EA bind to ERs (ERα, ERβ, or ER-related subtypes) and activate the transcription of estrogen-responsive genes, which leads to proliferation of MCF-7 cells.

Detailed methods for the MCF-7 assay are provided in Supplemental Material, (doi:10.1289/ehp.1003220). In brief, plastic resins or products were extracted using saline, a more polar solvent, or EtOH, a less polar solvent. Aliquots of the extracts were then diluted four to eight times to produce up to eight test concentrations. Each test chemical or extract at each concentration was added in triplicate or quadruplicate to 96-well plates containing MCF-7 cells in EA-free culture media. After 6 days of exposure, the amount of DNA per well, an indication of cell proliferation, was assayed using a microplate modification of the Burton diphenylamine assay (Burton 1956; Natarajan 1994).

The effect of a test chemical or extract on proliferation was expressed as the %E2, a percentage of the maximum DNA per well produced by the maximum response to 17β-estradiol (E2; positive control) corrected by the DNA response to the vehicle (negative) control [see Supplemental Material, Equation 1 (doi:10.1289/ehp.1003220)]. For estrogenic test chemicals, the concentration needed to obtain half-maximum stimulation of cell proliferation [half-maximal effective concentration (EC_{50}), a measure of binding affinity] was calculated from best fits to dose–response data that meet a well-defined set of criteria by Michaelis-Menton kinetics. The estrogenicity of extracts was calculated as the relative maximum %E2 (%RME2; a measure of response amplitude), a percentage of the maximum DNA per well produced by an extract at any dilution with respect to the maximum DNA per well produced by E2 at any dilution, corrected by the DNA response to the vehicle (negative) control (see Supplemental Material, Equation 2). If a test chemical had a positive response (> 15% RME2) but an EC_{50} could be calculated because not all criteria were met, then the estrogenicity of the test chemical was characterized simply as EA positive or by its %RME2.

The EA of a test chemical or extract was considered detectable if it produced cell proliferation > 15% of the maximum response to E2 (> 15% RME2), which is > 3SDs

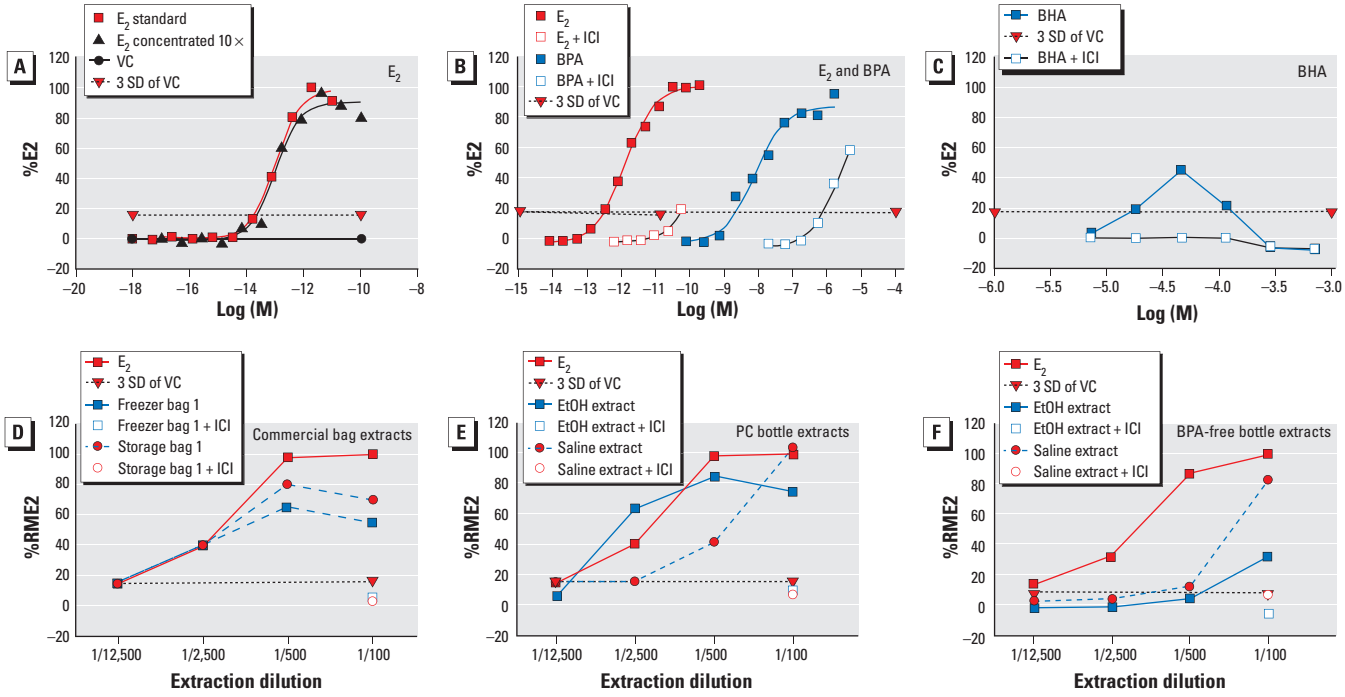


Figure 1. Results of MCF-7 assays shown as dilution response curves (%E2) for E2 (*A*), E2 and BPA (*B*), BHA (*C*), and %RME2 of extracts of plastic bags (*D*), a PC bottle (*E*), and a BPA-free bottle made from PETG (*F*). Abbreviations: PETG, PET glycol-modified polyethylene terephthalate; VC, vehicle control. Dotted lines represent 3 SD from the response. In *B–F*, the negative control (1% EtOH or saline) equals 0% E2. The E2 standard (10^{-9} M) is the positive control diluted as indicated in *C–F*. Each point plotted is the average of three or four replicates for each concentration whose SD is very small and falls within the space taken up by each data point. In (*A*), E2 was dissolved in EtOH (standard extract) or concentrated 10× and rediluted to show that the EtOH concentration protocol has very little effect on the EC_{50} of E2 (50% E2). The EC_{50} of E2 is approximately 1.3×10^{-13} M, and the threshold of detection (15% E2) is approximately 10^{-15} M. The maximum E2 response was attained at 10^{-11} M and remained constant at higher E2 concentrations. (*B*) The EC_{50} of both E2 (as in *A*) and BPA is approximately 6.6×10^{-8} M, and threshold detection is approximately 10^{-9} M, all suppressed by 10^{-8} M ICI. (*C*) BHA does not meet criteria needed for accurate calculation of EC_{50} [see Supplemental Material, pp. 5–7 (doi:10.1289/ehp.1003220)]. EA is positive; its maximum response is about 50% E2 (i.e., 50% RME2) and is suppressed by 10^{-8} M ICI. In *D*, commercially available plastic bags were extracted by 100% EtOH. Commercially available PC (*E*) and BPA-free (*F*) bottles were extracted with saline or EtOH as indicated.

from the historic control baseline response (about 10^{-15} M), which is a rather conservative measure of EA detectability. Stimulation of MCF-7 proliferation induced by the test chemical or extract was confirmed to be estrogenic (compared with nonspecific) in an EA confirmation study: If the stimulation of MCF-7 proliferation by a test chemical or extract was suppressed by coincubation with a strong antiestrogen [ICI 182,780 (ICI) at 10^{-7} to 10^{-8} M], the EA of the test chemical or extract was confirmed. Therefore, a test chemical or extract was classified as not having detectable EA if it did not induce MCF-7 cell proliferation or if it induced proliferation that could not be inhibited by ICI.

Figure 1 shows typical MCF-7 responses plotted as %E2. Figure 1A–C show responses to some test chemicals: E2 (positive control), BPA, and butylated hydroxyanisole (BHA; a common antioxidant). Figure 1D–F show %RME2 responses to test extracts of plastic food bags, PC bottles, and BPA-free baby bottles and their ICI-suppressed responses, confirming their EA. Some chemicals or products were also analyzed for anti-EA [for details, see Supplemental Material, pp. 7–8 (doi:10.1289/ehp.1003220)].

Purchase and analyses of plastic products in survey studies. For Tables 1 and 2, we purchased 455 plastic products used to contain foodstuffs from various commercial retailers from 2005 through 2008. The relative frequency of products having detectable EA did not change with later compared with earlier purchases. In some cases, we instructed undergraduate students or employees to purchase a mix of plastic items used to contain foodstuffs from a given large retailer (Albertsons, H-E-B, Randalls, Target, Wal-Mart, Trader Joe's, and Whole Foods) mainly in the Austin, Texas, or Boston, Massachusetts, areas, some of which market many "organic" products. In other cases, we purchased products of a particular plastic type (e.g., PE- or PP-based containers). We recorded the retailer, resin type [high-density PE (HDPE), PET, PC, PP, polystyrene (PS), polylactic acid], and product type (flexible packaging, food wrap, rigid packaging, baby bottle component, deli containers, plastic bags). In addition, because the contents of some plastic items might have added or extracted chemicals having EA from the plastic containers before we purchased and tested the products (Sax 2010), we recorded whether the plastic items had contents or were empty when purchased. For any plastic container having contents, we thoroughly washed out the container with distilled water before testing the plastic. Except for PC-based items, none of these products were known to contain BPA. (Plastic products typically do not list their chemical composition, which is proprietary to the manufacturer.) Samples were chosen in product areas where adverse health effects might occur if the samples leached chemicals having EA. Samples from each retailer generally included most of the product types listed above. In addition to surveying commercially available products, we tested plastic resins [e.g., PC, PET, glycol-modified PET (PETG)] that were purchased from M. Holland Company (Northbrook, IL) and individual chemicals used to manufacture plastic products [e.g., BPA, BHA, butylated hydroxytoluene (BHT), dimethyl terephthalate, etc.] that were purchased in their purest form from Sigma-Aldrich (St. Louis, MO).

Many plastic products have more than one plastic part. For example, baby bottles have 3–10 different plastic parts in various combinations [bottle, nipple, anticolic item(s), sealing ring(s), liner bag, cap, etc.], each part typically having different and rather unique combinations of 5–30 chemicals. Over the course of this entire study, we assayed > 100 component parts from > 20 different baby bottles, including many advertised as BPA free. Only some (13) of these component parts were purchased for the initial survey study (Tables 1 and 2).

Table 1. Percentage of unstressed plastic products having EA in at least one extract.

	Extraction solvent							
	EtOH		Concentrated EtOH		Saline		Any extract	
Plastic product	*n*	%D	*n*	%D	*n*	%D	*n*	%D
Resin type								
HDPE	13	69	11	55	18	56	30	70
PP	23	52	6	33	16	81	37	68
PET	30	40	17	94	34	76	57	75
PS	13	62	—	—	16	38	28	50
PLA	10	70	1	100	8	100	11	91
PC	1	0	1	100	2	100	2	100
Product type								
Flexible packaging	82	66	6	33	35	74	121	67
Food wrap	9	100	—	—	9	78	9	100
Rigid packaging	57	56	18	67	31	45	83	64
Baby bottle component	13	69	—	—	16	94	19	89
Deli containers	11	36	—	—	7	7	16	44
Plastic bags	33	97	1	100	23	96	43	98
Product retailer								
Large retailer 1	31	81	2	100	4	75	36	81
Large retailer 2	4	50	4	0	50	54	53	53
Large retailer 3	18	83	2	100	7	29	25	72
Large retailer 4	37	51	—	—	—	—	37	51
Large retailer 5	20	50	3	100	4	100	23	70
Organic retailer 1	28	71	5	60	5	80	32	81
Organic retailer 2	33	88	1	100	10	80	35	89
Total for extract	308	68	51	73	214	69	455	72

Abbreviations: —, not tested; %D, percent detectable (extract produced cell proliferation > 15% RME2; see "Materials and Methods"); *n*, total number of samples purchased (less than the sum of *n* values for individual extracts if some items were tested by more than one extraction protocol); PLA, polylactic acid. Data are percentages of samples for which EA was detected using a standard or concentrated EtOH extract, a saline extract, or one or more such extracts (any extract). Some individual items are listed in two or three categories (e.g., PET and baby bottles) but were counted only once for the extract total. Baby bottle components comprised 11 bottles and 2 sealant ring components.

Table 2. Percentage of unstressed plastic products having detectable EA (> 15% RME2) in two extracts.

		Extraction solvent			
Category	*n*	EtOH only	Saline only	Both EtOH and saline	Either EtOH or saline
HDPE	13	15	31	15	61
PET	21	19	29	52	100
PP	4	0	25	75	100
PLA	7	0	14	86	100
Bottles	38	13	34	42	89
Baby bottles	11	0	36	64	100
Rigid packaging	10	30	20	40	90
Food wrap	8	25	0	75	100
All products	102	17	21	54	92

PLA, polylactic acid. Values shown are percent (%) of unstressed plastic items (*n*) having detectable EA (> 15%RME2) only in an EtOH extract (and not in a saline extract), only in a standard saline extract (and not in an EtOH extract), in both EtOH and saline extracts, or in either EtOH or saline extracts. The last column is the sum of the three previous columns. "All products" is the total for each column when each product (*n* = 102) is only counted once (some products are listed in two categories). The standard EtOH extract was used for most (*n* = 81) products and the concentrated EtOH extract for the remainder (*n* = 21). If EA was detected in a saline or standard EtOH extract in survey studies such as those reported in Table 1, other extracts often were not performed. A concentrated EtOH extract was usually used to generate data shown in Tables 1 and 2 only if EA was not detected in a saline or standard EtOH extract. That is, samples listed for concentrated EtOH in Table 1 and EtOH in Table 2 had a selection bias for not having detectable EA.

Most of the samples (338 of 455) in the survey study (Tables 1 and 2) were extracted using only one extraction protocol. For the remaining samples (n = 102), both saline and EtOH extractions were used so that the efficacy of each protocol could be directly compared. We used a paired Student's t-test to test whether differences between pairs of samples were statistically significant ($p < 0.05$).

Protocols for common-use stresses of some plastic items. Given that common-use stresses can alter the complex chemical composition of plastics and/or increase the rate of leaching (Begley et al. 1990, 2005; De Meulenaer and Huyghebaert 2004), for some resins or products, we examined how leaching of chemicals having EA might be affected by exposure to microwave radiation, autoclaving (moist heat), and UV light. Additional plastic items, some of which are described in Figure 2 and Table 3, were purchased in 2008–2010 and subjected to common-use stresses. In addition, we tested a variety of resins (including PE- and PP-based resins; Table 3), antioxidants [see Supplemental Material, Table 3 (doi:10.1289/ehp.1003220)], and other additives or processing agents (see Supplemental Material, Table 4) identified by our laboratory as being free of detectable EA and hence possibly suitable for use to produce final products that would be EA free even after exposure to common-use stresses.

We used the following stresses:

- Samples were placed about 2 feet from a 254-nm fluorescent fixture for 24 hr, simulating repeated UV stress by sunlight (e.g., water bottles) or UV sterilizers (e.g., baby bottles and medical items)
- Samples were autoclaved at 134°C for 8 min, simulating moist heat stress in an automatic dishwasher
- We heated samples in a microwave 10 times for 2 min each, using a 1,000-W kitchen microwave oven set to "high," simulating heat and microwave radiation stress to reusable food containers.

Results

Release of chemicals having EA from unstressed plastics. Tables 1 and 2 show the percentage of samples in each category that had reliably detectable EA (> 15% RME2) in our survey of 455 commercially available plastic products. [For the %RME2 and content status of individual samples, as well as the average %RME2 for products classified by resins (HDPE, PP, PET, PS, polylactic acid, PC), product type (flexible packaging, food wrap, rigid packaging, baby bottle components, plastic bags), and retailer (large retailers 1–5 and large organic retailers 1 and 2), see Supplemental Material, Table 5 (doi:10.1289/ehp.1003220).] For example, 9 of 13 HDPE plastic products extracted by our standard EtOH protocol (69%) had detectable EA (Table 1), with a %RME2 (mean ± SD) of 66% ± 25% (see Supplemental Material, Table 5A). For PET products extracted by saline, 26 of 34 (76%) had detectable EA (Table 1) with a %RME2 of 64% ± 41% (see Supplemental Material, Table 5C). We found no consistent correlation between the percentage of items in a product type with detectable EA and their mean %RME2 (data not shown).

We found no significant difference ($p > 0.05$) in the percentage of items with detectable EA between those with contents and those with no contents (76%, n = 160) at the time of purchase based on the standard EtOH extraction protocol [67% vs. 70%; see Supplemental Material, Table 2A (doi:10.1289/ehp.1003220)], the standard saline protocol (62% vs. 75%; see Supplemental Material, Table 2C), or all extraction protocols combined (69% vs. 76%). Most important, items with no contents in all categories exhibited detectable EA in at least one protocol (see Supplemental Material, Tables 2 and 5), including 78% of items made from HDPE (n = 18), 57% from PP (n = 14), and 100% from PET (n = 6). Given all of these results, we present the data for all items shown in Tables 1 and 2 without regard to their content status.

Using different solvents increased the probability of detecting EA. Most (71%) unstressed plastic items released chemicals with reliably detectable EA in one or more extraction protocols, independent of resin type, product type, or retailer (Table 1). Results often differed between saline and EtOH extracts of the same unstressed plastic item, and EA was reliably detected most frequently (92% of all items listed in Table 2) when analyzed using both saline (more polar) and EtOH (less polar) extracts. For example, 15% of unstressed HDPE plastic items leached chemicals with detectable EA into both EtOH and saline extracts, 15% leached only into EtOH, and 31% leached only into saline (Table 2). That is, the leaching of a chemical with EA was significantly ($p < 0.01$) more likely to be detected if we used both polar and nonpolar solvents (61%) than if we used only one solvent (30% for EtOH only or 45% for saline

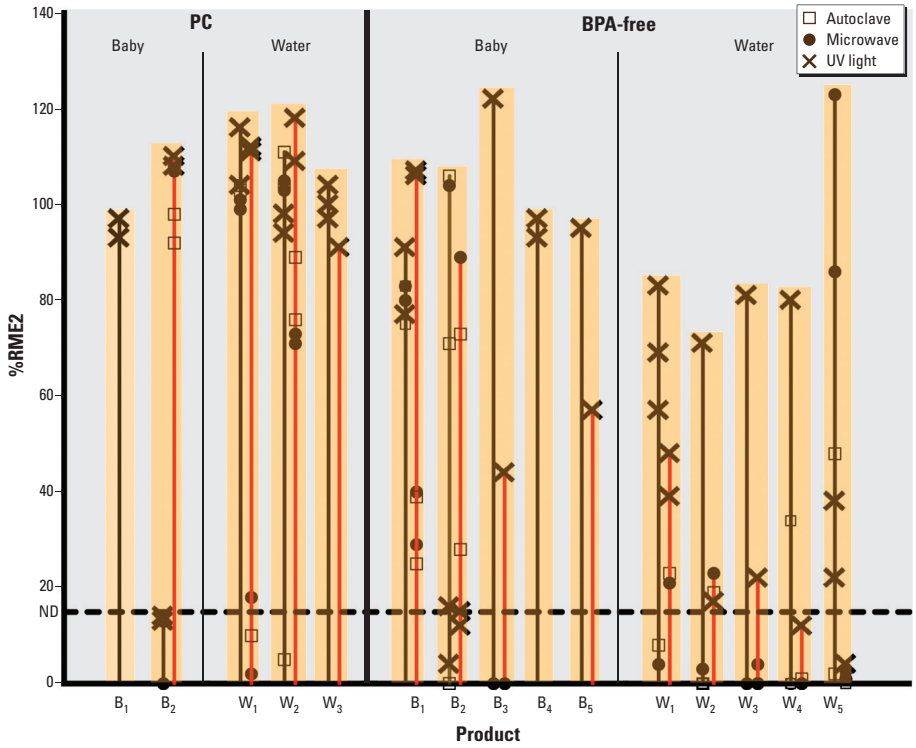


Figure 2. Total EA released by some PC and BPA-free water bottles (W) and baby bottles (B). The leaching of chemicals having EA (measured as %RME2; excluding caps, nipples, and other components) were extracted using saline or EtOH as solvents and exposed to autoclaving, microwaving, and/or UV light (see "Materials and Methods" for details). BPA-free water bottles W_1, W_2, W_3, and W_4 are PETG, and W_5 is PET. BPA-free baby bottles B_1 and B_2 are polyethersulfone; B_3 is PETG; and B_4 and B_5 are PP. Orange bars indicate the data set for each individual product. The %RME2 for saline extracts is represented by solid black lines and for EtOH as solid red lines. Symbols represent the %RME2 of chemicals released by each assay of a product after an autoclaving stress, microwaving stress, and UV light stress (see figure key). The dotted horizontal line at 15% RME2 is the rather conservative value below which EA was considered nondetectable (ND) for any assay. For some products shown (e.g., PC B_1, BPA-free B_4), if one solvent and/or stress condition showed reliably detectable EA, other solvents and stress conditions were not subsequently tested. Some values plotted as 0% RME2 actually had slightly negative %RME2 values (–1% to –7% RME2) due to cellular toxicity.

only). We obtained similar results for all types of plastic products (data not shown).

Assays of > 100 component parts from > 20 different baby bottles, including many advertised as BPA free, indicated that extracts of at least one bottle component of each baby bottle always had EA based on at least one assay (some data shown in Table 2 and Figure 2), as did at least one other component part (data not shown).

Stresses increased the release of chemicals having EA. Leaching of chemicals with EA was increased by common stresses. For example, one unstressed sample of an HDPE resin (P5 in Table 3) that had no detectable EA (i.e., RME2 < 15%) in two saline extracts and two EtOH extracts released chemicals with EA equivalent to 47% RME2 when extracted using EtOH after the resin was stressed with UV light. Similarly, two samples of low-density PE resins (LDPE resins 1 and 2) and PETG resins (PETG baby bottle and PETG resin 1) that had no detectable EA before stressing subsequently exhibited EA when stressed, especially by UV (Table 3). Samples (n > 10) of products made from PETG resins advertised as BPA free all released detectable EA when stressed, especially by UV light. Similarly, 25% of unstressed samples of PET and 50% of unstressed PS products surveyed did not have detectable EA in assays of EtOH and/or saline extracts (Table 1). However, when stressed and assayed using both saline and EtOH extracts, all PET (n > 10) and PS (n > 10) products released chemicals having detectable EA in at least one extracting solvent (Table 3).

EA-containing and EA-free monomers. Polymerization of monomers is rarely complete, and unpolymerized monomers are almost always released from polymer resins (Begley et al. 1990, 2005; De Meulenaer and Huyghebaert 2004). PE and PP polymers are often used to manufacture flexible and/or nontransparent rigid products (Figure 3). MCF-7 assays (n = 6) consistently showed that extracts of "barefoot" (no additives) polymers (e.g., LDPE resin P1 in Table 3) were EA free, even when stressed. (PP-based polymers require antioxidants to prevent severe degradation during their use in manufacturing plastic products.) Furthermore, PE- and PP-based resins containing appropriate additives to produce fit-for-use products could be constructed that remained EA free (n > 100 assays of > 10 resins), even when exposed to common-use stresses. Representative data from several such resins (LDPE resin P1, HDPE resin P2, PP homopolymer resin P3, PP copolymer resin P4) are shown in Table 3.

Figure 3 also shows other monomers and polymers that can or cannot be used to make hard-and-clear (HC) plastics. For example, HC PC plastics (n > 10) all released chemicals having EA (e.g., PC baby bottle B_1 and PC water bottle W_1 in Figure 2), almost certainly phenolics such as BPA (Figure 1B). The dimethyl terephthalate monomer used to make PET and PETG plastics exhibited anti-EA (n = 3 assays; data not shown; for anti-EA assay protocol, see Supplemental Material (doi:10.1289/ehp.1003220)]. Furthermore, breakdown products of dimethyl terephthalate, PET, and PETG resins probably contain and release phenolic moieties that have EA that account for some of the data for PET products in Tables 1 and 2. Polyethersulfone HC products also consistently released chemicals having EA or anti-EA, especially when stressed with UV light (data not shown), possibly from unreacted phenolic monomer residues or phenolic stress-degradation products. In contrast, some HC cyclic olefin polymer/cyclic olefin copolymer polymers produced from saturated cyclic olefin monomers contained no phenolics and did not release chemicals having detectable EA, even when stressed (Table 3).

Polymers that can be made EA free have a similar cost compared with polymers made from monomers that have EA. For example, currently, clarified PP having no additives that exhibit EA (even when stressed) that is suitable for molding bottles costs approximately $1.20/lb. PP resins containing additives that have EA also cost about $1.20/lb. Commodity resins such as PET, which are made from monomers having EA and are suitable for molding bottles, are priced at approximately $1.28/lb (Plastics News 2011).

EA-containing and EA-free additives. Many additives are physically, but not chemically, bound to a polymeric structure and hence can almost always leach from the polymer, especially when stressed (Begley et al. 1990, 2005; De Meulenaer and Huyghebaert 2004). Antioxidants are the most critical class of additives because they prevent or minimize plastic degradation due to oxidation that breaks polymer chains (chain scission) and/or causes cross-links (Kattas et al. 2000). The oldest and most common antioxidants deemed suitable for food contact belong to a chemical class known as HPs (hindered phenols), such as BHT and BHA, in large part because both are inexpensive and assumed to be nontoxic. However, BHT (n = 4 assays) had reliably detectable EA, as did BHA (n = 3 assays). [The EC_{50} of BHT and BHA (Figure 1C) could not be accurately calculated because both also exhibited cellular toxicity at higher concentrations (10^{-5} M).] Other commonly used HP antioxidants (n = 4/5) and organophosphines (n = 6/7) also exhibited reliably detectable EA, especially when exposed to moist heat, which presumably causes hydrolysis (data not shown). For example, proprietary antioxidants Phos (phosphate) OX 1 and HP AOX 2 had no detectable EA, whereas HP AOX 1 and Ph (bisphenol) AOX 1 had reliably detectable EA [see Supplemental Material, Table 3 (doi:10.1289/ehp.1003220)].

Many other additives (n > 50) with a phenolic group had reliably detectable EA, such as agents found in many base resins [tris(nonylphenyl) phosphite, octylphenol, nonylphenol, butylbenzene phthalate], colorants (especially blues or greens with

Table 3. Representative %RME2 values for stressed resins or parts made from flexible or HC polymers.

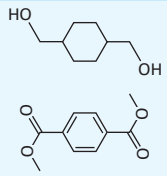
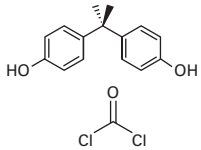
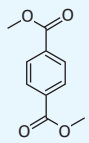
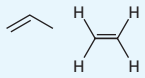
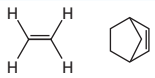
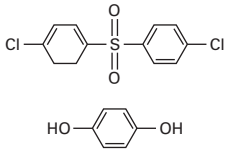
	Stress/extraction solvent					
	Microwave		UV		Autoclave	
Sample type	Saline	EtOH	Saline	EtOH	Saline	EtOH
Flexible polymers						
LDPE resin 1	5	7	0	4	4	30[a]
LDPE resin 2	3	7	26[a]	3	–1	27[a]
PET water bottle	100[a]	3	31[a]	2	47[a]	1
LDPE resin P1	2	3	0	0	4	5
HDPE resin P2	6	–4	2	–2	–1	–3
PPHO resin P3	0	–4	3	2	–6	–3
PPCO resin P4	3	7	–7	–6	–9	–3
HDPE resin P5	ND	ND	ND	47[a]	ND	ND
HC polymers						
Water bottle 1.1	3	23[a]	71[a]	17[a]	–1	19[a]
Water bottle 1.2	4	21[a]	57,[a] 69,[a] 98[a]	48,[a] 39[a]	8	23[a]
Water bottle 2.1	–7	–5	81[a]	22[a]	0	4
Water bottle 2.2	34[a]	–2	80[a]	12	–1	1
PETG baby bottle	0	–2	122[a]	44[a]	0	1
PETG resin 1	–8	17[a]	61[a]	111[a]	0	15[a]
PS 1	4	3	17[a]	45[a]	76[a]	0
COC 3	9	7	20[a]	20[a]	0	6
COC resin P18	4	1	9	11	1	–2
COC resin P19	6	2	6	–2	4	2

Abbreviations: COC, cyclic olefin copolymer; ND, not determined; PPCO, polypropylene copolymer; PPHO, polypropylene homopolymer. Numerical values are %RME2 responses of extract for several different baby bottle and other component parts. Resins designated with P (e.g., P1, P18) are EA-free formulations developed at PlastiPure. Resin P5 exhibited EA when stressed. Multiple values for water bottle 1.2 under UV stress are data for repeated analyses.
[a]Plastic items leaching chemicals having detectable EA > 15% RME2.

phthalocyanine groups), PS-based purge compounds, and mold-release agents [see Supplemental Material, Table 4 (doi:10.1289/ehp.1003220)]. In contrast, many metal-oxide–based inorganic pigments did not exhibit EA. However, these EA-free pigments are often mixed with dispersing agents and carrier resins that have EA to produce colorant masterbatch concentrates. Nevertheless, we have identified resins, dispersants, pigments, and antioxidants that are approved by the Food and Drug Administration for direct food contact (see Supplemental Material, Tables 3 and 4) to create colorant masterbatch concentrates (n > 100) that produce even colorant dispersion into plastics and that have no detectable EA, cellular toxicity, or adverse processing effects, even when stressed.

Because additives comprise a small fraction (typically 0.1–1% by weight) of plastic resins and compounds and because plastic resins and compounds using EA-free additives are processed during manufacture in a nearly identical manner as conventional resins and compounds containing chemicals with EA, the replacement of additives having EA with EA-free additives should have very little impact on the cost of the final product. Furthermore, EA-free additives have only a slightly higher or no additional cost compared with additives with EA, so that their cost impact is very small or nonexistent.

Figure 3. Properties of monomers and polymers used to make common resins.

Polymers	Monomers	Structures	EA	Toxicity[a]
Flexible polymers				
Low-density polyethylene (LDPE), linear low-density polyethylene (LLDPE), high density polyethylene (HDPE)	Ethylene		No	No
Polypropylene homopolymer (PPHO)	Propylene		No	No
HC polymers[b]				
Copolymer using terephthalate PETG	1,4-Cyclohexanedimethanol, dimethyl terephthalate[c]		Yes[d]	No
Polycarbonate (PC)	Bisphenol A,[e] phosgene		Yes	Yes
Polyethylene terephthalate (PET)	Dimethyl terephthalate[e]		Yes[d]	No
Polystyrene (PS)	Styrene		Yes[d]	No
Polypropylene copolymer (PPCO)	Propylene, ethylene		No	No
Cyclic olefin polymer (COP), cyclic olefin copolymer (COC)	Ethylene, norbornene		No	No
Polyacrylonitrile (PAN)	Acrylonitrile		No	Yes
Polyethersulfone (PES)	1,4-bis(4-Chlorophenyl) sulfone, 1,4-dihydroxybenzene[e]		Yes[d]	No

[a]Polymer exhibits other toxic effects (e.g., cellular damage or carcinogenicity), or toxic chemicals (e.g., phosgene and acrylonitrile) are used or produced during polymerization. [b]HC polymers generally have a glass transition temperature (T_g) above room temperature and limited or no ability to crystallize. [c]Monomer has anti-EA in MCF-7 assays. [d]Under certain conditions, degradation products exhibit EA. [e]Monomer has EA in MCF-7 assays.

Products currently marketed as BPA free are not EA free. In response to market and regulatory pressures to eliminate BPA in HC plastics, BPA-free HC materials have recently been introduced as replacements for PC resins. PET and PETG are two such resins, but HC plastic products made from these resins leached chemicals that had detectable EA (Tables 1–3, Figures 2 and 3), often in the absence of exposure to common-use stresses. Two popular brands of water bottles made from a PETG resin now marketed as an HC BPA-free replacement also released chemicals having significant EA (W1, W2, W3, and W4; Table 3, Figures 2 and 3), as did uncompounded PETG resins (Table 3). Most PE/PP-based plastic products were presumably BPA free but nevertheless had readily detectable EA (Tables 1 and 2), almost certainly due to one or more additives having EA. Many components of BPA-free baby bottles had reliably detectable EA (22–95% RME2) when extracted in either saline or EtOH, including the bottle, nipple, anticolic device, and liner (data not shown).

In fact, all BPA-replacement resins or products tested to date (n > 25) released chemicals having reliably detectable EA (data not shown), including polyethersulfone and PETG, sometimes having more total EA measured as %RME2 than many PC products when stressed. For example, the %RME2 released by various BPA-free baby and water bottle component parts extracted by saline or EtOH solutions and exposed to one or more common-use stresses can be greater than PC products under the same conditions (Figure 2). UV stress, in particular, often leads to the release of chemicals having greater EA than BPA-containing HC plastics currently sold. For example, saline extracts of BPA-free baby bottle B_3 (Figure 2) after exposure to UV showed greater EA than did any of the PC baby bottle extracts after any of the stresses. Saline extracts from BPA-free baby bottle B_1 after any of the stresses (microwave, autoclave, or UV) showed greater EA than did the saline extracts from PC baby bottle B_2 after any of the stresses. EtOH extracts from BPA-free baby bottle B_1 after UV stress showed greater EA than extracts from PC baby bottle B_1. Saline extracts from BPA-free baby bottle B_2 after microwave or autoclave stresses showed greater EA than did saline extracts from PC baby bottles B_1 or B_2 after any of the stresses. Note also in Figure 2 that multiple extracts of the same product using the same solvent/stress combination typically gave rather similar %RME2 data, but different solvent/stress combinations gave very different results, from very high EA to nondetectable EA. For example, EtOH extracts from PC baby bottle B_2

showed very high EA under all stress conditions, whereas saline extracts of the same bottle under the same stress conditions showed no detectable EA. Hence, to reliably detect EA, plastic resins or products must be extracted with both polar and nonpolar solvents and exposed to common-use stresses.

Discussion

Most plastic products release chemicals having EA. Our data show that both more polar (e.g., saline) and less polar (e.g., EtOH) solvents should be used to extract chemicals from plastics because the use of only one solvent significantly reduces the probability of detecting chemicals having EA. The ability to detect more polar and less polar chemicals having EA is important because plastic containers may hold either type of liquid or a liquid that is a mixture of more polar and less polar solvents (e.g., milk). When both more polar and less polar solvents are used, most newly purchased and unstressed plastic products release chemicals having reliably detectable EA independent of the type of resin used in their manufacture, type of product, processing method, retail source, and whether the product had contents before testing. However, the lack of significant difference in average percentage having detectable EA between plastic items with and without contents does not imply that the contents do not affect the total EA or specific chemicals having EA released by individual plastic items.

Our data show that most monomers and additives that are used to make many commercially available plastic items exhibit EA. Even when a "barefoot" polymer (no additives) such as PE or polyvinyl chloride does not exhibit EA, commercial resins and products from these polymers often release chemicals (almost certainly additives) having EA.

We found that exposure to one or more common-use stresses often increases the leaching of chemicals having EA. In fact, our data suggest that almost all commercially available plastic items would leach detectable amounts of chemicals having EA once such items are exposed to boiling water, sunlight (UV), and/or microwaving. Our findings are consistent with recently published reports that PET products release chemicals having EA (Wagner and Oehlmann 2009) and that different PET products leach different amounts of EA. For example, different PET products release different amounts of EA measured as %E2 or %RME2 [see Supplemental Material, Table 5C (doi:10.1289/ehp.1003220)], almost certainly because different PET copolymer manufacturers choose different monomers, additive packages, and synthetic processes to produce PET copolymer resins.

Our data are consistent with the hypotheses that the presence of a phenolic moiety is the best predictor of whether a chemical exhibits EA and that benzene moieties often probably convert to phenolic moieties when the monomer and/or polymer is exposed to one or more manufacturing or common-use stresses. For example, although in theory most organophosphites (antioxidants commonly used with HPs to provide synergistic oxidation protection) in their unaltered state should not bind to ERs [see Supplemental Material, Table 1 (doi:10.1289/ehp.1003220)], organophosphites are hydrolytically unstable and often produce phenols when exposed to water (Kattas et al. 2000). Most organophosphite antioxidants we tested exhibited detectable EA (data not shown).

Likewise, various additives that are high-molecular-weight HPs do not have EA, but if exposed to moist heat they can undergo hydrolysis and produce lower-molecular-weight phenolics that have EA. Therefore, antioxidants and other additives should be tested for EA both in their original, unstressed form and after stressing. We can identify monomers and additives (antioxidants, clarifiers, slip agents, colorants, inks, etc.) having no detectable EA for use at all stages of manufacturing processes to make flexible nontransparent or HC plastic items that are EA free, even after exposure to common-use stresses. All of our data suggest that, when both are manufactured in comparable quantities, carefully formulated EA-free plastic products could have all the fit-for-use properties of current EA-releasing products at minimal additional cost.

BPA free is not EA free. Although most items listed in Tables 1–3 would not be expected to contain BPA, nevertheless almost all stressed plastic items tested leached chemicals having reliably detectable EA measured as %RME2 if extracted with both more polar and less polar solvents. In response to market and regulatory pressures, BPA-free PET or PETG resins and products have recently been introduced as replacements for PC resins. However, all such replacement resins and products tested to date release chemicals having EA (measured as %RME2), sometimes having more EA than BPA-containing PC resins or products, especially when stressed by UV light (Figure 2, Table 3). Monomer or polymer breakdown products that have EA account for some of this EA, but the rest of the measured EA is almost certainly due to release of additives having EA in BPA-free products, including the bottle and many component parts of baby bottles advertised as BPA free.

Avoiding a potential health problem. We recognize that we quantitatively measured EA relative to E2 (EC_{50} or %RME2) using sensitive assay and extraction protocols. Furthermore, it is almost impossible to gauge how much EA anyone is exposed to, given such unknowns as the number of chemicals having EA, their relative EA, their release rate under different conditions, and their metabolic degradation products or half-lives *in vivo*. In addition, the appropriate levels of EA in males versus females at different life stages are currently unknown. Nevertheless, *a*) *in vitro* data overwhelmingly show that exposures to chemicals having EA (often in very low doses) change the structure and function of many human cell types (Gray 2008); *b*) many *in vitro* and *in vivo* studies document in detail cellular/molecular/systemic mechanisms by which chemicals having EA produce changes in various cells, organs, and behaviors (Gray 2008); and *c*) recent epidemiological studies (Gray 2008; Koch and Calafat 2009; Meeker et al. 2009; Swan et al. 2005; Talsness et al. 2009; Thompson et al. 2009) strongly suggest that chemicals having EA produce measurable changes in the health of various human populations (e.g., on the offspring of mothers given diethylstilbestrol, or sperm counts in Danish males and other groups correlated with BPA levels in body tissues).

Many scientists believe that it is not appropriate to bet our health and that of future generations on an assumption that known cellular effects of chemicals having EA released from most plastics will have no severe adverse health effects (Gray 2008; Talsness et al. 2009; Thompson et al. 2009). Because we can identify existing, relatively inexpensive monomers and additives that do not exhibit EA, even when stressed, we believe that plastics having comparable physical properties but that do not release chemicals having detectable EA could be produced at minimal additional cost.

References

Begley T, Castle L, Feigenbaum A, Franz R, Hinrichs K, Lickly T, et al. 2005. Evaluation of migration models that might be used in support of regulations for food-contact plastics. Food Addit Contam 22:73–90.

Begley TH, Dennison JL, Hollifield HC. 1990. Migration into food of polyethylene terephthalate (PET) cyclic oligomers from PET microwave packaging. Food Addit Contam 7:797–803.

Burton K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 62:315–323.

Della Seta D, Minder I, Belloni V, Aloisi AM, Dessi-Fulgheri F, Farabollini F. 2006. Pubertal exposure to estrogenic chemicals affects behavior in juvenile and adult male rats. Horm Behav 50:301–307.

De Meulenaer B, Huyghebaert A. 2004. Packaging and other food contact material residues. In: Handbook of Food Analysis, Vol 2 (Nollet LML, ed). 2nd ed. New York:Marcel Dekker, 1297–1330.

Gray J, ed. 2008. State of the Evidence: The Connection between Breast Cancer and the Environment. 5th ed. Breast Cancer Fund. Available: http://www.breastcancerfund.org/assets/pdfs/publications/state-of-the-evidence-2010.pdf [accessed 4 June 2011].

Hewitt SC, Deroo BJ, Korach KS. 2005. Signal transduction: a new mediator for an old hormone? Science 307:1572–1573.

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). 2003. ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays.

NIH Publication No. 03-4503. Available: http://iccvam.niehs.nih.gov/docs/endo_docs/edfinalrpt0503/edfinrpt.pdf [accessed 3 November 2010].

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). 2006. Addendum to ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays. NIH Publication No. 03-4503. Available: http://iccvam.niehs.nih.gov/docs/endo_docs/EDAddendFinal.pdf [accessed 3 November 2010].

Kabuto H, Amakawa M, Shishibori T. 2004. Exposure to bisphenol A during embryonic/fetal life and infancy increases oxidative injury and causes underdevelopment of the brain and testis in mice. Life Sci 74:2931–2940.

Kattas L, Gastrock F, Levin I, Cacciatore A. 2000. Plastics additives. In: Modern Plastics Handbook (Harper CA, ed). 1st ed. New York:McGraw-Hill, 4.1–4.69.

Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S, et al. 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a U.S. EPA-sponsored workshop. Environ Health Perspect (suppl 104):715–740.

Koch HM, Calafat AM. 2009. Human body burdens of chemicals used in plastics manufacture. Phil Trans R Soc B 364:2063–2078.

Leusch FDL, de Jager C, Levi Y, Lim R, Puijker L, Sacher F, et al. 2010. Comparison of five in vitro bioassays to measure estrogenic activity in environmental waters. Environ Sci Technol 44:3853–3860.

Matsushima A, Teramoto T, Okada H, Liu X, Tokunaga T, Kakuta Y, et al. 2008. ERRγ tethers strongly bisphenol A and 4-α-cumylphenol in an induced-fit manner. Biochem Biophys Res Commun 373(3):408–413.

Meeker JD, Sathyanarayana S, Swan SH. 2009. Phthalates and other additives in plastics: human exposure and associated health outcomes. Philos Trans R Soc Lond B Biol Sci 364:2097–2113.

Natarajan N, Shambaugh GE III, Elseth KM, Haines GK, Radosevich JA. 1994. Adaptation of the diphenylamine (DPA) assay to a 96-well plate tissue culture format and comparison with the MTT assay. BioTechniques 17:166–171.

National Research Council. 1999. Hormonally Active Agents in the Environment. Washington, DC:National Academies Press.

Newbold RR, Jefferson WN, Padilla-Banks E, Haseman J. 2004. Developmental exposure to diethylstilbestrol (DES) alters uterine response to estrogens in prepubescent mice: low versus high dose effects. Reprod Toxicol 18:399–406.

Patisaul HB, Fortino AE, Polston EK. 2006. Neonatal genistein or bisphenol-A exposure alters sexual differentiation of the AVPV. Neurotoxicol Teratol 28:111–118.

Patisaul HB, Todd KL, Mickens JA, Adewale HB. 2009. Impact of neonatal exposure to the ERα agonist PPT, bisphenol-A or phytoestrogens on hypothalamic kisspeptin fiber density in male and female rats. Neurotoxicology 30:350–357.

Plastics News. 2011. Resin Pricing Chart. 21 February: 21–22.

Sax L. 2010. Polyethylene terephthalate may yield endocrine disruptors. Environ Health Perspect 118:445–448.

Soto AM, Justicia H, Wray JW, Sonnenschein C. 1991. *p*-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. Environ Health Perspect 92:167–173.

Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect 103(suppl 7):113–122.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, et al. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect 113:1056–1061.

Talsness CE, Andrade AJ, Kuriyama SN, Taylor JA, vom Saal FS. 2009. Components of plastic: experimental studies in animals and relevance for human health. Philos Trans R Soc Lond B Biol Sci 364:2079–2096

Thompson RC, Swan SH, Moore CJ, vom Saal FS. 2009. Our plastic age. Philos Trans R Soc Lond B Biol Sci 364:1973–1976.

Till DE, Ehntholt DJ, Reid RC, Schwartz PS, Sidman KR, Schwope AD, et al. 1982. Migration of BHT antioxidant from high density polyethylene to foods and food simulants. Ind Eng Chem Prod Res Dev 21:106–113.

vom Saal FS, Nagel SC, Timms BG, Welshons WV. 2005. Implications for human health of the extensive bisphenol A literature showing adverse effects at low doses. Toxicology 212:244–252.

Wagner M, Oehlmann J. 2009. Endocrine disruptors in bottled mineral water: total estrogenic burden and migration from plastic bottles. Environ Sci Pollut Res 16:278–286.

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Proteomic Analysis of Pathways Involved in Estrogen-Induced Growth and Apoptosis of Breast Cancer Cells

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Abstract

Background: Estrogen is a known growth promoter for estrogen receptor (ER)-positive breast cancer cells. Paradoxically, in breast cancer cells that have been chronically deprived of estrogen stimulation, re-introduction of the hormone can induce apoptosis.

Methodology/Principal Findings: Here, we sought to identify signaling networks that are triggered by estradiol (E2) in isogenic MCF-7 breast cancer cells that undergo apoptosis (MCF-7:5C) versus cells that proliferate upon exposure to E2 (MCF-7). The nuclear receptor co-activator AIB1 (Amplified in Breast Cancer-1) is known to be rate-limiting for E2-induced cell survival responses in MCF-7 cells and was found here to also be required for the induction of apoptosis by E2 in the MCF-7:5C cells. Proteins that interact with AIB1 as well as complexes that contain tyrosine phosphorylated proteins were isolated by immunoprecipitation and identified by mass spectrometry (MS) at baseline and after a brief exposure to E2 for two hours. Bioinformatic network analyses of the identified protein interactions were then used to analyze E2 signaling pathways that trigger apoptosis versus survival. Comparison of MS data with a computationally-predicted AIB1 interaction network showed that 26 proteins identified in this study are within this network, and are involved in signal transduction, transcription, cell cycle regulation and protein degradation.

Conclusions: G-protein-coupled receptors, PI3 kinase, Wnt and Notch signaling pathways were most strongly associated with E2-induced proliferation or apoptosis and are integrated here into a global AIB1 signaling network that controls qualitatively distinct responses to estrogen.

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Introduction

Estrogen induces proliferation of estrogen receptor (ER)-positive breast cancer cells [1]. This response is consistent with the finding that antihormone therapies, such as tamoxifen or aromatase inhibitors, can enhance survivorship and reduce recurrence in patients with ER-positive breast cancers [2,3]. However, the majority of tumors eventually become unresponsive to antihormone treatments [4,5] and molecular mechanisms and markers of antihormone resistance have been described [6,7]. Once patients have failed on antihormone therapy, one treatment option has been the use of pharmacologic doses of estrogens [8,9] based on well-established findings that some breast cancers shrink during high dose estrogen treatment [10,11,12]. This phenomenon has also been observed in laboratory models of ER-positive breast cancer with acquired anti-hormone resistance that regress and undergo apoptosis in the presence of physiologic concentrations of estrogen [13,14] and was reviewed recently for its potential clinical implications [15].

Estrogen exerts diverse effects including genomic and non-genomic effects through multiple signaling pathways, that are significantly altered in anti-hormone resistant ER positive breast cancer cells. In antihormone resistant cells, for example, there is a general increase in EGFR and IGFR tyrosine kinase signaling [16,17], accompanied by increased ligand-independent phosphorylation of ER [18] and nuclear receptor co-activators such as AIB1/SRC3 (Amplified in Breast Cancer 1/Steroid Receptor Co-activator3) [19]. Overexpression and activation of AIB1 is associated with endocrine resistance in human breast cancer [20,21,22] and has been shown to be rate-limiting for estrogen-induced growth of breast cancer cells [23,24]. Beyond its role in these effects of estrogen, AIB1 was also shown to be rate-limiting for the growth of estrogen-insensitive breast cancer cells [25] as well as prostate cancer [26], pancreatic cancer [27] and lymphoma cells [28]. Furthermore, in AIB1 knockout mice, responses to hormones [29] as well as growth factor signaling [30] are blunted whereas overexpression of an AIB1 transgene leads to increased estrogen and growth factor responses resulting in

hyperplasia and neoplasia of mammary glands [31,32,33]. Thus, a large body of data support a crucial role for AIB1 in estrogen and growth factor signaling (reviewed in Refs [34,35]) and provides the rationale for the experimental paradigm used here.

To identify pathways that initiate estrogen-induced apoptosis versus growth, we used a combined proteomics and systems biology approach to elucidate triggering events and associated signaling pathways. We focused on changes of AIB1 interacting proteins, because of its central role in estrogen control of phenotypic behavior of breast cancer cells outlined above. AIB1 also coactivates IGF1R, EGFR and HER2 through modulation of tyrosine phosphorylation of these transmembrane receptors and phosphorylation of their subsequent signaling intermediaries [27,30,33,34]. Thus, to complement the analysis of direct AIB1 interacting proteins, we also monitored changes of phosphotyrosine (pY)-containing protein complexes, that are most likely regulated by growth factor signaling, as a means of discovering global intersecting pathways. As a model system, we used MCF-7 cells that proliferate in response to E2 [1], but also respond to EGF and heregulin [36] and have high levels of AIB1 protein due to gene amplification [37]. Wild-type MCF-7 cells were compared with MCF-7:5C cells that had been isolated under estrogen-free growth conditions [38,39]. MCF-7:5C cells were derived following long-term culture of MCF-7 cells in phenol red-free media. MCF-7:5C cells are ER-positive and undergo apoptosis after exposure to physiological concentrations of E2. In contrast, wild-type parental MCF-7 cells proliferate in the presence of the same concentration range of E2 [38,39]. The MCF-7:5C cells represent many of the characteristics of Phase II SERM resistant cells [40]. A parallel analysis after estrogen stimulation of these isogenic breast cancer cell lines served as a basis for the comparisons of signaling responses.

Here, we show that RNAi-mediated depletion of AIB1 reduces E2-induced growth of MCF-7 cells, and reverses the estrogen-induced apoptosis in MCF-7:5C cells. AIB1-interacting and pY-containing protein complexes were immunoprecipitated from short-term E2-treated cells, and the complexed proteins were identified by mass spectrometry (MS) analysis (Fig. 1A). From a comparison of the data sets obtained with MCF-7 versus MCF-7:5C cells treated with or without E2, and from a computationally-derived global AIB1-interacting network prediction, we identified pathways that participate in the differential response to E2 in these breast cancer cells. We found that a limited number of major cellular signaling pathways i.e. GPCR, PI3 kinase, Wnt, Notch

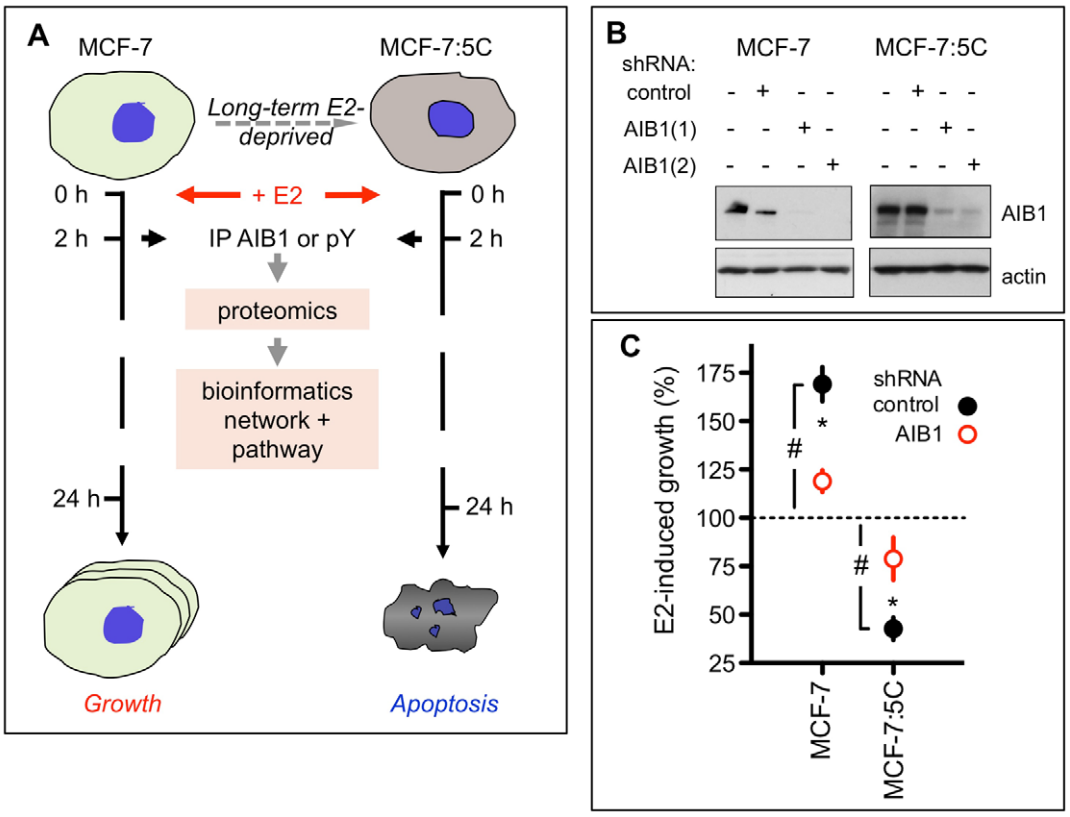

Figure 1. Phenotypic impact of AIB1 depletion on estradiol (E2) growth response in MCF-7 or MCF-7:5C cells. (*A*) The experimental paradigm. The differential responses to estradiol (E2) treatment of MCF-7 (cell growth) and long-term estrogen deprived MCF-7:5C cells (apoptosis) are indicated. Proteomics profiles of the two cell lines at baseline and after a brief (2 h) E2 treatment were generated using immunoprecipitations (IP). Proteins interacting with AIB1 or phosphotyrosine containing protein complexes were isolated by IP followed by mass spectrometry. Data were then subjected to an integrated bioinformatics analysis of signaling pathways and protein networks. (*B,C*) Reversal of E2-dependent effects on MCF-7 and MCF-7:5C after depletion of endogenous AIB1 protein using two different lentiviral shRNAs. MCF-7 or MCF-7:5C cells were infected with lentiviral particles expressing control or AIB1-targeting shRNAs. *(B)* RNAi-mediated knockdown was assayed by Western blot analysis for AIB1 relative to an actin loading control. *(C)* Cell growth was assayed 6 days after plating without or with E2. The E2 effect is shown relative to the respective untreated controls (mean ±S.E.M.). Closed circles: control shRNA; Open circles (red): AIB1 shRNA. #, $p<0.05$ E2 treatment effect vs. no treatment in control shRNA cells; *, $p<0.05$ E2 treatment effect in control shRNA cells vs. E2 treatment in AIB1 depleted cells. Representative data from one of at least three independent experiments are shown.
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and their associated molecules were involved in the control of estrogen induced proliferative or apoptotic responses. This information will be useful for determining appropriate targets to induce apoptosis in endocrine resistant human breast cancer.

Results and Discussion

Impact of AIB1 depletion on E2-induced growth effects in MCF-7 and MCF-7:5C cells

To determine the role of AIB1 in the E2-induced, distinct growth phenotypes of MCF-7:5C and wild-type MCF-7 cells, both cell lines were infected with lentiviral vectors that express control or two distinct AIB1-targeted shRNAs, and selected in puromycin for stable integrants. Both MCF-7 and MCF-7:5C cells were depleted of AIB1 protein, compared to uninfected and control shRNA infected cells with either of the shRNAs (Fig. 1B). Treatment with E2 significantly induced growth of control shRNA-infected MCF-7 cells and reduced the growth of MCF-7:5C cells (Fig. 1C, black symbols). In contrast to this, in AIB1-depleted, wild-type MCF-7 cells, E2 did not stimulate growth significantly above baseline and in AIB1 depleted MCF-7:5C, E2 lost its apoptosis-inducing effect (Fig. 1C, red symbols). These data suggest that AIB1 is a significant control hub of the E2-controlled growth phenotype in these ER-positive breast cancer cells.

Global analysis of AIB1- and phosphotyrosine-complexed proteins

Because AIB1 is rate-limiting for the E2-induced changes in the growth phenotype of MCF-7 and MCF-7:5C cells, we performed AIB1-specific immunoprecipitations of lysates from untreated and E2-treated (2 hrs) MCF-7 and MCF-7:5C cells to fractionate the respective proteome. Immunoprecipitation of phosphotyrosine-containing protein complexes was also performed to complement the AIB1-specific proteome fractionation (Fig. 1A). The immunoprecipitates were released from the beads, separated by denaturing gel electrophoreses (SDS-PAGE) and followed by Coomassie Blue staining of proteins in the gels (Fig. S7). Visible bands and the same region in parallel gel lanes were harvested and proteins present identified by mass spectrometry (MS). Stringent filtering of the initial proteomic data resulted in a subset of 101 proteins that either interacted with AIB1 (n = 58, Table S1) or are present in pY-protein complexes (n = 56, Table S2), with 13 proteins common to both.

The analytical approach emphasizes reliable identification of proteins by correlating mass spectrometry ID with the apparent molecular mass obtained from the SDS-PAGE (Fig. S7). This approach mimics Western blotting without having to rely on the availablility of antibodies, appropriate sensitivity, suitability for Western blotting and specificity. Still, we used Western blotting of some proteins identified by MS and show two examples in Fig. S8 (see below). To validate the mass spectrometry findings, separate experiments with independent mass spectrometry analyses were run. We found 48% of the proteins reported here in two and 16% in three or more independent experiments. This compares favorably with a recent HUPO study where only 7 of 27 laboratories identified all 20 proteins present at equimolar concentrations in a test sample [41]. In our experiments, the abundance of individual endogenous proteins captured in the immunoprecipitates covers a wide range (see Fig. S7). Thus, we expected that lower abundance proteins may drop below detection in repeat experiments. A combination of bioinformatics and mass spectrometry analysis was thus applied to meet this challenge as also described elsewhere [42,43].

The Venn diagrams of proteins pulled down with anti-AIB1 or anti-pY (Fig. 2) show the distribution of proteins between E2-treated and untreated, as well as wild-type MCF-7 versus MCF-7:5C cells (A and B), or between E2-treated and untreated cells regardless of cell type (C, *top*; and D, *top*), or between MCF-7 and MCF-7:5C cells regardless of treatment (C, *bottom*; and D, *bottom*). The number of pY-complexed proteins identified was affected very little by E2 treatment (18 vs. 25 proteins) with 13 proteins in either treatment group (Fig. 2D). In contrast, there was a significant, 4-fold higher number of AIB1-interacting proteins in the E2-treatment group (8 vs. 33 proteins; $p<0.05$, chi-square test; Fig. 2C) with 17 proteins not impacted in their interaction with AIB1. This suggests that AIB1-mediated protein-protein interactions are more responsive to E2 treatment, and new protein complexes are induced by E2 (Fig. 2A,C). In addition, the total number of proteins in complexes with AIB1 that overlap between MCF-7 and MCF-7:5C cells was not altered by the treatment, although the fraction of proteins per cell line that overlap decreases by 1/2 with E2-treatment (31% to 16%; Fig. 2A). Finally, while pathways activated by E2 gave rise to different sets of pY-containing protein complexes in both MCF-7 and MCF-7:5C cells, the percentage of proteins that overlap between cell lines remain almost constant regardless of treatment (4 vs. 5 in Fig. 2B).

Figure 3 shows the functional categories ascribed to the AIB1-associated (top) and pY-complexed (bottom) proteins. Tables S1 and S2 identify the proteins in each of these categories, cell lines (MCF-7 versus MCF-7:5C), and conditions (+/− E2) under which they were identified. Nearly half of the AIB1-interacting proteins fall into four categories, i.e. cytoskeleton and structural proteins, metabolism, transcription regulation, and signal transduction. Most of the pY-complexed proteins fall into four major functional categories: cytoskeleton and structural proteins, transcription regulation, signal transduction, and protein transport and vesicle trafficking. Thirteen proteins were found to be both AIB1-interacting and pY-complexed in MCF-7 and MCF-7:5C cells (Table S1).

Distinct profiles were observed for metabolism-related proteins between AIB1- and pY-complexed proteins, where the AIB1 complexes contained eight different enzymes in contrast to only one in the anti-pY group. This is consistent with studies demonstrating that AIB1 plays a role in the control of basal metabolic processes [44,45] that resulted in growth retardation and reduced hormonal responses in AIB1 knock-out mice [46]. Quite strikingly, all of these proteins were identified in E2 treated cells (e.g. 5-oxoprolinase in MCF-7:5C and fatty acid synthase in MCF-7 cells), whereas only three were identified in untreated as well as E2 treated cells. Seven AIB1-interacting proteins were detected in the categories of transcriptional regulation and chromatin complex, consistent with the role of AIB1 as a transcriptional coactivator. Interestingly, several proteins were found with pY immunoprecipitation that were unique to E2-treated MCF-7:5C cells, one of which was FAK1 (PTK2; Table S2). FAK1 is known to complex with EGFR as well as with an isoform of AIB1 and thus contribute to cellular signaling in breast cancer cells [47]. The MS based identification of FAK1 in the anti-pY immunoprecipitates was also seen by Western blot (Fig. S8A).

AIB1-containing protein complexes in E2-treated MCF-7:5C cells

We identified 18 proteins (CI >95%) that interact with AIB1 in E2-treated but not in untreated MCF-7:5C cells, 10 of which are also unique to MCF-7:5C cells (Table S1; Fig. 2A). These E2-induced AIB1-interacting proteins in MCF-7:5C cells mainly segregate in the category "transcriptional regulation" (6 of 18),

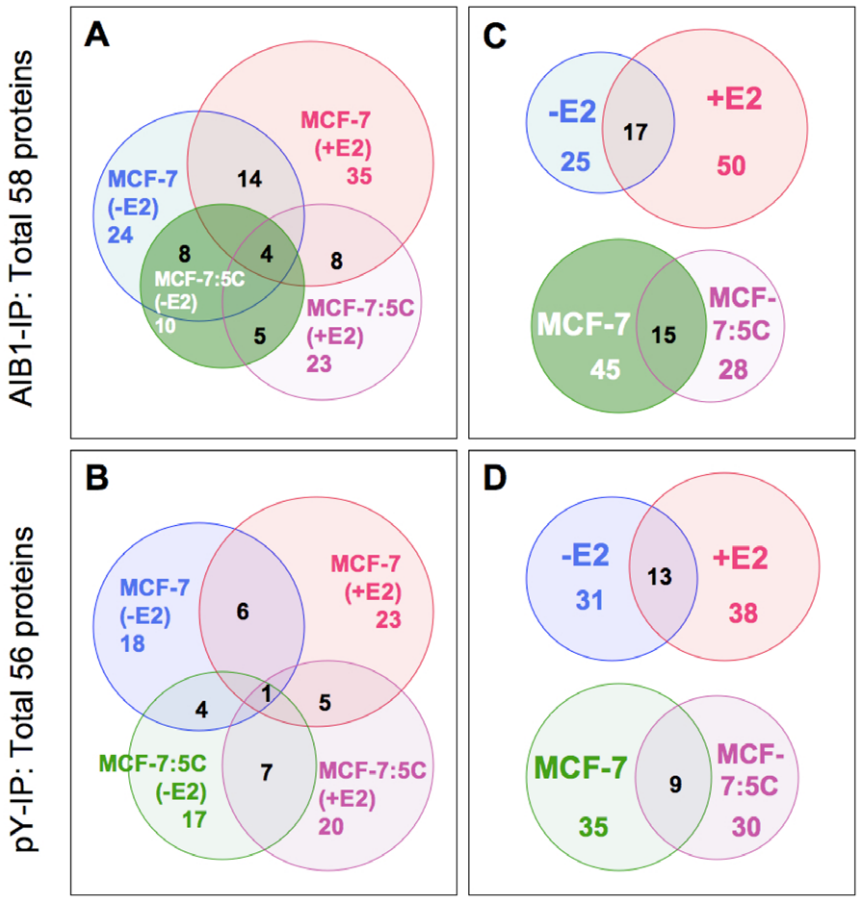

Figure 2. Summary of proteins identified under different conditions. Venn diagrams of proteins identified from anti-AIB1 (*A,C*) or anti-pY IP (*B,D*) experimental groups. (*C,D*) Proteins in combined AIB1-IP or pY-IP data sets. Individual proteins and subgroups are shown in Tables S1 & S2.
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several of which are also known to be involved in the control of apoptosis. For example, PRDM5, a PR domain and zinc-finger transcriptional regulator is a putative tumor suppressor and has been linked to cancer cell apoptosis [48]. TLE3, a transcriptional corepressor that binds to a number of transcription factors [49], can form a transcriptional repressor complex with RUNX3 [50], a known tumor suppressor that has been shown to be involved in apoptosis in gastric and colon cancer [51]. TLE3 has also been associated with the development of anti-estrogen resistance [52]. The MS identification of the 83 kDa TLE3 in AIB1 immunoprecipitations (IP) by was also seen by Western blot analysis (Fig. S8B). IASPP was identified in complex with AIB1 in both E2-treated MCF-7 and MCF-7:5C cells, but not in untreated cells. IASPP, a member of ASPP family of proteins, exerts anti-apoptosis effects through modulation of p53 [53,54,55]. Interestingly PRPF6, identified here as AIB1-interacting, is an U5 snRNP-associated protein involved in pre-mRNA splicing and has been shown to be a coactivator of the androgen receptor and mediates its ligand-independent AF-1 activation [56]. TLE3, PRDM5 and PRPF6 were all uniquely identified in E2-treated MCF-7:5C cells.

Potential pathways involved in E2-induced growth and apoptosis

To increase the potential of identifying pathways participating in E2-induced growth and apoptosis from the MS data sets, we not only analyzed proteins identified from MS with high confidence (CI $\geq$95%), but also took a global approach to include all proteins identified at various CI levels (see http://pir.georgetown.edu/iproxpress/coe2) by MS before filtering for pathway mapping with the Ingenuity^{TM} and GeneGO^{TM} pathway tools [43]. We hypothesized that if proteins identified at lower-level confidence by MS are found in known pathways that are consistent with the cellular phenotypes, they may provide valuable mechanistic insights. Also, supporting this approach are data from a recent study [57] with immunoprecipitation of nuclear extracts from MCF-7 cells that identified 13 of the 15 proteins we had seen at CI values in the lower range of 42–90%. The canonical pathway mapping analyses of all identified proteins suggest that several pathways are significantly represented both for proteins immunoprecipitated with anti-AIB1 and for those with anti-pY, including GPCRs, apoptosis, PI3K/AKT, and Wnt/β-catenin and Notch signaling pathways (Fig. S1, S2, S3, S4):

GPCR and growth factor signaling. Figure S1 depicts the GPCR-induced cell growth pathway, in which a number of proteins were identified in both AIB1 and pY-associated complexes. Gα(o) (GNAO2, IP-pY) and Rap1GAP (IP-AIB1) (Table S3), for example were identified exclusively in E2-treated MCF-7:5C cells. Gα(o) has been shown to directly bind to Rap1GAP resulting in the inhibition of the Ras-MAPK proliferation pathway [58]. In E2-treated MCF-7 cells, Gα(s) (GAS, GNAS) and CALM1 were coimmunoprecipitated with AIB1, while IP3R (ITPR3) was coimmunoprecipitated with AIB1 in both E2 treated MCF-7 and MCF-7:5C cells (Table S3). Each

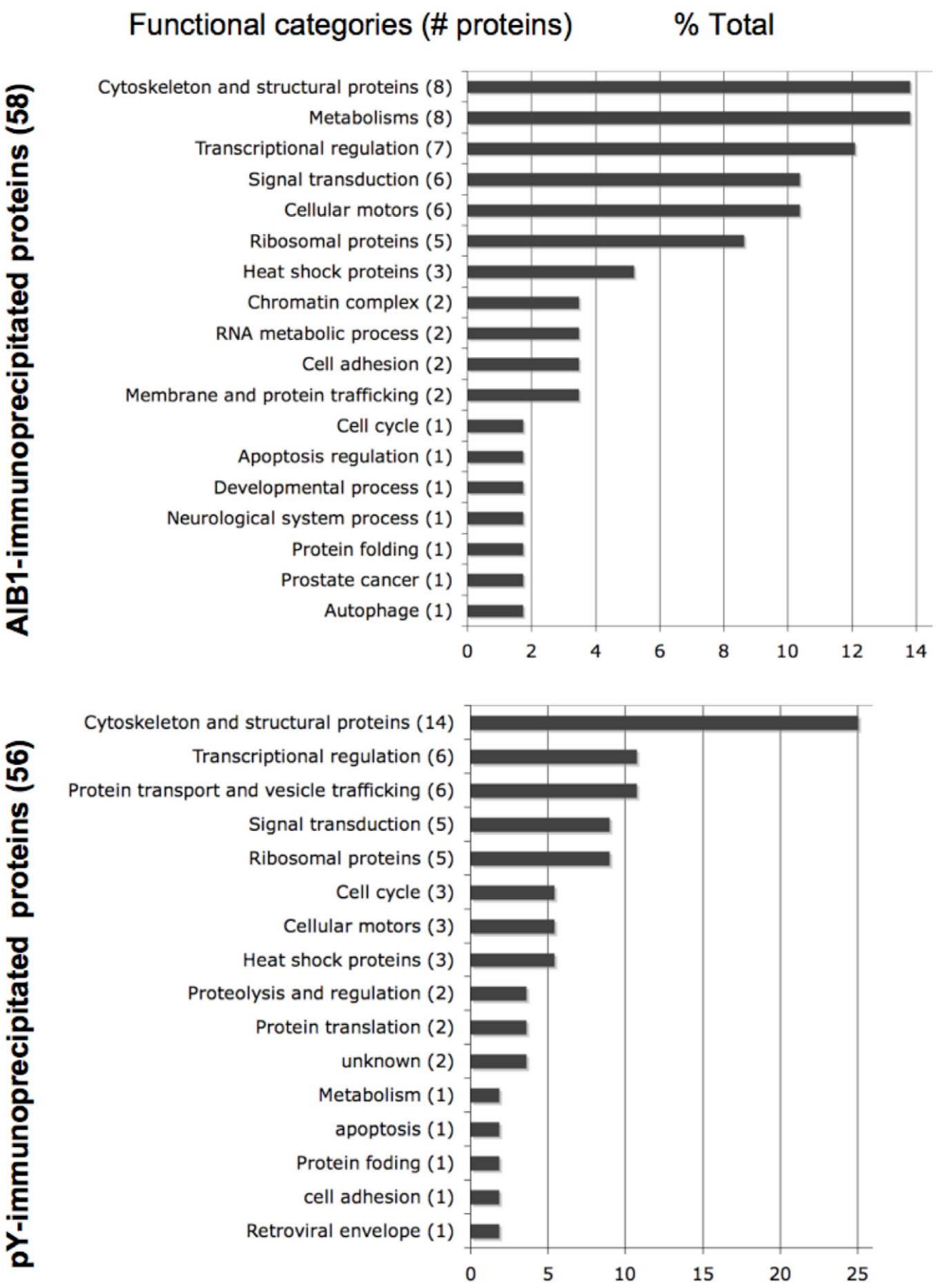
Functional categories (# proteins)
% Total
AIB1-immunoprecipitated proteins (58)
Cytoskeleton and structural proteins (8)
Metabolisms (8)
Transcriptional regulation (7)
Signal transduction (6)
Cellular motors (6)
Ribosomal proteins (5)
Heat shock proteins (3)
Chromatin complex (2)
RNA metabolic process (2)
Cell adhesion (2)
Membrane and protein trafficking (2)
Cell cycle (1)
Apoptosis regulation (1)
Developmental process (1)
Neurological system process (1)
Protein folding (1)
Prostate cancer (1)
Autophage (1)
0 2 4 6 8 10 12 14
pY-immunoprecipitated proteins (56)
Cytoskeleton and structural proteins (14)
Transcriptional regulation (6)
Protein transport and vesicle trafficking (6)
Signal transduction (5)
Ribosomal proteins (5)
Cell cycle (3)
Cellular motors (3)
Heat shock proteins (3)
Proteolysis and regulation (2)
Protein translation (2)
unknown (2)
Metabolism (1)
apoptosis (1)
Protein foding (1)
cell adhesion (1)
Retroviral envelope (1)
0 5 10 15 20 25

Figure 3. Functional categories of anti-AIB1 (upper) and anti-pY immunoprecipitated proteins (lower) from MCF-7 and MCF-7:5C breast cancer cells. Numbers in parenthesis are the number of proteins belonging to the respective category. Proteins profiled are those with CI values ≥95% from mass spectrometry.
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of these proteins is found downstream of GPCRs, and could lead to MAPK pathway activation and cell proliferation.

GPCRs and growth factors (IGF-1 and EGF) act via phosphorylation of the proapoptotic Bcl-2 family member BAD to regulate mitochondrial-mediated apoptosis (Fig. S2). BAD has been shown to be phosphorylated by Cdc2 (CDK1) at S128 [59] and Cdc2 was identified by anti-pY immunoprecipitation in E2-treated MCF-7:5C cells (Table S2). Also, two phosphatases, PP2B (PPP3CB) and PP2C (WIP1; Table S3, Fig. S2), associated with AIB1 only in MCF-7 cells. Both phosphatases can dephosphorylate BAD and thus modulate apoptosis [60]. In addition, RSK1 and RSK2, identified only in E2-treated cells (Table S3, Fig. S2), are also known to modulate cell survival [61,62].

Growth factors and cytokines can induce cellular growth and proliferation through PI3K-AKT signaling. A number of proteins complexed with AIB1 were identified in this pathway under different conditions (Fig. S3 and Table S3). The non-receptor tyrosine kinase TYK2 was detected in both MCF-7 and MCF-7:5C cells with or without E2 treatment. Both PI3K catalytic (p110) and regulatory (p85) subunits were pulled down only in E2-treated, not in untreated MCF-7 cells (Fig. S3C). PI3K/p110 was detected, additionally, in untreated but not treated MCF-7:5C cells (Fig. S3B). Thus, PI3K/p110 was isolated only under conditions that promoted proliferation in both cell lines. GSK3β, identified in AIB1 immunoprecipitates in E2-treated MCF-7 cells (Fig. S3C), can be activated by PI3K/AKT, and has also been shown to be a regulator of Wnt signaling (see below). Finally, BCL3, a member of the I-kappa-B family that regulates NFκB-mediated transcription [63,64], was only identified in E2-treated MCF-7 cells.

Wnt/β-catenin and Notch signaling. Our data indicate that Wnt/β-catenin, and Notch signaling pathways participate in E2 responses in both MCF-7 and MCF-7:5C cells (Fig. S4). Several key proteins in the pathway, such as Wnt ligands, cadherin, β-catenin, casein kinases and GSK3β were identified in distinct AIB1- and pY-containing complexes, amongst different cells and treatments (Fig. S4A, B and C). For example, in MCF-7:5C cells, Frizzled-7 (FZD7) and cadherin 22 (CDH22) were identified in pY-containing complexes after E2 treatment, while β-catenin associated with AIB1 regardless of E2 treatment (Table S3). In MCF-7 cells, the Wnt ligand Wnt-7a, CK1δ, and GSK3β were identified in AIB1 immunoprecipitates (Table S3). CK1δ was recently reported to modulate the transcriptional activity of ERα in an estrogen-dependent manner and regulates ER-AIB1 interactions [65]. An additional protein, δ-catenin, or $p120^{ctn}$, a member of armadillo/β-catenin superfamily [66], was identified in the AIB1 immunoprecipitates of E2-treated MCF-7 cells (Table S1).

Our results suggest that multiple proteins found in AIB1 associated complexes, that function in Wnt signaling, also crosstalk with Notch and growth factor-induced signaling in response to E2 treatment in breast cancer cells. TLE3 was detected only in E2-treated MCF-7:5C cells, and Notch1, Notch3, and Numb-like protein were identified only in E2-treated MCF-7 cells (Table S3). TLE3, the mammalian homolog of Gro [67], is a global corepressor mediating transcriptional repression targeted by a number of signal pathways. As shown in Fig. S4D, TLE3 connects the Notch and Wnt pathways [68,69]. In addition to the apoptosis related proteins discussed above (TLE3, PRDM5, CDK1), DBC1 was isolated from anti-pY immunoprecipitates in E2 treated MCF-7:5C cells (Table S2). Interestingly, DBC1 was recently reported to increase p53 mediated apoptosis in breast cancer cells [70]. Taken together, proteins from GPCR and PI3K/AKT-mediated growth signaling pathways were more prevalent in E2-stimulated MCF-7 cells, whereas proteins related to apoptosis pathways were more prevalent in E2-stimulated MCF-7:5C cells. The respective connectivity of the pathways is depicted in Figure 4.

Global AIB1 interaction networks

To extract further information from these experimental data, they were linked with an AIB1 interaction network generated from published data [43]. A computational global AIB1 protein interaction network can be constructed from 91 AIB1 interaction partners (first neighbors) based on the literature published since AIB1 was first described in 1997 [37]. These 91 proteins belong to several major functional categories that include transcription, cell communication, developmental processes and cell cycle regulation. The initial network was expanded to secondary interaction neighbors, based on protein-protein interaction data in the public domain. At this level, the network is composed of 1150 proteins, including 21 highly connected nodes that form major hubs (Fig. 5). These hubs include p53, BRCA1, BCL2, ABL1, CDK2, CDK4, EGFR, ER (=ESR1), p38, and MYC (Fig. 5 and S5). Closely related subnetworks of AIB1 (=NCOA3) shown in Figure S5 (*lower panel*), contain four hub proteins: BRCA1, MYC, CDK2 and PSME3. In the present study we identified 26 proteins that are part of the global AIB1 interaction network and function in signal transduction, transcriptional regulation, the cytoskeleton, and the heat shock response.

Eighteen of the proteins experimentally associated with tyrosine-phosphorylated protein complexes are also part of the global AIB1-interaction network. Of these, seven were identified as interacting with AIB1, including CALM1, ACTB, ACTG1, TUBGCP2, MYH9, HSPA1B, and HSPA9. These proteins correspond to interacting hubs, such as CDK4, MYC, PSME3 and CHUK. We conclude that these hubs may participate in the differential cellular responses to E2.

Connection of E2 transcriptome and proteome effects

An interesting question is to what extent the proteomic pathway mapping parallels mRNA expression profiling in MCF-7 and MCF-7:5C cells. Baseline mRNA expression profiles of these cell lines have been posted earlier (GSE10879; ncbi.nlm.nih.gov/). An analysis of mRNA expression regulation after 48 hrs of E2 treatment of the cells was analyzed and published recently [71]. In MCF-7 cells Bcl-2, a major anti-apoptosis gene, was found upregulated by E2 treatment whereas no change of bcl-2 was seen in MCF-7:5C cells. In our analysis Bcl-2 is one of the major hubs in the AIB1 interaction networks (Fig. 5 and S5). On the other hand, the pro-apototic Bcl-2 antagonists Bak, Bax and Bim mRNAs were found upregulated 2- to 7-fold after E2 treatment of MCF-7:5C cells whereas no mRNA expression change was seen in the MCF-7 cells. Our analysis shows that upstream regulators of the canonical intrinsic mitochondrial pathway such as RSKs, were identified in the proteomics approach (Fig. 4 and S2).

The most differentially regulated mRNA after E2 treatment was Gadd45beta that was found up-regulated 5-fold in MCF-7:5C cells but down-regulated 5-fold in MCF-7 cells [71]. Gadd45beta was described earlier as a hub of the MAP kinase signaling cascade and

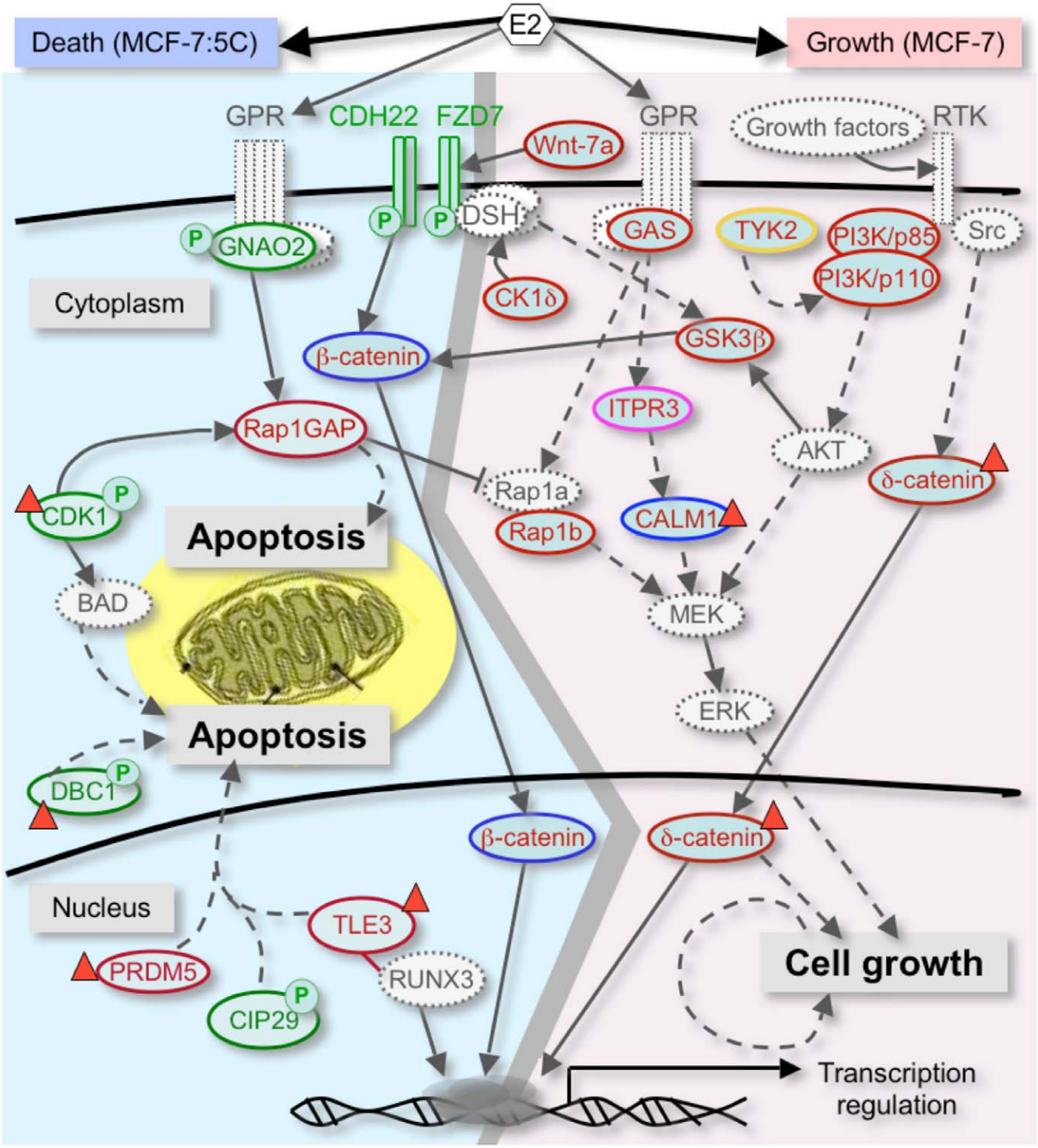
Death (MCF-7:5C)
E2
Growth (MCF-7)
GPR
CDH22
FZD7
Wnt-7a
GPR
Growth factors
RTK
P
GNAO2
P
P
DSH
GAS
TYK2
PI3K/p85
Src
PI3K/p110
Cytoplasm
CK1δ
GSK3β
β-catenin
ITPR3
Rap1GAP
AKT
δ-catenin
Rap1a
CDK1
P
Apoptosis
CALM1
Rap1b
BAD
MEK
ERK
Apoptosis
DBC1
P
β-catenin
δ-catenin
Nucleus
TLE3
PRDM5
RUNX3
Cell growth
CIP29
P
Transcription
regulation
AIB1-IPed:
+ E2, in given cells
+ E2, in both cells
-/+ E2, in given cells
-/+ E2, in both cells
pY-IPed + E2, in given cells
CI ≥95% from MS identification
Proteins in canonical pathways, but not observed in this study

Figure 4. Pathway overview map of proteins involved in E2-induced cell growth or apoptosis in MCF-7 versus MCF-7:5C breast cancer cells. The thick grey line in the middle provides an arbitrary boundary between the pathways. Anti-AIB1 immunoprecipitated (AIB1-IPed) and anti-pY-immunoprecipitated proteins (pY-IPed) are indicated by red or green circles respectively (keys at the bottom). The blue circled proteins are AIB1-IPed proteins from MCF-7 (CALM1) or MCF-7:5C cells (β-catenin) under both E2-treated and untreated conditions; the purple circled one (ITPR3) is an AIB1-IPed protein from both cells only under E2 treated condition, while the yellow circled one (TYK2) is an AIB1-IPed protein from both cells under both E2 treated and untreated conditions. Proteins circled in grey are from known canonical pathways (e.g. ERK in cell growth or BAD in apoptosis) but not identified here. Solid line arrows indicate direct interactions (e.g. CDK1 phosphorylates Rap1GAP) or translocations (e.g. catenins) of proteins, while dashed arrows indicate indirect actions of proteins (e.g. AKT activate MEK through several steps). Hammer-ended lines indicate inhibitory effects on the target. Detailled pathways are shown in Fig. S1, S2, S3, S4.
doi:10.1371/journal.pone.0020410.g004

connects to relA, the NFkappaB p65 subunit (see e.g. Ref. [72]) as well as cell survival in apoptosis resistant cells [73]. We isolated components of GPCR signaling in our proteomics analysis (Fig. 4 and Fig. S1) that can connect to these downstream effectors and can thus serve as trigger mechanisms. Interestingly, GPR30 mRNA was found upregulated in MCF-7:5C cells after estradiol treatment [40] and GPR30 was shown to rapidly transmit non-genomic effects of E2 in breast cancer cells [74]. Overall, the mRNA expression analyses and proteomics data show some interesting convergences especially in apoptotic regulatory pathways which may be functionally relevant as initiators of estradiol–induced apoptosis or cell survival.

Conclusions

The estrogen induced apoptotic response is most strongly associated with early signaling changes in G-protein coupled receptors, PI3 kinase, Wnt and Notch signaling and are integrated here into a global AIB1 signaling network that controls qualitatively distinct responses to estrogen.

Materials and Methods

The overall experimental design

We used combined proteomics and bioinformatics approaches [43] to identify the E2 induced signaling pathways and networks that are associated with AIB1 and/or tyrosine phosphorylated proteins and that differentiate the MCF-7 from MCF-7:5C cells in responses to E2 treatment (Fig. 1A). A single early time point after E2 treatment (2 hrs) was examined to capture signaling events that drive apoptosis or proliferation in these cells. Repeat independent proteomic experiments for each of the 4 experimental conditions and the two different immunoprecipitations were run.

Cell culture

MCF-7 (ATCC) human breast cancer cells and the MCF-7 variant MCF-7:5C [75] , which is a clonal variant of MCF-7 derived after longterm estrogen deprivation, were cultured in RPMI-1640 without Phenol Red (Invitrogen) supplemented with 10% FBS, or in RPMI-1640 supplemented with 10% charcoal/

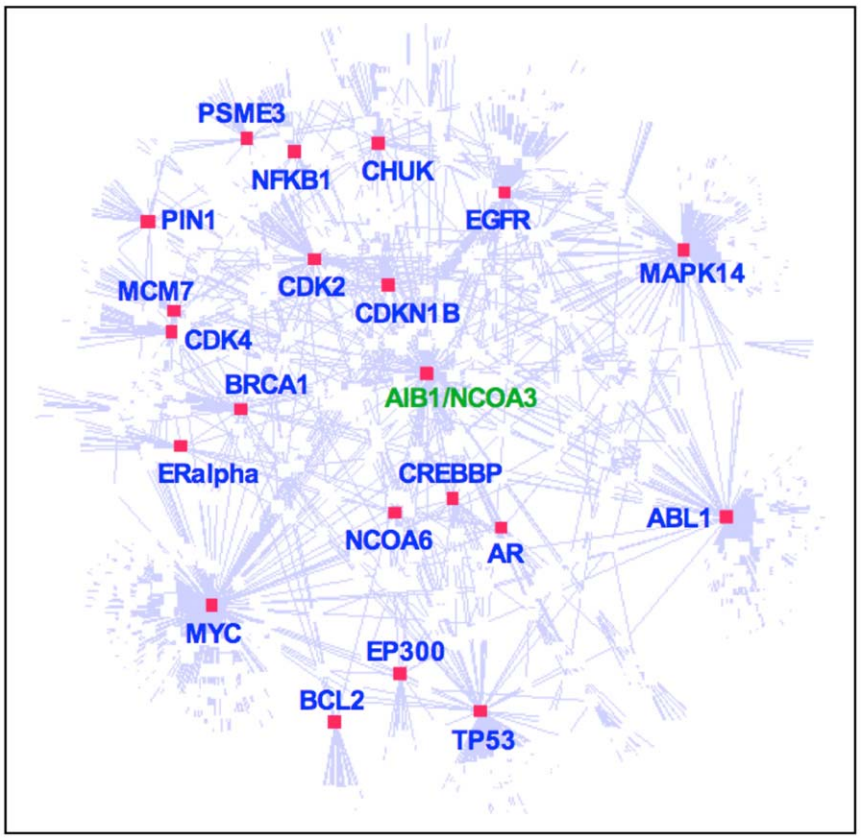

Figure 5. A global AIB1 interaction network showing the major hub proteins. Twenty-one hubs were identified using a cutoff of 20 node degrees. The full names of the respective gene symbols are provided in Table S8. Detailed nodes in the network are shown in Fig. S5.
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dextran-stripped FBS (Hyclone) and other supplements, respectively, as described previously [38]. MCF-7 or MCF-7:5C cells deprived of steroid hormones for 2 days were plated at a density of 2,000 and 3,000 cells per well, respectively, in 96-well cell culture plates. One day after plating, cells were treated with E2 (in ethanol) or vehicle (ethanol). To monitor the portion of viable cells after 6 days of growth, the CellTiter-Glo luminescent cell viability assay (Promega) or WST1 colorimetric cell proliferation assay (Roche) were used. Typical readings of baseline growth without E2 were 2.0×10^5 RLU (CellTiter-Glo) or an OD450 of 0.5 (WST1). Data are shown relative to the baseline.

Infection of MCF-7 and MCF-7:5C with lentiviral shRNA expression vectors

Prior to infection, MCF-7 and MCF-7:5C cells were plated at a density of 3×10^5 cells on 10 cm tissue culture dishes. 24 hrs later, cells were infected with lentiviral particles expressing control or AIB1-targeting shRNAs (in pLKO.1). The AIB1(1) shRNA was derived from an siRNA for AIB1 previously described [25], and the AIB1(2) shRNA was from Sigma (TRCN0000019703). The control shRNA used in the experiments is a scrambled sequence described previously [76]. Briefly, 1 ml of lentivirus-containing supernatant was added to 9 ml of growth medium and 8 ng/ml polybrene, and then added to cells for 24 hrs. Medium containing lentivirus was then replaced with growth medium without lentivirus. After two days, cells were treated for 48 hours with 5 μg/ml puromycin for the selection of lentiviral shRNA expression.

Western blot analysis, immunoprecipitation and protein isolation

Western blot analyses were done as previously described [25], using a monoclonal antibody for AIB1 (SRC3; clone 5E11, Cell Signaling). For the mass spectrometry analysis, protein lysates from cells treated for 2 hours with E2 or vehicle were subjected to immunoprecipitation using gamma-bind G-Sepharose beads and an anti-AIB1 monoclonal antibody (BD Biosciences) as described [77] or an anti-phosphotyrosine monoclonal antibody (4G-10, Millipore). The amount of protein input for immunoprecipitations ranged between 7 mg and 14 mg for each of the experimental conditions with bovine serum albumin used as the standard. It is noteworthy that over a 24 hour period of E2 treatment of cells the AIB1 protein expression levels varied less than 2-fold as illustrated in Figure S6. The immunoprecipitated proteins were separated by denaturing SDS-PAGE on 4–12% Nu-PAGE gels (Invitrogen). After electrophoresis, gels were stained with Coomassie blue overnight and washed with ddH2O overnight to remove background staining. Stained gels were imaged using a color scanner and visible bands were cut from the gels. The corresponding segments of lanes from the different treatments were also cut for analyses and served as controls. Figure S7 shows a representative set of stained gels with an overlay of the grid of segments harvested for the mass spectrometry analyses.

Mass spectrometry analysis

SDS-PAGE gel slices were subjected to tryptic digest and followed by MS and MS/MS on an ABI MALDI-TOF-TOF. Proteins in the MS or MS/MS analysis were identified based on searches of the Swiss-Prot database using the search engine Mascot 2.0. The Swiss-Prot database searched was based on its 9/24/2007 release (287,050 sequences). The database search parameters used were: 1) enzyme specificity considered, trypsin; 2) number of missed cleavages permitted, 1; 3) fixed modification(s), carbamidomethyl (C); 4) variable modification(s), oxidation (M); 5) mass tolerance for precursor ions, 75ppm; and 6) mass tolerance for fragment ions, 0.3 Da. Trypsin autolysis peaks were excluded from the peak list. GPS Explorer (Version 3.0) with default parameter setting was used to generate the peak list from raw data which were submitted to database searches using Mascot. The confidence interval (CI) for the peptide identification was calculated by GPS Explorer. A CI of $\geq$95% (or expectation value $\leq$0.05) was used as a cut off for the high CI proteins.

Bioinformatics Analysis

Protein data filtering. Proteins identified from mass spectrometry were subjected to extensive bioinformatics analysis, including protein data filtering, functional profiling and pathway mapping as described previously [78]. Protein identities from different experimental groups were assigned levels of identification confidence based on statistical processing by GPS Explorer™ of the MASCOT search results. It is commonly known that false negative identification is generated because low-scored proteins may result from factors such as database size, protein abundance and the type of mass spectrometry instrumentation. Therefore, in addition to analyzing the proteomic data based on the prioritized list of proteins with high Confidence Interval (CI; Tables S1, S2), we also used a global approach for pathway mapping on proteins identified at all confidence levels. We provide the identity, CI and spectra of those proteins as well as the reference to the respective pathway figures in Table S3.

We used the following criteria to filter the protein lists. (i) Proteins with MS confidence interval (CI) values smaller than 95% were removed to reduce false-positive results; (ii) Proteins described to be non-specific interactors e.g. HSPA5 and Desmoplakin [79] were removed; (iii) High abundant, non-specific proteins e.g. keratins were removed; (iv) Proteins migrating at an apparent mass in the SDS-PAGE that was different from the calculated mass or the experimentally described mass or the predicted mass were removed. A representative set of Coomassie stained gels after immunoprecipitations is shown in Fig. S7 to illustrate this latter consideration.

Protein annotation, profiling and pathway analysis. The iProXpress bioinformatics system (http://pir.georgetown.edu/iproxpress) was used for protein annotation, function and pathway profiling of the proteomics data. The experimental group(s) in which the proteins were identified was annotated for all proteins and integrated into the iProXpress system for direct functional comparison between selected groups, such as cell types, E2 treatment, and experimental repeats. The procedure of using iProXpress system has been described recently [43,78]. The data sets are accessible at http://pir.georgetown.edu/iproxpress/coe2/ . Pathway mapping and network visualization are assisted with Ingenuity Pathways Analysis (IPA) (www.ingenuity.com) and GeneGO MetaCore (www.GeneGO.com) software tools.

Data mining for known AIB1 interactors. The global AIB1 interaction network refers to a network of genes or proteins that directly or indirectly interact or are functionally associated with AIB1 regardless of cell/tissue types or species in which the interaction occurs. The network is was computationally generated based on two sources of data, i.e. the published literature (PubMed) and protein-protein interactions (PPI) available from public databases. A list of AIB1 synonyms included as query terms "AIB1 OR AIB-1 OR NCOA3 OR NCOA-3 OR SRC3 OR SRC-3 OR TRAM1 OR ACTR OR pCIP" to search PubMed and retrieved a total of about 650 papers related to AIB1. Of these papers about 250 papers that contain AIB1 interaction or functional association information were curated, and a total of

91 AIB1 interaction partners were thus obtained. The interaction types in the literature included physical interactions, such as "*binding*", "*complex*", "*interact*", "*phosphorylation*", etc., and functional associations, such as "*activation*", "*correlated expression*", "*lead to degradation*", "*modulate*", "*promoter binding*", "*suppression*", etc. These interacting proteins/genes reported for human as well as other species from mouse to *Xenopus*, were mapped to corresponding human orthologs based on UniProtKB database.

The protein/protein interaction (PPI) data annotated in bioinformatics databases were obtained from IntAct database [80], which contains high throughput PPI data from Y2H and IP in addition to literature data. The AIB1 interaction network was constructed based on the binary interactions of the curated 91 AIB1-interacting proteins and those from the PPI database. The network was clustered and filtered, and major hubs were selected using a cutoff of a node degree of 20. Cytoscape open source software was used to display the network for visual examinations.

Supporting Information

Figure S1 Proteins identified in GPCR signaling pathways. Canonical cell growth pathways initiated by GCPR signaling are depicted based on the MetaCore pathway tool of GeneGO. The AIB1- and pY-IPed proteins identified from the study were mapped to the pathway using MetaCore, which were manually re-annotated in the red-lined white boxes with black arrows pointing to the specific protein depictions. The corresponding experimental conditions under which the proteins were identified are indicated at the bottom. Proteins were AIB1-IPed under conditions indicated as A–D, or pY-IPed indicated by "p".
(TIF)

Figure S2 Proteins identified in apoptosis pathways. The canonical intrinsic mitochondrial apoptosis pathway is depicted based the MetaCore pathway tool of GeneGO. Similar to Fig. S3, the anti-AIB1- and pY-IPed proteins identified from the study were mapped to the pathway and were manually re-annotated with red-lined white boxes with the specific protein identified here.
(TIF)

Figure S3 Proteins identified in the PI3K/AKT pathway. The canonical PI3K/AKT pathway is depicted based on the Ingenuity pathway tool. AIB1-IPed proteins that were mapped to the canonical pathway are shown as orange-colored shapes in four panels, each representing the same PI3K/AKT pathway with different mapped proteins that were identified from untreated MCF-7 (A) or MCF-7:5C (B) and E2-treated MCF-7 (C) or MCF-7:5C (D) cells. Some proteins in the pathway were manually re-annotated with green-colored box to indicate the specific protein forms identified in this study that correspond to the protein classes represented in the canonical pathway, e.g. JAK refers to the non-receptor type tyrosine kinases, such as TYK2 here.
(TIF)

Figure S4 Proteins identified in the Wnt/β-catenin pathway. The canonical Wnt/β-catenin pathway is depicted based on the Ingenuity pathway tool. AIB1-IPed proteins that can be mapped to the canonical pathway are shown as orange-colored shapes in four panels, each representing the same Wnt/β-catenin pathway with different mapped proteins that were identified from untreated MCF-7 (A) or MCF-7:5C (B) and E2-treated MCF-7 (C) or MCF-7:5C (D) cells. Some proteins in the pathway were manually re-annotated with green-colored box to indicate the specific protein forms identified in the experiment that correspond to the classes represented in the canonical pathway, e.g. Wnt refers to class of Wnt ligands, such as Wnt-4 and Wnt-7a. Some proteins manually labeled with a "P" in red indicate that they were identified as pY-IPed.
(TIF)

Figure S5 AIB1 interaction network. A global AIB1 interaction network (upper) and the selected sub-networks (lower) are shown. The overall topology of the network is displayed with Spring-embedded layout using Cytoscape network visualization software before network clustering (image can be zoomed in to view individual node). Proteins that are identified with high confidence in this study are colored as green (AIB1-IPed), yellow (pY-IPed) or dark brown (both AIB1- and pY-IPed) nodes. Hub proteins that are subsequently clustered with AIB1 in several subnetworks are indicated with arrows (*upper*). Individual nodes in AIB1-clustered subnetworks are shown in the lower panel, with major functional categories labeled for the hub proteins.
(TIF)

Figure S6 Western blot analysis for AIB1. Cells treated with E2 for different times were harvested and Western blot analysis for AIB1 was performed as described in Materials and Methods.
(TIF)

Figure S7 Coomassie stained protein gels after anti-AIB1 or -pY immunoprecipitation (IP). MCF-7 and MCF-7:5C cells were treated or not with E2 for 2 hours, and proteins were extracted for IP. The immunoprecipitated proteins were separated by 4–12% Nu-PAGE, stained, washed with ddH20 and imaged using a color scanner. The images were magnified and analyzed visually on a screen. After identification, bands were cut from the gels and great care was taken to isolate the same segment of all lanes from the different treatments for a parallel MS analysis. Representative stained gels with the segments to be cut for analysis are indicated. Slices numbered 1–10 or 1–13 were cut from the gels for each segment that showed at least one distinctly regulated protein. Molecular masses of marker proteins are indicated (10–250 kDa).
(TIF)

Figure S8 Western blot analysis confirms that FAK1 and TLE3 are immunoprecipitated from E2 treated MCF7:5C cells. MCF-7:5C cells were treated or not with E2 for 2 hours, and proteins were extracted for IP/Western analysis A) Tyrosine-phosphorylated endogenous proteins were immunoprecipitated with anti-phosphotyrosine monoclonal antibody (4G-10, Millipore) and the immunoprecipitate was resolved on SDS-PAGE followed by Western analysis. The input is 5% of the amount of total cell lysates for IP. FAK1 was detected on the blot with an anti-FAK1 antibody (A-17, Santa Cruz). B) AIB1 interacting proteins were immunoprecipitated using an anti-AIB1 monoclonal antibody (BD Biosciences). The input is 5% of the amount of total cell lysates for IP. TLE3 was detected on the blot with a TLE3 antibody (Abcam).
(TIF)

Table S1 AIB1-interacting proteins with a CI value of ≥95%. AIB1-interacting proteins (n = 58) isolated from MCF-7 and MCF-7:5C cells identified by MALDI-MS/MS with a CI value of ≥95% are listed and assigned with functional categories. The number of peptides identified and % coverage are in Table S4. Various experimental groups in which AIB1-interacting proteins were identified, are shown in the right side columns (with vertical column names), and the number of total proteins in each group is given in parenthesis. Proteins are arranged by their functional categories (see Fig. 3) and the number of proteins in each experimental group of a given category is also indicated in

the same row of the category. The column furthest to the right shows AIB1-interacting proteins in this study that are also identified as part of the AIB1 protein interaction (int.) network. "X" indicates the presence of a given protein in a given experimental group or in the AIB1 interaction network. Asterisks by the protein accession indicate AIB1-interacting proteins that are also identified in pY complexes (see Table S2).
(DOC)

Table S2 Phosphotyrosine complexed proteins with a CI value of ≥95%. Proteins pulled down with anti-pY in MCF-7 and MCF-7:5C cells identified from MALDI-MS/MS with a CI value of ≥95% are listed and assigned with functional categories. The number of peptides identified and % coverage are in Table S5. Various experimental groups in which tyrosine-phosphorylated proteins are identified are shown in the right columns (with vertical column names), and the number of proteins in each group is given in parenthesis. Proteins are arranged by their functional categories (see Fig. 3) and the number of proteins in each experimental group of a given category is also indicated in the same row of the category. "X" indicates the presence of a given protein in a given experimental group or in the AIB1 interaction network. Asterisks by the protein accession indicate IP-pY complexes that are also identified as AIB1-interacting (see Table S1).
(DOC)

Table S3 Pathway mapping of proteins identified with a CI<95%. Proteins are listed alphabetically based on the "gene name" column for anti-AIB1 or anti-pY immunoprecipitated proteins. In the "Experiment" column A to D indicate: A, MCF-7 cells, no E2; B, MCF-7:5C cells, no E2; C, MCF-7 cells, +E2; and D, MCF-7:5C cells, +E2. The "Spec" column references the corresponding mass spectrum for single peptide MS/MS identification in the section "Single peptide spectral data" appended at the end of this table. The "Figures" column indicates in which figure(s) the proteins are depicted, except for a few only discussed in the main text (*text*). In the spectral data section, the underlined C and M in "peptide sequences" column represent fixed (carbamidomethyl) and variable (oxidation) modifications, respectively. *MALDI-TOF-MS generates peptides containing only one charge, and the precursor m/z is thus equal to the precursor mass. NA, not available.
(DOC)

Table S4 AIB1-complexed proteins identified by MALDI-TOF-TOF. Proteins were identified based on single MS (MS) or tandem MS (MS/MS) using the search engine Mascot 2.0 from the Swiss-Prot database. Note that the same proteins could be identified under different experimental ("Exp.") conditions: A, MCF-7 cells, no E2; B, MCF-7:5C cell, no E2; C, MCF-7 cell, +E2; D, MCF-7:5C cell, +E2. For proteins identified from single peptide MS/MS, spectral data (Spec.) are referenced using the labels (A1–A30) to correspond to those shown in Table S6. All spectra for single peptides shown here are manually inspected, including the one that shows CI of 93% but with good ion fragments. *The % coverage for single peptide MS/MS was only stated if the respective peptide covered ≥1% of the protein. The spectra and sequences are in Table S6.
(DOC)

Table S5 pY-complexed proteins identified by MALDI-TOF-TOF. Proteins were identified based on single MS (MS) or tandem MS (MS/MS) using the search engine Mascot 2.0 from the Swiss-Prot database. Note that the same proteins could be identified under different experimental ("Exp.") conditions: A, MCF-7 cells, no E2; B, MCF-7:5C cell, no E2; C, MCF-7 cell, +E2; D, MCF-7:5C cell, +E2. Proteins that were identified more than once from experimental repeats under the same conditions are labeled with * in the "Exp" column. For proteins identified from single peptide MS/MS, spectral data (Spec.) are referenced using the labels (Y1–Y38) to correspond to those shown in Table S7. All spectra for single peptides shown here were manually inspected, including those that show 90%≤ CI ≤95% but with good ion fragments. *The % coverage for single peptide MS/MS was only stated if the respective peptide covered ≥1% of the protein. The spectra and sequences are in Table S7.
(DOC)

Table S6 MS/MS spectra for single peptide identified AIB1-complexed proteins. The "No." column labels the spectra sequentially as referenced in Table S4. The "Exp." column indicates the experimental conditions under which the respective protein was identified: A, MCF-7 cells, no E2; B, MCF-7:5C cell, no E2; C, MCF-7 cell, +E2; D, MCF-7:5C cell, +E2. The underlined C and M in peptide sequences represent fixed (carbamidomethyl) and variable (oxidation) modifications, respectively. *MALDI-TOF-MS generates peptides containing only one charge and the precursor m/z (not shown) is thus equal to the precursor mass.
(DOC)

Table S7 MS/MS spectra for single peptide identified pY-complexed proteins. The "No." column labels the spectra sequentially as referenced in Table S5. The "Exp." column indicates the experimental conditions under which the protein was identified: A, MCF-7 cells, no E2; B, MCF-7:5C cell, no E2; C, MCF-7 cell, +E2; D, MCF-7:5C cell, +E2. The underlined C and M in peptide sequences represent fixed (carbamidomethyl) and variable (oxidation) modifications, respectively. *MALDI-TOF-MS generates peptides containing only one charge and the precursor m/z (not shown) is thus equal to the precursor mass.
(DOC)

Table S8 List of acronyms used.
(DOC)

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Author Contributions

Conceived and designed the experiments: Z-ZH VCJ ATR AW. Performed the experiments: BLK EAA LZ JVL. Analyzed the data: Z-ZH BLK DSR HH CW ATR AW. Wrote the paper: Z-ZH BLK ATR AW.

References

1. Lippman ME, Bolan G (1975) Oestrogen-responsive human breast cancer in long term tissue culture. Nature 256: 592–593.
2. EBCTCG (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. The Lancet 365: 1687–1717.
3. Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, et al. (2005) Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 365: 60–62.
4. Nicholson RI, Johnston SR (2005) Endocrine therapy–current benefits and limitations. Breast Cancer Res Treat 93 Suppl 1: S3–10.

5. Brauch H, Jordan VC (2009) Targeting of tamoxifen to enhance antitumour action for the treatment and prevention of breast cancer: the 'personalised' approach? Eur J Cancer 45: 2274–2283.
6. Fichtner I, Becker M, Zeisig R, Sommer A (2004) In vivo models for endocrine-dependent breast carcinomas: special considerations of clinical relevance. Eur J Cancer 40: 845–851.
7. Ariazi EA, Lewis-Wambi JS, Gill SD, Pyle JR, Ariazi JL, et al. (2006) Emerging principles for the development of resistance to antihormonal therapy: Implications for the clinical utility of fulvestrant. The Journal of Steroid Biochemistry and Molecular Biology 102: 128–138.
8. Lønning PE, Taylor PD, Anker G, Iddon J, Wie L, et al. (2001) High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat 67: 111–116.
9. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, et al. (2009) Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 302: 774–780.
10. Dodds EC, Goldberg L, Lawson W, Robinson R (1938) Estrogenic activity of certain synthetic compounds. Nature 141: 247–248.
11. Haddow A, Watkinson J, Paterson E (1944) Influence of synthetic oestrogens upon advanced malignant disease. BMJ 2: 393–398.
12. Carter AC, Sedransk N, Kelley RM, Ansfield FJ, Ravdin RG, et al. (1977) Diethylstilbestrol: recommended dosages for different categories of breast cancer patients. Report of the Cooperative Breast Cancer Group. JAMA 237: 2079–2078.
13. Jordan VC, Lewis JS, Osipo C, Cheng D (2005) The apoptotic action of estrogen following exhaustive antihormonal therapy: a new clinical treatment strategy. Breast 14: 624–630.
14. Song RX, Mor G, Naftolin F, McPherson RA, Song J, et al. (2001) Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 93: 1714–1723.
15. Jordan VC, Ford LG (2011) Paradoxical Clinical Effect of Estrogen on Breast Cancer Risk: A "New" Biology of Estrogen-Induced Apoptosis. Cancer prevention research (Philadelphia, Pa).
16. Macedo LF, Sabnis G, Brodie A (2009) Aromatase inhibitors and breast cancer. Ann N Y Acad Sci 1155: 162–173.
17. Musgrove EA, Sutherland RL (2009) Biological determinants of endocrine resistance in breast cancer. Nat Rev Cancer 9: 631–643.
18. Arpino G, Wiechmann L, Osborne C, Schiff R (2008) Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. Endocr Rev 29: 217–233.
19. Lahusen T, Henke R, Kagan B, Wellstein A, Riegel A (2009) The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer. Breast Cancer Research and Treatment 116: 225–237.
20. Zhao W, Zhang Q, Kang X, Jin S, Lou C (2009) AIB1 is required for the acquisition of epithelial growth factor receptor-mediated tamoxifen resistance in breast cancer cells. Biochem Biophys Res Commun 380: 699–704.
21. Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, et al. (2003) Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst 95: 353–361.
22. Schiff R, Massarweh S, Shou J, Osborne CK (2003) Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. Clin Cancer Res 9: 447S–454S.
23. List HJ, Lauritsen KJ, Reiter R, Powers C, Wellstein A, et al. (2001) Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells. J Biol Chem 276: 23763–23768.
24. Font de Mora J, Brown M (2000) AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. Mol Cell Biol 20: 5041–5047.
25. Oh A, List HJ, Reiter R, Mani A, Zhang Y, et al. (2004) The nuclear receptor coactivator AIB1 mediates insulin-like growth factor I-induced phenotypic changes in human breast cancer cells. Cancer Res 64: 8299–8308.
26. Zhou G, Hashimoto Y, Kwak I, Tsai SY, Tsai MJ (2003) Role of the steroid receptor coactivator SRC-3 in cell growth. Mol Cell Biol 23: 7742–7755.
27. Lahusen T, Fereshteh M, Oh A, Wellstein A, Riegel AT (2007) Epidermal growth factor receptor tyrosine phosphorylation and signaling controlled by a nuclear receptor coactivator, amplified in breast cancer 1. Cancer Res 67: 7256–7265.
28. Coste A, Antal M, Chan S, Kastner P, Mark M, et al. (2006) Absence of the steroid receptor coactivator-3 induces B-cell lymphoma. EMBO J 25: 2453–2464.
29. Xu J, Liao L, Ning G, Yoshida-Komiya H, Deng C, et al. (2000) The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. Proc Natl Acad Sci U S A 97: 6379–6384.
30. Fereshteh MP, Tilli MT, Kim SE, Xu J, O'Malley BW, et al. (2008) The nuclear receptor coactivator amplified in breast cancer-1 is required for Neu (ErbB2/HER2) activation, signaling, and mammary tumorigenesis in mice. Cancer Res 68: 3697–3706.
31. Torres-Arzayus MI, De Mora JF, Yuan J, Vazquez F, Bronson R, et al. (2004) High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. Cancer Cell 6: 263–274.
32. Torres-Arzayus MI, Yuan J, DellaGatta JL, Lane H, Kung AL, et al. (2006) Targeting the AIB1 oncogene through mammalian target of rapamycin inhibition in the mammary gland. Cancer Res 66: 11381–11388.
33. Tilli MT, Reiter R, Oh AS, Henke RT, McDonnell K, et al. (2005) Overexpression of an N-terminally truncated isoform of the nuclear receptor coactivator amplified in breast cancer 1 leads to altered proliferation of mammary epithelial cells in transgenic mice. Mol Endocrinol 19: 644–656.
34. Lahusen T, Henke RT, Kagan BL, Wellstein A, Riegel AT (2009) The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer. Breast Cancer Res Treat 116: 225–237.
35. Xu J, Wu R-C, O'Malley BW (2009) Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer 9: 615–630.
36. Konecny GE, Pegram MD, Venkatesan N, Finn R, Yang G, et al. (2006) Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. Cancer Res 66: 1630–1639.
37. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, et al. (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 277: 965–968.
38. Lewis JS, Osipo C, Meeke K, Jordan VC (2005) Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 94: 131–141.
39. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, et al. (2005) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97: 1746–1759.
40. Jordan VC, Lewis-Wambi J, Kim H, Cunliffe H, Ariazi E, et al. (2007) Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients. Breast 16 Suppl 2: S105–113.
41. Bell AW, Deutsch EW, Au CE, Kearney RE, Beavis R, et al. (2009) A HUPO test sample study reveals common problems in mass spectrometry-based proteomics. Nat Methods 6: 423–430.
42. Aebersold R (2009) A stress test for mass spectrometry-based proteomics. Nat Methods 6: 411–412.
43. Hu Z-Z, Huang H, Wu CH, Jung M, Dritschilo A, et al. (2011) Omics-based molecular target and biomarker identification. Methods Mol Biol 719: 547–571.
44. Louet JF, Coste A, Amazit L, Tannour-Louet M, Wu RC, et al. (2006) Oncogenic steroid receptor coactivator-3 is a key regulator of the white adipogenic program. Proc Natl Acad Sci U S A 103: 17868–17873.
45. Coste A, Louet JF, Lagouge M, Lerin C, Antal MC, et al. (2008) The genetic ablation of SRC-3 protects against obesity and improves insulin sensitivity by reducing the acetylation of PGC-1{alpha}. Proc Natl Acad Sci U S A 105: 17187–17192.
46. Xu J, Liao L, Ning G, Yoshida-Komiya H, Deng C, et al. (2000) The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. Proc Natl Acad Sci USA 97: 6379–6384.
47. Long W, Yi P, Amazit L, Lamarca HL, Ashcroft F, et al. (2010) SRC-3Delta4 Mediates the Interaction of EGFR with FAK to Promote Cell Migration. Molecular Cell 37: 321–332.
48. Deng Q, Huang S (2004) PRDM5 is silenced in human cancers and has growth suppressive activities. Oncogene 23: 4903–4910.
49. Brinkmeier ML, Potok MA, Cha KB, Gridley T, Stifani S, et al. (2003) TCF and Groucho-related genes influence pituitary growth and development. Mol Endocrinol 17: 2152–2161.
50. Nagahama Y, Ishimaru M, Osaki M, Inoue T, Maeda A, et al. (2008) Apoptotic pathway induced by transduction of RUNX3 in the human gastric carcinoma cell line MKN-1. Cancer Sci 99: 23–30.
51. Tong DD, Jiang Y, Li M, Kong D, Meng XN, et al. (2009) RUNX3 inhibits cell proliferation and induces apoptosis by TGF-beta-dependent and -independent mechanisms in human colon carcinoma cells. Pathobiology 76: 163–169.
52. van Agthoven T, Sieuwerts AM, Meijer-van Gelder ME, Look MP, Smid M, et al. (2009) Relevance of breast cancer antiestrogen resistance genes in human breast cancer progression and tamoxifen resistance. J Clin Oncol 27: 542–549.
53. Ahn J, Byeon IJ, Byeon CH, Gronenborn AM (2009) Insight into the structural basis of pro- and antiapoptotic p53 modulation by ASPP proteins. J Biol Chem 284: 13812–13822.
54. Liu ZJ, Cai Y, Hou L, Gao X, Xin HM, et al. (2008) Effect of RNA interference of iASPP on the apoptosis in MCF-7 breast cancer cells. Cancer Invest 26: 878–882.
55. Sullivan A, Lu X (2007) ASPP: a new family of oncogenes and tumour suppressor genes. Br J Cancer 96: 196–200.
56. Zhao Y, Goto K, Saitoh M, Yanase T, Nomura M, et al. (2002) Activation function-1 domain of androgen receptor contributes to the interaction between subnuclear splicing factor compartment and nuclear receptor compartment. Identification of the p102 U5 small nuclear ribonucleoprotein particle-binding protein as a coactivator for the receptor. J Biol Chem 277: 30031–30039.
57. Lanz RB, Bulynko Y, Malovannaya A, Labhart P, Wang L, et al. (2010) Global characterization of transcriptional impact of the SRC-3 coregulator. Mol Endocrinol 24: 859–872.
58. Jordan JD, Carey KD, Stork PJ, Iyengar R (1999) Modulation of rap activity by direct interaction of Galpha(o) with Rap1 GTPase-activating protein. J Biol Chem 274: 21507–21510.

59. Zhang J, Liu J, Yu C, Lin A (2005) BAD Ser128 is not phosphorylated by c-Jun NH2-terminal kinase for promoting apoptosis. Cancer Res 65: 8372–8378.
60. Klumpp S, Krieglstein J (2002) Serine/threonine protein phosphatases in apoptosis. Curr Opin Pharmacol 2: 458–462.
61. Fernando RI, Wimalasena J (2004) Estradiol abrogates apoptosis in breast cancer cells through inactivation of BAD: Ras-dependent nongenomic pathways requiring signaling through ERK and Akt. Mol Biol Cell 15: 3266–3284.
62. Roux PP, Richards SA, Blenis J (2003) Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity. Mol Cell Biol 23: 4796–4804.
63. Bundy DL, McKeithan TW (1997) Diverse effects of BCL3 phosphorylation on its modulation of NF-kappaB p52 homodimer binding to DNA. J Biol Chem 272: 33132–33139.
64. Mathas S, Johrens K, Joos S, Lietz A, Hummel F, et al. (2005) Elevated NF-kappaB p50 complex formation and Bcl-3 expression in classical Hodgkin, anaplastic large-cell, and other peripheral T-cell lymphomas. Blood 106: 4287–4293.
65. Castellano L, Giamas G, Jacob J, Coombes RC, Lucchesi W, et al. (2009) The estrogen receptor-alpha-induced microRNA signature regulates itself and its transcriptional response. Proc Natl Acad Sci USA 106: 15732–15737.
66. Zimmer A, Reynolds K (1994) Gene targeting constructs: effects of vector topology on co- expression efficiency of positive and negative selectable marker genes. Biochem Biophys Res Commun 201: 943–949.
67. Hoffman BG, Zavaglia B, Beach M, Helgason CD (2008) Expression of Groucho/TLE proteins during pancreas development. BMC Dev Biol 8: 81.
68. Liu Y, Dehni G, Purcell KJ, Sokolow J, Carcangiu ML, et al. (1996) Epithelial expression and chromosomal location of human TLE genes: implications for notch signaling and neoplasia. Genomics 31: 58–64.
69. Cuevas IC, Slocum AL, Jun P, Costello JF, Bollen AW, et al. (2005) Meningioma transcript profiles reveal deregulated Notch signaling pathway. Cancer Res 65: 5070–5075.
70. Hiraike H, Wada-Hiraike O, Nakagawa S, Koyama S, Miyamoto Y, et al. (2010) Identification of DBC1 as a transcriptional repressor for BRCA1. Br J Cancer 102: 1061–1067.
71. Lewis-Wambi JS, Jordan VC (2009) Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? Breast Cancer Res 11: 206.
72. Papa S, Zazzeroni F, Bubici C, Jayawardena S, Alvarez K, et al. (2004) Gadd45β mediates the NF-κB suppression of JNK signalling by targeting MKK7/JNKK2. Nat Cell Biol 6: 146–153.
73. Engelmann A, Speidel D, Bornkamm GW, Deppert W, Stocking C (2008) Gadd45 beta is a pro-survival factor associated with stress-resistant tumors. Oncogene 27: 1429–1438.
74. Ariazi EA, Brailoiu E, Yerrum S, Shupp HA, Slifker MJ, et al. (2010) The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. Cancer Res 70: 1184–1194.
75. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 90: 77–86.
76. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307: 1098–1101.
77. Oh AS, Lahusen JT, Chien CD, Fereshteh MP, Zhang X, et al. (2008) Tyrosine phosphorylation of the nuclear receptor coactivator AIB1/SRC-3 is enhanced by Abl kinase and is required for its activity in cancer cells. Mol Cell Biol 28: 6580–6593.
78. Hu Z-Z, Huang H, Cheema A, Jung M, Dritschilo A, et al. (2008) Integrated Bioinformatics for Radiation-Induced Pathway Analysis from Proteomics and Microarray Data. Journal of proteomics & bioinformatics 1: 47–60.
79. Han SJ, Jung SY, Malovannaya A, Kim T, Lanz RB, et al. (2006) A scoring system for the follow up study of nuclear receptor coactivator complexes. Nucl Recept Signal 4: e014.
80. Kerrien S, Alam-Faruque Y, Aranda B, Bancarz I, Bridge A, et al. (2007) IntAct–open source resource for molecular interaction data. Nucleic Acids Res 35: D561–565.

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The Selective Estrogen Receptor Modulator Bazedoxifene Inhibits Hormone-Independent Breast Cancer Cell Growth and Down-Regulates Estrogen Receptor α and Cyclin D1[S]

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ABSTRACT

Bazedoxifene (BZA) is a third-generation selective estrogen receptor modulator (SERM) that has been approved for the prevention and treatment of postmenopausal osteoporosis. It has antitumor activity; however, its mechanism of action remains unclear. In the present study, we characterized the effects of BZA and several other SERMs on the proliferation of hormone-dependent MCF-7 and T47D breast cancer cells and hormone-independent MCF-7:5C and MCF-7:2A cells and examined its mechanism of action in these cells. We found that all of the SERMs inhibited the growth of MCF-7, T47D, and MCF-7:2A cells; however, only BZA and fulvestrant (FUL) inhibited the growth of hormone-independent MCF-7:5C cells. Cell cycle analysis revealed that BZA and FUL induced G_1 blockade in MCF-7:5C cells; however, BZA down-regulated cyclin D1, which was constitutively overexpressed in these cells, whereas FUL suppressed cyclin A. Further analysis revealed that small interfering RNA knockdown of cyclin D1 reduced the basal growth of MCF-7:5C cells, and it blocked the ability of BZA to induce G_1 arrest in these cells. BZA also down-regulated estrogen receptor-α (ERα) protein by increasing its degradation and suppressing cyclin D1 promoter activity in MCF-7:5C cells. Finally, molecular modeling studies demonstrated that BZA bound to ERα in an orientation similar to raloxifene; however, a number of residues adopted different conformations in the induced-fit docking poses compared with the experimental structure of ERα-raloxifene. Together, these findings indicate that BZA is distinct from other SERMs in its ability to inhibit hormone-independent breast cancer cell growth and to regulate ERα and cyclin D1 expression in resistant cells.

Introduction

Bazedoxifene acetate (BZA) is a new third-generation selective estrogen receptor modulator (SERM) (Silverman et al., 2008) that is approved in Europe and is under regulatory review in the United States for the prevention and treatment of postmenopausal osteoporosis. In phase III clinical trials (Miller et al., 2008; Archer et al., 2009; Pinkerton et al., 2009) BZA (20 or 40 mg/daily) has been shown to prevent bone loss and to reduce bone turnover in postmenopausal women at risk for osteoporosis, with a favorable endometrial, ovarian, and breast safety profile. BZA also significantly reduces the risk of new vertebral fractures in postmenopausal women with osteoporosis compared with placebo (Silverman et al., 2008). In addition, recent studies indicate that BZA combined with conjugated estrogens relieves hot flashes and improves vulvovaginal atrophy and its symptoms (Kagan et al., 2010).

BZA is an indole-based ER ligand with unique structural characteristics with respect to tamoxifen (TAM) and raloxifene (RAL). It was assembled by using RAL as a template and substituting an indole ring for the benzothiophene core (Miller et al., 2001; Komm et al., 2005). BZA binds to both ERα and ERβ, with a slightly higher affinity for ERα; how-

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ABBREVIATIONS: BZA, bazedoxifene acetate; ER, estrogen receptor; SERM, selective estrogen receptor modulator; TAM, tamoxifen; RAL, raloxifene; E2, 17β-estradiol; FUL, fulvestrant; siRNA, small interfering RNA; 4OHT, 4-hydroxytamoxifen; ENDOX, endoxifen; Luc, luciferase; ERE, estrogen response element; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; IFD, Induced Fit Docking; SRC, steroid receptor coactivator; ICI 182,780, fulvestrant; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; PDB, Protein Data Bank.

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ever, it is less ERα-selective than RAL, with an affinity for ERα that is approximately 10-fold lower than 17β-estradiol (E2) (Miller et al., 2001). ERα is a well studied member of the steroid/nuclear receptor family of transcription regulators. ERα acts in the nucleus to regulate gene expression by binding to estrogen response elements (EREs) and related DNA sequences and through association with transcription factors bound at SP1 and AP-1 DNA binding sites. In response to high-affinity estrogen binding, ERα dimerizes, binds to ERE DNAs, and undergoes a conformational change in the ligand binding domain that facilitates the recruitment of coactivators. In contrast, antagonist-occupied ERα recruits corepressors. Although previous studies have reported that BZA antagonizes E2-dependent MCF-7 breast cancer cell proliferation in vitro (Komm et al., 2005), little is known about the actions of BZA on ERα expression and functionality. In addition, not known is whether BZA has antitumor activity in breast cancer cells that have acquired resistance to endocrine therapies.

We have reported previously the development of two ERα-positive human breast cancer cell lines, MCF-7:5C (Jiang et al., 1992; Lewis et al., 2005a) and MCF-7:2A (Pink et al., 1995; Lewis-Wambi et al., 2008b), that were clonally selected from hormone-dependent MCF-7 breast cancer cells after long-term (>1 year) estrogen deprivation. An interesting phenotype of MCF-7:5C and MCF-7:2A cells is that, unlike MCF-7 cells, which require estrogen to grow and are inhibited by antiestrogens, they do not require estrogen to grow and they undergo apoptosis when exposed to physiological levels of E2 (Lewis et al., 2005a; Jordan, 2008; Lewis-Wambi et al., 2008b). However, the effects of SERMs on MCF-7:5C and MCF-7:2A cells have not been fully examined. In this study, we investigated the effects of BZA, 4-hydroxytamoxifen (4OHT), endoxifen (ENDOX), RAL, and the pure antiestrogen fulvestrant (ICI 182,780) on the growth of MCF-7:5C and MCF-7:2A breast cancer cells and determined the mechanism of action of BZA in these cells. We found that all of the SERMs inhibited E2-stimulated MCF-7 and T47D breast cancer cell growth; however, only BZA and FUL significantly inhibited the hormone-independent growth of MCF-7:5C cells. The inhibitory effect of BZA was associated with cell cycle arrest and cyclin D1 and ERα down-regulation, which was reversed by small interfering RNA (siRNA) knockdown of cyclin D1 and ERα. It is noteworthy that we found that FUL also inhibited MCF-7:5C cell growth; however, this compound partially down-regulated cyclin D1. Together, these data show that BZA is distinct from the other members of the SERM family in its ability to inhibit the growth of breast cancer cells that are resistant to long-term estrogen deprivation.

Materials and Methods

Reagents and Cell Culture. E2, 4OHT (the active metabolite of TAM), and *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal (MG132) were purchased from Sigma-Aldrich (St. Louis, MO). Fulvestrant (ICI 182,780, Faslodex) was a generous gift from Dr. A. E. Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). ENDOX was a kind gift from Dr. James Ingle of the Mayo Clinic (Rochester, MN). RAL was a generous gift from Lilly Research Laboratories (Indianapolis, IN). BZA was synthesized by authors R.G. and M.A.S. using a protocol described previously (Miller et al., 2001). All of the compounds were dissolved in 100% ethanol except for MG132, which was dissolved in dimethyl sulfoxide. The compounds were added to the medium such that the total solvent concentration was never higher than 0.1%. An untreated group served as a control. The chemical structures of the compounds used in this study have been cited before (Komm et al., 2005; Jordan, 2007, 2009) and are shown in Supplemental Fig. 1.

MCF-7:WS8 and T47D:A18 human mammary carcinoma cells, clonally selected from their parental counterparts for sensitivity to growth stimulation by E2 (Pink and Jordan, 1996), were used in all experiments indicating MCF-7 and T47D cells. Cells were maintained in estrogenized medium (phenol red RPMI 1640 plus 10% fetal bovine serum), but 3 days before all experiments, they were cultured in steroid-free media as described previously (Pink and Jordan, 1996; Lewis et al., 2005a,b). MCF-7:5C (Jiang et al., 1992; Lewis et al., 2005a,b), and MCF-7:2A cells (Pink and Jordan, 1996; Lewis-Wambi et al., 2008b) were derived from the MCF-7 line by growth in estrogen-free media and two rounds of limiting dilution cloning and were maintained in phenol red-free RPMI 1640 medium containing 10% 3× dextran-coated charcoal-treated fetal bovine serum. MC2 cells were derived by stably transfecting ER-negative MDA-MB-231 breast cancer cells with the wild-type ERα (Jiang and Jordan, 1992), and these cells were grown in phenol red-free minimal essential medium supplemented with 5% 3× dextran-coated charcoal-treated calf serum, 0.5 mg/ml G-418. All cell culture reagents were from Invitrogen (Carlsbad, CA).

Cell Proliferation Assay. These procedures have been reported previously (Lewis et al., 2005; Lewis-Wambi et al., 2008). In brief, MCF-7 and T47D cells were grown in fully estrogenized medium, whereas MCF-7:5C and MCF-7:2A cells were grown in nonestrogenized media. Cells were seeded in 24-well plates (30,000/well), and after overnight incubation, cells were treated with various concentrations of the tested compounds for 7 days. Media were changed on days 3 and 5, the experiment was ended on day 7, and the DNA content of the cells was determined as described previously (Labarca and Paigen, 1980) using a Fluorescent DNA Quantitation kit (Bio-Rad Laboratories, Hercules, CA). Cell proliferation was also determined by cell counting using a hemocytometer.

Western Blot Analyses. Immunoblotting was performed using 30 μg of protein per well as described previously (Lewis et al., 2005a). Membranes were probed with primary antibodies against ERα, progesterone receptor, cyclin A, cyclin B1, or cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA) with β-actin (AC-15; Sigma-Aldrich) used to standardize loading. The appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was used to visualize the stained bands with an enhanced chemiluminescence visualization kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Bands were quantitated by densitometry using ImageQuant (GE Healthcare), and densitometric values were corrected for loading control.

Cell Cycle Analyses. MCF-7 and MCF-7:5C cells were treated with E2 or BZA for 24 and 48 h and then fixed using ice-cold 70% ethanol. Cell cycle distribution was determined by propidium iodide staining using a fluorescence-activated cell sorter (BD Biosciences, San Jose, CA) as described previously (Ariazi et al., 2010). Data were analyzed using FlowJo 7.2.5 for Windows (Tree Star, Ashland, OR).

Knockdown of ERα and Cyclin D1 by siRNA. MCF-7:5C cells were seeded at 10^5 cells/well in a 24-well plate overnight and then transfected with 100 nM nonspecific, ERα, or cyclin D1 siRNA (Dharmacon RNA Technologies, Lafayette, CO) using Lipofectamine 2000 (Invitrogen), as described previously (Lewis et al., 2005a). Transfected cells were either harvested for Western blot analysis or reseeded for cell growth or cell cycle analysis.

Quantitative Real-Time PCR. The detail procedures have been reported previously (Lewis et al., 2005). MCF-7 and MCF-7:5C cells were treated with either E2 (10^{-9} M) or BZA (10^{-7} M) for 48 h, and total RNA was isolated and then reverse-transcribed to cDNA using the SuperScript II RNase H reverse transcriptase system (Invitrogen). Aliquots of the cDNA were combined with the SYBR green kit (Superarray) and primers and assayed in triplicate by quantitative PCR over 40 cycles using a GeneAmp 5700 Sequence detection system (Applied Biosystems, Foster City, CA) as described previously

(Lewis et al., 2005a). Quantitation was done using the comparative CT method with 18S rRNA as the normalization gene, as described previously (Lewis-Wambi et al., 2008a). PCR primer sequences used were as follow: ERα forward, 5′-GGAGGGCAGGGGTGAA-3′; ERα reverse, 5′-GGCCAGGCTGTTCTTC TTAGA-3′; cyclin D1 forward, 5′-TCCTGTGCTGCGAAGTGGAAAC-3′; cyclin D1 reverse, 5′-AAATCGTGCGGGGTCATTGC-3; pS2 forward, 5′-GAGGCCCAGACAGAGACGTG-3′; and pS2 reverse, 5′-CCCTGCAGAAGTGTCTAAAATTCA-3′.

Transient Transfections and Luciferase Assays. Cells were cultured in estrogen-free RPMI 1640 media for 48 h before transfection. On the day of the experiment, cells were seeded in estrogen-free media at a density of 1.5×10^5 cells per well in 24-well plates. After 24 h, cells were transfected with the firefly luciferase reporter plasmid pERE(5×)TA-ffLuc (containing five copies of a consensus ERE and a TATA-box driving firefly luciferase) and the pTA-srLuc *Renilla reniformis* luciferase plasmid (containing a TATA-box element driving *R. reniformis* luciferase) (Promega, Madison, WI) using LT1 (Mirus) transfection reagent, according to the manufacturer's protocol. After 24 h, transfection reagents were removed, and fresh media were added. Cells were then treated with ethanol (vehicle), 10^{-9} M E2, 10^{-8} M BZA, or E2 + BZA combined for 24 h. At the indicated time point, cells were washed, lysed, and ERE luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's recommendations. Samples were then read on a Mithras MB540 luminometer (Berthold Technologies, Oak Ridge, TN).

For the cyclin D1 promoter assay, MCF-7:5C cells were transiently transfected with the full-length cyclin D1 promoter plasmid (−1745CD1-LUC) as described previously (Lewis et al., 2005c,d). The full-length cyclin D1 plasmid (−1745CD1-LUC) (Albanese et al., 1995) was a gift from Dr. Richard Pestell (Thomas Jefferson Kimmel Cancer Center, Philadelphia, PA).

Molecular Modeling. The molecular modeling performed in this study has been described previously (Maximov et al., 2010). In brief, the coordinates for the agonist and antagonist conformations of human ERα ligand binding domain cocrystallized with E2, RAL, and 4OHT were extracted from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) (Berman et al., 2000). Entries 1gwr for E2 (Wärnmark et al., 2002), 1err for RAL (Brzozowski et al., 1997), and 3ert for 4OHT (Shiau et al., 1998) were selected for further modeling, and these structures were prepared for docking using the Protein Preparation Workflow (Friesner et al., 2004; Guallar et al., 2004) implemented in Schrödinger suite and accessible from within the Maestro 8.5 program (Schrödinger, Cambridge, MA). To study the molecular basis of interaction of bazedoxifene in the antagonist conformation of ERα, the ligands were docked into the binding site of the receptor cocrystallized with RAL (PDB code 1err). For comparison reasons, RAL was also docked in its native protein structure.

The input geometries of the ligands were generated with CORINA (online demo, http://www.molecular-networks.com/online_demos/corina_demo) and were further prepared for docking using the LigPrep2.2 utility (Friesner et al., 2004; Guallar et al., 2004). The prepared structure of ERα cocrystallized with RAL was used to generate the scoring grid for docking simulations. A grid box of $26 \times 26 \times 26$ Å^3 centered on the ligand was created, using the default parameters and without constraints.

Flexible ligand docking simulations were carried out with Glide 5.0 (Friesner et al., 2004; Guallar et al., 2004) using the default settings, and the best 10 poses for each ligand were evaluated using Glide (Schrödinger) in Standard-Precision (GlideSP) and Extra-Precision (GlideXP) mode. The results obtained from the docking runs were compared, and GlideXP docking poses were selected for analysis.

Statistical Analysis. All quantitative experiments were performed in triplicate and/or repeated three times. Data were expressed as mean ± S.D. Statistical significances between vehicle treatment versus drug treatment were determined by one-way analysis of variance and the Student's t test. A value of $p < 0.05$ was considered statistically significant.

Results

BZA Inhibits the Growth of Hormone-Independent MCF-7:5C and MCF-7:2A Breast Cancer Cells. We first compared the growth characteristics of hormone-dependent MCF-7 and T47D breast cancer cells with those of long-term estrogen deprived MCF-7:5C and MCF-7:2A cells in the presence of E2. Cells were grown in estrogen-free media and then treated with 10^{-14} M to 10^{-8} M E2 for 7 days, and cellular DNA was measure as an index of growth. In parallel, cells were also treated with 10^{-9} M E2 for 2 to 12 days and then harvested and counted using a hemocytometer. Figure 1A shows that E2 treatment stimulated the growth of MCF-7 and T47D cells in a concentration-dependent manner with maximum stimulation at 10^{-9} M, whereas in MCF-7:5C and MCF-7:2A cells, E2 treatment had the opposite effect causing either complete growth inhibition in MCF-7:5C cells or partial growth inhibition in MCF-7:2A cells. This finding is consistent with our previous work (Lewis et al., 2005a; Lewis-Wambi et al., 2008b), which showed that physiological concentrations of E2 induced programmed cell death (apoptosis) in MCF-7:5C and MCF-7:2A cells through activation of the mitochondrial death pathway and suppression of glutathione synthesis, respectively. Specifically, we found that E2 induced apoptosis in MCF-7:5C cells by activating proapoptotic proteins Bax, Bak, Bim, and p53 and by suppressing antiapoptotic proteins. E2 also down-regulated survival proteins such as nuclear factor-κB, phospho-Akt, and Her2/neu, which were overexpressed in MCF-7:5C cells. In contrast, we found that MCF-7:2A cells underwent apoptosis after 10 to 12 days of E2 treatment and that these cells expressed elevated levels of the antioxidant glutathione as a result of overexpression of glutathione synthetase and glutathione peroxidase 2, the two main enzymes involved in glutathione synthesis. By selectively blocking the glutathione pathway in MCF-7:2A cells, we were able to sensitize these cells to E2-induced apoptosis, which was mediated by activation of the c-Jun NH_2-terminal kinase signaling pathway.

Next, we determined the inhibitory effects of BZA and other SERMs (see Supplemental Fig. 1 for chemical structures) on MCF-7, T47D, MCF-7:5C, and MCF-7:2A cells. For experiments, MCF-7 and T47D cells were grown in fully estrogenized media, and MCF-7:5C and MCF-7:2A cells were grown in estrogen-free media and then treated with 10^{-12} to 10^{-6} M BZA, RAL, FUL, 4OHT, or ENDOX for 7 days, and cellular DNA was measured as an index of growth. Figure 1B shows that all of the tested SERMs along with the pure antiestrogen FUL inhibited E2-stimulated growth in MCF-7 and T47D cells and hormone-independent growth in MCF-7:2A cells in a concentration-dependent manner; however, in MCF-7:5C cells, only BZA and FUL inhibited the growth of these cells with no effects observed with RAL, 4OHT, or ENDOX. BZA reduced the growth of MCF-7:5C cells in a concentration-dependent manner, causing an 80% reduction at 10^{-8} M, whereas FUL reduced the growth by 55% at a similar concentration.

BZA Down-Regulates ERα Protein in MCF-7:5C and MCF-7:2A Cells. Because BZA dramatically reduced the growth of MCF-7:5C cells, we next determined whether BZA

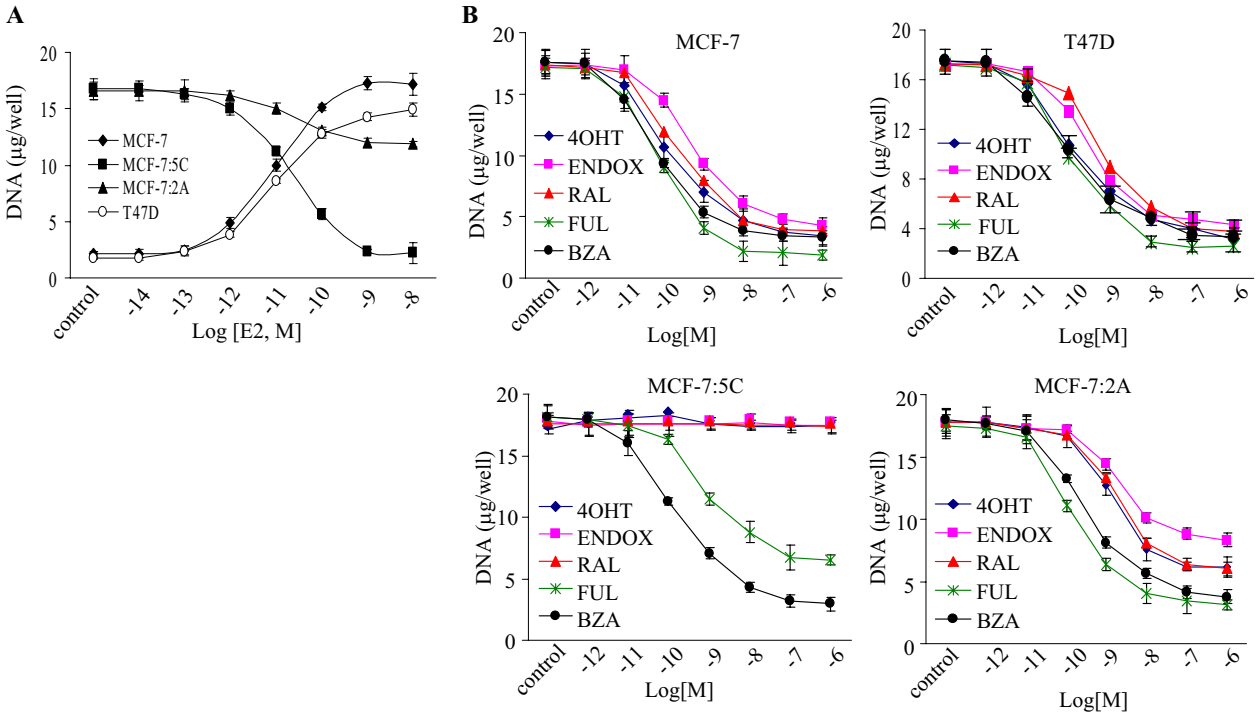

Fig. 1. Effects of E2 and SERMs on the growth of hormone-dependent MCF-7 and T47D cells versus hormone-independent MCF-7:5C and MCF-7:2A cells. A, MCF-7 and T47D cells were grown in phenol red-free RPMI medium supplemented with 10% charcoal-stripped fetal bovine serum for 3 days before the start of the experiment. On the day of the experiment, all cell lines were seeded in phenol red-free RPMI medium supplemented with 10% charcoal-stripped fetal bovine serum at 30,000 per well in 24-well dishes and after 24 h were treated with 10^{-14} to 10^{-8} M E2 for 7 days, with retreatment every other day. At the conclusion of the experiment, cells were harvested, and proliferation was assessed as cellular DNA mass (in micrograms per well) using a DNA quantitation kit. B, the effects of antihormones on the growth of hormone-dependent MCF-7 and T47D cells and hormone-independent MCF-7:5C and MCF-7:2A cells. Cells were seeded as described above, except MCF-7 and T47D cells were grown in fully estrogenized media and then treated with 10^{-12} to 10^{-6} M FUL, BZA, RAL, 4OHT, or ENDOX for 7 days with retreatment on alternate days. Proliferation was assessed as cellular DNA mass (in micrograms per well) as described under *Materials and Methods*. Each point represents the mean of three determinations ± S.E.M.

had actions similar to that of 4OHT or FUL at the level of ERα stability/degradation. We treated MCF-7:5C, MCF-7:2A, MCF-7, and T47D cells with 10^{-9} M E2 or 10^{-7} M FUL, 4OHT, RAL, or BZA for 24 h and monitored ERα protein level. As shown in Fig. 2A, ERα protein was highly expressed in MCF-7:5C and MCF-7:2A cells compared with MCF-7 and T47D cells and treatment with BZA markedly down-regulated ERα protein in MCF-7:5C and MCF-7:2A cells; however, it did not significantly reduce ERα levels in MCF-7 and T47D cells. The ability of BZA to down-regulate ERα in MCF-7:5C and MCF-7:2A cells was greater than that of RAL and almost comparable with that of the pure antiestrogen FUL, which strongly down-regulated ERα in all of the cell lines. E2 treatment also markedly down-regulated ERα protein in all of the cell lines including MCF-7:5C (Fig. 2A); however, 4OHT stabilized ERα against degradation in MCF-7 and T47D cells, as reported previously (Pink and Jordan, 1996), with marginal stabilization observed in MCF-7:5C and MCF-7:2A cells (Fig. 2A). We also examined the effect of the tamoxifen metabolite ENDOX on ERα expression in the different cell lines and found that endoxifen did not down-regulate ERα in any of the tested cell lines (Supplemental Fig. 2). Our finding differs from that of Wu et al. (2009), who reported that endoxifen degrades ERα in breast cancer cells.

We also performed dose-response studies in MCF-7, MCF-7:5C, and MCF-7:2A cells to determine the optimal concentration at which BZA down-regulated ERα protein. Figure 2B showed that BZA reduced ERα protein level in MCF-7:5C cells in a concentration-dependent manner with maximum inhibition at 10^{-6} M, whereas in MCF-7 and MCF-7:2A cells, BZA only marginally reduced ERα protein in these cells. It is noteworthy that the inhibitory effect of BZA on ERα protein was less pronounced than that observed with E2 or FUL, which almost completely reduced ERα protein level in MCF-7:5C cells. Time course studies revealed that BZA down-regulated ERα protein as early as 2 h after treatment with maximum suppression at 24 h (Fig. 2C, top). BZA also down-regulated ERα mRNA in MCF-7:5C cells to a level similar to that observed with E2 and FUL (Fig. 2C, bottom). To show that the decreased ERα protein by BZA was due to protein degradation, we used MG132 to inhibit the proteosome in MCF-7:5C and MCF-7 cells. We found that inhibition of proteosome activity completely blocked ERα degradation by BZA and E2 with partial reversal with fulvestrant (Fig. 2D). We further determined whether BZA might affect ERα protein expression by inhibiting its synthesis. We treated MCF-7:5C cells with 0.5 to 5 μM cycloheximide for 4 h to address this question. The impact of cycloheximide on ERα protein expression was much less dramatic than that of BZA (data not shown), which suggest that BZA-induced down-regulation of ERα protein is not likely to involve protein synthesis inhibition. Together, these data show that BZA differs from the other SERMs in its ability to regulate cell growth and ERα protein expression in MCF-7:5C cells.

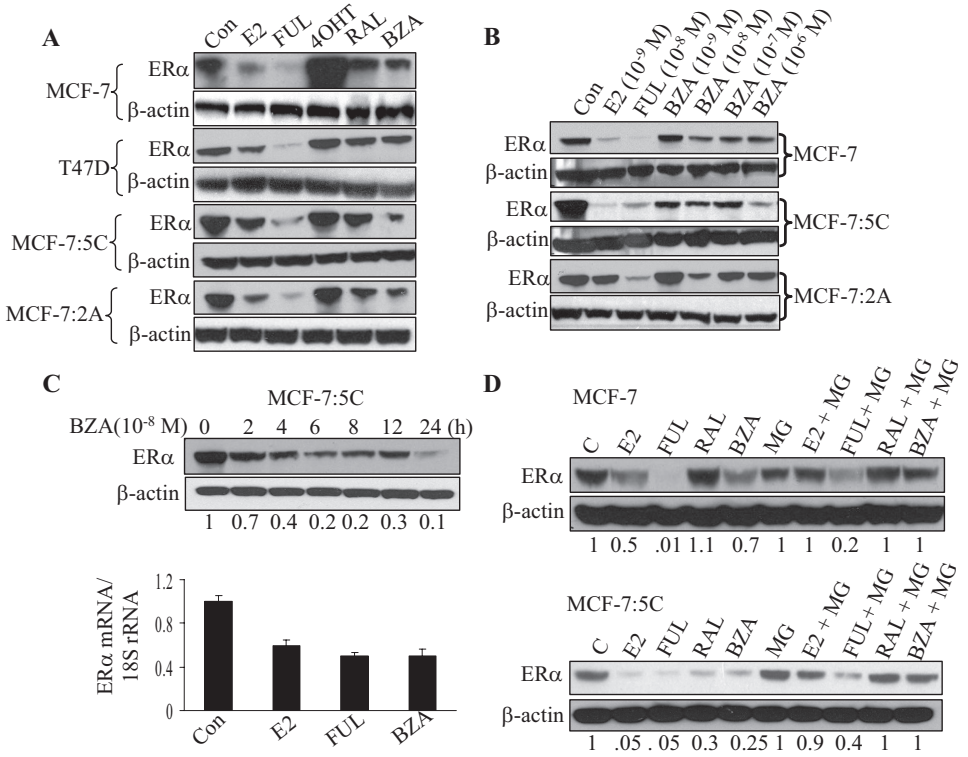

Fig. 2. Effects of SERMs on ERα expression and stability in hormone-dependent MCF-7 and T47D cells and hormone-independent MCF-7:5C and MCF-7:2A cells. A, Western blot analysis of ERα protein levels in MCF-7, T47D, MCF-7:5C, and MCF-7:2A cells in response to 24-h treatment with 10^{-9} M E2 or 10^{-7} M FUL, 4OHT, RAL, or BZA. β-actin was used as a loading control. B, Western blot analysis of ERα protein levels in MCF-7, MCF-7:5C, and MCF-7:2A cells after treatment with 10^{-9} to 10^{-6} M BZA for 24 h. For comparison, cells were also treated with 10^{-9} M E2 or 10^{-8} M FUL. C, Western blot analysis of ERα protein levels in MCF-7:5C cells in response to 10^{-8} M BZA treatment over a 24-h time period. Quantitated protein levels were normalized to β-actin. Densitometric quantitation relative to the control is shown on the bottom of the immunoreactive bands. Also shown is ERα mRNA levels in MCF-7:5C cells after treatment with E2 (10^{-9} M), FUL (10^{-8} M), or BZA (10^{-8} M) for 24 h. The amount of ERα mRNA was determined by real-time RT-PCR and normalized to the internal control 18S rRNA. Each data point represents the average of four biological replicates from three independent experiments. D, Western blot analysis of ERα protein levels in MCF-7 and MCF-7:5C cells pretreated with the proteosome inhibitor MG132 (4 μM) for 4 h and then treated as indicated for 8 h. β-Actin levels are shown as protein loading controls. Each point represents the mean of three determinations ± S.E.M.

BZA Inhibits ERα Transcriptional Activity in MCF-7:5C Cells. To determine whether BZA blocks ERα function, we next examined the transcriptional activation of an ERE in MCF-7, T47D, MCF-7:5C, and MCF-7:2A cells. Cells were transiently transfected with a 5× ERE-luciferase reporter plasmid and treated with 10^{-10} M E2, 10^{-8} M BZA, or E2 + BZA for 24 h. The results of these studies showed that basal ERE activity was elevated 5-fold in MCF-7:5C and 10-fold in MCF-7:2A cells compared with MCF-7 cells and treatment with BZA significantly reduced the basal ERE activity in these cells (Fig. 3A). E2 treatment further increased ERE activity in MCF-7:5C and MCF-7:2A cells by 1.5- and 2.5-fold, respectively; however, in MCF-7 and T47D cells, the response was markedly more robust with a 12- and 20-fold increase, respectively (Fig. 3A).

To further test whether BZA is able to block ERα-regulated genes, we analyzed the expression level of pS2 mRNA in MCF-7:5C cells using quantitative RT-PCR. The pS2 gene is often used as a prognostic marker in breast cancer cells and is frequently used in studies of ER action. Furthermore, it is suggested that estrogen regulates the expression of pS2 through an imperfect ERE in the pS2 promoter (Berry et al., 1989). Our results showed that basal pS2 mRNA level was ~3.5-fold higher in MCF-7:5C cells compared with wild-type MCF-7 cells, and E2 treatment increased pS2 mRNA level by ~5.5-fold in MCF-7 cells and MCF-7:5C cells, which was completely blocked by BZA (Fig. 3B). It is noteworthy that we also found that siRNA knockdown of ERα (Fig. 3C) significantly reduced the basal growth of MCF-7:5C cells and markedly reduced the inhibitory effect of BZA in these cells (Fig. 3C, bottom). In addition, suppression of ERα significantly reduced cyclin D1 protein in MCF-7:5C cells. Overall, these data indicate that in the absence of estrogen, the unliganded ERα drives the proliferation of hormone-independent breast cancer cells; however, in the presence of BZA, the ability to inhibit cell proliferation is dependent on receptor degradation.

BZA Blocks Cell Cycle Progression in MCF-7:5C Cells and Down-Regulates Cyclin D1. Because BZA significantly reduced the growth of MCF-7:5C cells, we next examined its effect on cell cycle progression. For experiment, MCF-7 and MCF-7:5C cells were treated with 10^{-9} M E2, 10^{-8} M BZA, or E2 plus BZA for 48 h followed by propidium iodide staining and flow cytometric analysis. The results showed that in MCF-7:5C cells, E2 treatment significantly reduced the percentage of cells in S phase from 33 to 17% and marginally increased the percentage of cells in G_1 phase from 60 (control) to 66%, whereas BZA treatment increased the proportion of cells in the G_1 phase from 60 to 81%, and it reduced the proportion of S phase cells from 33 to 9% at 48 h. In MCF-7 cells, treatment with E2 increased the proportion of S phase cells from 19 to 42% at 48 h with no effect observed with BZA alone (Fig. 4A). It is noteworthy that the inhibitory

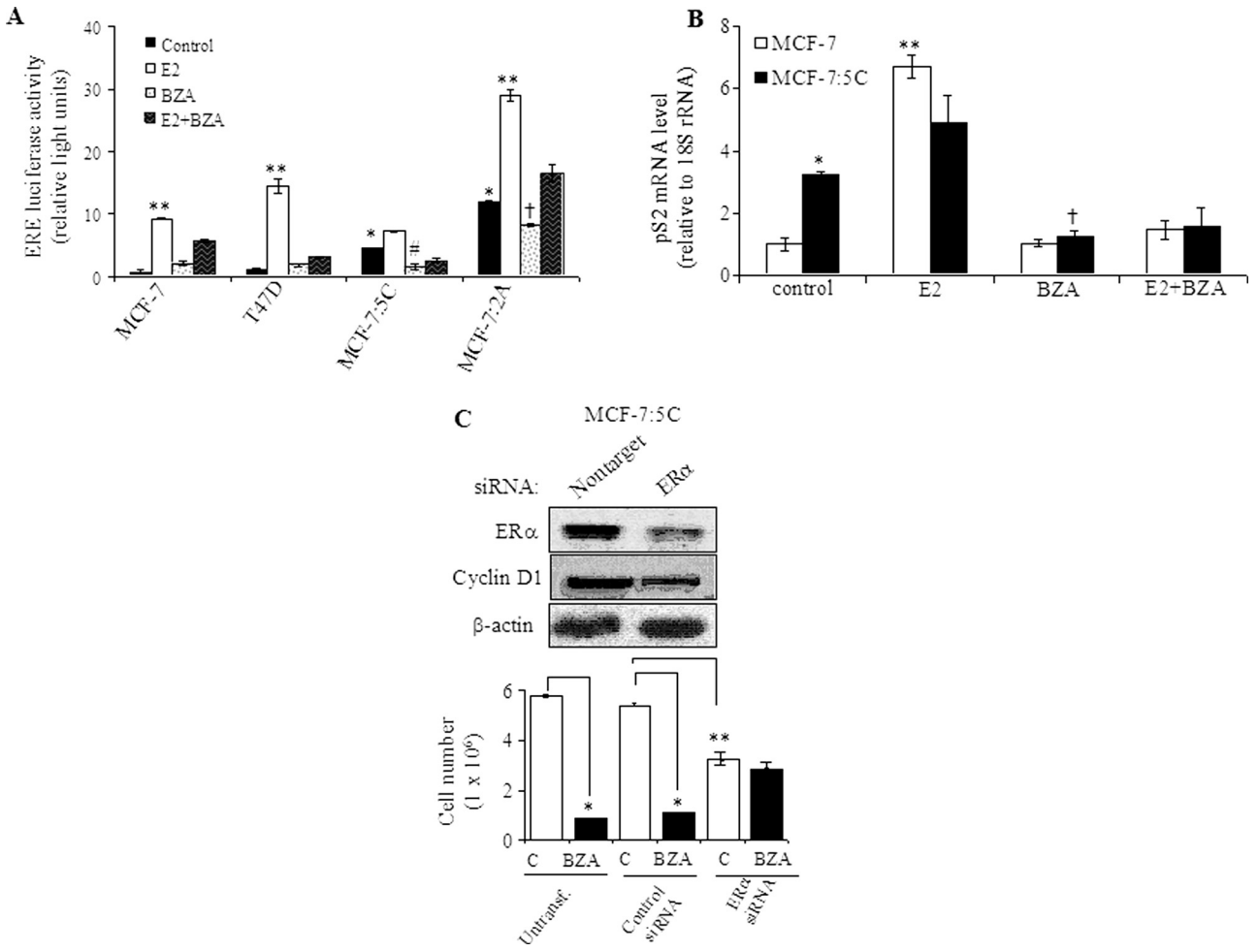

Fig. 3. BZA inhibits constitutive ERα transcriptional activity in hormone-independent and hormone-dependent breast cancer cells. A, ERE luciferase activity in hormone-dependent MCF-7 and T47D cells and hormone-independent MCF-7:5C and MCF-7:2A cells. For experiment, cells were transiently transfected with a 5× ERE-luciferase reporter construct and treated with 10^{-9} M E2, 10^{-7} M BZA, E2 + BZA, or nothing (control) for 24 h. Luciferase values for the treatment groups are reported as relative luciferase units. *, $p < 0.001$ compared with MCF-7 and T47D cells (control); **, $p < 0.0001$ compared with control for each cell line; #, $p < 0.01$ compared with untreated MCF-7:5C cells (control); †, $p < 0.05$ compared with untreated MCF-7:2A cells. B, real-time RT-PCR analysis of pS2 mRNA gene expression in MCF-7 and MCF-7:5C cells after treatments with E2 (10^{-9} M), BZA (10^{-7} M), or E2 + BZA for 24 h. Each data point represents the average of three biological replicates. *, $p < 0.01$ compared with untreated MCF-7 cells (control); **, $p < 0.001$ compared with untreated MCF-7 cells (control); †, $p < 0.001$ compared with untreated MCF-7:5C cells (control). C, MCF-7:5C cells were transfected with 100 nM nonspecific control or ERα siRNA for 48 h. Transfected cells were then harvested for Western blot analysis to detect ERα and cyclin D1 protein (top) or treated with 10^{-7} M BZA for an additional 4 days followed by cell counting using a hemocytometer (bottom). Data shown are representative of three independent experiments. *, $p < 0.001$ compared with untransfected control and nonspecific transfected cells; **, $p < 0.01$ compared with nonspecific transfected cells.

effect of BZA on cell cycle in MCF-7:5C cells was somewhat comparable with the pure antiestrogen fulvestrant; however, none of the other tested SERMs had any effect on cell cycle (data not shown).

Because BZA induced G_1-phase cell cycle block in MCF-7:5C cells, we further investigated the G_1-specific protein cyclin D1 in these cells. MCF-7 and MCF-7:5C cells were treated with BZA, E2, RAL, 4OHT, or FUL for 24 h, and lysates were prepared and analyzed by immunoblotting. Figure 4B shows that cyclin D1 was undetectable in untreated MCF-7 cells; however, treatment with E2 and, to a lesser extent, with 4OHT markedly increased cyclin D1 protein in these cells. In contrast, we found that cyclin D1 protein was constitutively overexpressed in MCF-7:5C and MCF-7:2A cells, and treatment with BZA completely reduced cyclin D1 protein in MCF-7:5C cells but not MCF-7:2A cells (Fig. 4B). It is noteworthy that none of the other SERMs inhibited cyclin D1 in MCF-7:5C cells; however, FUL significantly reduced cyclin D1 protein level at 96 h, and it markedly reduced cyclin A protein in these cells (Supplemental Fig. 3). Time course experiments revealed that BZA inhibited basal cyclin D1 protein in a time-dependent manner with measurable effects observed as early as 2 h after treatment and maximum reduction at 24 h (Fig. 4C, top). BZA also reduced cyclin D1 mRNA (Fig. 4C, bottom) and cyclin D1 promoter activity (Fig. 4C, top right) in MCF-7:5C cells. Finally, we found that siRNA knockdown of cyclin D1 (Fig. 5A) significantly reduced the hormone-independent growth of MCF-7:5C cells (Fig. 5B), and it significantly reduced the ability of BZA to induce G_1 blockade in these cells (Fig. 5C), thus confirming the importance of cyclin D1 in the inhibitory action of BZA in these cells.

Molecular Modeling and Docking of BZA into the Ligand Binding Site of ERα. Molecular modeling and docking studies were carried out in an attempt to predict the bioactive conformation of BZA and to understand the molec-

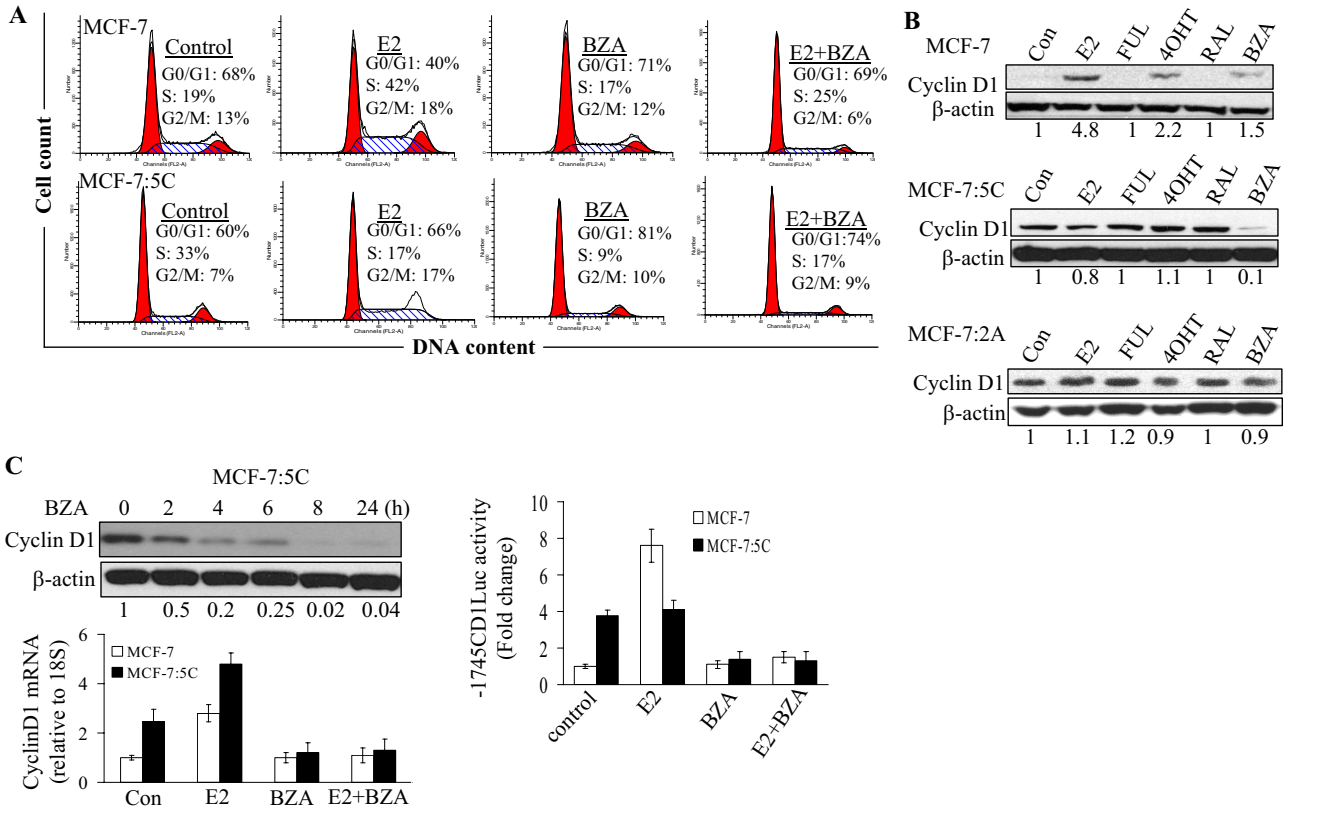

Fig. 4. Effects of BZA on cell cycle progression and cyclin D1 regulation in MCF-7 and MCF-7:5C cells. A, cell cycle distribution was determined by propidium iodide staining of DNA content and flow cytometry. Cells were treated with 10^{-9} M E2, 10^{-7} M BZA, or E2 plus BZA for 24 and 48 h. Thirty thousand cells per sample and three replicates per group were collected. Representative histograms are shown. B, Western blot analysis of cyclin D1 expression level in MCF-7 and MCF-7:5C cells after treatment with BZA and other SERMs. Before the experiment, MCF-7 cells were switched from fully estrogenized media to estrogen-free media for 3 days and then treated with ethanol vehicle (control), 10^{-9} M E2 alone, or 10^{-9} M E2 plus FUL (10^{-7} M), RAL (10^{-7} M), 4OHT (10^{-7} M), or BZA (10^{-7} M) for 24 h. MCF-7:5C cells, however, did not require a media switch because they are hormone-independent and are routinely grown in estrogen-free media. MCF-7:5C cells were treated as described above for MCF-7 cells. Quantitated protein levels normalized to β-actin are indicated. C, BZA regulation of cyclin D expression and promoter activity in MCF-7:5C cells. Cells were treated with 10^{-7} M BZA for the indicated time points. Cyclin D1 protein and mRNA levels were determined by Western blot and quantitative RT-PCR, respectively, with β-actin and 18S rRNA as internal controls. For cyclin D1 promoter activity experiment, MCF-7 and MCF-7:5C cells were cotransfected with a full-length cyclin D1 promoter plasmid (−1745CDLUC) and *Renilla reniformis* luciferase control plasmid overnight and then treated with 10^{-9} M E2, 10^{-8} M BZA, or E2 + BZA for 24 h. Luciferase activity was measured as described under *Materials and Methods*. Each point represents the mean of three determinations ± S.E.M.

ular basis of interaction of this ligand with ERα. Using the available X-ray crystallographic data, the flexible docking of BZA into the ligand binding domain of ERα cocrystallized with RAL was performed, and for comparison reasons, FUL and RAL were also docked in their native protein structure. The superimposition of the docked solution and experimental structure of RAL shows that the docking model recapitulates the orientation of the native ligand in the active site, and the same interactions with the key amino acids of the binding cavity are formed with a ligand root mean square deviation of 0.362 compared with the crystal structure (Fig. 6A). The experimental structure of ERα cocrystallized with E2 (PDB code 1gwr), the agonist conformation of the receptor, is displayed in Fig. 6B, whereas the experimental antagonist conformation of ERα bound to 4OHT and RAL are superimposed and presented in Fig. 6C. The docking results analysis reveals that BZA binds to ERα in an antagonist orientation similar with RAL (Fig. 6D) and has the tendency to form the same hydrophobic contacts with the amino acids lining the binding cavity. In addition, the same complex H-bond network is formed with Asp351, Glu353, Arg394, His524, and a highly ordered water molecule, located in the vicinity of residues Glu353 and Arg394 (Fig. 6D). However, we should note that a number of residues adopt different conformations in the Induced Fit Docking (IFD) poses compared with the experimental structure of ERα, PDB code 1err (Supplemental Fig. 4). The most significant difference has been observed for Leu539 of helix 12. The larger ring of BZA causes the side chain of Leu539 to be pushed away from its original position by approximately 1 Å. In all top-ranked IFD structures (four poses having the composite score of 0.5 kcal/mol), Leu529 side chain is moved up from its original orientation toward the ring of BZA to optimize the hydrophobic contacts between the ligand and residue side chain (Supplemental Fig. 4). We also compared the docked structure of BZA with the binding mode of 4OHT to ERα (Fig. 6C) and superimposed it in the binding site of 4OHT-ERα complex (Fig. 6E). The 4OHT bound receptor shows that the H-bond between BZA and H524 is missing (Fig. 6E) because of the different orientation of this amino acid in the binding site compared with the RAL-ERα complex (Fig. 6C). When FUL was docked to RAL-ERα complex (Fig. 7A), the H-bond network was recapitulated with one exception: the interaction with Asp351 is missing, whereas the flexible side chain of FUL fills the groove between helix 3 and helix 12 (Fig. 7B).

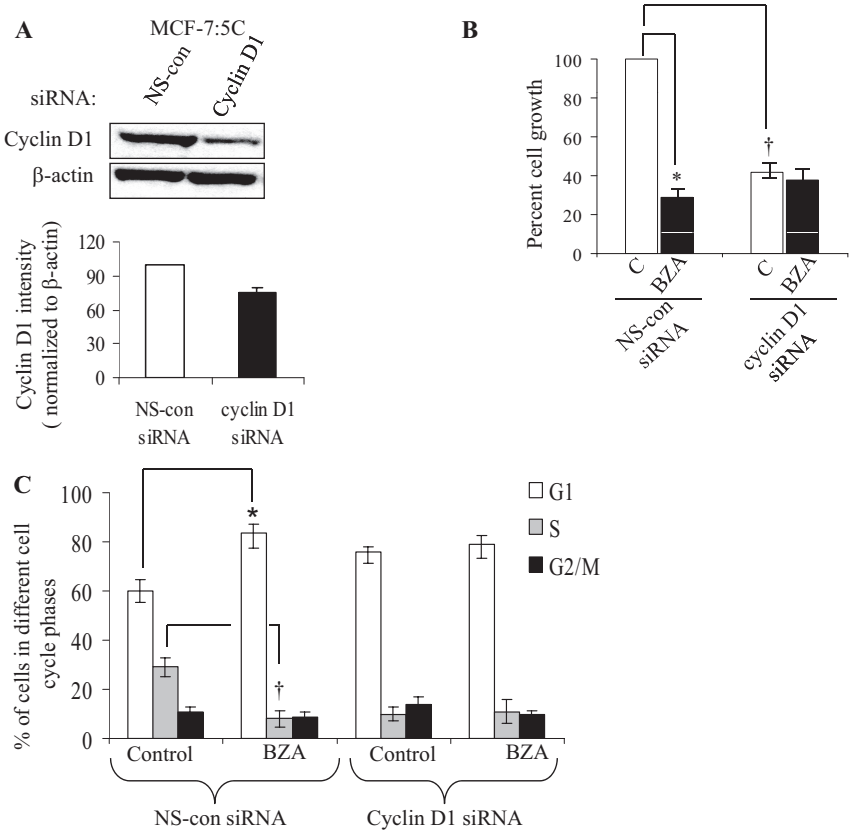

Fig. 5. Effect of cyclin D1 knockdown on proliferation and cell cycle in MCF-7:5C cells. A, Western blot analysis of cyclin D1 protein expression in MCF-7:5C cells transfected with 100 nM cyclin D1 siRNA or the nonspecific (NS)-control siRNA, as determined 72 h after transfection. B, cell growth of transfected cells treated with 100 nM BZA or vehicle (control). Transfected cells (30,000/well) were seeded in 24-well dishes overnight and then treated with BZA for 5 days. After treatment, cells were collected and counted using a hemocytometer. Data is presented as percentage and is based on the mean from three independent experiments with duplicate (∗, $p < 0.01$ versus nontarget transfected cells). C, cell cycle analysis of cyclin D1 siRNA-transfected and control siRNA transfected MCF-7:5C cells after treatment with BZA for 48 h. Data are based on the mean from three independent experiments with duplicate. ∗, $p < 0.01$; ∗∗, $p < 0.001$.

Overall, these findings indicate that the alignment of BZA in the binding pocket of ERα predicted by the IFD is similar with that predicted via the rigid docking method (Glide) and with the alignment of RAL in the experimental structure, PDB code 1err. However, there are a few differences in the orientation of some residues in the binding site when the docking of BZA is performed with IFD protocol, and these differences might help to explain the different biological effects of BZA versus RAL in our cell model.

Discussion

In the present study, we report for the first time that BZA inhibits the growth of breast cancer cells that have acquired resistance to long-term estrogen deprivation (i.e., hormone-independent/aromatase inhibitor resistant). Specifically, we found that BZA at 10^{-8} M inhibited the growth of hormone-independent MCF-7:5C and MCF-7:2A breast cancer cells by 80 and 55%, respectively. The inhibitory effect of BZA in MCF-7:5C cells was associated with G_1 arrest and cyclin D1 and ERα down-regulation, whereas in MCF-7:2A cells, BZA suppressed cyclin A with marginal effects on cyclin D1. The pure antiestrogen FUL also inhibited the growth of MCF-7:5C cells by inducing G_1 arrest; however, it did not down-regulate cyclin D1 until 96 h, which was 48 h after its effect on cell cycle. Strikingly, RAL, 4OHT, and ENDOX failed to inhibit cyclin D1 expression in MCF-7:5C cells, and these compounds did not have any growth-inhibitory effect in MCF-7:5C cells. Although it is not entirely clear why BZA was more potent than fulvestrant at inhibiting the growth of MCF-7:5C cells, one possibility might be due to the fact that BZA down-regulated both ERα and cyclin D1, whereas FUL down-regulated ERα and had marginal effects on cyclin D1, which was observed at 96 h. Molecular modeling studies indicated that BZA bound the ligand binding domain of ERα in an antagonist orientation similar to RAL (Fig. 6D) but distinct from 4OHT (Fig. 6E) and fulvestrant (Fig. 7). However, a few differences were noticed in the orientation of some residues in the binding site when the docking of BZA was performed with the IFD protocol. The most significant difference was observed for the Leu539 of helix 12. The larger ring of BZA caused the side chain of Leu539 to be pushed away from its original position by approximately 1 Å. This alteration in the orientation of Leu539 side chain could trigger a conformational change of helix 12, which in turn could lead to the recruitment of other proteins by the BZA-ERα compared with the RAL-ERα complex. Indeed, these findings help to further distinguish BZA from the other SERMs such as TAM and RAL, and they support the concept that subtle but moderate structural differentiation can dramatically affect the ability of a ligand to regulate cell proliferation.

Previous research has indicated that deregulation of ERα expression is a driving force in the initiation and progression of estrogen-sensitive breast tumors (Garcia-Closas and Chanock, 2008; Garcia-Closas et al., 2008). It has been suggested that alterations in pathways leading to ERα synthesis and/or degradation underlie the deregulation of ERα and its consequent manifestations, including enhanced proliferation in breast tumors (Sommer and Fuqua, 2001). ERα is the predominant receptor isoform expressed in breast cancer cells, and increased numbers of ERα-expressing cells can be observed at the earliest stages of breast tumorigenesis. We have shown previously that ERα mRNA and protein levels are significantly elevated in breast cancer cells that have been adapted to grow in an estrogen-depleted environment

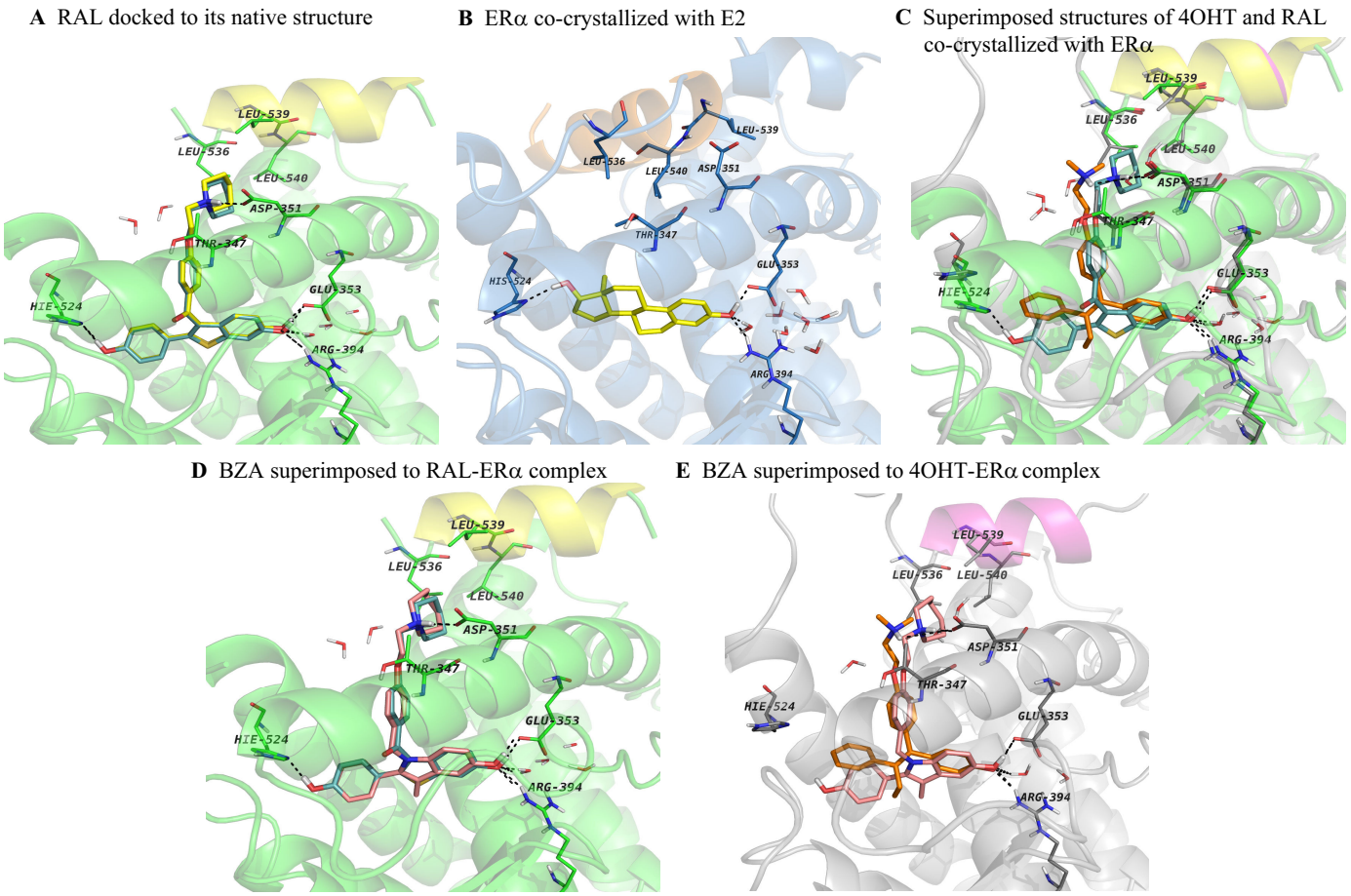

Fig. 6. Molecular modeling of ERα binding site with various ligands. A, comparison between the experimental (yellow sticks) and top ranked docking pose (cyan sticks) of RAL to ERα binding site. The docking pose recapitulates very well the alignment of the cocrystallized ligand in the receptor binding site having a ligand root mean square deviation of 0.36 Å. B, agonist conformation of ERα cocrystallized with E2; helix 12 is depicted in orange and lays over the binding site sealing the ligand inside it. The antagonist conformations of the receptor are shown in C, D, and E. X-ray structures of ERα cocrystallized with 4OHT (C), raloxifene (D), and bazedoxifene (E) docked into the ERα-raloxifene crystal structure. Helix 12 is depicted in magenta for 4OHT bound conformation and yellow for raloxifene and bazedoxifene. In addition, the key amino acids lining the binding site are displayed and the network of hydrogen bonds in which they are involved with the ligands is shown in black dashed lines. Carbon atoms are colored in yellow for E2, orange for 4OHT, cyan for raloxifene, and pink for bazedoxifene. These images show the differences between the agonist (B) and antagonist conformation (C, D, and E) of ERα and present the alignment of bazedoxifene in the binding site of ERα, which is similar to raloxifene's orientation, and the same interactions with the key amino acids of the binding cavity are encountered.

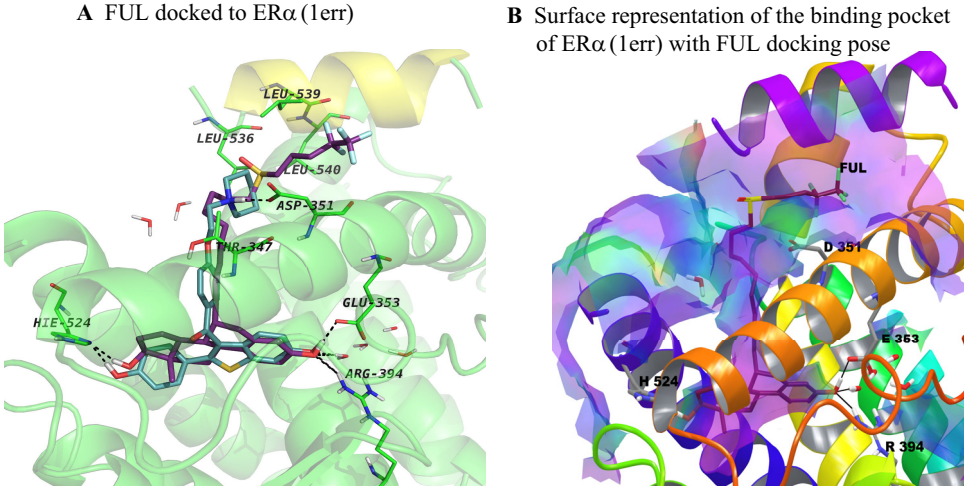

Fig. 7. Simplified representations of the ERα binding site with fulvestrant. A, representation of the ERα binding site with the best docking pose for fulvestrant (FUL, purple sticks). B, surface representation of ERα binding site accommodating FUL. Hydrophobic areas are mapped in purple, whereas the hydrophilic parts are colored in light yellow-green. The binding site accommodates very well the ligand, which forms the H-bond contacts with the same amino acids like E2 or RAL, whereas the aliphatic side chain protrudes from the binding site and lies in the groove between helix 3 (orange cartoon) and helix 12 (purple cartoon). Only the key amino acids underlying the binding site are shown.

(Murphy et al., 1990; Pink et al., 1996; Lewis et al., 2005a). This particular type of regulation in which ERα levels are increased after estrogen deprivation has been described as a model I response (Pink and Jordan, 1996). A model I response is characterized by an ERα that is expressed at high levels in the absence of estrogen and is subsequently downregulated after estrogen binding, primarily through repression of the steady-state level of the mRNA. In the present

study, we found that basal ERα protein levels were up-regulated greater than 3-fold in hormone-independent MCF-7:5C and MCF-7:2A breast cancer cells compared with MCF-7 and T47D cells, and treatment with BZA (10^{-8} M) induced proteasome-mediated degradation of ERα in these cells, which was reversed by the proteasome inhibitor MG132. The ability of BZA to degrade ERα in MCF-7:5C cells was rapid and robust, occurring as early as 4 h after treatment with maximum degradation at 24 h. It is noteworthy that BZA and fulvestrant were the only compounds that markedly reduced the growth of both MCF-7:5C and MCF-7:2A breast cancer cells, and blocking BZA-induced ERα degradation with MG132 dramatically reduced its growth inhibitory effects on these cells (data not shown). The importance of ERα in mediating the antagonist effects of BZA in hormone-independent MCF-7:5C cells was further confirmed by siRNA knockdown experiments, which showed a 60% reduction in the ability of BZA to inhibit the growth of these cells. Suppression of ERα also significantly reduced the basal growth of MCF-7:5C cells and E2-induced growth in wild-type MCF-7 cells, which is consistent with recent findings by Ariazi et al. (2010). It should be noted, however, that degradation or suppression of ERα is not the only mechanism by which an antagonist can inhibit cell proliferation. For example, TAM has been shown to stabilize ERα protein against degradation in breast cancer cells (Murphy et al., 1990; Pink et al., 1995, 1996; Pink and Jordan, 1996); however, it is a potent antagonist in the breast with the ability to block E2-stimulated proliferation and E2-induced ERE activity in these cells.

Apart from ERα, BZA also significantly reduced cyclin D1 expression in hormone-independent MCF-7:5C breast cancer cells. Cyclin D1 is a breast cancer oncogene whose overexpression has been linked to poor prognosis in ERα and progesterone receptor-positive breast cancers (Lammie and Peters, 1991). It is a multifunctional G_1-phase cyclin whose regulatory effects are particularly important in breast development and cancer (Sutherland and Musgrove, 2004). Cyclin D1 is highly induced by estrogen (Said et al., 1997), and it contributes to poor treatment response of ER-positive tumors by acting downstream to promote hormone agonist- and antagonist-independent proliferation (Wilcken et al., 1997). We found that cyclin D1 protein was constitutively elevated by 3-to 5-fold in hormone-independent MCF-7:5C and MCF-7:2A cells compared with wild-type MCF-7 and T47D cells, and treatment with BZA reduced it to an undetectable level in MCF-7:5C cells but not MCF-7:2A cells. In addition, we found that suppression of cyclin D1 in MCF-7:5C cells reduced the hormone-independent growth of these cells, and it significantly reduced the ability of BZA to inhibit cell growth and induce cell cycle arrest in these cells. Suppression of cyclin D1 also significantly reduced ERα protein levels in MCF-7:5C cells with similar effects observed after ERα suppression, thus suggesting a link between cyclin D1 and ERα in these cells. Indeed, a connection between ER and cyclin D1 was demonstrated previously when cyclin D1 was shown to interact directly with the ligand-binding domain of ER and stimulate ER transactivation in a ligand-independent fashion (Zwijsen et al., 1997). More recently, cyclin D1 was shown to interact with coactivators of the SRC-1 family through a motif that resembles the leucine-rich coactivator binding motif of nuclear receptors. By acting as a bridging factor between ER and SRCs, it is believed that cyclin D1 can recruit SRC family coactivators to ER in the absence of ligand. It is worth noting that hormone-independent MCF-7:5C cells express elevated levels of SRC-1 protein compared with hormone-dependent MCF-7 cells, and BZA treatment significantly reduces basal SRC-1 levels in these cells (data not shown).

Although cyclin D1 gene transcription is directly induced by estrogen, there is no estrogen response element in it. Instead, the cyclin D1 promoter contains multiple regulatory elements, including binding sites for activator protein-1, signal transducer and activator of transcription 5, nuclear factor-κB, cAMP response element, SP1, and E2F. A fragment between −994 and −136 of the cyclin D1 promoter was shown previously to be estrogen-responsive, and this region has binding sites for AP-1 and SP-1 (Altucci et al., 1996). We have reported that estrogen-induced cyclin D1 transactivation in MCF-7 breast cancer cells was mediated by the CRE region, which is known to bind activating transcription factor 2 (Lewis et al., 2005c,d). A notable finding of our study was that basal cyclin D1 promoter activity was significantly elevated in hormone-independent MCF-7:5C cells compared with hormone-dependent MCF-7 cells and treatment with BZA completely reduced the promoter activity in these cells to the level seen in the untreated MCF-7 cells. In contrast, E2 did not induce cyclin D1 expression or promoter activity in hormone-independent MCF-7:5C cells, whereas in hormone-dependent MCF-7 cells, it increased cyclin D1 protein level by 3-fold and its promoter activity by 4-fold, which is consistent with its function as a proapoptotic agent in MCF-7:5C cells versus an agonist in MCF-7 cells.

In conclusion, it is clear from clinical data that BZA in combination with conjugated estrogens represents a new form of therapeutic agents for the treatment of postmenopausal symptoms and prevention of postmenopausal osteoporosis. The fact that it does not stimulate the breast or endometrium and is very effective at inhibiting the proliferation of endocrine-resistant breast cancer cells highlights its widespread therapeutic potential and demonstrates that not all SERMs are alike. Our data also suggest that the overexpression of ERα and cyclin D1 in MCF-7:5C cells might be driving the hormone-independent growth of these cells and that the ability of BZA to down-regulate ERα and cyclin D1 is critical to treat and possibly reverse antihormone resistance in breast cancer.

Acknowledgments

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Authorship Contributions

Participated in research design: Lewis-Wambi and Jordan.
Conducted experiments: Lewis-Wambi and Kim.
Contributed new reagents or analytic tools: Curpan, Grigg, and Sarker.
Performed data analysis: Lewis-Wambi.
Wrote or contributed to the writing of the manuscript: Lewis-Wambi and Jordan.

References

Albanese C, Johnson J, Watanabe G, Eklund N, Vu D, Arnold A, and Pestell RG (1995) Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J Biol Chem* **270:**23589–23597.

Altucci L, Addeo R, Cicatiello L, Dauvois S, Parker MG, Truss M, Beato M, Sica V, Bresciani F, and Weisz A (1996) 17beta-Estradiol induces cyclin D1 gene transcription, p36D1–p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells. *Oncogene* **12:** 2315–2324.

Archer DF, Pinkerton JV, Utian WH, Menegoci JC, de Villiers TJ, Yuen CK, Levine AB, Chines AA, and Constantine GD (2009) Bazedoxifene, a selective estrogen receptor modulator: effects on the endometrium, ovaries, and breast from a randomized controlled trial in osteoporotic postmenopausal women. *Menopause* **16:** 1109–1115.

Ariazi EA, Brailoiu E, Yerrum S, Shupp HA, Slifker MJ, Cunliffe HE, Black MA, Donato AL, Arterburn JB, Oprea TI, et al. (2010) The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. *Cancer Res* **70:**1184–1194.

Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, and Bourne PE (2000) The Protein Data Bank. *Nucleic Acids Res* **28:**235–242.

Berry M, Nunez AM, and Chambon P (1989) Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence. *Proc Natl Acad Sci USA* **86:**1218–1222.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engström O, Ohman L, Greene GL, Gustafsson JA, and Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389:**753–758.

Friesner RA, Banks JL, Murphy RB, Halgren TA, Klicic JJ, Mainz DT, Repasky MP, Knoll EH, Shelley M, Perry JK, et al. (2004) Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J Med Chem* **47:**1739–1749.

Garcia-Closas M and Chanock S (2008) Genetic susceptibility loci for breast cancer by estrogen receptor status. *Clin Cancer Res* **14:**8000–8009.

Garcia-Closas M, Hall P, Nevanlinna H, Pooley K, Morrison J, Richesson DA, Bojesen SE, Nordestgaard BG, Axelsson CK, Arias JI, et al. (2008) Heterogeneity of breast cancer associations with five susceptibility loci by clinical and pathological characteristics. *PLoS Genet* **4:**e1000054.

Guallar V, Jacobson M, McDermott A, and Friesner RA (2004) Computational modeling of the catalytic reaction in triosephosphate isomerase. *J Mol Biol* **337:** 227–239.

Jiang SY and Jordan VC (1992) Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. *J Natl Cancer Inst* **84:**580–591.

Jiang SY, Wolf DM, Yingling JM, Chang C, and Jordan VC (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* **90:**77–86.

Jordan VC (2007) New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer. *Steroids* **72:**829–842.

Jordan VC (2008) The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer–survival or death? *J Clin Oncol* **26:**3073–3082.

Jordan VC (2009) A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. *Cancer Res* **69:**1243–1254.

Kagan R, Williams RS, Pan K, Mirkin S, and Pickar JH (2010) A randomized, placebo- and active-controlled trial of bazedoxifene/conjugated estrogens for treatment of moderate to severe vulvar/vaginal atrophy in postmenopausal women. *Menopause* **17:**281–289.

Komm BS, Kharode YP, Bodine PV, Harris HA, Miller CP, and Lyttle CR (2005) Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity. *Endocrinology* **146:**3999–4008.

Labarca C and Paigen K (1980) A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* **102:**344–352.

Lammie GA and Peters G (1991) Chromosome 11q13 abnormalities in human cancer. *Cancer Cells* **3:**413–420.

Lewis-Wambi JS, Cunliffe HE, Kim HR, Willis AL, and Jordan VC (2008a) Overexpression of CEACAM6 promotes migration and invasion of oestrogen-deprived breast cancer cells. *Eur J Cancer* **44:**1770–1779.

Lewis-Wambi JS, Kim HR, Wambi C, Patel R, Pyle JR, Klein-Szanto AJ, and Jordan VC (2008b) Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* **10:**R104.

Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, and Jordan VC (2005a) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* **97:**1746–1759.

Lewis JS, Osipo C, Meeke K, and Jordan VC (2005b) Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* **94:**131–141.

Lewis JS, Thomas TJ, Pestell RG, Albanese C, Gallo MA, and Thomas T (2005c) Differential effects of 16alpha-hydroxyestrone and 2-methoxyestradiol on cyclin D1 involving the transcription factor ATF-2 in MCF-7 breast cancer cells. *J Mol Endocrinol* **34:**91–105.

Lewis JS, Vijayanathan V, Thomas TJ, Pestell RG, Albanese C, Gallo MA, and Thomas T (2005d) Activation of cyclin D1 by estradiol and spermine in MCF-7 breast cancer cells: a mechanism involving the p38 MAP kinase and phosphorylation of ATF-2. *Oncol Res* **15:**113–128.

Maximov PY, Myers CB, Curpan RF, Lewis-Wambi JS, and Jordan VC (2010) Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. *J Med Chem* **53:**3273–3283.

Miller CP, Collini MD, Tran BD, Harris HA, Kharode YP, Marzolf JT, Moran RA, Henderson RA, Bender RH, Unwalla RJ, et al. (2001) Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. *J Med Chem* **44:**1654–1657.

Miller PD, Chines AA, Christiansen C, Hoeck HC, Kendler DL, Lewiecki EM, Woodson G, Levine AB, Constantine G, and Delmas PD (2008) Effects of bazedoxifene on BMD and bone turnover in postmenopausal women: 2-yr results of a randomized, double-blind, placebo-, and active-controlled study. *J Bone Miner Res* **23:**525–535.

Murphy CS, Pink JJ, and Jordan VC (1990) Characterization of a receptor-negative, hormone-nonresponsive clone derived from a T47D human breast cancer cell line kept under estrogen-free conditions. *Cancer Res* **50:**7285–7292.

Pink JJ, Bilimoria MM, Assikis J, and Jordan VC (1996) Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. *Br J Cancer* **74:**1227–1236.

Pink JJ, Jiang SY, Fritsch M, and Jordan VC (1995) An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* **55:**2583–2590.

Pink JJ and Jordan VC (1996) Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* **56:**2321–2330.

Pinkerton JV, Archer DF, Utian WH, Menegoci JC, Levine AB, Chines AA, and Constantine GD (2009) Bazedoxifene effects on the reproductive tract in postmenopausal women at risk for osteoporosis. *Menopause* **16:**1102–1108.

Said TK, Conneely OM, Medina D, O'Malley BW, and Lydon JP (1997) Progesterone, in addition to estrogen, induces cyclin D1 expression in the murine mammary epithelial cell, in vivo. *Endocrinology* **138:**3933–3939.

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, and Greene GL (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95:**927–937.

Silverman SL, Christiansen C, Genant HK, Vukicevic S, Zanchetta JR, de Villiers TJ, Constantine GD, and Chines AA (2008) Efficacy of bazedoxifene in reducing new vertebral fracture risk in postmenopausal women with osteoporosis: results from a 3-year, randomized, placebo-, and active-controlled clinical trial. *J Bone Miner Res* **23:**1923–1934.

Sommer S and Fuqua SA (2001) Estrogen receptor and breast cancer. *Semin Cancer Biol* **11:**339–352.

Sutherland RL and Musgrove EA (2004) Cyclins and breast cancer. *J Mammary Gland Biol Neoplasia* **9:**95–104.

Wärnmark A, Treuter E, Gustafsson JA, Hubbard RE, Brzozowski AM, and Pike AC (2002) Interaction of transcriptional intermediary factor 2 nuclear receptor box peptides with the coactivator binding site of estrogen receptor alpha. *J Biol Chem* **277:**21862–21868.

Wilcken NR, Prall OW, Musgrove EA, and Sutherland RL (1997) Inducible overexpression of cyclin D1 in breast cancer cells reverses the growth-inhibitory effects of antiestrogens. *Clin Cancer Res* **3:**849–854.

Wu X, Hawse JR, Subramaniam M, Goetz MP, Ingle JN, and Spelsberg TC (2009) The tamoxifen metabolite, endoxifen, is a potent antiestrogen that targets estrogen receptor alpha for degradation in breast cancer cells. *Cancer Res* **69:**1722–1727.

Zwijsen RM, Wientjens E, Klompmaker R, van der Sman J, Bernards R, and Michalides RJ (1997) CDK-independent activation of estrogen receptor by cyclin D1. *Cell* **88:**405–415.

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Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time

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In laboratory studies, acquired resistance to long-term antihormonal therapy in breast cancer evolves through two phases over 5 y. Phase I develops within 1 y, and tumor growth occurs with either 17β-estradiol (E_2) or tamoxifen. Phase II resistance develops after 5 y of therapy, and tamoxifen still stimulates growth; however, E_2 paradoxically induces apoptosis. This finding is the basis for the clinical use of estrogen to treat advanced antihormone-resistant breast cancer. We interrogated E_2-induced apoptosis by analysis of gene expression across time (2–96 h) in MCF-7 cell variants that were estrogen-dependent (WS8) or resistant to estrogen deprivation and refractory (2A) or sensitive (5C) to E_2-induced apoptosis. We developed a method termed differential area under the curve analysis that identified genes uniquely regulated by E_2 in 5C cells compared with both WS8 and 2A cells and hence, were associated with E_2-induced apoptosis. Estrogen signaling, endoplasmic reticulum stress (ERS), and inflammatory response genes were overrepresented among the 5C-specific genes. The identified ERS genes indicated that E_2 inhibited protein folding, translation, and fatty acid synthesis. Meanwhile, the ERS-associated apoptotic genes Bcl-2 interacting mediator of cell death (BIM; BCL2L11) and caspase-4 (CASP4), among others, were induced. Evaluation of a caspase peptide inhibitor panel showed that the CASP4 inhibitor z-LEVD-fmk was the most active at blocking E_2-induced apoptosis. Furthermore, z-LEVD-fmk completely prevented poly (ADP-ribose) polymerase (PARP) cleavage, E_2-inhibited growth, and apoptotic morphology. The up-regulated proinflammatory genes included IL, IFN, and arachidonic acid-related genes. Functional testing showed that arachidonic acid and E_2 interacted to superadditively induce apoptosis. Therefore, these data indicate that E_2 induced apoptosis through ERS and inflammatory responses in advanced antihormone-resistant breast cancer.

aromatase inhibitor | antihormonal resistance | estrogen receptor | gene expression microarrays | selective estrogen receptor modulator

Elucidation of the basic structure function relationships of synthetic estrogens based on either stilbene (1) or triphenylethylene (2) was a landmark achievement that continues to have major therapeutic implications to this day. The first successful chemical therapy for the treatment of any cancer was the use of high-dose synthetic estrogen for the treatment of metastatic breast cancer (3). Response rates for patients who were more than a decade beyond menopause were about 30%. Importantly, treatment near menopause was ineffective, and therefore, tumor responsiveness was related to the duration of estrogen deprivation. In 1970, Alexander Haddow commented that "the extraordinary extent of tumor regression observed in perhaps 1% of postmenopausal cases [with oestrogen] has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us" (4). High-dose estrogen therapy using diethylstilbestrol (DES) remained the standard of care for the treatment of metastatic breast cancer in postmenopausal women for 30 y (1950s to late 1970s in the United States). However, triphenylethylene-based estrogens evolved into nonsteroidal antiestrogens (5), where the initial interest focused on their potential as postcoital antifertility agents. This application failed, and the compounds were subsequently reinvented as antiestrogens targeted to estrogen receptor (ER) for the treatment of all stages of breast cancer (6, 7). Subsequently, the nonsteroidal antiestrogens would again evolve and be reinvented as selective ER modulators (SERMs) (8). This new drug class exploited the observations that they blocked breast cancer development and growth as antiestrogens but lowered circulating cholesterol and maintained bone density as estrogens. This finding led to the idea that the treatment and prevention of osteoporosis would simultaneously prevent breast cancer (5, 9). Raloxifene is the first SERM of the class used to prevent both osteoporosis and breast cancer (10, 11).

The strategy of targeting ER and using long-term adjuvant tamoxifen therapy for breast cancer treatment (7) has increased 15-y survival rates (12, 13) and contributed significantly to the national reduction breast cancer mortality (14). From 1975 to 1990, breast cancer mortality rates held roughly steady, but from 1990 to 2000, they declined by 19.6%. It is estimated that about two-thirds of this reduction is because of therapy and one-third is because of mammography screening. Specifically, in ER-positive tumors, 5 y of tamoxifen therapy was estimated to have reduced the hazard of breast cancer mortality by 37% (14). Tamoxifen remains the antihormone treatment of choice for the adjuvant treatment of

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See Profile on page 18876.

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breast cancer in premenopausal patients, despite the development of the aromatase inhibitors (AIs) for the adjuvant treatment of postmenopausal patients with ER-positive breast cancer. The AIs provide a modest, but significant, improvement in disease-free survival for patients and a significant decrease in the incidence of both endometrial cancer and thromboembolism associated with tamoxifen therapy in postmenopausal women (15). Nevertheless, tamoxifen remains an important and cheap lifesaving drug, available in countries without a sophisticated healthcare infrastructure.

Despite the ability of long-term adjuvant antihormone therapy to enhance breast cancer patient survivorship, the consequence of any sustained therapy to control tumor growth is the development of resistance. Studies in vivo with MCF-7 cells inoculated into athymic mice showed that, although tamoxifen initially blocked tumor growth, eventually tumors would grow, despite continued tamoxifen treatment (16). Similar studies showed that tamoxifen, in fact, stimulated growth of resistant MCF-7 tumors (17). A new form of acquired drug resistance was described for breast cancer that grew in response to ER activation through either tamoxifen or the natural ligand 17β-estradiol (E_2). This finding explained the observed resistance to tamoxifen in ER-positive metastatic breast cancer patients after ~1–2 y of therapy but was inconsistent with the clinical observation that patients with stages I and II breast cancer could be routinely treated with 5 or more y of adjuvant tamoxifen therapy without developing tumor recurrence. A possible explanation would emerge from studies of acquired resistance to antihormone therapy that, at the same time, would expose a vulnerability of breast cancer cells and explain the mechanism of high-dose estrogen therapy for the treatment of breast cancer.

The continuous passage of MCF-7 tumors for more than 5 y in tamoxifen-treated athymic mice results in a reconfiguration of survival signaling pathways. Although tumors remain tamoxifen stimulated for growth, physiologic E_2 now causes rapid tumor regression rather than growth (18, 19). Indeed, some tumors that regress and then regrow during continuous E_2 treatment are exclusively E_2-dependent, because tamoxifen or E_2 withdrawal will impair tumor growth (19). The evolution of acquired resistance to SERMs (20) naturally raised the concern of the development of resistance to the new standard of care for adjuvant treatment of ER-positive breast cancer in postmenopausal patients, the AIs.

Parallel studies to replicate the clinical expression of acquired resistance to estrogen deprivation (i.e., resistance to an AI) (21, 22) were initiated in vitro 20 y ago using ER-positive MCF-7 breast cancer cells. When cells were grown under long-term estrogen-deprived conditions (>1 y), cells lost their dependency on estrogen for proliferation but maintained expression of ER. Subsequent studies of E_2 action on the growth of long-term estrogen-deprived MCF-7 cells in vitro at high (23) and low concentrations in vitro and in vivo (24, 25) indicated that the concept of "an estrogen purge" (19) to destroy antihormone-resistant cells could be applied to the treatment of breast cancer. This concept has now been translated to clinical trials.

A pivotal study of high-dose DES therapy (15 mg daily) in 32 patients with metastatic breast cancer who had been treated exhaustively with antihormonal therapies produced a 30% objective response rate (26). There were 4 of 32 complete responses, and one patient maintained a complete response for an additional 7 y, even after stopping estrogen (27). A recent study in patients whose breast cancer had responded but then failed AI treatment (28) showed that low-dose E_2 treatment (6 mg daily) would produce the same clinical benefit as high-dose E_2 (30 mg daily) but with fewer toxic side effects. Thus, laboratory observations with low doses of estrogen treatment translate to clinical practice, and a mechanism is now emerging to explain the original observations by Haddow (3, 4). The goal of future translational research is to discover molecular mechanisms to amplify the estrogen-induced apoptotic trigger.

The question arises as to the precise sequence of events that lead to E_2-induced apoptosis. By describing and defining these molecular events, refractory cells may be manipulated to respond to estrogen-induced apoptosis. To begin to address the question, we have developed a series of MCF-7 variants that are either estrogen-dependent for growth (MCF-7:WS8 cells) (29–31) or resistant to estrogen deprivation (ED) and refractory (MCF-7:2A) (25, 30, 31) or sensitive (MCF-7:5C) (24, 29, 32) to E_2-induced apoptosis. We previously reported changes in gene expression among these cell lines by Affymetrix-based microarray analysis under estrogen-free conditions (33). Thus, these identified differentially expressed genes were associated with progression to an ED-resistant phenotype. We have also recently reported a proteomic analysis of 5C compared with WS8 cells after 2 h of E_2 exposure to identify proteins that may initiate apoptosis (34). Here, we seek to identify genes differentially regulated by E_2 over a 2–96 h time course, which overlaps with actively occurring apoptosis. Therefore, we interrogated these models for changes in E_2-regulated global gene expression as a function of time using Agilent 4 × 44 K oligonucleotide microarrays. We developed a method termed differential area under the curve (dAUC) analysis to identify genes that exhibited significantly altered regulation by E_2 across time specifically in the apoptosis-sensitive 5C cells compared with both the estrogen-dependent WS8 and apoptosis-refractory 2A cells. Examination of the identified 5C-specific genes and functional testing indicated that E_2-elicited endoplasmic reticulum stress (ERS) and inflammatory stress responses that led to apoptosis.

Results and Discussion

Cell Line Characterization. Before gene expression microarray studies were carried out, the estrogen-dependent WS8 (29–31), ED-resistant but apoptosis-refractory 2A (25, 30, 31), and apoptosis-sensitive 5C cells (24, 29, 32) were characterized to confirm previously reported growth responses, biomarker status, and estrogen response element (ERE) -regulated transcriptional activity (*SI Results and Discussion*, *SI Methods*, and Fig. S1). The apoptotic responses of 5C cells to E_2 were also characterized according to loss of plasma membrane integrity (*SI Results and Discussion*, *SI Methods*, and Fig. S2). The 5C cells exhibited an EC_{50} for apoptosis of 3×10^{-11} M E_2 after 96 h of exposure (Fig. S2*B*). Additionally, 10^{-9} M E_2, the concentration used for the microarray studies, caused apoptosis ranging from ~30% to 42% of the 5C cells, depending on the experiment (Fig. S2 *B* and *C*). The pure antiestrogen fulvestrant completely blocked apoptosis induced by E_2 and DES, showing that apoptosis was ER-dependent (Fig. S2*C*).

Global Gene Expression Across Time. To identify genes and pathways/processes associated with E_2-induced apoptosis, differential regulation of global gene expression in response to E_2 was interrogated in ED-resistant/apoptotic-sensitive 5C cells vs. estrogen-dependent WS8 and ED-resistant/apoptotic-refractory 2A cells. Each cell line was treated with 10^{-9} M E_2 or vehicle control over a 96-h time course consisting of seven time points (2, 6, 12, 24, 48, 72, and 96 h) using six biological replicates per condition. cRNA probes from individual E_2-treated samples were competitively hybridized against time-matched pooled control probes using two-color Agilent 4 × 44 K human oligonucleotide microarrays. The resulting gene expression values were $\log_2$ ratios of mRNA levels in E_2/control-treated cells that, when plotted across time, form a curve. A measure of change in E_2-mediated regulation of expression over a defined time interval was then calculated as the difference in AUCs or dAUCs for a given gene between two cell lines. Genes that showed a 50% change in AUCs between two cell lines (corresponding to a dAUC = 0.58 on a $\log_2$ scale) at a P value < 0.00005 (*Methods* has details on P value determination) were defined as significantly different. The dAUC method was

applied to identify differentially regulated genes at 2–96, 2–24, and 24–96 h to identify overall, relatively early, and late-responding genes, respectively.

To identify genes specifically associated with E_2-induced apoptosis, genes were selected with regulation that differed significantly with E_2 in the 5C cells vs. both the WS8 and 2A cells. A total of 1,142 genes were identified as significantly differentially regulated by E_2 specifically in the 5C cells (Dataset S1). These genes were examined for overrepresentation of those genes mapping to a particular curated pathway/network (Fig. S3*A*). As expected, estrogen signaling and apoptosis genes were significantly enriched. Within the apoptosis category, ERS was the most enriched apoptosis subcategory (Fig. S3*B*). Inflammatory response genes were also enriched. The overlapping distribution of genes mapping to estrogen signaling (Dataset S2), apoptosis (Dataset S3), and inflammatory responses (Dataset S4) is shown in the Venn diagram in Fig. S3*C*.

Estrogen Signaling Genes. Estrogen signaling genes selectively regulated by E_2 in 5C cells relative to both WS8 and 2A cells are listed in Dataset S2, and examples discussed are shown in Fig. 1. Multiple genes were differentially regulated by E_2 in 5C cells compared with WS8 and 2A cells, which would diminish ERα activity and hence, the apoptotic stimulus. For example, genes that negatively modulate ERα activity (i.e., AR, CYP1B1, FHL2, HSD17B11, INHBA, NR2F1/COUP-TF1, SNAI1/Snail 1, and THRA/TRα) were selectively up-regulated, whereas those genes that promote ERα activity were selectively down-regulated (AREG, CAV1, and PIK3CB) by E_2 in 5C cells. The up-regulated estrogen metabolizing enzymes CYP1B1 and HSD17B11 would decrease intracellular E_2 pools. SETD7/SET7/SET9 methylates ER to stabilize the protein (Dataset S5, ref. 1); hence, its down-regulation by E_2 in 5Cs would accelerate ERα protein degradation. ERα activity would be suppressed by up-regulation of transcription factors that repress ERα RNA expression (i.e., FHL2 and Snail-1) (Dataset S5, refs. 2 and 3) or compete with ERα for binding-extended ERE half-sites, which overlap with many natural EREs (i.e., COUP-TF1 and TRα) (Dataset S5, refs. 4 and 5). AR failed to down-regulate in response to E_2 in 5C cells, allowing greater AR activity. AR and ERα interact in complexes, and androgens inhibit E_2-stimulated growth of MCF-7 cells (Dataset S5, ref. 6); thus, AR can oppose ERα. ERα activity can also be suppressed by activin-A, a TGFβ superfamily ligand, in a SMAD3-dependent manner in MCF-7 cells (Dataset S5, ref. 7). Both INHBA and SMAD3 were selectively induced in 5C cells, and INHBA homodimerizes to form activin-A, which signals to SMAD3; SMAD3 interacts with ERα at promoters to repress transcription. AREG and PIK3CB failed to increase in response to E_2 in 5C cells. This failure to increase may have prevented increased ERα activity, because AREG activates EGFR, which leads to ERα-Ser118 phosphorylation, and PIK3CB is the catalytic subunit of PI3K, which through Akt, targets ERα-Ser167 phosphorylation (Dataset S5, ref. 8). CAV1 expression also failed to increase, which again prevents increased ERα activity, because CAV1 interacts with and promotes activity of membrane-localized ERα (Dataset S5, ref. 9). However, not all of ERα's activities were suppressed. In particular, ERα interacts with and directs transcription through AP-1 transcription complexes in addition to EREs (Dataset S5, ref. 10). AP-1 complexes consist of FOS, JUN, and JUND subunits, which were all selectively induced by E_2 in 5C cells [FOS and JUN were verified by quantitative PCR (qPCR) in Fig. S4]. Importantly, AP-1 complexes play important roles in apoptosis and inflammatory responses (discussed later), and thus, ERα interaction with AP-1 provides a mechanism for E_2 to target such genes.

Apoptosis Genes. The identified apoptosis genes are listed in Dataset S3, and discussed examples are shown in Fig. 2. Enrichment analysis indicated ERS-mediated apoptosis as the top-scoring individual pathways within the apoptosis category (Fig. S3). The endoplasmic reticulum is a key site for protein folding. When cellular stresses perturb energy levels, the redox state, or Ca^{2+} concentrations, unfolded proteins accumulate and protein aggregation occurs; this condition is referred to as ERS (Dataset S5, refs. 11 and 12). To relieve ERS, an unfolded protein response (UPR) is triggered by the chaperone HSPA5/GRP78/BiP. In addition to binding unfolded proteins, GRP78 binds and prevents oligomerization of the endoplasmic reticulum transmembrane receptors EIF2AK3/PERK, IRE1α/ERN1, and ATF6. When unfolded proteins accumulate, GRP78 is released from binding the transmembrane receptors, allowing them to oligomerize and autophosphorylate to initiate a UPR signal. The UPR signals to attenuate protein translation, induce expression of additional chaperones, and export misfolded proteins to the cytosol for degradation. If the UPR fails

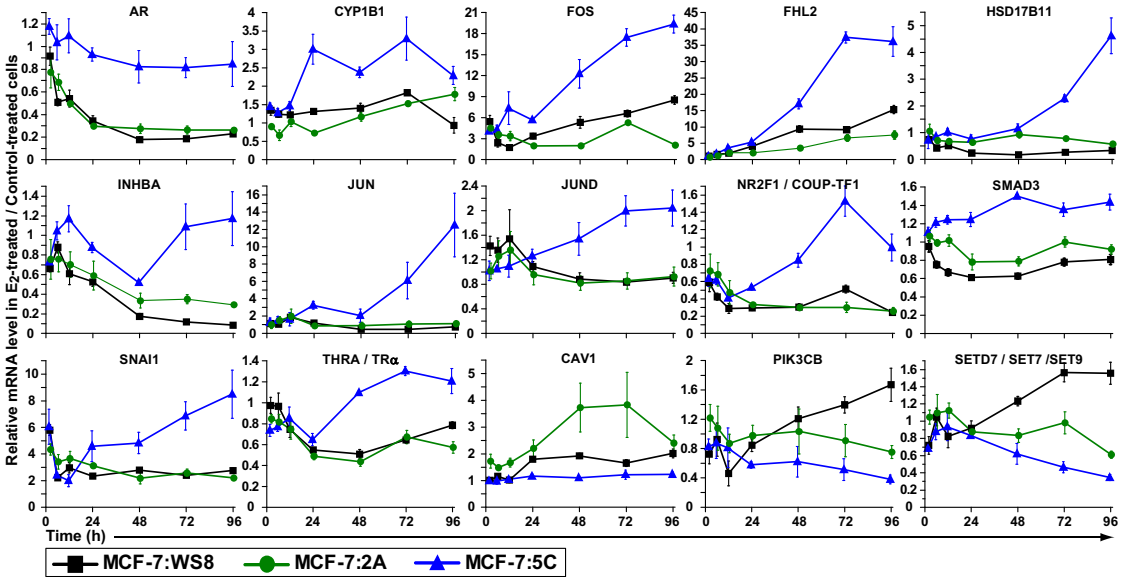

Fig. 1. Examples of estrogen signaling genes. Full annotation, dAUC values, and *P* values of all estrogen signaling genes are given in Dataset S2.

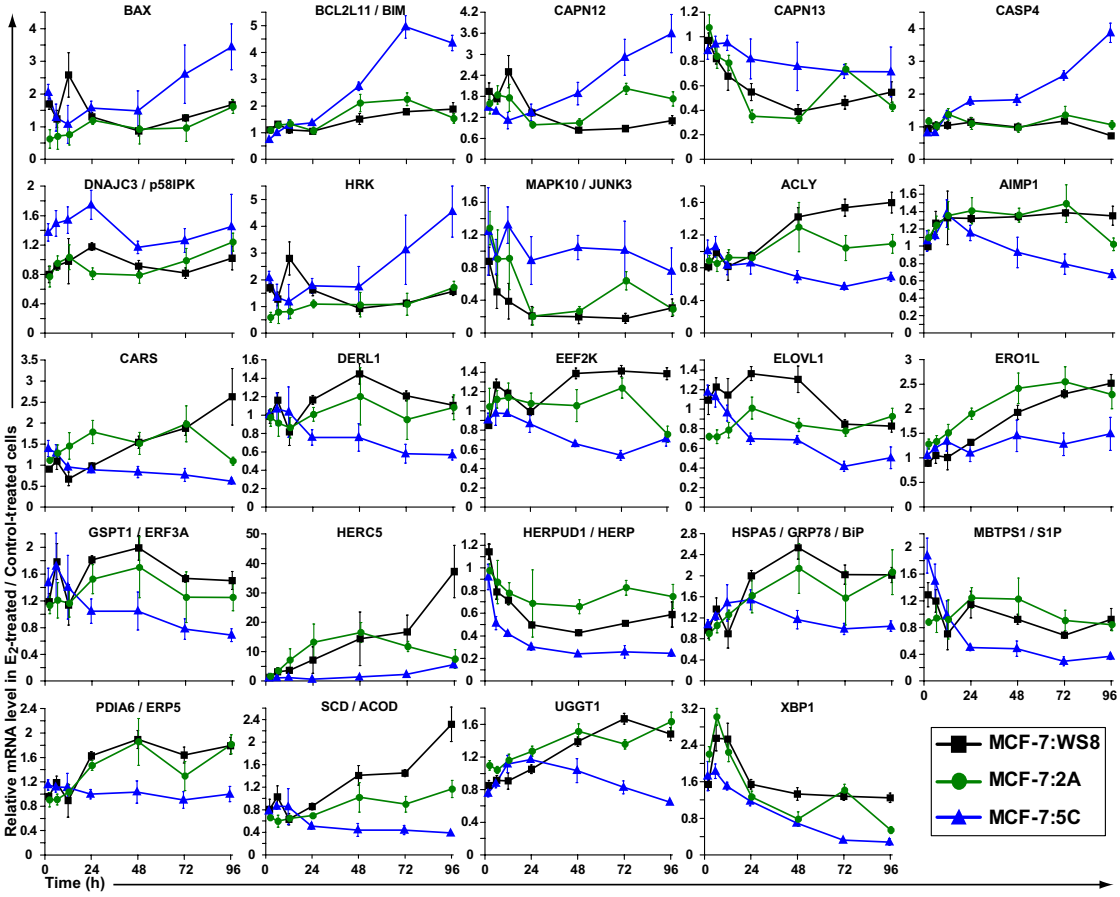

Fig. 2. Examples of apoptosis genes. Full annotation, dAUC values, and *P* values of all apoptosis genes are given in Dataset S3.

to relieve the stress, the function of the UPR switches from promoting cell survival to promoting cell death. Thus, excessive or prolonged ERS typically induces apoptosis.

Growth stimulation of hormonally responsive cells by E_2 leads to increases in requirements for folding nascent polypeptides and clearance of malfolded proteins. However, in 5C cells compared with WS8 and 2A cells, E_2-regulated expression changes indicated a deficiency in these functions. In 5C cells, E_2 failed to sufficiently up-regulate endoplasmic reticulum-localized protein folding genes, including GRP78 (verified by qPCR in Fig. S4), ERO1L, PDIA6, and UGGT1. Cytoplasmic protein folding genes, including HSP90AB1/HSP90B, PPIAL4A, and PPIF (also FKBP10), also failed to up-regulate. Additionally, in 5C cells, E_2 preferentially down-regulated HERPUD1/HERP1 and DERL1, factors that promote degradation of endoplasmic reticulum-resident proteins. A deficiency in up-regulating UPR genes in 5C cells may have resulted in part by the pronounced E_2-mediated repression of MBTPS1/S1P, which cleaves ATF6, activating its translocation to the nucleus to induce transcription of UPR genes, including XBP1. Thus, decreased S1P may have led to decreased ATF6 and XBP1 activity, thereby preventing induction of multiple UPR genes.

E_2-mediated gene expression alterations in 5C cells indicated widespread inhibition of protein translation compared with E_2-treated WS8 and 2A cells. Within 2 h, E_2 had up-regulated DNAJC3/p58^{IPK}, which binds to and inactivates EIF2AK3/PERK, leading to reduced global translational initiation (Dataset S5, ref. 12). The aminoacyl tRNA synthetase interacting protein AIMP1 and tRNA synthetases, including CARS (also LARS, SARS, and YARS) failed to increase in response to E_2 in 5Cs. Other translational factors that failed to induce in 5C cells include EEF2K and GSPT1/ERF3A (also EEF1A1, ETF1, and PABPC4).

Under severe ERS, the UPR can shut down lipogenesis as cells commit to death (Dataset S5, ref. 12). This was likely the case in E_2-treated 5C cells since they showed a lack of induction of critical genes involved in fatty acid synthesis, including ACLY, SCD/ACOD, and ELOVL1. ACLY is the primary enzyme responsible for synthesis of acetyl-CoA, the basic building block of fatty acids. SCD introduces a C-C double bond in fatty acyl-CoA substrates, including stearoyl-CoA and palmitoyl-CoA, a key step in producing monounsaturated fatty acids. ELOVL1 condenses both saturated and monounsaturated fatty acids. Notably, SCD and ELOVL1 are localized to the endoplasmic reticulum membrane.

In response to severe ERS, specific BCL2 and Bcl-2 homology domain 3 (BH3) -only family members are targeted to initiate apoptosis (Dataset S5, ref. 11). Prototypical BCL2 inhibits cell death by binding and inactivating proapoptotic members such as BAX. BH3 only-containing proteins like BCL2L11/BIM indirectly activate BAX by binding BCL2 (through the BH3 motif), thereby releasing BAX from the complex. BAX then permeabolizes the mitochondrial outer membrane, allowing cytochrome C release to the cytoplasm. Under ERS, BAX also interacts with and activates IRE1α. IRE1α then signals to JNK to simultaneously activate BIM and inhibit BCL2 (Dataset S5, ref. 11). A variety of ERS inducers stimulate BIM expression, and BIM is essential in ERS-induced apoptosis in a wide range of cell types (Dataset S5, ref. 13). This apoptotic pathway was likely activated by E_2 in 5C cells. E_2 failed to repress MAPK10 (JNK3) in 5C cells, indicating higher JNK3 activity. Meanwhile, E_2 selectively up-regulated BAX, BIM (verified

by qPCR in Fig. S4), and another BH3-only proapoptotic factor, HRK (also BBC3/PUMA but PUMA did not make the significance cutoff) (Fig. S5). Importantly, E_2 repressed BCL2 in 5C cells but induced it in WS8 cells. However, E_2 also repressed BCL2 in 2A cells, and therefore, it was not a 5C-specific gene (Fig. S5). We previously verified the importance of BAX and BIM by showing that they were selectively induced by E_2 at the protein level in 5C vs. WS8 cells and that their depletion by RNAi blocked E_2-induced apoptosis (31). Therefore, ERS may have triggered mitochondrial-mediated apoptotic cell death in E_2-treated 5C cells.

After prolonged ERS, specific caspases are activated to enact cell death. Examination of the caspases revealed that only CASP4 met the stringent statistical significance criteria in the microarray data. CASP1, CASP5, and CASP8 also showed up-regulation in 5C cells but did not meet our significance threshold (Fig. S5). CASP4 along with CASP1 and CASP5 are inflammatory caspases, because they are involved in cytokine maturation (Dataset S5, ref. 14). CASP4 specifically localizes to the endoplasmic reticulum and undergoes cleavage in response to ERS-inducing agents/proteins but not other apoptotic agents, and its blockade using z-LEVD-fmk or depletion by RNAi can prevent endoplasmic stress-induced apoptosis in multiple model systems (Dataset S5, refs. 15–20). Importantly, CASP4 autoactivates by dimerizing and undergoing interdomain cleavage (Dataset S5, ref. 21), and thus, simply overexpressing CASP4 is sufficient to induce cleavage of downstream caspases (Dataset S5, ref. 22) and cause apoptosis (Dataset S5, ref. 23). Under ERS, CASP4 can also be activated by calpain (Dataset S5, refs. 24 and 25), and CAPN12 and CAPN13 were selectively up-regulated in 5C cells.

Inflammatory Response Genes. The inflammatory response genes are listed in Dataset S4, and discussed examples are shown in Fig. 3. In 5C cells, E_2 elicited up-regulation of many proinflammatory cytokine/cytokine receptors, including IL-4R (verified by qPCR in Fig. S4), IL-6R, IL-6ST/gp130, IL-17RD/Sef, and VEGFA. IL-4R was induced with early kinetics, indicating that it may be a primary response. IL-6R was up-regulated shortly after IL-4R, whereas IL-6ST/gp130, also an IL-4R subunit, was already up-regulated by 2 h. Hence, IL-6 signaling was likely activated in 5Cs. IL-17RD/Sef not only mediates IL-17 signaling, but its overexpression also leads to JNK activation and apoptosis (Dataset S5, ref. 26), which links inflammatory responses and ERS. VEGFA also leads to activation of JNK in tamoxifen-resistant MCF-7 cells (Dataset S5, ref. 27). An IFN response was likely activated, because the IFN IFNL1 and the IFN-responsive genes IFI6 and IFI16 (Dataset S3) were up-regulated. CASP4 can also be induced by IFN (Dataset S5, ref. 28).

A number of other proinflammatory genes, such as CEBPB, NTN1 (verified by qPCR in Fig. S4), and UNC5C, were selectively up-regulated in E_2-treated 5C cells with relatively early kinetics, indicating possible mechanistic roles. CEBPB is important in induction of IL-6, is activated by ERS (Dataset S5, ref. 29), is required for nuclear import of the key ERS protein CHOP/GADD153 (Dataset S5, ref. 30), and enhances NF-κB signaling (Dataset S5, refs. 31 and 32). NTN1 is a secreted inflammatory marker, but it protects tissues from inflammatory injury by suppressing cytokine production, repulsing leukocyte infiltration, and acting as an antiinflammatory and antiapoptotic ligand of its receptors DCC and the UNC-5 family members (Dataset S5, refs. 33 and 34). In the context of E_2-induced apoptosis, NTN1 may have been up-regulated to limit or resolve the inflammatory response. Interestingly, E_2 rapidly down-regulated UNC5C in WS8 and 2A cells within 6 h but failed to do so in 5C cells, resulting in higher UNC5C expression. UNC5C may have a proinflammatory role, because synovial cells from patients with rheumatoid arthritis and osteoarthritis dramatically overexpress UNC5C (769-fold) compared with those cells of healthy donors (Dataset S5, ref. 35).

Arachidonic acid (AA; 20:4n-6) is a polyunsaturated fatty acid that plays a key role as an inflammatory mediator. Enzymes involved in AA biosynthesis were up-regulated by E_2 in 5C cells, including FADS1 (verified by qPCR in Fig. S4), FADS3, PLA2G10, PLCD3, MGLL/MAGL, PPAP2A/LPP1 (verified by qPCR in Fig. S4), and SGMS1/SMS1. FADS3 and FADS1 catalyze the first and last steps in AA biosynthesis by introducing C-C double bonds in linoleic acid, producing γ-linolenic acid (18:3n-6), and dihomo-γ-linolenic acid (20:3n-6), producing AA. PLA2s hydrolyze phospholipids, releasing AA, whereas PLCD3 cleaves AA from diacylglycerol. MGLL converts monoacylglycerides such as 2-arachidonoylglycerol to free fatty acids including AA. PPAP2A/LPP1 converts phosphatidic acid to diacylglycerol, providing increased substrate levels for PLCD3 to release AA. As an inflammatory mediator, AA is used as a precursor by cyclooxygenase and lipoxygenase to generate inflammatory prostaglandins and leukotrienes, respectively. However, the cyclooxygenase pathway was unlikely to have been involved in E_2-induced apoptosis, because induction of PTGES failed in 5C cells compared with WS8 and 2A cells. In hormone-dependent breast cancer cells, E_2 is known to induce PTGES expression through an ERE, which may promote breast cancer proliferation, because the increased prostaglandin E_2 may enhance aromatase expression and also promote local productions of estrogens (Dataset S5, ref. 36). Thus, a failure to induce PTGES may, ultimately, have served to prevent any potential increases in estrogen concentrations in 5C cells. Considering that ERS likely led to a block of fatty acid synthesis and conversion to monounsaturated fatty acids (i.e., no induction of ACLY and SCD), the selective increases in AA-related genes likely indicate the importance of AA in promoting an inflammatory response in E_2-induced apoptosis.

Cross-Talk Between ERS and Inflammatory Stress. As mentioned previously, ERS and inflammatory pathways intersect. The key ERS genes IRE1α, ATF6, and PERK can all activate NF-κB, which serves as a master regulator of inflammatory response gene transcription (Dataset S5, refs. 12 and 37). Many of the identified cytokine/cytokine receptors signal through NF-κB pathways. Other genes selectively induced by E_2 in 5C cells, including BCL10 (Dataset S5, ref. 38), CXXC5 (Dataset S5, ref. 39), LTB (verified by qPCR in Fig. S4 and Dataset S5, ref. 40), and ITGB2 (Dataset S4; Dataset S5, ref. 41), activate NF-κB signaling as well. Additionally, SETD7/SET7/SET9, which negatively regulates NF-κB activity by methylating the RelA subunit to induce its degradation (Dataset S5, ref. 42), was down-regulated by E_2 in 5Cs (Fig. 1). Furthermore, multiple 5C-specific genes are NF-κB–responsive, including BIM (Dataset S5, refs. 43 and 44), CASP4 (Dataset S5, ref. 45), CEBPB (Dataset S5, ref. 46), CP (Dataset S4; Dataset S5, ref. 47), NTN1 (Dataset S5, ref. 48), and VEGFA (Dataset S5, ref. 49). Moreover, ERα and NF-κB can interact to transcriptionally regulate promoters, providing a direct mechanism for E_2 to target a diverse array of inflammatory and apoptotic genes. Therefore, NF-κB signaling was very likely involved in E_2-induced apoptosis, and we are pursuing this hypothesis in future studies.

ERS also intersects with inflammatory responses through JNK. As mentioned, the ERS sensor IRE1α (Dataset S5, ref. 12) and the IL receptor 17RD/Sef can activate JNK (Dataset S5, ref. 26). The orphan TNF receptor TNFRSF19/TAJ, which failed to down-regulate in response to E_2 in 5C cells, also activates JNK (Dataset S5, ref. 50). JNK then phosphorylates AP-1 complexes to induce expression of inflammatory response genes (Dataset S5, ref. 12). As mentioned earlier, the AP-1 subunits JUN, JUND, and FOS were selectively induced in E_2-treated 5C cells.

Functional Involvement of AA and CASP4 in E_2-Induced Apoptosis. The involvement of ERS and inflammatory stress in E_2-induced apoptosis was functionally examined. We first tested

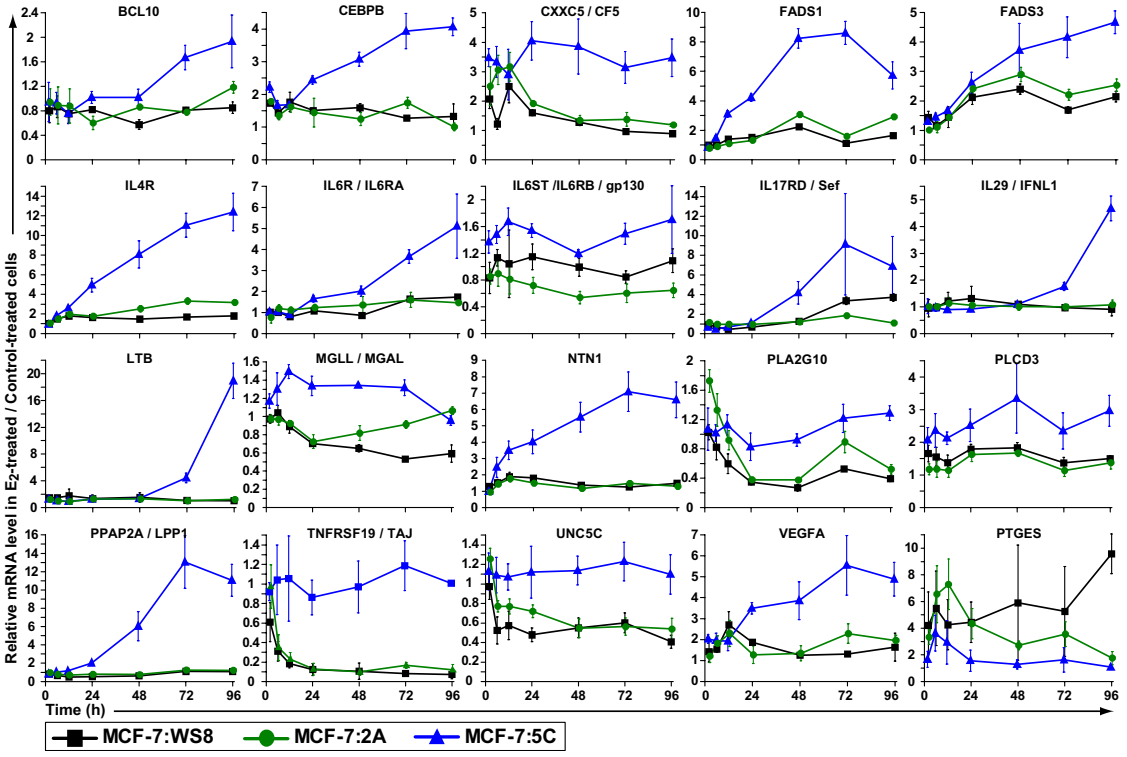

Fig. 3. Examples of inflammatory response genes. Full annotation, dAUC values, and *P* values of all inflammatory response genes are given in Dataset S4.

whether E_2-induced apoptosis could be promoted by AA. AA was chosen, because (*i*) it is widely recognized as a proinflammatory agent; (*ii*) it induces apoptosis (Dataset S5, ref. 51), at least in part by depleting the endoplasmic reticulum of Ca^{2+} and inhibiting protein translation, thereby likely eliciting ERS (Dataset S5, ref. 52); (*iii*) it can activate NF-κB in mammary epithelial cells (Dataset S5, ref. 53); and (*iv*) several genes, which increase AA levels (e.g., FADS1 and PLA2G10), were up-regulated in response to E_2 in 5C vs. WS8 and 2A cells. 5C cells were exposed to varying concentrations of both AA and E_2 in a factorial design, and then, apoptosis was measured by flow cytometric analysis of YO-PRO-1 and 7-aminoactinomycin D staining (Fig. 4*A*). Because E_2-induced apoptosis occurs maximally with 10^{-9} M E_2 after 96 h of exposure, E_2 was used at low concentrations of 2.5 and 5×10^{-11} M, and apoptosis was assayed at 72 h to allow observation of potential additional AA effects. The combination of AA plus E_2 at all varied concentrations increased the percentage of apoptotic plus dead cells in a greater than additive manner relative to either agent alone. Fitting the data to a multiple regression model showed the rate of increase (slope) in apoptotic plus dead cells progressively and significantly increased comparing E_2 alone with E_2 + 10 μM AA or E_2 + 20 μM AA. Therefore, AA and E_2 interacted to superadditively induce apoptosis, indicating that their pathways functionally intersect.

The importance of CASP4 was evaluated using a panel of irreversible caspase peptide inhibitors selectively targeting caspases-1 to -9 (except CASP3, which is not expressed in MCF-7 cells) (Dataset S5, ref. 54). 5C cells were treated with 10^{-9} M E_2 plus each caspase inhibitor as indicated for 96 h to induce apoptosis, which was measured by altered plasma membrane permeability (Fig. 4*B*). The broad spectrum caspase inhibitor z-VAD-fmk was used as a positive control, because we previously reported that this inhibitor completely blocks E_2-induced apoptosis (31), whereas the inactive inhibitor z-FA-fmk was used as a negative control. In an effort to prevent off-target caspase inhibition, the blockers were used at 10 μM, which was the concentration that reduced apoptosis by approximately one-half by the pan inhibitor z-VAD-fmk. The most active inhibitor was the CASP4 blocker z-LEVD-fmk, which was slightly more effective than the pan CASP inhibitor (Fig. 4*B*). The CASP8 inhibitor z-IETD-fmk was the next most active blocker but was significantly less potent than z-LEVD-fmk (*P* value = 0.0026). Therefore, in an unbiased comparison of caspases-1 to -9, CASP4 was validated as functionally critical in E_2-induced apoptosis.

The functional activity of CASP4 was also studied. Real-time qPCR and immunoblotting confirmed induction of CASP4 expression at the mRNA and protein levels, respectively, occurred specifically in 5C cells in response to E_2 (Fig. 5 *A* and *B*). Importantly, in 5C cells, z-LEVD-fmk at 20 μM completely blocked E_2-induced PARP cleavage (Fig. 5*B*), reversed E_2-inhibited growth (Fig. 5*C*), and prevented morphologic alterations associated with apoptosis in 5C cells (Fig. 5*D*). Because z-LEVD-fmk was used at 20 rather than 10 μM, we do not discount the possibility that some caspases in addition to CASP4 were also inhibited and that other caspases could still play an important role. Yet, our data establishes a critical role for CASP4 in E_2-induced apoptosis.

Concluding Remarks. We have interrogated E_2-induced apoptosis by identifying differentially regulated genes across time associated with this process compared with E_2-stimulated and -independent growth using a method we developed termed dAUC analysis. Overrepresentation analysis of the identified genes indicated that 5C cells respond to E_2 by suppressing ERα signaling and producing endoplasmic reticulum and inflammatory stress. Estrogen signaling was suppressed by metabolically reducing intracellular E_2 concentrations (increased CYP1B1 and HDS17B11) and up-regulating genes that antagonize ERα activity (SETD7, FHL2, Snail 1, COUP-TF1, TRα, AR, INHBA, and SMAD3) or repressing genes that promote ERα activity (AREG, PIK3CB, and CAV1). ERS was indicated by a deficiency in up-regulating genes involved in initiating a UPR (GRP78, XBP1, and S1P), protein

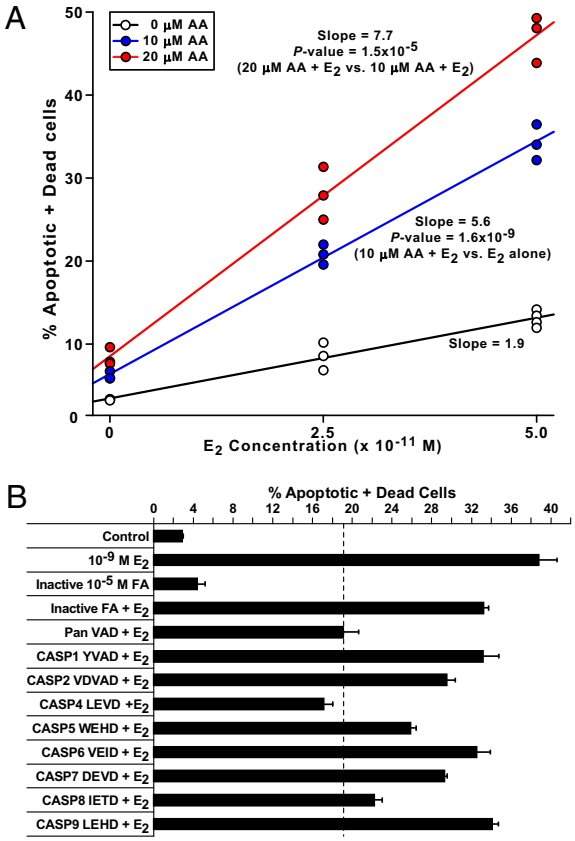

Fig. 4. Functional interrogation of E_2-induced apoptosis. (*A*) AA and E_2 interact to superadditively induce apoptosis. 5C cells were treated with combinations of AA and E_2 as indicated for 72 h. (*B*) Screening of selective CASP inhibitors. The selectivity of the inhibitors for individual caspases is indicated according to the manufacturer. 5C cells were treated with 10^{-9} M E_2 and 10 μM of each CASP inhibitor as indicated for 96 h. (*A* and *B*) Apoptosis according to altered plasma membrane permeability was determined by flow cytometric analysis of cells stained with the DNA-specific binding dyes YO-PRO-1 and 7-aminoactinomycin D. Double-negative staining cells were defined as viable, double-positive staining cells were defined as dead, and intermediately staining cells were defined as apoptotic. Data shown in *B* represent triplicates and associated SDs.

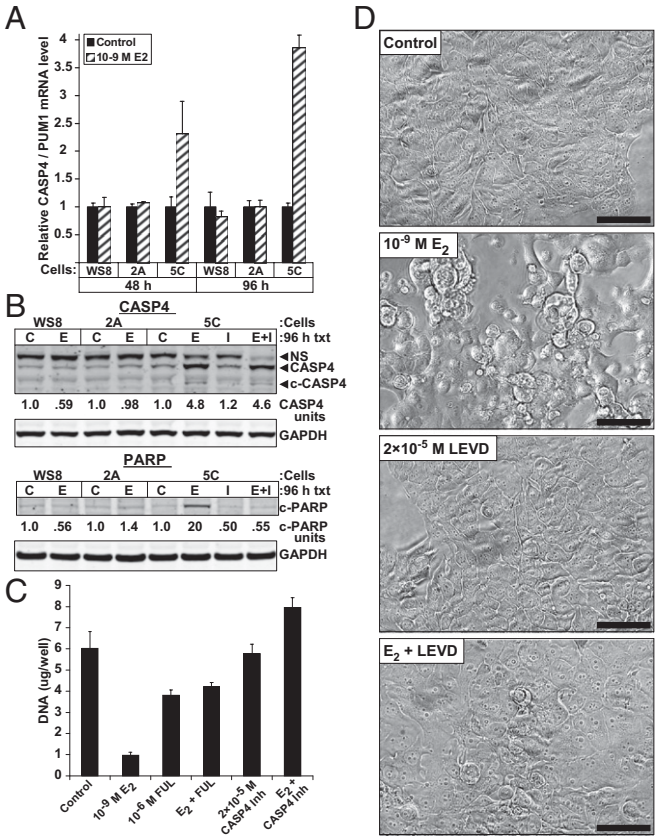

Fig. 5. Functional involvement of CASP4 in E_2-induced apoptosis. E_2-induced CASP4 at the (*A*) mRNA and (*B*) protein levels in 5C cells but not in WS8 or 2A cells. CASP4 mRNA and protein levels were measured by qPCR and immunoblotting, respectively. (*B*) In 5C cells, E_2 led to cleavage of the apoptotic marker PARP, which was blocked by the CASP4 inhibitor z-LEVD-fmk. C, control; E, E_2; I, inhibitor (z-LEVD-fmk); c-CASP4, cleaved CASP4; NS, nonspecific band. (*C*) E_2-inhibited growth of 5C cells was completely reversed by z-LEVD-fmk. Proliferation was determined after 6 d of 10^{-9} M E_2 exposure and measured by DNA mass per well. CASP4 Inh, CASP4 inhibitor z-LEVD-fmk. (*D*) Morphologic alterations after 96 h of 10^{-9} M E_2 in 5C cells were completely reversed by z-LEVD-fmk (LEVD). (Scale bar: 100 microns.) (*A–D*) E_2 was used at 10^{-9} M and z-LEVD-fmk at 2×10^{-5} M. Data in *A* and *C* represent the average and SDs of four and eight replicates, respectively.

folding (GRP78, PDIA6, and UGGT1), and degradation of malfolded proteins (HERP1 and DERL1), which would lead to accumulation of unfolded/misfolded proteins. Meanwhile, expression profiles indicated a widespread inhibition of protein translation (increased $p58^{IPK}$ and decreased aminoacyl tRNA synthetases, EEF2K, and ERF3A) and fatty acid synthesis (decreased ACLY and SCD), which combined with accumulation of unfolded proteins, would also promote stress and apoptosis. ERS was also indicated by induction of BIM, BAX, and the inflammatory caspase CASP4. We previously showed that depletion of BIM or BAX blocked E_2-induced apoptosis (31), and here, we showed that blocking CASP4 with z-LEVD-fmk also blocked E_2-induced apoptosis. Inflammatory stress was indicated by up-regulation of cytokines/cytokine receptors (IL-4R, IL-6R, IL-6ST/gp130, IL-17RD, and LTB), IFN/IFN responsive genes (IFNL1, IFI6, and IFI16), AA biosynthetic genes (FADS1 and PLA2G10), and other inflammatory markers (CEBPB, NTN1, and UNC5C). These findings indicate that inflammatory and ERS responses leading to apoptosis are highly interrelated and may cross-talk in part through NF-κB, JNK, and AP-1. Thus, additional stimulation of ERS and inflammatory responses by AA interacted with E_2 to superadditively induce apoptosis.

It should be noted that the differentially expressed genes identified here are associated with E_2-induced apoptosis; hence, their causal role in apoptosis needs to be functionally validated. Additional characterization and functional validation of genes and pathways regulating E_2-mediated apoptosis in 5C cells is currently being investigated using genome-wide, high-throughput RNAi profiling. Also, the results presented here are based on only MCF-7 derivative cell lines; hence, the findings may have limited applicability to the clinic. However, the MCF-7 cell line has accurately predicted clinical responses to antihormonal therapy in breast cancer (35). We and others have reported antihormonal-resistant MCF-7–based models besides 5C cells that exhibit E_2-induced apoptosis in vitro and in vivo (18, 19, 23, 36). We are aware of only one other breast cancer model not derived from MCF-7 cells that exhibits this behavior (i.e., T47D cells stably expressing PKCα), but only when grown in vivo as xenograft tumors (37). Therefore, molecular markers of ERS and inflammatory stress need to be confirmed in low-dose E_2 responding compared with nonresponding tumors in patients with estrogen-deprived metastatic breast cancer.

The identified 5C-specific genes may serve as biomarkers to predict response to estrogen therapy (e.g., the secreted factors

IFNL1, LTB, and NTN1 could be readily measured in patients). The identified 5C-specific genes also provide the basis for potentially improving clinical response rates to estrogen by combining it with agents that promote ERS and/or tumor-specific inflammation. For example, neutralizing NTN1 antibodies, AA, or its precursor, conjugated LA (Dataset S5, ref. 51), may increase response rates without engaging systemic inflammatory responses. Furthermore, these findings lead to the hypothesis that antiinflammatory agents prescribed for ancillary clinical problems should not be used during antitumor estrogen therapy.

Methods

Generation and Validation of RNA Samples for Microarrays. Each cell line was treated with or without 10^{-9} M E_2 using six replicates per treatment for 2, 6, 12, 24, 48, 72, and 96 h. To validate that each isolated RNA sample was derived from cells appropriately treated with or without E_2, expression of two classical E_2-responsive genes, MYC and TFF1 (pS2), were measured using real-time qPCR. MYC exhibited early kinetics, and TFF1 exhibited later kinetics of E_2 induction; together, induction of these markers spanned the entire time course. Successfully validated samples are shown in Figs. S6–S8.

dAUC Analysis. Differentially labeled fluorescent cRNA probes for each individual E_2-treated RNA sample (Cy3) and time point-matched, pooled, control-treated RNA samples (Cy5) were competitively hybridized to Agilent 4×44 K oligonucleotide microarrays using standard Agilent protocols. Gene expression values were extracted from arrays as relative $\log_2$ ratios of E_2/control-treated cells. To determine whether a gene's regulation by E_2 was significantly different between two cell lines, a method termed dAUC analysis was developed. In this method, the quantity of interest (dAUC) for a given probe is calculated as the signed area between the expression profiles for the two cell lines (using the average observed values at each time point). The null hypothesis is that dAUC is zero, and the distribution of dAUC values under the null hypothesis can be obtained by repeatedly permuting (n = 20,000) the cell line to which each $\log_2$ ratio value was assigned, while keeping the time points fixed. The two-sided P value of the observed dAUC can then be calculated as the proportion of permutations yielding a dAUC that exceeds the observed dAUC in absolute value. A probe was considered significantly different between two cell lines if the magnitude of the observed dAUC exceeded that obtained in all permutations (i.e., $P < 0.00005$). To exclude probes with statistically significant but numerically small differences, we imposed an additional condition that the probe's dAUC must have exhibited an average $\log_2$ fold change of 0.58 (1.5-fold on a linear scale) across a given time period. The dAUCs of each probe were calculated using all pairwise combinations of the three cell lines over the entire 2–96 h time course, and to delineate relatively early and late response genes, they were calculated over 2–24 and 24–96 h time periods.

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1. Dodds EC, Goldberg L, Lawson W, Robinson R (1938) Estrogenic activity of certain synthetic compounds. *Nature* 141:247–248.
2. Robson JM, Schönberg A (1937) Oestrous reactions including mating produced by triphenylethylene. *Nature* 140:196.
3. Haddow A, Watkinson JM, Paterson E, Koller PC (1944) Influence of synthetic oestrogens on advanced malignant disease. *Br Med J* 2:393–398.
4. Haddow A (1970) David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer* 26:737–754.
5. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: Eighth Cain memorial award lecture. *Cancer Res* 50:4177–4189.
6. Jordan VC (2003) Tamoxifen: A most unlikely pioneering medicine. *Nat Rev Drug Discov* 2:205–213.
7. Jordan VC (2008) Tamoxifen: Catalyst for the change to targeted therapy. *Eur J Cancer* 44:30–38.
8. Jordan VC (2001) Selective estrogen receptor modulation: A personal perspective. *Cancer Res* 61:5683–5687.
9. Jordan VC, Phelps E, Lindgren JU (1987) Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 10:31–35.
10. Cummings SR, et al. (1999) The effect of raloxifene on risk of breast cancer in postmenopausal women: Results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* 281:2189–2197.
11. Vogel VG, et al. (2006) Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: The NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* 295:2727–2741.
12. Early Breast Cancer Trialists' Collaborative Group (1998) Tamoxifen for early breast cancer: An overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. *Lancet* 351:1451–1467.
13. Early Breast Cancer Trialists' Collaborative Group (EBCTCG) (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. *Lancet* 365:1687–1717.
14. Berry DA, et al. (2006) Modeling the impact of treatment and screening on U.S. breast cancer mortality: A Bayesian approach. *J Natl Cancer Inst Monogr* 36:30–36.
15. Dowsett M, et al. (2010) Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. *J Clin Oncol* 28:509–518.
16. Osborne CK, Coronado EB, Robinson JP (1987) Human breast cancer in the athymic nude mouse: Cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol* 23:1189–1196.
17. Gottardis MM, Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 48:5183–5187.
18. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res* 127:23–33.
19. Yao K, et al. (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 6:2028–2036.
20. Jordan VC (2004) Selective estrogen receptor modulation: Concept and consequences in cancer. *Cancer Cell* 5:207–213.
21. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y (1987) Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res* 47:4355–4360.
22. Welshons WV, Jordan VC (1987) Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *Eur J Cancer Clin Oncol* 23:1935–1939.
23. Song RX, et al. (2001) Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *J Natl Cancer Inst* 93:1714–1723.
24. Lewis JS, et al. (2005) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 97:1746–1759.
25. Lewis-Wambi JS, et al. (2008) Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* 10:R104.
26. Lønning PE, et al. (2001) High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 67:111–116.
27. Lønning PE (2009) Additive endocrine therapy for advanced breast cancer—back to the future. *Acta Oncol* 48:1092–1101.
28. Ellis MJ, et al. (2009) Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: A phase 2 randomized study. *JAMA* 302:774–780.
29. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* 90:77–86.
30. Pink JJ, Jiang SY, Fritsch M, Jordan VC (1995) An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* 55:2583–2590.
31. Pink JJ, Jordan VC (1996) Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 56:2321–2330.
32. Lewis JS, Osipo C, Meeke K, Jordan VC (2005) Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 94:131–141.
33. Lewis-Wambi JS, Cunliffe HE, Kim HR, Willis AL, Jordan VC (2008) Overexpression of CEACAM6 promotes migration and invasion of oestrogen-deprived breast cancer cells. *Eur J Cancer* 44:1770–1779.
34. Hu ZZ, et al. (2011) Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. *PLoS One* 6:e20410.
35. Levenson AS, Jordan VC (1997) MCF-7: The first hormone-responsive breast cancer cell line. *Cancer Res* 57:3071–3078.
36. Liu H, et al. (2003) Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*. *J Natl Cancer Inst* 95:1586–1597.
37. Chisamore MJ, Ahmed Y, Bentrem DJ, Jordan VC, Tonetti DA (2001) Novel antitumor effect of estradiol in athymic mice injected with a T47D breast cancer cell line overexpressing protein kinase Calpha. *Clin Cancer Res* 7:3156–3165.

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Differential DNase I hypersensitivity reveals factor-dependent chromatin dynamics

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Transcription factor cistromes are highly cell-type specific. Chromatin accessibility, histone modifications, and nucleosome occupancy have all been found to play a role in defining these binding locations. Here, we show that hormone-induced DNase I hypersensitivity changes (ΔDHS) are highly predictive of androgen receptor (AR) and estrogen receptor I (ESRI) binding in prostate cancer and breast cancer cells, respectively. While chromatin structure prior to receptor binding and nucleosome occupancy after binding are strikingly different for ESRI and AR, ΔDHS is highly predictive for both. AR binding is associated with changes in both local nucleosome occupancy and DNase I hypersensitivity. In contrast, while global ESRI binding is unrelated to changes in nucleosome occupancy, DNase I hypersensitivity dynamics are also predictive of the ESRI cistrome. These findings suggest that AR and ESRI have distinct modes of interaction with chromatin and that DNase I hypersensitivity dynamics provides a general approach for predicting cell-type specific cistromes.

[Supplemental material is available for this article.]

In eukaryotes, transcription is regulated in a cell-type and condition-specific manner through the association of transcription factors with chromatin. The genome-wide binding sites of transcription factors, or the transcription factor cistromes, are influenced by the active protein levels of the transcription factors, chromatin structure, and DNA sequence. The nucleosome is the fundamental unit of chromatin structure and has been thought to compete with transcription factors for occupancy at thermodynamically favorable genomic loci. By comparing nucleosome occupancy maps generated from nucleosome-resolution H3K4me2 ChIP-seq, we found that nucleosome occupancy changes can be predictive of transcription factor cistromes. In particular, the binding of androgen receptor (AR) in prostate cancer LNCaP cells leads to an increased occupancy of nucleosomes flanking the AR binding site and decreased nucleosome occupancy in the position of the binding site itself (He et al. 2010). This approach also correctly predicted the binding of two factors, POU2F1 and NKX3-1, which are part of the secondary cellular response to androgens (He et al. 2010). This phenomenon is not unique to the LNCaP AR system; it has also been observed with CDX2, HNF4A, and GATA6 binding in intestinal differentiation (Verzi et al. 2010) and with GATA1 in hematopoiesis (Hu et al. 2011).

DNase I hypersensitivity is an alternative measure of chromatin accessibility (Wu 1980). DNase I hypersensitive sites (DHS), short regions of chromatin that are highly sensitive to cleavage by DNase I, typically occur in nucleosome free regions and frequently arise as a result of transcription factor binding. DNase I digestion followed by high-throughput sequencing (DNase-seq) has evolved into a powerful technique for identifying genome-wide DNase hypersensitive sites (Ling et al. 2010; John et al. 2011; Siersbaek et al. 2011). Because transcription factor binding sites tend to be DNase I hypersensitive and DNase-seq does not require a factor-specific antibody, DNA sequence motif analysis on DHS data has been proposed as a method for discovering the binding sites of multiple transcription factors in a single experiment (Pique-Regi et al. 2011; Song et al. 2011).

To analyze the effects of androgen receptor (AR) and estrogen receptor 1 (ESR1) binding on DHS, we conducted genome-wide DNase-seq in both unstimulated and hormone-stimulated conditions. Using a quantitative measurement of DHS changes (ΔDHS) between these conditions, we were able to predict the ESR1 and AR cistromes. Although they are related members of the steroid receptor family, AR and ESR1 display distinct DHS profiles. Binding of both ESR1 and AR are frequently associated with significant increases in DHS signal upon hormone stimulation; however, ESR1 sites show strong DHS prior to binding and AR sites do not. Following hormone stimulation, FOXA1 binding sites that lacked AR or ESR1 binding are associated with a significant decrease in DHS. In MCF-7 cells, this change in DHS is linked not to a change in FOXA1 binding but rather to a decrease in the binding of the ESR1 coactivator, NCOA3, supporting a model of physiologic squelching. This study demonstrates that ΔDHS is a more effective and general approach to predict perturbation-induced transcription factor binding sites than either static DHS or nucleosome resolution H3K4me2 ChIP-seq.

Results

Estrogen receptor binding in breast cancer cells is not associated with significant nucleosome depletion

Based on our earlier work demonstrating the association between AR binding and nucleosome depletion (He et al. 2010), we carried out an H3K4me2 ChIP-seq experiment on MNase digested chromatin in the MCF-7 breast cancer cell line comparing unstimulated

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(Veh) cells with cells grown under conditions of estrogen stimulation (E2). Consistent with previous studies (Barski et al. 2007; He et al. 2010), the H3K4me2 sites in both samples were mainly located in intergenic and intronic regions and were also found to be enriched in promoter regions (Fig. 1A). Over 64% of estrogen receptor 1 (ESR1) binding sites overlapped with regions enriched in H3K4me2 (estrogen-stimulated) (Fig. 1B). We examined the distribution of H3K4me2 signals relative to the center of all ESR1 binding sites. Although in some cases ESR1 binds to regions depleted of H3K4me2 signal (Supplemental Fig. 1A), in both the vehicle and stimulated conditions the overall pattern shows a peak in the H3K4me2 signal that overlaps with the ESR1 binding sites (Fig. 1C).

We systematically assessed ESR1 binding as a function of the nucleosome stabilization-destabilization (NSD) score, a measure of nucleosome occupancy changes established in previous studies (He et al. 2010). The fraction of ESR1 binding sites located in high NSD scoring regions was no greater than the fraction in regions with low NSD scores (Fig. 1D). This pattern is significantly different from that observed in AR binding (Supplemental Fig. 1B). In AR binding an H3K4me2 tag density peak at the AR binding site becomes a trough after androgen stimulation, resulting in high NSD scoring regions being highly predictive of AR binding (He et al. 2010). Whereas in MCF-7 the distributions of NSD scores at ER and non-ER sites are not significantly different (Supplemental Fig. 1C, *P*-value = 0.25), the distributions of NSD scores in LNCaP AR and non-AR sites are significantly different (Supplemental Fig. 1D, *P*-value = 2.2×10^{-16}).

In order to determine whether the differences in the behavior of AR in LNCaP cells and ESR1 in MCF7 cells were due to a difference in the transcription factors or the cell lines, we analyzed H3K4me2 enrichment at AR, ESR1, and FOXA1 sites together (Fig. 2A,B; Supplemental Fig. 2A,B). We included the winged helix transcription factor FOXA1 in the analysis as it acts as a "pioneer factor" in breast cancer cells and is required for ESR1 binding to a large proportion of its binding sites (Carroll et al. 2005; Lupien et al. 2008). The role of FOXA1 in AR action in prostate cancer cells is more complex, though a significant number of AR-bound sites are also bound by FOXA1 (Lupien et al. 2008; Wang et al. 2011). Consistent with our previous findings (He et al. 2010), sites bound by FOXA1 alone in either LNCaP or MCF7 cells show a pair of stimulus-independent peaks that flank a trough directly over the FOXA1 binding site (Fig. 2A,B, right panels).

When we examined the H3K4me2 signal at sites bound by AR or ESR1 that lacked FOXA1, we observed very different patterns. In LNCaP cells, AR binding sites that do not bind FOXA1 had a broad peak of H3K4me2 prior to hormone stimulation that resolved into

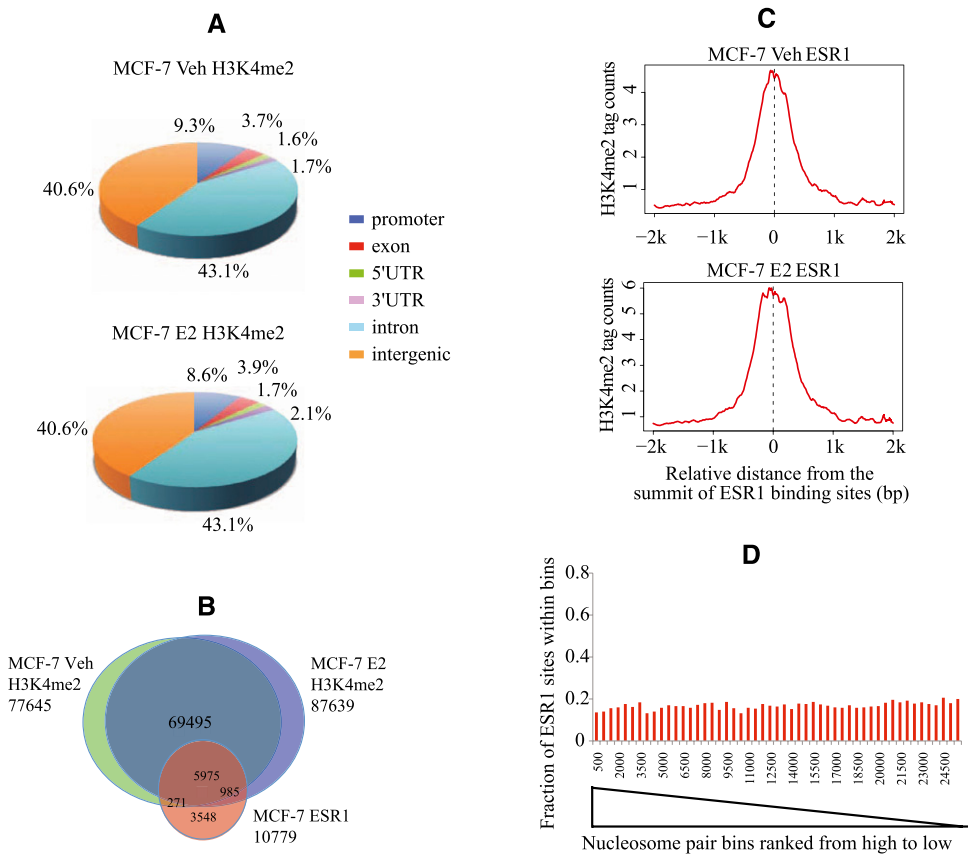

Figure 1. Characteristics of H3K4me2 ChIP-seq in MCF-7 cells. (*A*) Location of H3K4me2 ChIP-enriched peaks relative to gene annotations in unstimulated (Veh) and estrogen-stimulated (E2) conditions. (*B*) Venn diagram of ESR1 binding loci in relation to H3K4me2-enriched regions. (*C*) Distribution of H3K4me2 ChIP-seq signal at non-promoter (>1 kb from TSSs) ESR1 binding sites under unstimulated and estrogen-stimulated conditions. (*D*) The fraction of ESR1 binding sites in paired nucleosome bins sorted in descending order by NSD score (stimulated vs. unstimulated). Paired nucleosome regions are ranked by the NSD score that represents the differences in the H3K4me2 tag counts before and after estrogen treatment. These ranked regions are grouped into bins of 500 to calculate the proportion of real binding sites as a function of rank. (*Y*-axis) Fraction of the regions in each bin that overlap with ESR1 ChIP-seq enriched regions.

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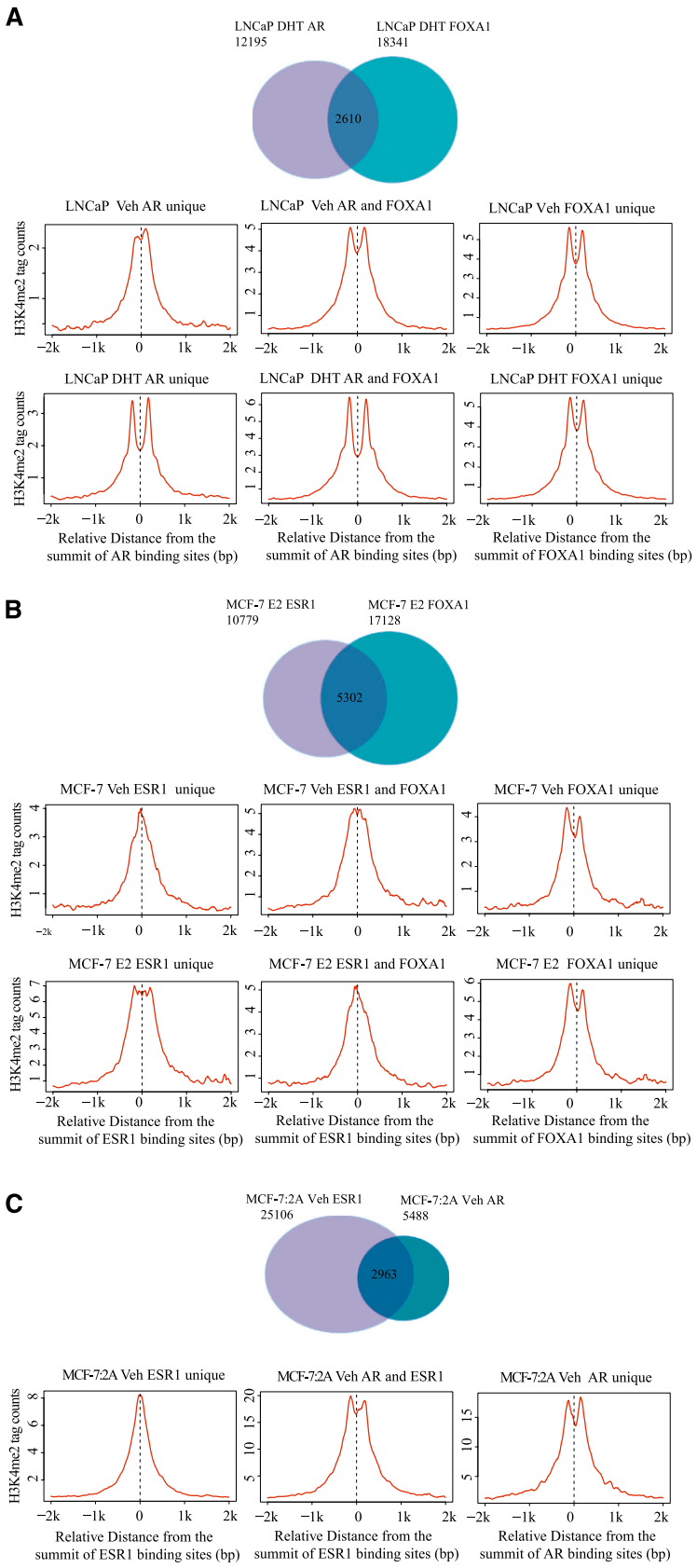
two sharp peaks flanking the AR binding site upon AR activation (Fig. 2A, left panels). In contrast, ESR1 binding sites in MCF7 cells that lack FOXA1 had a broad peak of H3K4me2 centered over the ESR1 binding site both before and after ESR1 activation (Fig. 2B, left panels). The pattern of H3K4me2 at the shared AR/FOXA1 and ESR1/FOXA1 sites was also distinct. H3K4me2 signal at the AR/FOXA1 bound sites indicates nucleosome depletion and better positioned flanking nucleosomes after AR activation (Fig. 2A, center panels). In contrast, the pattern at ESR1/FOXA1 sites is similar to the ESR1-unique sites and has a single broad peak both before and after ESR1 activation (Fig. 2B, center panels). NPS, an algorithm that predicts nucleosome position (Zhang et al. 2008b), also predicts clearly different nucleosome distributions relative to ESR1-unique binding sites, FOXA1-unique binding sites, and shared sites (Supplemental Fig. 1C–E). At sites of ESR1 binding with or without FOXA1, the predicted nucleosomes more frequently overlap the ESR1 binding site (Supplemental Fig. 1E,F) while FOXA1 sites that lack ESR1 binding sites have a peak of binding that is in a region removed from a nucleosome center (Supplemental Fig. 1G).

To further test whether the differences between ESR1 and AR are intrinsic to the transcription factors, we examined the MCF-7-derived hormone-independent breast cancer cell line MCF-7:2A (Pink et al. 1995; Ariazi et al. 2011). While MCF-7:2A cells grow in the absence of estrogen or androgen, their growth is inhibited by silencing of either ESR1 or AR (data not shown). Sixty-five percent of the ESR1 binding sites in MCF-7 under the E2-stimulated condition overlap with those of MCF-7:2A in the absence of estrogen (Supplemental Fig. 2C). While there is significant overlap in the ESR1 and AR binding sites in MCF-7:2A, there are also many ESR1- and AR-unique sites (Fig. 2C, Venn diagram). MNase digested H3K4me2 ChIP-seq in MCF-7:2A was performed, and the distribution of H3K4me2 at ESR1-unique, AR-unique, and shared sites was determined (Fig. 2C; Supplemental Fig. 2D). At the ESR1-unique binding sites, H3K4me2 formed a sharp, unimodal peak at the binding site (Fig. 2C, left panel). In contrast, the AR-unique sites are associated with a broader H3K4me2 tag distribution with two modes that flank the AR binding site (Fig. 2C, right panel). Shared ESR1 and AR binding sites had an H3K4me2 profile with an intermediate



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distribution between that of the ESR1-unique and AR-unique sites (Fig. 2C, middle panel). These results suggest that, although AR binding involves depletion of a nucleosome directly over the AR binding site, ESR1 binding does not.

Quantitative measures of DNase I dynamics are predictive of TF binding

Given our finding that ESR1 binding could not be predicted by changes in the occupancy of H3K4me2 marked nucleosomes, we investigated stimulus-dependent changes in DNase I hypersensitivity (DHS) as a complement to nucleosome occupancy. An analysis of DHS under unstimulated (Veh) and androgen-stimulated (DHT) conditions in the LNCaP cell line demonstrated that 51% of AR binding sites overlap with androgen-stimulated DHS regions (Fig. 3A), as would be expected from our prior work on nucleosome occupancy. When we analyzed DHS in MCF-7 cells in unstimulated and estrogen-stimulated (E2) conditions, we found that ~63% of ESR1 binding sites overlap with stimulated DHS regions (Fig. 3B). In LNCaP cells, increasing the sequencing depth from 50 M to 70 M increased the proportion of AR sites that overlapped a DHS site from 51% to 55% (Supplemental Fig. 3A). Similarly, increasing the sequencing depth in MCF-7 cells from 28 M to 70 M raised the proportion of ESR1 sites that overlap with DHS from 63% to 71% (Supplemental Fig. 3B). ESR1 and AR sites that are not associated with DHS show significantly lower levels of binding than those that are associated with DHS (Supplemental Fig. 3C,D).

DHS regions encompass genomic locations that are associated with a variety of transcription factors and other chromatin-associated complexes; therefore, we investigated whether changes in DHS between conditions can be used to enhance the specificity of transcription factor binding site prediction. Starting with the set of DHS regions that were detected under hormone-stimulated conditions, we ranked the regions by three criteria: the DHS tag count in the unstimulated condition, the DHS tag count in the stimulated condition, and a score representing the change in the number of tag counts between the two conditions (ΔDHS) (Fig. 3C,D). The results for the LNCaP AR and MCF-7 ESR1 systems were quite distinct. In LNCaP cells, the level of DHS is not a strong predictor of AR binding in either the unstimulated or stimulated condition, although in both cases it is somewhat informative. In contrast, the change in DHS, ΔDHS, is a very strong predictor of AR binding (Fig. 3C). Interestingly, in the MCF-7 system, the level of DHS under unstimulated conditions is slightly predictive of ESR1 binding; however, estrogen-stimulated DHS and, most significantly, ΔDHS are progressively superior at predicting ESR1 binding (Fig. 3D). These results suggest on a genome-wide scale that at AR and ESR1 binding sites DHS increases upon receptor binding.

On a genomic scale, DNA sequence recognition motifs alone are poor predictors of in vivo ESR1 and AR binding. However, within DHS regions, DNA sequence motifs may be useful for identifying the DHS sites associated with the binding of a particular transcription factor. Starting with the set of DHS regions detected in the hormone-stimulated condition, we ranked the regions by three criteria: ΔDHS, strength of the AR or ESR1 DNA sequence motif, and a combination of the sequence motif and ΔDHS. In both the LNCaP and MCF7 systems, the nuclear receptor binding motifs are capable of discerning the binding locations of the specific factors from the remainder of the open chromatin regions (Supplemental Fig. 4A,B). Therefore, while DNA sequence motifs may not be reliable predictors of transcription factor binding across the entire genome (Carroll et al. 2006), they are reliable predictors within the regions of open chromatin. The best prediction of AR or ESR1 binding, however, was obtained by combining ΔDHS and motif based rankings. To further assess the ability of our approach to predict genome-wide receptor binding sites, we carried out precision-recall analysis for ESR1 (Fig. 3E) and AR (Supplemental Fig. 5). Precision is the fraction of predicted binding sites that are true positives and recall is the fraction of true binding sites identified. As seen for ESR1 in MCF-7 cells, DNA sequence motif alone is a poor predictor of binding. Combining static DHS peaks with motif yields a significantly better prediction, while combining ΔDHS with motif is most predictive. Interestingly when we plotted the precision-recall value for the ESR1 binding sites predicted by the CENTIPEDE algorithm (Pique-Regi et al. 2011) we found a point-prediction (see Methods) that is very similar to what we find using static DHS plus motif. Thus ΔDHS plus motif provides a powerful computational model for TF binding site prediction.

DNase I hypersensitivity is dependent on combinations of bound transcription factors

We further investigated the influence of combinations of ESR1 and AR binding with FOXA1 on ΔDHS. We found that the majority of FOXA1 sites are DHS in the LNCaP (72%) and MCF-7 (64%) cell lines (Supplemental Fig. 6). Interestingly, while DHS tends to increase at shared nuclear receptor FOXA1 sites, FOXA1 sites that do not overlap with AR or ESR1 loci after stimulation are associated with a decrease in DHS (Fig. 3F,G). In addition, ΔDHS at nuclear receptor binding loci are modified by the presence of FOXA1 in a cell line dependent fashion. In MCF-7, ESR1 sites that overlap with FOXA1 loci tend to show larger increases in DHS than the non-FOXA1 binding site containing ESR1 sites (Fig. 3G). In contrast, we observe a larger ΔDHS in non-FOXA1 AR binding sites than in the AR sites that overlap with FOXA1 (Fig. 3F) in LNCaP cells, despite the fact that the hormone-stimulated DHS signals in both cell lines are greatest at the shared nuclear receptor-FOXA1 shared sites (Supplemental Fig. 7).

Coactivator activity is detected by ΔDHS

One motivation for generating cistromes is to gain insight into the regulation of gene expression. To determine if ΔDHS can inform transcriptional regulation, we compared published LNCaP gene expression data (Wang et al. 2009) and MCF-7 GRO-seq data (Hah et al. 2011) with three sets of DHS sites: hormone-increased (top 5000 ΔDHS), hormone-diminished (bottom 5000 ΔDHS), and hormone-unchanged (middle 5000 ΔDHS). In both LNCaP and MCF-7 cells, the ratio of up-regulated genes to non-regulated genes (odds ratio) has a strong positive association with the hormone-increased DHS sites within 20 kb of the transcription start site

Figure 2. Mono-nucleosome level H3K4me2 ChIP-seq at nuclear receptor and FOXA1 binding loci in the MCF-7 (*A*), LNCaP (*B*), and MCF-7:2A (*C*) cell lines. (*A*) (*Top* panel) Venn diagram of AR binding in relation to FOXA1 binding. (*Middle* panel) Distribution of H3K4me2 signal centered on AR-unique, AR/FOXA1 shared, and FOXA1-unique sites in the unstimulated condition. (*Bottom* panel) Distribution of H3K4me2 signal centered on the AR-unique, AR/FOXA1 shared, and FOXA1-unique sites under conditions of androgen stimulation. (*B*) (*Top* panel) Venn diagram of ESR1 binding in relation to FOXA1 binding. (*Middle* panel) Distribution of H3K4me2 signal centered on ESR1-unique, ESR1/FOXA1 shared and FOXA1-unique sites in unstimulated cells. (*Bottom* panel) Distribution of H3K4me2 signal centered on ESR1-unique, ESR1/FOXA1 shared, and FOXA1-unique sites in estrogen stimulated cells. (*C*) (*Top* panel) Venn diagram of ESR1 binding in relation to AR binding. (*Bottom* panel) Distribution of H3K4me2 signal centered on ESR1-unique, ESR1/AR shared, and AR-unique sites in unstimulated cells.

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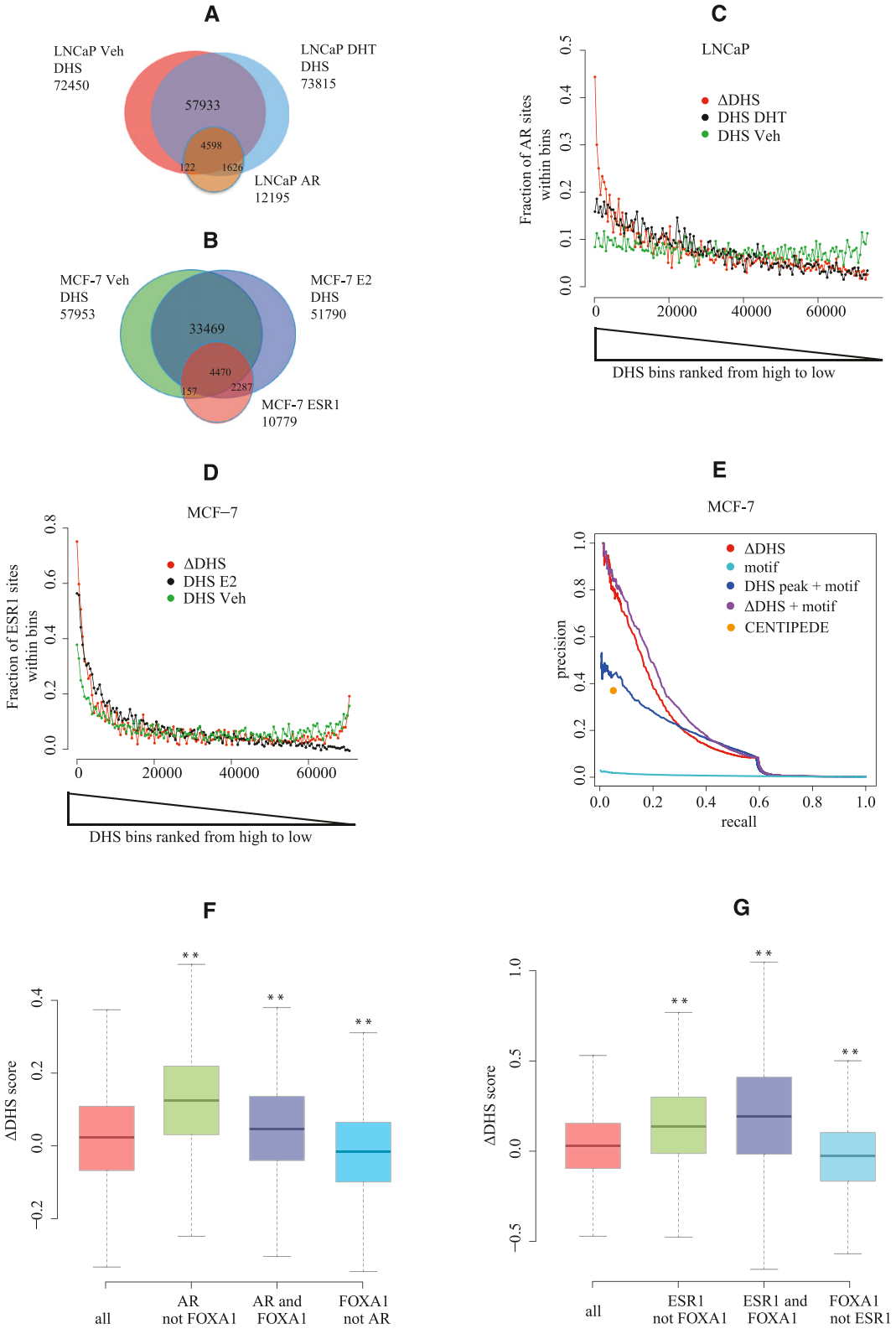
(TSS) (Fig. 4A, red bars). In contrast, there is no positive association between hormone-unchanged or -diminished DHS sites with increased gene expression (Fig. 4A, blue and green bars).

We have previously shown using ESR1 ChIP-chip and gene expression microarrays in MCF-7 that early up-regulated genes, which increased after 3 h of hormone stimulation, are strongly associated



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with ESR1 binding, whereas the early down-regulated genes are not (Carroll et al. 2006). These findings were confirmed by Hah and colleagues using GRO-seq (Hah et al. 2011). Interestingly, we find a strong association of early down-regulated genes with the hormone-diminished DHS sites (Fig. 4B, green bars). Motif analysis shows that, while the hormone-induced DHS regions are enriched for motifs for ESR1, forkhead and AP-1, the hormone-diminished DHS sites are enriched primarily for the forkhead motif and not the ESR1 motif (Table 1). We confirmed that FOXA1 binding is enriched at the sites with both the highest and lowest ΔDHS using FOXA1 ChIP-seq data (Fig. 5A). Interestingly, the FOXA1 sites lacking ESR1 are only strongly associated with the sites with the lowest ΔDHS (Fig. 5B). One explanation for these findings would be that, at sites where FOXA1 is bound in the absence of ESR1, FOXA1 binding is reduced upon estrogen stimulation.

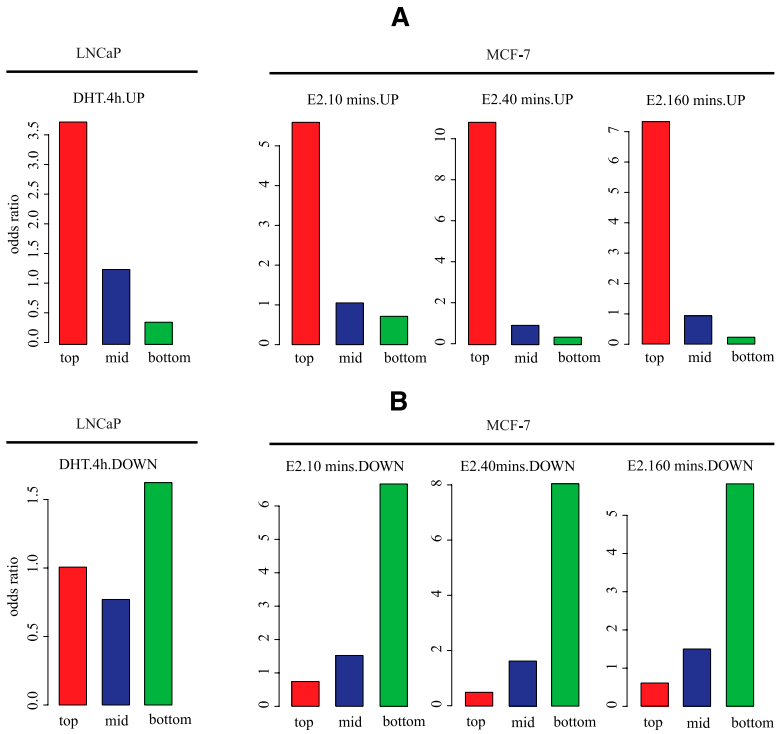

Figure 4. Association between dynamic DNase-seq and differentially expressed genes. Three groups of DHS are represented in LNCaP and MCF-7 cells: hormone-induced DHS sites (red); hormone-unchanged DHS sites (blue); and hormone-diminished DHS sites (green). (*Y*-axis) Odds ratio calculated by the following formula: (up-regulated genes with at least one nearby site/non-regulated genes with at least one nearby site)/(up-regulated genes with no nearby site/non-regulated genes with no nearby site). In this definition, "nearby" means within 20 kb of the TSS. The hormone-induced sites are associated with up-regulated genes (*A*), while the hormone-depleted sites are associated with down-regulated genes (*B*).

To investigate whether FOXA1 sites without ESR1 binding have reduced enrichment upon stimulation, we compared the FOXA1 ChIP-seq reads under vehicle and stimulated conditions (Joseph et al. 2010) within the three categories of 5000 DHS sites (Fig. 5C). Starting with DHS regions detected in the E2-stimulated condition we counted the number of FOXA1 tags obtained from ChIP-seq in unstimulated and E2-stimulated conditions. If we restrict the set of DHS regions to include only the middle 5000 hormone-unchanged regions and plot the FOXA1 tag count for the stimulated condition as a function of that for the unstimulated condition, we see a linear trend, represented by the blue regression line in Figure 5C. In a similar way if we select the top 5000 hormone-increased DHS sites, we again see a linear trend but the slope of the regression line (red) for this trend is greater. This indicates that there is more hormone-stimulated FOXA1 binding in the hormone-increased set than in the hormone-unchanged set. If we select the top 5000 hormone-diminished sites and plot a regression line (green), we see the slope of the regression line through the hormone-diminished set is not significantly lower than that of the hormone-unchanged set. A reduction in FOXA1 binding does not, therefore, appear to explain the decrease in DHS.

Physiological squelching (Meyer et al. 1989) has been postulated to be an important mode of early estrogen down-regulation (Carroll et al. 2006). This phenomenon occurs when multiple factors in the same cell share a common factor, such as a coactivator that is present at a limiting concentration. The transcription factors interfere with each other, "squelching" each other's influence. Of the numerous known ESR1 coactivators, NCOA3 has been shown to have a particularly strong synergy with ESR1 in enhancing gene expression (Torchia et al. 1997). As with FOXA1, NCOA3 binding was associated with both the highest and lowest ΔDHS sites overall and with only the lowest ΔDHS sites at loci lacking ESR1 (Supplemental Fig. 8).

Figure 3. Characteristics of DNase I hypersensitivity sequencing. (*A*) Venn diagram of the DHS and AR peaks in LNCaP. The DNase-seq sequencing depth was normalized to the lower sequencing depth for the unstimulated (50 M) and androgen-stimulated (70 M) conditions. (*B*) Venn diagram of the DHS and ESR1 peaks in MCF-7. DNase-seq sequencing depth was normalized to the lower sequencing depth of the unstimulated (28 M) and estrogen-stimulated (70 M) conditions. (*C*,*D*) The fraction of LNCaP AR (*C*) or MCF-7 ESR1 (*D*) binding sites in bins ranked by three measures: DNase-seq tag counts in stimulated and unstimulated conditions and a score, ΔDHS, representing the change in DNase I hypersensitivity between the two conditions. The DNase-seq peak regions under the stimulated condition are ranked by these measures. To calculate the proportion of real binding sites as a function of rank, these ranked regions are grouped into bins of 500. (*Y*-axis) Fraction of regions in each bin that overlap with AR (*C*) or ESR1 (*D*) ChIP-seq enriched regions. (*E*) The precision-recall curves for prediction power of MCF-7 ESR1 binding sites were calculated by five measures: ΔDHS, ESR1 motif, ESR1 motif in E2 DHS, sqrt([ΔDHS rank]*[motif rank]), and results generated by the CENTIPEDE algorithm on ENCODE MCF-7 DNase-seq data (see Methods). (*F*,*G*) Box plots showing the distribution of the DNase-seq change (ΔDHS) between the unstimulated and stimulated conditions in LNCaP (*F*) and MCF-7 (*G*) cells. "All" represents all the DHS sites in MCF-7 and LNCaP; "AR not FOXA1" and "ESR1 not FOXA1" represent AR and ESR1 binding sites that do not overlap with FOXA1; "AR and FOXA1" and "ESR1 and FOXA1" represent AR and ESR1 binding sites that overlap with FOXA1; "FOXA1 not AR" and "FOXA1 not ESR1" represent FOXA1 binding sites that do not overlap with AR and ESR1. (**) Wilcoxon rank-sum test *P*-values <0.01, comparing "all" with the other categories.

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Table 1. Top 20 motifs enriched in the top 5000 and bottom 5000 MCF-7 ΔDHS regions

Top 5000 ΔDHS				Bottom 5000 ΔDHS			
Motif ID	Gene symbol	Number of hits	*P*-value	Motif ID	Gene symbol	Number of hits	*P*-value
M00959	*ESR1*	2002	1.00×10^{-30}	M00724	*FOXA1*	3806	1.00×10^{-30}
M00515	*PPARG*	997	1.00×10^{-30}	M00131	*FOXA2*	3782	1.00×10^{-30}
M00925	*JUN/FOS*	1557	1.00×10^{-30}	M01012	*FOXM1*	4283	1.00×10^{-30}
M00156	*RORA*	2797	1.00×10^{-30}	M00269	*FOXA2*	4803	1.00×10^{-30}
M00037	*NFE2*	1696	1.00×10^{-30}	M00292	*FOXD1*	3250	1.00×10^{-30}
M00285	*NFE2L1*	4634	1.00×10^{-30}	M00422	*FOXJ2*	4171	1.00×10^{-30}
M00495	*BACH1*	972	1.00×10^{-30}	M00290	*FOXF2*	4927	1.00×10^{-30}
M00490	*BACH2*	875	1.00×10^{-30}	M00291	*FOXC1*	4891	1.00×10^{-30}
M00239	*NR1D1*	1531	1.00×10^{-30}	M00289	*FOXI1*	4747	1.00×10^{-30}
M00727	*SF1*	4133	1.00×10^{-30}	M00266	*CROCC*	4693	1.00×10^{-30}
M00511	*SLC7A1*	3291	1.00×10^{-30}	M00268	*XFD2*	4962	1.00×10^{-30}
M01138	*ROR1*	3723	1.00×10^{-30}	M01137	*FOXO3*	4496	1.00×10^{-30}
M00292	*FOXD1*	4559	1.00×10^{-30}	M00809	*FOX* factors	3571	1.00×10^{-30}
M00157	*ROR2*	1568	1.00×10^{-30}	M00472	*FOXO4*	4543	1.00×10^{-30}
M00204	*GCN4*	808	1.00×10^{-30}	M00742	*FOXJ1*	4768	1.00×10^{-30}
M00821	*NFE2L2*	2809	1.00×10^{-30}	M00267	*XFD1*	4583	1.00×10^{-30}
M00035	*MAF*	2896	1.00×10^{-30}	M00951	*GRHL3*	3561	1.00×10^{-30}
M00269	*FOXA2*	2247	2.06×10^{-27}	M00294	*FOXF1*	4859	1.00×10^{-30}
M00724	*FOXA1*	4452	1.34×10^{-25}	M00475	*FOXO3*	4259	1.00×10^{-30}
M00983	*MAF*	949	2.89×10^{-25}	M00473	*FOXO1*	4800	1.00×10^{-30}

Using published MCF-7 NCOA3 ChIP-seq data (Joseph et al. 2010; Lanz et al. 2010), we compared NCOA3 and FOXA1 cistromes, finding 61% of FOXA1 binding sites overlap with NCOA3 (Supplemental Fig. 9). Analyzing the three categories of DHS sites using this NCOA3 ChIP-seq data, we found that NCOA3 binding associated with hormone-diminished DHS loci was distributed in a clearly distinct pattern from the hormone-unchanged sites (Fig. 5D). The slope of the regression line of the hormone-diminished set was significantly lower than that of the hormone-unchanged set (Fig. 5D). As ESR1 directly interacts with NCOA3, these data support the hypothesis that ESR1 competes with FOXA1 for limited amounts of NCOA3 that are either directly associated with FOXA1 or associated with other transcription factors whose binding is facilitated by FOXA1.

If physiological squelching is responsible for the E2-stimulated loss of NCOA3 at FOXA1 binding sites, then higher concentrations of NCOA3 in the nucleus should result in a reduced E2-stimulated NCOA3 loss. We tested this by overexpressing NCOA3 (Supplemental Fig. 10A), selecting six FOXA1 non-ESR1 binding sites from hormone-diminished DHS and determining NCOA3 and FOXA1 binding strength by ChIP-qPCR (Supplemental Fig. 10). The control confirms what we found in the ChIP-seq data: FOXA1 binding does not significantly change on E2 stimulation and there is NCOA3 loss (Fig. 5E). In the NCOA3 overexpression experiment, however, we find no significant change in either FOXA1 or NCOA3 binding on E2 stimulation (Fig. 5F). We also examined the effect of NCOA3 overexpression on the expression of five genes downregulated by estrogen and found that NCOA3 overexpression reduced the extent of these expression changes (Supplemental Fig. 11). These results are consistent with the physiological squelching mechanism in which E2-induced ESR1 binding sites compete with FOXA1 sites for the NCOA3 coregulator.

Discussion

Using genome-wide DNase-seq and H3K4me2 ChIP-seq analyses, we have mapped important features of enhancer-associated chromatin. We observed systematic differences in nucleosome occupancy patterns and DHS associated with different transcription factors in LNCaP and MCF-7 cell lines. While AR binding in LNCaP cells has large effects on nucleosome occupancy, ESR1 binding in MCF-7 cells is not strongly influenced by, nor does it influence, nucleosome occupancy. In LNCaP cells, it has been reported that a knockdown of FOXA1 expression causes a dramatic change in AR binding locations, including the gain of numerous sites that are not observed under normal FOXA1 conditions (Wang et al. 2011). Notably, these new AR binding sites were not associated with observable nucleosome remodeling but were more like the ESR1 binding we observed in MCF-7 cells.

Thermodynamic equilibrium has been proposed to explain experimentally observed genome-wide in vivo nucleosome occupancy patterns. In this model, both nucleosomes and transcription factors have an intrinsic affinity for DNA sequence that is dependent on sequence composition (Segal and Widom 2009). Transcription factors compete with nucleosomes for DNA, and thermodynamic equilibrium determines the configuration of nucleosomes and transcription factors. In addition, nucleosome occupancy is likely to be shaped by kinetic elements, in particular, chromatin-remodeling factors using the energy derived from ATP hydrolysis to actively modify DNA-histone interactions. The importance of ATP-dependent factors was demonstrated in a recent study that showed that ATP is essential for creating the strongly positioned nucleosome arrays observed near TSSs in *Saccharomyces* (Zhang et al. 2011). Experimental evidence shows that different chromatin remodeling enzymes are recruited to enhancer loci by sequence-specific transcription factors (Peterson and Workman 2000), such as nuclear receptors. For example, BRG-1, the active component of human SWI/SNF chromatin-remodeling complexes, has been shown to be a key factor that potentiates AR- and ESR1-regulated transcription (DiRenzo et al. 2000; Dai et al. 2008). Both AR and ESR1 are known to interact directly with BAF57, a component of the SWI/SNF remodeling complexes (Belandia et al. 2002; Link et al. 2005). Several modes of chromatin remodeling have been suggested, including nucleosome sliding, nucleosome eviction, and looping of DNA away from the histone core. We speculate that the distinct mechanisms of the different classes

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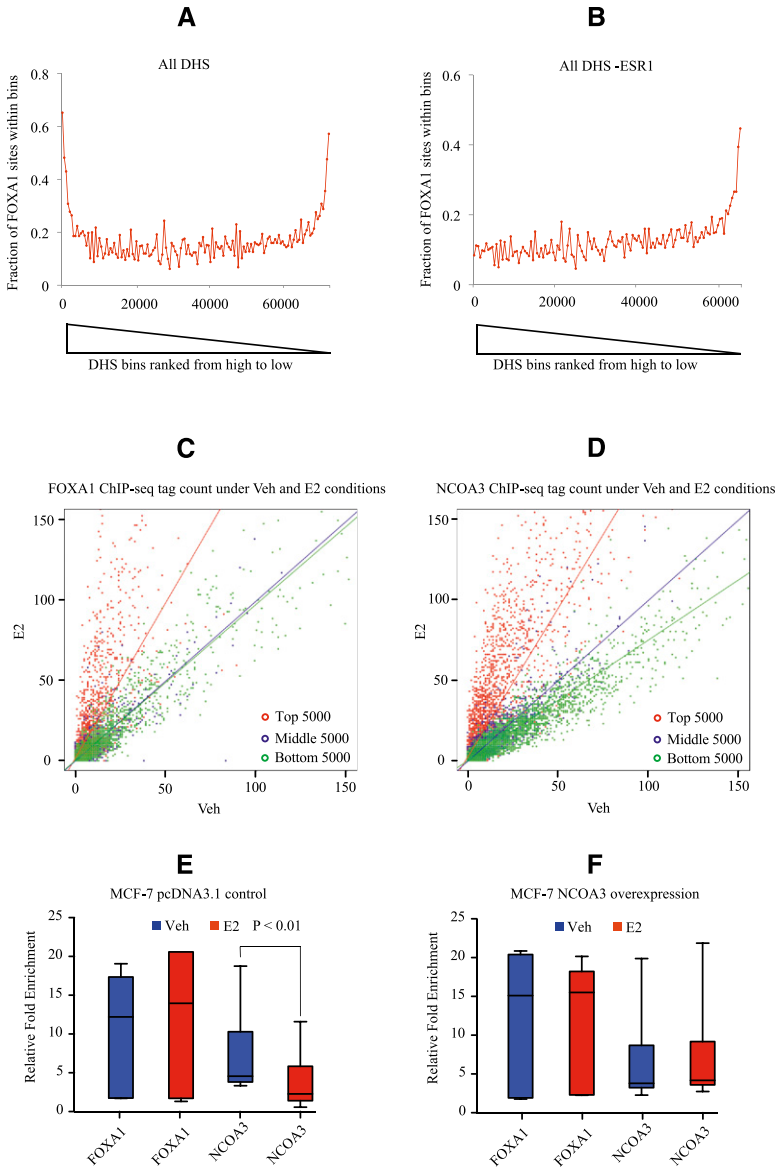

Figure 5. DNase I hypersensitivity changes at FOXA1 and NCOA3 sites. Association of ΔDHS with FOXA1 sites in the presence (*A*) and absence (*B*) of ESR1 binding. MCF-7 DHS in the estrogen-stimulated condition were ranked in descending order based on the ΔDHS score. These ranked regions are grouped into bins of 500. (*Y*-axis) Fraction of regions that overlap with FOXA1 ChIP-seq enriched regions. Scatter plots of FOXA1 (*C*) and NCOA3 (*D*) ChIP-seq tag counts in the stimulated condition compared with counts in the unstimulated condition. Three groups of 5000 DHS sites were selected from the MCF-7 estrogen-stimulated DHS sites: DHS-increased (red), DHS-unchanged (blue), and DHS-diminished (green). Regression lines were drawn for each of the groups. The steeper the slope of a regression line, the greater the binding of the factor in the E2-stimulated condition relative to the unstimulated condition. While the slope for FOXA1 in the DHS-diminished category is not significantly different from that in the DHS-unchanged category, the slope for NCOA3 in the DHS-diminished category is less than that for the DHS-unchanged category. This means that within the DHS-diminished category NCOA3 binding tends to decrease on E2 stimulation while FOXA1 binding is maintained at the same level. Changes of FOXA1 and NCOA3 binding strength at FOXA1 binding sites in the overexpression control (*E*) and NCOA3 overexpression (*F*) samples under stimulated and unstimulated conditions. Six FOXA1 binding sites were selected from the hormone-diminished DHS sites. Box plots were generated from the ChIP-qPCR data of the six sites tested. The individual ChIP-qPCR assays are shown in Supplemental Figure 10.

of ATP-dependent remodeling enzymes may explain the differential chromatin effects seen in our experiments. Our study demonstrates how MNase digestion and DHS chromatin assays provide complementary information on chromatin structure.

Our differential DNase I hypersensitivity experiments revealed a surprising link between coregulator and chromatin structure. Significantly, this link was not merely a consequence of FOXA1 binding itself. NCOA3 ChIP-seq in MCF-7 cells under vehicle and estrogen-induced conditions revealed that, although a high overlap between NCOA3 and ESR1 was observed, an unexpectedly high overlap between FOXA1 binding sites and NCOA3-enriched loci was also found (Lanz et al. 2010). Previously, coregulators and chromatin remodeling activity had been shown to act synergistically in the AR and ESR1 systems in collaboration with the AR and ESR1 factors themselves (Metivier et al. 2003; Wang et al. 2005). Here, we find evidence for a chromatin remodeling–coregulator synergy that is associated with FOXA1 in the absence of ESR1 or AR. Our experiment supports the hypothesis that physiological squelching is an important mechanism involved in the down-regulation of genes at early time points following estrogen treatment.

According to our current understanding, DNase I hypersensitivity occurs in nucleosome free regions that are close to transcription factor binding sites. Although we do observe many DHS in non-nucleosomal DNA, DHS sites sometimes occur in regions having high nucleosome occupancy. In particular, we identified a set of DHS sites that were associated with ESR1 binding to nucleosomal DNA. The different nucleosome occupancy and DNase I hypersensitivity patterns that we observed are likely dependent on not only the details of the transcription factor–DNA interaction but also on the chromatin environment at the binding site. Relevant aspects of the chromatin environment may include post-translational histone modifications, the composition of the nucleosomes themselves, and the presence of other protein complexes. Histone post-translational modifications may influence transcription factor binding by enhancing the affinity of transcription factor related protein complexes for the modified histone or by reducing the affinity of the histone octamer for DNA. The structure of the nucleosome cores may also determine nucleosomes as being more or less permissive to transcription factor binding as histones that constitute nucleosomes come in variants, such as H2A.Z, that have been reported to alter nucleosome properties (Jin et al. 2009).

In our analysis of genome-wide dynamic DNase-seq, we noted three important factors that contribute to DNase I hypersensitivity.

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First, in agreement with the standard view, the majority of DHS sites occur in nucleosome-free regions. Second, DHS frequently arises as a result of transcription factor binding; however, they do not necessarily occur in nucleosome-free regions. Third, DHS can change with the addition or removal of cofactors. We demonstrated that dynamic DNase-seq is an effective and informative approach that can be used to locate enhancers that regulate a cell's transcriptional response to stimuli.

Methods

Cell line and culture conditions

The prostate cancer cell line LNCaP was obtained from the American Type Culture Collection. LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. The hormone-independent breast cancer cell line MCF-7:2A and the parental MCF-7 cell line were from V. Craig Jordan's lab. MCF-7 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 1× NEAA, and 6 μg/L insulin. MCF-7:2A cells were maintained in phenol-red-free RPMI 1640 medium supplemented with 10% charcoal stripped FBS. LNCaP and MCF-7 cells were starved in phenol-red-free medium supplemented with 10% charcoal stripped FBS for 3 d before hormone stimulation.

ChIP and ChIP-seq

The ChIP experiments were performed as previously described (He et al. 2010). We used antibodies to ESR1 (Ab-10 from Neomarkers; HC-20 from Santa Cruz), AR (N-20 from Santa Cruz), FOXA1 (ab23738 from Abcam), and H3K4me2 (07-030 from Millipore). Library construction was performed using the Illumina ChIP-seq DNA sample Prep Kit according to the manufacture's instruction; the libraries were sequenced at a length of 35 bp with the Illumina Genome Analyzer. Model-based Analysis of ChIP-seq (MACS) software (Zhang et al. 2008a) was used to detect ChIP-seq peak regions. Nucleosome Positioning from Sequencing (NPS) software (Zhang et al. 2008b) was used to identify nucleosome positions based on the H3K4me2 ChIP-seq data. Binding Inference from Nucleosome Occupancy Changes (BINOCh) software (Meyer et al. 2011) was used to predict transcription factor binding events from the H3K4me2 NPS data.

DNase hypersensitivity mapping

DNase hypersensitivity mapping was performed as previously described with brief modifications (Ling et al. 2010; John et al. 2011). LNCaP cells were starved for 3 d in phenol-red-free medium supplemented with 10% charcoal stripped FBS and then treated with ethanol or active androgen 5α-dihydrotestosterone (DHT) at a final concentration of 10 nM for 4 h. MCF-7 cells were starved the same way and then treated with ethanol or 17β-estrodial (E2) at a final concentration of 10 nM for 45 min. The cells were trypsinized and pelleted prior to washing and resuspension in buffer A (15 mM Tris-Cl [pH 8.0], 15 mM NaCl, 60 mM KCl, 1 mM EDTA [pH 8.0], 0.5 mM EGTA [pH 8.0], 0.5 mM spermidine, and 0.15 mM spermine) to a final concentration of 2 × 10 M cells/mL. Nuclei were extracted by adding buffer A containing NP-40. The nuclei were washed with buffer A and resuspended in prewarmed lysis buffer (13.5 mM Tris-HCl [pH 8.0], 87 mM NaCl, 54 mM KCl, 6 mM CaCl2, 0.9 mM EDTA, 0.45 mM EGTA) at a concentration of 5 M/mL and then digested with different amounts of DNase I (Roche, 0–75 U) for 5 min at 37°C. The reactions were terminated by the addition of an equal volume of stop buffer (1 M Tris-Cl [pH 8.0], 5 M NaCl, 20% SDS, 0.5 M EDTA [pH 8.0], and 10 μg/mL of RNase A [Roche]) and incubated at 55°C. After 15 min, Proteinase K (final concentration of 20 μg/mL) was added to each digestion reaction and incubated for 2 h at 55°C. DNA was extracted by careful phenol-chloroform purification. The isolated DNA was run out on a gel, and DNA fragments between 100 and 400 bp long were gel-selected. The libraries were prepared following the Illumina library preparation protocol. DNase-seq libraries were sequenced at the Beijing Genomic Institute and the Center for Cancer Computational Biology (CCCB) at the Dana-Farber Cancer Institute.

NCOA3 overexpression experiments

A total of 12 μg of pcDNA3.1–NCOA3 construct or the control empty vector were transfected in MCF-7 cells in 10-cm culture dishes using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 72 h of transfection, cells were treated with estrogen or ethanol control for 45 min and then processed for ChIP-qPCR. For RT-qPCR, 3 μg of the pcDNA3.1–NCOA3 or the empty vector were transfected in MCF-7 cells in six-well plates. After 72 h of transfection, cells were treated with estrogen or ethanol control for 3 h. RNA was isolated using RNeasy mini kit (Qiagen) following the manufacturer's instructions. PCR primers used in this work are listed in Supplemental Table 1.

Model for identifying differential DNase I hypersensitivity locations

DNase I hypersensitive regions were identified using MACS with the default parameters. A tag was considered to belong to a genomic interval if, when shifted 100 bp in a strand-directed direction, the entire tag fell within that interval. Each peak *i* from the set of *m* MACS peaks was then given a DHS change score (ΔDHS) by the formula:

$$\Delta DHS_i = \sqrt{n_i^{treat} \Big/ \left(\sum_{k=1}^{m} n_k^{treat}\right)/m} - \sqrt{n_i^{control} \Big/ \left(\sum_{k=1}^{m} n_k^{control}\right)/m}.$$

In this formula, n_i is the tag count in a 600-bp interval centered on the *i*-th MACS peak. The superscripts *treat* and *control* refer to the hormone-stimulated and vehicle conditions, respectively. We use the square root transformation to stabilize the variance of the score, allowing regions with high counts to be compared with those having low counts. Peaks within 1 kb of any RefSeq TSS were excluded from all analyses so as not to confound transcription factor binding effects with transcriptional ones. All analyses involving motifs enriched in the peak regions were identified using the BINOCh motif analysis software.

Precision recall analysis

To evaluate the ability of our method to predict TF binding we defined a set of bound and unbound genomic locations. We defined the bound set as the summits of MACS peaks determined from ChIP-seq data and located >1 kb from the nearest RefSeq TSS. To define the unbound set, we downloaded a file of "mappable" genomic locations, "wgEncodeCrgMapabilityAlign50mer.bw.gz" from http://hgdownload.cse.ucsc.edu/goldenPath/hg18/encodeDCC/wgEncodeMapability/ and selected a set of 850,000 non-bound, non-TSS sites by randomly sampling genomic locations that had a mappability index >0.9. These locations were filtered to not lie within 1 kb of any RefSeq TSS, TF ChIP-seq summit or other random location. The background was then scaled up to cover 2 Gb,

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the size of the mappable genome. A DHS or ChIP-seq region was considered to be a true positive if its center was within 250 bp of a TF summit and a false positive if its center was within 250 bp of a background site. For motif analysis 200 bp from the center of the DHS or ChIP-seq region was scanned using the BINOCh software (Meyer et al. 2011). CENTIPEDE predictions (Pique-Regi et al. 2011) for ESR1 binding in MCF-7 were downloaded from http://centipede.uchicago.edu/SimpleMulti/. In the performance evaluation CENTIPEDE predictions were treated the same way as our DHS regions. Since the result we retrieved from the website contains no scoring information for the sites predicted by CENTIPEDE, a single point was drawn for the performance evaluation.

DHS boxplots

Tag counting under DHS peaks was carried out as before. Peaks were considered to be overlapping if their summits were within 600 bp of each other. Box plots were produced using R with default parameters. The outliers beyond the whiskers are not shown. The *P*-values were calculated using the Mann-Whitney test.

Gene expression data

Affymetrix U133 Plus 2.0 microarray data (GSE7868) (Wang et al. 2007) in LNCaP cells and the processed GRO-seq gene expression data (GSE27463) (Hah et al. 2011) in MCF-7 cells were used in this study. The microarray data were analyzed using the RMA algorithm (Irizarry et al. 2003) using a custom CDF probe (v11) mapping to the RefSeq genes (Dai et al. 2005). The statistical significance was calculated using limma software (Smyth and Speed 2003).

Data access

MCF-7 H3K4me2 ChIP-seq, LNCaP, and MCF-7 DNase-seq raw sequence tags, and processed bed files have been submitted to the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE33216.

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Author contributions: H.H.H., C.A.M., X.S.L., and M.B. designed the experiments and wrote the manuscript. V.C.J. provided the hormone-resistant breast cancer cells and revised the manuscript. H.H.H. and M.W.C. performed the experiments. C.A.M. and H.H.H. conducted the data analysis.

References

Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, Tapia C, Kim HR, Yerrum S, Sharma CGN, et al. 2011. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *Proc Natl Acad Sci* **108:** 18879–18886.

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. 2007. High-resolution profiling of histone methylations in the human genome. *Cell* **129:** 823–837.

Belandia B, Orford RL, Hurst HC, Parker MG. 2002. Targeting of SWI/SNF chromatin remodelling complexes to estrogen-responsive genes. *EMBO J* **21:** 4094–4103.

Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoute J, Shao W, Hestermann EV, Geistlinger TR, et al. 2005. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* **122:** 33–43.

Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J, Brodsky AS, Keeton EK, Fertuck KC, Hall GF, et al. 2006. Genome-wide analysis of estrogen receptor binding sites. *Nat Genet* **38:** 1289–1297.

Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, Bunney WE, Myers RM, Speed TP, Akil H, et al. 2005. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. *Nucleic Acids Res* **33:** e175. doi: 10.1093/nar/gni179.

Dai Y, Ngo D, Jacob J, Forman LW, Faller DV. 2008. Prohibitin and the SWI/SNF ATPase subunit BRG1 are required for effective androgen antagonist-mediated transcriptional repression of androgen receptor-regulated genes. *Carcinogenesis* **29:** 1725–1733.

DiRenzo J, Shang Y, Phelan M, Sif S, Myers M, Kingston R, Brown M. 2000. BRG-1 is recruited to estrogen-responsive promoters and cooperates with factors involved in histone acetylation. *Mol Cell Biol* **20:** 7541–7549.

Hah N, Danko CG, Core L, Waterfall JJ, Siepel A, Lis JT, Kraus WL. 2011. A rapid, extensive, and transient transcriptional response to estrogen signaling in breast cancer cells. *Cell* **145:** 622–634.

He HH, Meyer CA, Shin H, Bailey ST, Wei G, Wang Q, Zhang Y, Xu K, Ni M, Lupien M, et al. 2010. Nucleosome dynamics define transcriptional enhancers. *Nat Genet* **42:** 343–347.

Hu G, Schones DE, Cui K, Ybarra R, Northrup D, Tang Q, Gattinoni L, Restifo NP, Huang S, Zhao K. 2011. Regulation of nucleosome landscape and transcription factor targeting at tissue-specific enhancers by BRG1. *Genome Res* **21:** 1650–1658.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4:** 249–264.

Jin C, Zang C, Wei G, Cui K, Peng W, Zhao K, Felsenfeld G. 2009. H3.3/H2A.Z double variant-containing nucleosomes mark 'nucleosome-free regions' of active promoters and other regulatory regions. *Nat Genet* **41:** 941–945.

John S, Sabo PJ, Thurman RE, Sung MH, Biddie SC, Johnson TA, Hager GL, Stamatoyannopoulos JA. 2011. Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. *Nat Genet* **43:** 264–268.

Joseph R, Orlov YL, Huss M, Sun W, Kong SL, Ukil L, Pan YF, Li G, Lim M, Thomsen JS, et al. 2010. Integrative model of genomic factors for determining binding site selection by estrogen receptor-α. *Mol Syst Biol* **6:** 456. doi: 10.1038/msb.2010.109.

Lanz RB, Bulynko Y, Malovannaya A, Labhart P, Wang L, Li W, Qin J, Harper M, O'Malley BW. 2010. Global characterization of transcriptional impact of the SRC-3 coregulator. *Mol Endocrinol* **24:** 859–872.

Ling G, Sugathan A, Mazor T, Fraenkel E, Waxman DJ. 2010. Unbiased, genome-wide *in vivo* mapping of transcriptional regulatory elements reveals sex differences in chromatin structure associated with sex-specific liver gene expression. *Mol Cell Biol* **30:** 5531–5544.

Link KA, Burd CJ, Williams E, Marshall T, Rosson G, Henry E, Weissman B, Knudsen KE. 2005. BAF57 governs androgen receptor action and androgen-dependent proliferation through SWI/SNF. *Mol Cell Biol* **25:** 2200–2215.

Lupien M, Eeckhoute J, Meyer CA, Wang Q, Zhang Y, Li W, Carroll JS, Liu XS, Brown M. 2008. FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell* **132:** 958–970.

Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, Gannon F. 2003. Estrogen receptor-α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* **115:** 751–763.

Meyer ME, Gronemeyer H, Turcotte B, Bocquel MT, Tasset D, Chambon P. 1989. Steroid hormone receptors compete for factors that mediate their enhancer function. *Cell* **57:** 433–442.

Meyer CA, He HH, Brown M, Liu XS. 2011. BINOCh: Binding inference from nucleosome occupancy changes. *Bioinformatics* **27:** 1867–1868.

Peterson CL, Workman JL. 2000. Promoter targeting and chromatin remodeling by the SWI/SNF complex. *Curr Opin Genet Dev* **10:** 187–192.

Pink JJ, Jiang SY, Fritsch M, Jordan VC. 1995. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* **55:** 2583–2590.

Pique-Regi R, Degner JF, Pai AA, Gaffney DJ, Gilad Y, Pritchard JK. 2011. Accurate inference of transcription factor binding from DNA sequence and chromatin accessibility data. *Genome Res* **21:** 447–455.

Segal E, Widom J. 2009. Poly(dA:dT) tracts: Major determinants of nucleosome organization. *Curr Opin Struct Biol* **19:** 65–71.

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Siersbaek R, Nielsen R, John S, Sung MH, Baek S, Loft A, Hager GL, Mandrup S. 2011. Extensive chromatin remodelling and establishment of transcription factor 'hotspots' during early adipogenesis. *EMBO J* **30:** 1459–1472.

Smyth GK, Speed T. 2003. Normalization of cDNA microarray data. *Methods* **31:** 265–273.

Song L, Zhang Z, Grasfeder LL, Boyle AP, Giresi PG, Lee BK, Sheffield NC, Graf S, Huss M, Keefe D, et al. 2011. Open chromatin defined by DNaseI and FAIRE identifies regulatory elements that shape cell-type identity. *Genome Res* **21:** 1757–1767.

Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, Rosenfeld MG. 1997. The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* **387:** 677–684.

Verzi MP, Shin H, He HH, Sulahian R, Meyer CA, Montgomery RK, Fleet JC, Brown M, Liu XS, Shivdasani RA. 2010. Differentiation-specific histone modifications reveal dynamic chromatin interactions and partners for the intestinal transcription factor CDX2. *Dev Cell* **19:** 713–726.

Wang Q, Carroll JS, Brown M. 2005. Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. *Mol Cell* **19:** 631–642.

Wang Q, Li W, Liu XS, Carroll JS, Janne OA, Keeton EK, Chinnaiyan AM, Pienta KJ, Brown M. 2007. A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. *Mol Cell* **27:** 380–392.

Wang Q, Li W, Zhang Y, Yuan X, Xu K, Yu J, Chen Z, Beroukhim R, Wang H, Lupien M, et al. 2009. Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. *Cell* **138:** 245–256.

Wang D, Garcia-Bassets I, Benner C, Li W, Su X, Zhou Y, Qiu J, Liu W, Kaikkonen MU, Ohgi KA, et al. 2011. Reprogramming transcription by distinct classes of enhancers functionally defined by eRNA. *Nature* **474:** 390–394.

Wu C. 1980. The 5′ ends of *Drosophila* heat shock genes in chromatin are hypersensitive to DNase I. *Nature* **286:** 854–860.

Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nussbaum C, Myers RM, Brown M, Li W, et al. 2008a. Model-based analysis of ChIP-Seq (MACS). *Genome Biol* **9:** R137. doi: 10.1186/gb-2008-9-9-r137.

Zhang Y, Shin H, Song JS, Lei Y, Liu XS. 2008b. Identifying positioned nucleosomes with epigenetic marks in human from ChIP-Seq. *BMC Genomics* **9:** 537. doi: 10.1186/1471-2164-9-537.

Zhang Z, Wippo CJ, Wal M, Ward E, Korber P, Pugh BF. 2011. A packing mechanism for nucleosome organization reconstituted across a eukaryotic genome. *Science* **332:** 977–980.

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Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines

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Abstract ***Purpose:*** c-Src is an important adapter protein with oestrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2), which validates it as an attractive target for the treatment of breast cancer. A specific c-Src inhibitor, 4-amino-5-(4-chlorophenyl)-7 (t-butyl)pyrazolo[3,4-d]pyrinidine (PP2), was utilised to block c-Src activity to identify targeted vulnerabilities affected by ER and HER2 in a panel of breast cancer cell lines.
Methods: ER, growth factor receptors and signalling pathways were detected by Western-blot. The DNA content of the cells was determined by using a DNA fluorescence quantitation kit. Cell cycles were analysed by flow cytometry.
Results: The antiproliferative effect of PP2 closely correlated with the inhibition of c-Src mediated extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and/or phosphoinositide 3-kinase (PI3K)/Akt growth pathways. Inhibition of c-Src tyrosine kinase predominantly blocked ER negative breast cancer cell growth, particularly the triple (i.e. ER, progesteron receptor (PR), and HER2) negative cells. In contrast, ER negative Sk-Br-3 cells with highest HER2 phosphorylation were resistant to PP2, in which hyper-activated HER2 directly regulated growth pathways. However, blocking c-Src recovered ER expression and down-regulated HER2 which made Sk-Br-3 cells regain responsiveness to 4-hydroxytamoxifen. The majority of ER positive cells were not sensitive to PP2 regardless of wild-type or endocrine resistant cell lines.
Conclusions: c-Src mediates the essential role of growth pathways in ER negative breast cancer cells. The ER positive and HER2 over-activation are two important predictive biomarkers for the resistance to a c-Src inhibitor. These data provided an important therapeutic rationale for patient selection in clinical trials with c-Src inhibitors in breast cancer.
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1. Introduction

Targeting oestrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) are two successful therapies in the treatment of breast cancer patients expressing relevant target molecules.[1,2] c-Src is a ubiquitously expressed intracellular tyrosine kinase that regulates protein–protein interactions and participates as a convergence point in different signalling pathways.[3] c-Src functions as an important adapter protein between ER and receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR) and HER2 in breast cancer.[4–6] In this regard, c-Src acts as a critical component of the signalling cascades initiated by ER and HER2 to activate the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt pathways,[6,7] both of which cause ER phosphorylation and ER-dependent gene transcription.[7]

Observations *in vitro* also support that multiple levels of association exist among ER, HER2 and c-Src in breast cancer. Targeting ER with tamoxifen increases c-Src activity which enhances cellular invasion and motility in breast cancer cells.[8,9] Furthermore, c-Src is shown to be critical in mediating tamoxifen resistance since blocking its activity reverses tamoxifen resistance.[10] A recent report indicates that c-Src is a common node downstream of multiple trastuzumab (targeting HER2) resistance pathways.[11] These observations highlight c-Src as an important therapeutic target for the treatment of human breast cancer.

Dasatinib, a potent oral inhibitor of c-Src family tyrosine kinase, is approved for clinical use in imatinib-resistant and -intolerant chronic myeloid leukaemia and solid tumour.[12,13] Preclinical studies in breast cancer cell lines have shown that basal like triple negative (i.e. ER, PR and HER2) breast cancer may have preferential sensitivity to the c-Src inhibitor.[14,15] Two parallel phase II monotherapy studies of dasatinib in breast cancer were initiated in different breast cancer subtypes. In patients with triple-negative breast cancer (TNBC), dasatinib has good tolerability and modest activity,[16] whereas dasatinib has limited single-agent activity in patients with HER2 positive and/or hormone receptors (HR) positive advanced breast cancer.[17] These findings imply that HR and HER2 may prevent the therapeutic effects of the c-Src inhibitor in breast cancer. Thus, there is a need to identify patients who are unlikely to respond to the c-Src inhibitor treatment. More importantly, factors that cause c-Src inhibitor resistance will serve as molecular targets to improve the action of c-Src inhibitors. Unfortunately, there is little understanding of resistance to the c-Src inhibitors in breast cancer cells. Chen et al.[18] have demonstrated that acquired resistance to AZD 0530 (a c-Src inhibitor) can be mediated through activation of mitogen-activated protein kinase kinase (MEK) and PI3K pathways thus these may prove to be future therapeutic targets to improve the c-Src inhibitor sensitivity.

The goal of this study is to identify biological markers of resistance to a c-Src inhibitor in a panel of wild-type and long-term oestrogen deprived breast cancer cell lines. We demonstrate that c-Src has an essential role in mediating the growth pathways of ER negative breast cancer cells. ER positive and HER2 over-activation reduce the responsiveness to the c-Src inhibitor. Indeed, c-Src controls oestrogen action in ER positive long-term oestrogen deprived resistant cells. Our data provide an important therapeutic rationale for patient selection in future clinical trials of c-Src inhibitors in breast cancer.

2. Materials and methods

2.1. Materials

The c-Src inhibitor PP2 was purchased from CalBiochem (San Diego, CA). Sources of antibodies for Western blot are as follows: ERα (sc-544) and PR (sc-810) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Total MAPK antibody (#9102), phosphorylation MAPK (#9101), total Akt (#9272), phosphorylated AktSer473 (#9271), phosphorylated c-SrcTyr416 (#2101L) antibodies and secondary antibodies conjugated with horseradish peroxidase (rabbit #7074, mouse #7076) were from Cell Signalling Technology (Beverly, MA). Phosphorylated HER2 Tyr1248 and total c-Src mouse (GD11) antibodies were from Millipore (Temecula, CA). Antibodies to HER2 (Ab18) and EGFR (Ab15) were from NeoMarkers (Fremont, CA).

2.2. Cells and culture conditions

Briefly, MCF-7:WS8 and T47D:A18 human mammary carcinoma cells, clonally selected from their parental counterparts for sensitivity to growth stimulation by E_2,[19] were used in all experiments indicating MCF-7 and T47D cells. ZR-75-1, BT474 and Sk-Br-3 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-231(10A) cells,[20] clonally selected from parental MDA-MB-231 cells (obtained from ATCC), were used in this study indicating MDA-MB-231 cells. MCF-7:5C and MCF-7:2A cells were cloned from E_2 deprived MCF-7 cells and maintained in E_2-free Roswell Park Memorial Institute (RPMI) medium which is phenol red-free RPMI 1640 supplemented with 10% dextran-coated charcoal-stripped foetal bovine serum (SFS).[21,22] T47D:C42 cells were cloned from E_2 deprived T47D cells and maintained in E_2-free RPMI 1640 medium.[23] Pure antioestrogen fulvestrant resistant cell line MCF-7/F was derived

from MCF-7 which was maintained in phenol red RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS).[24]

2.3. Cell proliferation assays

Cell DNA content was determined as a measure of cell proliferation using the Fluorescent DNA Quantitation Kit (Bio-Rad, Hercules, CA)[25]

2.4. Immunoblotting

Proteins were extracted in cell lysis buffer (Cell Signalling Technology, Beverly, MA) supplemented with Protease Inhibitor Cocktail (Roche, Indianapolis, IN) and Phosphatase Inhibitor Cocktail Set I and Set II (Calbiochem, San Diego, CA). Total protein content of the lysate was determined by a standard BCA assay using the reagent from Bio-Rad Laboratories (Hercules, CA). Fifty micrograms of total protein was separated on 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with primary antibodies followed by incubation with secondary antibody conjugated with HRP and reaction with Western Lighting™ plus-ECL enhanced chemiluminescent substrate (PerkinElmer Inc., Waltham MA). Protein bands were visualised by exposing the membrane to X-ray film.

2.5. Cell cycles analysis

Briefly, Sk-Br-3, BT474, T47D:C42 and MDA-MB-231 cells were cultured in dishes. They were treated with vehicle (0.1% dimethyl sulfoxide (DMSO)), lapatinib (1 μM) and PP2 (5 μM) for 24 h respectively. Cells were harvested and gradually fixed with 75% EtOH on ice. After staining with propidium iodide (PI), cells were analysed using a fluorescence-activated cell sorter (FACS) flow cytometer (Becton Dickinson, San Jose, CA), and the data were analysed with CellQuest software.

2.6. Quantitative real-time RT-PCR

Cells were harvested in TRIzol. Total RNA, isolated with an RNeasy Micro kit (Qiagen, Valencia, CA), was converted to first-strand cDNA using a kit from Applied Biosystem (Foster City, CA). Quantitative real-time polymerase chain reaction (RT-PCR) assays were done with the SYBR Green PCR Master Mixes (Applied Biosystems, Foster City, CA) and a 7900HT Fast Real-time PCR System (Applied Biosystems, Foster City, CA). The PUM1 forward primer was 5′-AATGCAGGCGCGAGAAAT-3′, PUM1 reverse primer was 5′-TTGTGCAGCTGAGGAACTAATGA-3′. The ERα forward primer was 5′-GGAGGGCAGGGGTGAA-3′, ERα reverse primer was 5′-GGCCAGGCTGTTCTTCTTAGA-3′. All the data were normalised by PUM1.

2.7. Statistical Analysis

All reported values are the means ± SE. Statistical comparisons were determined with two-tailed Student's *t* tests. Results were considered statistically significant if the *P* value was <0.05.

3. Results

3.1. Baseline levels of ER, HER2 and c-Src activation in a panel of breast cancer cell lines

We addressed the question whether expression of ER and growth factor receptors would affect the therapeutic effects of the c-Src inhibitors in breast cancer cells. To answer this question, a panel of wild-type (MCF-7, T47D, ZR-75-1, BT474, MDA-MB-231 and Sk-Br-3), long-term oestrogen deprived (MCF-7:5C, MCF-7:2A and T47D:C42) and pure antioestrogen ICI 182,780 resistant (MCF-7/F) breast cancer cell lines were investigated. Baseline levels of ER, HER2, EGFR and c-Src were measured by immunoblot analysis. They all keep their biological characteristics with differential levels of ER, PR, HER2 and EGFR (Supplementary Fig. S1A and S1B). All cell lines expressed detectable levels of total c-Src, whereas they manifested different levels of phosphorylated c-Src (Supplementary Fig. S1C). Although there is no clear relationship between c-Src phosphorylation and HR expression (Supplementary Fig. S1D) after being normalised by total c-Src among tested cell lines, interestingly, we observe that c-Src is activated in resistant cell lines compared with respective parental cell lines (MCF-7:5C, MCF-7:2A and MCF-7/F versus MCF-7, T47D:C42 versus T47D). The DNA fingerprinting pattern of all cell lines is consistent with the report by the ATCC (Supplementary Fig. S2).

3.2. Inhibitory effects of the c-Src inhibitor on ER positive wild-type breast cancer cells

All ER positive wild-type breast cancer cells were cultured in oestrogenised medium. The specific c-Src inhibitor, PP2, effectively blocked phosphorylation of c-Src in all cell lines (Fig. 1A). However, PP2 could not inhibit all cell growth (Fig. 1B). T47D and BT474 cells were responsive to PP2 with 50% and 40% inhibition after 7 days treatment, respectively (Fig. 1B), whereas MCF-7 and ZR-75-1 cells were resistant to PP2 treatment (Fig. 1B). Further investigation showed that antiproliferative effects of PP2 were correlated with inhibition of extracellular signal-regulated kinase/mitogen-activated protein kinase ERK/MAPK and/or phosphoinositide 3-kinase (PI3K)/Akt pathways. PP2 could not continuously block growth pathways in resistant

cells such as MCF-7 and ZR-75-1 (Fig. 1C). In contrast, PP2 effectively inhibited both signalling pathways in T47D and BT474 cells (Fig. 1C).

3.3. Inhibitory effects of the c-Src inhibitor varied under conditions with or without basal E_2 in ER positive wild-type breast cancer cells

Since basal oestrogen levels in the culture medium affect the biological function of the ER positive wild-type breast cancer cells[19] (Supplementary Fig. S3), we investigated inhibitory effects of the c-Src inhibitor on ER positive wild-type cells under conditions with (10% FBS) or without (10% SFS) basal oestrogen. Two distinct modulations of c-Src phosphorylation existed in ER positive wild-type cells after short-term absence of E_2. MCF-7 and ZR-75-1 cells had the same pattern with enhanced c-Src phosphorylation, conversely, c-Src phosphorylation was down-regulated in T47D and BT474 cells (Fig. 2A). The PP2 effectively blocked c-Src phosphorylation in four wild-type breast cancer cells under conditions with 10% SFS (Fig. 2B). However, inhibition by PP2 varied in ER positive wild-type cells under these two conditions (Fig. 2C). MCF-7 cells were effectively responsive to PP2 under conditions without basal E_2 (10% SFS), conversely, T47D cells were completely resistant to PP2 in phenol red free medium (Fig. 2C). Four ER positive wild-type breast cancer cells were stimulated by E_2 to grow with different sensitivity (Fig. 2D). Notably, PP2 could not block the proliferation induced by E_2 in MCF-7 and ZR-75-1 cells but partially abolished E_2 stimulation in T47D and BT474 cells (Fig. 2D). These results indicated that c-Src might play a distinct role in mediating E_2 signalling in wild-type cells.[4,26]

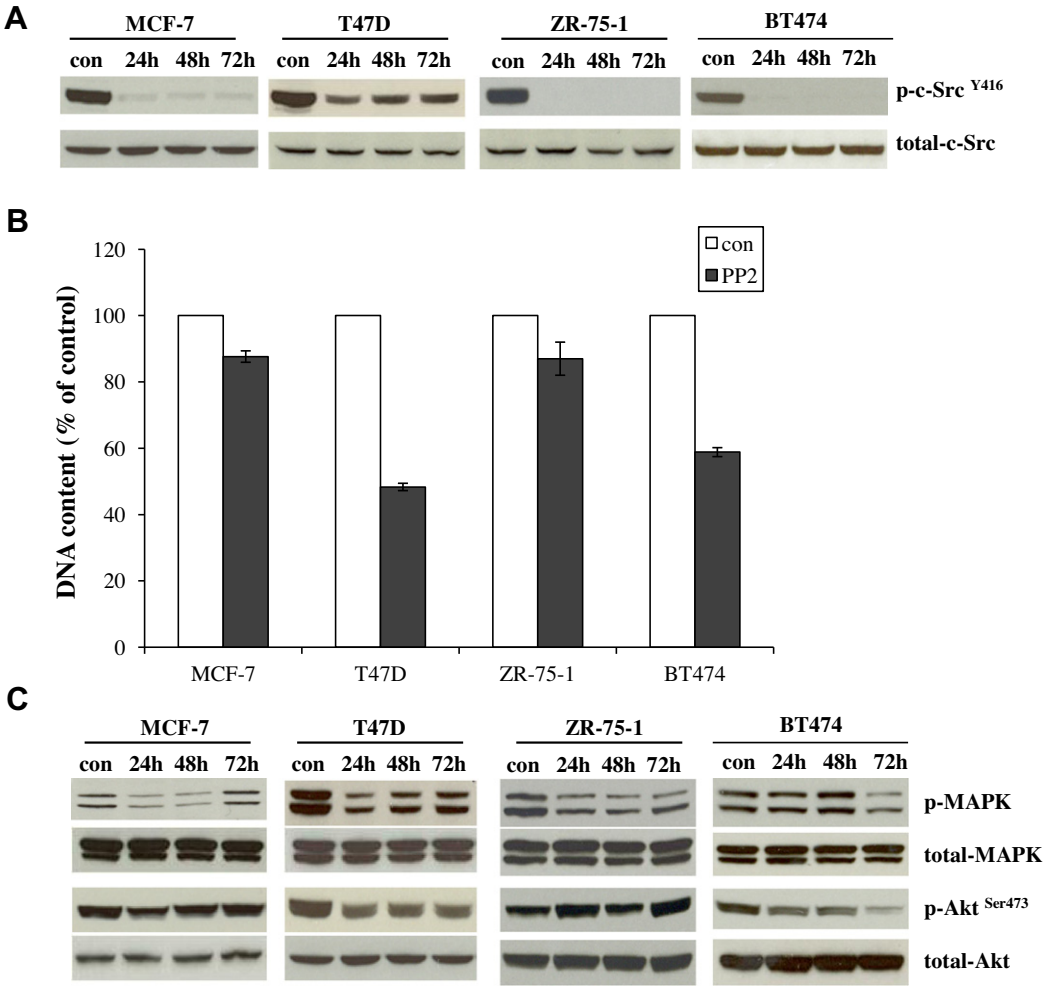

Fig. 1. Effects of the c-Src inhibitor on oestrogen receptor (ER) positive wild-type cell lines. (A) Blocking c-Src phosphorylation in ER positive wild-type cell lines by PP2. ER positive wild-type cells were treated with PP2 (5 μM) in oestrogenised medium at time points as indicated and cell lysates were harvested. Phosphorylated c-Src was detected by immunoblotting with primary antibody. Immunoblotting for total c-Src was used for loading control. (B) Inhibitory effects of PP2 on wild-type ER positive cells. Wild-type ER positive cells were seeded in 24-well plates in triplicate in oestrogenised medium. After 1 day, the cells were treated with vehicle (0.1% DMSO) and PP2 (5 μM) respectively. The cells were harvested after 7 days treatment and total DNA was determined using a DNA fluorescence quantitation kit. (C) Signalling pathways change in ER positive wild-type cells after PP2 treatment. Cell lysates were harvested as above. Phosphorylated mitogen-activated protein kinase (MAPK) and Akt were examined by immunoblotting with primary antibodies. Immunoblotting for total MAPK and Akt was used for loading controls.

3.4. Effects of the c-Src inhibitor on ER positive long-term oestrogen deprived breast cancer cells

In two long-term oestrogen deprived breast cancer cells (MCF-7:5C and MCF-7:2A), that overexpress ER, PP2 could block c-Src activation (Fig. 3A) and abolished about 25% of proliferation in MCF-7:5C cells but without any inhibition in MCF-7:2A cells (Fig. 3B). The inhibitory effects of PP2 were consistent with blocking growth pathways in different cells. Phosphorylated Akt was abolished in MCF-7:5C cells but without continuous inhibition of MAPK. PP2 could not continuously block both growth pathways in MCF-7:2A cells (Fig. 3C). Our previous data showed that E_2 has therapeutic function to induce apoptosis in long-term E_2 deprived breast cancer cells.[25] We reasoned that a combination of PP2 with E_2 would enhance E_2-induced apoptosis. Surprisingly, PP2 did not enhance the growth inhibitory effects of E_2 on these two cell lines but blocked the growth inhibition induced by E_2 (Fig. 3D). These data implied that E_2-triggered apoptosis might be utilising c-Src tyrosine kinase as an important signalling pathway. We are currently investigating the mechanisms of how the c-Src inhibitor blocks E_2-triggered apoptosis.

3.5. The c-Src inhibitor effectively blocked ER negative breast cancer cell growth

The inhibitory effects of the c-Src inhibitor, PP2, on ER negative breast cancer cell lines were examined in two wild-type MDA-MB-231 and Sk-Br-3 and two resistant cell lines MCF-7/F (ICI 182,780 resistance) and T47D:C42 (long-term oestrogen deprived). PP2 blocked the phosphorylation of c-Src in all ER negative cells (Fig. 4A). However, the growth inhibitory effects of the c-Src inhibitor were different. PP2 could inhibit 80% of cell growth in MDA-MB-231 cells. In contrast,

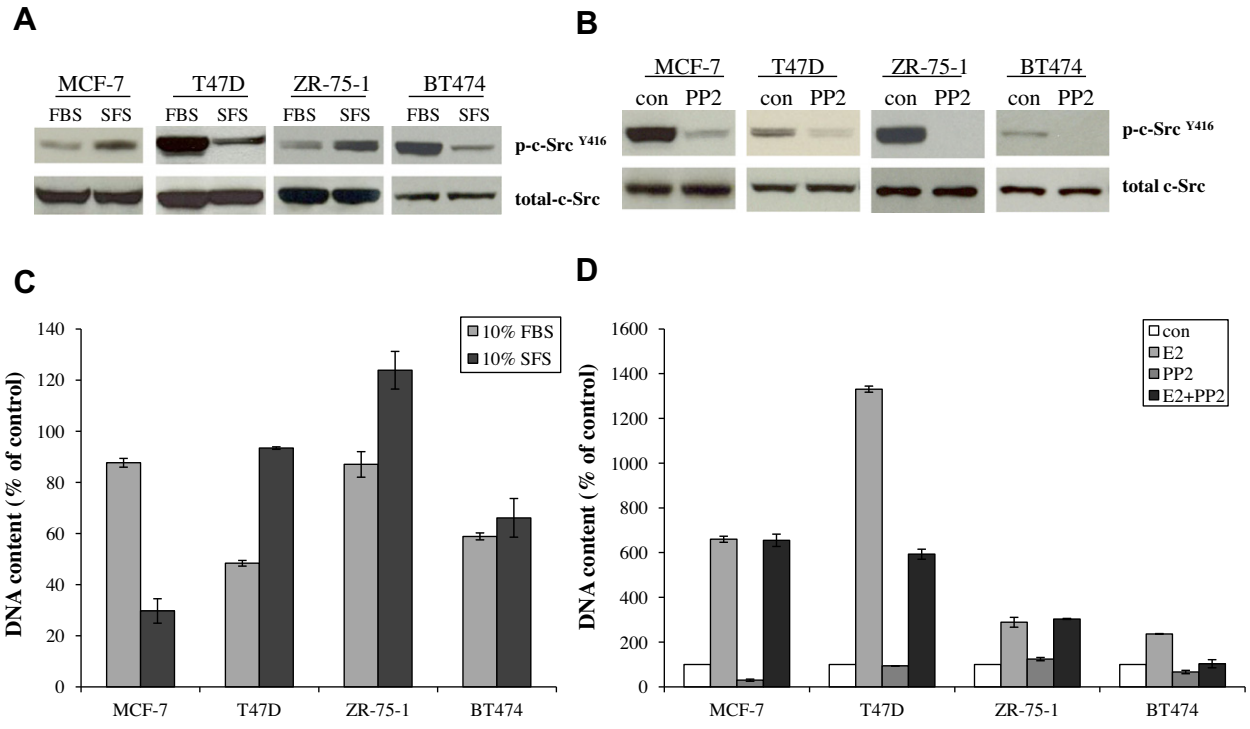

Fig. 2. Effects of the c-Src inhibitor on oestrogen receptor (ER) positive wild-type cell lines under conditions with or without basal E_2. (A) c-Src phosphorylation changed after short-term absence of E_2 in ER positive wild-type cells. Wild-type ER positive cells were cultured under conditions with basal oestrogen (10% foetal bovine serum (FBS)) or without basal oestrogen (10% dextran-coated charcoal-stripped foetal bovine serum (SFS)) for 3 days, respectively. Cell lysates were harvested. Phosphorylated c-Src was examined by immunoblotting with primary antibody. Immunoblotting for total c-Src was determined as loading control. (B) Blocking c-Src phosphorylation in ER positive wild-type cell lines by PP2 under the conditions without basal oestrogen. Wild-type ER positive cells were cultured under the conditions without basal oestrogen (10% SFS) for 3 days. Then cells were treated with PP2 (5 μM) in 10% SFS medium for 24 h and cell lysates were harvested. Phosphorylated c-Src was detected by immunoblotting with primary antibody. Immunoblotting for total c-Src was used for loading control. (C) Growth inhibitory effects of PP2 on ER positive wild-type cells under conditions with or without basal E_2. Wild-type ER positive cells were cultured under conditions with basal oestrogen (10% FBS) or without basal oestrogen (10% SFS) for 3 days, respectively. Then, they were seeded in 24-well plates in triplicate. After one day, the cells were treated with vehicle (0.1% DMSO) and PP2 (5 μM) in oestrogenised medium (10% FBS) or E_2 free medium (10%SFS), respectively. The cells were harvested after 7 days treatment and total DNA was determined as above. (D) The PP2 had different effects on E_2 stimulation in ER positive wild-type cells. Wild-type ER positive cells were changed to E_2 free medium for 3 days. Then, they were seeded in 24-well plates. After one day, the cells were treated with vehicle (0.1% EtOH), E_2 (10^{-9} mol/L), PP2 (5 μM) and E_2 (10^{-9} mol/L) plus PP2 (5 μM) respectively in E_2 free culture medium. The cells were harvested after 7 days treatment and total DNA was determined as above.

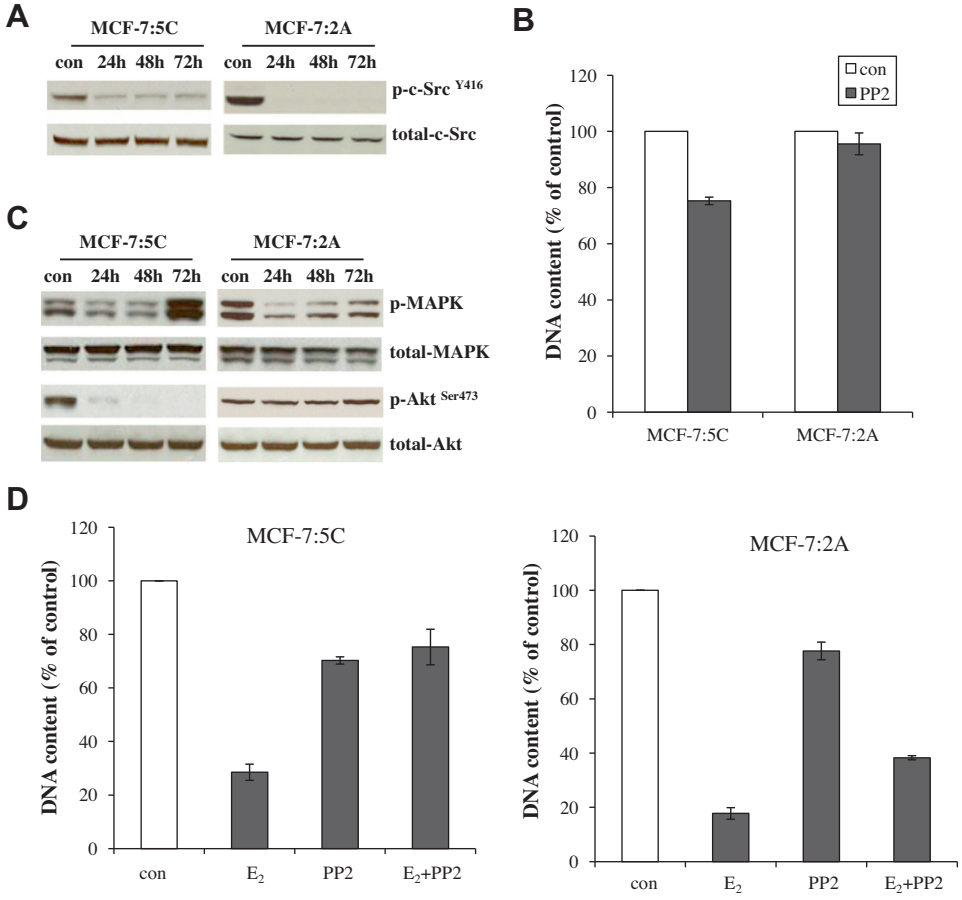

Fig. 3. Effects of the c-Src inhibitor on oestrogen receptor (ER) positive endocrine resistant cell lines. (A) Blocking c-Src phosphorylation in endocrine resistant ER positive cells. MCF-7:5C and MCF-7:2A cells were treated with PP2 (5 μM) at time points as indicated and cell lysates were harvested. Phosphorylated c-Src was detected by immunoblotting with primary antibody. Immunoblotting for total c-Src was used for loading control. (B) Growth inhibitory effects of PP2 on endocrine resistant ER positive cells. MCF-7:5C and MCF-7:2A cells were seeded in 24-well plates in triplicate. After one day, the cells were treated with vehicle (0.1% DMSO) and PP2 (5 μM) respectively in culture medium. The cells were harvested after 7 days treatment and total DNA was determined as above. (C) Signalling pathways change in endocrine resistant ER positive cells after PP2 treatment. Cell lysates were harvested as above. Phosphorylated mitogen-activated protein kinase (MAPK) and Akt were examined by immunoblotting with primary antibodies. Immunoblotting for total MAPK and Akt was used for loading controls. (D) The PP2 blocked E_2-induced inhibition in MCF-7:5C and MCF-7:2A cells. MCF-7:5C cells were seeded in 24-well plates as above. After one day, the cells were treated with vehicle (0.1% EtOH), E_2 (10^{-9} mol/L), PP2 (5 μM) and E_2 (10^{-9} mol/L) plus PP2 (5 μM) respectively. The cells were harvested after 7 days treatment and total DNA was determined as above. MCF-7:2A cells were seeded in 6-well plates. After 1 day, the cells were similarly treated as in MCF-7:5C cells. The cells were harvested after 14 days treatment and total DNA was determined as above.

PP2 exerted no inhibitory effects on Sk-Br-3 cells with HER2 overexpression (Fig. 4B). Inhibition of c-Src could efficiently suppress around 60% of cell growth in both resistant cells, MCF-7/F and T47D:C42 (Fig. 4B). The triple negative MDA-MB-231 cell line was the most sensitive to PP2. These results demonstrated that HER2 amplification might be an indicator for resistance to the c-Src inhibitors in clinical trials. Further investigation indicated that PP2 effectively blocked the MAPK and Akt pathways in the c-Src inhibitor sensitive cells, whereas MAPK and Akt phosphorylation were increased in Sk-Br-3 cells (Fig. 4C). The data implied that HER2 might drive the growth pathways in Sk-Br-3 cells.

3.6. Activation status of HER2 determined the inhibitory effects of the c-Src inhibitor

HER2 overexpression leads to a very aggressive cancer phenotype and poor patient survival.[27] c-Src is known to bind to HER2 and is thus activated in HER2-overexpressing cancer cells.[28,29] BT474 and Sk-Br-3 cells overexpress endogenous HER2 (Supplementary Fig. S1B), however, they had different responses to PP2 (Figs. 1B and 4B). To examine whether HER2 activation affects the inhibitory rate of PP2, phosphorylation of HER2 was evaluated. Among tested cell lines, Sk-Br-3, BT474 and T47D:C42 cells had elevated though different levels of HER2 activation. As a

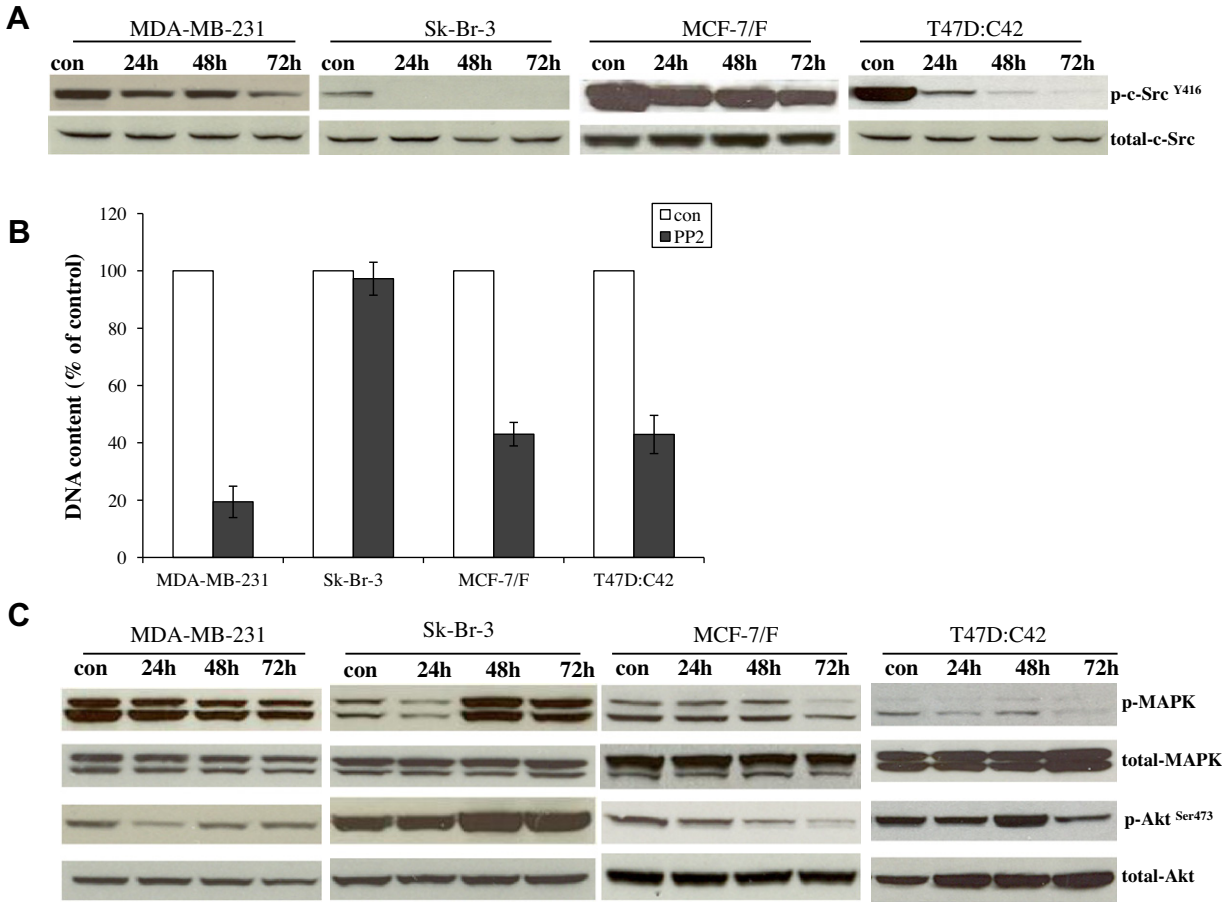

Fig. 4. Effects of the c-Src inhibitor on oestrogen receptor (ER) negative cell lines. (A) Blocking c-Src phosphorylation in ER negative cell lines by PP2. ER negative cells were treated with PP2 (5 μM) for different times as indicated and cell lysates were harvested. Phosphorylated c-Src was detected by immunoblotting with primary antibody. Immunoblotting for total c-Src was used for loading control. (B) Inhibitory effects of PP2 on ER negative cells. ER negative cells were seeded in 24-well plates in triplicate. After 1 day, the cells were treated with vehicle (0.1% DMSO) and PP2 (5 μM) in 10% SFS medium. The cells were harvested after 7 days treatment and total DNA was determined as above. (C) Signalling pathways were changed in ER negative cells after PP2 treatment. ER negative cells were treated with PP2 (5 μM) for different times as indicated and cell lysates were harvested. Phosphorylated mitogen-activated protein kinase (MAPK) and Akt were examined by immunoblotting with primary antibodies. Immunoblotting for total MAPK and Akt was determined for loading controls.

control, HER2 was undetectable in MDA-MB-231 cells (Fig. 5A). HER2 was highly activated in Sk-Br-3 cells compared with BT474 cells which made it hypersensitive to lapatinib, a dual tyrosine kinase inhibitor of HER2 and EGFR (Fig. 5B). The growth inhibitory effects by lapatinib corresponded to the levels of phosphorylated HER2 (Fig. 5B). We observed that HER2 hyper-activation rendered breast cancer cell completely resistant to PP2, the higher HER phosphorylation, the lower responsive rate to PP2 (Fig. 5B). This was further confirmed by S phase changes through flow cytometric analysis (Fig. 5C and Supplementary Fig. S4). Lapatinib reduced S phase in cells with higher HER2 phosphorylation, conversely, PP2 was effective in cells with lower HER2 phosphorylation (Fig. 5C and Supplementary Fig. S4). Lapatinib's antitumour activity was associated with blocking phosphorylation of HER2 and the subsequent inhibition of its downstream signalling pathways (Fig. 5D and Supplementary Fig. S5). Lapatinib blocked MAPK and Akt pathways in Sk-Br-3 and BT474 cells, but it exerted no inhibition in MDA-MB-231 cells (Supplementary Fig. S5), which demonstrated that antiproliferative effects of lapatinib also correlated with inhibitory ability of growth pathways.

3.7. Blocking c-Src tyrosine kinase recovered ERα expression and reduced HER2 levels in ER negative Sk-Br-3 cells

c-Src may drive oestrogen-dependent ERα proteolysis in a subset of ER negative breast cancer.[30] c-Src did not play a critical role in mediating growth pathways in Sk-Br-3 cells (Fig. 4B). To study whether the c-Src inhibitor can regulate ER turn-over in breast cancer cells with HER2 amplification, we found that PP2 could recover ERα expression in Sk-Br-3 cells (Fig. 6A). Real-time PCR analysis showed that mRNA levels of ERα was increased after PP2 treat-

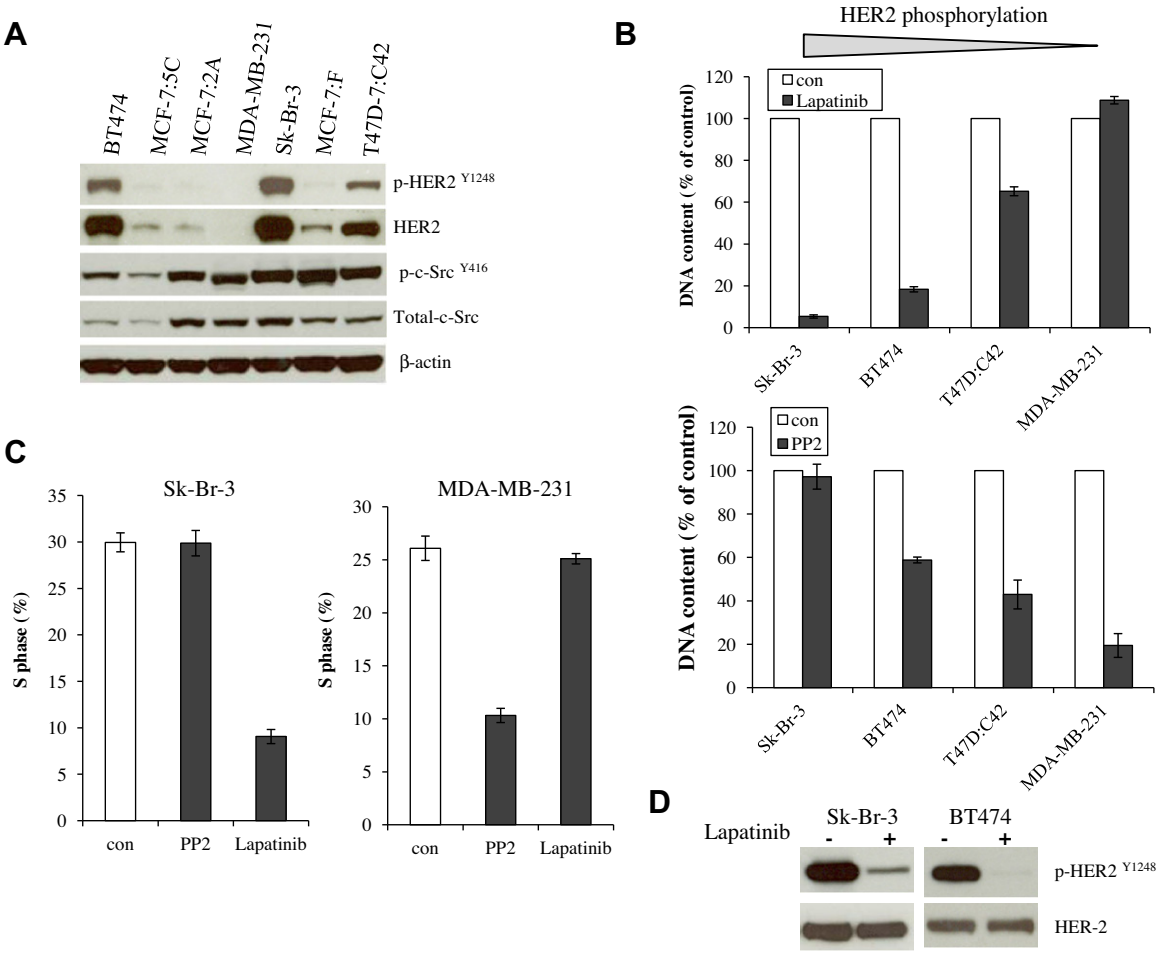

Fig. 5. Activation status of human epidermal growth factor receptor 2 (HER2) determined the inhibitory effects of the c-Src inhibitor. (A) Baseline HER2 phosphorylation in different cell lines. Cell lysates were harvested from different cells. Phosphorylated HER2 and total HER2 were examined by immunoblotting with primary antibodies. Immunoblotting for β-actin was determined for loading control. (B) Inhibitory effects of the HER2 inhibitor and the c-Src inhibitor on cells with elevated HER2 phosphorylation. Sk-Br-3, BT474, T47D:C42 and MDA-MB-231 cells were seeded in 24-well plates in triplicate. After one day, the cells were treated with vehicle (0.1% DMSO), lapatinib (1 μM) and PP2 (5 μM) in 10% SFS medium. The cells were harvested after 7 days treatment and total DNA was determined as above. (C) S phase changes after lapatinib and PP2 treatment. Sk-Br-3 and MDA-MB-231 cells were treated with vehicle (0.1% DMSO), lapatinib (1 μM) and PP2 (5 μM) for 24 h. Cells were harvested and fixed with 75% EtOH. Cell cycles were analysed through flow cytometery. (D) Blocking HER2 phosphorylation after lapatinib treatment. Sk-Br-3 and BT474 cells were treated with vehicle (0.1% DMSO) and lapatinib (1 μM) for 24 h. HER2 phosphorylation was examined by immunoblotting with primary antibody. Immunoblotting for total HER2 was determined for loading control.

ment in Sk-Br-3 cells (Fig. 6B) which implied that c-Src was involved in the regulation of ERα not only in the protein level but also at the transcription level. We further demonstrated that PP2 decreased HER2 levels in Sk-Br-3 cells after extending treatment time (Fig. 6C). This result also implied a complicated feedback loop existed between c-Src and HER2 in Sk-Br-3 cells. Importantly, Sk-Br-3 cells acquired responses to 4-hydroxytamoxifen and ICI 182,780 after short-term treatment with PP2 (Fig. 6D and Supplementary Fig. S6). Therefore, it is plausible that the simultaneous interruption of c-Src tyrosine kinase and targeting ER might be an effective treatment for breast cancer cells with HER2 amplification.[31]

4. Discussion

We employed a panel of well characterised breast cancer cell lines (MCF-7, T47D, ZR-75-1, BT474, MDA-MB-231 and Sk-Br-3) and resistant cell lines (MCF-7:5C, MCF-7:2A, MCF-7/F and T47D:C42) to identify biomarkers associated with the inhibitory actions of a specific c-Src inhibitor, PP2. PP2 blocked c-Src tyrosine kinase activity in all cell lines tested. However, the antiproliferative effects of PP2 were associated with the inhibition of ERK/MAPK and/or PI3K/Akt growth pathways. ER positive and HER2 hyperactivation were two important clinically related markers that were associated with the inability of PP2 to inhibit both

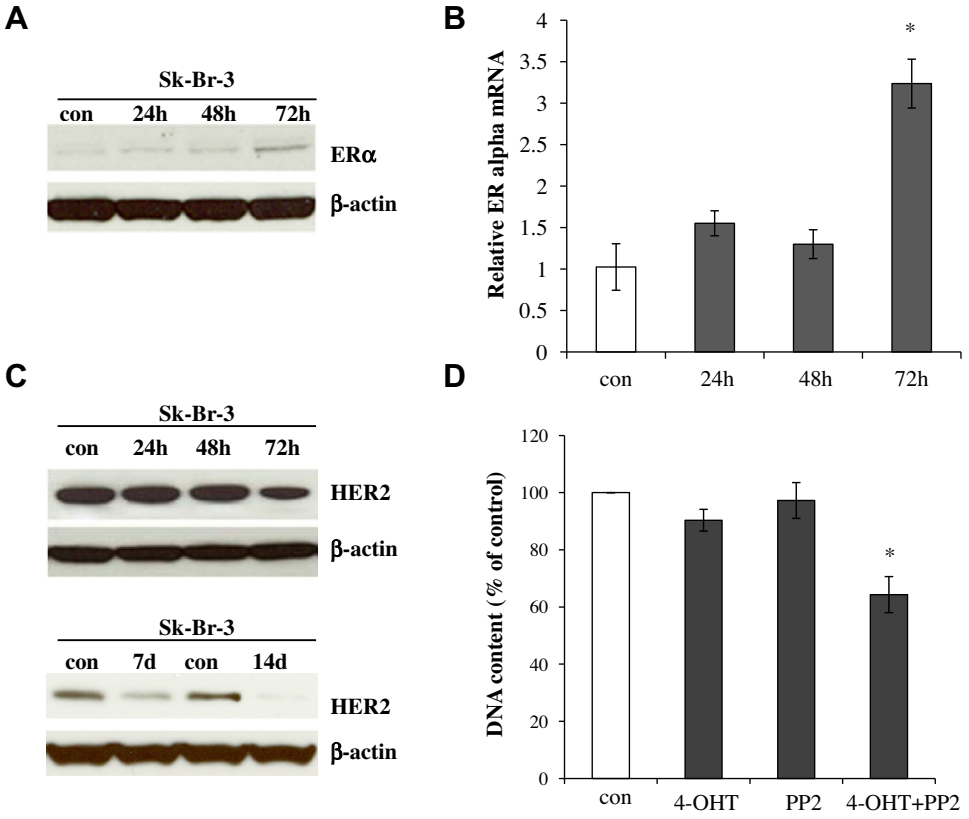

Fig. 6. Blocking c-Src sensitised cell to antioestrogen in Sk-Br-3 cells. (A) ERα expression was elevated in Sk-Br-3 cells after PP2 treatment. Sk-Br-3 cells were treated with PP2 as indicated. ERα expression was examined by immunoblotting with primary antibody. Immunoblotting for β-actin was determined for loading control. (B) ERα mRNA was increased in Sk-Br-3 cells after PP2 treatment. Sk-Br-3 cells were treated with PP2 (5 μM) for the times as indicated. RNA was harvested in TRIzol for real-time polymerase chain reaction (PCR) analysis. $^{*}P < 0.05$, compared with control. (C) HER2 expression was down regulated in Sk-Br-3 cells after PP2 treatment. Sk-Br-3 cells were treated with PP2 for the times as indicated. HER2 was examined by immunoblotting with primary antibody. Immunoblotting for β-actin was determined for loading control. (D) The PP2 sensitised Sk-Br-3 cells to 4-hydroxytamoxifen. Sk-Br-3 cells were treated with vehicle, 4-OHT (1 μM), PP2 (5 μM) and 4-OHT (1 μM) plus PP2 (5 μM) in 10% foetal bovine serum (FBS) medium. The cells were harvested after 7 days treatment and total DNA was determined as above. $^{*}P < 0.05$, compared with control.

wild-type and different resistant breast cancer cells. Triple-negative breast cancer cells, defined by a lack of expression of oestrogen, progesterone and HER2 receptors, were the most sensitive to the c-Src inhibitor.

The therapeutic mechanisms of the c-Src inhibitor are to block its phosphorylation and subsequent growth pathways.[32] It has been reported that cancer cells which do not manifest detectable c-Src phosphorylation are resistant to the c-Src inhibitor.[33] Generally, cells with higher c-Src activity were more sensitive to PP2 (Fig. 4B), but not all cells with elevated c-Src tyrosine kinase activity were able to be effectively inhibited by the c-Src inhibitor such as ZR-75-1, MCF-7:2A and Sk-Br-3 cells (Figs. 1B and 4B). Thus, the level of c-Src phosphorylation is not sufficient to distinguish responsive cells from cells resistant to the c-Src inhibitor. Growth inhibition also depends on whether c-Src directly mediates growth pathways in a special type of cell. We consistently found that the levels of MAPK phosphorylation and/or Akt phosphorylation were reduced by PP2 in responsive cell lines but not in resistant cell lines (Figs. 1C, 3C, and 4C).

The non-receptor tyrosine kinase c-Src acts as a critical molecule in relaying ER signalling, including non-genomic and genomic actions.[4,26] Its activity is modulated by E_2 through multiple mechanisms, leading to breast cancer cell proliferation, invasion and metastasis.[3,7] Consistently, the growth inhibitory effects by the c-Src inhibitor on ER positive cells appear to be more complex than on ER negative cells in present work. Most ER negative breast cancer cells were sensitive to the inhibition by PP2 (Fig. 4B). However, the majority of ER positive cells were not sensitive to PP2 regardless of whether they were wild-type or long-term oestrogen deprived cells (Figs. 1B and 3B). Although PP2 had moderate ability to inhibit some ER positive wild-type cell growth (Fig. 1B), inhibitory effects by it varied under conditions with or without basal E_2 (Fig. 2C). Our results also demonstrated that c-Src mainly mediated E_2 responses which included E_2-stimualted growth and

E_2-induced apoptosis in ER positive cells (Figs. 2D and 3D). These functions might disturb the therapeutic effects of the c-Src inhibitor on ER positive cells. Although the c-Src inhibitor shows limited activity in ER positive cells as a single-agent, c-Src is consistently activated after ER targeting treatment with tamoxifen[8–10] which plays a critical role in mediating migration and invasion in tamoxifen resistant cells.[8,9] Therefore, combined together the c-Src inhibitor and ER blockade may delay endocrine resistance and increase the therapeutic effects.[34]

The function of c-Src has been linked to its association with the HER2/Neu epidermal growth factor receptor family members.[35] In this study, increased expression of EGFR (MDA-MB-231 and MCF-7/F) did not affect the inhibitory effects of PP2, but HER2 overexpression was an indicator for the resistance to PP2 (Fig. 4B). Finn et al.[15] also reported HER2 amplification was a predictive marker for resistance to a c-Src inhibitor, dasatinib, in breast cancer cells. However, both BT474 and Sk-Br-3 cells overexpress endogenous HER2, they had differential responses to PP2 (Figs. 1B and 4B). Further investigation demonstrated that status of HER2 activation determined the inhibitory rate of PP2, the higher HER2 phosphorylation, the lower inhibitory rate of PP2 (Fig. 5B and C). HER2 was highly activated in Sk-Br-3 cells compared with BT474 cells which made it hypersensitive to the HER2 inhibitor but not the c-Src inhibitor (Fig. 5A and B). Therefore, status of HER2 activation may be a better predictive biomarker for resistance to the c-Src inhibitor than currently available total HER2 determined by immunohistochemistry (IHC) or fluorescent *in situ* hybridisation (FISH).[16]

The triple negative MDA-MB-231 cells are characterised by a point mutation at codon 13 in the *K-RAS* gene.[36] This mutation is responsible for the constitutive phosphorylation of ERK1/2 which leads to a very aggressive cancer phenotype.[37] Among tested cell lines, we observed that PP2 could not completely block c-Src phosphorylation in MDA-MB-231 cells within the first 24 h (Figs. 1A, 2B, 3A, and 4A). But the level of c-Src phosphorylation was gradually decreased (Fig. 4A). We prolonged treatment time to 4 days, the level of c-Src phosphorylation was clearly decreased (supplementary Fig. S7). It is unclear how EGFR and *K-RAS* regulate the function of c-Src in MDA-MB-231 cells. The c-Src inhibitor, PP2, effectively suppressed growth in MDA-MB-231cells, which demonstrated that triple negative breast cancer cells depend on c-Src to proliferate (Fig. 4B). Two independent studies support our observation by showing that the majority of dasatinib sensitive breast cancer cell lines were 'basal' type or 'triple-negative'.[14,15] The hyper-sensitivity to the c-Src inhibitors provides a good therapeutic option for the clinical triple negative breast cancer (TNBC) patient. However, the TNBC is actually a highly diverse group of cancer,[38] so that the determination of ER, PR and HER2 is not a precise classification to subtype this aggressive disease. MDA-MB-231 cells can not represent clinical TNBC model. Recent Phase II clinical trial shows that single-agent dasatinib has limited activity in unselected patients with TNBC,[17] which suggests that a strategy of better patient selection with gene signatures is required to further evaluate the potential of the c-Src inhibitors in TNBC patient.[38]

In summary, this study demonstrated a complex association exists among ER, HER2 and c-Src in different breast cancer cell lines. Moreover, our results underscored that ER expression and HER2 overexpression (especially over-activation) might be causes of resistance to a c-Src inhibitor in breast cancer. Our findings may be of value for future clinical investigation to determine the therapeutic efficacy of c-Src inhibitors in ER negative breast cancer with or without HER2 over-activation.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2012.04.020.

References

1. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* 2007;**72**:7–25.
2. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001;**344**:783–92.
3. Thomas SN, Brugge JS. Cellular functions regulated by Src family kinases. *Ann Rev Cell Dev Biol* 1997;**13**:513–609.
4. Wong CW, McNally C, Nickbarg E, Komm BS, Cheskis BJ. Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proc Natl Acad Sci USA* 2002;**99**:14783–8.
5. Belsches-Jablonski AP, Biscardi JS, Peavy DR, et al. Src family kinases and HER2 interactions in human breast cancer cell growth and survival. *Oncogene* 2001;**20**:1465–75.
6. Bromann PA, Korkaya H, Courtneidge SA. The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene* 2004;**23**:7957–68.
7. Shupnik MA. Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. *Oncogene* 2004;**23**:7979–89.
8. Hiscox S, Morgan L, Green TP, et al. Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. *Breast Cancer Res Treat* 2006;**97**:263–74.
9. Zhao Y, Planas-Silva MD. Mislocalizaion of cell-cell adhesion complexes in tamoxifen-resistant breast cancer cells with elevated c-Src tyrosine kinase activity. *Cancer Lett* 2009;**275**:204–12.
10. Fan P, Wang J, Santen RJ, Yue W. Long-term treatment with tamoxifen facilitates translocation of estrogen receptor alpha out of the nucleus and enhances its interaction with EGFR in MCF-7 breast cancer cells. *Cancer Res* 2007;**67**:1352–60.
11. Zhang S, Huang WC, Li P, et al. Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. *Nat Med* 2011;**17**:461–9.
12. Talpaz M, Shah NP, Kantarjian H, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. *N Engl J Med* 2006;**354**:2531–41.
13. Demetri GD, Lo Russo P, Macpherson IR, et al. Phase I dose-escalation and pharmacokinetic study of dasatinib (BMS-354825), a Src and multi-kinase inhibitor, in patients with advanced solid tumors. *Clin Cancer Res* 2009;**15**:6232–40.
14. Huang F, Reeves K, Han X, et al. Identification of candidate molecular markers predicting sensitivity in solid tumors to dasatinib: rationale for patient selection. *Cancer Res* 2007;**67**:2226–38.
15. Finn RS, Dering J, Ginther C, et al. Dasatinib, an orally active small molecule inhibitor of both the src and abl kinases, selectively inhibits growth of basal-type/"triple-negative" breast cancer cell lines growing in vitro. *Breast Cancer Res Treat* 2007;**105**:319–26.
16. Finn RS, Bengala C, Ibrahim N, et al. Dasatinib as a single agent in triple-negative breast cancer: results of an open-label phase 2 study. *Clin Cancer Res* 2011;**17**:6905–13.
17. Mayer E, Baurain JF, Sparano JA, et al. A phase 2 trial of dasatinib in patients with advanced HER2-positive and/or hormone receptor-positive breast cancer. *Clin Cancer Res* 2011;**17**:6897–904.
18. Chen Y, Guggisberg N, Jorda M, et al. Combined Src and aromatase inhibition impairs human breast cancer growth in vivo and bypass pathways are activated in AZD0530-resistant tumors. *Clin Cancer Res* 2009;**15**:3396–405.
19. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 1996;**56**:2321–30.
20. Jiang SY, Jordan VC. Growth regulation of estrogen receptor negative breast cancer cells transfected with complementary DNAs for estrogen receptor. *J Natl Cancer Inst* 1992;**84**:580–91.
21. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* 1992;**90**:77–86.
22. Pink JJ, Jiang SY, Fritsch M, Jordan VC. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* 1995;**55**:2583–90.
23. Murphy CS, Pink JJ, Jordan VC. Characterization of a receptor-negative, hormone-nonresponsive clone derived from a T47D human breast cancer cell line kept under estrogen-free conditions. *Cancer Res* 1990;**50**:7285–92.
24. Liu H, Cheng D, Weichel AK, et al. Cooperative effect of gefitinib and fumitremorgin c on cell growth and chemosensitivity in estrogen receptor alpha negative fulvestrant-resistant MCF-7 cells. *Int J Oncol* 2006;**29**:1237–46.
25. Lewis JS, Meeke K, Osipo C, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 2005;**97**:1746–59.
26. Faivre EJ, Lange CA. Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells. *Mol Cell Biol* 2007;**27**:466–80.
27. Yu D, Hung MC. Overexpression of ErbB2 in cancer and ErbB2-targeting strategies. *Oncogene* 2000;**19**:6115–21.
28. Muthuswamy SK, Muller WJ. Activation of Src family kinases in Neu-induced mammary tumors correlates with their association with distinct sets of tyrosine phosphorylated proteins in vivo. *Oncogene* 1995;**11**:1801–10.
29. Belsches-Jablonski AP, Biscardi JS, Peavy DR, et al. Src family kinases and HER2 interactions in human breast cancer cell growth and survival. *Oncogene* 2001;**20**:1465–75.
30. Chu I, Arnaout A, Loiseau S, et al. Src promotes estrogen-dependent estrogen receptor alpha proteolysis in human breast cancer. *J Clin Invest* 2007;**117**:2205–15.
31. Page K, Hava N, Ward B, et al. Detection of HER2 amplification in circulating free DNA in patients with breast cancer. *Br J Cancer* 2011;**104**:1342–8.
32. Luttrell DK, Lee A, Lansing TJ, et al. Involvement of pp 60c-src with two major signaling pathways in human breast cancer. *Proc Natl Acad Sci USA* 1994;**91**:83–7.
33. Okamoto W, Okamoto I, Yoshida T, et al. Identification of c-Src as a potential therapeutic target for gastric cancer and of MET activation as a cause of resistance to c-Src inhibition. *Mol Cancer Ther* 2010;**9**:1188–97.
34. Chen Y, Alvarez EA, Azzam D, et al. Combined Src and ER blockade impairs human breast cancer proliferation in vitro and in vivo. *Breast Cancer Res Treat* 2011;**128**:69–78.
35. Tan M, Li P, Klos KS, et al. ErbB2 promotes Src synthesis and stability: novel mechanisms of Src activation that confer breast cancer metastasis. *Cancer Res* 2005;**65**:1858–67.
36. Eckert LB, Repasky GA, Ülkü AS, et al. Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoikis. *Cancer Res* 2004;**64**:4585–92.
37. Toulany M, Baumann M, Rodemann HP. Stimulated PI3K-AKT signaling mediated through ligand or radiation-induced EGFR depends indirectly, but not directly, on constitutive K-Ras activity. *Mol Cancer Res* 2007;**5**:863–72.
38. Lehmann BD, Bauer JA, Chen X, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest* 2011;**121**:2750–67.

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The 2012 Hormone Therapy Position Statement of The North American Menopause Society

Peter Schmidt

Abstract

Objective—This position statement aimed to update the evidence-based position statement published by The North American Menopause Society (NAMS) in 2010 regarding recommendations for hormone therapy (HT) for postmenopausal women. This updated position statement further distinguishes the emerging differences in the therapeutic benefit-risk ratio between estrogen therapy (ET) and combined estrogen-progestogen therapy (EPT) at various ages and time intervals since menopause onset.

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This position statement was developed by The North American Menopause Society (NAMS) 2012 Hormone Therapy Position Statement Advisory Panel consisting of representatives of the NAMS Board of Trustees and other experts in women's health: Margery L.S. Gass, MD, NCMP (Co-Chair); JoAnn E. Manson, MD, DrPH, NCMP (Co-Chair); Felicia Cosman, MD; Francine Grodstein, ScD; V. Craig Jordan, OBE, PhD, DSc, FMedSci; Richard H. Karas, MD, PhD; Andrew M. Kaunitz, MD; Pauline M. Maki, PhD; Peter J. Schmidt, MD; Jan L. Shifren, MD, NCMP; Cynthia A. Stuenkel, MD, NCMP; and Wulf H. Utian, MD, PhD, DSc(Med), NCMP. The Board of Trustees approved the position statement on January 15, 2012.

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Methods—An Advisory Panel of expert clinicians and researchers in the field of women's health was enlisted to review the 2010 NAMS position statement, evaluate new evidence, and reach consensus on recommendations. The Panel's recommendations were reviewed and approved by the NAMS Board of Trustees as an official NAMS position statement.

Results—Current evidence supports the use of HT for perimenopausal and postmenopausal women when the balance of potential benefits and risks is favorable for the individual woman. This position statement reviews the effects of ET and EPT on many aspects of women's health and recognizes the greater safety profile associated with ET.

Conclusions—Recent data support the initiation of HT around the time of menopause to treat menopause-related symptoms and to prevent osteoporosis in women at high risk of fracture. The more favorable benefit-risk ratio for ET allows more flexibility in extending the duration of use compared with EPT, where the earlier appearance of increased breast cancer risk precludes a recommendation for use beyond 3 to 5 years.

Keywords

Biodentical hormones; Breast cancer; Cardiovascular disease; Cognitive decline; Coronary heart disease; Dementia; Depression; Diabetes mellitus; Endometrial cancer; Estrogen; Estrogen-progestogen therapy; Estrogen therapy; Hormone therapy; Menopause; Mood; The North American Menopause Society; Osteoporosis; Ovarian cancer; Perimenopause; Postmenopause; Premature menopause; Premature ovarian insufficiency; Progestogen; Sexual function; Stroke; Total mortality; Urinary health; Quality of life; Vaginal atrophy; Vaginal health; Vasomotor symptoms; Venous thromboembolism; Women's Health Initiative

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The intent of The North American Menopause Society (NAMS) 2012 Hormone Therapy Position Statement is to clarify the benefit-risk ratio of estrogen therapy (ET) versus estrogen-progestogen therapy (EPT) for both treatment of menopause-related symptoms and disease prevention at various time intervals since menopause. The availability of long-term data related to the effects of hormone therapy (HT) both during and after use of HT prompted the NAMS Board of Trustees to update its position statement. NAMS convened a seventh Advisory Panel to provide recommendations. The Panel's recommendations were reviewed and approved by the 2011-2012 NAMS Board of Trustees.

The term HT is used to encompass both ET and EPT when outcomes are not specific to one or the other treatment.

These statements do not represent codified practice standards as defined by regulating bodies and insurance agencies.

METHODS

An Advisory Panel of clinicians and researchers expert in the field of women's health was enlisted to review the previous position statement of July 2010 (available at http://www.menopause.org/PSHT10.pdf), evaluate the literature published subsequently, and conduct an evidence-based analysis with the goal of reaching consensus on recommendations.

NAMS acknowledges that no single trial data can be extrapolated to all women. However, because the Women's Health Initiative (WHI) is, for some outcomes, the only large long-term randomized controlled trial (RCT) of postmenopausal women using HT, these findings were given prominent consideration among all the studies reviewed in the development of this position statement. Nonetheless, the WHI hormone trials had several characteristics that

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limit generalizing the findings to all postmenopausal women. These include the use of only one route of administration (oral), only one formulation of estrogen (conjugated estrogens [CEs]), and only one progestogen (medroxyprogesterone acetate). Unlike most HT studies that focused on symptomatic, recently postmenopausal women, the WHI enrolled generally healthy postmenopausal women aged 50 to 79 years in a prevention trial. These parameters should be taken into consideration when applying the WHI findings to clinical practice as should be the findings from observational studies with their known limitations. In general, the panel gave more weight to RCTs.

BENEFITS AND RISKS OF HORMONE THERAPY

Vasomotor symptoms

ET with or without a progestogen is the most effective treatment of menopause-related vasomotor symptoms and their potential consequences, such as diminished sleep quality, irritability, difficulty concentrating, and subsequently reduced quality of life (QOL).[1,2] Treatment of moderate to severe vasomotor symptoms remains the primary indication for HT. Almost all systemic HT products except for the ultralow-dose estradiol transdermal patch (approved for the prevention of osteoporosis) have government approval for this indication.[3] Progestogen alone also reduces vasomotor symptoms but not as effectively as estrogen does.[4]

Vaginal symptoms

ET is the most effective treatment of moderate to severe symptoms of vulvar and vaginal atrophy (eg, vaginal dryness, dyspareunia, and atrophic vaginitis).[5] Many systemic HT products and all local vaginal ET products have government approval for treating symptomatic vaginal atrophy. Some low-dose systemic regimens may be inadequate for the relief of vaginal symptoms and may require the addition of low-dose local ET to achieve the desired results. When ET is considered solely for treatment of vaginal atrophy, local vaginal ET is advised. Lower doses of vaginal ET than previously used, with less frequent administration, often yield satisfactory results.[6]

A progestogen is generally not indicated when ET at the recommended low doses is administered locally for vaginal atrophy, although clinical trial data supporting endometrial safety beyond 1 year are lacking.[7] Because endometrial hyperplasia increases with increasing dose and duration of estrogen exposure, thorough evaluation of any uterine bleeding in women using low-dose local ET is advised.

Sexual function

A significant effect of ET on sexual interest, arousal, and orgasmic response independent from its role in treating menopausal symptoms is not supported by current evidence.[8] Low-dose local ET may improve sexual satisfaction by improving lubrication and increasing blood flow and sensation in vaginal tissues. In an analysis of the persistence of sexual activity in the WHI, HT was not correlated with longer persistence of sexual activity.[9] HT is not recommended as the sole treatment of other problems of sexual function, including diminished libido.[10]

Urinary tract health

Local ET may benefit some women with overactive bladder.[11] One RCT found that an estradiol ring had a clinical benefit equivalent to that of oxybutynin among women with overactive bladder.[12] Systemic HT may worsen or provoke stress incontinence.[13-15] Ultralow-dose transdermal estradiol therapy neither increased nor decreased incontinence.[16] A large RCT reported an increased risk of kidney stones with HT.[17]

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Two studies reported a decreased risk of recurrent urinary tract infection through the use of intravaginal estrogen.[18,19] Only ET administered by the vaginal route has been shown to be effective for this purpose. No HT product has government approval for any urinary health indication.

Quality of life

Although no HT product has government approval for enhancing QOL, use of HT can result in an improvement in health-related QOL (HQOL) in symptomatic women through the alleviation of symptoms.[1,2,20] There is no clear evidence that HT improves HQOL in asymptomatic women.[20-23] With regard to physical functioning as a measure of HQOL, data from the WHI found no benefit of HT in women 65 years or older when measured for grip strength, chair standing, and walking.[24]

Osteoporosis

There is RCT evidence that standard-dose HT reduces postmenopausal osteoporotic fractures, including hip, spine, and all nonspine fractures, even in women without osteoporosis.[25,26] Low doses are effective in maintaining or improving bone mineral density. No HT product currently has government approval for the treatment of osteoporosis. Many systemic HT products, however, have government approval for the prevention of postmenopausal osteoporosis.

When alternate osteoporosis therapies are not appropriate or cause adverse effects, the extended use of HT is an option for women who are at high risk of osteoporotic fracture. There is no evidence that HT stops working with long-term treatment; however, the benefits of HT on bone mass and fracture reduction dissipate quickly after the discontinuation of treatment,[27,28] necessitating a transition to a different osteoporosis treatment (or prevention strategy) to preserve bone mass. Within a few years of the discontinuation of ET in the WHI, the cumulative incidence of hip fracture was the same in the ET and placebo groups.[28]

Unless there is a contraindication, women experiencing an early menopause who require prevention of bone loss are probably best served by the administration of HT or oral contraceptives, rather than other bone-specific treatments, until they reach the normal age of menopause at which time treatment may be reassessed. The presumed increased risk of fracture in older women who had an early menopause, however, was not substantiated in a recent report from the Study of Osteoporotic Fractures.[29] Women older than 65 years with a history of early menopause and no HT use did not sustain more fractures than did the group who had menopause at the average age. Removal of both ovaries at the time of hysterectomy compared with ovarian conservation was similarly found not to increase the subsequent rate of hip fracture.[30]

Cardiovascular effects

The cardiovascular effects discussed are coronary heart disease (CHD), carotid intima media thickness, coronary artery calcium, stroke, and venous thromboembolism (VTE).

Coronary heart disease—Most observational studies (primarily composed of women who began HT around the time of menopause) support the potential benefits of systemic HT in reducing the risk of CHD.[31] Most RCTs do not.[31,32] However, it is understood that the characteristics of women participating in observational studies are markedly different from those of many women enrolled in RCTs designed to evaluate the cardiovascular effects of HT.[33-35] These demographic and biologic differences can influence baseline cardiovascular risks and may modify the overall observed effects of HT on cardiovascular risk. In the WHI clinical trials, overall CHD risk was estimated to be increased by eight cases per 10,000

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women per year in the EPT arm; in the ET arm, overall CHD risk was estimated to be decreased by three cases per 10,000 women per year[36] (see "Dose and route of administration").

Timing of initiation: Secondary analyses of the WHI data indicate that the disparity in findings between observational studies and RCTs is related partly to the timing of initiation of HT in relation to age and proximity to menopause.[36,37] Most participants in the observational studies of CHD risk were younger than 55 years at the time HT was initiated and within 2 to 3 years of menopause. On the other hand, women enrolled, to date in RCTs with clinical cardiovascular endpoints have an age of 63 to 64 years and are more than 10 years beyond menopause. When analyzed by age and time since menopause at initiation of HT, the ET arm of the WHI[38] is in general agreement with observational studies[39] suggesting that ET may reduce CHD risk (coronary revascularization and composite outcomes including myocardial infarction [MI] and coronary death) when initiated in younger and more recently postmenopausal women without a uterus.[36] These findings for ET were even stronger with extended follow-up of the cohort and inclusion of 4 years after stopping. For women ages 50 to 59 years, the hazard ratio (HR) for CHD was 0.59 (95% CI, 0.38-0.90); for total MI, it was 0.54 (95% CI, 0.34-0.85; *P* for interaction by age = 0.05 and 0.007, respectively).[28]

Combined data incorporating both the ET and EPT trials of the WHI show a statistical trend of an HT effect relative to placebo on CHD by time since menopause, indicating that the women who initiate HT more than 10 years beyond menopause are at increased risk for CHD, and those women who initiate HT within 10 years of menopause tend to have a lower risk of CHD.[36] However, statistical modeling of the combined WHI data, including data from the WHI observational studies, did not find that CHD risks varied by the timing of HT initiation.[36,40]

Coronary artery calcium: Some observational studies,[41,42] but not all,[43] suggest that long-term HT is associated with less accumulation of coronary artery calcium, which is strongly correlated with atheromatous plaque burden and future risk of clinical CHD events. In an ancillary substudy of younger women (<60 y) in the WHI ET trial, after an average of 7 years of treatment, women who had been randomized to ET had lower levels of coronary artery calcium than did those randomized to placebo.[44] Although the effect in older women was not evaluated, these findings suggest that ET initiated by recently postmenopausal women may slow the development of calcified atherosclerotic plaque.

Carotid intima media thickness: Observational studies[45-47] demonstrate less accumulation of carotid plaque as measured through ultrasound in women taking HT. Two RCTs reported contradictory findings with regard to carotid plaque.[48,49]

Stroke—The WHI EPT and ET trials demonstrated an increased risk of ischemic stroke and no effect on the risk of hemorrhagic stroke.[50,51] In these trials, when the entire cohort was analyzed, there were eight additional strokes per 10,000 women per year of EPT and 11 additional strokes per 10,000 women per year of ET. In recent analyses that combined results from the WHI EPT and ET trials, HT in younger women (ages 50-59 y) at study entry had no significant effect on risk of stroke (relative risk [RR], 1.13; 95% CI, 0.73-1.76).[36,40]

Although stroke was not increased in the group ages 50 to 59 years in the combined analysis of the WHI, it was almost doubled in the ET group less than 10 years since menopause. This apparent contradiction in the data is hard to explain but may be caused by relatively few events and the difficulty in accurately timing the onset of menopause in the ET group. In

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both the ET and EPT trials, excess stroke risk dissipated rapidly after discontinuation of HT.[27,28]

In women randomized in the WHI within 5 years of menopause, there were three additional strokes per 10,000 women per year of EPT, which is not statistically significant.[36] The excess risk of stroke in this age group observed in the WHI studies would fall into the rare-risk category. Stroke risk was not significantly increased in the Heart and Estrogen/Progestin Replacement Study[52] and the Women's Estrogen for Stroke Trial secondary prevention trials.[53] The Women's International Study of long Duration Oestrogen after Menopause RCT found no excess risk of stroke in EPT users compared with women on placebo in 1 year.[54]

The results of observational studies on the risk of stroke with HT have been inconsistent. Several studies (including the Nurses' Health Study [NHS], the largest and longest prospective cohort study of women's health) indicated an increased risk of ischemic stroke consistent with the findings from the WHI,[55] whereas other studies showed no effect on stroke risk.[56-58] In the NHS, among women ages 50 to 59 years, the RR of stroke for current EPT users was not significantly elevated (RR, 1.34; 95% CI, 0.84-2.13), but it was significantly increased for current users of ET among women ages 50 to 59 years (RR, 1.58; 95% CI, 1.06-2.37).[55] The lowest dose of estrogen (eg, 0.3 mg CE) was not associated with an increased risk in the NHS, although this was based on the relatively few women who were taking that dose (see "Dose and route of administration").

Venous thromboembolism—Data from both observational studies and RCTs consistently demonstrate an increased risk of VTE with oral HT.[59,60] In the WHI trials, when the entire cohort was analyzed, there were 18 additional VTEs per 10,000 women per year of EPT[60] and 7 additional VTEs per 10,000 women per year of ET.[61] VTE risk in RCTs emerges soon after HT initiation (ie, during the first 1-2 y), and the magnitude of the excess risk seems to decrease somewhat in time. In the WHI trials, the absolute excess VTE risk associated with either EPT or ET was lower in women who started HT before age 60 years than in older women who initiated HT after age 60 years. In women ages 50 to 59 years who were randomized to HT,[61] there were 11 additional VTEs per 10,000 women per year of EPT and 4 additional VTEs per 10,000 women per year of ET. These risks fall into the rare-risk category. The baseline risk of VTE also increased relative to body mass index (BMI). For obese women (BMI, >30 kg/m^2), the baseline risk was almost threefold greater. At any BMI, the risk of VTE doubled with HT and returned to baseline soon after HT discontinuation.[27,28]

Women with a previous history of VTE, obese women, or women who possess a factor V Leiden mutation are at increased risk of VTE with HT use.[60,62,63] There are limited observational data suggesting lower risks of VTE with transdermal than with oral ET,[64-66] but there are no comparative RCT data on this subject. Lower doses of oral ET may also confer less VTE risk than higher doses, but no comparative RCT data are available to confirm this assumption. Studies that have evaluated the contribution of various progestogens to clotting suggest that norpregnanes may be more thrombogenic.[67,68]

HT is currently not recommended for coronary protection in women of any age. Initiation of HT by women ages 50 to 59 years or by those within 10 years of menopause to treat typical menopausal symptoms does not seem to increase the risk of CHD events. There is emerging evidence that the initiation of ET in early postmenopause may reduce coronary artery disease and CHD risk. Two ongoing studies of early HT intervention may provide further information on this topic: the Early versus Late Intervention Trial with Estradiol and the Kronos Early Estrogen Prevention Study.

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Diabetes mellitus

Large RCTs demonstrate that HT reduces the diagnosis of new onset type 2 diabetes mellitus (T2DM), although no HT product has government approval to prevent T2DM. Women who received active treatment in the WHI EPT arm had a statistically significant 21% reduction (HR, 0.79; 95% CI, 0.67-0.93) in the incidence of T2DM requiring treatment, which indicates 15 fewer cases per 10,000 women per year of therapy.[69] A similar statistically significant risk reduction was also noted in the Heart and Estrogen/Progestin Replacement Study trial (HR, 0.65; 95% CI, 0.48-0.89).[70] In the WHI ET trial, there was a 12% reduction (HR, 0.88; 95% CI, 0.77-1.01) in incident T2DM or 14 fewer cases per 10,000 women per year of ET.[71] Unfortunately, none of these trials included an oral glucose tolerance test to evaluate postchallenge glucose levels. In the Postmenopausal Estrogen and Progestin Intervention trial, fasting glucose levels were reduced in women assigned to HT; however, 2-hour postchallenge glucose levels, which may be associated with CHD risk, were elevated.[72] There is inadequate evidence to recommend HT for the sole or primary indication of the prevention of T2DM in perimenopausal or postmenopausal women.

Endometrial cancer

Unopposed systemic ET in postmenopausal women with an intact uterus is associated with increased endometrial cancer risk related to the ET dose and duration of use. A metaanalysis reported a summary RR of 2.3 (95% CI, 2.1-2.5) overall and an RR of 9.5 if used for more than 10 years.[73] This increased risk persisted for several years after ET discontinuation. To negate this increased risk, adequate concomitant progestogen is recommended for women with an intact uterus when using systemic ET (see “Progestogen indication”). In general, HT is not recommended in women with a history of endometrial cancer. Progestogen alone could be considered for the management of vasomotor symptoms but no long-term data are available.

Breast cancer

Estrogen-progestogen therapy—Diagnosis of breast cancer increases with EPT use beyond 3 to 5 years.[74] In the WHI overall, this increased risk, in absolute terms, was eight additional breast cancers per 10,000 women using EPT for 5 or more years. Studies have not clarified whether the risk differs between continuous and sequential use of progestogen, with observational studies suggesting that risk may be greater with continuous use of progestogen. It is also not clear whether there is a class effect with progestogens or whether the specific agent used influences the degree of breast cancer risk. Data from a large observational study suggest that EPT with micronized progesterone carries a low risk of breast cancer with short-term use but carries an increased risk of breast cancer with all EPT formulations with long-term use.[75]

EPT and, to a lesser extent, ET increase breast cell proliferation, breast pain, and mammographic density, and EPT may impede the diagnostic interpretation of mammograms, therein delaying the diagnosis of breast cancer.[74,76] Evolving but not conclusive evidence suggests that the increased risk of breast cancer with EPT may be a result of the promotion of preexisting cancers that are too small to be diagnosed by imaging studies or clinical examination. Some of these small cancers may never progress without the stimulation of HT. Long-term follow-up found that the risk of new diagnosis of breast cancer dissipated in the 3 years after cessation of EPT.[77] However, the follow-up also revealed that breast cancer mortality was increased in EPT users in the WHI who were followed for 11 years after study initiation. The breast cancer death rates with EPT were two additional deaths per 10,000 women per year attributed to breast cancer and two additional deaths per 10,000 women per year attributed to all-cause mortality.[78]

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In the WHI, the initial reports suggested that the increase in breast cancer risk was limited to those who had used EPT before enrollment.[79] Because most women initiate EPT shortly after menopause, a reanalysis of the data examined the effect of a "gap time" (duration of time between onset of menopause and start of EPT) on breast cancer risk. In a combined analysis of the WHI observational study and the EPT clinical trial, those starting EPT shortly after menopause had an HR of 2.75 for breast cancer with more than 5 years of use, whereas those with a gap time of greater than 5 years did not.[80] A detailed secondary analysis reported that women who experienced a hiatus in their exposure to hormones before randomization to EPT were found to have a delayed increase in breast cancer compared with previous EPT users.[81] The French E3N (a prospective cohort study of French women that examined the potential relationship between premenopausal and postmenopausal breast cancer occurrence) also reported a greater risk of breast cancer in those women with a short (<3 y) as opposed to those with a long gap time.[75] The Million Women Study (MWS) investigators reported an increased risk in women initiating HT shortly after menopause.[82]

These data on breast cancer (potentially more harm with early postmenopausal HT use) are in contrast with the findings on CHD, stroke, VTE, and all-cause mortality that suggest greater safety in younger women closer to menopause. For all outcomes, the absolute risk of events in younger women is lower than that for older women.

Estrogen therapy—Women in the ET arm of the WHI demonstrated no increase in risk of breast cancer after an average of 7.1 years of use, with six fewer cases of invasive breast cancer per 10,000 women per year of ET use, which is not statistically significant.[76] The decrease in risk was observed in all three age groups studied (ages 50-59, 60-69, and 70-79 y). Other findings in the ET group included a reduction in ductal carcinomas (HR, 0.71; 95% CI, 0.52-0.99).[76] In analyses based on extended follow-up of the WHI ET trial, including after stopping, the HR for breast cancer was 0.77 (95% CI, 0.62-0.95).[28] However, in women assigned to CE who developed invasive breast cancer, fewer breast cancers presented with localized disease (HR, 0.69; 95% CI, 0.51-0.95), and tumors were larger and more likely to be node positive compared with those in women assigned to placebo.[76]

The hypothesis for the decreased incidence of breast cancer with use of CE in the WHI is the apoptotic effect that estrogen has on breast cancer cells in a low-estrogen environment. Although the use of CE in the WHI did not show an age-related difference in the reduction of breast cancer, all laboratory evidence suggests that the longer breast cancer cells are estrogen-deprived, the more probable that physiologic estrogen will have a tumoricidal effect.[83]

The decreased risk of breast cancer as seen in the ET arm of the WHI was not observed in the MWS.[82] The RR for breast cancer in the MWS was increased in women who started ET within 5 years after menopause, with an absolute increased risk of 13 cases per 10,000 women per year.[82] Whether the difference between these findings and the WHI ET arm reflects differences in the timing of ET initiation, the types of ET, study populations, increased mammographic surveillance of women using HT, or other factors not controlled for in an observational study has not been determined.

When ET was extended beyond 15 years in the NHS, breast cancer risk increased.[84-86] A large meta-analysis of 67,370 women in observational studies found no increased risk with less than 5 years of ET use and RRs of 1.31 for 5 to 9 years of use, 1.24 for 10 to 14 years of use, and 1.56 for more than 15 years of use.[87] The possibility of differences in mammographic surveillance for breast cancer in users and nonusers of HT in observational studies cannot be excluded.

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HT after breast cancer—Controversy surrounds the use of HT in survivors of breast cancer. Some observational studies suggest that HT use may not increase the risk of recurrent breast cancer.[88-94] These reports have been questioned because of the potential bias from the selection of women at lower risk of recurrence for HT use. An RCT of HT use in women with a history of breast cancer and bothersome vasomotor symptoms was terminated early, after 2 years of follow-up, when significantly more new breast cancer events were diagnosed in women randomized to HT.[95] These data would indicate that HT use in breast cancer survivors may be associated with an increased risk of recurrence.

Ovarian cancer

Published data on the role of HT and risk of ovarian cancer are conflicting. Some studies did not find an association.[96,97] There is a relatively large volume of observational trial data that points to an association between HT and increased ovarian cancer risk, particularly with long-term use.[98-109] In the National Institutes of Health American Association of Retired Persons Diet and Health Cohort, no elevated risk of ovarian cancer was seen with less than 10 years of ET use, but a significantly increased risk was seen after 10 years.[107] One meta-analysis reported an increase in annual ovarian cancer risk for EPT of 1.11-fold (95% CI, 1.02-1.21), and a 1.28-fold (95% CI, 1.18-1.40) increase was reported for ET.[110] A second meta-analysis reported RRs of 1.24 (95% CI, 1.15-1.34) for cohort studies and 1.19 (95% CI, 1.02-1.40) for case-control studies with use of any HT.[111] The use of HT for less than 5 years was associated with a significant RR of 1.03, whereas use for more than 10 years was associated with an RR of 1.21 ($P < 0.05$ for both RRs). ET was associated with a higher risk of ovarian cancer than EPT.

In the WHI, the only RCT to date to study ovarian cancer, EPT was not associated with a statistically significant increase in ovarian cancer after a mean of 5.6 years of use.[112] There were 4.2 cases per 10,000 for HT users and 2.7 cases per 10,000 per year for the placebo group.

The association between ovarian cancer and EPT use beyond 5 years would fall into the rare- or very rare–risk category. Women at increased risk of ovarian cancer (eg, those with a family history or a *BRCA* mutation) should be counseled about this potential association.

Lung cancer

In a post hoc analysis of the EPT arm of the WHI that included data from a mean of 7.1 years of intervention plus approximately 1 year of postintervention follow-up (total mean years of data, 7.9), the incidence of non–small-cell lung cancer (which accounts for about 80% of lung cancer) was not significantly increased (HR, 1.28; 95% CI, 0.94-1.73; $P = 0.12$), but the number of lung cancer deaths (from non–small-cell lung cancer) increased (HR, 1.87; 95% CI, 1.22-2.88; $P = 0.004$), and the number of poorly differentiated and metastatic tumors increased in the treatment group (HR 1.87; 95% CI, 1.22-2.88; $P = 0.004$).[77] The cases were essentially limited to past and current smokers and to women older than 60 years. The absolute rates of death from non-small-cell lung cancer were small: nine per 10,000 per year on EPT and five per 10,000 on placebo. Because the WHI was not designed to assess lung cancer and chest imaging was not part of the study protocol, the findings are preliminary and require validation in further studies.

In the WHI ET trial, no increase in lung cancer incidence or mortality was observed in the treatment compared with the placebo group.[113] There was no significant treatment effect related to age. Mortality from lung cancer was increased in current smokers in both treatment and placebo groups compared with nonsmokers and former smokers.

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Reports from observational trials are mixed.[114-122] One large observational study reported an increase in incident lung cancer associated with increasing duration of EPT use (50% increase after 10 y of therapy); there was no association with duration of ET use.[123] One meta-analysis reported an increased risk of adenocarcinoma of the lung.[124] Another meta-analysis reported a possible protective effect against lung cancer for users of HT with the exception of current smokers.[125]

These findings underscore the need to encourage the cessation of smoking and possibly to increase surveillance in older smokers who are current or past users of EPT.

Mood and depression

For postmenopausal women without clinical depression, evidence is mixed concerning the effects of HT on mood. Several small short-term trials among middle-aged women with vasomotor symptoms suggested that HT improves mood, whereas other trial results showed no change. Progestogens in EPT may worsen mood in some women, possibly in those with a history of premenstrual syndrome, premenstrual depressive disorder, or clinical depression.

Only a few RCTs have examined the effects of HT in middle-aged or older women who have depression. One small RCT involving depressed perimenopausal and postmenopausal women found no short-term benefit from ET, but post hoc analyses revealed that higher estradiol levels were associated with decreased depressive symptoms in perimenopausal women but not postmenopausal women.[126] Two small RCTs support the antidepressant efficacy of short-term ET in depressed perimenopausal women,[127,128] whereas one RCT failed to demonstrate the antidepressant efficacy of ET in depressed women who were 5 to 10 years into postmenopause.[129] It is controversial whether ET might, in some circumstances, augment the antidepressant effects of selective serotonin reuptake inhibitors.[130,131]

Although HT might have a positive effect on mood and behavior, HT is not an antidepressant and should not be considered as such. Evidence is insufficient to support HT use in the treatment of depression.

Cognitive aging and dementia

Very small clinical trials support the use of ET for cognitive benefits when initiated immediately after surgical menopause.[132,133] To date, clinical trials of ET have demonstrated no substantial effect on episodic memory or executive function at the time of menopause.[134] Reports from the longitudinal Study of Women's Health Across the Nation suggest that natural menopause has a significant but small effect on some aspects of cognitive function that may be time limited. This effect is not explained by menopausal symptoms.[135,136] Recent literature suggests a transient negative effect of the menopausal transition on cognition, but it is a negligible longterm effect.[134,135]

The NHS found no benefit on cognitive function from longterm use of HT among women who had started HT in early menopause; rather, there was a suggestion of a more rapid cognitive decline among HT users.[137] Conversely, in the Study of Women's Health Across the Nation, women who initiated hormones (oral contraceptives or HT) after enrollment but before their final menstrual period and then discontinued the hormones had a beneficial cognitive effect, whereas women who initiated hormones after the final menstrual period had a detrimental effect on cognitive performance.[135]

For postmenopausal women older than 65 years, findings from several large well-designed clinical trials indicate that HT does not improve memory or other cognitive abilities and that EPT is harmful for memory.[138-140] The WHI Memory Study of women aged 65 to 79 years

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reported an increase in dementia incidence with HT use.[141] The estimate of dementia cases attributed to HT was 12 per 10,000 persons per year of ET use and 23 per 10,000 persons per year of EPT use. The effect was not statistically significant for ET but was for EPT and the combined ET and EPT groups.[141]

Evidence from the WHI Study of Cognitive Aging, an ancillary study of WHI and WHI Memory Study that enrolled women aged 66 years or older, indicated a worsening of verbal memory but a trend toward a positive effect on figural memory among women using EPT compared with those using placebo.[140] There are currently no placebo-controlled trial data comparing the effects of different progestogens on memory or dementia in younger or older postmenopausal women. Overall, the RCTs of ET demonstrate no adverse impact on memory. The WHI Study of Cognitive Aging found neither benefit nor persistent negative impact of HT on memory during a 2.7-year interval.[142]

A number of observational studies have reported associations between HT and reduced risk of developing Alzheimer disease (AD).[143] HT exposure in observational studies is more likely to involve ET use by younger women closer to menopause, suggesting an early window during which HT use might reduce AD risk. However, recall bias and the healthyuser bias may account for protective associations in the observational studies. Similarly, an increased risk of dementia observed with early oophorectomy, countered by use of estrogen until age 50 years,[144] may be at least partially caused by demographic differences between groups.[145] HT exposure in observational studies is also more likely to involve women on ET rather than EPT. For women with AD, limited clinical results suggest that ET has no substantial effect.

In summary, available data do not adequately address whether HT used soon after menopause increases or decreases the rate of cognitive decline or later dementia risk. In the absence of more definitive findings, HT cannot be recommended at any age for preventing or treating cognitive aging or dementia.

Premature menopause and primary ovarian insufficiency

Women experiencing premature menopause (age ≤40 y) or primary ovarian insufficiency (POI) are medically a distinctly different group from women who reach menopause at the median age of 51.3 years. Premature menopause and POI are associated with a lower risk of breast cancer and earlier onset of estrogen-related bone loss. Other conditions that have been associated with premature menopause, such as CHD and Parkinson disease, may be the result of other factors responsible for both premature menopause and the specific condition. For example, mutations found in the gene encoding mitochondrial DNA polymerase gamma have been reported to be associated with both premature menopause and Parkinson disease.[146]

Some observational reports suggest an increased risk of CHD with early natural or surgical menopause in the absence of HT and a reduced risk when HT is administered.[147] Analysis of the Framingham data revealed that women who had an earlier menopause also had more CHD risk factors.[148] The authors concluded that CHD risk factors may cause earlier menopause and not the converse. Both a history of heart disease and smoking have been associated with earlier menopause.[149] Another extensive analysis of three birth cohorts from three different countries concluded that there is no change in the rate of increase in CHD mortality at menopause. The rate of increase is constant during a woman's lifetime.[150]

The existing data regarding HT in women experiencing menopause at the median age should not be extrapolated to women experiencing premature menopause and initiating HT at that time. The well-documented safety of supraphysiologic doses of HT in the form of oral

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contraceptives in young women suggests that physiologic dosing of HT for women with POI or premature menopause would convey minimal risk. Given the potential harmful effects of estrogen deficiency on bone mass in young women who may still be building their peak bone mass and the severity of vasomotor symptoms in younger women, the benefits of HT are potentially greater in this age group (see “Osteoporosis”).

The lack of clinical trials on this topic necessitates clinical judgment. In the absence of contraindications, NAMS recommends the use of HT or oral contraceptives until the median age of natural menopause, with periodic reassessment.

Total mortality

The WHI trials are consistent with observational studies and meta-analyses[151] indicating that HT may reduce total mortality when initiated soon after menopause. The WHI suggests that both ET and EPT nonsignificantly reduce total mortality by 30% when initiated in women younger than 60 years and that when data from the ET and EPT arms were combined, that reduction was statistically significant.[36] There were 10 fewer deaths per 10,000 women aged 50 to 59 years, compared with 16 additional deaths among those aged 70 to 79 years.[36] The mortality advantage for younger women did not remain significant when evaluated by years since menopause.[36]

PRACTICAL THERAPEUTIC ISSUES

Class versus specific product effect

All estrogens have some common features and effects as well as potentially different properties. The same is true of all progestogens. However, in the absence of RCTs designed to compare clinical outcomes of various estrogens and progestogens, clinicians will be required to generalize the clinical trial results, tempered by emerging reports from observational studies (as addressed in individual sections of this report), for one agent to all agents within the same hormonal family. On a theoretical basis, however, there are likely to be differences within each family based on factors such as relative potency of the compound, androgenicity, glucocorticoid effects, bioavailability, and route of administration.

Progestogen indication

The primary menopause-related indication for progestogen use is to negate the increased risk of endometrial cancer from systemic ET use. All women with an intact uterus who use systemic ET should also be prescribed adequate progestogen. With occasional exceptions (eg, history of extensive endometriosis), postmenopausal women without a uterus should not be prescribed a progestogen with systemic ET.[152-154]

A progestogen is generally not indicated when ET is administered locally in a low dose for vaginal atrophy, although trials to date have been limited to only 1 year.[155] Although one 2-year study of the ultralow-dose estradiol patch found no statistically significant increase in endometrial hyperplasia,[156] intermittent progestogen probably should be used with longterm use of any systemic ET, including the ultralow-dose patch, which carries that recommendation in the product information sheet (see “Dose and route of administration”).

Concomitant progestogen may improve the efficacy of low-dose ET in treating vasomotor symptoms. Some women who use EPT may experience dysphoria from the progestogen component. A combination of estrogen with an estrogen agonist/antagonist is currently under investigation and may become an alternate option to progestogen.

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Dose and route of administration

The lowest effective dose of estrogen consistent with treatment goals, benefits, and risks for the individual woman should be the therapeutic goal, with an appropriate dose of progestogen added to counter the adverse effects of systemic ET on the uterus. Among the lower doses typically used when initiating systemic ET are 0.3 mg to 0.45 mg oral CE, 0.5 mg oral micronized 17A-estradiol, and 0.014 mg to 0.0375 mg transdermal 17A-estradiol patch. Low-dose formulations of estradiol are available in approved topical gels, creams, and sprays. Estrogen doses less than those traditionally prescribed (<0.625 mg CE) often require longer duration of treatment upon initiation to achieve maximal efficacy in reducing vasomotor symptoms.[157,158] Tailoring the dose to a woman's individual needs represents an appropriate strategy in HT management.

Lower HT doses generally have fewer adverse effects, such as breast tenderness and uterine bleeding, and may have a more favorable benefit-risk ratio than standard doses. In a nested case-control study from the UK General Practice Research database, the risk of stroke was not increased with low-dose transdermal estrogen (≤0.05 mg) but did increase with oral therapies and with higher transdermal doses.[159] Lower doses of HT have not been tested in long-term trials with clinical outcomes to support an assumed more favorable benefit-risk ratio.

All routes of administration of ET can effectively treat menopausal symptoms. Nonoral routes of administration including transdermal, vaginal, and intrauterine systems may offer both advantages and disadvantages compared with the oral route, but the long-term benefit-risk ratio has not been demonstrated in RCTs with clinical outcomes. There are differences related to the role of the first-pass hepatic effect, the hormone concentrations in the blood achieved by a given route, and the biologic activity of ingredients. With transdermal therapy, there is no significant increase in triglycerides, C-reactive protein, or sex hormone–binding globulin and little effect on blood pressure. With cutaneous therapies, caution should be exercised to avoid inadvertent transfer to children and animals.[160]

There is growing observational evidence that transdermal ET may be associated with a lower risk of deep vein thrombosis, stroke, and MI.[64,65,68,161]

There are multiple progestogen dosing-regimen options for endometrial safety. The dose varies based on the progestogen used and the estrogen dose, typically starting at the lowest effective doses of 1.5 mg medroxyprogesterone acetate, 0.1 mg norethindrone acetate, 0.5 mg drospirenone, or 100 mg micronized progesterone. Different doses may have different health outcomes. A long-term Finnish observational study reported that continuous use of EPT reduced the risk of endometrial neoplasia compared to no use of HT, and sequential progestogen therapy with ET increased the risk, particularly with long-cycle progestogen.[162] In this study, all progestogens performed similarly within a given regimen.

Oral progestogens, combined with systemic estrogen, and combined progestogen-estrogen matrix patches have demonstrated endometrial protection and are government approved. A progestin-containing intrauterine system and a vaginal progesterone cream are government approved for use in premenopausal women; however, neither has been approved for use in postmenopausal women. A small study reported that when used with systemic ET in perimenopausal and postmenopausal women, the progestin-containing intrauterine system was found to provide endometrial protection equivalent to protection provided by systemic progestogen administered continuously and superior protection compared with progestogen given sequentially.[163]

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Bioidentical hormones

The term bioidentical hormones is most often used to describe custom-made HT formulations (called bioidentical hormone therapy [BHT]) that are compounded for an individual according to a healthcare provider's prescription. The term is used by proponents of BHT to convey that the hormones they use are identical to the hormones made by the ovaries. In that regard, the term can also be used to refer to many well-tested, government-approved, brand-name HT products containing hormones chemically identical to those produced by women (primarily in the ovaries), such as 17A-estradiol and progesterone.

Custom-compounding of HT may combine several hormones (eg, estradiol, estrone, and estriol) and use nonstandard routes of administration (eg, subdermal implants). Some of the hormones are not government approved (estriol) or monitored and some of the compounded therapies contain non-hormonal ingredients (eg, dyes, preservatives) that some women cannot tolerate. Use of BHT has escalated in recent years, along with the use of salivary hormone testing, which has been proven to be inaccurate and unreliable. There may be increased risks to the women using these products. Custom-compounded formulations, including BHT, have not been tested for efficacy or safety; product information is not consistently provided to women along with their prescription, as is required with commercially available HT; and batch standardization and purity may be uncertain. The dosing of compounded progesterone is particularly difficult to assess because the levels in serum, saliva, and tissue are markedly different.[164] Custom-compounded drug formulations are not government approved.

The US Food and Drug Administration has ruled that some compounding pharmacies have made claims about the safety and effectiveness of BHT unsupported by clinical trial data and considered to be false and misleading.[165] Pharmacies have been instructed not to use estriol without an investigational new drug authorization. The Food and Drug Administration also states that there is no scientific basis for using saliva testing to adjust hormone levels.

NAMS recommends that BHT products include a patient package insert identical to that required for products that have government approval. In the absence of efficacy and safety data for BHT, the generalized benefit-risk ratio data of commercially available HT products should apply equally to BHT. For most women, government-approved HT will provide appropriate therapy without the risks of custom preparations. Therefore, NAMS does not generally recommend compounded EPT or ET unless necessary because of allergies to ingredients contained in government-approved products.

TREATMENT ISSUES

Duration of use

One of the most challenging issues regarding HT is the duration of use. Long-term follow-up data from the WHI have clarified the increased risk of breast cancer and breast cancer mortality with 4 to 5 years of EPT used at the time of menopause and a slightly later onset of breast cancer if used after a hiatus in estrogen exposure.[74,78] Regarding ET, there was no increase in risk of breast cancer with early postmenopausal use in the WHI or NHS, and there was decrease in breast cancer incidence when used after a hiatus in estrogen exposure in the WHI.[76,85] Long-term use of ET (15-20 y in the NHS) can be expected to increase breast cancer, but to a lesser degree than EPT.[85]

Potential coronary artery disease and CHD benefits were also seen with early use of ET. In the WHI ET trial, women ages 50 to 59 years had a significantly lower risk of combined endpoints including CHD and total MI and no elevation in breast cancer risk.[28]

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Observational studies suggest that longer duration of HT use is associated with a reduced risk of CHD and related mortality.[166] The WHI RCTs and observational study suggest a pattern of lower risk of CHD among women who used HT for 5 or more years,[40] but this is not conclusive and should be considered in light of other factors altered by duration of therapy, such as breast cancer. In contrast, both ET and EPT are associated with an initial increase in CHD risk among women who are more distant from menopause at the time of HT initiation.[38,167,168]

These findings allow for longer duration of use with ET based on a woman's symptoms, preferences, and current benefit-risk profile.

Provided that the woman is well aware of the potential benefits and risks and has clinical supervision, extending EPT use with the lowest effective dose is acceptable under some circumstances, including (1) for the woman who has determined that the benefits of menopause symptom relief outweigh risks, notably after failing an attempt to stop EPT, and (2) for the woman at high risk of fracture for whom alternate therapies are not appropriate or cause unacceptable adverse effects.

Discontinuation of HT

Data from long-term follow-up of women who discontinued ET and EPT have increased our understanding of the sequelae of discontinuing HT. In the WHI, women in the EPT group who had stopped HT for 3 years had a rate of cardiovascular events, fractures, and colon cancers equivalent to that of women who had been assigned to placebo.[27] The only statistical difference was an increase in the rates of all cancer in women who had been assigned to EPT, with an excess of 30 cancers per 10,000 women per year of EPT, including a number of fatal lung cancers.[27,77] For women without a uterus, when followed for 3 years after stopping ET, there was no overall increased or decreased risk of CHD, deep-vein thrombosis, stroke, hip fracture, colorectal cancer, or total mortality. A statistically significant decreased risk of invasive breast cancer persisted (8 fewer cases/10,000 women).[28] Discontinuance of HT will lead to a transient increased incidence of fracture, including hip fracture.[169] After 4 years of follow-up in the ET arm of the WHI, cumulative fracture rates were similar for both ET and placebo groups.[28]

HRs for all-cause mortality, reflecting the balance of all of the above and other outcomes, tended to be neutral in both the EPT and ET arms of the WHI (HR, 0.98 and 1.04, respectively). During the 3-year postintervention phase of the EPT trial, mortality rates were borderline elevated (HR, 1.15; 95% CI, 0.95-1.39) primarily because of the aforementioned increase in cancer. During the entire EPT follow-up period (active treatment plus poststopping phases), the HR for allcause mortality in the EPT arm was 1.04 (95% CI, 0.91-1.18)[27] and 1.02 (95% CI, 0.91-1.15) in the ET arm.[28]

Regarding other outcomes after discontinuance of EPT, an initial analysis of data from the National Cancer Institute's Surveillance, Epidemiology, and End Results registries showed that the age-adjusted incidence rate of breast cancer in women in the United States fell sharply (by 6.7%) in 2003, as compared with the rate in 2002.[170] The decrease was evident only in women who were 50 years or older and was more evident in cancers that were estrogen receptor positive, which represent most breast cancers. It was theorized that the drop could be related to the large number of women discontinuing HT after the termination of the EPT arm of the WHI.

Vasomotor symptoms have an approximately 50% chance of recurring when HT is discontinued, independent of age and duration of use.[171,172] In one RCT, tapering the dose of HT for 1 month and abruptly discontinuing HT had a similar impact on vasomotor

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symptoms.[173] The decision to continue HT should be individualized based on the severity of symptoms and current benefit-risk ratio considerations.

CONCLUSIONS AND RECOMMENDATIONS

- Individualization is of key importance in the decision to use HT and should incorporate the woman's health and quality of life priorities as well as her personal risk factors, such as risk of venous thrombosis, CHD, stroke, and breast cancer.
- The recommendation for duration of therapy differs for EPT and ET. For EPT, duration is limited by the increased risk of breast cancer and breast cancer mortality associated with 3 to 5 years of use; for ET, a more favorable benefitrisk profile was observed during a mean of 7 years of use and 4 years of follow-up, a finding that allows more flexibility in duration of use.
- ET is the most effective treatment of symptoms of vulvar and vaginal atrophy; low-dose, local vaginal ET is advised when only vaginal symptoms are present.
- Women with premature or early menopause who are otherwise appropriate candidates for HT can use HT at least until the median age of natural menopause (age 51 y). Longer duration of treatment can be considered if needed for symptom management.
- Although ET did not increase breast cancer risk in the WHI, there is a lack of safety data supporting the use of ET in breast cancer survivors, and one RCT reported a higher increase in breast cancer recurrence rates.
- Both transdermal and low-dose oral estrogen have been associated with lower risks of VTE and stroke than standard doses of oral estrogen, but RCT evidence is not yet available.

SUMMARY

In the decade since the first publication of results from the WHI EPT study, much has been learned. There is a growing body of evidence that HT formulation, route of administration, and the timing of therapy produce different effects. Constructing an individual benefit-risk profile is essential for every woman considering any HT regimen. A woman's interest in using HT will vary depending on her individual situation, particularly the severity of her menopausal symptoms and their effect on her QOL. The absolute risks known to date for use of HT in healthy women ages 50 to 59 years are low. In contrast, long-term HT or HT initiation in older women is associated with greater risks.

Recommendations for duration of use differ between ET and EPT. Given the more favorable safety profile of ET, it could be considered for longer duration of therapy in the absence of adverse effects and risk factors. Women experiencing premature menopause are at increased risk of osteoporosis and, possibly, cardiovascular disease, and they often experience more intense symptoms than do women reaching menopause at the median age. Therefore, HT generally is advised for these young women until the median age of menopause when treatment should be reassessed.

Additional research is needed to understand the different effects of ET and EPT and how they apply to individual women. Further research is also needed to more clearly delineate the role of aging versus menopause and the effects of genetics, lifestyle, and individual clinical characteristics on midlife women's health.

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REFERENCES

1. Barnabei VM, Cochrane BB, Aragaki AK, et al. for the Women's Health Initiative Investigators. Menopausal symptoms and treatment-related effects of estrogen and progestin in the Women's Health Initiative. Obstet Gynecol. 2005; 105:1063–1073. [PubMed: 15863546]
2. National Institutes of Health. State-of-the-Science Panel. National Institutes of Health State-of-the-Science Conference statement: management of menopause-related symptoms. Ann Intern Med. 2005; 142:1003–1013. [PubMed: 15968015]
3. Maclennan A, Broadbent J, Lester S, Moore V. Oral oestrogen and combined oestrogen/progestogen therapy versus placebo for hot flushes. Cochrane Database Sys Rev. 2004; 4:CD002978.
4. Schiff I, Tulchinsky D, Cramer D, Ryan KJ. Oral medroxyprogesterone in the treatment of postmenopausal symptoms. JAMA. 1980; 244:1443–1445. [PubMed: 6775094]
5. Suckling J, Kennedy R, Lethaby A, Roberts H. Local oestrogen for vaginal atrophy in postmenopausal women. Cochrane Database Sys Rev. 2006; 4:CD001500.
6. The North American Menopause Society. The role of local vaginal estrogen for treatment of vaginal atrophy in postmenopausal women: 2007 position statement of The North American Menopause Society. Menopause. 2007; 14:357–369.
7. Sturdee DW, Panay N, on behalf of the International Menopause Society Writing Group. Recommendations for the management of postmenopausal vaginal atrophy. Climacteric. 2010; 13:509–522. [PubMed: 20883118]
8. Wierman ME, Nappi RE, Avis N, et al. Endocrine aspects of women's sexual function. J Sex Med. 2010; 7:561–585. [PubMed: 20092453]
9. Gass M, Cochrane BB, Larson JC, et al. Patterns and predictors of sexual activity among women in the Hormone Therapy trials of the Women's Health Initiative. Menopause. 2011; 18:1160–1171. [PubMed: 21983008]
10. Davis SR, Guay AT, Shifren JL, Mazer NA. Endocrine aspects of female sexual dysfunction. J Sex Med. 2004; 1:82–86. [PubMed: 16422987]
11. Cody JD, Richardson K, Moehrer B, Hextall A, Glazener CMA. Oestrogen therapy for urinary incontinence in postmenopausal women. Cochrane Database Sys Rev. 2009; 4:CD001405.
12. Nelken RS, Ozel BZ, Leegant AR, Felix JC, Mishell DR. Randomized trial of estradiol vaginal ring versus oral oxybutynin for the treatment of overactive bladder. Menopause. 2011; 18:962–966. [PubMed: 21532512]
13. Townsend MK, Curhan GC, Resnick NM, Grodstein F. The incidence of urinary incontinence across Asian, black, and white women in the United States. Am J Obstet Gynecol. 2010; 202:378.e1–378.e7. [PubMed: 20042169]
14. Hendrix SL, Cochrane BB, Nygaard IE, et al. Effects of estrogen with and without progestin on urinary incontinence. JAMA. 2005; 293:935–948. [PubMed: 15728164]
15. Grady D, Brown JS, Vittinghoff E, Applegate W, Varner E, Snyder T, for the HERS Research Group. Postmenopausal hormones and incontinence: the Heart and Estrogen/Progestin Replacement Study. Obstet Gynecol. 2001; 97:116–120. [PubMed: 11152919]
16. Waetjen LE, Brown JS, Vittinghoff E, et al. for the Ultra Low Dose Transdermal estRogen Assessment (ULTRA) Study. The effect of ultralow-dose transdermal estradiol on urinary incontinence in postmenopausal women. Obstet Gynecol. 2005; 106:946–952. [PubMed: 16260511]
17. Maalouf NM, Sato AH, Welch BJ, et al. Postmenopausal hormone use and the risk of nephrolithiasis: results from the Women's Health Initiative hormone therapy trials. Arch Intern Med. 2010; 170:1678–1685. [PubMed: 20937929]
18. Raz R, Stamm WE. A controlled trial of intravaginal estriol in postmenopausal women with recurrent urinary tract infections. N Engl J Med. 1993; 329:753–756. [PubMed: 8350884]
19. Eriksen BC. A randomized, open, parallel-group study on the preventive effect of an estradiol-releasing vaginal ring (Estring) on recurrent urinary tract infections in postmenopausal women. Am J Obstet Gynecol. 1999; 80:1072–1079. [PubMed: 10329858]
20. Barnabei VM, Grady D, Stovall DW, et al. Menopausal symptoms in older women and the effects of treatment with hormone therapy. Obstet Gynecol. 2002; 100:1209–1218. [PubMed: 12468165]

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21. Hlatky MA, Boothroyd D, Vittinghoff E, Sharp P, Whooley MA, for the Heart and Estrogen/progestin Replacement Study (HERS) Research Group. Quality-of-life and depressive symptoms in postmenopausal women after receiving hormone therapy: results from the Heart and Estrogen/Progestin Replacement Study (HERS) trial. JAMA. 2002; 287:591–597. [PubMed: 11829697]
22. Hays J, Ockene JK, Brunner RL, et al. for the Women's Health Initiative Investigators. Effects of estrogen plus progestin on health-related quality of life. N Engl J Med. 2003; 348:1839–1854. [PubMed: 12642637]
23. Brunner RL, Gass M, Aragaki A, et al. for the Women's Health Initiative Investigators. Effects of conjugated equine estrogen on health-related quality of life in postmenopausal women with hysterectomy: results from the Women's Health Initiative Randomized Clinical Trial. Arch Intern Med. 2005; 165:1976–1986. [PubMed: 16186467]
24. Michael YL, Gold R, Manson JE, Keast EM, et al. Hormone therapy and physical function change among older women in the Women's Health Initiative: a randomized controlled trial. Menopause. 2010; 17:295–302. [PubMed: 19858764]
25. Cauley JA, Robbins J, Chen Z, et al. for the Women's Health Investigators. Effects of estrogen plus progestin on risk of fracture and bone mineral density: the Women's Health Initiative Randomized Trial. JAMA. 2003; 290:1729–1738. [PubMed: 14519707]
26. Jackson RD, LaCroix AZ, Gass M, et al. for the Women's Health Investigators. Calcium plus vitamin D supplementation and the risk of fractures. N Engl J Med. 2006; 354:669–683. [PubMed: 16481635]
27. Heiss G, Wallace R, Anderson GL, et al. for the WHI Investigators. Health risks and benefits 3 years after stopping randomized treatment with estrogen and progestin. JAMA. 2008; 299:1036–1045. [PubMed: 18319414]
28. LaCroix AZ, Chlebowski RT, Manson JE, et al. for the WHI Investigators. Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. JAMA. 2011; 305:1305–1314. [PubMed: 21467283]
29. Vesco, KK.; Marshall, LM.; Nelson, HD.; et al. for the Study of Osteoporotic Fractures. Surgical menopause and nonvertebral fracture risk among older U.S. women [e-pub]. Menopause: 2011.
30. Jacoby VL, Grady D, Wactawski-Wende J, et al. Oophorectomy vs ovarian conservation with hysterectomy: cardiovascular disease, hip fracture, and cancer in the Women's Health Initiative Observational Study. Arch Intern Med. 2011; 171:760–768. [PubMed: 21518944]
31. Ouyang P, Michos ED, Karas RH. Hormone replacement therapy and the cardiovascular system lessons learned and unanswered questions. J Am Coll Cardiol. 2006; 47:1741–1753. [PubMed: 16682298]
32. Toh S, Hernández-Díaz S, Logan R, Rossouw JE, Hernán MA. Coronary heart disease in postmenopausal recipients of estrogen plus progestin therapy: does the increased risk ever disappear? A randomized trial. Ann Intern Med. 2010; 152:211–217. [PubMed: 20157135]
33. Barrett-Connor E, Bush TL. Estrogen and coronary heart disease in women. JAMA. 1991; 265:1861–1867. [PubMed: 2005736]
34. Matthews KA, Kuller LH, Wing RR, Meilahn EN, Plantinga P. Prior to use of estrogen replacement therapy, are users healthier than nonusers? Am J Epidemiol. 1996; 143:971–978. [PubMed: 8629615]
35. Barrett-Connor E, Wenger NK, Grady D, et al. Coronary heart disease in women, randomized clinical trials, HERS and RUTH. Maturitas. 1998; 31:1–7. [PubMed: 10091198]
36. Rossouw JE, Prentice RL, Manson JE, et al. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. JAMA. 2007; 297:1465–1477. [PubMed: 17405972]
37. Prentice RL, Langer R, Stefanick ML, et al. for Women's Health Initiative Investigators. Combined postmenopausal hormone therapy and cardiovascular disease: toward resolving the discrepancy between observational studies and the Women's Health Initiative clinical trial. Am J Epidemiol. 2005; 162:404–414. [PubMed: 16033876]
38. Hsia J, Langer RD, Manson JE, et al. for the Women's Health Initiative Investigators. Conjugated equine estrogens and coronary heart disease: the Women's Health Initiative. Arch Intern Med. 2006; 166:357–365. [PubMed: 16476878]

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39. Salpeter SR, Walsh JM, Greyber E, Salpeter EE. Brief report: coronary heart disease events associated with hormone therapy in younger and older women. A meta-analysis. J Gen Intern Med. 2006; 21:363–366. [PubMed: 16686814]
40. Prentice RL, Manson JE, Langer RD, et al. Benefits and risks of postmenopausal hormone therapy when it is initiated soon after menopause. Am J Epidemiol. 2009; 170:12–23. [PubMed: 19468079]
41. Akhrass F, Evans AT, Wang Y, et al. Hormone replacement therapy is associated with less coronary atherosclerosis in postmenopausal women. J Clin Endocrinol Metab. 2003; 88:5611–5614. [PubMed: 14671142]
42. Barrett-Connor E, Laughlin GA. Hormone therapy and coronary artery calcification in asymptomatic postmenopausal women: the Rancho Bernardo Study. Menopause. 2005; 12:40–48. [PubMed: 15668599]
43. Schisterman EF, Gallagher AM, Bairey Merz CN, et al. The association of hormone replacement therapy and coronary calcium as determined by electron beam tomography. J Womens Health Gend Based Med. 2002; 11:631–638. [PubMed: 12396895]
44. Manson JE, Allison MA, Rossouw JE, et al. for the WHI and WHI-CACS Investigators. Estrogen therapy and coronary-artery calcification. N Engl J Med. 2007; 356:2591–2602. [PubMed: 17582069]
45. Tremollieres FA, Cigagna F, Alquier C, Cauneille C, Pouilles J, Ribot C. Effect of hormone replacement therapy on age-related increase in carotid artery intima-media thickness in postmenopausal women. Atherosclerosis. 2000; 153:81–88. [PubMed: 11058702]
46. Grady D, Rubin SM, Petitti DB, et al. Hormone therapy to prevent disease and prolong life in postmenopausal women. Ann Intern Med. 1992; 117:1016–1037. [PubMed: 1443971]
47. Barrett Connor E, Grady D. Hormone replacement therapy, heart disease, and other considerations. Annu Rev Public Health. 1998; 19:55–72. [PubMed: 9611612]
48. Hodis HN, Mack WJ, Lobo RA, Shoupe D, et al. for the Estrogen in Prevention of Atherosclerosis Trial Research Group. Estrogen in the prevention of atherosclerosis: a randomized, double-blind, placebo-controlled trial. Ann Intern Med. 2001; 135:939–953. [PubMed: 11730394]
49. Angerer P, Stork S, Kothny W, Schmitt P, von Schacky C. Effect of oral postmenopausal hormone replacement on progression of atherosclerosis: a randomized, controlled trial. Arterioscler Thromb Vasc Biol. 2001; 21:262–268. [PubMed: 11156863]
50. Wassertheil-Smoller S, Hendrix SL, Limacher M, et al. for the WHI Investigators. Effect of estrogen plus progestin on stroke in postmenopausal women. The Women's Health Initiative: a randomized trial. JAMA. 2003; 289:2673–2684. [PubMed: 12771114]
51. Hendrix SL, Wassertheil-Smoller S, Johnson KC, et al. for the Women's Health Initiative Investigators. Effects of estrogen plus progestin on stroke in the Women's Health Initiative: a randomized trial. Circulation. 2006; 113:2425–2434. [PubMed: 16702472]
52. Simon JA, Hsia J, Cauley JA, et al. Postmenopausal hormone therapy and risk of stroke: the Heart and Estrogen-progestin Replacement Study (HERS). Circulation. 2001; 103:638–642. [PubMed: 11156873]
53. Viscoli CM, Brass LM, Kernan WN, Sarrel PM, Suissa S, Horwitz RI. A clinical trial of estrogen-replacement therapy after ischemic stroke. N Engl J Med. 2001; 345:1243–1249. [PubMed: 11680444]
54. Vickers MR, MacLennan AH, Lawton B, et al. Main morbidities recorded in Women's International Study of long Duration Oestrogen after Menopause (WISDOM): a randomised controlled trial of hormone replacement therapy in postmenopausal women. BMJ. 2007; 335:239–251. [PubMed: 17626056]
55. Grodstein F, Manson JE, Stampfer MJ, Rexrode K. Postmenopausal hormone therapy and stroke: role of time since menopause and age at initiation of hormone therapy. Arch Intern Med. 2008; 168:861–866. [PubMed: 18443262]
56. Arana A, Varas C, González-Pérez A, Gutiérrez L, Bjerrum L, García Rodríguez LA. Hormone therapy and cerebrovascular events: a population-based nested case-control study. Menopause. 2006; 13:730–736. [PubMed: 16946686]

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57. Lobo RA. The risk of stroke in postmenopausal women receiving hormonal therapy. Climacteric. 2009; 12:81–85. [PubMed: 19811248]
58. Lisabeth L, Bushnell C. Stroke risk in women: the role of menopause and hormone therapy. Lancet Neurol. 2012; 11:82–91. [PubMed: 22172623]
59. Canonico M, Oger E, Plu-Bureau G, et al. for the Estrogen and Thromboembolism Risk (ESTHER) Study Group. Hormone therapy and venous thromboembolism among postmenopausal women. Circulation. 2007; 115:840–845. [PubMed: 17309934]
60. Cushman M, Kuller LH, Prentice R, et al. for the Women's Health Initiative Investigators. Estrogen plus progestin and risk of venous thrombosis. JAMA. 2004; 292:1573–1580. [PubMed: 15467059]
61. Curb JD, Prentice RL, Bray PF, et al. Venous thrombosis and conjugated equine estrogen in women without a uterus. Arch Intern Med. 2006; 166:772–780. [PubMed: 16606815]
62. Høibraaten E, Qvigstad E, Arnesen H, Larsen S, Wickstrøm E, Sandset PM. Increased risk of recurrent venous thromboembolism during hormone replacement therapy—results of the randomized, double-blind, placebo-controlled Estrogen in Venous Thromboembolism Trial (EVTET). Thromb Haemost. 2000; 84:961–967. [PubMed: 11154141]
63. Herrington DM, Vittinghoff E, Howard TD, et al. Factor V Leiden, hormone replacement therapy, and risk of venous thromboembolic events in women with coronary disease. Arterioscler Thromb Vasc Biol. 2002; 22:1012–1017. [PubMed: 12067913]
64. Scarabin PY, Oger E, Plu-Bureau G, on behalf of the EStrogen and THromboEmbolism Risk (ESTHER) Study Group. Differential association of oral and transdermal oestrogen-replacement therapy with venous thromboembolism risk. Lancet. 2003; 362:428–432. [PubMed: 12927428]
65. Canonico M, Plu-Bureau G, Lowe GD, Scarabin PY. Hormone replacement therapy and risk of venous thromboembolism in postmenopausal women: systematic review and meta-analysis. BMJ. 2008; 336:1227–1231. [PubMed: 18495631]
66. Laliberte F, Dea K, Duh MS, Kahler KH, Rolli M, Lefebvre P. Does the route of administration for estrogen hormone therapy impact the risk of venous thromboembolism? Estradiol transdermal system versus oral estrogen-only hormone therapy. Menopause. 2011; 17:1052–1059. [PubMed: 21775912]
67. Canonico M, Alhenc-Gelas M, Plu-Bureau G, Olie V, Scarabin PY. Activated protein C resistance among postmenopausal women using transdermal estrogens: importance of progestogen. Menopause. 2010; 17:1122–1127. [PubMed: 20613675]
68. Canonico M, Plu-Bureau G, Scarabin PY. Progestogens and venous thromboembolism among postmenopausal women using hormone therapy. Maturitas. 2011; 70:354–360. [PubMed: 22024394]
69. Margolis KL, Bonds DE, Rodabough RJ, et al. Effect of oestrogen plus progestin on the incidence of diabetes inpostmenopausal women: results from the Women's Health Initiative Hormone Trial. Diabetologia. 2004; 47:1175–1187. [PubMed: 15252707]
70. Kanaya AM, Herrington D, Vittinghoff E, et al. Glycemic effects of postmenopausal hormone therapy: the Heart and Estrogen/Progestin Replacement Study. A randomized, doubleblind, placebo-controlled trial. Ann Intern Med. 2003; 138:1–9. [PubMed: 12513038]
71. Bonds DE, Lasser N, Qi L, et al. The effect of conjugated equine oestrogen on diabetes incidence: the Women's Health Initiative randomised trial. Diabetologia. 2006; 49:459–468. [PubMed: 16440209]
72. Espeland MA, Hogan PE, Fineberg SE, et al. Effect of postmenopausal hormone therapy on glucose and insulin concentrations. Diabetes Care. 1998; 21:1589–1595. [PubMed: 9773716]
73. Grady D, Gebretsadik T, Kerlikowske K, Ernster V, Petitti D. Hormone replacement therapy and endometrial cancer risk: a meta-analysis. Obstet Gynecol. 1995; 85:304–313. [PubMed: 7824251]
74. Chlebowski RT, Hendrix SL, Langer RD, et al. for the WHI Investigators. Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. JAMA. 2003; 289:3243–3253. [PubMed: 12824205]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

75. Fournier A, Mesrine S, Boutron-Ruault MC, Clavel-Chapelon F. Estrogen-progestogen menopausal hormone therapy and breast cancer: does delay from menopause onset to treatment initiation influence risks? J Clin Oncol. 2009; 27:5138–5143. [PubMed: 19752341]

76. Stefanick ML, Anderson GL, Margolis KL, et al. for the WHI Investigators. Effects of conjugated equine estrogens on breast cancer and mammography screening in postmenopausal women with hysterectomy. JAMA. 2006; 295:1647–1657. [PubMed: 16609086]

77. Chlebowski RT, Schwartz AG, Wakelee H, et al. for the Women's Health Initiative Investigators. Oestrogen plus progestin and lung cancer in postmenopausal women (Women's Health Initiative trial): a post-hoc analysis of a randomised trial. Lancet. 2009; 374:1243–1251. [PubMed: 19767090]

78. Chlebowski RT, Anderson GL, Gass M, et al. Estrogen plus progestin and breast cancer incidence and mortality in postmenopausal women. JAMA. 2010; 304:1684–1692. [PubMed: 20959578]

79. Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. JAMA. 2002; 288:321–333. [PubMed: 12117397]

80. Prentice RL, Chlebowski RT, Stefanick ML, et al. Conjugated equine estrogens and breast cancer risk in the Women's Health Initiative Clinical Trial and Observational Study. Am J Epidemiol. 2008; 167:1407–1415. [PubMed: 18448442]

81. Anderson GL, Chlebowski RT, Rossouw JE, et al. Prior hormone therapy and breast cancer risk in the Women's Health Initiative randomized trial of estrogen plus progestin. Maturitas. 2006; 55:103–115. [PubMed: 16815651]

82. Beral V, Reeves G, Bull D, Green J, for the Million Women Study Collaborators. Breast cancer risk in relation to the interval between menopause and starting hormone therapy. J Natl Cancer Inst. 2011; 103:296–305. [PubMed: 21278356]

83. Jordan VC, Ford LG. Paradoxical clinical effect of estrogen on breast cancer risk: a 'new' biology of estrogen-induced apoptosis. Cancer Prev Res (Phila). 2011; 4:633–637. [PubMed: 21478501]

84. Colditz GA, Hankinson SE, Hunter DJ, et al. The use of estrogens and progestins and the risk of breast cancer in postmenopausal women. N Engl J Med. 1995; 332:1589–1593. [PubMed: 7753136]

85. Colditz GA, Rosner B. Cumulative risk of breast cancer to age 70 years according to risk factor status: data from the Nurses' Health Study. Am J Epidemiol. 2000; 152:950–964. [PubMed: 11092437]

86. Chen W, Manson J, Hankinson S, et al. Unopposed estrogen therapy and the risk of invasive breast cancer. Arch Intern Med. 2006; 166:1027–1032. [PubMed: 16682578]

87. World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception. Acute myocardial infarction and combined oral contraceptives: results of an international multicentre case-control study. Lancet. 1997; 349:1202–1209. [PubMed: 9130941]

88. Col NF, Kim JA, Chlebowski RT. Menopausal hormone therapy after breast cancer: a meta-analysis and critical appraisal of the evidence. Breast Cancer Res. 2005; 7:R535–R540. [PubMed: 15987460]

89. Durna E, Wren B, Heller G, Leader L, Sjoblom P, Eden J. Hormone replacement therapy after a diagnosis of breast cancer: cancer recurrence and mortality. Med J Aust. 2002; 177:347–351. [PubMed: 12358575]

90. Decker DA, Pettinga JE, VanderVelde N, Huang RR, Kestin L, Burdakin JH. Estrogen replacement therapy in breast cancer survivors: a matched-controlled series. Menopause. 2003; 10:277–285. [PubMed: 12851510]

91. Beckmann MW, Jap D, Djahansouzi S, et al. Hormone replacement therapy after treatment of breast cancer: effects on postmenopausal symptoms, bone mineral density and recurrence rates. Oncology. 2001; 60:199–206. [PubMed: 11340370]

92. Marttunen M, Hietanen P, Pyrhonen S, Tiitinen A, Ylikorkala O. A prospective study on women with a history of breast cancer and with or without estrogen replacement therapy. Maturitas. 2001; 39:217–225. [PubMed: 11574181]

93. DiSaia P, Brewster W, Ziogas A, Anton-Culver H. Breast cancer survival and hormone replacement therapy: a cohort analysis. Am J Clin Oncol. 2000; 23:541–545. [PubMed: 11202792]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

94. Ursic-Vrscaj M, Bebar S. A case-control study of hormone replacement therapy after primary surgical breast cancer treatment. Eur J Surg Oncol. 1999; 25:146–151. [PubMed: 10218456]

95. Holmberg L, Iversen OE, Rudenstam CM, et al. for the HABITS Study Group. Increased risk of recurrence after hormone replacement therapy in breast cancer survivors. J Natl Cancer Inst. 2008; 100:475–482. [PubMed: 18364505]

96. Kaufman DW, Kelly JP, Welch WR, et al. Noncontraceptive estrogen use and epithelial ovarian cancer. Am J Epidemiol. 1989; 130:1142–1151. [PubMed: 2589307]

97. Sit AS, Modugno F, Weissfeld JL, Berga SL, Ness RB. Hormone replacement therapy formulations and risk of epithelial ovarian carcinoma. Gynecol Oncol. 2002; 86:118–123. [PubMed: 12144815]

98. Weiss NS, Lyon JL, Krishnamurthy S, Dietert SE, Liff JM, Daling JR. Noncontraceptive estrogen use and the occurrence of ovarian cancer. J Natl Cancer Inst. 1982; 68:95–98. [PubMed: 6948131]

99. Purdie DM, Bain CJ, Siskind V, et al. Hormone replacement therapy and risk of epithelial ovarian cancer. Br J Cancer. 1999; 81:559–563. [PubMed: 10507786]

100. Risch HA. Estrogen replacement therapy and risk of epithelial ovarian cancer. Gynecol Oncol. 1996; 63:254–257. [PubMed: 8910636]

101. Rodriguez C, Patel AV, Calle EE, Jacob EJ, Thun MJ. Estrogen replacement therapy and ovarian cancer mortality in a large prospective study of US women. JAMA. 2001; 285:1460–1465. [PubMed: 11255422]

102. Lacey JV Jr, Mink PJ, Lubin JH, et al. Menopausal hormone replacement therapy and risk of ovarian cancer. JAMA. 2002; 288:334–341. [PubMed: 12117398]

103. Riman T, Dickman PW, Nilsson S, et al. Hormone replacement therapy and the risk of invasive epithelial ovarian cancer in Swedish women. J Natl Cancer Inst. 2002; 94:497–504. [PubMed: 11929950]

104. Glud E, Kjaer SK, Thomsen BL, et al. Hormone therapy and the impact of estrogen intake on the risk of ovarian cancer. Arch Intern Med. 2004; 164:2253–2259. [PubMed: 15534163]

105. Moorman PG, Schildkraut JM, Calingaert B, Halabi S, Berchuck A. Menopausal hormones and risk of ovarian cancer. Am J Obstet Gynecol. 2005; 76:82.

106. Folsom AR, Anderson JP, Ross JA. Estrogen replacement therapy and ovarian cancer. Epidemiology. 2004; 15:100–104. [PubMed: 14712153]

107. Lacey JV Jr, Brinton LA, Leitzmann, et al. Menopausal hormone therapy and ovarian cancer risk in the National Institutes of Health-AARP Diet and Health Study Cohort. J Natl Cancer Inst. 2006; 98:1397–1405. [PubMed: 17018786]

108. Rossing MA, Cushing-Haugen KL, Wicklund KG, Doherty JA, Weiss NS. Menopausal hormone therapy and risk of epithelial ovarian cancer. Cancer Epidemiol Biomarkers Prev. 2007; 16:2548–2556. [PubMed: 18086757]

109. Mørch LS, Løkkegaard E, Andreasen AH, Krüger-Kjaer S, Lidegaard O. Hormone therapy and ovarian cancer. JAMA. 2009; 302:298–305. [PubMed: 19602689]

110. Greiser CM, Greiser EM, Dören M. Menopausal hormone therapy and risk of ovarian cancer: systematic review and meta-analysis. Hum Reprod Update. 2007; 13:453–463. [PubMed: 17573406]

111. Zhou B, Sun Q, Cong R, et al. Hormone replacement therapy and ovarian cancer risk: a meta-analysis. Gynecol Oncol. 2008; 108:641–651. [PubMed: 18221779]

112. Anderson GL, Judd HL, Kaunitz AM, et al. for the Women's Health Initiative Investigators. Effects of estrogen plus progestin on gynecologic cancers and associated diagnostic procedures. The Women's Health Initiative Randomized Trial. JAMA. 2003; 290:1739–1748. [PubMed: 14519708]

113. Chlebowski RT, Anderson GL, Manson JE, et al. Lung cancer among postmenopausal women treated with estrogen alone in the Women's Health Initiative Randomized Trial. J Natl Cancer Inst. 2010; 102:1–9.

114. Rodriguez C, Spencer Feigelson H, et al. Postmenopausal hormone therapy and lung cancer risk in the cancer prevention study II nutrition cohort. Cancer Epidemiol Biomarkers Prev. 2008; 17:655–660. [PubMed: 18349283]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

115. Ramnath N, Menezes RJ, Loewen G, et al. Hormone replacement therapy as a risk factor for non-small cell lung cancer: results of a casecontrol study. Oncology. 2007; 73:305–310. [PubMed: 18493157]

116. Schwartz AG, Wenzlaff AS, Prysak GM, et al. Reproductive factors, hormone use, estrogen receptor expression and risk of non small-cell lung cancer in women. J Clin Oncol. 2007; 25:5785–5792. [PubMed: 18089876]

117. Elliott AM, Hannaford PC. Use of exogenous hormones by women and lung cancer: evidence from the Royal College of General Practitioners' Oral Contraception Study. Contraception. 2006; 73:331–335. [PubMed: 16531161]

118. Liu Y, Inoue M, Sobue T, Tsugane S. Reproductive factors, hormone use and the risk of lung cancer among middle-aged never-smoking Japanese women: a large-scale population-based cohort study. Int J Cancer. 2005; 117:662–666. [PubMed: 15929081]

119. Schabath MB, Wu X, Vassilopoulou-Sellin R, Vaporciyan AA, Spitz MR. Hormone replacement therapy and lung cancer risk: a case-control analysis. Clin Cancer Res. 2004; 10:113–123. [PubMed: 14734459]

120. Kreuzer M, Gerken M, Heinrich J, Kreienbrock L, Wichmann HE. Hormonal factors and risk of lung cancer among women? Int J Epidemiol. 2003; 32:263–271. [PubMed: 12714547]

121. Olsson H, Bladström A, Ingvar C. Are smoking-associated cancers prevented or postponed in women using hormone replacement therapy? Obstet Gynecol. 2003; 102:565–570. [PubMed: 12962944]

122. Taioli E, Wynder EL. Endocrine factors and adenocarcinoma of the lung in women. J Natl Cancer Inst. 1994; 86:869–870. [PubMed: 8182770]

123. Slatore CG, Chien JW, Au DH, Satia JA, White E. Lung cancer and hormone replacement therapy: association in the vitamins and lifestyle study. J Clin Oncol. 2010; 28:1540–1546. [PubMed: 20159813]

124. Greiser CM, Greiser EM, Dören M. Menopausal hormone therapy and risk of lung cancer—systematic review and meta-analysis. Maturitas. 2010; 65:198–204. [PubMed: 20031346]

125. Oh SW, Myung SK, Park JY, Lym YL, Ju W. Hormone therapy and risk of lung cancer: a meta-analysis. J Womens Health (Larchmt). 2010; 19:279–288. [PubMed: 20095904]

126. Joffe H, Petrillo LF, Koukopoulos A, et al. Increased estradiol and improved sleep, but not hot flashes, predict enhanced mood during the menopausal transition. J Clin Endocrinol Metab. 2011; 96:E1044–E1054. [PubMed: 21525161]

127. Schmidt PJ, Nieman L, Danaceau MA, et al. Estrogen replacement in perimenopause-related depression: a preliminary report. Am J Obstet Gynecol. 2000; 183:414–420. [PubMed: 10942479]

128. Soares CN, Almeida OP, Joffe H, Cohen LS. Efficacy of estradiol for the treatment of depressive disorders in perimenopausal women: a double-blind, randomized, placebo-controlled trial. Arch Gen Psychiatry. 2001; 58:529–534. [PubMed: 11386980]

129. Morrison MF, Kallan MJ, Ten Have T, Katz I, Tweedy K, Battistini M. Lack of efficacy of estradiol for depression in postmenopausal women: a randomized, controlled trial. Biol Psychiatry. 2004; 55:406–412. [PubMed: 14960294]

130. Rasgon NL, Dunkin J, Fairbanks L, et al. Estrogen and response to sertraline in postmenopausal women with major depressive disorder: a pilot study. J Psychiatr Res. 2007; 41:338–343. [PubMed: 16697413]

131. Cohen LS, Soares CN, Vitonis AF, Otto MW, Harlow BL. Risk for new onset of depression during the menopausal transition: the Harvard study of moods and cycles. Arch Gen Psychiatry. 2006; 63:385–390. [PubMed: 16585467]

132. Phillips SM, Sherwin BB. Effects of estrogen on memory function in surgically menopausal women. Psychoneuroendocrinology. 1992; 17:485–495. [PubMed: 1484915]

133. Sherwin BB. Estrogen and/or androgen replacement therapy and cognitive functioning in surgically menopausal women. Psychoneuroendocrinology. 1988; 13:345–357. [PubMed: 3067252]

134. Henderson VW. Gonadal hormones and cognitive aging: a midlife perspective. Womens Health (Lond Engl). 2011; 7:81–93. [PubMed: 21175393]

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135. Greendale GA, Huang MH, Wight RG, et al. Effects of the menopause transition and hormone use on cognitive performance in midlife women. Neurology. 2009; 72:1850–1857. [PubMed: 19470968]
136. Greendale GA, Wight RG, Huang MH, et al. Menopause-associated symptoms and cognitive performance: results from the study of women's health across the nation. Am J Epidemiol. 2010; 171:1214–1224. [PubMed: 20442205]
137. Kang JH, Weuve J, Grodstein F. Postmenopausal hormone therapy and risk of cognitive decline in community-dwelling aging women. Neurology. 2004; 63:101–107. [PubMed: 15249618]
138. Binder EF, Schechtman KB, Birge SJ, Williams DB, Kohrt WM. Effects of hormone replacement therapy on cognitive performance in elderly women. Maturitas. 2001; 38:137–146. [PubMed: 11306202]
139. Grady D, Yaffe K, Kristof M, Lin F, Richards C, Barrett-Connor E. Effect of postmenopausal hormone therapy on cognitive function: the Heart and Estrogen/progestin Replacement Study. Am J Med. 2002; 113:543–548. [PubMed: 12459399]
140. Resnick SM, Maki PM, Rapp SR, et al. Effects of combination estrogen plus progestin hormone treatment on cognition and affect. J Clin Endocrinol Metab. 2006; 91:1802–1810. [PubMed: 16522699]
141. Shumaker S, Legault C, Rapp S, et al. Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: The Women's Health Initiative Memory Study: a randomized controlled trial. JAMA. 2003; 289:2651–2662. [PubMed: 12771112]
142. Resnick SM, Espeland MA, An Y, et al. Effects of conjugated equine estrogens on cognition and affect in postmenopausal women with prior hysterectomy. J Clin Endocrinol Metab. 2009; 94:4152–4161. [PubMed: 19850684]
143. Yaffe K, Sawaya G, Lieberburg I, Grady D. Estrogen therapy in postmenopausal women: Effects on cognitive function and dementia. JAMA. 1998; 279:688–695. [PubMed: 9496988]
144. Rocca WA, Bower JH, Maraganore DM, et al. Increased risk of cognitive impairment or dementia in women who underwent oophorectomy before menopause. Neurology. 2007; 69:1074–1083. [PubMed: 17761551]
145. Henderson VW, Sherwin BB. Surgical versus natural menopause: cognitive issues. Menopause. 2007; 14:572–579. [PubMed: 17476147]
146. Luoma P, Melberg A, Rinne JO, et al. Parkinsonism, premature menopause, and mitochondrial DNA polymerase gamma mutations: clinical and molecular genetic study. Lancet. 2004; 364:875–882. [PubMed: 15351195]
147. Parker WH, Broder MS, Chang E, et al. Ovarian conservation at the time of hysterectomy and long-term health outcomes in the Nurses' Health Study. Obstet Gynecol. 2009; 113:1027–1037. [PubMed: 19384117]
148. Kok HS, van Asselt KM, van der Schouw YT, et al. Heart disease risk determines menopausal age rather than the reverse. J Am Coll Cardiol. 2006; 47:1976–1983. [PubMed: 16697313]
149. Gold EB, Bromberger J, Crawford S, et al. Factors associated with age at natural menopause in a multiethnic sample of midlife women. Am J Epidemiol. 2001; 153:865–874. [PubMed: 11323317]
150. Vaidya D, Becker DM, Bittner V, Mathias RA, Ouyang P. Ageing, menopause, and ischaemic heart disease mortality in England, Wales, and the United States: modelling study of national mortality data. BMJ. 2011; 343:d5170. [PubMed: 21896608]
151. Salpeter SR, Cheng J, Thabane L, Buckley NS, Salpeter EE. Bayesian meta-analysis of hormone therapy and mortality in younger postmenopausal women. Am J Med. 2009; 122:1016–1022. [PubMed: 19854329]
152. Efthymiou CA. Endometriosis-associated intestinal tumours: a consequence of long-term unopposed oestrogen? Ann R Coll Surg Engl. 2009; 91:259–260. [PubMed: 19220946]
153. Giudice LC. Clinical practice. Endometriosis. N Engl J Med. 2010; 362:2389–2398. [PubMed: 20573927]
154. Pachman DR, Jones JM, Loprinzi CL. Management of menopause-associated vasomotor symptoms: Current treatment options, challenges and future directions. Int J Womens Health. 2010; 2:123–135. [PubMed: 21072305]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

155. Ettinger B, Ensrud KE, Wallace R, et al. Effects of ultralow-dose transdermal estradiol on bone mineral density: a randomized clinical trial. Obstet Gynecol. 2004; 104:443–451. [PubMed: 15339752]

156. Johnson R, Ettinger B, Macer J, et al. Uterine and vaginal effects of unopposed ultralow-dose transdermal estradiol. Obstet Gynecol. 2005; 105:779–787. [PubMed: 15802405]

157. Utian WH, Shoupe D, Bachmann G, Pinkerton JV, Pickar JH. Relief of vasomotor symptoms and vaginal atrophy with lower doses of conjugated equine estrogens and medroxyprogesterone acetate. Fertil Steril. 2001; 75:1065–1079. [PubMed: 11384629]

158. Bachmann GA, Schaefers M, Uddin A, Utian WH. Lowest effective transdermal 17A-estradiol dose for relief of hot flushes in postmenopausal women: a randomized controlled trial. Obstet Gynecol. 2007; 110:771–779. [PubMed: 17906008]

159. Renoux C, Dell'aniello S, Garbe E, Suissa S. Transdermal and oral hormone replacement therapy and the risk of stroke: a nested casecontrol study. BMJ. 2010; 340:c2519. [PubMed: 20525678]

160. Steunkel CA, Gass M. Results from 2010 NAMS Survey on secondary transfer of transdermal estrogen preparations: Abstract P-85. Menopause. 2011; 18:1371.

161. Lokkegaard E, Andreasen AH, Jacobsen RK, Nielsen LH, Agger C, Lidegaard O. Hormone therapy and risk of myocardial infarction: a national register study. Eur Heart J. 2008; 29:2660–2668. [PubMed: 18826989]

162. Jaakkola S, Lyytinen H, Pukkala E, Ylikorkala O. Endometrial cancer in postmenopausal women using estradiol-progestin therapy. Obstet Gynecol. 2009; 114:1197–1204. [PubMed: 19935019]

163. Somboonporn W, Panna S, Temtanakitpaisan T, Kaewrudee S, Soontrapa S. Effects of the levonorgestrel-releasing intrauterine system plus estrogen therapy in perimenopausal and postmenopausal women: systematic review and meta-analysis. Menopause. 2011; 18:1060–1066. [PubMed: 21720280]

164. Bhavnani BR, Stanczyk FZ. Misconception and concerns about bioidentical hormones used for custom-compounded hormone therapy [e-pub]. J Clin Endocrinol Metab. 2011

165. Food and Drug Administration. [Accessed January 10, 2012] FDA takes action against compounded menopause hormone therapy drugs. 2008. Available at: http://www.fda.gov/newsevents/newsroom/pressannouncements/2008/ucm116832.htm

166. Grodstein F, Manson JE, Colditz GA, et al. A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. Ann Intern Med. 2000; 133:933–941. [PubMed: 11119394]

167. Hulley S, Grady D, Bush T, et al. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. JAMA. 1998; 280:605–613. [PubMed: 9718051]

168. Manson JE, Hsia J, Johnson KC, et al. for the Women's Health Initiative Investigators. Estrogen plus progestin and the risk of coronary heart disease. N Engl J Med. 2003; 349:523–534. [PubMed: 12904517]

169. Karim R, Dell RM, Greene DF, Mack WJ, Gallagher JC, Hodis HN. Hip fracture in postmenopausal women after cessation of hormone therapy: results from a prospective study in a large health management organization. Menopause. 2011; 18:1172–1177. [PubMed: 21775911]

170. National Cancer Institute. [Accessed January 10, 2012] Surveillance, Epidemiology, and End Results (SEER). Available at: http://seer.cancer.gov/statfacts/html/breast.html

171. Ockene JK, Barad DH, Cochrane BB, et al. Symptom experience after discontinuing use of estrogen plus progestin. JAMA. 2005; 294:183–193. [PubMed: 16014592]

172. Brunner RL, Aragaki A, Barnabei V, et al. Menopausal symptom experience before and after stopping estrogen therapy in the Women's Health Initiative randomized, placebo-controlled trial. Menopause. 2010; 17:946–954. [PubMed: 20505547]

173. Haimov-Kochman R, Barak-Glantz E, et al. Gradual discontinuation of hormone therapy does not prevent the reappearance of climacteric symptoms: a randomized prospective study. Menopause. 2006; 13:370–376. [PubMed: 16735933]

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DNA profiling analysis of endometrial and ovarian cell lines reveals misidentification, redundancy and contamination

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ABSTRACT

Objectives. Cell lines derived from human ovarian and endometrial cancers, and their immortalized non-malignant counterparts, are critical tools to investigate and characterize molecular mechanisms underlying gynecologic tumorigenesis, and facilitate development of novel therapeutics. To determine the extent of misidentification, contamination and redundancy, with evident consequences for the validity of research based upon these models, we undertook a systematic analysis and cataloging of endometrial and ovarian cell lines.

Methods. Profiling of cell lines by analysis of DNA microsatellite short tandem repeats (STR), p53 nucleotide polymorphisms and microsatellite instability was performed.

Results. Fifty-one ovarian cancer lines were profiled with ten found to be redundant and five (A2008, OV2008, C13, SK-OV-4 and SK-OV-6) identified as cervical cancer cells. Ten endometrial cell lines were analyzed, with RL-92, HEC-1A, HEC-1B, HEC-50, KLE, and AN3CA all exhibiting unique, uncontaminated STR profiles. Multiple variants of Ishikawa and ECC-1 endometrial cancer cell lines were genotyped and analyzed by sequencing of mutations in the p53 gene. The profile of ECC-1 cells did not match the EnCa-101 tumor, from which it was reportedly derived, and all ECC-1 isolates were genotyped as Ishikawa cells, MCF-7 breast cancer cells, or a combination thereof. Two normal, immortalized endometrial epithelial cell lines, HES cells and the hTERT-EEC line, were identified as HeLa cervical carcinoma and MCF-7 breast cancer cells, respectively.

Conclusions. Results demonstrate significant misidentification, duplication, and loss of integrity of endometrial and ovarian cancer cell lines. Authentication by STR DNA profiling is a simple and economical method to verify and validate studies undertaken with these models.

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Introduction

Cell lines, immortalized from normal human tissues or derived from tumors, are widely used models to address molecular mechanisms underlying the physiology and pathology of the female reproductive tract, and to evaluate novel therapeutics or preventive strategies [1–3]. Verification of the provenance and integrity of such cell lines is clearly of paramount importance, but historically, has rarely been undertaken by investigators. The problem of cross-contamination, identified and characterized by examination of isozyme patterns, karyotyping, and cytogenetics, dates back to the establishment of the prototypical HeLa cell line in culture in 1951 and remains a significant concern [4–7]. Over one-third (18–50%) of cell lines may be mixtures, misidentified or intra-species contaminants [2,8–15]. Furthermore, there are many examples of redundancy among reportedly unique cell lines, and instances of contamination during original derivations, such that the intended novel cell line was never established [5,10,16–19]. Thus, it is evident that authentication of cell line origins and integrity is crucial to validate results and conclusions obtained using these model systems.

Short tandem repeat (STR) profiling or 'DNA fingerprinting' identifies variants in tetranucleotide microsatellite loci on multiple human chromosomes and is the accepted international standard for genetic analysis of cell lines for authentication by comparison to established STR databases [20–24].

A comprehensive analysis of cell lines commonly used in the study of ovarian and endometrial cancer had not been undertaken, particularly with respect to those cell lines not obtained from established cell repositories. We used STR profiling, sequencing of p53 mutations, and

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human papilloma virus screening to examine cell lines of purported ovarian and endometrial origins. We observed examples of cross-contamination, misidentification of lines and/or tissue of origin, and redundancy among established cancer cells, and found evidence that immortalized normal endometrial epithelial cell lines are genetically identical to previously established cervical and breast cancer cells. We provide reference DNA profiles for women's cancer cell lines that are not currently in public cell banks and extend the number of loci for profiles currently available through central repositories.

Materials and methods

DNA isolation and STR profiling

Cell lines were grown in appropriate specific standard media. Genomic DNA was isolated from 0.5 to 5×10^6 cells using a Zymo Research ZR genomic DNA II kit and quantified by gel electrophoresis and ethidium bromide staining by comparison to a DNA mass ladder. Multiplex PCR amplified products were generated using 1–2 ng of genomic DNA with an Applied Biosystems Identifiler kit and ABI 3730 capillary sequencer as described [2,18]. STR loci were analyzed with Gene Mapper 4.0. Profiles were compared to published reports [22,25], consolidated (ATCC, DSMZ, JCRB and RIKEN) databases, and an in-house database, using a custom search algorithm designed to facilitate comparison of cell lines with related profiles and identify individual cell lines in a mixture (C. Korch and J. West, Vanderbilt University, unpublished). STR profiles of the ovarian and endometrial cancer cells analyzed in this study are available online at http://DNAsequencingcore.UCDenver.edu.

TP53 sequence analysis and microsatellite instability assays

PCR amplification was used to generate overlapping products spanning the Variable Number Tandem Repeat (VNTR; a pentanucleotide repeat of A_4T) in intron 1, through the protein encoding exons 2–11, including intervening introns 2–8 and 10 [26]. Sequencing primers and p53 gene structure are shown in Fig. S1. DNAs were screened for microsatellite instability [27] using Promega MSI analysis system version 1.2 according to the manufacturers' protocol.

HPV testing

Aliquots of cells were placed into ThinPrep (Hologic) solution. DNA was isolated and tested in the University of Colorado Hospital Clinical Laboratory using the hybrid capture PCR, Digene HC2 High Risk HPV test (Qiagen).

Ovarian and endometrial cell lines

We obtained cell lines from multiple institutions in the United States, Europe and Japan, including, where possible, the originating laboratories. Multiple independent samples of the earliest available passages from each institution were analyzed and, if available, profiles of each individual cell line were compared from several sources. Ovarian cancer cell lines are listed in Table S1. Ishikawa cells were obtained from Dr. K.K. Leslie (University of Iowa), Dr. B.A. Lessey (Greenville Hospital System, SC), Dr. M. Brown (Dana Farber Cancer Institute, Harvard University) and Drs. H. Philpott and P. Thraves (European Collection of Cell Cultures, ECACC). ECC-1 cells were from Drs. B.A. Lessey, M. Brown and V.C. Jordan (Lombardi Comprehensive Cancer Center, Georgetown University). EnCa-101 tumors were provided by Drs. V.C. Jordan and G. Balburski (Fox Chase Cancer Center). HES cells were from Dr. D. Kniss (Ohio State University) and hTERT-EEC cells from Dr. T. Klonisch (University of Manitoba, Canada). KLE and HEC-50 cells were from Dr. K.K. Leslie. RL-95-2, HEC-1A, HEC-1B and AN3CA cells were from the American Type Culture Collection (ATCC, Manassas, VA).

Results

Analysis of endometrial cancer cell lines

Endometrial carcinomas are derived from glandular epithelium and are typically divided into two subtypes based on clinical, histological and molecular characteristics [28–30]. Cell lines derived from type I (Ishikawa, ECC-1 and RL-95-2) and type II (HEC-1, HEC-50, KLE and AN3CA) tumors have been widely used as models to investigate molecular genetics and mechanisms underlying their development, progression and response to therapeutics [31–35].

HEC-1B cells, the first to be derived from a human endometrial carcinoma [32,36,37], exhibited a unique profile (Table S3). HEC-1A, derived from the same patient, cells are predominantly diploid, while the HEC-1B line is tetraploid [38,39]. HEC-50 cells [38,40], also have a unique profile consistent with that on file with the Japanese Collection of Research Bioresources (JCRB: 1145).

Similarly, KLE (CRL-1622) and AN3CA (HTB-111) cells, originating from peritoneal and lymph node metastases, respectively [34,41,42], and RL-95-2 cells (CRL-1671) derived from a moderately differentiated (Grade 2) endometrial adenosquamous carcinoma [35], all have STR profiles consistent with those reported by the ATCC (Table S3).

Ishikawa cells were established from the epithelial component of a moderately differentiated, stage 2, endometrial adenocarcinoma [43,44]. At least three variants of Ishikawa cells, the original line, 3-H-4 and 3-H-12, differing in their reported degree of differentiation, relative expression of estrogen (ER) and progesterone (PR) receptors, growth and colony formation rates, were distributed to investigators [45].

We profiled multiple isolates of the original Ishikawa cells and 3-H-12 variants obtained from a number of laboratories as detailed in the Materials and methods section. Samples with unique profiles, which may represent the 3-H-4 variant based upon their date of origin are designated '3-H-4'. The results are summarized in Table 1.

Overall the Ishikawa cell lines exhibit very similar profiles, indicative of their origin from the same patient. Identical alleles were present at several loci (CSF1PO, D5S818, D16S539, D21S11, THO1 and TPOX). Others reflect loss or gain of alleles (D8S1179, D13S317 and FGA) or alterations in the number of repeats (D2S1338, D3S1358, D19S433 and vWA). At the D7S820 locus, the original Ishikawa isolate exhibits 8.3- and 11-repeat alleles, while subsequent sublines display 9- or 10-repeats. The D18S51 locus was found to be highly polymorphic in most Ishikawa lines.

Minor differences in the number of repeats at certain loci are consistent with the known microsatellite instability (MSI) of these lines, due to mutations in mismatch repair systems [46–48], and suggest that these variants arose by genetic drift between different clonal isolates over hundreds of cell passages. Accordingly, all Ishikawa cell lines exhibited high variability/instability at microsatellite loci (Table S2). Defective mismatch repair also underlies allelic variation in AN3CA cells (Table S3) [49]. In contrast, EnCa-101 tumors and MCF-7 cells were MSI stable.

We also profiled a variant of Ishikawa cells lacking ER [50]. Previous reports implied that these cells, also known as Ishikawa B, were derived from a different patient [51,52]. The STR profile of ER-negative Ishikawa cells exhibits minor variations from other Ishikawa sublines (Table 1), but overlap at the majority of loci indicates a common origin.

A second type 1, ER and PR positive cell line, ECC-1, was established from a grade 2, well-differentiated, endometrial carcinoma adenocarcinoma [42,53,54]. The line was derived by passage of the tumor, designated EnCa-101, in nude mice and subsequent isolation of PR positive cells from an epithelial monolayer culture [42,55]. ECC-1 cells were described as a well-differentiated, steroid responsive line with a phenotype characteristic of luminal surface epithelium, distinct from Ishikawa cells, which expressed markers of glandular endometrial epithelium [33].

Table 1
Summary of STR profiles of Ishikawa and ECC-1 endometrial cancer cells and EnCa-101 tumor.

Cell line	Amelogenin	CSF1PO	D2S1338	D3S1358	D5S818	D7S820	D8S1179	D13S317	D16S539	D18S51	D19S433	D21S11	FGA	THO1	TPOX	vWA
Ishikawa original	X	11, 12	18, 20	17, 18	10, 11	8.3, 11	12, 16	9, 12	9	14, (19) 20, 21 polymorphic	12.2, 14	28	21	9, 10	8	14, 18
Ishikawa '3-H-4'	X	11, 12	19, 20	16, 17	10, 11	9, 10	12, 16	9, 12	9	13, 21, 22	12.2, 14	28	21, 22	9	8	14, 17
Ishikawa 3-H-12	X	11, 12, (13)	19, 20	16, 17	10, 11	9, 10	12, 13, 16	9, 12, 13	9	12, 19, 20	13.2, 14	28	20, 21	9, 10	8	14, 17
Ishikawa 3-H-12	X	11, 12	20	16, 17, (18)	10, 11, (12)	9, 10	12, (13), 16	9, 12	9, (10)	13, 20	12.2, 14, (15)	28	21	9, 10 or 11	8	14, 17 or 18
Ishikawa ER -ve	X	11, 13	20	16, 17	10, 11	9, 10	12, 13, 16	9, 12	8, 9	13, 19	12.2, 14	28	20	9,10	8	14, 17
Ishikawa, ECACC, this report	X	11,12, (13)	20	15, 17	10, 11, 12	9, 10	12, 16	9, 12, 13	9	13, 19, (14, 20)	12.2, 14	28	21	9, 10	8	14, 17
Ishikawa, ECACC	X	11,12	NT	NT	10, 11	9, 10	NT	9, 12	9	NT	NT	NT	NT	9, 10	8	14, 17
ECC-1	X	11, 12	20	16, 17	10, 11	9, 10	13, 16	9, 12,	9	12, 19	12.2, 14 or 15	28	21	9, 10	8	14, 17
ECC-1 ATCC CRL-2923	X	11, 12	NT	NT	10, 11	9, 10	NT	9, 12	9	NT	NT	NT	NT	9, 10	8	14, 17
EnCa-101	X	13, 14	23, 27	15, 21	14, 15	11.3, 12	18, 21	10, 13	12, 13	16	13.2, 14 or 15	27, 30	21	9, 9.3	8	18, 23

Number of STRs at each of 16 surveyed loci. Numbers after decimal point indicate number of bases in an incomplete STR. Commas separate allele calls for multiple peaks. Alleles in parenthesis indicate low amplitude peaks suggesting only a minor fraction of the cells in the population carry that allele. ECACC: DNA profile from European Collection of Cell Cultures; ATCC: DNA profile from American Type Culture Collection. NT: locus not tested. X: only the amelogenin allele on the X chromosome was detected.

Upon STR and MSI analyses, ECC-1 samples exhibited DNA profiles essentially identical to Ishikawa 3-H-12 cells (Tables 1 and S2). In addition, the ATCC profile for ECC-1 also closely matched that of earlier Ishikawa cells on file with the European Collection of Cell Cultures (ECACC). Other 'ECC-1' cell lines were found to be identical to MCF-7 breast cancer cells or consist of a mixture of Ishikawa and MCF-7 cells (not shown). Unfortunately, following the death of Dr. Satyaswaroop, records and cell lines from his laboratory were lost or destroyed (Zaino, R. and Lessey, B., personal communication). Thus, we could not obtain reference samples of the original ECC-1 line or EnCa-101 tumor from which it was purportedly derived. However, the EnCa-101 tumor has been continuously maintained in mice [56] and we obtained and analyzed 3 independent samples. Profiling of these tumors showed minor variations, but results indicated that they were derived from the same human patient. In contrast, the unique EnCa-101 profiles did not match ECC-1, Ishikawa or MCF-7 cell lines (Table 1). These data are inconsistent with the reported origins of ECC-1 cells and suggest that the original line has been lost. Our results show that currently available ECC-1 cells are Ishikawa cells, MCF-7 breast cancer cells, or a mixture of both.

Sequencing of p53 mutations in endometrial cancer cells

To confirm the apparent equivalence of Ishikawa and ECC-1 cells, we screened for p53 mutations by PCR amplification and sequencing of the Variable Number Tandem Repeat (VNTR) region in intron 1, and the protein encoding exons and introns (Fig. S1). Table 2 lists the observed p53 mutations and SNPs compared to the reference/normal sequence.

In agreement with previous reports [31,57], Ishikawa original and 3-H-12 cells harbor a Met 246 Val mutation in exon 7. These two lines are also homozygous in the VNTR region with 8 repeats of A_4T, heterozygous in exon 4 for the Asp 49 Val mutation (nucleotide G12069S), and heterozygous in intron 10 for deletion of the seventh T in a heptanucleotide repeat (17822delT). The original Ishikawa sample has two additional heterozygous mutations, 12724insA (intron 4) and 13764delA (intron 6), which are not present in the 3-H-12 line (Table 2).

Possible '3-H-4' sublines have a similar profile, but lack the intronic 12724insA and 13764delA mutations of poly A stretches, present in the original Ishikawa lines (Table 2). An additional heterozygous mutation in intron 4 (G12299K (G + T)) was detected in some Ishikawa 3-H-12 sublines. Interestingly, consistent with their closely matched STR profiles, the ER-negative Ishikawa cells, despite their purported distinct origin, exhibit TP53 mutations identical to Ishikawa 3-H-12 and '3-H-4' (not shown). TP53 mutations unique to the original Ishikawa lines are insertions or deletions in homopolymer A or T stretches, which are consistent with microsatellite instability due to mutations in the mismatch repair system [46].

In agreement with their identical STR profiles, ECC-1 cells show the same TP53 mutations as Ishikawa 3-H-12 lines, further evidence that ECC-1 cells are misidentified Ishikawa cells. In contrast, EnCa-101 tumors have completely different TP53 mutations from the Ishikawa and ECC-1 lines (Table 2), again demonstrating that ECC-1 cells are not derived from the EnCa-101 tumor. 'ECC-1' cells shown to be contaminated with or identical to MCF-7 cells were not subjected to TP53 analysis.

Finally, our data suggest that only one copy of the p53 gene is expressed in Ishikawa cells. In the genomic DNA, both the A14063R (A + G) and G12069S (G + C) positions are heterozygous. However, only the 14063G mutation is present in the cDNA sequence [31,57], suggesting that the G12069C mutation is in the unexpressed copy of the gene.

Analysis of normal endometrial epithelial cells

Immortalized, non-transformed endometrial epithelial cells are a potentially valuable resource to investigate normal uterine physiology and tumorigenesis. We profiled two such lines, human endometrial (HES) cells [58] and hTERT-EEC [59], obtained from their developers, which have been extensively used as models of normal endometrium. Neither cell line was authenticated as they exhibited DNA profiles corresponding to HeLa and MCF-7 cancer cells, respectively.

HES cells were established, in 1989, from a primary culture of benign proliferative endometrium, which apparently underwent spontaneous transformation after serial passage [58,60]. Profiling of these cells

Table 2
Summary of TP53 mutations and single nucleotide polymorphisms (SNPs).

TP53 reference sequence	Ishikawa original	Ishikawa '3-H-4'	Ishikawa 3-H-12	Ishikawa 3-H-12	ECC-1	EnCa-101 tumor
Intron 1: VNTR A_4T repeats	Homozygous 8 repeats	Homozygous 8 repeats	Homozygous 8 repeats	Homozygous 8 repeats	Homozygous 8 repeats	Heterozygous 7 and 9 repeats
Exon 4: G12069 Asp 49	Heterozygous G12069S Asp49His	Heterozygous G12069S Asp49His	Heterozygous G12069S Asp49His	Heterozygous G12069S Asp49His	Heterozygous G12069S Asp49His	
Intron 4: G12299			Heterozygous G12299K			
Intron 4: Poly A_7 12718–12724	Heterozygous 12724insA Poly A_7/A_8					
Intron 5: G12786						Homozygous G12786T SNP
Intron 5: C13253						Heterozygous C13253Y SNP
Intron 6: G13642						Heterozygous G13462K SNP
Intron 6: Poly A_9 13756–13764	Heterozygous 13764delA Poly A_9/A_8					
Exon 7: A14063 Met246	Heterozygous A14063R Met246Val	Heterozygous A14063R Met246Val	Heterozygous A14063R Met246Val	Heterozygous A14063R Met246Val	Heterozygous A14063R Met246Val	
Intron 10: Poly T_7 17816–17822	Heterozygous 17822delT Poly T_7/T_6	Heterozygous 17822delT Poly T_7/T_6	Heterozygous 17822delT Poly T_7/T_6	Heterozygous 17822delT Poly T_7/T_6	Heterozygous 17822delT Poly T_7/T_6	Homozygous/Hemizygous Poly T_7

Tumor protein p53 (TP53) genomic DNA, from multiple independent samples of each cell line, was sequenced as described in the Materials and methods section. The normal reference normal is GenBank HSP53G, a.k.a. X54156, which is used by the International Agency for Research on Cancer IARC (http://www-p53.iarc.fr). A blank cell in the table indicates the DNA sequence that matches the reference/normal sequence. VNTR: Variable Number Tandem Repeat. Symbols — K: G and T; R: A and G; S:G and C; Y:C and T; del: nucleotide deletion; ins: nucleotide insertion.

(Table 3) indicated that they are identical at all loci to HeLa cervical carcinoma cells, specifically the HeLaS3 variant. HES cells are also identical to WISH cells, a cell line originally described as derived from human amnion [61] but subsequently also identified as HeLa [7,62,63]. These results were independently confirmed by the STR fragment analysis facility at Johns Hopkins University (D. Kniss, Ohio State University; personal communication).

hTERT-EECs were isolated from normal proliferative phase endometrial epithelium and immortalized by stable transfection with the catalytic subunit of human telomerase (hTERT) [59]. Replicate STR

Table 3
Summary of STR profiles of normal immortalized endometrial epithelial cells.

Cell line	Amelogenin	CSF1PO	D2S1338	D3S1358	D5S818	D7S820	D8S1179	D13S317	D16S539	D18S51	D19S433	D21S11	FGA	THO1	TPOX	vWA
hTERT-EEC-B37	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
hTERT-EEC-15	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
hTERT-EEC-17	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
hTERT-EEC-38	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
hTERT-EEC-49	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
MCF-7 (HTB-22) this report	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
MCF-7 NCI-60	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
MCF-7 ATCC (HTB-22)	X	10	NT	NT	11, 12	8, 9	NT	11	11, 12	NT	NT	NT	NT	6	9, 12	14, 15
HES	X	9, 10	17	15, 18	11, 12	8, 12	12, 13	13.3	9, 10	16	13, 14	27, 28	21	7	8, 12	16, 18
HeLa this report	X	9, 10	17	15, 18	11, 12	8, 12	12, 13	12, 13.3	9, 10	16	13, 14	27, 28	18, 21	7	8, 12	16, 18
HeLa ATCC (CCL-2)	X	9, 10	NT	NT	11, 12	8, 12	NT	12, 13.3	9, 10	NT	NT	NT	NT	7	8, 12	16, 18
HeLaS3 ATCC (CCL-2.2)	X	9, 10	NT	NT	11, 12	8, 12	NT	13.3	9, 10	NT	NT	NT	NT	7	8, 12	16, 18
WISH ATCC (CCL-25)	X	9, 10	NT	NT	11, 12	8, 12	NT	13.3	9, 10	NT	NT	NT	NT	7	8, 12	16, 18

Number of STRs at each of 16 surveyed loci. Numbers after decimal point indicate number of bases in an incomplete STR. Commas separate allele calls for multiple peaks. NT: not tested. Numbers following hTERT-ECC indicate clones. Samples were analyzed in duplicate independent reactions. MCF-7 breast cancer cells reference STR profiles from ATCC (HTB-22) and NCI-60 panel [25]. HeLa and WISH reference profiles from ATCC database. X: only the amelogenin allele on the X chromosome was detected.

profiling of the earliest available passages of multiple clonal lines indicated all isolates of hTERT-EEC cells to be genetically identical to MCF-7 breast cancer cells (Table 3). As for HES cells, this was not attributable to contamination as no other profiles were detected in the samples.

Analysis of ovarian cancer cell lines

We obtained and genotyped fifty-one ovarian cancer cell lines (Table S1), many of which are not available from public repositories. Two of the lines (IGROV1 and OVCAR-10) gave mixed genotypes indicating cross-contamination and were excluded from further analysis. The mixed genotype for IGROV1 was confirmed in multiple isolates including those obtained directly from the National Cancer Institute.

Several purported 'ovarian cancer' lines were genotypically identical to other known, non-ovarian, cancer cells: BG-1[64] was identified as MCF-7 breast cancer cells, and CH1, CH1cisR, and 222 as the teratocarcinoma line PA1. C13, A2008 and OV2008 were identical to the ME-180 (ATCC: HTB-33) cervical cancer cell line, and confirmed to be HPV positive (Table 4). The genotypically distinct 2008 cell line [65], obtained directly from the originating laboratory of Dr. Peter Disaia [66], was HPV negative. Finally, SK-OV-4 and SK-OV-6 lines matched HPV-negative C-33A (HTB-31) cervical cancer cells (Table 4).

Two 'normal ovarian' cell lines, NOSE06 and NOSE07, were genotyped as the ovarian cancer line DOV-13. Similarly, Caov-2 was identical to the earlier NIH:OVCAR-2 line (Table S4) and some samples of COLO-720E were found to be COLO-704 (not shown). Ovary1847 cells were genotyped as NIH:OVCAR-8.

The remaining ovarian cancer cell lines exhibited unique, uncontaminated genotypes and are listed with their STR profiles in Table S4.

We noted disparate genotypes for several cell lines with similar names; 2008 cells are distinct from A2008 and OV2008, and 167 differs from OV167 cells. In contrast, the TOV-112D cell line is identical to TOV-21D, which appears to have arisen via transposition of numbers and letters in the name. Some isolates of TOV-112D were misidentified and matched TOV-21G cells.

The heterogeneity of ovarian tumor cells in ascitic fluid has previously lead to the establishment of several cell lines with different phenotypic

Table 4
STR profiles of cervical and other cancer cell lines misclassified as ovarian.

Cell line	Amelogenin	CSF1PO	D2S1338	D3S1358	D5S818	D7S820	D8S1179	D13S317	D16S539	D18S51	D19S433	D21S11	FGA	THO1	TPOX	vWA	HPV
A2008	X	11	18	16	12	9, 10	14	11, 12	12, 13	12	13, 15.2	30, 31	23	8, 9.3	8, 10	15, 17	+
C13	X	11	18	16	12	9	14	11, 12	12, 13	12	15.2	30, 31	23	8, 9.3	8, 10	15, 17	NT
ME-180	X	11	18	16	12	9, 10	14	11, 12	12, 13	12	13, 15.2	30, 31	23	8, 9.3	8, 10	15, 17	NT
OV2008	X	11	18	16	12	9, 10	14	11, 12	12, 13	12	13, 15.2	30, 31	23	8, 9.3	8, 10	15, 17	+
ME-180 ATCC (HTB-33)	X	11	NT	NT	12	9, 10	NT	11, 13	12, 13	NT	NT	NT	NT	8, 9.3	8, 10	15, 17	+
SKOV4	X	12	23, 25	16	11, 12	10	10, 14	13	13, 14	15, (17), 18	11, 13	29, 31, 32	21, 26	7, 8	9	18, 20 (19)	NT
SKOV6	X	12	23, 25	16	11, 12	10	10, 14	13	13, 14	15, (17),18	11, 13	29, 30, 31, 32	21, 26	7, 8	9	18, 20	NT
C-33 A	X	12	23, 25	16	11, 12	10	10, 14	13	13, 14	15, (17), 18	11, 13, 14	29, 30, 31	21, 26	7, 8	9	18, 20	NT
C-33 A ATCC (HTB-31)	X	12	NT	NT	11, 12	10	NT	13	13, 14	NT	NT	NT	NT	7, 8	9	18, 20	−
BG-1	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 24, 25	6	9, 12	14, 15	NT
MCF-7 NCI-60	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15	NT
MCF-7 ATCC (HTB-22)	X	10	NT	NT	11, 12	8, 9	NT	11	11, 12	NT	NT	NT	NT	6	9, 12	14, 15	NT
CH1	X	9, 12, 13	24	15	11	9	14, 15	9, 10	9, 12	15, 18	13	29, 31.2	24	7, 9	11	15, 17	NT
CH1-cisR	X	9, 13	24	15	11	9	14, 15	9, 10	9, 12	15, 18	13	29, 31.2	24	7, 9	11	15, 17	NT
222	X	9, 13	24	15	11	9	14, 15	9, 10	9, 12	15, 18	13	29, 31.2	24	7, 9	11	15, 17	NT
PA-1 JCRB (9061)	X	9, 12	NT	NT	11	9	NT	9, 10	9, 12	NT	NT	NT	NT	7, 9	11	15, 17	NT
NOSE06	X	8, 10	20, 24	14, 16	11	10	14	11	10, 13	12, 16	13, 14	32.2, 33.2	21, 24	6, 9.3	6, 8	19	NT
NOSE07	X	8, 10	20, 24	14, 16	11	10	14	11	10, 13	12, 16	13, 14	32.2, 33.2	21, 24	6, 9.3	6, 8	19	NT
DOV-13	X	8, 10	20, 24	14, 16	11	10	14	11	10, 13	12, 16	13, 14	32.2, 33.2	21, 24	6, 9.3	6, 8	19	NT

Number of STRs at each of 16 surveyed loci. Numbers after decimal point indicate number of bases in an incomplete STR. Commas separate allele calls for multiple peaks. Alleles in parentheses indicate low amplitude peaks suggesting only a minor fraction of the cells in the population carry that allele. NT: allele not tested. ATCC is a reference DNA profile from the American Type Culture Collection. HPV: human papilloma virus status (+: positive; −: negative). MCF-7 breast cancer cells reference STR profiles from ATCC (HTB-22) and NCI-60 panel. X: only the amelogenin allele on the X chromosome was detected.

characteristics [67]. We profiled very early passages of OV429 and OV433 [68,69] and found identical genotypes, indicative of either a common patient origin or early cross-contamination (Table S4). Of historical note, OV433 was the cell line used originally to select for reactivity to the OC125 monoclonal antibody to the ovarian tumor marker CA125.

The cluster of PEO1/PEO4/PEO6 cells is known to originate from the same patient [70], and genotype accordingly. Similarly, HEY/HEYA8/HEYC2 cells [71] are derived from the same original line, and share identical genotypes (Table S4).

Chemotherapy resistant derivatives mirror parental cell line genotypes

We tested five original and cisplatin-resistant paired cell lines and all five parent and derivative combinations were confirmed by genotyping. However, as shown earlier (Table 4), the OV2008/C13 cells are cervical, not ovarian cancer cells and the CH1/CH1cisR lines [72] are PA1 teratocarcinoma cells. Table S5 shows STR profiles of the matched cisplatin-sensitive/-resistant ovarian cancer cell lines. The 41M/41McisR, TYKnu/TYKnucisR and A2780/A2780cisR pairs each have unique profiles. The paired lines demonstrate some genetic instability, consistent with cisplatin-induced MSI [73]. Cisplatin-resistant A2780 cells have lost alleles at the D3S1358, FGA, D8S1179. D5S818, D7S820, CSF1PO, and D2S1338 loci, and gained an allele at the D18S51 locus. The 41M/41McisR pair is more stable, with the cisplatin-resistant line differing only at the vWA locus. The original derivation of the 41M cisplatin-resistant lines lists three isolates (41McisR2, 41McisR4 and 41McisR6), which differed in their IC_{50} [74]. The subline profiled herein is unknown, as the identifying number has been lost.

Discussion

Gynecologic cancer research is critically dependent on the use of cell culture models, to investigate molecular mechanisms underlying the development and progression of tumors, to design and test novel therapeutic strategies, and to identify potential diagnostic or prognostic markers. In this report, we profiled the most widely used endometrial and ovarian cell lines and discovered several examples of misidentification, redundancy and cross-contamination.

Genotyping and HPV testing of ovarian cancer cell lines identified eight (BG-1 [64], CH1/CH1cisR [72], 222 [75], C13 [76], A2008 [77,78], OV2008, SKOV-4 and SKOV-6 [79]) as previously existing, breast cancer, teratocarcinoma or cervical cancer cell lines. In addition, two 'normal ovarian' cell lines, NOSE06 and NOSE07 [80], were genotyped as the ovarian cancer line DOV-13 [81]. We also highlight the possibility for confusion of several ovarian cancer cell lines with similar names, but distinct genotypes; e.g. 167 and OV167, 2008 and A2008/OV2008.

We profiled a number of variants of Ishikawa endometrial cancer cells. Results are consistent with a common origin for these sublines, with variations and polymorphisms in some STR loci attributable to genetic instability, mismatch repair defects, and high passage number [75–77]. Analyses of mutations in the p53 gene (TP53) are consistent with previous reports [31,57] and provide additional genetic markers to perhaps distinguish the original, 3-H-4 and 3-H-12 Ishikawa lines. Furthermore, STR profiling, TP53 sequencing, and MSI analysis confirm that currently available isolates of ECC-1 cells are not authentic but are identical to Ishikawa cells, specifically the 3-H-12 line. This conclusion is reinforced by evidence that the EnCa-101 tumor, from which the original EEC-1 line was purportedly derived [42,55], is genetically distinct from both Ishikawa and ECC-1 cells. We also observed several ECC-1 isolates to be misidentified MCF-7 cells or a cross-contaminated mixture of Ishikawa and MCF-7 lines.

ECC-1 cells were initially characterized as distinct from Ishikawa lines based on differential expression of cytokeratin 13 and osteopontin [33]. However, both markers were present in the two lines, which otherwise showed identical patterns of expression of steroid hormone receptors and their coactivators [33]. The karyotypes of Ishikawa and ECC-1 cells also exhibit some apparent differences [31,33], but chromosomal number and structural rearrangements in both lines were complex with high intercellular variability [31,33]. Comparative cytogenetic analysis found that, given the evident heterogeneity and differential capabilities of the techniques used (FISH or SKY) to detect abnormalities in small chromosomal segments, the karyotypic similarity was likely underestimated, and is consistent with the two lines sharing a common origin.

Thus, we conclude that the original ECC-1 cell line has been lost, although the persistence of the EnCa-101 tumor [56] provides an opportunity for its re-derivation. ECC-1 cells have been extensively used as models of ER positive, type 1, endometrial cancers. Since Ishikawa cells are also representative of such endometrioid tumors, our evidence that the two lines are identical may not significantly impact conclusions drawn from these studies, beyond the use of two redundant cell lines. However, the possible misidentification of MCF-7 breast cancer cells as ECC-1, or cross contamination with the former, should be considered in interpreting results using ECC-1 cells.

We identified the normal endometrial epithelial cell line (HES) as HeLa cervical carcinoma cells. HES cells have been used as a model of benign endometrial epithelium to study mucosal immunity [82], implantation [83,84], decidualization [85] and endometriosis [86], and have served as 'normal' controls for novel chemotherapeutics [87,88] and analysis of signaling pathways in the endometrium [89–93]. Similarly, the telomerase immortalized endometrial epithelial cell line, hTERT-EEC [59], was an exact genotypic match to MCF-7 breast cancer cells. hTERT-EEC has been proposed as model to study steroids in normal endometrial physiology, including, endometriosis and implantation [59,94,95]. Clearly, conclusions derived from studies utilizing HES cells (HeLa) or hTERT-EEC (MCF-7) should be interpreted with caution, in the light of evidence that they are neither normal nor endometrial in origin.

Cell line authentication is essential for their meaningful use in research. We recommend that cell lines be quarantined and authenticated by DNA profiling prior to use, and periodically evaluated by STR genotype, to check for cross-contamination and validate construction of stably transfected, genetically modified or clonally selected variants. Derivation of novel cell lines should be accompanied, where possible, by STR profiles of the patient germ line, tumor or tissue, and cell line DNA. We also suggest the use of histological or phenotypic markers to verify the tissue of origin, since STR profiling cannot provide this information resulting in debate as to the tissue type of some cancer cell lines [2,96].

The origins and mechanisms of cell line contamination, including poor tissue culture technique, inadequate quality control, clerical and labeling errors, and aerosol transfer of cells, have been reviewed previously [63] and, despite best laboratory practices, are probably unavoidable. Accordingly, even among cell lines that exhibited unique profiles, we found examples, from all sources, of individual aliquots that were misidentified or contaminated, indicating a widespread and pervasive problem. STR profiling is a simple, widely available and relatively inexpensive method to document and authenticate cell lines, and has been recommended as an internationally accepted standard for human cells [22,63,97,98]. Despite repeated calls for journals to require DNA profiling of cells for publication, this practice has not been widely adopted [63,99]. Complacency and denial of the existence and extent of the problem with validation and authenticity of cell lines, while prevalent [7,24,63,99], are antithetical to the conduct of responsible research in gynecologic oncology.

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Conflict of interest statement

No conflict of interest.

Acknowledgments

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References

[1] Burdall SE, Hanby AM, Lansdown MR, Speirs V. Breast cancer cell lines: friend or foe? Breast Cancer Res 2003;5:89–95.
[2] Schweppe RE, Klopper JP, Korch C, Pugazhenthi U, Benezra M, Knauf JA, Fagin JA, Marlow LA, Copland JA, Smallridge RC, Haugen BR. Deoxyribonucleic acid profiling analysis of 40 human thyroid cancer cell lines reveals cross-contamination resulting in cell line redundancy and misidentification. J Clin Endocrinol Metab 2008;93:4331–41.
[3] Vollmer G. Endometrial cancer: experimental models useful for studies on molecular aspects of endometrial cancer and carcinogenesis. Endocr Relat Cancer 2003;10:23–42.
[4] Lucey BP, Nelson-Rees WA, Hutchins GM. Henrietta Lacks, HeLa cells, and cell culture contamination. Arch Pathol Lab Med 2009;133:1463–7.
[5] MacLeod RA, Dirks WG, Matsuo Y, Kaufmann M, Milch H, Drexler HG. Widespread intraspecies cross-contamination of human tumor cell lines arising at source. Int J Cancer 1999;83:555–63.
[6] McCormick D. Sleeper cell. Biotechniques 2008;45:11.
[7] Masters JR. HeLa cells 50 years on: the good, the bad and the ugly. Nat Rev Cancer 2002;2:315–9.
[8] Identity crisis. Nature 2009;457:935–6.
[9] Chatterjee R. Cell biology. When 60 lines don't add up. Science 2007;315:929.
[10] Chatterjee R. Cell biology. Cases of mistaken identity. Science 2007;315:928–31.
[11] Freshney RI. Authentication of cell lines: ignore at your peril! Expert Rev Anticancer Ther 2008;8:311–4.
[12] Hughes P, Marshall D, Reid Y, Parkes H, Gelber C. The costs of using unauthenticated, over-passaged cell lines: how much more data do we need? Biotechniques 2007;43:575 [577–8, 581–2 passim.].
[13] Nelson-Rees WA, Daniels DW, Flandermeyer RR. Cross-contamination of cells in culture. Science 1981;212:446–52.
[14] Rae JM, Creighton CJ, Meck JM, Haddad BR, Johnson MD. MDA-MB-435 cells are derived from M14 melanoma cells—a loss for breast cancer, but a boon for melanoma research. Breast Cancer Res Treat 2007;104:13–9.
[15] Capes-Davis A, Theodosopoulos G, Atkin I, Drexler HG, Kohara A, MacLeod RA, Masters JR, Nakamura Y, Reid YA, Reddel RR, Freshney RI. Check your cultures! A list of cross-contaminated or misidentified cell lines. Int J Cancer 2010;127:1–8.
[16] Lacroix M. Persistent use of "false" cell lines. Int J Cancer 2008;122:1–4.
[17] van Bokhoven A, Varella-Garcia M, Korch C, Hessels D, Miller GJ. Widely used prostate carcinoma cell lines share common origins. Prostate 2001;47:36–51.
[18] van Bokhoven A, Varella-Garcia M, Korch C, Johannes WU, Smith EE, Miller HL, Nordeen SK, Miller GJ, Lucia MS. Molecular characterization of human prostate carcinoma cell lines. Prostate 2003;57:205–25.
[19] van Bokhoven A, Varella-Garcia M, Korch C, Miller GJ. TSU-Pr1 and JCA-1 cells are derivatives of T24 bladder carcinoma cells and are not of prostatic origin. Cancer Res 2001;61:6340–4.
[20] Dirks WG, Drexler HG. Online verification of human cell line identity by STR DNA typing. Methods Mol Biol 2011;731:45–55.
[21] Dirks WG, Faehnrich S, Estella IA, Drexler HG. Short tandem repeat DNA typing provides an international reference standard for authentication of human cell lines. ALTEX 2005;22:103–9.
[22] Masters JR, Thomson JA, Daly-Burns B, Reid YA, Dirks WG, Packer P, Toji LH, Ohno T, Tanabe H, Arlett CF, Kelland LR, Harrison M, Virmani A, Ward TH, Ayres KL, Debenham PG. Short tandem repeat profiling provides an international reference standard for human cell lines. Proc Natl Acad Sci U S A 2001;98:8012–7.
[23] Parson W, Kirchebner R, Muhlmann R, Renner K, Kofler A, Schmidt S, Kofler R. Cancer cell line identification by short tandem repeat profiling: power and limitations. FASEB J 2005;19:434–6.
[24] Cell line misidentification. the beginning of the end. Nat Rev Cancer 2010;10:441–8.
[25] Lorenzi PL, Reinhold WC, Varma S, Hutchinson AA, Pommier Y, Chanock SJ, Weinstein JN. DNA fingerprinting of the NCI-60 cell line panel. Mol Cancer Ther 2009;8:713–24.
[26] Mouchawar J, Korch C, Byers T, Pitts TM, Li E, McCredie MR, Giles GG, Hopper JL, Southey MC. Population-based estimate of the contribution of TP53 mutations to subgroups of early-onset breast cancer: Australian Breast Cancer Family Study. Cancer Res 2010;70:4795–800.
[27] Gologan A, Sepulveda AR. Microsatellite instability and DNA mismatch repair deficiency testing in hereditary and sporadic gastrointestinal cancers. Clin Lab Med 2005;25:179–96.
[28] Hecht JL, Mutter GL. Molecular and pathologic aspects of endometrial carcinogenesis. J Clin Oncol 2006;24:4783–91.
[29] Lax SF. Molecular genetic pathways in various types of endometrial carcinoma: from a phenotypical to a molecular-based classification. Virchows Arch 2004;444:213–23.
[30] Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E, Vergote I. Endometrial cancer. Lancet 2005;366:491–505.
[31] Albitar L, Pickett G, Morgan M, Davies S, Leslie KK. Models representing type I and type II human endometrial cancers: Ishikawa H and Hec50co cells. Gynecol Oncol 2007;106:52–64.
[32] Kuramoto H, Hamano M, Imai M. HEC-1 cells. Hum Cell 2002;15:81–95.
[33] Mo B, Vendrov AE, Palomino WA, DuPont BR, Apparao KB, Lessey BA. ECC-1 cells: a well-differentiated steroid-responsive endometrial cell line with characteristics of luminal epithelium. Biol Reprod 2006;75:387–94.
[34] Richardson GS, Dickersin GR, Atkins L, MacLaughlin DT, Raam S, Merk LP, Bradley FM. KLE: a cell line with defective estrogen receptor derived from undifferentiated endometrial cancer. Gynecol Oncol 1984;17:213–30.
[35] Way DL, Grosso DS, Davis JR, Surwit EA, Christian CD. Characterization of a new human endometrial carcinoma (RL95-2) established in tissue culture. In Vitro 1983;19:147–58.
[36] Kuramoto H, Tamura S, Notake Y. Establishment of a cell line of human endometrial adenocarcinoma in vitro. Am J Obstet Gynecol 1972;114:1012–9.
[37] Kuramoto H. Studies of the growth and cytogenetic properties of human endometrial adenocarcinoma in culture and its development into an established line. Acta Obstet Gynaecol Jpn 1972;19:47–58.
[38] Kuramoto H, Nishida M, Morisawa T, Hamano M, Hata H, Kato Y, Ohno E, Iida T. Establishment and characterization of human endometrial cancer cell lines. Ann N Y Acad Sci 1991;622:402–21.
[39] Satyaswaroop PG, Fleming H, Bressler RS, Gurpide E. Human endometrial cancer cell cultures for hormonal studies. Cancer Res 1978;38:4367–75.
[40] Kuramoto H, Suzuki K. Effects of progesterone on the growth kinetics and the morphology of a human endometrial cancer cell-line. Acta Obstet Gynaecol Jpn 1976;23:123–32.
[41] Dawe CJ, Banfield WG, Morgan WD, Slatick MS, Curth HO. Growth in continuous culture, and in hamsters, of cells from a neoplasm associated with acanthosis nigricans. J Natl Cancer Inst 1964;33:441–56.
[42] Satyaswaroop PG. Endometrial cancer. In: Masters JRW, Palsson B, editors. Human cell culture. Kluwer Academic Publishers; 1999. p. 71–8.
[43] Nishida M, Kasahara K, Kaneko M, Iwasaki H, Hayashi K. Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors. Nihon Sanka Fujinka Gakkai Zasshi 1985;37:1103–11.
[44] Nishida M, Kasahara K, Oki A, Satoh T, Arai Y, Kubo T. Establishment of eighteen clones of Ishikawa cells. Hum Cell 1996;9:109–16.
[45] Nishida M. The Ishikawa cells from birth to the present. Hum Cell 2002;15:104–17.
[46] Lewis KA, Mullany S, Thomas B, Chien J, Loewen R, Shridhar V, Cliby WA. Heterozygous ATR mutations in mismatch repair-deficient cancer cells have functional significance. Cancer Res 2005;65:7091–5.
[47] Inoue M. Current molecular aspects of the carcinogenesis of the uterine endometrium. Int J Gynecol Cancer 2001;11:339–48.
[48] Risinger JI, Berchuck A, Kohler MF, Watson P, Lynch HT, Boyd J. Genetic instability of microsatellites in endometrial carcinoma. Cancer Res 1993;53:5100–3.
[49] Umar A, Boyer JC, Thomas DC, Nguyen DC, Risinger JI, Boyd J, Ionov Y, Perucho M, Kunkel TA. Defective mismatch repair in extracts of colorectal and endometrial cancer cell lines exhibiting microsatellite instability. J Biol Chem 1994;269:14367–70.
[50] Ignar-Trowbridge DM, Teng CT, Ross KA, Parker MG, Korach KS, McLachlan JA. Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. Mol Endocrinol 1993;7:992–8.
[51] Collins F, MacPherson S, Brown P, Bombail V, Williams AR, Anderson RA, Jabbour HN, Saunders PT. Expression of oestrogen receptors, ERalpha, ERbeta, and ERbeta variants, in endometrial cancers and evidence that prostaglandin F may play a role in regulating expression of ERalpha. BMC Cancer 2009;9:330.
[52] Wenger SL, Senft JR, Sargent LM, Bamezai R, Bairwa N, Grant SG. Comparison of established cell lines at different passages by karyotype and comparative genomic hybridization. Biosci Rep 2004;24:631–9.
[53] Clarke CL, Feil PD, Satyaswaroop PG. Progesterone receptor regulation by 17 beta-estradiol in human endometrial carcinoma grown in nude mice. Endocrinology 1987;121:1642–8.
[54] Satyaswaroop PG, Sivarajah A, Zaino RJ. Hormonal control of growth of human endometrial carcinoma in nude mouse model. In: Brescani F, King RG, Lippman ME, editors. Progress in cancer research and therapy. New York: Raven Press; 1988. p. 430–55.
[55] Satyaswaroop PG, Tabibzadeh SS. Extracellular matrix and the patterns of differentiation of human endometrial carcinomas in vitro and in vivo. Cancer Res 1991;51:5661–6.
[56] Patel RR, Sengupta S, Kim HR, Klein-Szanto AJ, Pyle JR, Zhu F, Li T, Ross EA, Oseni S, Fargnoli J, Jordan VC. Experimental treatment of oestrogen receptor (ER) positive breast cancer with tamoxifen and brivanib alaninate, a VEGFR-2/FGFR-1 kinase inhibitor: a potential clinical application of angiogenesis inhibitors. Eur J Cancer 2010;46:1537–53.

[57] Yaginuma Y, Westphal H. Analysis of the p53 gene in human uterine carcinoma cell lines. Cancer Res 1991;51:6506–9.
[58] Desai NN, Kennard EA, Kniss DA, Friedman CI. Novel human endometrial cell line promotes blastocyst development. Fertil Steril 1994;61:760–6.
[59] Hombach-Klonisch S, Kehlen A, Fowler PA, Huppertz B, Jugert JF, Bischoff G, Schluter E, Buchmann J, Klonisch T. Regulation of functional steroid receptors and ligand-induced responses in telomerase-immortalized human endometrial epithelial cells. J Mol Endocrinol 2005;34:517–34.
[60] Kniss DA, Zimmerman PD, Garver CL, Fertel RH. Interleukin-1 receptor antagonist blocks interleukin-1-induced expression of cyclooxygenase-2 in endometrium. Am J Obstet Gynecol 1997;177:559–67.
[61] Hayflick L. The establishment of a line (WISH) of human amnion cells in continuous cultivation. Exp Cell Res 1961;23:14–20.
[62] Kniss DA, Xie Y, Li Y, Kumar S, Linton EA, Cohen P, Fan-Havard P, Redman CW, Sargent IL. ED(27) trophoblast-like cells isolated from first-trimester chorionic villi are genetically identical to HeLa cells yet exhibit a distinct phenotype. Placenta 2002;23:32–43.
[63] Masters JR. False cell lines: the problem and a solution. Cytotechnology 2002;39: 69–74.
[64] Geisinger KR, Kute TE, Pettenati MJ, Welander CE, Dennard Y, Collins LA, Berens ME. Characterization of a human ovarian carcinoma cell line with estrogen and progesterone receptors. Cancer 1989;63:280–8.
[65] DiSaia PJ, Sinkovics JG, Rutledge FN, Smith JP. Cell-mediated immunity to human malignant cells. A brief review and further studies with two gynecologic tumors. Am J Obstet Gynecol 1972;114:979–89.
[66] Orth K, Hung J, Gazdar A, Bowcock A, Mathis JM, Sambrook J. Genetic instability in human ovarian cancer cell lines. Proc Natl Acad Sci U S A 1994;91:9495–9.
[67] Woods LK, Morgan RT, Quinn LA, Moore GE, Semple TU, Stedman KE. Comparison of four new cell lines from patients with adenocarcinoma of the ovary. Cancer Res 1979;39:4449–59.
[68] Bast Jr RC, Feeney M, Lazarus H, Nadler LM, Colvin RB, Knapp RC. Reactivity of a monoclonal antibody with human ovarian carcinoma. J Clin Invest 1981;68:1331–7.
[69] Berkowitz R, Kabawat S, Lazarus H, Colvin R, Knapp R, Bast Jr RC. Comparison of a rabbit heteroantiserum and a murine monoclonal antibody raised against a human epithelial ovarian carcinoma cell line. Am J Obstet Gynecol 1983;146:607–12.
[70] Langdon SP, Lawrie SS, Hay FG, Hawkes MM, McDonald A, Hayward IP, Schol DJ, Hilgers J, Leonard RC, Smyth JF. Characterization and properties of nine human ovarian adenocarcinoma cell lines. Cancer Res 1988;48:6166–72.
[71] Buick RN, Pullano R, Trent JM. Comparative properties of five human ovarian adenocarcinoma cell lines. Cancer Res 1985;45:3668–76.
[72] Hills CA, Kelland LR, Abel G, Siracky J, Wilson AP, Harrap KR. Biological properties of ten human ovarian carcinoma cell lines: calibration in vitro against four platinum complexes. Br J Cancer 1989;59:527–34.
[73] Watanabe Y, Koi M, Hemmi H, Hoshai H, Noda K. A change in microsatellite instability caused by cisplatin-based chemotherapy of ovarian cancer. Br J Cancer 2001;85:1064–9.
[74] Loh SY, Mistry P, Kelland LR, Abel G, Harrap KR. Reduced drug accumulation as a major mechanism of acquired resistance to cisplatin in a human ovarian carcinoma cell line: circumvention studies using novel platinum (II) and (IV) ammine/amine complexes. Br J Cancer 1992;66:1109–15.
[75] Nio Y, Zighelboim J, Berek JS, Bonavida B. Sensitivity of fresh and cultured ovarian tumor cells to tumor necrosis factor, interferon-alpha 2, and OK-432. Cancer Immunol Immunother 1988;27:246–54.
[76] Andrews PA, Murphy MP, Howell SB. Differential potentiation of alkylating and platinating agent cytotoxicity in human ovarian carcinoma cells by glutathione depletion. Cancer Res 1985;45:6250–3.
[77] Eva A, Robbins KC, Andersen PR, Srinivasan A, Tronick SR, Reddy EP, Ellmore NW, Galen AT, Lautenberger JA, Papas TS, Westin EH, Wong-Staal F, Gallo RC, Aaronson SA. Cellular genes analogous to retroviral onc genes are transcribed in human tumour cells. Nature 1982;295:116–9.
[78] Ozols RF, Hogan WM, Young RC. Direct cloning of human ovarian cancer in soft agar: clinical limitations and pharmacologic applications. Recent Results Cancer Res 1984;94:41–50.
[79] Yuan Y, Kim WH, Han HS, Lee JH, Park HS, Chung JK, Kang SB, Park JG. Establishment and characterization of human ovarian carcinoma cell lines. Gynecol Oncol 1997;66:378–87.
[80] Berchuck A, Rodriguez G, Olt G, Whitaker R, Boente MP, Arrick BA, Clarke-Pearson DL, Bast Jr RC. Regulation of growth of normal ovarian epithelial cells and ovarian cancer cell lines by transforming growth factor-beta. Am J Obstet Gynecol 1992;166:676–84.
[81] Young TN, Rodriguez GC, Moser TL, Bast Jr RC, Pizzo SV, Stack MS. Coordinate expression of urinary-type plasminogen activator and its receptor accompanies malignant transformation of the ovarian surface epithelium. Am J Obstet Gynecol 1994;170:1285–96.
[82] King AE, Fleming DC, Critchley HO, Kelly RW. Regulation of natural antibiotic expression by inflammatory mediators and mimics of infection in human endometrial epithelial cells. Mol Hum Reprod 2002;8:341–9.
[83] Banerjee P, Sapru K, Strakova Z, Fazleabas AT. Chorionic gonadotropin regulates prostaglandin E synthase via a phosphatidylinositol 3-kinase-extracellular regulatory kinase pathway in a human endometrial epithelial cell line: implications for endometrial responses for embryo implantation. Endocrinology 2009;150:4326–37.
[84] Wang P, Julian JA, Carson DD. The MUC1 HMFG1 glycoform is a precursor to the 214D4 glycoform in the human uterine epithelial cell line, HES. Biol Reprod 2008;78:290–8.
[85] Menkhorst E, Salamonsen L, Robb L, Dimitriadis E. IL11 antagonist inhibits uterine stromal differentiation, causing pregnancy failure in mice. Biol Reprod 2009;80: 920–7.
[86] Bukulmez O, Hardy DB, Carr BR, Auchus RJ, Toloubeydokhti T, Word RA, Mendelson CR. Androstenedione up-regulation of endometrial aromatase expression via local conversion to estrogen: potential relevance to the pathogenesis of endometriosis. J Clin Endocrinol Metab 2008;93:3471–7.
[87] Cho JH, Lee JG, Yang YI, Kim JH, Ahn JH, Baek NI, Lee KT, Choi JH. Eupatilin, a dietary flavonoid, induces G2/M cell cycle arrest in human endometrial cancer cells. Food Chem Toxicol 2011;49:1737–44.
[88] Kim JH, Yang YI, Lee KT, Park HJ, Choi JH. Costunolide induces apoptosis in human endometriotic cells through inhibition of the prosurvival Akt and nuclear factor kappa B signaling pathway. Biol Pharm Bull 2011;34:580–5.
[89] Khorram O, Han G, Magee T. Cigarette smoke inhibits endometrial epithelial cell proliferation through a nitric oxide-mediated pathway. Fertil Steril 2010;93:257–63.
[90] Carino C, Olawaiye AB, Cherfils S, Serikawa T, Lynch MP, Rueda BR, Gonzalez RR. Leptin regulation of proangiogenic molecules in benign and cancerous endometrial cells. Int J Cancer 2008;123:2782–90.
[91] Jabbour HN, Boddy SC. Prostaglandin E2 induces proliferation of glandular epithelial cells of the human endometrium via extracellular regulated kinase 1/2-mediated pathway. J Clin Endocrinol Metab 2003;88:4481–7.
[92] Luo X, Xu J, Chegini N. Gonadotropin releasing hormone analogue (GnRHa) alters the expression and activation of Smad in human endometrial epithelial and stromal cells. Reprod Biol Endocrinol 2003;1:125.
[93] Luo X, Xu J, Chegini N. The expression of Smads in human endometrium and regulation and induction in endometrial epithelial and stromal cells by transforming growth factor-beta. J Clin Endocrinol Metab 2003;88:4967–76.
[94] Aboussahoud W, Aflatoonian R, Bruce C, Elliott S, Ward J, Newton S, Hombach-Klonisch S, Klonisch T, Fazeli A. Expression and function of Toll-like receptors in human endometrial epithelial cell lines. J Reprod Immunol 2010;84:41–51.
[95] Willing C, Peich M, Danescu A, Kehlen A, Fowler PA, Hombach-Klonisch S. Estrogen-independent actions of environmentally relevant AhR-agonists in human endometrial epithelial cells. Mol Hum Reprod 2011;17:115–26.
[96] Chambers AF. MDA-MB-435 and M14 cell lines: identical but not M14 melanoma? Cancer Res 2009;69:5292–3.
[97] O'Brien SJ. Cell culture forensics. Proc Natl Acad Sci U S A 2001;98:7656–8.
[98] Barallon R, Bauer SR, Butler J, Capes-Davis A, Dirks WG, Elmore E, Furtado M, Kline MC, Kohara A, Los GV, MacLeod RA, Masters JR, Nardone M, Nardone RM, Nims RW, Price PJ, Reid YA, Shewale J, Sykes G, Steuer AF, Storts DR, Thomson J, Taraporewala Z, Alston-Roberts C, Kerrigan L. Recommendation of short tandem repeat profiling for authenticating human cell lines, stem cells, and tissues. In Vitro Cell Dev Biol Anim 2010;46: 727–32.
[99] Katsnelson A. Biologists tackle cells' identity crisis. Nature 2010;465:537.

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RESEARCH PAPER

Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells

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BACKGROUND AND PURPOSE

Oestrogen receptor alpha (ERα) binds to different ligand which can function as complete/partial oestrogen-agonist or antagonist. This depends on the chemical structure of the ligands which modulates the transcriptional activity of the oestrogen-responsive genes by altering the conformation of the liganded-ERα complex. This study determined the molecular mechanism of oestrogen-agonistic/antagonistic action of structurally similar ligands, bisphenol (BP) and bisphenol A (BPA) on cell proliferation and apoptosis of ERα + ve breast cancer cells.

EXPERIMENTAL APPROACH

DNA was measured to assess the proliferation and apoptosis of breast cancer cells. RT-PCR and ChIP assays were performed to quantify the transcripts of *TFF1* gene and recruitment of ERα and SRC3 at the promoter of *TFF1* gene respectively. Molecular docking was used to delineate the binding modes of BP and BPA with the ERα. PCR-based arrays were used to study the regulation of the apoptotic genes.

KEY RESULTS

BP and BPA induced the proliferation of breast cancer cells; however, unlike BPA, BP failed to induce apoptosis. BPA consistently acted as an agonist in our studies but BP exhibited mixed agonistic/antagonistic properties. Molecular docking revealed agonistic and antagonistic mode of binding for BPA and BP respectively. BPA treatment resembled E2 treatment in terms of PCR-based regulation of apoptotic genes whereas BP was similar to 4OHT treatment.

CONCLUSIONS AND IMPLICATIONS

The chemical structure of ERα ligand determines the agonistic or antagonistic biological responses by the virtue of their binding mode, conformation of the liganded-ERα complex and the context of the cellular function.

Abbreviations

4OHT, 4-hydroxy tamoxifen; BP, bisphenol; BPA, bisphenol A; ChIP, chromatin-immunoprecipitation assay; DES, diethylstilbestrol; E2, 17β-oestradiol; ERα, oestrogen receptor alpha; LBD, ligand binding domain; RAL, raloxifene; RT-PCR, real time PCR; SRC3, steroid coactivator 3; *TFF1*, *trefoil factor 1*

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Introduction

Oestrogen receptor alpha (ERα) mediates its action in cells and tissues by binding to its cognate ligands and function as a 'ligand-activated' transcription factor (Jordan and O'Malley, 2007). Apart from its natural ligands, many different compounds can bind to ERα and thus can function as its ligand (Sengupta and Jordan, 2008). However, depending upon the chemical structures of these ligands, they can either function as a complete/partial oestrogen- agonist or antagonist. Broadly, the oestrogenic compounds can be classified as class I and class II depending upon their planar or non-planar chemical structures respectively (Jordan *et al.*, 2001). Different ligands bind to the same core of the ligand binding domain (LBD) of ERα protein but can evoke distinct three-dimensional conformation of the liganded-ERα complex which can either interact with the coactivators or the corepressors (collectively known as coregulators) at the promoters of oestrogen-responsive genes (Jordan and O'Malley, 2007). Consequently, this complex modulates the transcriptional activity of the various oestrogen-responsive genes and eventually determines the outcome of the ERα-dependent physiological responses of a particular cell or tissue type. The molecular basis of this differential recruitment of the coregulators has been attributed to the ability of the liganded-ERα to reorient the helix 12 (H12) of the LBD in such a manner that the complex can interact with the coactivators at the structural interface formed by H3, H4 and H5 helices; when ERα is bound to an agonist [17β-oestradiol (E2) or diethylstilbestrol (DES)] (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998), but this interaction is completely blocked when the ERα is bound to antagonists, such as 4-hydroxy-tamoxifen (4OHT) (Brzozowski *et al.*, 1997) or raloxifene (RAL) (Shiau *et al.*, 1998). Interestingly, when ERα is liganded with an antagonist, such as 4OHT, an active metabolite of tamoxifen, which is extensively used in treatment and prevention of breast cancers (Jordan, 1993), it can now interact with the corepressors and can inhibit the transcriptional activity from the oestrogen-responsive genes (Metivier *et al.*, 2002; Shang and Brown, 2002; Liu and Bagchi, 2004). Besides the interaction of coregulators with the liganded ERα, the levels of coactiavtors and corepressors in a given cell can also determine the physiological responses to different ligands of ERα (Shang and Brown, 2002).

Earlier studies from our laboratory have identified that the amino acid aspartate at 351 (which is in the H3) of the ERα LBD is critically important for maintaining the integrity of antioestrogenic activity of keoxifene (RAL) and 4OHT (Levenson *et al.*, 1997; 1998). Earlier, the mutation of ERα encoding amino acid 351 which substituted the aspartate to tyrosine amino acid was detected in one of the xenograft tumours stimulated by tamoxifen in the athymic mice (Wolf and Jordan, 1994). Further investigations have revealed that changing the amino acid aspartate 351 of the ERα to glycine (D351G) abolishes the oestrogenic effect of 4OHT but does not affect oestradiol action on *TGFα* gene activation in the ER negative breast cancer cells stably transfected with either wild type ERα or D351G mutated ERα (MacGregor Schafer *et al.*, 2000). Using these models, oestrogens were classified as either type I, which have the planar structures or type II, which have the angular or non-planar structures (Jordan *et al.*, 2001; Bentrem *et al.*, 2003). A recent confirmatory study evaluated the ability of several type I and II liganded ERα to associate with the specific peptide motif 'LXXLL' which coactivators use to interact with the ERα (Bourgoin-Voillard *et al.*, 2010).

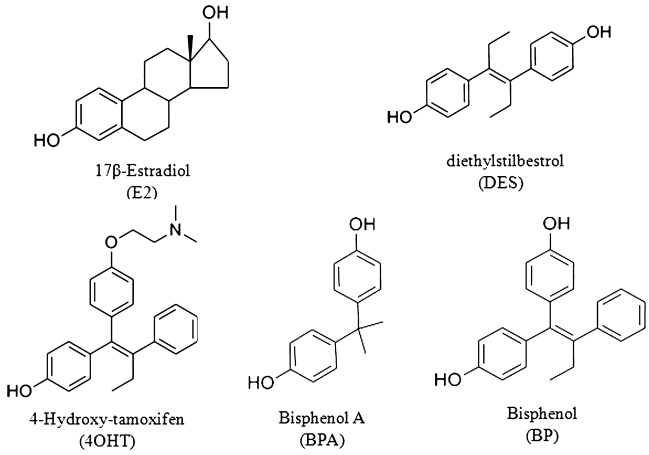

Figure 1

Chemical structures of 17β-oestradiol (E2), Diethylstilbestrol (DES), 4-Hydroxy tamoxifen (4OHT), Bisphenol (BP) and Bisphenol A (BPA).

A previous study (Maximov *et al.*, 2011) from our laboratory indicated that the conformation of the ERα complex can govern the oestrogen-induced apoptosis in the MCF7 : 5C breast cancer cells. The present study dissects the ERα mediated effect of two structurally similar oestrogenic ligands, namely, bisphenol (BP) and bisphenol A (BPA) (Figure 1), on two critical physiological responses, that is growth and apoptosis in the breast cancer cells. BP is structurally related to 4OHT with E2-like agonistic properties, whereas BPA has been characterized as an endocrine disruptor with weak oestrogenic properties. Using various investigative tools, this study underscore the fact that minor difference in the shape of the ERα-liganded complex has profound modulation on oestrogen-induced apoptosis but not on oestrogen-induced replication of breast cancer cells.

Materials and methods

Cell culture and reagents

Cell culture media were purchased from Invitrogen Inc. (Grand Island, NY, USA) and fetal calf serum (FCS) was obtained from HyClone Laboratories (Logan, UT, USA). Compounds E2, 4OHT and BPA were obtained from Sigma-Aldrich (St. Louis, MO, USA). BP was synthesized and the details of the synthesis have been reported previously (Maximov *et al.*, 2010). The ER positive breast cancer cells MCF-7 : WS8 (hereafter mentioned as MCF7) and oestrogen-deprived MCF7 : 5C were derived from MCF7 cells obtained from the Dr. Dean Edwards, San Antonio, TX, USA as reported previously (Jiang *et al.*, 1992). MCF7 cells were maintained in RPMI media supplemented with 10% FCS, 6 ng·mL^{-1} bovine insulin and penicillin and streptomycin. MCF7 : 5C cells were maintained in phenol red-free RPMI media containing 10% char-

coal dextran treated FCS, 6 ng·mL^{-1} bovine insulin and penicillin and streptomycin. Three to four days prior to harvesting the MCF7, cells were cultivated in phenol red-free media containing 10% charcoal dextran treated FCS. The cells were treated with indicated compounds (with media changes every 48 h) for the specified time and were subsequently harvested for growth assay. MDA-MB-231 cells stably transfected with wild type ERα (MC2) or D351G ERα (JM6) were grown in minimal essential medium without phenol red in the presence of 5% charcoal dextran treated calf serum, glutamine, bovine insulin, penicillin, streptomycin, nonessential amino acids and 500 µg·mL^{-1} G418 as described previously (MacGregor Schafer *et al.*, 2000). All the experiments were repeated at least three times, in triplicate to confirm the results.

Cell growth assay

The cell growth was monitored by measuring the total DNA content per well in 24 well plates. Fifteen thousand cells were plated per well and treatment with indicated concentrations of compounds was started after 24 h, in triplicate. Media containing the specific treatments were changed every 48 h. On day 6 (144 h post treatment), the cells were harvested and total DNA was assessed using a fluorescent DNA quantitation kit (Cat # 170–2480; Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. Briefly, the cells were harvested using hypotonic buffer solution and were subsequently sonicated. The DNA content was estimated using a fluorescent dye (Hoechst 33258) provided in the kit.

RNA isolation and real-time PCR (RT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNAeasy kit according to the manufacturer's instructions. RT-PCR was performed as previously described (Sengupta *et al.*, 2010). Briefly, high capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA, USA) was used to generate cDNA was using 1 ug of total RNA in a total volume of 20 uL. The cDNA was subsequently diluted to 500 uL and RT-PCR was performed using ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). In each well, 20 uL reaction volume included 10 uL SYBR green PCR master mix (Applied Biosystems), 125 nM each of forward and reverse primers and 5 uL of diluted cDNA. The change in expression of transcripts was determined as described previously and used the ribosomal protein 36B4 mRNA as the internal control (Sengupta *et al.*, 2010).

Chromatin-immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously (Maximov *et al.*, 2011). Briefly, cells were treated with indicated compounds for 45 min and cross-linked using 1.25% paraformaldehyde for 15 min and subsequently stopped cross-linking with 2 M glycine. Cells were collected, followed by nuclei isolation by centrifugation. Isolated nuclei were resuspended in SDS-lysis buffer followed by sonication and centrifugation at 14 000× *g* for 20 min at 4°C. The supernatant were diluted 1 : 10 with ChIP dilution buffer. Normal rabbit IgG and Magna ChIP protein A magnetic bead (Upstate Cell Signaling Solutions, Temecula CA, USA) were used to immunoclear the supernatant followed by immunoprecipitation with antibodies against ERα (1:1 mixture of cat# sc-543 and sc-7207; Santa Cruz Biotechnology Inc., Dallas, TX, USA) and steroid receptor coactivator-3 (SRC3) (cat# 13066; Santa Cruz Biotechnology, Inc.). Immunocomplexes were pulled down using protein A magnetic beads and a magnet. The beads bound to immunocomplexes were washed using different buffers as described previously (Maximov *et al.*, 2011). Precipitates were finally extracted twice using freshly made 1% SDS and 0.1 M $NaHCO_3$ followed by de-crosslinking. The DNA fragments were purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA). RT-PCR was performed using 2 µL isolated DNA, using primers specific for *PS2* promoter (Maximov *et al.*, 2011). The data are presented as percent input of starting chromatin input after subtracting the percent input pull down of the negative control (normal rabbit IgG).

Molecular modelling

A commonly used method to evaluate the docking method efficiency is to dock the cocrystallized ligand to its native experimental structure. The expected outcome would be a docking solution, pose, which recapitulates the binding mode of the ligand in the binding site of the experimental structure. For this reason, 3D-conformations of E2, DES and 4OHT were generated, optimized with MMFF94 force field and then subjected to preparation for docking using the LigPrep utility. The same protocol was followed for BPA and BP. Protein Preparation Workflow (Schrödinger, LLC, New York, NY, 2011) was employed to prepare the proteins for molecular docking. The residues well known to be important for biological activity D351 and E353 were kept charged in all three receptors, the free rotation of hydroxyl group for T347 was allowed and H524 residue was protonated at the epsilon nitrogen atom in the complexes 1GWR and 3ERT based on the available literature data. In the case of 3ERD complex, two structures were prepared for docking runs having H524 protonated at epsilon (3ERD_ε) and delta (3ERD_δ) nitrogen.

The best docking poses were selected based on the composite score, Emodel, which accounts not only for the binding affinity but also for the energetic terms, such as ligand strain energy and interaction energy. When E2, DES and 4OHT were docked to their native structures the top ranked docking solutions have a ligand RMSD of 0.353 for E2, 0.416 for DES docked to 3ERD_ε and 0.372 when docked to 3ERD_δ and 0.629 for 4OHT.

Real time profiler assay for apoptosis

RT-PCR profiler assay kits for apoptosis was used from a commercial vendor which uses 384 well plates to profile the expression of 370 apoptosis related human genes (Qiagen; SABiosciences Corp, Fredrick, MD, USA; Cat#330231 PAHS-3012E). All the procedures were followed as per the manufacturer's instructions. Briefly, MCF7 : 5C cells were treated with E2 (10^{-9} M) for 24, 48 and 72 h or with indicated compounds (in triplicate) for 48 h and total RNA was isolated using the method mentioned earlier. Two micrograms of total RNA was reverse transcribed and RT-PCR was performed using ABI 7900HT. The fold change was calculated by ΔΔCt method and volcano plots were generated using the web based tool, RT^2 profile PCR array data analysis version 3.5 (Qiagen; SABiosciences Corp.).

Statistics

Statistical significance of our data was assessed using the Student's *t*-test wherever relevant. A *P*-value of <0.05 was considered as statistically significant.

Results

Differential effect of BP and BPA in inducing apoptosis in MCF7 : 5C cells but not growth in MCF7 cells

BP (Figure 1) a triphenylethylene (TPE) is a known partial oestrogenic ligand which can induce growth of the ERα positive breast cancer cells (Maximov *et al.*, 2010) and can also partially initiate prolactin synthesis from primary culture of cells from immature rat pituitary glands (Jordan and Lieberman, 1984). Another compound with similar chemical structure, BPA (Figure 1) is also a well-characterized but weak oestrogenic ligand (Routledge *et al.*, 2000). Here, we evaluated the ability of these two oestrogenic compounds to induce growth and apoptosis in MCF7 and MCF7 : 5C cells, respectively as both these responses are dependent on oestrogen-agonistic action. As expected, BP as well as BPA was able to induce the concentration dependent growth in the MCF7 cells (Figure 2A). BPA was less potent compared to BP as maximal growth was achieved by BP at 10^{-9} M concentra-

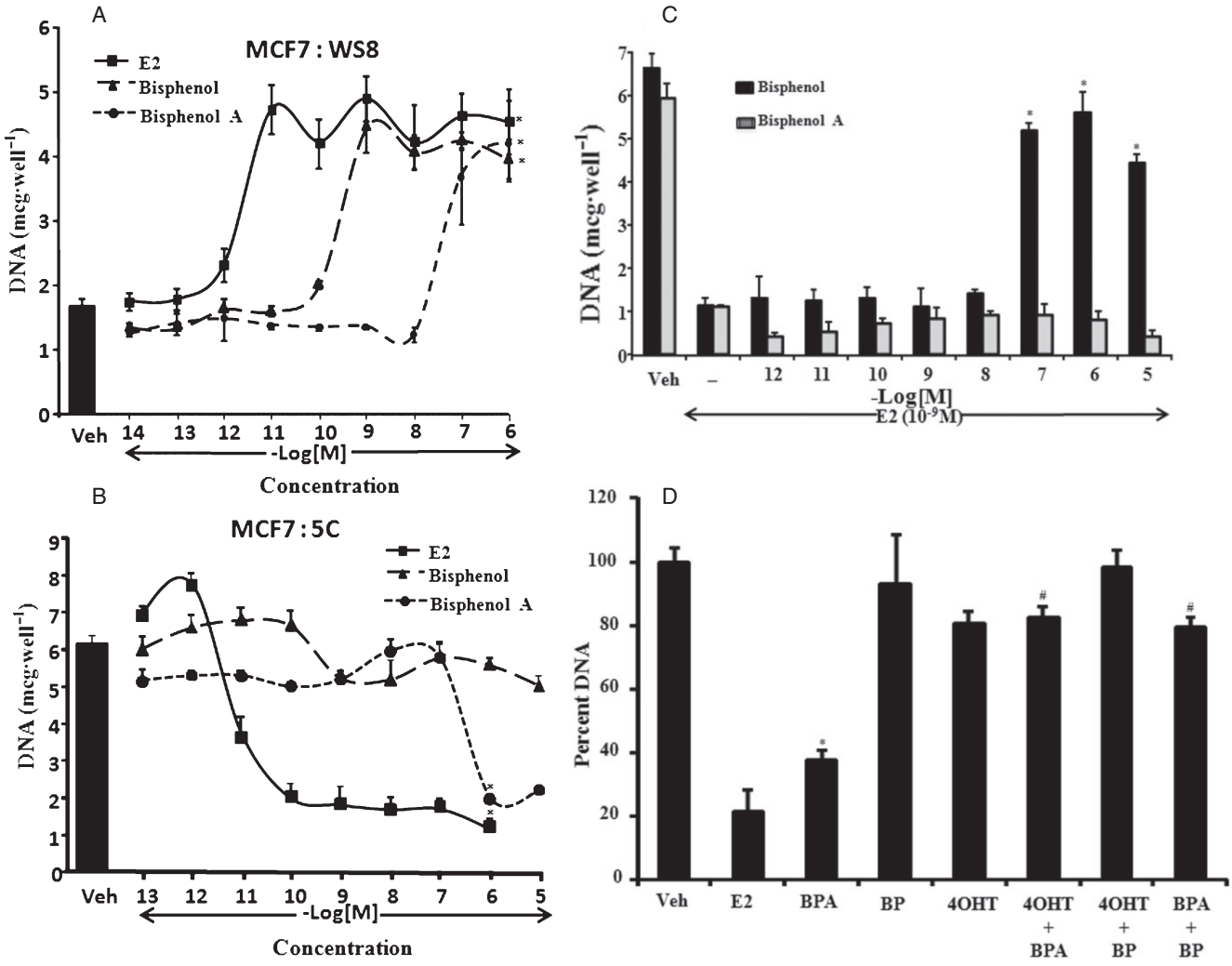

Figure 2

Differential effect of bisphenol (BP) and bisphenol A (BPA) on growth and apoptosis of ERα positive breast cancer cells. (A) Dose-dependent effects of BP, BPA and (oestradiol) E2 on growth of MCF7 cells treated for 6 days as indicated. The black bar denotes the level of DNA in vehicle treated cells over a 6-day period. The growth is measured as amount of DNA present in each well. (*P < 0.05 vs. vehicle treatment) (B) Dose-dependent effect of BP, BPA and E2 on apoptosis of MCF7 : 5C cells treated for 6 days as indicated. The black bar denotes the level of DNA in vehicle treated cells over a 6-day period. The growth is measured as amount of DNA present in each well. (*P < 0.05 vs. vehicle treatment) (C) Dose dependent effect of BP and BPA on E2 (1 nM)-induced apoptosis in MCF7 : 5C cells, treated over a six day period. The growth is measured as amount of DNA present in each well. (*P < 0.05 vs. 1 nM E2 treatment) (D) Effect of BP (10^{-6} M) and 4OHT (10^{-6} M) on BPA (10^{-6} M) induced apoptosis in MCF7 : 5C cells over 6-day period. (*P < 0.05 vs. vehicle treatment; $^{\#}P$ < 0.05 vs. BPA treatment) The data are presented as percent of growth considering the vehicle treated cells as 100 percent. Each value is average of at least three replicates ±SD.

tion as compared to 10^{-6} M for BPA. By comparison, E2 induced maximal growth at 10^{-11} M concentration in the MCF7 cells. In the case of MCF7 : 5C cells, which undergo apoptosis with E2 treatment (Lewis *et al.*, 2005; Ariazi *et al.*, 2011), a marked contrast was observed between BP and BPA in the induction of apoptosis. BPA was able to induce apoptosis to the same extent as E2 in these cells at a higher (10^{-6} M) concentration (Figure 2B) as compared to E2 which achieved maximal effect at 10^{-10} M. However, BP failed to induce apoptosis even at 10^{-5} M concentration (Figure 2B). We further investigated that if BP was actually binding to the ERα in the MCF7 : 5C cells by treating these cells with BP in combination with 10^{-9} M of E2. BP was able to block the effect of E2 in the MCF7 : 5C cells (Figure 2C and Supporting Information Figure S3) in a concentration dependent manner indicating that the effect of BP was through the ERα, thus inhibiting the E2 action. On the other hand, BPA was not able to block the effect E2 action (Figure 2C). In addition, we also show that the oestrogenic effect of BPA (10^{-6} M) in inducing apoptosis in MCFF7 : 5C cells was completely blocked by BP (10^{-6} M) as well as 10^{-6} M of 4OHT (Figure 2D).

Regulation of oestrogen-responsive gene trefoil factor 1(TFF1 or PS2) by BP and BPA

We next investigated the transcriptional regulation of a well-characterized oestrogen-regulated gene, *TFF1* (*PS2*) (Metivier *et al.*, 2003) by BP and BPA and compared it with E2 and 4OHT. MCF7 cells were treated for 4 h with the 0.1% ethanol (veh), E2 (10^{-9} M), 4OHT (10^{-6} M), BP (10^{-6} M and 10^{-5} M) or BPA (10^{-6} M and 10^{-5} M) and the transcripts levels of *PS2* gene were measured using RT-PCR. Two different concentrations (10^{-6} M and 10^{-5} M) were used for BP and BPA, because BPA is a weak oestrogen and we wanted to evaluate the concentration dependent regulation of these compounds. As expected, *PS2* mRNA was up-regulated around fivefold by E2 (10^{-9} M) compared to vehicle treatment and 4OHT (10^{-6} M) which completely failed to induce the levels of *PS2* mRNA (Figure 3A). On the other hand, BP treatment at 10^{-6} M concentration moderately (~2 fold) up-regulated the *PS2* mRNA levels and higher concentration (10^{-5} M) of BP failed to further increase the levels of *PS2* (Figure 3A). Conversely, cells treated with BPA exhibited concentration dependent increase in up-regulation of the *PS2* mRNA and the magnitude of up-regulation with high concentration (10^{-5} M) of BP was equivalent to the E2-mediated up-regulation of *PS2* mRNA (Figure 3A).

Recruitment of ERα and SRC3 at the promoter of TFF1 gene after treatment with BP and BPA

To understand the differences in the molecular mechanism of the transcriptional activation of *PS2* gene *in vivo* by BP and BPA in comparison to E2 and 4OHT treatment, we performed ChIP assay to evaluate the recruitment of ERα and SRC3 at the promoter region of *TFF1* (*PS2*) gene (Figure 3B) which has a well-characterized functional oestrogen-responsive element (ERE) (Metivier *et al.*, 2002). MCF7 cells were treated with either 0.1% ethanol (veh), E2 (10^{-9} M), 4OHT (10^{-6} M), BP (10^{-6} M or 10^{-5} M) or BPA (10^{-6} M or 10^{-5} M) for 45 min and thereafter harvested for ChIP assay. The results (Figure 3C) reveal that both concentrations of BPA (10^{-6} M and 10^{-5} M) recruited ERα to the *PS2* promoter with ERE in a concentration-dependent manner which was equivalent to results obtained with E2 treatment. In contrast, BP did not show a concentration-related effect and the levels of ERα plateaued at 50% of either E2 or BPA (Figure 3C). Recruitment of the coactivator, SRC3 (AIB1), which plays a key role in transcriptional activation of several oestrogen-regulated genes, including *PS2* gene (Shao *et al.*, 2004; Labhart *et al.*, 2005), followed the similar pattern as the ERα (Figure 3D). BPA treatment at both the concentrations (10^{-6} M or 10^{-5} M) recruited SRC3 in a concentration-dependent manner to become equivalent to levels observed with E2 treatment whereas BP treatment (both concentration) plateaued at 50% of E2 or BPA recruitment levels (Figure 3D). As expected, 4OHT treatment did not recruit SRC3 and was comparable to vehicle treatment. The ChIP data correlates very well with the observed pattern of transcriptional activation of *PS2* gene (Figure 3A) under same treatment conditions.

Differential induction of transforming growth factor alpha (TGFα) gene by BP and BPA in MDA : MB-231 cells stably transfected with wild-type (wt) ERα or D351G mutant ERα

Previous studies from our laboratory have established an *in vitro* system to evaluate and differentiate the conformation of liganded ERα induced by planar and non-planar ligands (Jordan *et al.*, 2001). Activation of *TGFα* gene in MDA : MB 231 cells stably transfected with wt ERα (MC2 cells) or mutant ERα (JM6 cells, D351G; which has the aspartate substituted with glycine at amino acid 351), is used as a marker to distinguish the ERα interactions between planar and non-planar oestrogen ligands (Jordan *et al.*, 2001). We treated the MC2 and JM6 cells with increasing concentrations of BP and BPA and measured the *TGFα* induction in these cells. E2 was used as a positive control. In MC2 cells, (wt ERα), all the tested ligands induced *TGFα* transcripts level to similar levels (Figure 4A). Induction of *TGFα* by BPA was observed at higher concentrations whereas BP and E2 had similar effects (Figure 4A). On the other hand, in JM6 cells (mutant; D351G ERα), BP failed to induce *TGFα* transcription even at higher concentrations (Figure 4B), whereas E2 and BPA treatment induced TGFα (Figure 4B), although the maximal induction with BPA was observed at higher concentration (10^{-5} M) which was less than 50% of E2 treatment. We further confirmed that E2-induced *TGFα* stimulation in JM6 cells was completely blocked by BP and 4OHT in a dose-dependent manner; whereas co-treatment of BPA in presence of E2 failed to inhibit it (Figure 4C).

Molecular docking of BP and BPA to the LBD of ERα

To determine the binding mode of BPA and BP to ERα, the ligands were docked to the agonist and antagonist conformations of the receptor. The experimental structure, 3ERT, was selected from protein database for the antagonist conformation of ERα (Figure 5A) containing 4OHT, while for the agonist conformation, two experimental structures were selected, namely the receptor cocrystallized with E2, 1GWR (Figure 5B) and DES, 3ERD (Figure 5C) respectively.

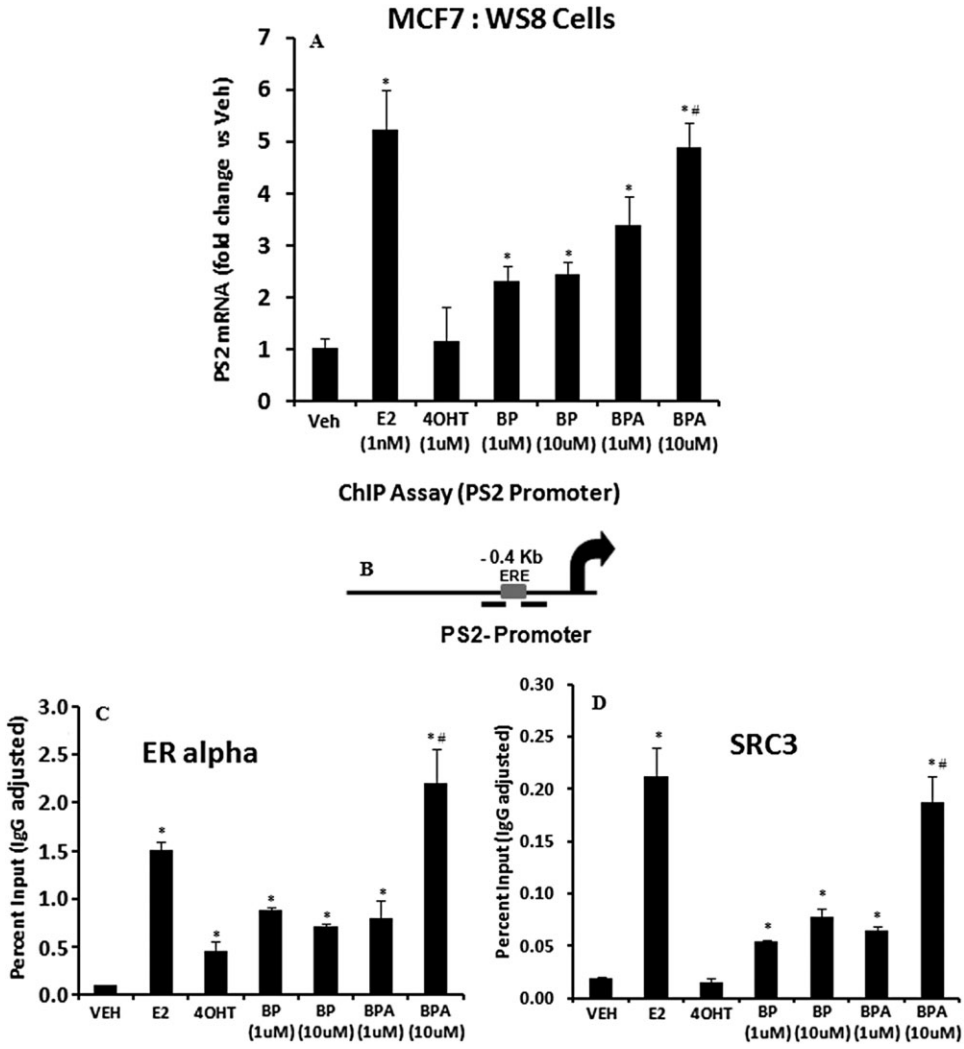

Figure 3

Regulation of *PS2* (*TFF1*) gene by bisphenol (BP), bisphenol A (BPA) compared with 17β-oestradiol (E2) and 4-hydroxy-tamoxifen (4OHT) and recruitment of oestrogen receptor alpha (ER alpha) and steroid receptor coactivator-3 (SRC3) at the oestrogen-responsive element (ERE) of proximal promoter of *PS2* gene followed by 45 min treatments of bisphenol (BP), bisphenol A (BPA) compared with 17β-oestradiol (E2) and 4-hydroxy-tamoxifen (4OHT) in MCF7 cells. (A) MCF7 cells were treated with indicated treatments for 4 h and harvested for total RNA. Total RNA was reverse transcribed and assessed for *PS2* gene expression levels using RT-PCR. *36B4* gene was used as an internal control. All values are represented in terms of fold difference versus vehicle treatment. (*$P < 0.05$ vs. vehicle treatment; $^{\#}P < 0.05$ vs. 1 μM BPA and 10 μM BP treatment) (B) Schematic representation of the *PS2* proximal promoter containing an ERE (grey box) and the black bars represent the primers used for RT-PCR. (C) Recruitment of ERα at the *PS2* proximal promoter, by ChIP assay after 45 min of indicated treatment. (*$P < 0.05$ vs. vehicle treatment; $^{\#}P < 0.05$ vs. 1 μM BPA and 10 μM BP treatment) (D) Recruitment of SRC3 at the *PS2* proximal promoter, by ChIP assay after 45 min of indicated treatment. All the values are representated as percent input of the starting chromatin material and after subtracting the IgG control for each sample. (*$P < 0.05$ vs. vehicle treatment; $^{\#}P < 0.05$ vs. 1 μM BPA and 10 μM BP treatment.)

When BPA is docked to the antagonist conformation, 3ERT, it is oriented perpendicular with the binding pocket and in this alignment it has the propensity to form the H-bond network involving E353, R394 and a water molecule (Figure 5D). Additionally, a hydrogen bond with the hydroxyl group of T347 is formed. In this alignment, the binding site is poorly occupied and the hydrophobic contacts with the amino acids lining the bottom of the binding site are missing.

In the case of BPA, two highly probable binding modes have been identified. The first one has been mostly predicted when the ligand has been docked into the binding sites of ERα cocrystallized with E2 and DES, the structure 3ERD_ε using the SP mode. The ligand is placed across the binding site in a similar orientation with the native ligands, having the two methyl groups involved in hydrophobic contacts with the side chains of amino acids W383, L384, L525 and L540. Also, BPA forms H-bonds with H524 and E353 (Figure 5E). When docking calculations have been run in the XP mode of Glide a second alignment of the top tanked poses in the binding site of 3ERD_ε and 3ERD_δ has been noticed. This orientation involves the formation of H-bonds between

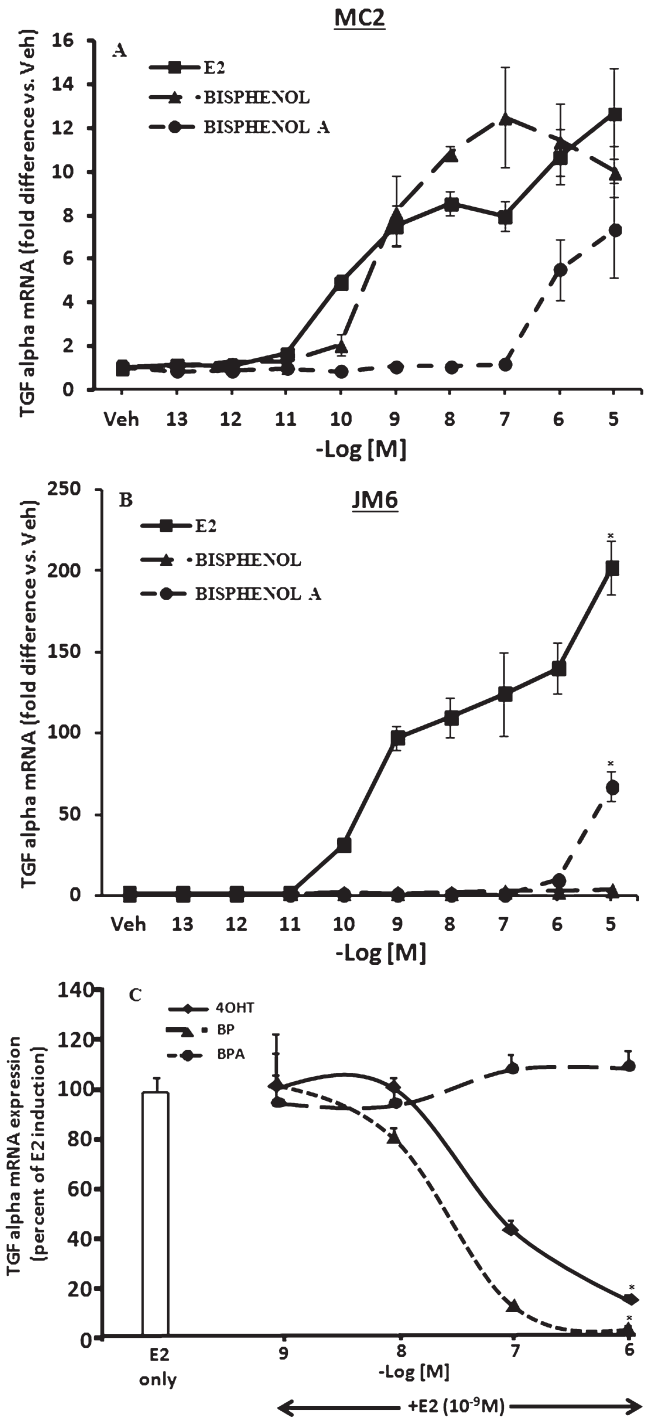

Figure 4

Induction of TGFα mRNA by 17β-oestradiol (E2), bisphenol (BP) and bisphenol A (BPA) in MDA : MB 231 cells stably transfected with wild type ERα (MC2 cells) or D351G mutant ERα (JM6 cells). (A) MC2 cells were treated with (E2), (BP) or BPA at indicated concentration for 48 h and cells were harvested for total RNA. Total RNA was reverse transcribed and real time PCR (RT-PCR) was performed to assess the expression of *TGFα* using *36B4* as an internal control. The values are presented as fold difference versus vehicle treated cells. (B) JM6 cells were treated with (E2), (BP) or (BPA) at indicated concentrations for 48 h and cells were harvested for total RNA. Total RNA was reverse transcribed and RT-PCR was performed to assess the expression of *TGFα* using *36B4* as an internal control. The values are presented as fold difference versus vehicle treated cells. (**P* < 0.05 vs.10^{-5} M BP treatment) (C) JM6 cells were treated with E2 alone or in combination with different concentration of BP, BPA or 4OHT as indicated for 48 h. The values are presented as percentage of expression of *TGFα* mRNA considering the E2-induced levels as 100%. (**P* < 0.05 vs. 1 nM E2 and 1 nM E2 +10^{-6} M BPA treatment.)

the hydroxyl groups of BPA and amino acids G521, E353 and R394 (Figure 5F). Apart from the H-bonds formation, the methyl groups are involved in hydrophobic contacts with amino acids L346, F404 and L428. Also, this binding mode has been encountered for 6 out of 10 poses resulted from the docking of BPA into the experimental structure 1GWR.

The predicted binding modes of BP to the open and closed conformation of ER are similar, forming the H-bond network between E353, R394 and the highly ordered water molecule and an additional H-bond with the hydroxyl group of T347 (Figure 5G–I). The composite score, Emodel, shows that BP is better accommodated in the binding site of the open or antagonist conformation of ERα and it is more likely for the ligand to bind at this conformation of ER. Similar results have been obtained using the induced fit docking method, which accounts for both the ligand and protein flexibility (Maximov *et al.*, 2010).

The comparative analysis of the composite score Emodel for the agonist and antagonist top ranked docking poses of BPA has shown that the binding mode predicted for the antagonist conformation is highly improbable and it is more likely for BPA to bind to a conformation of ERα closely related with the agonist one. Two distinct binding modes of BPA to the agonist conformations of ERα have been predicted with tight Emodel scores and cannot be clearly discriminated which alignment is correct or at least with the highest probability of being right. The docking scores calculated for E2, DES and BPA shows the binding affinity of BPA to ERα is much lower when compared with the binding affinities of E2 or DES to ERα.

Comparative analysis of regulation of apoptotic genes by BP, BPA, 4OHT and E2 in MCF7 : 5C cells using apoptotic gene RT-PCR profiler

We thereafter determined the effect of BP and BPA treatment in regulating the apoptosis related genes in MCF7 : 5C cells and compared it with E2 and 4OHT as a positive and negative inducer of apoptosis respectively. We used the RT-PCR profiler assay kits for apoptosis from a commercial vendor which uses 384 well plates to profile the expression of 370 apoptosis related human genes (Qiagen; SABiosciences Corp.; Cat#330231 PAHS-3012E). To select a single time point of treatment with the ligands, we first treated the MCF7 : 5C cells with E2 (10^{-9} M) for 24, 48 and 72 h (in triplicate) and created an apoptotic gene signature throughout these time points after comparing them with vehicle treatment (Supporting Information Figure S1A, B, C and Supporting Information Table S1). This gene signature was generated by comparing the expression level of all the genes with vehicle

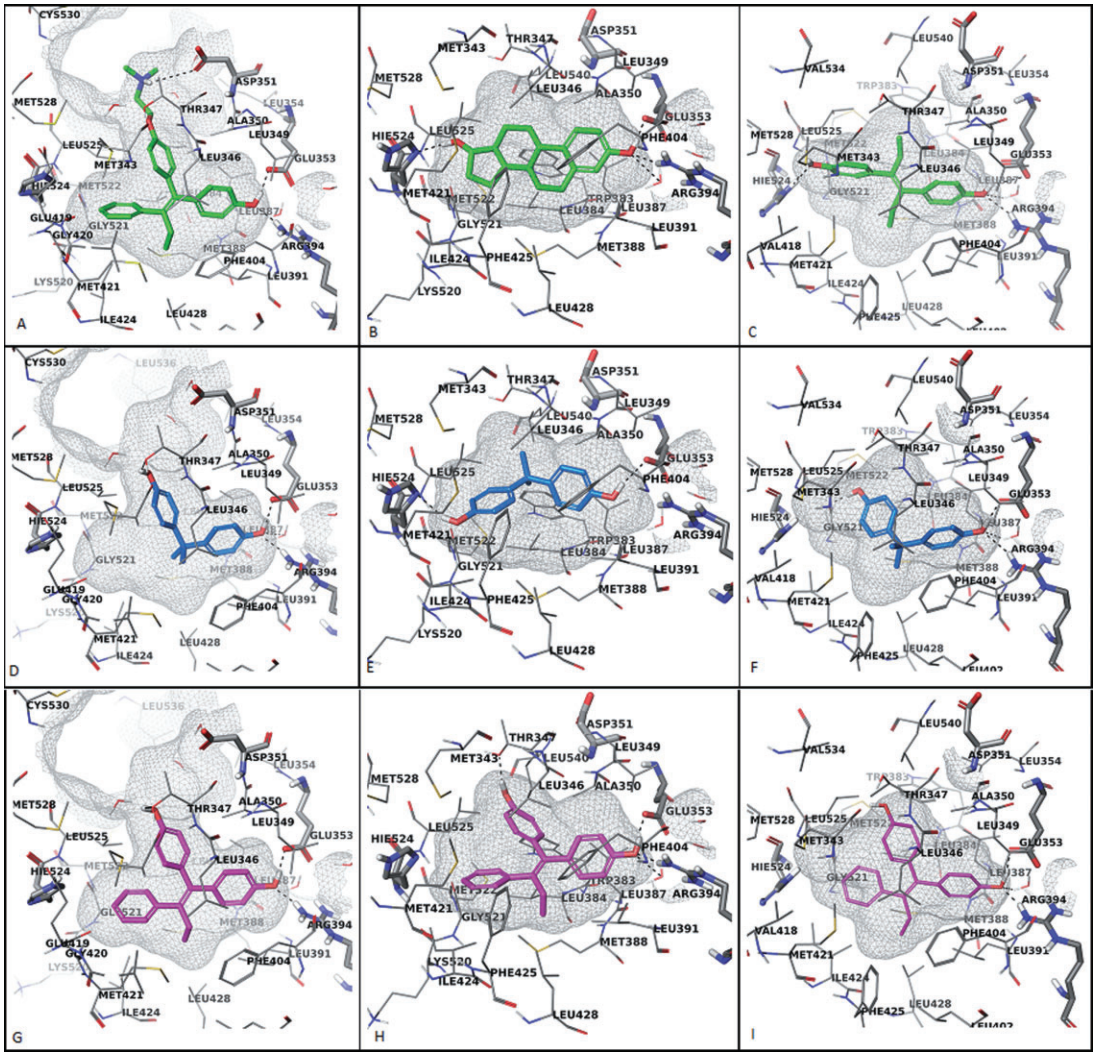

Figure 5

Molecular docking of bisphenol (BP) and bisphenol A (BPA) with ERα ligand binding domain. Cross-sectional representations of ERα binding sites in the antagonist (A) with 4OHT and agonist (B, C) with 17β-oestradiol and DES conformations. The top ranked docking poses of BPA into the binding site of 3ERT (D), 1GWR (E), 3ERD (F) are displayed with C atoms coloured in magenta while the best docking solutions of BP computed for 3ERT (G), 1GWR (H), 3ERD (I) are represented with C atoms coloured in blue. The amino acids involved in H-bond contacts are depicted as sticks and the rest of the amino acids lining the binding site are shown as lines having the C atoms coloured in gray. Only polar hydrogen atoms are shown, for simplicity.

treatment and selecting the genes which were at least 2.5-fold overexpressed or underexpressed as compared to vehicle treated cells. The fold change was calculated by delta-delta Ct method using the web based tool, RT^2 profile PCR array data analysis version 3.5 (Qiagen; SABiosciences Corp.).

After carefully analysing the gene list generated by E2 treatments over the above said time period, we selected 48 h as the time point to treat MCF7 : 5C cells with BP, BPA and 4OHT and compare the expression of the apoptosis related genes with the gene signature of the E2 treatment at 48 h. This particular time point was selected because the MCF7 : 5C cells undergo apoptotic changes after E2 treatment during this time period (Lewis *et al.*, 2005) and also because after 48 h of E2 treatment, the cells are committed to apoptosis, as 4OHT treatment cannot rescue these cells after this time point (unpublished observations).

Next, we analysed the changes in the overall expression profiles of apoptotic genes by E2, 4OHT, BP and BPA versus vehicle (Veh) treatment at 48 h (Supporting Information Figure S2A, B, C and D respectively) using the same apoptosis RT profiler. For any gene to be considered as differentially expressed, we set the cut-off as 2.5-fold up- or down-regulation versus the vehicle treatment. Using this criterion, we created a gene list for up-regulated and down-regulated genes for each treatment group (Supporting Information Table S2). We thereafter generated a heat map (Figure 6) in which we selected all the genes which were at least 2.5-fold up- or down-regulated by E2 treatment and compared it with other ligand treatments. This heat map clearly demonstrates that the genes which are up-regulated at least 2.5-fold after 48 h of E2 treatment are not up-regulated in 4OHT or BP treatment. In contrast, the majority of the genes up-regulated

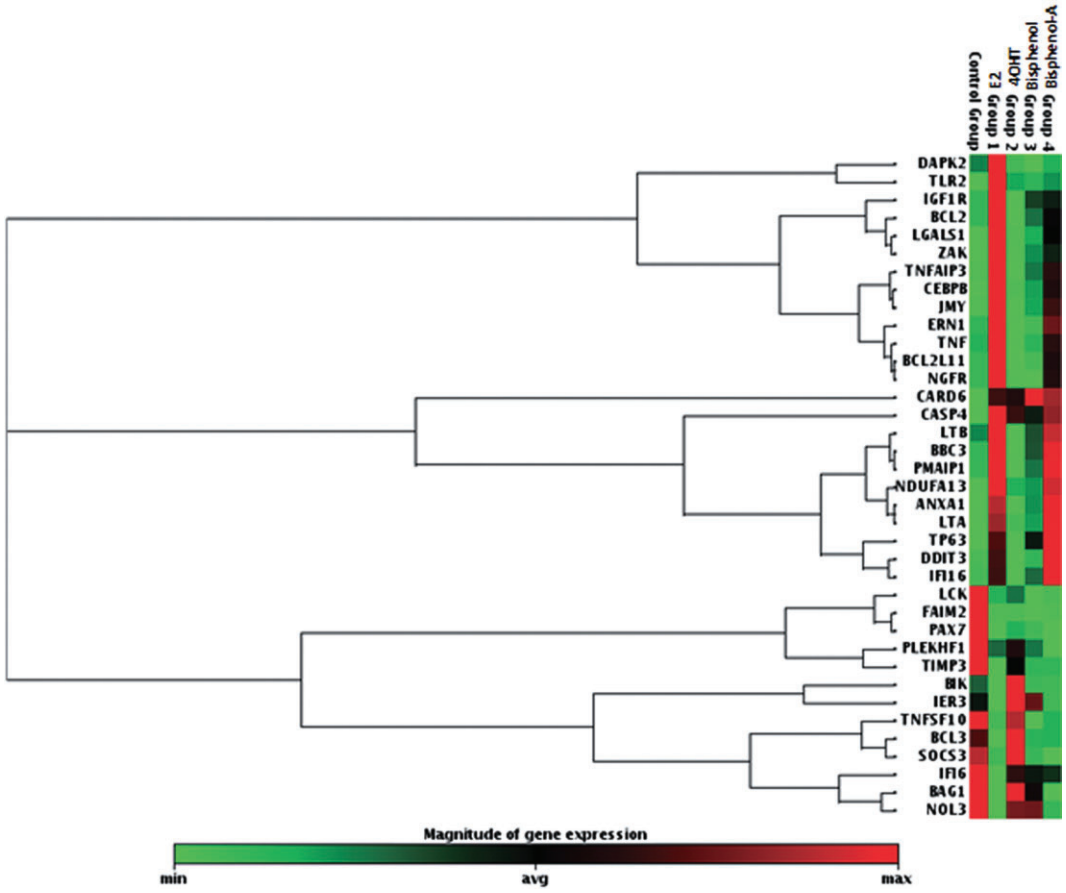

Figure 6

Heat map of apoptotic genes which are at least 2.5-fold up- or down-regulated by 48 h of treatment of 17β-oestradiol 10^{-9} M (E2), versus vehicle and its relative comparison of their expression with 4-hydroxy tamoxifen, 10^{-6} M (4OHT), bisphenol, 10^{-6} M (BP) and bisphenol A, 10^{-6} M (BPA) treatment after 48 h in MCF7 : 5C cells. The maximum expressed level of any given gene is represented by red colour and minimum levels are presented as green colour. Control group and group 1, 2, 3, 4 are the representation of the vehicle, E2, 4OHT, BP and BPA treatments respectively. The gene expression levels in each treatment group are the average of three independent biological replicates.

by BPA treatment were shown to be the same genes up-regulated by the E2 treatment. Many of these genes are up-regulated by BPA to the similar extent as E2 and others show a distinct trend of overexpression as compared to vehicle (Figure 6). Nevertheless, down-regulated genes follow a different pattern. The pattern of genes down-regulated by BP treatment resembles the pattern observed with E2 and BPA treatment and not with the pattern of 4OHT treatment (Figure 6 and Supporting Information Table S2). Approximately, 53 and 61% of down-regulated genes are in common with E2 treatment and with the treatment of BP and BPA respectively (Supporting Information Table S2).

Discussion

The chemical structures of the ligands which bind to ERα are critical in determining the biological effects in the oestrogen-responsive cells and tissues. Minor changes in the ligand structures can alter the way these ligands interact with the ERα protein and transform the conformation of the liganded –ERα complex in the cells. Structure-function relationships have been studied extensively using various biological end-points, such as modulation of prolactin gene expression in primary cell cultures of rat pituitary glands (Jordan and Lieberman, 1984; Jordan *et al.*, 1984; 1986), or *TGFα* activation in stably transfected wt and mutant ERα in MDA : MB 231 cells (Jordan *et al.*, 2001). The current study dissects, compares and contrasts the mechanism of action of BP and BPA, two structurally similar ligands of ERα, which have opposing effects on apoptosis but not on the growth of oestrogen-responsive breast cancer cells.

The results of this study established that unlike BPA and E2, BP was not functioning as an oestrogen-agonist in inducing apoptosis in MCF7 : 5C cells while both compounds (BPA and BP) were oestrogenic in inducing growth in MCF7 cells. This clearly indicated differential requirement of ERα mediated molecular action to achieve two distinct physiological responses in the breast cancer cells. Activation of oestrogen-responsive gene *PS2* by these compounds in MCF7 cells suggested that higher concentrations of BPA was as effective as E2 but BP treatment failed to achieve E2-like stimulation,

even with higher concentration. This phenomenon was observed because BP has a high ERα binding affinity and can maximally induce *PS2* gene at lower concentration and raising the concentration did not enhance the induction because it failed to recruit sufficient coactivator (SRC3) at the *PS2* gene promoter. This was most likely due to insufficient ERα recruitment at the promoter and inaccessibility of the coactivator interacting surface of BP-liganded ERα. A recent study (Bourgoin-Voillard *et al.*, 2010) however, suggested that BP-liganded ERα cannot bind to a peptide containing the coactivator interacting domain. This discrepancy can be attributed to the fact that our studies were performed in live cells chromatin as opposed to using an *in vitro* ELISA based system. This indicates that binding of liganded ERα and its interaction with other coregulators can be modulated by other factors involved in transcriptional complex.

On the other hand, BPA at higher concentration engaged SRC3 to a similar level as E2 treatment. The fact that higher concentration of BPA was required to recruit ERα and SRC3 to the similar levels as E2 treatment is because its binding affinity with ERα is very low (RBA, 0.073) (Routledge *et al.*, 2000) and therefore higher concentrations of the ligand is required to drive the kinetics towards the activated state. In the case of BP, it has a strong binding affinity to the ERα (RBA, 96.0) (Jordan *et al.*, 1984) and therefore maximal activation is achieved at lower concentration and increasing concentration do not enhance the activation. Overall, these results indicate that binding mode of BPA and E2 are similar whereas BP might bind differently to ERα. Indeed, our molecular docking studies determined that BPA binds to the ERα in two possible ways, both similar to agonistic mode of binding. Also docking scores calculated in this study predicted very low binding affinity of BPA to ERα, which is in excellent agreement with previous reports (Gould *et al.*, 1998; Kuiper *et al.*, 1998; Kitamura *et al.*, 2005). In contrast, modelling studies suggested antagonistic mode of binding (as in 4OHT) for BP to the ERα. To confirm the molecular modelling, we used a biological model system which can distinguish between planar and angular oestrogen ligands (Jordan *et al.*, 2001; Bentrem *et al.*, 2003) by measuring the transcriptional activation of *TGFα* in MDA : MB 231 cells stably transfected with wt ERα (MC2 cells) or mtERα (D351G) (JM6 cells). Results (Figure 4B) show that BP treatment failed to activate *TGFα* transcription similar to 4OHT (Jordan *et al.*, 2001) in JM6 cells whereas BPA treatment was similar to E2 action, albeit with lower potency. This consolidated our finding that the mode of action of BP is more like 4OHT rather than E2. Importantly, the structure of BP is identical to 4OHT except for the basic dimethylamine-ethoxy side chain. The absence of the side chain contributes towards the enhanced oestrogenic properties of BP with AF-1 fully engaged in ER responses to stimulate growth, as H12 of the ERα protein liganded with BP may not be properly restrained. This contrasts with 4OHT or RAL, where the restricted structure of the coactivator-interacting interface for binding of SRC3 or the other coactivators now has limited AF-1 and AF-2 activity for growth. Of note, 4OHT and BP-liganded ERα was less efficiently recruited to the *PS2* promoter ERE which may also contribute towards lesser recruitment of SCR3 for BP as recruitment of ERα precedes the coactivator binding (Metivier *et al.*, 2003).

The fact that SRC3 is essential for E2-induced apoptosis in the MCF7 : 5C cells (Hu *et al.*, 2011) as well as E2-mediated growth of MCF7 cells (List *et al.*, 2001) coupled with the findings of this study, leads to the hypothesis that the oestrogen-mediated growth of MCF7 cells is more sensitive and can be induced even if the conformation of the liganded-ERα complex allows only partial interaction of coactivators as in case of BP binding. In contrast, complete and robust interaction of coactivator with the liganded-ERα complex must be needed for rapid induction of apoptosis in MCF7 : 5C cells.

Indeed, using an 'apoptosis' pathway focused RT-PCR based profiler consisting of 370 genes, this study further illustrated that apoptosis related genes were similarly up-regulated by E2 and BPA treatments after 48 h of treatment whereas BP and 4OHT showed very few up-regulated genes and the TPE based compounds did not have a similar profile of up-regulated genes during this time frame. By comparing the gene list (Supporting Information Table S2), which includes all the genes up- or down-regulated at least 2.5-fold by the treatments, it is evident that 66% of up-regulated genes are common between E2 and BPA treatment, whereas only 8% genes are commonly up-regulated by BP or 4OHT treatment.

Interestingly, a different pattern was observed for the down-regulated genes as both BP and BPA treatment exhibited common down-regulated genes as E2 and distinctly different from 4OHT. This suggests that the conformational requirement of liganded ERα may be different for up-regulation and down-regulation of genes. Furthermore, it indicates that the up-regulated apoptotic genes are responsible for triggering and executing apoptosis since up-regulated genes are differentially regulated by BP and BPA but not the down-regulated genes. These observations merits further investigations.

By employing structurally related ligands and using MCF7 : 5C and parental MCF7 cells, we have demonstrated that depending upon the biological response, the same molecule can function as an E2-antagonist or agonist respectively. Based on these data, it is reasonable to speculate that genistein and related phytoestrogens may also induce apoptosis in MCF7 : 5C cells as their binding to ERα LBD is similar as E2 and DES (Gao *et al.*, 2012) and function as type I oestrogens (Bentrem *et al.*, 2003). In conclusion, this study provides evidence that binding of ERα with different ligands that programme conformational changes of the liganded-ERα, determines the transcriptional profile of the responsive genes by virtue of interaction with coregulators.

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Conflicts of interest

None.

References

Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P *et al.* (2011). Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci U S A 108: 18879–18886.

Bentrem D, Fox JE, Pearce ST, Liu H, Pappas S, Kupfer D *et al.* (2003). Distinct molecular conformations of the estrogen receptor alpha complex exploited by environmental estrogens. Cancer Res 63: 7490–7496.

Bourgoin-Voillard S, Gallo D, Laios I, Cleeren A, Bali LE, Jacquot Y *et al.* (2010). Capacity of type I and II ligands to confer to estrogen receptor alpha an appropriate conformation for the recruitment of coactivators containing a LxxLL motif-Relationship with the regulation of receptor level and ERE-dependent transcription in MCF-7 cells. Biochem Pharmacol 79: 746–757.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O *et al.* (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389: 753–758.

Gao L, Tu Y, Agren H, Eriksson LA (2012). Characterization of agonist binding to His524 in the estrogen receptor alpha ligand binding domain. J Phys Chem B 116: 4823–4830.

Gould JC, Leonard LS, Maness SC, Wagner BL, Conner K, Zacharewski T *et al.* (1998). Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. Mol Cell Endocrinol 142: 203–214.

Hu ZZ, Kagan BL, Ariazi EA, Rosenthal DS, Zhang L, Li JV *et al.* (2011). Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. PLoS ONE 6: e20410.

Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC (1992). An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 90: 77–86.

Jordan VC (1993). Fourteenth Gaddum Memorial Lecture. A current view of tamoxifen for the treatment and prevention of breast cancer. Br J Pharmacol 110: 507–517.

Jordan VC, Lieberman ME (1984). Estrogen-stimulated prolactin synthesis in vitro. Classification of agonist, partial agonist, and antagonist actions based on structure. Mol Pharmacol 26: 279–285.

Jordan VC, O'Malley BW (2007). Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 25: 5815–5824.

Jordan VC, Lieberman ME, Cormier E, Koch R, Bagley JR, Ruenitz PC (1984). Structural requirements for the pharmacological activity of nonsteroidal antiestrogens in vitro. Mol Pharmacol 26: 272–278.

Jordan VC, Koch R, Mittal S, Schneider MR (1986). Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. Br J Pharmacol 87: 217–223.

Jordan VC, Schafer JM, Levenson AS, Liu H, Pease KM, Simons LA *et al.* (2001). Molecular classification of estrogens. Cancer Res 61: 6619–6623.

Kitamura S, Suzuki T, Sanoh S, Kohta R, Jinno N, Sugihara K *et al.* (2005). Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. Toxicol Sci 84: 249–259.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT *et al.* (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139: 4252–4263.

Labhart P, Karmakar S, Salicru EM, Egan BS, Alexiadis V, O'Malley BW *et al.* (2005). Identification of target genes in breast cancer cells directly regulated by the SRC-3/AIB1 coactivator. Proc Natl Acad Sci U S A 102: 1339–1344.

Levenson AS, Catherino WH, Jordan VC (1997). Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. J Steroid Biochem Mol Biol 60: 261–268.

Levenson AS, Tonetti DA, Jordan VC (1998). The oestrogen-like effect of 4-hydroxytamoxifen on induction of transforming growth factor alpha mRNA in MDA-MB-231 breast cancer cells stably expressing the oestrogen receptor. Br J Cancer 77: 1812–1819.

Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T *et al.* (2005). Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97: 1746–1759.

List HJ, Lauritsen KJ, Reiter R, Powers C, Wellstein A, Riegel AT (2001). Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells. J Biol Chem 276: 23763–23768.

Liu XF, Bagchi MK (2004). Recruitment of distinct chromatin-modifying complexes by tamoxifen-complexed estrogen receptor at natural target gene promoters in vivo. J Biol Chem 279: 15050–15058.

MacGregor Schafer J, Liu H, Bentrem DJ, Zapf JW, Jordan VC (2000). Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. Cancer Res 60: 5097–5105.

Maximov P, Sengupta S, Lewis-Wambi JS, Kim HR, Curpan RF, Jordan VC (2011). The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: a hypothesis. Horm Mol Biol Clin Investig 5: 27–34.

Maximov PY, Myers CB, Curpan RF, Lewis-Wambi JS, Jordan VC (2010). Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. J Med Chem 53: 3273–3283.

Metivier R, Stark A, Flouriot G, Hubner MR, Brand H, Penot G *et al.* (2002). A dynamic structural model for estrogen receptor-alpha activation by ligands, emphasizing the role of interactions between distant A and E domains. Mol Cell 10: 1019–1032.

Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M *et al.* (2003). Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 115: 751–763.

Routledge EJ, White R, Parker MG, Sumpter JP (2000). Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) alpha and ERbeta. J Biol Chem 275: 35986–35993.

Sengupta S, Jordan VC (2008). Selective estrogen modulators as an anticancer tool: mechanisms of efficiency and resistance. Adv Exp Med Biol 630: 206–219.

Sengupta S, Sharma CG, Jordan VC (2010). Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells. Horm Mol Biol Clin Investig 2: 235–243.

Shang Y, Brown M (2002). Molecular determinants for the tissue specificity of SERMs. Science 295: 2465–2468.

Shao W, Keeton EK, McDonnell DP, Brown M (2004). Coactivator AIB1 links estrogen receptor transcriptional activity and stability. Proc Natl Acad Sci U S A 101: 11599–11604.

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA *et al*. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95: 927–937.

Wolf DM, Jordan VC (1994). The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31: 129–138.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Representation of E2 (1 nM) regulated apoptotic genes in MCF7 : 5C cells at 24, 48 and 72 h of treatment versus vehicle treatment using volcano plots.

Figure S2 Representation of 17β-oestradiol 10^{-9} M (E2), 4-hydroxy tamoxifen, 10^{-6} M (4OHT), bisphenol, 10^{-6} M (BP) and bisphenol A, 10^{-6} M (BPA) regulated apoptotic genes in MCF7 : 5C cells after 48 h of treatment versus vehicle using volcano plots.

Figure S3 Dose dependent effect of BP (at various concentrations between 10^{-8} M and 10^{-7} M) on E2 (1 nM)-induced apoptosis in MCF7 : 5C cells, treated over a 6-day period. The growth is measured as percent of DNA present in each well; vehicle treated cells were considered as 100%.

Table S1 Gene list of E2 (1 nM) regulated apoptotic genes in MCF7 : 5C cells at 24, 48 and 72 h of treatment versus vehicle treatment.

Table S2 Gene list of 17β-oestradiol, 10^{-9} M (E2), 4-hydroxy tamoxifen, 10^{-6} M (4OHT), bisphenol, 10^{-6} M (BP) and bisphenol A, 10^{-6} M (BPA) regulated apoptotic genes in MCF7 : 5C cells after 48 h of treatment versus vehicle.

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5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells☆

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ABSTRACT

Tamoxifen (Tam) is a selective estrogen receptor modulator (SERM) that remains one of the major drugs used in the hormonotherapy of breast cancer (BC). In addition to its SERM activity, we recently showed that the oxidative metabolism of cholesterol plays a role in its anticancer pharmacology. We established that these effects were not regulated by the ER but by the microsomal antiestrogen binding site/cholesterol-5,6-epoxide hydrolase complex (AEBS/ChEH). The present study aimed to identify the oxysterols that are produced under Tam treatment and to define their mechanisms of action. Tam and PBPE (a selective AEBS/ChEH ligand) stimulated the production and the accumulation of 5,6α-epoxy-cholesterol (5,6α-EC), 5,6α-epoxy-cholesterol-3β-sulfate (5,6-ECS), 5,6β-epoxy-cholesterol (5,6β-EC) in MCF-7 cells through a ROS-dependent mechanism, by inhibiting ChEH and inducing sulfation of 5,6α-EC by SULT2B1b. We showed that only 5,6α-EC was responsible for the induction of triacylglycerol (TAG) biosynthesis by Tam and PBPE, through the modulation of the oxysterol receptor LXRβ. The cytotoxicity mediated by Tam and PBPE was triggered by 5,6β-EC through an LXRβ-independent route and by 5,6-ECS through an LXRβ-dependent mechanism. The importance of SULT2B1b was confirmed by its ectopic expression in the SULT2B1b(-) MDA-MB-231 cells, which became sensitive to 5,6α-EC, Tam or PBPE at a comparable level to MCF-7 cells. This study established that 5,6-EC metabolites contribute to the anticancer pharmacology of Tam and highlights a novel signaling pathway that points to a rationale for re-sensitizing BC cells to Tam and AEBS/ChEH ligands.

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☆ This work is part of the PhD thesis of GS.

Abbreviations: AEBS, antiestrogen binding site; ChEH, cholesterol epoxide hydrolase; Tam, tamoxifen, *trans*-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-*N,N*-dimethylethylamine; 4OH-tam, 4-[(Z)-1-[4-(2-dimethylaminoethyloxy)phenyl]-2-phenylbut-1-enyl]phenol; Ralox, raloxifene, [6-hydroxy-2-(4-hydroxyphenyl)-1-benzothiophen-3-yl]-[4-(2-piperidin-1-ylethoxy)phenyl]methanone; Clom, clomiphene, 2-[4-[(Z)-2-chloro-1,2-di(phenyl)ethenyl]phenoxy]-N,N-diethylethanamine; BZA, bazedoxifene1-[[4-[2-(azepan-1-yl)ethoxy]phenyl]methyl]-2-(4-hydroxyphenyl)-3-methylindol-5-ol; Tesm, tesmilifene (DPPE), N,N′-diethylamino-4-(phenylmethylphenoxy)ethanamine-HCl; PBPE, 1-(2-(4-benzylphenoxy)ethyl)pyrrolidin-HCl; PCPE, 1-(2-(4-(2-phenylpropan-2-yl)phenoxy)ethyl)pyrrolidine; MBPE, 4-(2-(4-benzylphenoxy)ethyl)morpholine; MCPE, 4-(2-(4-(2-phenylpropan-2-yl)phenoxy)ethyl)morpholine; BD-1008, N-(3,4-dichlorophenethyl)-N-methyl-2-(pyrrolidin-1-yl)ethanemine; SR-31747A, (E)-N-(4-(3-chloro-4-cyclohexylphenyl) but-3-enyl)-N-ethylcyclohexanamine; FPT, flupenthixol, 2-[4-[(3Z)-3-[2-(trifluoromethyl)thio xanthen-9-ylidene]propyl]piperazin-1-yl]ethanol; CLP, chlorpromazine, 3-(2-chlorophenothiazin-10-yl)-N,N-dimethylpropan-1-amine; TFP, trifluoroperazine, 10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)phenothiazine; U-18666A, 3-beta-(2-(diethylamino)ethoxy)androst-5-en-17-one; AY-9944, *trans*-1,4-Bis(2-chlorobenzaminomethyl)cyclohexane; Triparanol, 2-(4-chlorophenyl)-1-(4-(2-(diethylamino)ethoxy)phenyl)-1-p-tolylethanol; D8D7I, 3β-hydroxysterol-Δ^8-Δ^7-isomerase; DHCR7, 3β-hydroxysterol-Δ^7-reductase; 7-KC, 7-ketocholesterol; 7α-HC, 7α-hydroxycholesterol; 7β-HC, 7β-hydroxycholesterol; 4β-HC, 4β-hydroxycholesterol; 5,6-EC, 5,6-epoxy-cholesterol; CT, cholestane-3β,5α,6β-triol; 5,6-αEC, 5,6α-epoxy-5α-cholestestan-3β-ol; 5,6β-EC, 5,6β-epoxy-5β-cholestestan-3β-ol; 25-HC, 25-hydroxycholesterol; 27-HC, 27-hydroxycholesterol; 5,6-ECS, 5,6α-epoxy-5α-cholestestan-3β-sulfate; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; LXRα, Liver-X-Receptor alpha; LXRβ, Liver-X-Receptor beta; SULT2B1, Steroid sulfotransferase 2B1; SCD1, Stearoyl-CoA desaturase 1; ACC, Acetyl-CoA carboxylase; siRNA, small interfering RNA; siSC, siRNA scrambled.

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1. Introduction

Breast cancer (BC) is the most common cancer in women affecting more than 1 million women world-wide and with about 400,000 deaths due to this disease every year [1]. Tamoxifen (Tam) is one of the major drugs used as an adjuvant treatment to prevent BC recurrence and as a therapy to extend the lives of patients with metastatic disease [2]. Tam is a selective estrogen receptor modulator (SERM) that can compete with 17β-estradiol (E2) at the estrogen receptor (ER) on ER positive breast cancers and block its mitogenic action. This mechanism constitutes the rational for its clinical use [3]. It is now emerging that Tam displays a complex pharmacology and may exert additional ER-independent mechanisms [4]. Cholesterol metabolism is reportedly involved in breast cancer development in mice [5–7] and in resistance to Tam in patients [8,9]. At the molecular level, Tam has been shown to modulate cholesterol metabolism through its interaction with the microsomal antiestrogen binding site (AEBS) [4]. The AEBS binds selective estrogen modulators (SERMs) such as Tam, 4-hydroxytamoxifen, raloxifene and clomiphene [10]. Diphenylmethane (DPM) compounds such as 1-(2-(4-benzylphenoxy)ethyl)pyrrolidin-HCl (PBPE) and N,N'-diethylamino-4-(phenylmethylphenoxy)ethanamine-HCl (tesmilifene) have been developed to selectively bind to the AEBS/ChEH complex [11–14]. DPM were used for the molecular characterization the AEBS/ChEH complex [15–21] and the definition of its functional role in the pharmacology of its cognate ligands [12–14,16,22–27]. Despite a lack of a clear understanding of its mechanism of action at that time, tesmilifene was evaluated positively for the treatment of breast and prostate cancer in phase II and II clinical trials [28–31]. However, a pivotal phase III clinical trial was aborted because of the lack of a therapeutic outcome [32]. It is clear that a better understanding of its mechanism of action would have warranted a better selection of patients and an improved clinical response to tesmilifene.

We established that the AEBS is a hetero-oligomeric complex consisting of 3β-hydroxysteroid-Δ^8-Δ^7-isomerase (D8D7I, EBP) and 3β-hydroxysteroid-Δ^7-reductase (DHCR7) [16] with both enzymes being involved in the post-lanosterol cholesterol biosynthesis pathway. In addition, we showed that the AEBS carried out cholesterol-5,6-epoxide hydrolase (ChEH) activity [15]. ChEH catalyzes the *trans*-hydration of 5,6α-epoxy-cholesterol (5,6α-EC) and 5,6β-epoxy-cholesterol (5,6β-EC) into cholestane-3β,5α,6β-triol (CT) [33]. We showed that the interaction of the AEBS/ChEH complex with its cognate ligands induced: (1) the intracellular accumulation of free cholesterol precursors due to a non-competitive inhibition of cholesterogenic enzymes that are involved in the AEBS [8,16]; (2) the competitive inhibition of ChEH that could lead to the accumulation of 5,6-EC [15,33]. We established that free sterols accumulated in cells in multilamellar bodies (MLB) [23,24] and were responsible for the induction of a survival autophagy [8,10,23,34,35].

We previously showed that SERMs and other AEBS/ChEH ligands induced BC cell differentiation and cytotoxicity in a concentration- and time-dependent manner [12,23–25]. We found that these effects occurred independently of the ER through the modulation of the oxidative metabolism of cholesterol, and these effects were inhibited by the antioxidant vitamin E (Vit E, alpha-tocopherol) [10,23,24,34]. We found that the exposure of MCF-7 cells to 1–5 μM Tam or 10–20 μM PBPE for 3 days led to the appearance of BC cell characteristics of differentiation with no cytotoxicity [24], while 10 μM Tam and 40 μM PBPE triggered cell death [23,24]. Cytotoxicity required the expression of new genes and new proteins in BC cells establishing that transcription factors were involved in this process [23].

ROS include different reactive oxygen species, and some of them are known to produce different oxysterols [36]. NAD(P)H oxidase (NOX) is a ROS producing enzyme that induces the production of superoxide anion ($O_2^{\bullet -}$) which can be transformed by superoxide dismutase into H_2O_2 [37]. H_2O_2 produces 5,6-ECs as major cholesterol oxidation products [38]. We reported earlier that Tam and PBPE stimulated ROS production in MCF-7 cells [23] and other groups have reported that Tam induced ROS production in different cell lines including BC cells such as MCF-7 and MDA-MB-231 cells [39–42]. Consistent with these data, it was recently reported that the induction of TAG by Tam in MCF7 was inhibited by catalase [40], the enzyme that destroys H_2O_2, suggesting the formation of unknown endogenous mediator. We postulated that the stimulation of ROS by Tam can induce the production of 5,6-ECs. One of the major characteristics of BC cell differentiation induced by AEBS ligands is the stimulation of triacylglycerol (TAG) biosynthesis [23,24,40,43]. TAG biosynthesis is known to be under control of the oxysterol receptors Liver-X-Receptors (LXR) [44], and LXR were shown to be modulated by 5,6α-EC [45], suggesting that LXR could contribute to the oxysterol-dependent activity of Tam. The impact of Tam and AEBS/ChEH ligands on 5,6-EC metabolism in BC cells remains to be studied. 5,6-EC are known to be present in low amounts in mammals [36] and their presence in BC cells has never been studied. We designed the present study to identify the cholesterol autoxidation species that are produced under Tam- and AEBS ligand-treatment and to determine the molecular pathways involved in the induction of TAG biosynthesis by these oxysterols and Tam and PBPE and cytotoxicity in BC cells.

2. Materials and methods

2.1. Chemicals and reagents

Raloxifene, RU-39411 and SR-31747A were from Sanofi-Aventis. Triparanol was from Dr C Wolf (University Paris 06, France). BD-1008 was kindly given by Pr W D Bowen (Brown University, RI, USA). Bazedoxifene was synthesized as previously described [46] as were other compounds [11,15]. 5,6α-EC, 5,6β-EC, d7-5,6α-EC, d7-5,6β-EC, [^{14}C]-5,6α-EC and [^{14}C]-5,6β-EC were synthesized as described previously [15,47]. Other deuterated oxysterols were from Avanti Polar Lipids (Alabaster, AL). All other chemicals were from Sigma–Aldrich (St. Louis, MO).

2.2. Detection and quantification of 5,6-EC

Cells were grown to 70% confluence, and then pre-treated for 30 min with the appropriate amount of drug or solvent vehicle in the presence or absence of 500 μM Vit E. After a 48 h incubation, the cells were washed and scraped in cold PBS and the neutral lipids were extracted with chloroform/methanol/8.8% aqueous KCl (2:1:1 v/v) as reported previously [48]. 10^8 cells were used for analysis. The organic phase was evaporated to dryness under an argon stream and the residues dissolved in 50 μl of ethanol. 100 pmol of deuterated oxysterols as internal standards (IS) were added under argon flux, the samples saponified with KOH 1 N for 1 h at 55 °C, and oxysterols extracted with chloroform/methanol/8.8% aqueous KCl (2:1:1, v/v/v). Oxysterol purification was accomplished using a 100 mg Sep-Pak Silica Vac RC Cartridge equilibrated with hexane. Samples were applied to the silica cartridge, washed with hexane (5 ml), 12% methylterbutyl ether (MTBE) in hexane (5 ml), 23% MTBE in hexane (7 ml), 40% MTBE in acetone (5 ml) and the oxysterol fraction was eluted with 5 × 2 ml of MeOH. Under these conditions of preparation, no 5,6-EC were formed as artifacts. We measured a 98% yield in 5,6-EC recovery using [^{14}C]-5,6-EC without cholesterol and vit E contaminations.

Oxysterols were derivatized using pyridine–hexamethyldisilazane–trimethylchlorosilane (3:2:1) and analyzed by GC/MS. Quantification of oxysterols was carried out using stable isotope dilution mass spectrometry. For GC–MS analysis, samples were redissolved in 100 μl methylene chloride and 1 μl was used for analysis in a trace gas chromatographer (Thermo Fisher Scientific, Austin, TX) coupled to a mass spectrometer (Polaris Q, Thermo Fisher Scientific) (GC–MS). Samples were separated on an RTX-5MS fused silica column (15 m × 250 μm × 0.25 μm). The oven temperature program was as follows: 180 °C for 1 min, 20 °C/min to 250 °C and then 5 °C/min to 300 °C where the temperature was kept for 6 min. Helium was used as the carrier gas, with a flow rate of 1 ml/min. The molecules were ionized by electron impact at 70 eV. 5,6-EC were monitored with ions at mass/charge ratio (*m*/*z*) 472 (7-KC), 479 (*d*7-KC); 474 (5,6α-EC and 5,6β-EC), 481 (*d*7-5,6α-EC and *d*7-5,6β-EC); 546 (CT), 553 (*d*7-CT); 456 (27-HC, 25-HC), 462 (*d*6-27-HC, *d*6-25-HC); 456 (4β-HC, 7α-HC, 7β-HC), 463 (*d*7-4β-HC, *d*7-7α-HC, *d*7-7β-HC). Quantitative GC/MS determinations were calculated from triplicate injections and from the linear response range of standard curves established for oxysterol/IS pairs.

2.3. Cholesterol oxidation analysis

MCF-7 cells were seeded in 100 mm plates at 0.5×10^6, then incubated with [^{14}C]-5,6-EC (final concentration 0.6 μM; 20 μCi/μmol) in the presence of solvent vehicle (EtOH 0.1%), and 5 μM Tam for 72 h. The cells were scraped and pelleted by centrifugation for 10 min at 1500 rpm, and then extracted as described above. Samples were spotted onto Fluka 20 × 20 silica gel plates previously heated for 1 h at 100 °C and developed using chloroform/acetone/MeOH:2/acetic acid/H_2O (8/4/2/2/1). The radioactive metabolites were identified on TLC plates by co-migration with authentic standards by autoradiography using Kodak Biomax MS film (Sigma–Aldrich) and quantified by liquid scintillation counting of the 5,6-EC and CT regions. The *Rf* for 5,6-EC and CT were 0.94 and 0.82 respectively.

2.4. Measurement of ChEH activity

Cells were seeded in 6-well plates (100,000 cells/well) in RPMI 1640 medium with 5% FCS. Cells were incubated for 24 h with 0.6 μM [^{14}C]-5,6α-EC in the presence of increasing concentrations of drugs. Cells were scraped, resuspended in PBS and pelleted by centrifugation 10 min at 800 × *g* and then extracted with 200 μl of chloroform/methanol (2:1). The organic layer was reduced to dryness under a flux of argon, and the residue was resuspended in 30 μl of ethanol. More than 95% of the radioactivity was recovered in the organic layers. Samples were applied to TLC plates that had been heated previously for 1 h at 100 °C and were developed using ethyl acetate. The radioactive metabolites were visualized by autoradiography using Kodak Biomax MS film (Sigma–Aldrich) and quantified by liquid scintillation counting of the 5,6-EC region. The concentration of a compound required to inhibit ChEH by 50% (IC_{50}) was calculated using Graphpad Prism software, version 4.0 (GraphPad Software Inc., San Diego, CA). The IC_{50} values were calculated with data from triplicate assays at each drug concentration.

2.5. Cell culture

MCF-7 and MDA-MB-231 cells were from the American Type Culture Collection (ATCC) and cultured until passage 30. MCF-7 and MDA-MB-231 cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), penicillin and streptomycin (50 U/ml) in a humidified atmosphere with 5% CO_2 at 37 °C.

2.6. Luciferase assay

One day after seeding in 100-mm dishes, MCF-7 or MDA-MB-231 cells were transfected with the LXRE-luciferase construct using polyethyleneimine (PEI). For each dish, a transfection solution with 4.3 μg of PEI and 5 μg of the construct in 2 ml of OptiMEM was prepared and incubated with the cells. After 5 h, the medium was replaced by RPMI 1640 with 5% FCS. Cells were incubated at 37 °C in a humidified 5% CO_2 incubator. One day after transfection, cells were seeded in 12-well plates (50,000 cells/well) in RPMI 1640 with 5% FCS. After 4 h, the cells were treated with the test compounds dissolved in ethanol. Before treatment with the test compounds the medium was replaced by phenol red-free medium without FCS. At the end of the treatment, the cells were lysed in 100 μl of passive lysis buffer (Promega, Charbonnières, France). Luciferase (Luc) activity was measured using the Luc assay reagent (Promega), according to the manufacturer's instructions. Protein concentrations were measured using the Bradford technique to normalize the Luc activity data as reported earlier [49]. For each condition, the mean Luc activity was calculated from the data of three independent wells.

2.7. Western blot analysis

Immunoblotting was carried out as previously described [23]. Proteins were separated on 10% SDS-PAGE gels, electro-transferred onto PVDF membranes and incubated overnight at 4 °C with goat anti-LXRβ (Y-16, sc-34341, Santa Cruz) or anti-SULT2B1b (AB82865, Abcam) or anti-Actin (C4, MAB1501, Millipore). Visualization was carried out using an ECL plus kit (Pierce), and chemiluminescence was detected by autoradiography (Amersham Biosciences).

2.8. Oil Red O staining procedures and TAG quantification

A stock solution was prepared as follows: 0.5 g of Oil Red O (ORO) (Sigma) was dissolved in isopropanol. Before use, the solution was diluted 3:2 with distilled water and then filtered through Whatman paper. Cells were grown on glass coverslips and treated with drugs for 48 h and then fixed with 3.7% paraformaldehyde for 1 h at room temperature followed by washing twice with PBS (Euromedex). Cells were incubated with 2 ml of 60% (v/v) isopropyl alcohol in water for 5 min. 2 ml of ORO working solution was added to each well for 5 min. Cells were rinsed with tap water until the rinsing water was clear. Finally, the cells were counter-stained with 2 ml hematoxilin stain for 1 min. Quantifications of lipid accumulation and TAG quantification were done as exactly as previously reported [24].

2.9. Transmission electron microscopy

Cells were fixed with 2% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.4) for 1 h and washed with the Sorensen's phosphate buffer (0.1 M) for 12 h. The cells were then post-fixed with 1% OsO_4 in Sorensen's phosphate buffer (Sorensen's phosphate 0.05 M, glucose 0.25 M, OsO_4 1%) for 1 h, washed twice with distilled water, and pre-stained with an aqueous solution of 2% uranyl acetate for 12 h. Samples were then treated exactly as previously described [24].

2.10. RNA isolation and qPCR analysis

The detailed procedures have been published previously [50]. Total RNA was extracted from cultured cells using an RNA Extraction kit (Qiagen) according to manufacturer's instructions. LXRα: forward primer 5′-ACACCTACATAGCGTCGCAAG-3′, reverse primer 5′-GACGAGCTTCTCGATCATAGCC-3′; LXRβ: forward primer

5′-CTACAGCAAGGACGACTT-3′, reverse primer 5′-AGATAGTTAGATAGGCGATAGAG-3′; Sterol Regulatory Element Binding Protein 1c (SREBP-1c): forward primer 5′-CAGCCCCACTTCATCAAGG-3′, reverse primer 5′-ACTAGTTAGCCAAGATAGGTTCCG-3′; Stearoyl-Coenzyme A Desaturase 1 (SCD1): forward primer 5′-ACCGCTCTTACAAAGCTCGG-3′, reverse primer 5′-CCACGTCGGGAATTATAGAGGAT-3′; Acetyl-Coenzyme A Carboxylase (ACC): forward primer 5′-GCCCACGGTTATCATAGGACC-3′, reverse primer 5′-GTCAGGCGAATAGTTAGATTTTCAG-3′; Sulfotransferase 2B1 (SULT2B1): forward primer 5′-CGGGACGACGACATCTTTAT-3′, reverse primer 5′-CACCCACAATAGGTCTCACAC-3′; ATP-Binding Cassette Transporter A1 (ABCA1): forward primer 5′-ATAGAGGACAACAACTACAAAGCC-3′, reverse primer 5′-GGGAAAGAGGACTAGACTCCAAA-3′; ATP-Binding Cassette Transporter G1 (ABCG1): forward primer 5′-TTTAGAGGGATTTAGGGTCTAGAAC-3′, reverse primer 5′-CCCCTTTAATCGTTTTAGTCTAGCT-3′; low density lipoprotein receptor (LDLR): forward primer 5′-GGACCAACGAATAGCTTAGGACA-3′, reverse primer 5′-CTAGGCACTTAGTAGCCACCC-3′; Steroid Sulfatase (STS): forward primer 5′-TAGGGATCTCTTTAGACCAATCTAGA-3′, reverse primer 5′-CAGCAAGGGTAAGGAGGGTAG-3′; Cylophilin A (CycA): forward primer 5′-GCATACGGGTCCTAGGCATCTTAGTC-3′, reverse primer 5′-ATAGGTAGATCTTCTTAGCTAGGTCTTAGC-3′. First-strand cDNA was synthesized with iScript Reverse Transcriptase (Bio-Rad). 25 ng of cDNA were amplified using SyBR Supermix (Bio-Rad). Quantitative PCR analyses were performed on an iCycler (Bio-Rad). The threshold cycle (Ct) values of genes of interest were normalized with the Ct values of CycA.

2.11. Knock-down of LXRβ and SULT2B1b by siRNA

Gene expression of endogenous LXRβ or SULT2B1b was suppressed with a pool of 4 siRNAs for LXRβ (siLXRβ, M-003412, Dharmacon) or 4 siRNAs for SULT2B1b (siSULT2B1b, M-009488, Dharmacon) along with a control scrambled sequence siRNA (siSC, D-001210, Dharmacon). MCF-7 cells were seeded in 100-mm dishes in RPMI medium containing 5% FBS. After 24 h of seeding, cells were transfected in Opti-MEM with 50 nM siSC, siLXRβ or siSULT2B1b using DharmaFECT1 (T-2001, Dharmacon) following the procedure recommended by the manufacturer. After transfection, the cells were grown in RPMI 1640 5% FCS at 37 °C in a humidified 5% CO_2 incubator.

2.12. Transfection of MDA-MB-231 cells with SULT2B1b

One day after seeding in 100-mm dishes, MDA-MB-231 cells were transfected with the construct pCMV6-XL5-SULT2B1b (SULT2B1b) (SC123353, Origene) or the empty pCMV6-XL5 (Mock) using polyethyleneimine (PEI) as described above. One day after transfection, cells were seeded in 12-well plates (50,000 cells/well) in RPMI 1640 with 5% FCS and grown at 37 °C in a humidified 5% CO_2 incubator.

2.13. Cell death assay

Cells were seeded in RPMI 1640 with 5% FBS into 6-well plates at 100,000 cells/well. The cells were then treated with solvent vehicle (0.1% ethanol), 10 μM Tam or 40 μM PBPE for 72 h. Cell death was determined by the trypan blue exclusion assay. The cells were scraped and resuspended in the trypan blue solution (0.25%, w/v in PBS) and counted in a Malassez cell under a light microscope.

2.14. Chemical synthesis of oxysterol-sulfates

5,6α-Epoxy-5α-cholestestan-3β-sulfate (5,6-ECS) and cholestane-5α,6β-diol-3β-sulfate (CTS) were synthesized using a published method [51]. The purity of the synthesized steroids was determined by thin-layer chromatography, nuclear magnetic resonance spectroscopy, and mass spectrometry and was greater than 95%.

2.15. Biosynthesis of oxysterol-sulfates in cells

0.5×10^6 cells were incubated with [^{14}C]-5,6α-EC (final concentration 0.6 μM; 20 μCi/μmol) in the presence of solvent vehicle (EtOH 0.1%), 5 μM Tam or 10 μM PBPE for 72 h. The cells were then scraped and pelleted by centrifugation for 10 min at 1500 rpm, and then extracted under the conditions previously described for 5,6-EC. Samples were spotted onto Fluka 20 × 20 silica gel plates previously heated for 1 h at 100 °C and developed using chloroform/acetone/MeOH/acetic acid/H_2O (8/4/2/2/1). The radioactive metabolites were identified and quantified as described above. The *Rf* for 5,6-EC, CT, 5,6-ECS and CTS were 0.94, 0.82, 0.56 and 0.46 respectively.

2.16. Statistical analysis

Values are the mean ± S.E. of three independent experiments each carried out in duplicate. Statistical analysis was carried out using a Student's *t*-test for unpaired variables. * and ** in the figures refer to statistical probabilities (*P*) of <0.001 and <0.0001, respectively, compared with control cells that received solvent vehicle alone.

3. Results

3.1. Tam and PBPE stimulate the production and the accumulation of 5,6α-EC, and 5,6β-EC in MCF-7 cells

We investigated the nature of sterol oxidation products induced by Tam and PBPE in MCF-7 cells using isotope dilution gas chromatography–mass spectrometry (GC–MS) to monitor the different oxysterol species. The treatment of cells for 3 days with 5 μM Tam and 10 μM PBPE (a concentration required for the induction of BC cell differentiation) drastically increased the content of 5,6α-EC and 5,6β-EC. Other ring B oxysterols such as 7-KC, 7α-HC and 7β-HC (Fig. 1A) were detectable but did not increase under Tam or PBPE treatment. 4β-HC, 25-HC and 27-HC were not detectable in MCF-7 cells at the basal level and were not increased under Tam or PBPE treatment. This increase in 5,6-EC biosynthesis by Tam or PBPE was inhibited by the antioxidant Vit E establishing that 5,6-EC were produced through a ROS-mediated mechanism induced by Tam and PBPE (Fig. 1A). 5,6α-EC and 5,6β-EC are products of epoxidation on the delta 5 double bond of cholesterol (Fig. 1A). Kinetic analysis indicated (Fig. 1B) that a 6 h treatment with Tam was sufficient to induce cholesterol epoxidation. The production of 5,6α-EC increased up to 12 h and then decreased after 24 h suggesting possible metabolism of 5,6α-EC in MCF-7 cells. The production of 5,6β-EC increased up to 24 h and then plateaued at 72 h. The absence of an increase in CT, the product of hydration of 5,6-EC by ChEH, is consistent with the inhibition of ChEH by Tam and PBPE in BC cells (Fig. 1A). Indeed, we previously reported that Tam and AEBS ligands were potent inhibitors of the rat liver microsomal ChEH and that MCF-7 cells expressed ChEH [15]. We next showed that Tam and PBPE inhibited ChEH in intact MCF-7 cells in a concentration-dependent manner giving IC_{50} of 48.5 ± 7 nM and 1.23 ± 0.9 μM respectively (Fig. 1C) explaining why no increase in CT was found in treated cells despite the increase in 5,6-EC. These data established that Tam and PBPE induced the accumulation of 5,6α-EC and 5,6β-EC in MCF-7 cells through a dual mechanism involving a ROS-mediated cholesterol epoxidation and the inhibition of ChEH (Fig. 1D).

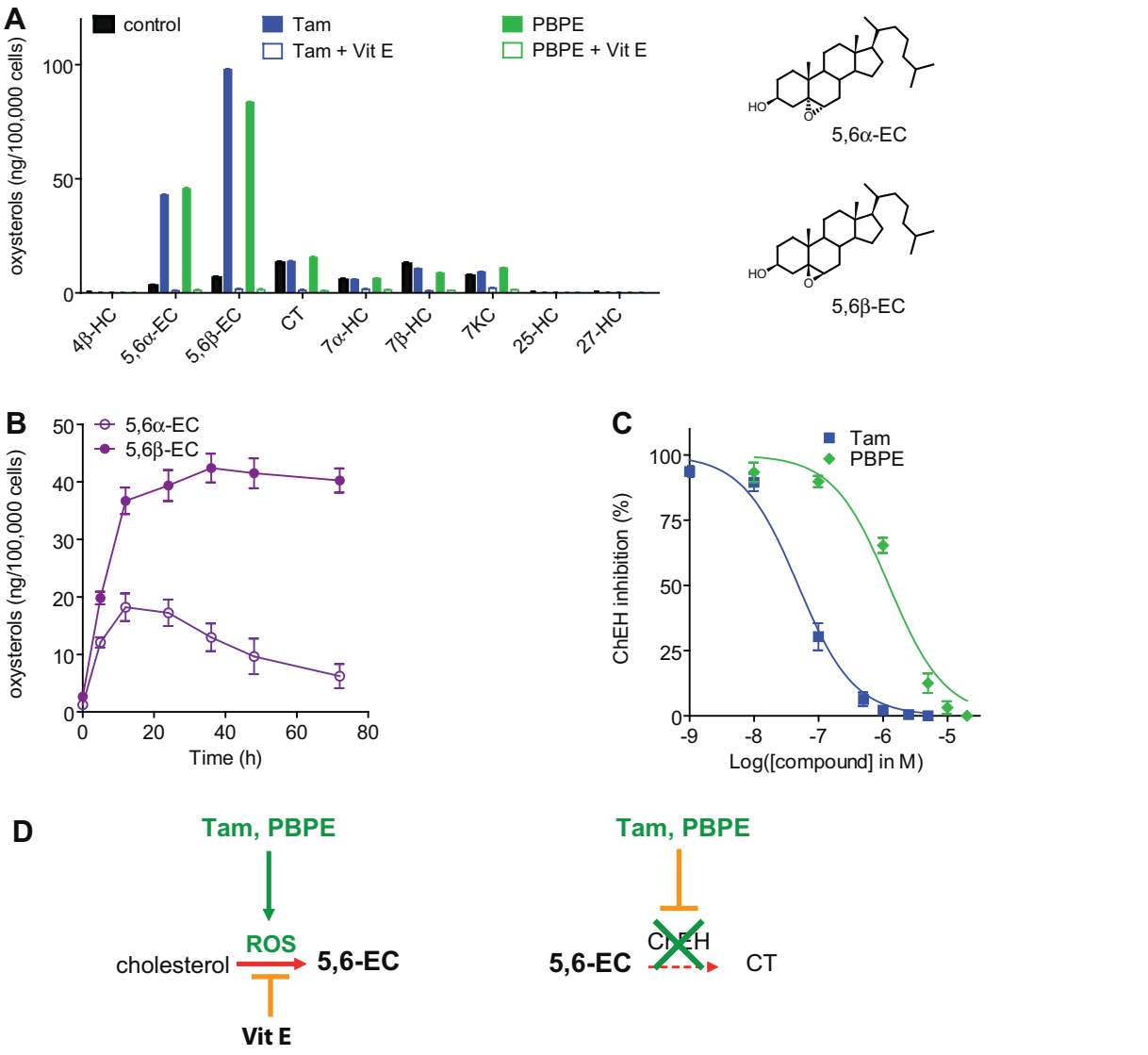

Fig. 1. Analyses of the ROS-dependent stimulation of oxysterol biosynthesis by Tam and PBPE in MCF-7 cells. (A) Quantification of oxysterol in MCF-7 cells incubated for 48 h with 10 μM Tam or 40 μM PBPE in the absence or presence of 500 μM Vit E. 9 different oxysterols were quantified by gas chromatography using a dilution isotope methodology as described in Section 2. 4β-HC: 4β-hydroxycholesterol; 5,6α-EC: 5,6α-epoxy-cholesterol; 5,6β-EC: 5,6β-epoxy-cholesterol; CT: cholestane-3β,5α,6β-triol; 7α-HC: 7α-hydroxycholesterol; 7β-OH: 7β-hydroxycholesterol; 7KC: 7-ketocholesterol; 25-HC: 25-hydroxycholesterol; 27-HC: 27-hydroxycholesterol. The results are reported in ng of oxysterol per 10^5 cells. 5,6α-EC and 5,6β-EC are drawn the right part of the figure. (B) Kinetic study on the accumulation 5,6α-EC and 5,6β-EC in MCF-7 cells after treatment with Tam for 0, 6, 12, 24, 48 and 72 h. 8×10^7 to 10^8 cells were used per condition. (C) Inhibition of cholesterol-5,6-epoxide hydrolase activity (ChEH) by Tam and PBPE in MCF-7 cells. Cells were incubated with [^{14}C]-5,6-EC (0.6 μM; 20 μCi/μmol) and were treated with increasing concentrations of Tam or PBPE ranging from 10 nM to 10 μM over 24 h. The positions of the 5,6-EC and CT were determined using [^{14}C]-5,6-EC and [^{14}C]-CT as standards. The data presented are the means ± S.E. of three independent experiments. (D) Scheme summarizing 5,6-EC formation and accumulation under Tam and PBPE treatment in MCF-7 cells.

3.2. 5,6α-EC, Tam and PBPE stimulated an LXRβ-dependent TAG biosynthesis in MCF-7 cells

AEBS/ChEH ligands were reported to stimulate TAG biosynthesis in MCF-7 cells, which can be revealed by the accumulation of ORO positive vesicles [23,24]. TAG biosynthesis can be controlled at the transcriptional level by nuclear receptors such as LXRα and LXRβ [52] that are known to be modulated by 5,6α-EC [45]. Since Tam and PBPE stimulate the accumulation of 5,6α-EC, we evaluated whether 5,6-EC, Tam and PBPE stimulate TAG biosynthesis in an LXR-dependent manner. We initially examined whether LXR isoforms were expressed in MCF-7 cells and found that LXRβ was predominant in MCF-7 cells ($Ct_{LXR\alpha}$ = 32.1, $Ct_{LXR\beta}$ = 25.1) and that LXRα was not detectable at the protein level (data not shown). MCF-7 cells were transfected with a plasmid encoding a luciferase (Luc) coding gene under the control of a promoter containing an LXR-response element (LXRE). Analysis at 12 h of treatment showed that the LXR agonist T0901317 activated the expression of Luc with an EC_{50} of 0.21 ± 0.06 μM and established that 5,6α-EC stimulated Luc activity in a concentration-dependent manner with an EC_{50} of 8.9 ± 0.1 μM (Fig. 2B). In contrast, 5,6β-EC was inefficient at stimulating Luc activity. Tam and PBPE stimulated Luc activity, but as opposed to direct LXR modulators such as T0901317 and 5,6α-EC, the stimulation required 48 h of treatment (Fig. 2C). Both Tam and PBPE stimulated Luc activity dose-dependently with EC_{50} values of 1.5 ± 0.3 μM and 9.9 ± 0.4 μM respectively at 48 h of treatment (Fig. 2D). The stimulation of Luc activity by Tam and PBPE was completely blocked by vit E, whereas vit E did not inhibit

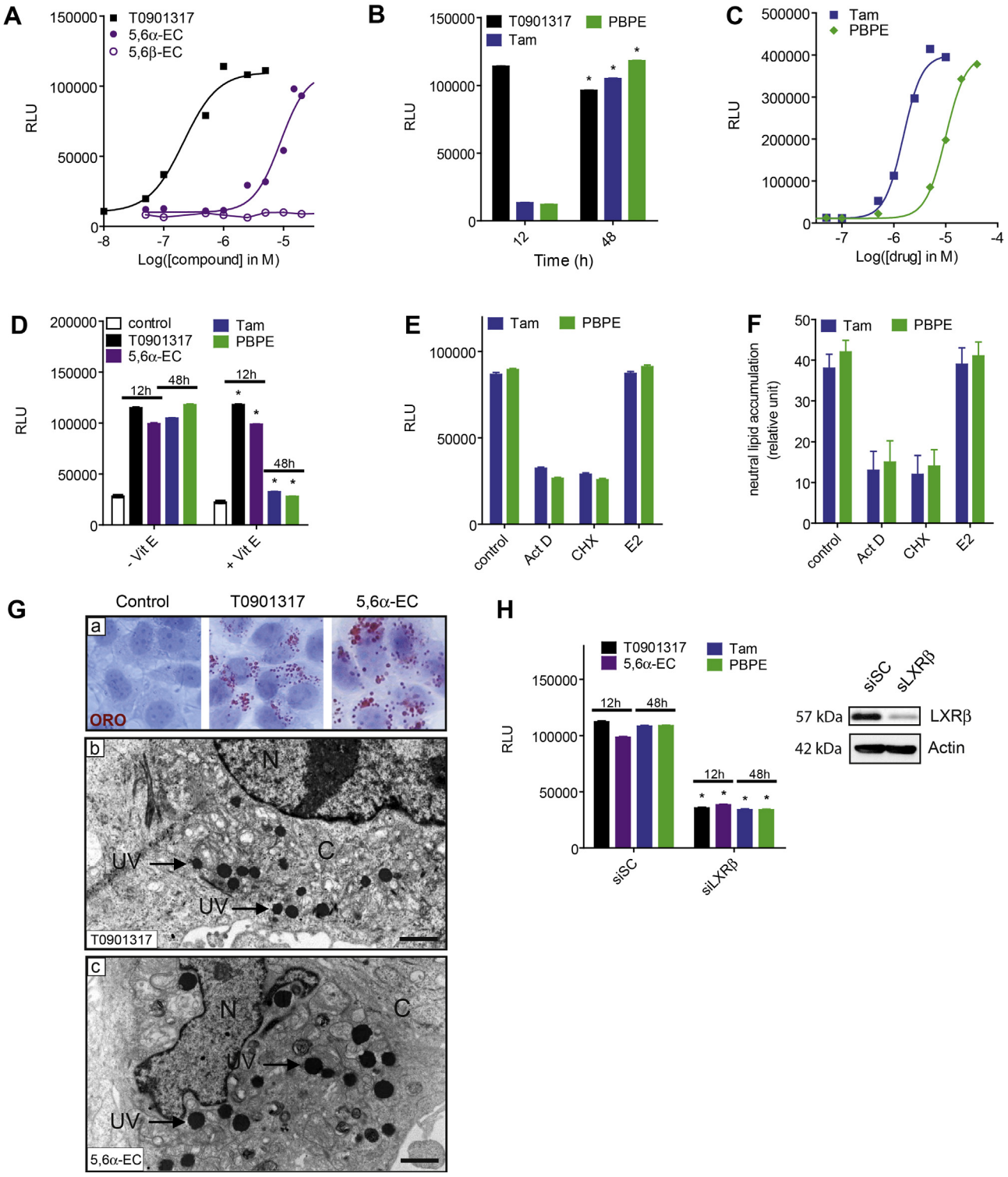

Fig. 2. Impact of 5,6α-EC, 5,6β-EC, Tam and PBPE treatment on the LXR-dependent transcription in MCF-7 cells. (A) Effect of T0901317, 5,6α-EC, and 5,6β-EC on LXRE-Luc. MCF-7 cells transfected with an LXRE-Luc plasmid were treated with increasing concentrations of T0901317, 5,6α-EC, 5,6β-EC for 12 h and assayed for luciferase activity (Luc) and expressed as relative luciferase unit (RLU) as described in Section 2. (B) Effect of T0901317, Tam, and PBPE on LXRE-Luc at 12 h and 48 h. Cells were incubated with 1 μM T0901317, 5 μM Tam and 20 μM PBPE and assayed for Luc activity at 12 h and 48 h after treatment. (C) Dose–response study of Tam and PBPE on MCF-7 cells transfected with an LXRE-Luc plasmid. Cells were incubated with solvent vehicle or increasing concentrations (50 nM–50 μM) of each drug for 40 h and assayed for Luc activity. (D) Effect of Vit E on LXRE-luc activity stimulated by solvent vehicle (control), T0901317, 5,6α-EC, Tam and PBPE in MCF-7 cells. MCF-7 cells were treated for 12 h with 1 μM T0901317 or 20 μM 5,6α-EC, 48 h with 5 μM Tam or 10 μM PBPE, with or without 500 μM Vit E, and assayed for Luc activity. Effect of actinomycin D (Act D), cycloheximide (CHX), and 17β-estradiol (E2) on the stimulation of LXR-dependent Luc activity by Tam and PBPE. MCF-7 cells were incubated for 40 h with 5 μM Tam and 20 μM PBPE in the absence or in the presence of 1 μg/ml Act D, 2.5 μg/ml CHX or 100 nM E2 and (E) assayed for Luc activity and F) for ORO staining and quantification. (G) The effect of LXR ligands T0901317 and 5,6α-EC on neutral lipid accumulation was assessed by ORO staining and ultrastructure analyses were done by electron microscopy. (a) Staining of neutral lipids with ORO in MCF-7 cells treated with solvent vehicle, 1 μM T0901317, or 20 μM 5,6α-EC for 24 h. Cells were stained with ORO and counterstained with Meyer's hematoxylin as described in Section 2. Electron micrographs of MCF-7 cells treated with: (b) 1 μM T0901317, (c) 20 μM 5,6α-EC for 24 h. Unilamellar vesicles (UV) were found in the cytoplasm of treated cells. *N*, nucleus; *C*, cytoplasm. Bars, 6.6 μm. (F) Effect of T0901317, 5,6α-EC, Tam and PBPE on the LXRβ-dependent stimulation of Luc activity in MCF-7 cells. MCF-7 cells were transfected with siRNA scrambled (siSC) or siRNA targeting LXRβ (siLXRβ). LXRβ expression was verified by Western blot

the stimulation of Luc by the direct LXR modulators T0901317 and 5,6α-EC (Fig. 2E). We found that actinomycin D (Act D) and cycloheximide (CHX) inhibited the stimulation of LXR-dependent Luc activity (Fig. 2E) and the stimulation of ORO positive vesicles by Tam and PBPE in MCF-7 cells (Fig. 2F), while co-treatment with E2 did not interfere with drug effects (Fig. 2E–F). This indicates that transcription and translation were required for induction of TAG biosynthesis by Tam and PBPE (Fig. 2A) while the ER was not involved. These data support the implication of 5,6α-EC as the endogenous mediator of the stimulation of Luc by Tam and PBPE. T0901317 and 5,6α-EC induced the accumulation of neutral lipid in MCF-7 cells as judged by the appearance of cytoplasmic ORO-positive vesicles (Fig. 2G(a)) as previously observed with MCF-7 cells treated with Tam or PBPE [24]. Ultrastructure analysis of MCF-7 cells by electron microscopy showed the accumulation of unilamellar vesicles (UV) in cells treated with T0901317 (Fig. 2G(b)) or 5,6α-EC (Fig. 2G(c)) which reflect the accumulation of neutral lipids such as TAG [24]. We next performed the knock-down of LXRβ in MCF-7 cells using an siRNA approach to define the implication of this receptor in the LXR-dependent stimulation of Luc activity by 5,6-EC and drugs. Knock-down of LXRβ was confirmed by Western blotting (Fig. 2H, insert) and drastically decreased the stimulation of Luc activity by T0901317, 5,6α-EC, Tam and PBPE (Fig. 2H). ORO staining of MCF-7 cells transfected with the scrambled control siRNA (siSC) showed that treatment with Tam or PBPE induced the accumulation of ORO-positive vesicles, while in cells transfected with siRNA against LXRβ, Tam and PBPE did not stimulate the accumulation of ORO-positive vesicles (Fig. 3A). Lipid analysis showed that knock-down of LXRβ completely inhibited the production of TAG induced by T0901317, 5,6α-EC, Tam and PBPE (Fig. 3B). We next used qPCR to investigate if Tam and PBPE modulated LXR-responsive genes and found that Tam and PBPE slightly repressed the expression of ABCA1, SREBP-1c and stimulated the expression of SCD1, ACC, ABCG1 and LDLR (Fig. 3C) showing a modulatory activity rather than an agonist activity as reported by Berrodin et al. in other cell lines [45]. This modulation of gene expression was abrogated in the presence of vit E consistent with what was seen for ORO positive accumulation and TAG biosynthesis [23,24] and for LXRβ-dependent Luc activity (Fig. 2E). We next studied the expression by qPCR of LXR-responsive genes in MCF-7 cells treated with the LXR modulator T0901317 and 5,6α-EC or the inducors of 5,6-EC accumulation, Tam and PBPE. We found that T0901317 stimulated the expression of ABCA1, SREBP-1c, SCD1, ACC, ABCG1, and repressed the expression of LDLR (Fig. 3D). 6 h of treatment of MCF-7 cells with 5,6α-EC induced a repression of the expression of ABCA1, SREBP-1c, had little impact on ACC expression and stimulated the expression of SCD1 and LDLR (Fig. 3D), consistent with the peculiar modulatory activity of LXR-dependent transcription reported with 5,6α-EC [45]. Knock down of LXRβ abrogated these effects showing that these transcriptional modulations were LXRβ-dependent (Fig. 3D). Tam and PBPE slightly repressed the expression of ABCA1, SREBP-1c and stimulated the expression of SCD1, ACC, ABCG1 and LDLR (Fig. 3D) similarly to what we found with 5,6α-EC in an LXRβ-dependent manner. Altogether, these data demonstrate the role of LXRβ in the induction of TAG biosynthesis by T0901317, 5,6α-EC, Tam and PBPE in MCF-7 cells. The induction of TAG biosynthesis by Tam and PBPE required a longer treatment (48 h) than 5,6α-EC (24 h), and the production of ROS which are involved in 5,6-EC production, strongly suggesting that 5,6α-EC was the endogenous mediator in Tam and PBPE stimulation of TAG biosynthesis.

3.3. 5,6α-EC is sulfated by SUL2B1b in MCF-7 cells treated with Tam and PBPE

MCF-7 cells have been reported to over-express the sterol sulfotransferase (SULT2B1b), and 5,6α-EC was shown to be the preferred substrate of this enzyme [53] to form 5,6α-epoxy-5α-cholestestan-3β-sulfate (5,6-ECS) that can be produced in MCF-7 cells treated with Tam or PBPE. Since our data suggested a possible metabolism of 5,6α-EC (Fig. 1B), we studied 5,6α-EC metabolism in MCF-7 cells and observed that 5,6α-EC was metabolized into 5,6-ECS when ChEH was inhibited by Tam or PBPE (Fig. 4A). The knock-down of SULT2B1b expression and activity (Fig. 4B) confirmed the inhibition of 5,6-ECS biosynthesis thus establishing the implication of SULT2B1b in 5,6-ECS formation in MCF-7 cells. We next evaluated the impact of 5,6-ECS on LXR-responsive genes in MCF-7 cells (Fig. 4C) and found that 5,6-ECS gave a similar profile as 5,6α-EC (Fig. 3D). However, the knock-down of SULT2B1b did not inhibit the stimulation of TAG biosynthesis by 5,6α-EC, Tam and PBPE (Fig. 4C) showing that the production of 5,6-ECS was not necessary for the induction of TAG biosynthesis by 5,6α-EC, Tam or PBPE. These data established that the inhibition of ChEH by Tam and PBPE induced the accumulation of 5,6α-EC that is metabolized into 5,6-ECS by SULT2B1b (Fig. 4E).

3.4. Importance of LXRβ and SULT2B1b in the cytotoxicity induced by Tam, PBPE, 5,6α-EC, 5,6β-EC and 5,6-ECS in MCF-7 cells

We next compared the cytotoxicity of Tam, PBPE and 5,6-EC metabolites in MCF-7 cells in which LXRβ (MCF7/siLXRβ) or SULT2B1b (MCF7/siSULT) were knocked down by transfection with small interfering RNA (siRNA) and compared to MCF-7 transfected with control scrambled siRNA (MCF7/siSC). Tam, PBPE, 5,6α-EC, 5,6-ECS and 5,6β-EC induced cytotoxicity in MCF-7 with EC_{50} of 2.5, 10.9, 22.4, 10.4 and 12.6 μM respectively (Table 1). MCF7/siLXRβ showed a 2.2- and 1.7-fold decrease in sensitivity to Tam and PBPE compared to the control MCF7/siSC demonstrating that LXRβ was involved in their cytotoxicity (Table 1). The loss of sensitivity to 5,6α-EC and 5,6-ECS in MCF7/siLXRβ indicated that LXRβ mediated their cytotoxicity. We observed a 2.3- and 1.8-fold diminution of sensitivity to Tam and PBPE respectively in MCF7/siSULT compared to MCF7/siSC showing that the presence of SULT2B1b contributed to the cytotoxicity of the drugs (Table 1). Interestingly, the contribution of SULTB1b was equivalent to that of LXRβ in the cytotoxicity induced by Tam and PBPE suggesting that the sulfation of 5,6α-EC into 5,6-ECS was required for cytotoxicity. This was confirmed by the observation that MCF7/siSULT cells that did not produce 5,6-ECS (Fig. 4B) lost their sensitivity to 5,6α-EC ($EC_{50} > 40$ μM) (Table 1). No change in the sensitivity to 5,6β-EC was measured in MCF7/siLXRβ and MCF7/siSULT compared to MCF7/siSC demonstrating that the cytotoxicity of 5,6β-EC was independent of LXRβ and SULT2B1b (Table 1). We had previously shown that Tam and PBPE activity was diminished by the over-expression of the anti-apoptotic protein Bcl2 in MCF-7 cells (MCF7/Bcl2) [23]. Measurement of their EC_{50} on MCF7/Bcl2 showed a 4.9- and 3.2-fold decreased sensitivity to Tam and PBPE respectively compared to control cells (MCF-7/Neo)

using anti-LXRβ antibodies. 24 h after cell transfection with siRNA, cells were transfected with the LXRE-luc plasmid. Cells were treated with solvent vehicle, 12 h with 1 μM T0901317, 12 h with 20 μM 5,6α-EC, 48 h with 5 μM Tam or 20 μM PBPE and assayed for Luc activity. H) Effect of T0901317, 5,6α-EC, Tam and PBPE on the LXRβ-dependent stimulation of TAG biosynthesis in MCF-7 cells. MCF-7 cells transfected with siSC or siLXRβ were treated with solvent vehicle, 12 h with 1 μM T0901317, 12 h with 20 μM 5,6α-EC, 48 h with 5 μM Tam or 20 μM PBPE. TAG quantification was done as described in Section 2. In all experiments the values are the mean ± S.E. of three independent experiments performed in triplicate. $^{*}P < 0.001$.

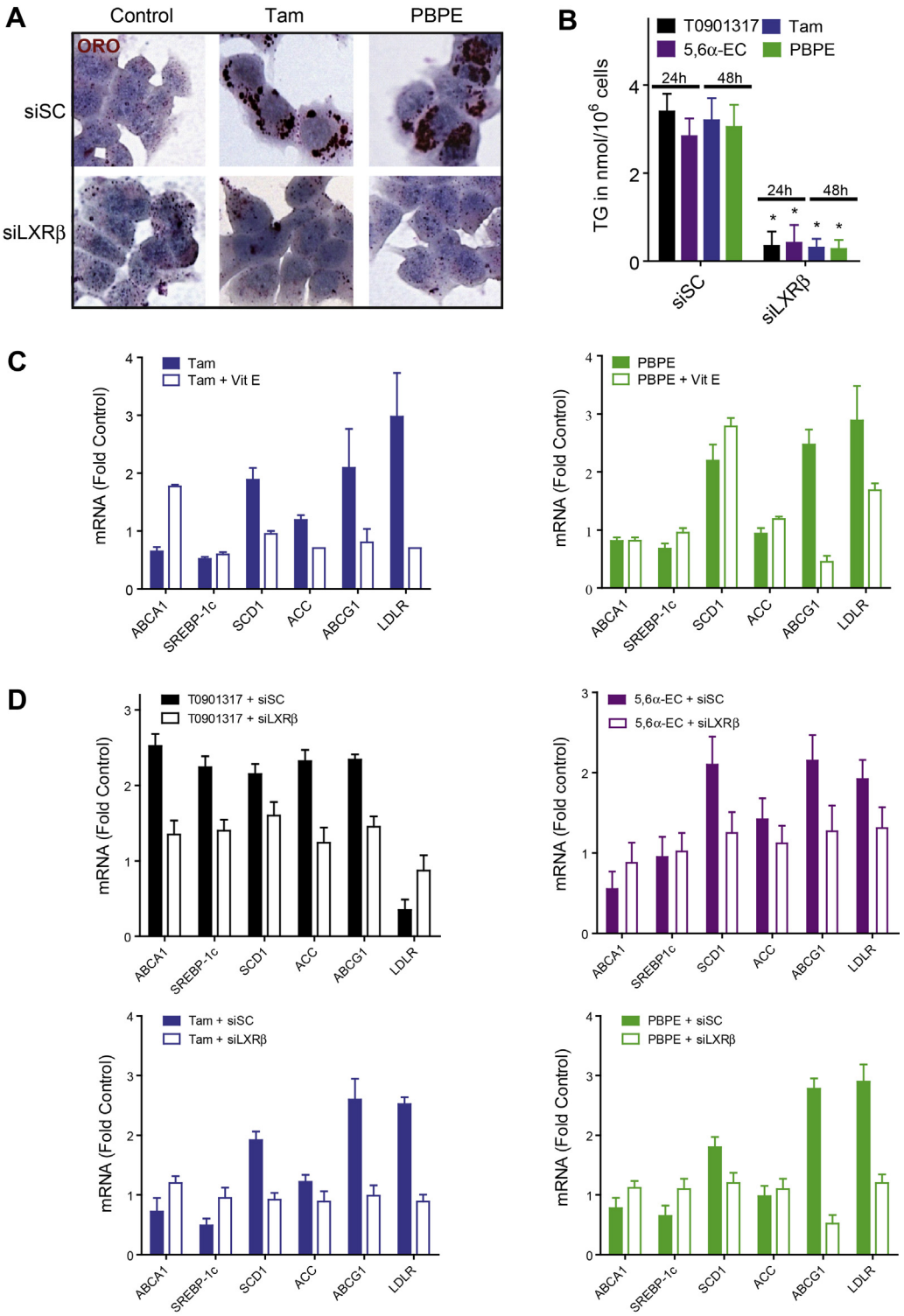

Fig. 3. Importance of LXRβ in the stimulation by Tam and PBPE of TAG biosynthesis in MCF-7 cells. (A) MCF-7 cells were transfected with siRNA scrambled (siSC) or siRNA targeting LXRβ (siLXRβ) and incubated 48 h with 5 μM Tam or 10 μM PBPE. Cells were stained for neutral lipids with ORO and countersained with Meyer's hematoxylin as described in Section 2. (B) MCF-7 cells transfected with siSC or siLXRβ were incubated 24 h with 1 μM T0901317, 20 μM 5,6α-EC or incubated 48 h with 5 μM Tam or 10 μM PBPE. TAG quantification was done as described in Section 2. (C) Importance of oxidation in the regulation of the expression of LXR-responsive genes by Tam and PBPE. MCF-7 cells were treated 40 h with 5 μM Tam or 10 μM PBPE in the presence or absence of 500 μM vit E. Expression of the LXR target was measured by quantitative RT-PCR as described in Section 2. (D) Importance of LXRβ in the regulation of the expression of LXR-responsive genes by T01901317, 5,6α-EC, Tam and PBPE. MCF-7 cells transfected with siSC or siLXRβ were incubated 6 h with 1 μM T0901317, 20 μM 5,6α-EC or incubated 40 h with 5 μM Tam or 10 μM PBPE. The expression of LXR-responsive genes was measured by quantitative RT-PCR as described in Section 2. Values are means of three independent experiments.

(Table 1). There were no changes in sensitivity to 5,6α-EC and 5,6-ECS in MCF7/Bcl2 cells, establishing that the Bcl2-controlled cytotoxicity of Tam and PBPE did not involve the production of 5,6α-EC and 5,6-ECS (Table 1). MCF7/Bcl2 cells showed decreased sensitivity to 5,6β-EC ($EC_{50} > 40$ μM) establishing that 5,6β-EC was responsible for the Bcl2-controlled cytotoxicity of Tam and PBPE (Table 1).

Altogether, these data established that, in MCF-7 cells, the cytotoxicity of Tam and PBPE can be decomposed into two

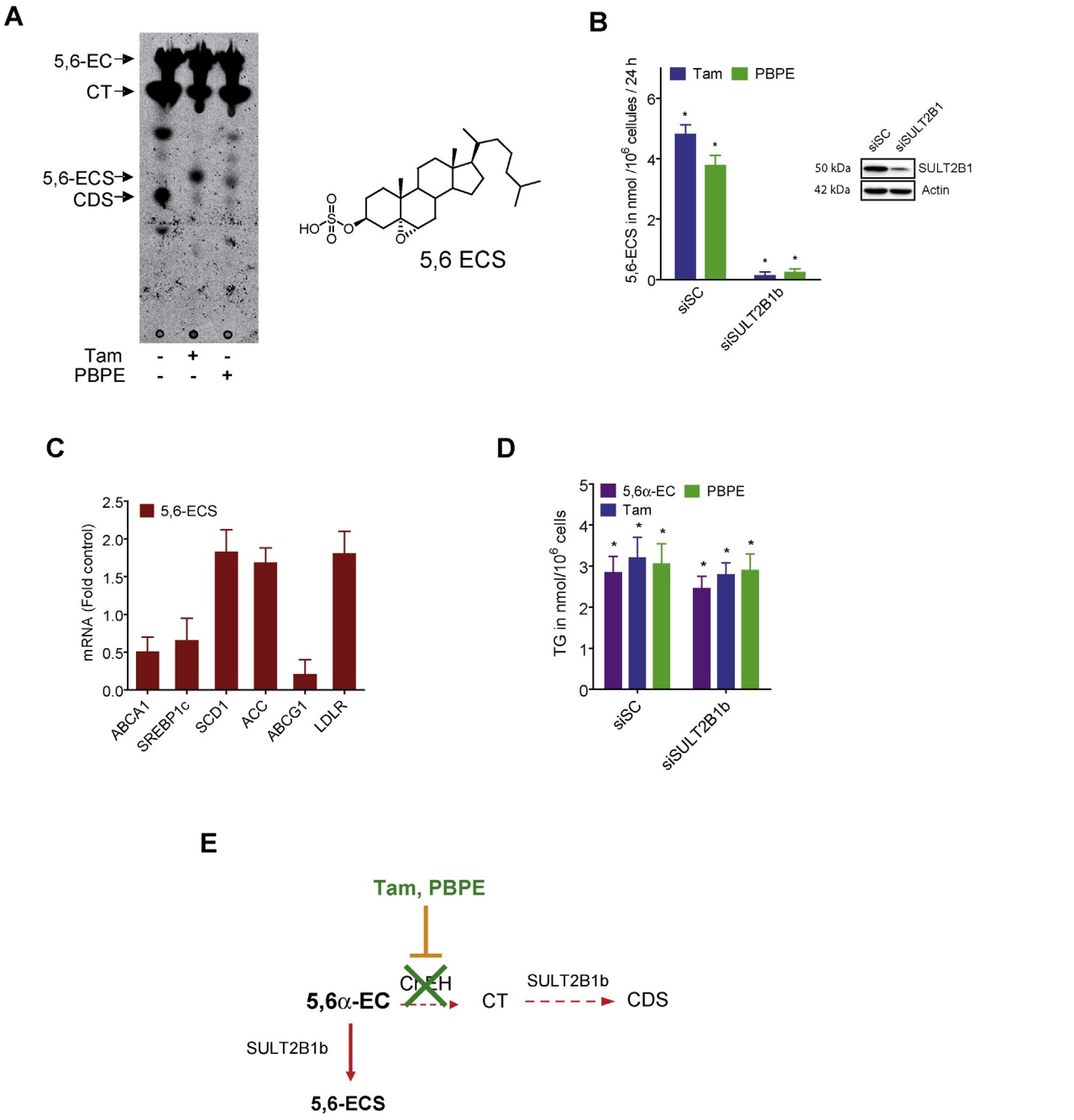

Fig. 4. Effect of Tam and PBPE on 5,6-EC sulfation in MCF-7 cells, the impact of 5,6-ECS on LXRβ-dependent TAG biosynthesis, and the importance of SULT2B1b. (A) MCF-7 cells were incubated with [^{14}C]-5,6-EC (0.6 μM; 20 μCi/μmol) in the presence of solvent vehicle (EtOH 0.01%), 5 μM Tam or 20 μM of PBPE for 48 h. The lipids were extracted and separated by silica TLC plates as described in Section 2. The TLC plates were developed by autoradiography and the positions of 5,6α-EC metabolites were determined using authentic standards. A representative autoradiogram of the TLC from three independent experiments is shown. The chemical structure of 5,6-ECS is given. (B) Impact of the knock-down of SULT2B1b on SULT2B1b protein expression and 5,6-ECS biosynthesis in MCF-7 cells. MCF-7 cells were transfected with siSC or siSULT2B1b. SULT2B1b expression was verified by Western blot using an anti-SULT2B1b antibody. 24 h after transfection, the cells were incubated with [^{14}C]-5,6-EC (0.6 μM; 20 μCi/μmol) for 48 h. 5,6-ECS biosynthesis was quantified as described in Section 2. Values are the mean ± S.E. of three independent experiments performed in triplicate. *$P < 0.001$. (C) Effect of 5,6-ECS on the modulation of endogenous responsive genes in MCF-7 cells. Cells were treated 6 h in the presence of 20 μM 5,6-ECS. The expression of LXR-responsive genes was measured by quantitative RT-PCR as described in Section 2. Values are means of three independent experiments. (D) Impact of the knock-down of SULT2B1b on TAG biosynthesis induced by 5,6α-EC, Tam and PBPE in MCF-7 cells. 24 h after MCF-7 cells were transfected with siSC or siSULT2B1b they were treated for 24 h with 20 μM 5,6α-EC or 48 h with 5 μM Tam or 20 μM PBPE for TAG quantification. Quantifications were performed as described in the caption of Fig. 2. Values are the mean ± S.E. of three independent experiments performed in triplicate. *$P < 0.001$. (E) Scheme summarizing 5,6-ECS formation under Tam and PBPE treatment in MCF-7 cells.

mechanisms: (1) a SULT2B1b- and LXRβ-dependent cytotoxicity mediated by 5,6-ECS, the sulfated metabolite of 5,6α-EC and (2) a Bcl2-controlled cytotoxicity mediated by 5,6β-EC. These data show that 5,6-EC epimers are cytotoxic through different mechanisms and account for both the mechanisms responsible for the cytotoxicity induced by Tam and PBPE against MCF-7 cells.

3.5. AEBS/ChEH ligands of different structural and pharmacological classes had similar effects on MCF-7 cells as Tam and PBPE

MCF-7 cells were treated with other AEBS/ChEH ligands showing they were potent inducers of 5,6-EC and 5,6-ECS accumulation (Table 1). We established that drugs that are known

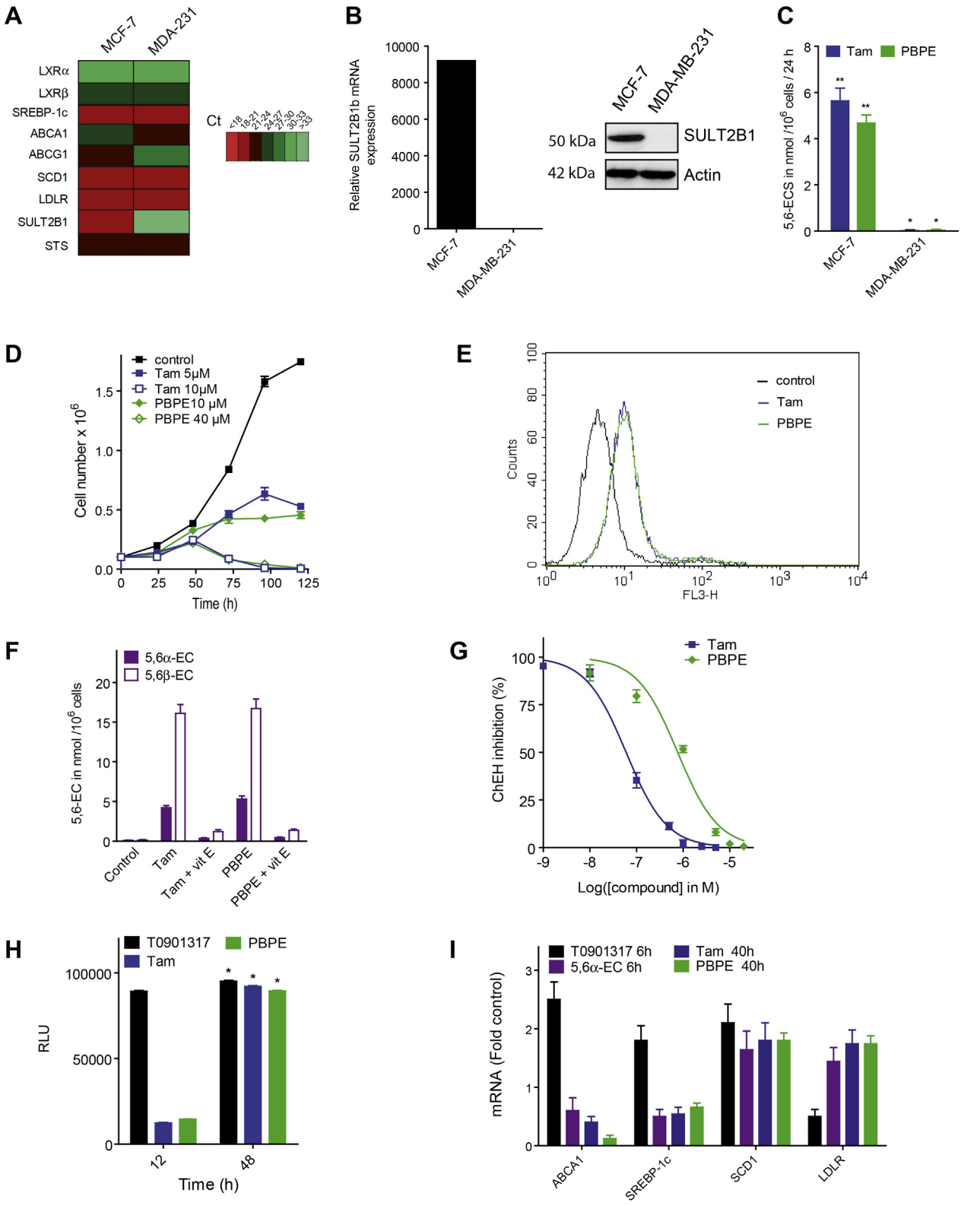

Fig. 5. Impact of Tam and PBPE on the modulation of cholesterol oxidative metabolism in MDA-MB-231 cells and of the modulation of 5,6-ECS. (A) Expression of mRNA encoding LXR isoforms, LXR-responsive genes, sterol sulfotransferase SULT2B1b and steroid sulfatase (STS) in MCF-7 cells and MDA-MB-231 cells. The expression of mRNA was measured by qPCR. (B) Expression of SULT2B1b in MCF-7 and MDA-MB-231 cells was measured at the mRNA level by qPCR and at the protein level by Western blotting. (C) The sulfation of 5,6α-EC into 5,6-ECS was measured in MCF-7 and MDA-MB-231 cells by incubating cells with 5,6α-EC for 48 h. Quantification was done as described in Section 2. (D) Measurement of the antiproliferative index. MDA-MB-231 cells were plated into 6-well plates and treated 48 h after plating with 5 and 10 μM Tam or 10 and 40 μM PBPE or the solvent vehicule (0.1% EtOH) for 5 days. The drugs and media were changed every 48 h. Cells were counted daily. Cells were harvested by trypsinization and counted on a Coulter counter. Experiments were repeated in triplicate. (E) Tam and PBPE induced the production of reactive oxygen species in MDA-MB-231 cells. MDA-MB-231cells were treated for 48 h with the solvent vehicle (0.1% EtOH), 5 μM Tam or 20 μM PBPE. ROS production was determined by flow cytometry analysis on cells stained with dihydroethidine probe (2.5 μM) as described in Section 2. Experiments were repeated at least three times in duplicate with comparable results. (F) Quantification of oxysterol in MDA-MB-231 cells incubated for 48 h with 10 μM Tam or 40 μM PBPE in the absence or presence of 500 μM Vit E. The results are reported in ng

Table 1
Effect of AEBS/ChEH ligands on 5,6-EC and 5,6-ECS biosynthesis, neutral lipid accumulation, and inhibition of ChEH on MCF-7 cells after 48 h treatment with drugs. 5,6-EC (5,6α-EC and 5,6β-EC) biosynthesis were performed by studying the metabolism of [^{14}C]-cholesterol in MCF-7 cells treated for 48 h with 10 μM of drugs as described in Section 2. 5,6-ECS biosynthesis was measured as described in the legend of Fig. 2. Stimulation of neutral lipid accumulation was monitored after treatment of MCF-7 cells and revealed by staining with Oil Red O (ORO) and visualized by light microscopy. Cytotoxicity (Cytx) was measure by the trypan blue exclusion methodology for a 72 h exposure of MCF-7 cells with 10 μM (SERMs), 20 μM (selective AEBS/ChEH ligands (AEBS/ChEH), sigma receptor ligands (σ-R) and cholesterol biosynthesis inhibitors (CBI)). The inhibition by Vit E (−) included the production 5,6-EC, 5,6-ECS, neutral lipid accumulation and cytotoxicity. + means a stimulation, − means inhibition.

Compound	Class	5,6-EC	5,6-ECS	ORO	Cytx	Vit E
Tam	SERM	+	+	+	+	−
4OHTam		+	+	++	+	−
Ralox		+	+	+	+	−
BZA		+	++	+	+	−
Clom		+	+	+	+	−
RU-39411		+	+	++	+	−
PBPE	AEBS/ChEH	+	+	+	+	−
PCPE		+	+	+	+	−
Tesm		+	+	+	+	−
MBPE		+	+	+	+	−
MCPE		+	+	+	+	−
BD-1008	Misc	+	+	+	+	−
SR-31747A		+	+	+	+	−
FPT		+	+	+	+	−
CLP		+	+	+	+	−
TFP		+	+	+	+	−
U-18666A	CBI	+	+	+	+	−
AY-9944		+	+	+	+	−
Triparanol		+	+	++	+	−

to inhibit ChEH [15,33], stimulated the biosynthesis of TAG and were cytotoxic to MCF-7 cells (Table 1). SERMs such as raloxifene and clomiphene compounds of the DPN family such as tesmilifene, cholesterol biosynthesis inhibitors such as U-18666A, triparanol and AY-9944, sigma receptor ligands BD-1008 and SR-31747A and tricyclic antidepressants such as trifluoroperazine, flupenthixol and chlorpromazine showed similar characteristics as Tam and PBPE on MCF-7 cells (Table 1). Altogether these data established that AEBS/ChEH ligands belonging to different structural and pharmacological classes induced the accumulation of 5,6-EC and 5,6-ECS, stimulated the accumulation of neutral lipid and were cytotoxic to MCF-7 cells. All these effects were inhibited by vit E.

3.6. Effect of Tam and PBPE on MDA-MB-231 cells

To confirm the importance of SULT2B1b in Tam and PBPE activity, we tested them on MDA-MB-231 cells. MDA-MB-231 cells were found to be similar to MCF-7 cells in their expression level of LXRβ, LXRα, SREBP-1c, SCD1, and LDLR. They expressed a higher amount of ABCA1 and a weaker amount of ABCG1 compared to MCF-7 cells (Fig. 5A). Interestingly, in contrast to MCF-7 cells, MDA-MB-231 did not express SULT2B1b while both cell lines expressed the steroid sulfatase (STS) in equal amount. STS being responsible for the de-sulfation of steroid- and sterol-sulfates [54]. The absence of SULT2B1b at the protein level was confirmed by Western blotting while SULT2B1b was detected in MCF-7 cells (Fig. 5B). Analyses of 5,6-EC sulfation in cells showed that, while in the presence of Tam and PBPE, MCF-7 cells produced 5,6-ECS, no production of 5,6-ECS was found under the same conditions of treatment in MDA-MB-231 cells (Fig. 5C). To study further the sensitivity of MDA-MB-231 to Tam and PBPE, kinetic and dose response studies were carried out. In Fig. 5D it can be seen that drugs induced a concentration- and time-dependent growth control and cytotoxicity. Treatment of cells with Tam and PBPE induced the production of ROS (Fig. 5D) as observed in MCF-7 cells [23]. We found that Tam and PBPE stimulated the biosynthesis of 5,6-EC in MDA-MB-231 cells (Fig. 5F). The antioxidant Vit E inhibited the stimulation of 5,6-EC production by Tam and PBPE (Fig. 5F) strongly suggesting that this epoxidation was ROS-dependent. Tam and PBPE were found to inhibit ChEH with IC_{50} of 59.9 ± 8 nM and 765 ± 12 nM respectively (Fig. 5G). To determine if LXRβ was modulated by Tam and PBPE in MDA-MB-231 cells, cells were transfected with the LXRE-Luc plasmid. T0901317, but neither Tam nor PBPE activated the expression of Luc at 12 h (Fig. 5H). At 48 h of treatment, Tam and PBPE stimulated Luc activity, as observed in MCF-7 cells (Fig. 2B). We next used qPCR to study the expression of endogenous LXR-responsive genes in MDA-MB-231 cells treated with the LXR modulator T0901317, 5,6α-EC, Tam and PBPE. We found that T0901317 stimulated the expression of ABCA1, SREBP-1c, SCD1, and repressed the expression of LDLR (Fig. 5I). 6 h of treatment of cells with 5,6α-EC and 40 h treatment with Tam or PBPE induced a similar effect of repression ABCA1 and SREBP-1c, and stimulation of the expression of SCD1 and LDLR (Fig. 5I).

Looking at neutral lipid metabolism, we found that Tam and PBPE induced the accumulation of ORO positive vesicles in MDA-MB-231 cells and knock-down of LXRβ inhibited this stimulation (Fig. 6A) establishing that this stimulation was LXRβ-dependent. We next showed that Tam and PBPE stimulated the accumulation of TAG in MDA-MB-231 cells (Fig. 6B) and it was ROS- and LXRβ-dependent (Fig. 6C). The LXR modulators T0901317 and 5,6α-EC stimulated the biosynthesis of TAG in an LXR-dependent manner in MDA-MB-231 cells (Fig. 6C). The expression of SULT2B1b did not modify the induction of TAG biosynthesis by 5,6α-EC, Tam or PBPE, indicating that TAG biosynthesis does not necessarily require SULT2B1b and the production of 5,6-ECS as observed in MCF-7 cells. 5,6-ECS was found to be cytotoxic to these cells and improved the cytotoxicity of Tam and PBPE (Fig. 6E). Altogether, these data established that MDA-MB-231 are SULT2B1b negative cells that cannot produce 5,6-ECS. We established that Tam and PBPE induced similar events in these cells as in MCF-7 cells in terms of ROS, 5,6-EC, and the induction of LXR-dependent TAG biosynthesis. The addition of 5,6-ECS to cells strongly sensitized MDA-MB-231 cells to Tam and PBPE.

3.7. Expression of SULT2B1b in MDA-MB-231 cells sensitized them to cytotoxicity induced by Tam and PBPE

MDA-MB-231 cells were found 1.9- and 2.1-fold less sensitive than in MCF-7 cells to Tam and PBPE (Table 2). These cells were insensitive to 5,6α-EC while their sensitivity to 5,6-ECS and 5,6β-EC was equivalent to that measured in MCF-7 cells (Table 2). Interestingly, the sensitivity of MDA-MB-231 to Tam and PBPE was similar to that of MCF-7/siLXRβ and MCF-7/siSULT suggesting an

of oxysterol per 10^6 cells. (G) Inhibition of ChEH activity by Tam and PBPE in MDA-MB-231 cells. Cells were incubated with [^{14}C]-5,6-EC (0.6 μM; 20 μCi/μmol) and were treated with increasing concentrations of Tam or PBPE ranging from 10 nM to 10 μM over 24 h. The positions of the 5,6-EC and CT were determined using [^{14}C]-5,6-EC and [^{14}C]-CT as standards. (H) Effect of T0901317, Tam, and PBPE on LXRE-Luc at 12 h and 48 h. MDA-MB-231 cells transfected with an LXRE-Luc plasmid were incubated with 1 μM T0901317, 5 μM Tam and 20 μM PBPE and assayed for Luc activity at 12 h and 48 h after treatment (Luc), expressed as relative luciferase unit (RLU) as described in Section 2. (I) Effect of T0901317, 5,6α-EC, Tam and PBPE on the expression of LXR-responsive genes in MDA-MB-231. MDA-MB-231 cells were incubated 6 h with 1 μM T0901317, 20 μM 5,6α-EC or incubated 40 h with 5 μM Tam or 10 μM PBPE. The expression of LXR-responsive genes was measured by quantitative RT-PCR as described in Section 2. Values are means of three independent experiments.

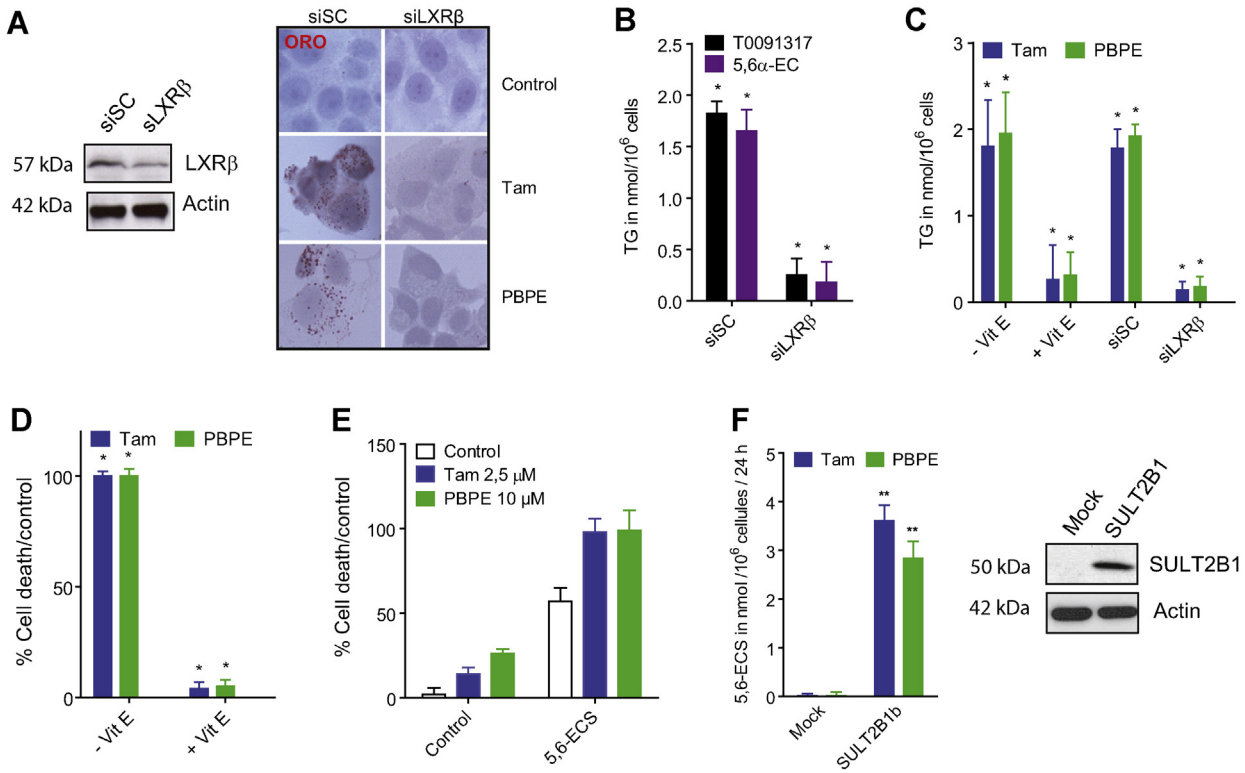

Fig. 6. Importance of LXRβ and ROS in the induction of TAG by Tam and PBPE in MDA-MB-231cells and effect of 5,6-ECS complementation on the cytotoxicity induced by Tam and PBPE. (A) Importance of LXRβ in the induction by Tam and PBPE of neutral lipid accumulation in MDA-MB-231 cells. Cells were transfected with siSC or siLXRβ and incubated 48 h with 5 μM Tam or 10 μM PBPE. LXRβ expression was verified by Western blot using anti-LXRβ antibodies. Cells were stained for neutral lipids with ORO and counterstained with Meyer's hematoxylin as described in Section 2. (B) Effect of Tam and PBPE on ROS- and LXRβ-dependent stimulation of TAG biosynthesis in MDA-MB-231 cells. MDA-MB-231 cells were transfected or not with siSC or siLXRβ and 24 h later treated for 48 h with solvent vehicle, 5 μM Tam or 20 μM PBPE in the presence or absence of 500 μM Vit E. TAG quantification was done as described in Section 2. (C) Effect of T0901317 and 5,6α-EC on the LXRβ-dependent stimulation of TAG biosynthesis in MDA-MB-231 cells. MDA-MB-231 cells were transfected with siSC or siLXRβ. TAG quantification was done as described in Section 2. (D) Effect of Vit E on the induction of cytotoxicity by Tam and PBPE in MDA-MB-231 cells. MDA-MB-231 cells were incubated 72 h in the presence of 10 μM Tam or 40 μM PBPE in the presence or absence of 500 μM Vit E. Cell death was quantified using the trypan blue exclusion methodology as described in Section 2. (E) Effect of 5,6-ECS on the induction of cytotoxicity by Tam and PBPE in MDA-MB-231 cells. Cells were treated 72 with solvent vehicle, 2.5 μM Tam or 10 μM PBPE in the absence or presence of 10 μM 5,6-ECS. Cell death was quantified using the trypan blue exclusion methodology as described in Section 2. (F) Ectopic expression of SULT2B1b activity in MDA-MB-231 cells. Cells were transfected with a plasmid encoding human SULT2B1b. Expression of SULT2B1b was verified by Western blotting and the functionality of the enzymes was confirmed by transformation of 5,6α-EC into 5,6-ECS as described above. The data presented here are the means ± S.E. of four independent experiments in triplicate. $^{*}P < 0.001$, $^{**}P < 0.0001$.

absence of LXRβ-dependent cytotoxicity due to the absence SULT2B1b expression and the absence of 5,6-ECS formation. Knock-down of LXRβ (MDA-MB-231/siLXRβ) confirmed the absence of LXRβ-depentent cytotoxicity of Tam and PBPE showing no significant impact on the EC_{50} of Tam and PBPE compared to MDA-MB-231 control cells (MDA-MB-231/siSC). Interestingly a loss of 5,6-ECS sensitivity was found in MDA-MB-231/siLXRβ ($EC_{50} > 40$ μM) confirming the LXRβ-dependent cytotoxicity of 5,6-ECS. As expected, there was no change in the cytotoxicity induced by 5,6β-EC in MDA-MB-231/siLXRβ compared to MDA-MB-231/siSC showing that 5,6β-EC induced a similar LXRβ-independent cytotoxicity in MDA-MB-231 as in MCF-7 cells (Table 2). To determine the importance of SULT2B1b in Tam, PBPE and 5,6α-EC cytotoxicity, MDA-MB-231 cells were transfected with a plasmid encoding SULT2B1b (MDA-MB-231/SULT) which led to the expression of the enzyme at the protein level (Fig. 6F) and enabled the production of 5,6-ECS when the cells were treated with Tam or PBPE (Fig. 6F). SULT2B1b expression sensitized cells by 1.9- and 2.2-fold to Tam and PBPE respectively compared to control cells (MDA-MB-231/MOCK) and MDA-MB-231/SULT became sensitive to 5,6α-EC (Table 2). Interestingly, MDA-MB-231/SULT cells were as sensitive to Tam, PBPE and 5,6α-EC as MCF-7 cells (Table 2) establishing a sensitization of cells to Tam, PBPE and 5,6α-EC. Altogether, these data showed that the induction of cytotoxicity by Tam and PBPE in MDA-MB-231 is LXR-independent and is mediated by 5,6β-EC. Importantly, the ectopic expression of SULT2B1b in MDA-MB-231 cells restored the sensitivity of cells to Tam and PBPE to the same level as MCF-7 cells.

4. Discussion

The aim of the present study was first to identify the cholesterol autoxidation species that are produced under Tam- and AEBS/ChEH ligands-treatment of BC cells and to determine the molecular pathways involved in the induction of TAG biosynthesis and cytotoxicity.

Here we report for the first time that Tam and other AEBS ligands induced the production 5,6-EC diastereoisomers, 5,6α-EC and 5,6β-EC in a 1/3 ratio in MCF-7 cells. No other oxysterols from the series studied were found to be stimulated. The production of 5,6-EC diastereoisomers was totally blocked by Vit E establishing that they were produced through a ROS mediated mechanism.

Tam has been reported to stimulate NOX in MCF-7 and MDA-MB-231 cells [41] which can induce H_2O_2 production required for cholesterol epoxidation. The mechanism involved in NOX activation has not been studied in BC cells but a hypothesis can be formulated. AEBS/ChEH ligands have been shown to inhibit cholesterol biosynthesis at the AEBS level which led to the accumulation of free sterol in cells [16], producing the appearance of multilamellar bodies [23,24]. The presence of MLB recapitulates

Table 2
Evaluation of the cytotoxicity of Tam, PBPE, 5,6α-EC, 5,6-ECS and 5,6β-EC on wild type and genetically modified MCF-7 cells and MDA-MB-231 cells. Cells were exposed 72 h to drugs (Tam, PBPE) or oxysterols (5,6α-EC, 5,6-ECS, 5,6β-EC) with increasing concentrations ranging from 1 to 100 μM. Cytotoxicity was measure by the trypan blue exclusion methodology. EC_{50} corresponds to the concentration required to kill 50% of cells. MCF-7/*siSC*, MCF-7/*siLXRβ* and MCF-7/*siSULT* are MCF-7 cells transfected with a scrambled small interfering RNA (siRNA), an siRNA against LXRβ and with an siRNA against SULT2B1b respectively. MCF7/*neo* and MCF-7/*bcl2* are MCF-7 cells transfected permanently with the pZip-*neo* and pZip-*bcl2* vectors [23]. MDA-MB-231/*siSC* and MDA-MB-231/*siLXRβ* are MDA-MB-231 cells transfected with siSC or siLXRβ. MDA-MB-231/MOCK and MDA-MB-231/SULT are MDA-MB-231 cells transfected with an empty pCMV6-XL5 plasmid or a pCMV6-XL5-SULT2B1b encoding the human SULT2B1b. Results are the mean of 3 independent experiments in triplicate.

	Tam	PBPE	5,6α-EC	5,6-ECS	5,6β-EC
			EC_{50} in μM		
MCF-7	2.5 ± 0.6	10.9 ± 2.1	22.4 ± 1.5	10.4 ± 0.6	12.6 ± 0.6
MCF-7/*siSC*	2.2 ± 0.8	10.2 ± 1.8	21.6 ± 2.2	11.4 ± 1.4	12.4 ± 1.7
MCF-7/*siLXRβ*	4.8 ± 0.8	17.8 ± 1.8	>40	>40	12.5 ± 2.5
MCF-7/*siSULT*	5.1 ± 0.7	18.1 ± 1.7	>40	11.1 ± 1.3	13.5 ± 2.6
MCF-7/*neo*	2.1 ± 0.8	10.1 ± 1.7	22.4 ± 1.5	9.1 ± 1.3	11.5 ± 3.6
MCF-7/*bcl2*	10.2 ± 1.1	32. ± 2.1	25.6 ± 3.2	12. 1 ± 1.4	>40
MDA-MB-231	4.8 ± 1.3	23.2 ± 1.1	>40	9.6 ± 1.8	14.2 ± 1.1
MDA-MB-231/*siSC*	4.7 ± 1.2	21.0 ± 1.3	>40	10.1 ± 1.5	13.6 ± 1.6
MDA-MB-231/*siLXRβ*	4.6 ± 1.4	22.1 ± 1.4	>40	>40	13.4 ± 1.8
MDA-MB-231/*MOCK*	4.9 ± 1.3	23.5 ± 1.4	>40	9.4 ± 2.1	13.9 ± 1.4
MDA-MB-231/*SULT*	2.6 ± 1.4	10.6 ± 1.3	24.4 ± 2.5	9.2 ± 2.8	14.1 ± 2.5

Niemann-Pick C (NPC) diseases characterized by the accumulation of free sterols in cells [55] and which is known to be induced by U18666A [56], an AEBS/ChEH ligand [15]. NPC1 diseases are associated with a stimulation of oxidative stress and with the production in the blood of patients [57] of increased amounts of 5,6-EC and CT, produced by hydration by ChEH NPC diseases are associated with the accumulation of lipid rafts in cells [58], and these lipid rafts were reported to control the activation of NOX in MCF-7 cells [59].

Altogether, these data strongly suggested the implication of free sterol accumulation in NOX activation and ROS production as a result of the treatment of BC cells by Tam and other AEBS/ChEH ligands. Therefore, the precise determination of the mechanism involved in ROS formation in BC cells treated with Tam and PBPE deserves further investigations.

We reported that 5,6-EC are extensively hydrated by ChEH in MCF-7 cells to give CT [15]. We have previously shown that Tam and PBPE were potent inhibitors of ChEH from rat liver microsomes and show in the present study that these drugs were also potent inhibitors of the human ChEH present in MCF-7 and MDA-MB-231 cells. As observed for ChEH from rat liver microsomes, AEBS ligands inhibited the human ChEH from BC cells at pharmacologic and therapeutic concentrations. The consequence of ChEH inhibition is the blockage of CT formation and the accumulation of 5,6-EC diastereoisomers. CT has been reported to be metabolized into a tumor promoter suggesting that blocking its production might *per se* constitute a protection against tumorigenic processes [33,38]. Since the sterol and oxysterol sulfotransferase SULT2B1b was reported to be over expressed in MCF-7 cells [60] and used 5,6α-EC as preferred substrate among cholesterol and several ring B oxysterol [53], we studied 5,6α-EC metabolism in BC cells. As expected we found that under Tam and PBPE treatment, 5,6α-EC was sulfated into 5,6-ECS. We then evaluated whether the accumulation of 5,6-EC and 5,6-ECS was involved in the ROS dependent induction of TAG biosynthesis and the cytotoxicity triggered by Tam and AEBS/CHEH ligands in BC cells. Activation of TAG biosynthesis constitutes one the major markers of BC re-differentiation which is indicative of the reactivation of lactation [24,40,43]. TAG biosynthesis is tightly regulated and several enzymes involved in its biosynthesis have been reported to be under the transcriptional control of nuclear receptors including members of the oxysterol liver-X-receptors (LXRα and LXRβ) [61]. We established that Tam, AEBS/ChEH ligands, and 5,6α-EC induced TAG biosynthesis through an LXRβ-dependent mechanism leading to the up-regulation of lipogenic enzymes involved in TAG biosynthesis (ACC and SCD1). The fact that the inhibition of 5,6α-EC biosynthesis by Vit E and the knock-down of LXRβ blocked the biosynthesis of TAG induced by AEBS/ChEH ligands established that 5,6α-EC is the oxysterol that mediates TAG biosynthesis in BC cells stimulated by Tam and PBPE.

5,6α-EC was reported to be an LXRα and LXRβ modulator that displays agonist, antagonist and inverse agonist properties in a cell type-dependent manner [45]. We found here that 5,6α-EC showed a similar effect on the regulation of LXR-responsive genes in MCF-7 cells confirming the observations that Berrodin et al. made in other cell lines [45]. 5,6-ECS had a similar profile of gene regulation as 5,6-EC on BC cells. This established that if 5,6-ECS is a direct regulator of LXR, than this compound is not a full antagonist on LXR as reported earlier [51,62], but rather a modulator depending on the target gene.

In this current study, we demonstrate that the cytotoxicity induced by Tam and AEBS ligands implicated both 5,6α-EC and 5,6β-EC as endogenous mediators but through different mechanisms. 5,6α-EC was cytotoxic in an LXRβ-dependent manner after being sulfated by SULT2B1b to produce 5,6-ECS in MCF-7 cells. Consistent with these data, 5,6α-EC was not cytotoxic in SULT2B1b negative cells such as MDA-MB-231 cells. The cytotoxicity of 5,6-ECS was found to be LXRβ-dependent establishing that 5,6-ECS was the oxysterol that mediates the LXRβ-dependent cytotoxicity induced by Tam and AEBS/ChEH ligands in MCF-7 cells. 5,6β-EC was the most prominent oxysterol produced in both MCF-7 and MDA-MB-231 cells. Its cytotoxicity was not LXRβ- and SULT2B1b-dependent. 5,6β-EC has been reported to induce apoptosis in

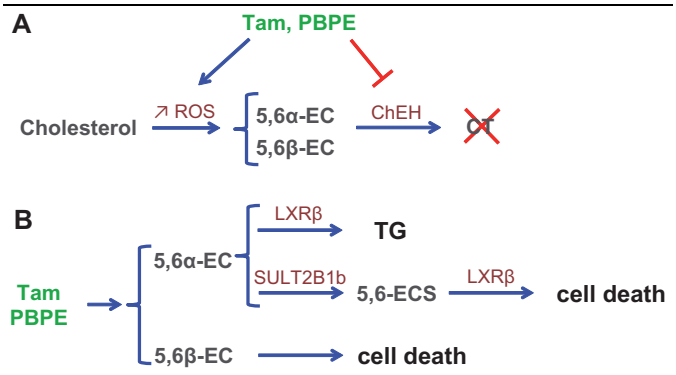

Fig. 7. Proposed molecular mechanism explaining the induction of TAG biosynthesis and cytotoxicity by Tam and PBPE in MCF-7 cells.

tumor cells through a mechanism involving mitochondria [63] but the determination of the precise mechanism of action of 5,6β-EC deserves further studies.

Several groups have shown that Vit E blocked the cytoxicity of Tam on breast cancer cell lines such as MCF-7 cells and MDA-MB-231 [23,24,64,65] and the mechanism described here gives a rationale to explain these effects. Fig. 7 provides a scheme describing the molecular mechanisms of the action of Tam and PBPE in the stimulation of TAG biosynthesis and cytotoxicity. These data indicate that the LXR-dependent cytotoxicity of Tam and AEBS/ChEH ligands depends on the presence of 5,6-EC and its product of sulfation (5.6-ECS) by SULT2B1b. On the other hand, we showed for the first time that the expression of SULT2B1b in the triple negative and SULT2B1b negative MDA-MB-231 cells, sensitizes the cells to the cytotoxicity of Tam and AEBS/ChEH ligands to the level of MCF-7 cells.

We found that sulfation of 5,6α-EC into 5,6-ECS by SULT2B1b was possible when 5,6α-EC accumulated in cells when ChEH was inhibited. Together these data established that 5,6-ECS formation contributes to the sensitivity of BC cells to Tam cytotoxicity. The fact that the ectopic expression of SUT2B1b in MDA-MB-231 cells or addition of 5,6-ECS sensitizes cells to Tam and PBPE strongly suggests that the expression of SULT2B1b in cells could be a predictor of the sensitivity of BC cells to Tam and AEBS/ChEH ligands. Furthermore the association of SERMs or DPM compounds with 5,6-ECS could represent an alternative to treat SULT2b1b negative triple negative untreatable BC. Further investigations will be carried to determine if these *in vitro* observations are reproduced *in vivo* on BC cell xenographs in mice.

The transcriptional modulation of 5,6-ECS was found comparable to that of 5,6α-EC suggesting that other LXR-responsive genes could be differentially modulated and this could explain the higher cytotoxicity of 5,6-ECS compared to 5,6-EC. This is supported by the observation that 5,6-ECS was found to be an antagonist in LXRE-Luc (data not shown and [51,62]) despite a similar modulation of several LXR-responsive genes, but further investigations are warranted.

The effect of other ChEH/AEBS selective inhibitors was observed with tesmilifene on the induction of BC cell differentiation and death. Tesmilifene was shown to significantly improve the overall survival in a phase III randomized trial for metastatic breast cancer when given with doxorubicin [32]. These effects were proposed to be due to the killing of tumor initiating cells (TIC) observed at therapeutic doses in four different models of breast cancer [22]. Since tesmilifene is a selective AEBS ligand and inhibitor of ChEH [8,15], the mechanisms detailed in the present study are likely to be involved in these effects giving a rationale for its use.

Tam has been reported in some clinical studies to induce a reversible stimulation of TAG production during the time of treatment and to lower circulating LDL cholesterol [66,67]. The liver is one of the tissues richest in AEBS/ChEH [15] and a tissue that produces 5,6α-EC [36], thus it is reasonable to propose that Tam could cause a decrease in LDL cholesterol through a previously observed inhibition of cholesterol esterification [68] and a stimulation of the expression of LDLR [69] through an LXR-dependent mechanism. On the other hand the hyper-triacylglycerolemia can be explained by the LXR-dependent mechanism we report on this paper, because LXRβ is known to control the biosynthesis of TAG [70]. This is supported by the observation that Vit E blocks hyper-triacylglycerolemia in patients treated with Tam [71], which is similar to what we found in MCF-7 cells in the present study. Thus, variations in the control of the LXR signaling pathway could explain the variations in the severity of the hyper-triacylglycerolemia observed in patients and again this deserves further study.

This current study established for the first time that 5,6-EC metabolites and LXRβ play a role in the induction of cell differentiation and death by Tam, other SERMs and AEBS/ChEH ligands in BC cells.These mechanisms now have to be taken into account in the development of new SERMs or other AEBS/ChEH ligands for anticancer applications.

Competing interest

PDM and MRP are employed by the company Affichem.

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References

[1] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225–49.
[2] Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ. Strategies for subtypes—dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 2011;22:1736–47.
[3] Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nat Rev Cancer 2007;7:46–53.
[4] de Medina P, Favre G, Poirot M. Multiple targeting by the antitumor drug tamoxifen: a structure-activity study. Curr Med Chem Anticancer Agents 2004;4:491–508.
[5] Llaverias G, Danilo C, Mercier I, Daumer K, Capozza F, Williams TM, et al. Role of cholesterol in the development and progression of breast cancer. Am J Pathol 2011;178:402–12.
[6] Danilo C, Frank PG. Cholesterol and breast cancer development. Curr Opin Pharmacol 2012;12:677–82.
[7] Alikhani N, Ferguson RD, Novosyadlyy R, Gallagher EJ, Scheinman EJ, Yakar S, et al. Mammary tumor growth and pulmonary metastasis are enhanced in a hyperlipidemic mouse model. Oncogene 2013;32:961–7.
[8] Poirot M, Silvente-Poirot S, Weichselbaum RR. Cholesterol metabolism and resistance to tamoxifen. Curr Opin Pharmacol 2012;12:683–9.
[9] Pitroda SP, Khodarev NN, Beckett MA, Kufe DW, Weichselbaum RR. MUC1-induced alterations in a lipid metabolic gene network predict response of human breast cancers to tamoxifen treatment. Proc Natl Acad Sci USA 2009;106:5837–41.
[10] de Medina P, Paillasse MR, Segala G, Khallouki F, Brillouet S, Dalenc F, et al. Importance of cholesterol and oxysterols metabolism in the pharmacology of tamoxifen and other AEBS ligands. Chem Phys Lipids 2011;164:432–7.
[11] Poirot M, De Medina P, Delarue F, Perie JJ, Klaebe A, Faye JC. Synthesis, binding and structure-affinity studies of new ligands for the microsomal anti-estrogen binding site (AEBS). Bioorg Med Chem 2000;8:2007–16.
[12] Delarue F, Kedjouar B, Mesange F, Bayard F, Faye JC, Poirot M. Modifications of benzylphenoxy ethanamine antiestrogen molecules: influence affinity for antiestrogen binding site (AEBS) and cell cytotoxicity. Biochem Pharmacol 1999;57:657–61.
[13] Fargin A, Bayard F, Faye JC, Traore M, Poirot M, Klaebe A, et al. Further evidence for a biological role of anti-estrogen-binding sites in mediating the growth inhibitory action of diphenylmethane derivatives. Chem Biol Interact 1988;66:101–9.
[14] Brandes LJ. A diphenylmethane derivative selective for the anti-estrogen binding site may help define its biological role. Biochem Biophys Res Commun 1984;124:244–9.
[15] de Medina P, Paillasse MR, Segala G, Poirot M, Silvente-Poirot S. Identification and pharmacological characterization of cholesterol-5,6-epoxide hydrolase as a target for tamoxifen and AEBS ligands. Proc Natl Acad Sci USA 2010;107:13520–25.
[16] Kedjouar B, de Medina P, Oulad-Abdelghani M, Payre B, Silvente-Poirot S, Favre G, et al. Molecular characterization of the microsomal tamoxifen binding site. J Biol Chem 2004;279:34048–61.
[17] Mesange F, Sebbar M, Capdevielle J, Guillemot JC, Ferrara P, Bayard F, et al. Identification of two tamoxifen target proteins by photolabeling with 4-(2-morpholinoethoxy)benzophenone. Bioconjug Chem 2002;13:766–72.
[18] Mesange F, Sebbar M, Kedjouar B, Capdevielle J, Guillemot JC, Ferrara P, et al. Microsomal epoxide hydrolase of rat liver is a subunit of theanti-oestrogen-binding site. Biochem J 1998;334(Pt 1):107–12.

[19] Poirot M, Chailleux C, Mesange F, Bayard F, Faye JC. Characterization of the membranous antiestrogen binding protein: II. Purification to homogeneity. J Recept Res 1994;14:37–46.
[20] Chailleux C, Poirot M, Mesange F, Bayard F, Faye JC. Characterization of the membranous antiestrogen binding protein: I. Partial purification of the protein in its active state. J Recept Res 1994;14:23–35.
[21] Poirot M, Chailleux C, Fargin A, Bayard F, Faye JC. A potent and selective photoaffinity probe for the anti-estrogen binding site of rat liver. J Biol Chem 1990;265:17039–43.
[22] Deng T, Liu JC, Pritchard KI, Eisen A, Zacksenhaus E. Preferential killing of breast tumor initiating cells by N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine/tesmilifene. Clin Cancer Res 2009;15:119–30.
[23] de Medina P, Payre B, Boubekeur N, Bertrand-Michel J, Terce F, Silvente-Poirot S, et al. Ligands of the antiestrogen-binding site induce active cell death and autophagy in human breast cancer cells through the modulation of cholesterol metabolism. Cell Death Differ 2009;16:1372–84.
[24] Payre B, de Medina P, Boubekeur N, Mhamdi L, Bertrand-Michel J, Terce F, et al. Microsomal antiestrogen-binding site ligands induce growth control and differentiation of human breast cancer cells through the modulation of cholesterol metabolism. Mol Cancer Ther 2008;7:3707–18.
[25] Kedjouar B, Daunes S, Vilner BJ, Bowen WD, Klaebe A, Faye JC, et al. Structural similitudes between cytotoxic antiestrogen-binding site (AEBS) ligands and cytotoxic sigma receptor ligands. Evidence for a relationship between cytotoxicity and affinity for AEBS or sigma-2 receptor but not for sigma-1 receptor. Biochem Pharmacol 1999;58:1927–39.
[26] Poirot M, Garnier M, Bayard F, Riviere I, Traore M, Wilson M, et al. The antiproliferative properties of 4-benzylphenoxy ethanamine derivatives are mediated by the anti-estrogen binding site (ABS), whereas the anti-estrogenic effects of trifluopromazine are not. Biochem Pharmacol 1990;40:425–9.
[27] Brandes LJ, Gerrard JM, Bogdanovic RP, Lint DW, Reid RE, LaBella FS. Correlation of the antiproliferative action of diphenylmethane-derivative antiestrogen binding site ligands with antagonism of histamine binding but not of protein kinase C-mediated phosphorylation. Cancer Res 1988;48:3954–8.
[28] Raghavan D, Brandes LJ, Klapp K, Snyder T, Styles E, Tsao-Wei D, et al. Phase II trial of tesmilifene plus mitoxantrone and prednisone for hormone refractory prostate cancer: high subjective and objective response in patients with symptomatic metastases. J Urol 2005;174:1808–13 [discussion 13].
[29] Khoo K, Brandes L, Reyno L, Arnold A, Dent S, Vandenberg T, et al. Phase II trial of N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine.HCl and doxorubicin chemotherapy in metastatic breast cancer: a National Cancer Institute of Canada clinical trials group study. J Clin Oncol 1999;17:3431–7.
[30] Brandes LJ, Bracken SP, Ramsey EW. N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine in combination with cyclophosphamide: an active, low-toxicity regimen for metastatic hormonally unresponsive prostate cancer. J Clin Oncol 1995;13:1398–403.
[31] Brandes LJ, Simons KJ, Bracken SP, Warrington RC. Results of a clinical trial in humans with refractory cancer of the intracellular histamine antagonist, N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine-HCl, in combination with various single antineoplastic agents. J Clin Oncol 1994;12:1281–90.
[32] Reyno L, Seymour L, Tu D, Dent S, Gelmon K, Walley B, et al. Phase III study of N,N-diethyl-2-[4-(phenylmethyl) phenoxy]ethanamine (BMS-217380-01) combined with doxorubicin versus doxorubicin alone in metastatic/recurrent breast cancer: National Cancer Institute of Canada Clinical Trials Group Study MA.19. J Clin Oncol 2004;22:269–76.
[33] Silvente-Poirot S, Poirot M. Cholesterol epoxide hydrolase and cancer. Curr Opin Pharmacol 2012;12:696–703.
[34] de Medina P, Silvente-Poirot S, Tamoxifen Poirot M. AEBS ligands induced apoptosis and autophagy in breast cancer cells through the stimulation of sterol accumulation. Autophagy 2009;5:1066–7.
[35] Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 2012;8:445–544.
[36] Schroepfer Jr. GJ. Oxysterols: modulators of cholesterol metabolism and other processes. Physiol Rev 2000;80:361–554.
[37] Cai H. NAD(P)H oxidase-dependent self-propagation of hydrogen peroxide and vascular disease. Circ Res 2005;96:818–22.
[38] Poirot M, Silvente-Poirot S. Cholesterol-5,6-epoxides. Chemistry, biochemistry, metabolic fate and cancer. Biochimie 2013;95:622–31.
[39] Shah VP, Chegini HA, Vishneski SR, Weatherman RV, Blackmore PF, Dobrydneva Y. Tamoxifen promotes superoxide production in platelets by activation of PI3-kinase and NADPH oxidase pathways. Thromb Res 2012;129:36–42.
[40] Chakraborty A, Bodipati N, Demonacos MK, Peddinti R, Ghosh K, Roy P. Long term induction by pterostilbene results in autophagy and cellular differentiation in MCF-7 cells via ROS dependent pathway. Mol Cell Endocrinol 2012;355:25–40.
[41] Kallio A, Zheng A, Dahllund J, Heiskanen KM, Harkonen P. Role of mitochondria in tamoxifen-induced rapid death of MCF-7 breast cancer cells. Apoptosis 2005;10:1395–410.
[42] Lee YS, Kang YS, Lee SH, Kim JA. Role of NAD(P)H oxidase in the tamoxifen-induced generation of reactive oxygen species and apoptosis in HepG2 human hepatoblastoma cells. Cell Death Differ 2000;7:925–32.
[43] Munster PN, Troso-Sandoval T, Rosen N, Rifkind R, Marks PA, Richon VM. The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces differentiation of human breast cancer cells. Cancer Res 2001;61:8492–7.
[44] Jakobsson T, Treuter E, Gustafsson JA, Liver Steffensen KR. X receptor biology and pharmacology: new pathways, challenges and opportunities. Trends Pharmacol Sci 2012;33:394–404.
[45] Berrodin TJ, Shen Q, Quinet EM, Yudt MR, Freedman LP, Nagpal S. Identification of 5{alpha},6{alpha}-epoxycholesterol as a novel modulator of liver X receptor activity. Mol Pharmacol 2010;78:1046–58.
[46] Lewis-Wambi JS, Kim H, Curpan R, Grigg R, Sarker MA, Jordan VC. The selective estrogen receptor modulator bazedoxifene inhibits hormone-independent breast cancer cell growth and down-regulates estrogen receptor alpha and cyclin D1. Mol Pharmacol 2011;80:610–20.
[47] de Medina P, Paillasse MR, Payre B, Silvente-Poirot S, Poirot M. Synthesis of new alkylaminooxysterols with potent cell differentiating activities: identification of leads for the treatment of cancer and neurodegenerative diseases. J Med Chem 2009;52:7765–77.
[48] Paillasse MR, de Medina P, Amouroux G, Mhamdi L, Poirot M, Silvente-Poirot S. Signaling through cholesterol esterification: a new pathway for the cholecystokinin 2 receptor involved in cell growth and invasion. J Lipid Res 2009;50:2203–11.
[49] de Medina P, Casper R, Savouret JF, Poirot M. Synthesis and biological properties of new stilbene derivatives of resveratrol as new selective aryl hydrocarbon modulators. J Med Chem 2005;48:287–91.
[50] de Medina P, Genovese S, Paillasse M, Mazaheri M, Subra-Caze S, Bystricky K, et al. Auraptene is an inhibitor of cholesterol esterification and a modulator of estrogen receptors. Mol Pharmacol 2010;78:827–36.
[51] Song C, Hiipakka RA, Liao S. Auto-oxidized cholesterol sulfates are antagonistic ligands of liver X receptors: implications for the development and treatment of atherosclerosis. Steroids 2001;66:473–9.
[52] Viennois E, Mouzat K, Dufour J, Morel L, Lobaccaro JM, Baron S. Selective liver X receptor modulators (SLiMs): what use in human health. Mol Cell Endocrinol 2012;351:129–41.
[53] Fuda H, Javitt NB, Mitamura K, Ikegawa S, Strott CA. Oxysterols are substrates for cholesterol sulfotransferase. J Lipid Res 2007;48:1343–52.
[54] Reed MJ, Purohit A, Woo LW, Newman SP, Potter BV. Steroid sulfatase: molecular biology, regulation, and inhibition. Endocr Rev 2005;26:171–202.
[55] Liao G, Yao Y, Liu J, Yu Z, Cheung S, Xie A, et al. Cholesterol accumulation is associated with lysosomal dysfunction and autophagic stress in Npc1 −/− mouse brain. Am J Pathol 2007;171:962–75.
[56] Pacheco CD, Kunkel R, Lieberman AP. Autophagy in Niemann-Pick C disease is dependent upon Beclin-1 and responsive to lipid trafficking defects. Hum Mol Genet 2007;16:1495–503.
[57] Porter FD, Scherrer DE, Lanier MH, Langmade SJ, Molugu V, Gale SE, et al. Cholesterol oxidation products are sensitive and specific blood-based biomarkers for Niemann-Pick C1 disease. Sci Transl Med 2010;2:56ra81.
[58] Simons K, Gruenberg J. Jamming the endosomal system: lipid rafts and lysosomal storage diseases. Trends Cell Biol 2000;10:459–62.
[59] Rao Malla R, Raghu H, Rao JS. Regulation of NADPH oxidase (Nox2) by lipid rafts in breast carcinoma cells. Int J Oncol 2010;37:1483–93.
[60] He D, Meloche CA, Dumas NA, Frost AR, Falany CN. Different subcellular localization of sulphotransferase 2B1b in human placenta and prostate. Biochem J 2004;379:533–40.
[61] Wagner M, Zollner G, Trauner M. Nuclear receptors in liver disease. Hepatology 2011;53:1023–34.
[62] Argmann CA, Edwards JY, Sawyez CG, O'Neil CH, Hegele RA, Pickering JG, et al. Regulation of macrophage cholesterol efflux through hydroxymethylglutaryl-CoA reductase inhibition: a role for RhoA in ABCA1-mediated cholesterol efflux. J Biol Chem 2005;280:22212–21.
[63] Ryan L, O'Callaghan YC, O'Brien NM. Comparison of the apoptotic processes induced by the oxysterols 7beta-hydroxycholesterol and cholesterol-5beta,6-beta-epoxide. Cell Biol Toxicol 2004;20:313–23.
[64] Mandlekar S, Yu R, Tan TH, Kong AN. Activation of caspase-3 and c-Jun NH2-terminal kinase-1 signaling pathways in tamoxifen-induced apoptosis of human breast cancer cells. Cancer Res 2000;60:5995–6000.
[65] Gundimeda U, Chen ZH, Gopalakrishna R. Tamoxifen modulates protein kinase C via oxidative stress in estrogen receptor-negative breast cancer cells. J Biol Chem 1996;271:13504–14.
[66] Love RR, Newcomb PA, Wiebe DA, Surawicz TS, Jordan VC, Carbone PP, et al. Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 1990;82:1327–32.
[67] Love RR, Wiebe DA, Feyzi JM, Newcomb PA, Chappell RJ. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women after 5 years of treatment. J Natl Cancer Inst 1994;86:1534–9.
[68] de Medina P, Payre BL, Bernad J, Bosser I, Pipy B, Silvente-Poirot S, et al. Tamoxifen is a potent inhibitor of cholesterol esterification and prevents the formation of foam cells. J Pharmacol Exp Ther 2004;308:1165–73.
[69] Suarez Y, Fernandez C, Gomez-Coronado D, Ferruelo AJ, Davalos A, Martinez-Botas J, et al. Synergistic upregulation of low-density lipoprotein receptor activity by tamoxifen and lovastatin. Cardiovasc Res 2004;64:346–55.
[70] Patel R, Patel M, Tsai R, Lin V, Bookout AL, Zhang Y, et al. LXRbeta is required for glucocorticoid-induced hyperglycemia and hepatosteatosis in mice. J Clin Invest 2011;121:431–41.
[71] Babu JR, Sundravel S, Arumugam G, Renuka R, Deepa N, Sachdanandam P. Salubrious effect of vitamin C and vitamin E on tamoxifen-treated women in breast cancer with reference to plasma lipid and lipoprotein levels. Cancer Lett 2000;151:1–5.

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Tumor and Stem Cell Biology

Cancer Research

c-Src Modulates Estrogen-Induced Stress and Apoptosis in Estrogen-Deprived Breast Cancer Cells

Ping Fan[1], Obi L. Griffith[2], Fadeke A. Agboke[1], Pavana Anur[4], Xiaojun Zou[1], Russell E. McDaniel[1], Karen Creswell[1], Sung Hoon Kim[3], John A. Katzenellenbogen[3], Joe W. Gray[2,4], and V. Craig Jordan[1]

Abstract

The emergence of anti-estrogen resistance in breast cancer is an important clinical phenomenon affecting long-term survival in this disease. Identifying factors that convey cell survival in this setting may guide improvements in treatment. Estrogen (E_2) can induce apoptosis in breast cancer cells that have been selected for survival after E_2 deprivation for long periods (MCF-7:5C cells), but the mechanisms underlying E_2-induced stress in this setting have not been elucidated. Here, we report that the c-Src kinase functions as a key adapter protein for the estrogen receptor (ER, *ESR1*) in its activation of stress responses induced by E_2 in MCF-7:5C cells. E_2 elevated phosphorylation of c-Src, which was blocked by 4-hydroxytamoxifen (4-OHT), suggesting that E_2 activated c-Src through the ER. We found that E_2 activated the sensors of the unfolded protein response (UPR), IRE1α (*ERN1*) and PERK kinase (*EIF2AK3*), the latter of which phosphorylates eukaryotic translation initiation factor-2α (eIF2α). E_2 also dramatically increased reactive oxygen species production and upregulated expression of heme oxygenase HO-1 (*HMOX1*), an indicator of oxidative stress, along with the central energy sensor kinase AMPK (*PRKAA2*). Pharmacologic or RNA interference–mediated inhibition of c-Src abolished the phosphorylation of eIF2α and AMPK, blocked E_2-induced ROS production, and inhibited E_2-induced apoptosis. Together, our results establish that c-Src kinase mediates stresses generated by E_2 in long-term E_2-deprived cells that trigger apoptosis. This work offers a mechanistic rationale for a new approach in the treatment of endocrine-resistant breast cancer. *Cancer Res; 73(14); 4510–20. ©2013 AACR.*

Introduction

Developing drugs that target the estrogen receptor (ER) either directly (tamoxifen) or indirectly (aromatase inhibitors) has improved the prognosis of breast cancer (1, 2). Although aromatase inhibitors show considerable advantages over tamoxifen with respect to patient disease-free survival and tolerability, acquisition of resistance to all forms of endocrine treatments is inevitable (3, 4). Multiple mechanistic changes are involved in antihormone resistance, which provides the scientific rationale for the clinical development of additional targeted therapies (5, 6). It is well-known that the biologic actions of E_2 are mediated through the ER, which functions in the nucleus as ligand-dependent transcription factors to promote gene transcription and stimulation of cell growth (7). Paradoxically, laboratory evidence shows that E_2 can induce apoptosis in sensitive antihormone-resistant cells *in vivo* (8–10). This new targeted strategy provides novel therapeutic approaches to endocrine-resistant breast cancer. A recent phase II clinical trial reports that E_2 provides a clinical benefit for patients with aromatase inhibitor–resistant advanced breast cancer (11). In addition, the laboratory results on E_2 -induced apoptosis using antihormone-treated MCF-7 cells have been used to explain the reduction of breast cancer and the reduction in mortality observed in postmenopausal hysterectomized women in their 60s treated with conjugated equine estrogen (CEE) when compared with a placebo-treated control (12). The antitumor action of CEE is observed, not only during CEE treatment but also for 6 years after treatment. These data suggest a cidal effect for CEE and has been noted recently (13). These encouraging clinical results prompted us to investigate the mechanisms underlying E_2-induced apoptosis to increase the therapeutic benefits of E_2 in aromatase inhibitor–resistant breast cancer.

Experimental evidence has established the oncogene, c-Src, as a critical component of multiple signaling pathways that regulate proliferation, survival, angiogenesis, and metastasis (14, 15). Increased c-Src activity is believed to play an important role in the development and progression of breast cancer

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(16), and c-Src has been considered as a survival signal for endocrine resistant breast cancer cells (17). Therefore, a c-Src inhibitor administered as a single-agent or in combination with other antihormone therapy has the potential to enhance the inhibitory effects of antihormones and delay antihormone resistance (18). These observations highlight c-Src as an important therapeutic target for the treatment of human breast cancer.

Mitochondria are important intracellular organelles involved in apoptosis via an intrinsic pathway (19). Although the molecular mechanisms of E_2-induced apoptosis are not fully understood, evidence indicates that mitochondria-related caspase pathways are involved (20, 21). Similarly, a variety of events in apoptosis focus on mitochondria, including the loss of mitochondrial transmembrane potential, release of cytochrome *c*, and participation of pro- and antiapoptotic Bcl-2 family proteins (22, 23). However, accumulating evidence suggests that the endoplasmic reticulum where members of the Bcl-2 family of proteins localize is also a major point of integration of pro-apoptotic signaling or damage sensing (24, 25). The endoplasmic reticulum senses local stress such as unfolded protein (UPR) through a set of pathways known as the UPR (26), which activates 3 transmembrane sensors PRK-like endoplasmic reticulum kinase (PERK), inositol-requiring 1 alpha (IRE-1α), and activating transcription factor 6 (ATF-6) in endoplasmic reticulum (26). Depending on the duration and degree of stress, the UPR can provide either survival signals by activating adaptive and antiapoptotic signals or death signals by inducing cell death programs (27, 28).

We have found that E_2 changes the cell number according to the treatment period in long-term E_2-deprived breast cancer cell lines MCF-7:5C and MCF-7:2A (25). E_2 has the capacity to decrease around 80% of cell number in MCF-7:5C cells after 7 days treatment, whereas in MCF-7:2A cells after 2-week treatment (29). Unexpectedly, the c-Src inhibitor effectively rescues the decreasing of cell number by E_2 in 2 long-term E_2-deprived cell lines (29). The goal of this study is to identify the mechanisms underlying the early stage of E_2-induced apoptosis and the function of c-Src in the process of E_2-initiated apoptosis. To that end, we show that E_2 triggers endoplasmic reticulum stress and oxidative stress, which activate 2 main apoptotic pathways, the mitochondrial (intrinsic) and death receptor (extrinsic) pathways, whereas c-Src plays an essential role in mediating stress responses induced by E_2 in MCF-7:5C cells. These findings have important clinical implications for the appropriate application of combination therapies in advanced aromatase inhibitor–resistant breast cancer.

Materials and Methods

Materials

Estradiol was purchased from Sigma-Aldrich. c-Src inhibitor PP2 was purchased from CalBiochem. ERα antibody was from Santa Cruz Biotechnology. Total mitogen-activated protein kinase (MAPK), phosphorylated MAPK, phosphorylated c-Src, phosphorylated eIF2α, total eIF2α, and IRE1α antibodies were from Cell Signaling Technology. Total c-Src mouse antibody was from Millipore. Estrogen dendrimer conjugate (EDC) was a kind gift by Dr. J.A. Katzenellenbogen (University of Illinois at Urbana-Champaign, Urbana, IL).

Cell culture conditions and cell proliferation assays

Estrogen-deprived MCF-7:5C cells were maintained in estrogen-free RPMI-1640 medium supplemented with 10% dextran-coated charcoal-stripped FBS as previously described (20). The DNA fingerprinting pattern of cell line is consistent with the report by the American Type Culture Collection (29). The DNA content of the cells, a measure of proliferation, was determined by using a DNA fluorescence quantitation kit (29).

Cell-cycle analysis

Briefly, MCF-7:5C cells were treated with vehicle (0.1% EtOH) and E_2 (10^{-9} mol/L), respectively. Cells were harvested and gradually fixed with 75% EtOH on ice. After staining with propidium iodide (PI), cells were analyzed using a FACSort flow cytometer (Becton Dickinson), and the data were analyzed with ModFit software.

Annexin V analysis of apoptosis

The FITC Annexin V Detection Kit I (BD Pharmingen) was used to quantify apoptosis by flow cytometry according to the manufacturer's instructions. In brief, MCF-7:5C cells were treated with different compounds, respectively. Cells were suspended in 1× binding buffer and 1×10^5 cells were stained simultaneously with fluorescein isothiocyanate (FITC)-labeled Annexin V (FL1-H) and PI (FL2-H). Cells were analyzed using FACSort flow cytometer (Becton Dickinson).

Mitochondrial/TRANSMEMBRANE potential ($\Delta\psi_m$) detection

Mitochondrial membrane potential was measured by flow cytometry using the cationic lipophilic green fluorochrome rhodamine-123 (Rh123; Molecular Probes) as previously described (20). Disruption of $\Delta\psi_m$ is associated with a lack of Rh123 retention and a decrease in fluorescence.

Detection of oxidative stress

Intracellular reactive oxygen species (ROS) were detected by fluorescent dye 2′,7′-dichlorofluorescein diacetate (H_2DCFDA, Invitrogen; ref. 30). Briefly, MCF-7:5C cells were treated with E_2 for different time points using vehicle (0.1% EtOH) cells as control. Cells were loaded with 1 μmol/L CM-H_2DCFDA for 10 minutes and washed with PBS twice. Then, cells were monitored at fluorescence 530 nm and an excitation wavelength of 488 nm through flow cytometry.

Immunoblotting

Proteins were extracted in cell lysis buffer (Cell Signaling Technology) supplemented with Protease Inhibitor Cocktail (Roche) and Phosphatase Inhibitor Cocktail Set I and Set II (Calbiochem). The immunoblotting was conducted as previously described (29).

Transient transfection reporter gene assays

Transient transfection assay was conducted using a dual-luciferase system (Promega). To determine ER transcriptional

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activity, cells were transfected with an estrogen response element (ERE)-regulated (pERE (5×) TA-ffLuc plus pTA-srLuc) dual-luciferase reporter gene sets. The cells were treated with E_2 for 24 hours following the transfection. Then, the cells were harvested and processed for dual-luciferase reporter activity, in which the firefly luciferase activity was normalized by *Renilla* luciferase activity.

Quantitative real-time reverse transcription PCR

Total RNA, isolated with an RNeasy Micro Kit (Qiagen), was converted to first-strand cDNA using a kit from Applied Biosystems. Quantitative real-time PCR assays were done with the SYBR Green PCR Master Mixes (Applied Biosystems) and a 7900HT Fast Real-time PCR System (Applied Biosystems). All primers were synthesized in Integrated DNA Technologies. The sequence of primers is shown in the Supplementary Table S1. All the data were normalized by 36B4.

RNA sequencing analysis

MCF-7:5C cells were treated with different compounds for 72 hours. Cells were harvested in TRIzol. Total RNA was isolated with an RNeasy Micro Kit. These long RNA samples were first converted into a library of cDNA fragments. Sequencing adaptors were subsequently added to each cDNA fragment and a 2×100 bp paired-end sequence was obtained from each cDNA using high-throughput sequencing technology (Illumina GAII). An average of 73.8 million such reads was produced for each sample. The resulting sequence reads were aligned to reference genome build hg19 using TopHat 1.3.0 (31), a splice junction aligner. Transcript abundance was estimated as Fragments Per Kilobase of exon per Million fragments mapped (FPKM), using Cufflinks 1.0.3 (32). Additional analysis was conducted with the alternative expression analysis by sequencing (Alexa-seq) software package as previously described (33). Gene expression measures were compared between Cufflinks and Alexa-seq for the set of 17,993 overlapping genes. Correlations were excellent with Spearman correlations of 0.955 to 0.971 for the 6 samples. Pathway analysis was conducted with DAVID (34) on lists of differentially expressed gene lists.

Statistical analysis

All reported values are the means ± SE. Statistical comparisons were determined with 2-tailed Student *t* tests. Results were considered statistically significant if the $P < 0.05$.

Results

c-Src mediated estrogen-activated growth pathways in long-term estrogen-deprived breast cancer cells MCF-7:5C

It is well-documented that E_2 stimulates growth and prevents apoptosis in wild-type breast cancer cells and estrogen-responsive osteoblast cells (35, 36). In contrast, physiologic concentrations of E_2 induce apoptosis in long-term E_2-deprived breast cancer cells (20, 21). c-Src plays a critical role in relaying ER signaling pathways in breast cancer cells (37). To investigate the function of E_2 and c-Src in long-term E_2-deprived breast cancer cells MCF-7:5C, a specific c-Src tyrosine kinase inhibitor, PP2, was used to block phosphorylation of c-Src (Fig. 1A). It also effectively abolished the growth pathways including the MAPK and phosphoinositide 3-kinase (PI3K)/AKT pathways in MCF-7:5C cells (Fig. 1A). E_2 activated c-Src through ER as 4-hydroxytamoxifen (4-OHT) completely suppressed phosphorylation of c-Src (Fig. 1B). Although our previous finding showed that E_2 initiates apoptosis in MCF-7:5C cells (20), E_2 was able to activate nongenomic (Supplementary Fig. S1A) and genomic pathways in MCF-7:5C cells (Fig. 1C). These actions were blocked by the c-Src inhibitor, PP2 (Fig. 1C and Supplementary Fig. S1A). Even though the characteristic E_2-induced apoptosis occurs after 72-hour treatment (20), cell numbers were initially increased by E_2 with a high percentage in S-phase (Fig. 1D). All of these results suggested that E_2 caused an imbalance between growth and apoptosis in MCF-7:5C cells.

Inhibition of c-Src suppressed estrogen-induced apoptosis in MCF-7:5C cells

We have shown that long-term E_2 deprivation increases c-Src activity (29). Therefore, we addressed the question of whether the c-Src inhibitor, PP2, in combination with E_2 would enhance apoptosis in MCF-7:5C cells. Unexpectedly, the c-Src inhibitor blocked apoptosis initiated by E_2 (Fig. 2A and Supplementary Fig. S1D). To confirm that inhibition of c-Src could block E_2-induced apoptosis, a specific siRNA was used to knock down c-Src in MCF-7:5C cells (Fig. 2B), which reduced the percentage of Annexin V binding induced by E_2 (Fig. 2C). Further experiments showed that E_2 disrupted mitochondrial membrane potential ($\Delta\psi_m$) after 48-hour treatment, which was measured by flow cytometry using Rh123 (Fig. 2D). The c-Src inhibitor PP2 and 4-OHT both prevented reduction of Rh123 retention induced by E_2 (Fig. 2D). These data showed that E_2-triggered apoptosis use the c-Src tyrosine kinase pathway. To evaluate the role of the nongenomic pathway in E_2-induced apoptosis, studies were completed with a synthetic ligand, EDC, that only activates the nongenomic pathway at certain concentration (38). The results showed that EDC (10^{-8} mol/L) activated the nongenomic pathway incorporating c-Src (Supplementary Fig. S2). Importantly, EDC had no capacity to activate endogenous E_2 target gene pS2 and did not induce apoptosis in MCF-7:5C cells (Supplementary Fig. S2). All of these findings suggested that the nongenomic pathway does not play a critical role in triggering E_2-induced apoptosis.

Suppression of E_2-induced apoptosis by the c-Src inhibitor was independent of the classical ERE-regulated transcriptional genes in MCF-7:5C cells

The ER is the initial site for E_2 to induce apoptosis as antiestrogens ICI 182,780 and 4-OHT completely block apoptosis triggered by E_2 (ref. 20 and Supplementary Fig. S3A). In addition to the mediation of ER growth pathways, c-Src is involved in the process of ligand-activated ER ubiquitylation (39). Therefore, blockade of c-Src tyrosine kinase with PP2 further increased ERα protein and mRNA expression levels in MCF-7:5C cells (Fig. 3A). E_2 activated ERE activity, which could be blocked by 4-OHT but not by PP2 (Fig. 3B). It was interesting to find that the c-Src inhibitor alone could upregulate

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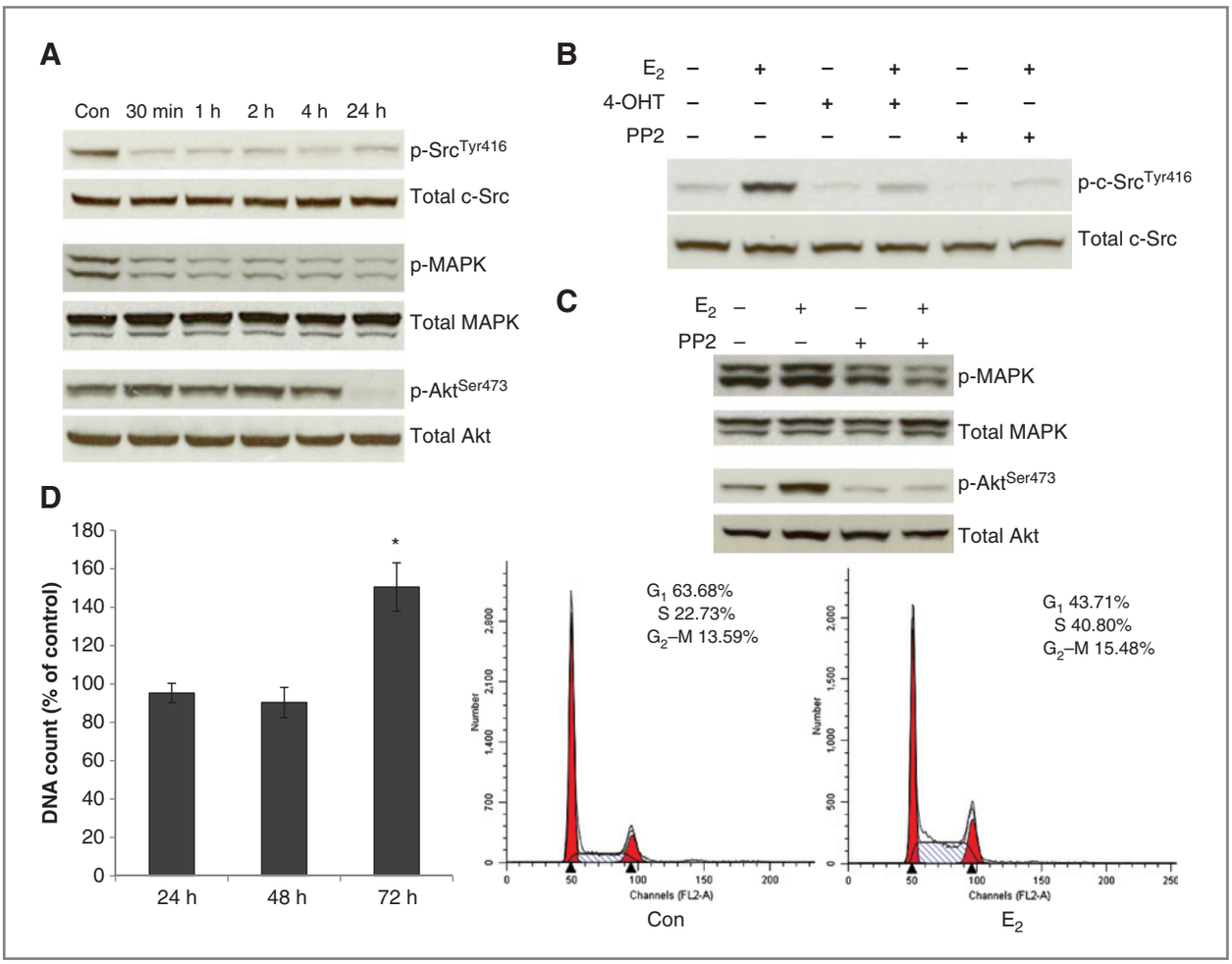

Figure 1. c-Src mediated estrogen-activated growth pathways in MCF-7:5C cells. A, MCF-7:5C cells were treated with vehicle (0.1% dimethyl sulfoxide) and PP2 (5×10^{-6} mol/L) for different durations. Phosphorylated c-Src, MAPK, and Akt were detected by immunoblotting. Total c-Src, MAPK, and Akt were used for loading controls. B, MCF-7:5C cells were treated with vehicle (0.1% dimethyl sulfoxide), E_2 (10^{-9} mol/L), 4-OHT (10^{-6} mol/L), E_2 (10^{-9} mol/L) plus 4-OHT (10^{-6} mol/L), PP2 (5×10^{-6} mol/L), E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L), respectively for 48 hours. Phosphorylated c-Src was detected by immunoblotting. Total c-Src was used for loading control. C, MCF-7:5C cells were treated with E_2 or combined with PP2, respectively, for 24 hours. Phosphorylated MAPK and Akt were examined by immunoblotting. Total MAPK and Akt were used for loading controls. D, MCF-7:5C cells were treated with vehicle and E_2 for different durations. Total DNA was determined using a DNA fluorescence quantitation kit. As a parallel experiment, MCF-7:5C cells were treated with vehicle and E_2 for 72 hours. Cells were fixed for cell-cycle analysis. *, $P < 0.05$, compared with respective control.

E_2-inducible gene pS2 and was additive with E_2 to elevate pS2 mRNA level (Fig. 3C). Another important ER target gene progesterone receptor (PR) has been regarded as an indicator of a functional ER pathway, as expression of PR is regulated by E_2. Although the c-Src inhibitor alone did not elevate PR expression, it dramatically synergized with E_2 to upregulate PR mRNA (Fig. 3D). All of these results showed that blockade of c-Src increased expression of classical ER target genes. It also implied that classical ER pathway might not directly involve in the E_2-induced apoptosis.

c-Src was involved in the process of triggering apoptosis-related genes by E_2 in MCF-7:5C cells

To further investigate the mechanisms of the suppression of E_2-induced apoptosis by PP2, RNA-seq analysis was conducted to examine the genes regulated by E_2 to trigger apoptosis in MCF-7:5C cells. A wide range of apoptosis-related genes was activated by E_2 (Fig. 4A), which were functionally classified into 3 groups: *TP53*-related genes (such as *TP63*, *PMAIP1*, and *CYFIP2*), stress-related genes (such as *HMOX1*, *PPP1R15A*, *ZAK*, *NUAK2*, etc.), and inflammatory response–related genes (such as *LTB*, *FAS*, *TNFRSF21*, *CXCR4*, etc.). Most were stress-related genes (Supplementary Fig. S3B). Consistent with the biologic experiments, 4-OHT and PP2 both blocked apoptosis-related genes induced by E_2 but to a different extent in MCF-7:5C cells (Fig. 4A). The majority of these apoptosis-related genes were confirmed by real-time PCR with similar changes noted as in RNA-seq analysis (Fig. 4B–D and Supplementary Fig. S4). E_2 dramatically increased p63 mRNA levels (Fig. 4B) but did not arrest cells in the G_1 phase. In fact, S-phase was markedly elevated in MCF-7:5C cells (Fig. 1D). Heme oxygenase 1 (*HMOX1*), which is active at high concentrations of heme,

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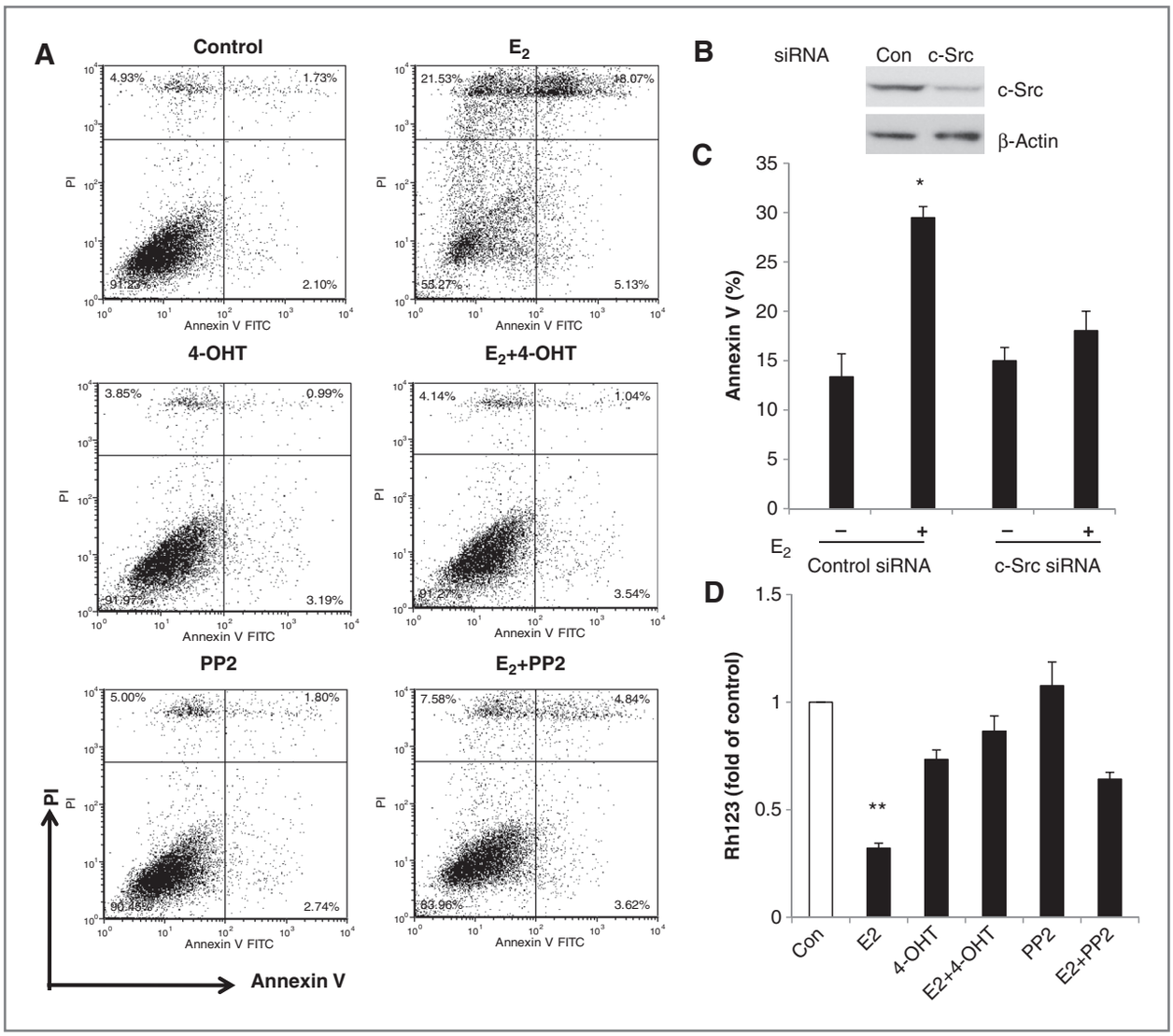

Figure 2. Inhibition of c-Src suppressed estrogen-induced apoptosis in MCF-7:5C cells. A, MCF-7:5C cells were treated with different compounds respectively as above for 72 hours and Annexin V binding assay was used to detect apoptosis. B, MCF-7:5C cells were transfected with siRNA of c-Src for 72 hours using nontarget siRNA as control. c-Src was detected by immunoblotting. The β-actin was used for loading control. C, MCF-7:5C cells were transfected with c-Src siRNA and nontarget siRNA as above. Then, they were treated with vehicle (0.1% EtOH) and E_2 (10^{-9} mol/L), respectively, for 72 hours. Apoptosis was detected through Annexin V binding assay. *, $P < 0.05$, compared with control. D, MCF-7:5C cells were treated with different compounds respectively as above for 48 hours and cells were harvested to detect mitochondrial potential through Rh123. **, $P < 0.001$, compared with control.

catalyzes the degradation of heme and is thought to function as an oxidative stress indicator (40). In breast cancer cells, cytochrome *c* is a major source of heme protein found in the inner membrane of the mitochondrion. E_2 markedly increased *HMOX1* in MCF-7:5C cells (Fig. 4C), thereby confirming that E_2 may damage the mitochondria and caused cytochrome *c* release. In contrast to MCF-7:5C cells, E_2 decreased *HMOX1* levels in wild-type MCF-7 cells (Supplementary Fig. S5A) and clearly did not change *HMOX1* expression in another long-term E_2-deprived cell line MCF-7:2A (Supplementary Fig. S5B), both of MCF-7 and MCF-7:2A do not undergo apoptosis after exposure to E_2 in the first 3 days. In addition, E_2 upregulated TNF family members (such as TNFα, LTA, and LTB), which were abolished by 4-OHT and PP2 (Fig. 4D and Supplementary Fig. S6A and S6B). Low dose of TNFα activated pro-apoptotic pathways in MCF-7:5C cells and inhibited cell growth (Supplementary Fig. S6C and S6D). All of these data suggested that E_2 widely activated intrinsic and extrinsic apoptosis pathways and c-Src was directly involved in mediating apoptosis.

The c-Src inhibitor blocked estrogen-induced oxidative stress in MCF-7:5C cells

ROSs are the product of oxidative stress by mitochondria, whereas an increase in ROS contributes to degenerative changes in mitochondrial function (41). Under physiologic conditions, cellular ROS levels are tightly controlled by

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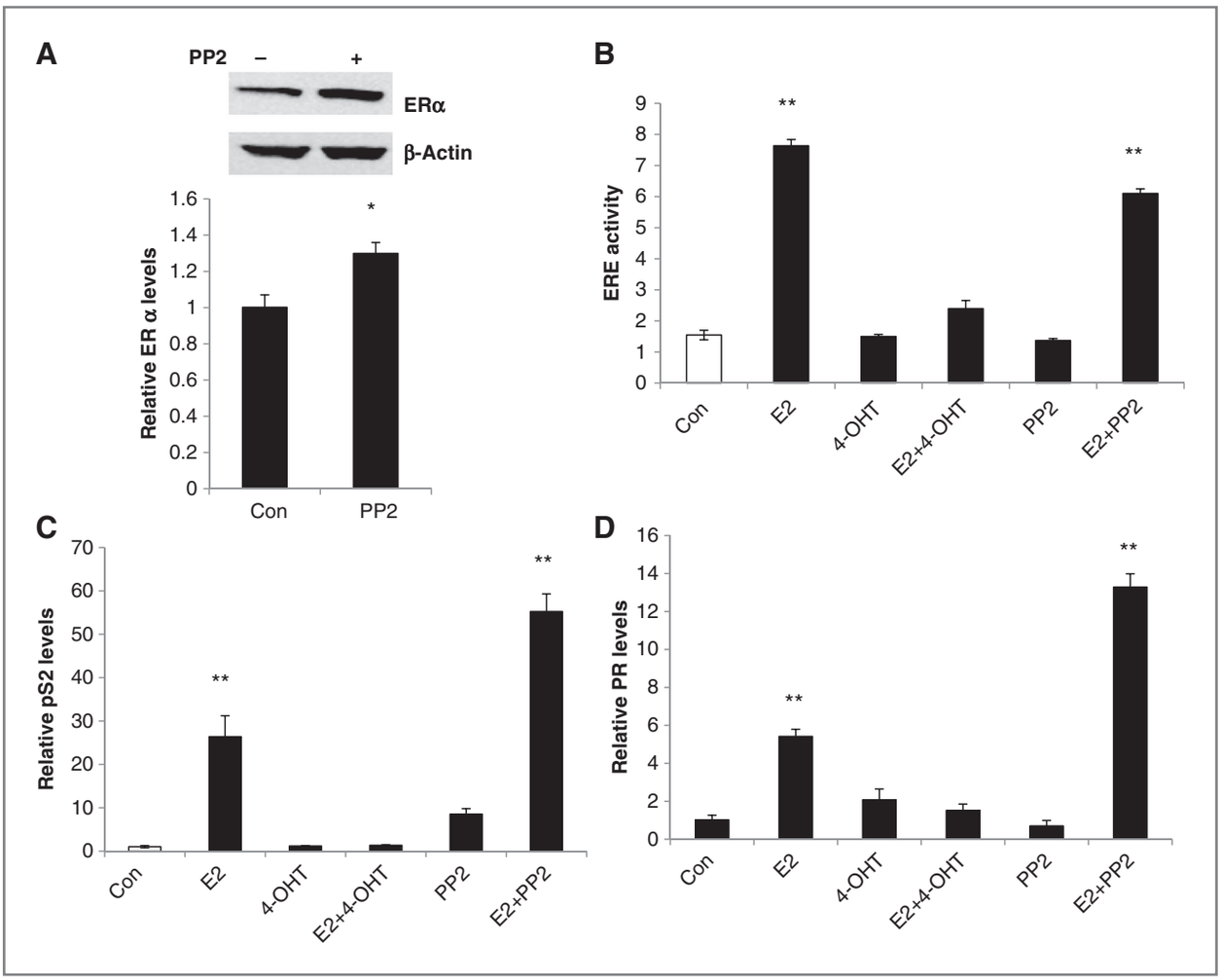

Figure 3. Suppression of E_2-induced apoptosis by the c-Src inhibitor was independent of the classical ERE-regulated transcriptional genes in MCF-7:5C cells. A, MCF-7:5C cells were treated with vehicle (0.1% dimethyl sulfoxide) and PP2 (5×10^{-6} mol/L), respectively, for 24 hours. ERα protein was detected by immunoblotting. ERα mRNA was quantified with quantitative PCR (qPCR). *, $P < 0.05$, compared with control. B, MCF-7:5C cells were transfected with ERE firefly luciferase plasmid plus *Renilla* luciferase plasmid. Then, cells were treated with different compounds respectively for 24 hours to detect ERE activity. **, $P < 0.001$, compared with control. C, MCF-7:5C cells were treated with different compounds respectively for 24 hours. The pS2 mRNA was quantified with qPCR. **, $P < 0.001$, compared with control. D, MCF-7:5C cells were treated with different compounds respectively for 72 hours. The PR mRNA was quantified with qPCR. **, $P < 0.001$, compared with control.

low-molecular-weight radical scavengers and by a complex intracellular network of enzymes such as catalases (*CAT*) and superoxide dismutases (*SOD*). Under conditions of lethal stress, ROSs are considered as key effectors of cell death (42). Intracellular ROSs were detected by CM-H_2DCFDA through flow cytometry (Fig. 5A). Detectable ROS appeared after 48 hours of treatment with E_2. The production of ROS reached a peak after 72-hour treatment (Fig. 5A and B). Blocking ER (by 4-OHT) and c-Src (by PP2) abolished ROS generation induced by E_2 (Fig. 5C), indicating that both ER and c-Src were upstream signals of ROS. Free-radical scavengers Mn-TBAP, catalase, and sodium formate (SF) that respectively act on superoxide radical (O_2^-), H_2O_2, and hydroxyl radical (OH^-) were used to suppress the production of ROS. Our results suggested that H_2O_2 and OH^- were the major sources of ROS induced by E_2. This conclusion was based on the observation that catalase and sodium formate inhibited E_2 -induced apoptosis, whereas Mn-TBAP was less effective (Fig. 5D). The RNA-seq analysis showed that E_2 did not significantly regulate antioxidant enzymes such as catalases (*CAT*) and superoxide dismutases (*SOD*) in MCF-7:5C cells (data not shown). Our results suggest that E_2 has the potential to damage mitochondria to cause oxidative stress.

c-Src was involved in estrogen-induced endoplasmic reticulum stress in MCF-7:5C cells

Our previous global gene array data show that E_2 activates genes related to endoplasmic reticulum stress in MCF-7:5C cells (25). To relieve stress, sensors of UPRs are activated as initial responses (43). In this study, a significant induction of UPR sensors, inositol-requiring protein 1 alpha (IRE1α) and PERK/eukaryotic translation initiation factor-2α (eIF2α), by E_2

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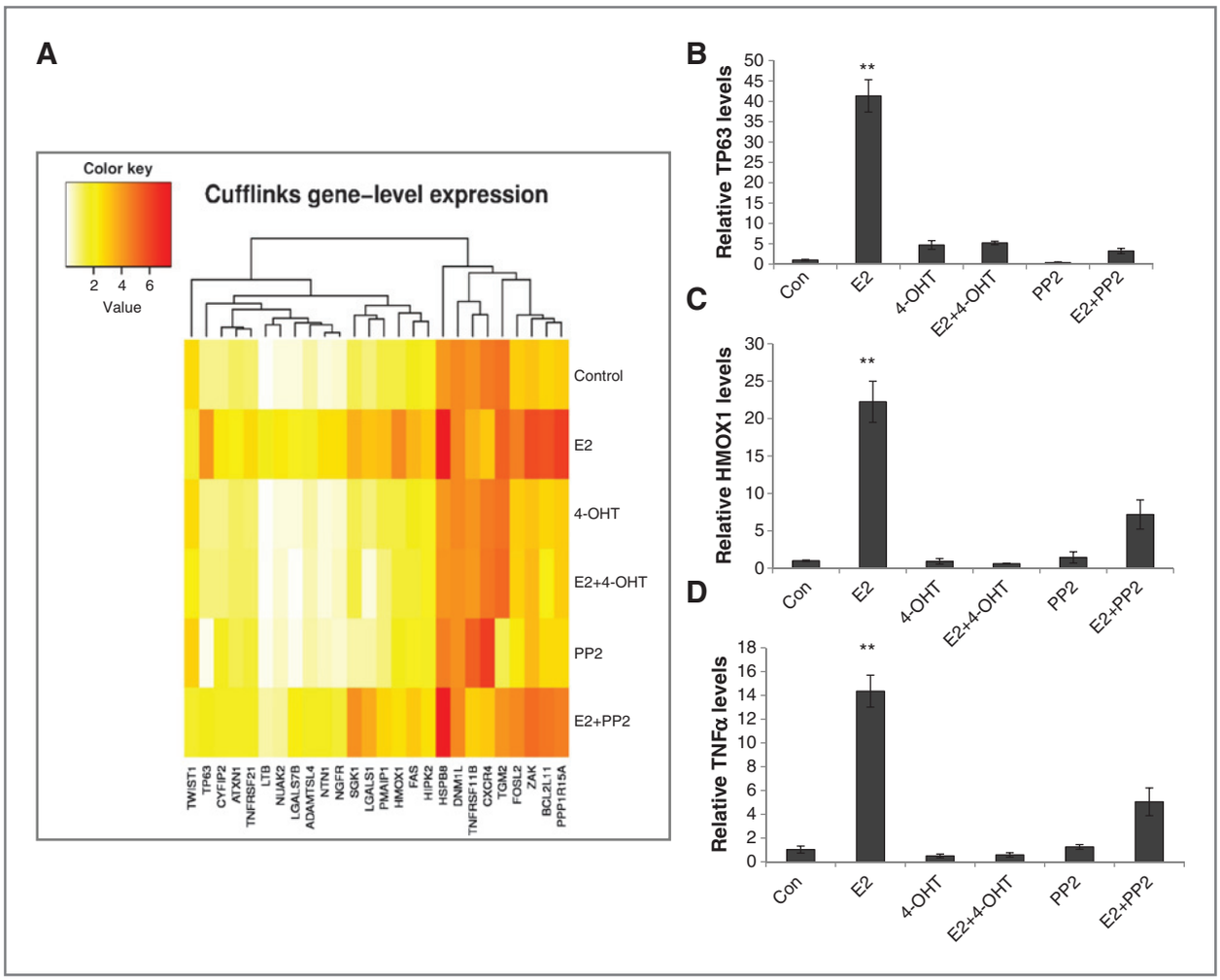

Figure 4. c-Src was involved in the process of triggering apoptosis-related genes by E_2 in MCF-7:5C cells. A, MCF-7:5C cells were treated with vehicle and different compounds respectively as above for 72 hours. Cells were harvested in TRIzol for RNA-seq analysis. B, MCF-7:5C cells were treated with different compounds as above. TP63 mRNA was quantified with quantitative PCR (qPCR). **, $P < 0.001$, compared with control. C, HMOX1 mRNA was quantified with qPCR. **, $P < 0.001$, compared with control. D, TNFα mRNA was quantified with qPCR. **, $P < 0.001$, compared with control.

occurred after 24 hours of treatment and was further increased by prolonging treatment times in MCF-7:5C cells (Fig. 6A). The anti-estrogen 4-OHT completely abolished the response (Fig. 6A). The PERK inhibitor blocked phosphorylation of eIF2α and prevented E_2-induced apoptosis (Fig. 6B and C), confirming that endoplasmic reticulum stress was important in the apoptosis initiated by E_2. Phosphorylated eIF2α closely associates with an important cellular energy sensor, adenosine monophosphate (AMP)-activated protein kinase (AMPK) to regulate protein translation and apoptosis (44). AMPK, which phosphorylates many metabolic enzymes to stimulate catabolic pathways and increases the capacity of cells to produce ATP (45), was significantly activated after 48-hour treatment with E_2 (Fig. 6D). The c-Src inhibitor, PP2, blocked the phosphorylation of eIF2α but not IRE1α induced by E_2 (Fig. 6E). PP2 also prevented the activation of AMPK after E_2 treatment (Fig. 6F). All of these data indicate that c-Src acts as an important transducer in the protein kinase pathways (eIF2α and AMPK) of stress response (Fig. 6E and F) that result in apoptosis.

Discussion

We have previously investigated the inhibitory effects of E_2 on long-term endocrine-resistant breast cancer tumor growth *in vivo* (8–10). And we have confirmed that this therapeutic effect is related with the apoptosis induced by E_2 (20). This scientific discovery has been used in the clinical trials to treat aromatase inhibitor–resistant patients with breast cancer and 30% of patients receive benefit (11). The potential limitation on translational research in the treatment of hormone-responsive breast cancer is that only 4 ER-positive breast cancer cell lines are available to use routinely (46). Only MCF-7 of the 4 produces the phenotype of E_2-induced apoptosis observed clinically (20, 21). The purpose of establishing long-term E_2 deprivation *in vitro* models is to mimic administration of an aromatase inhibitor that reduces levels of circulating estrogen

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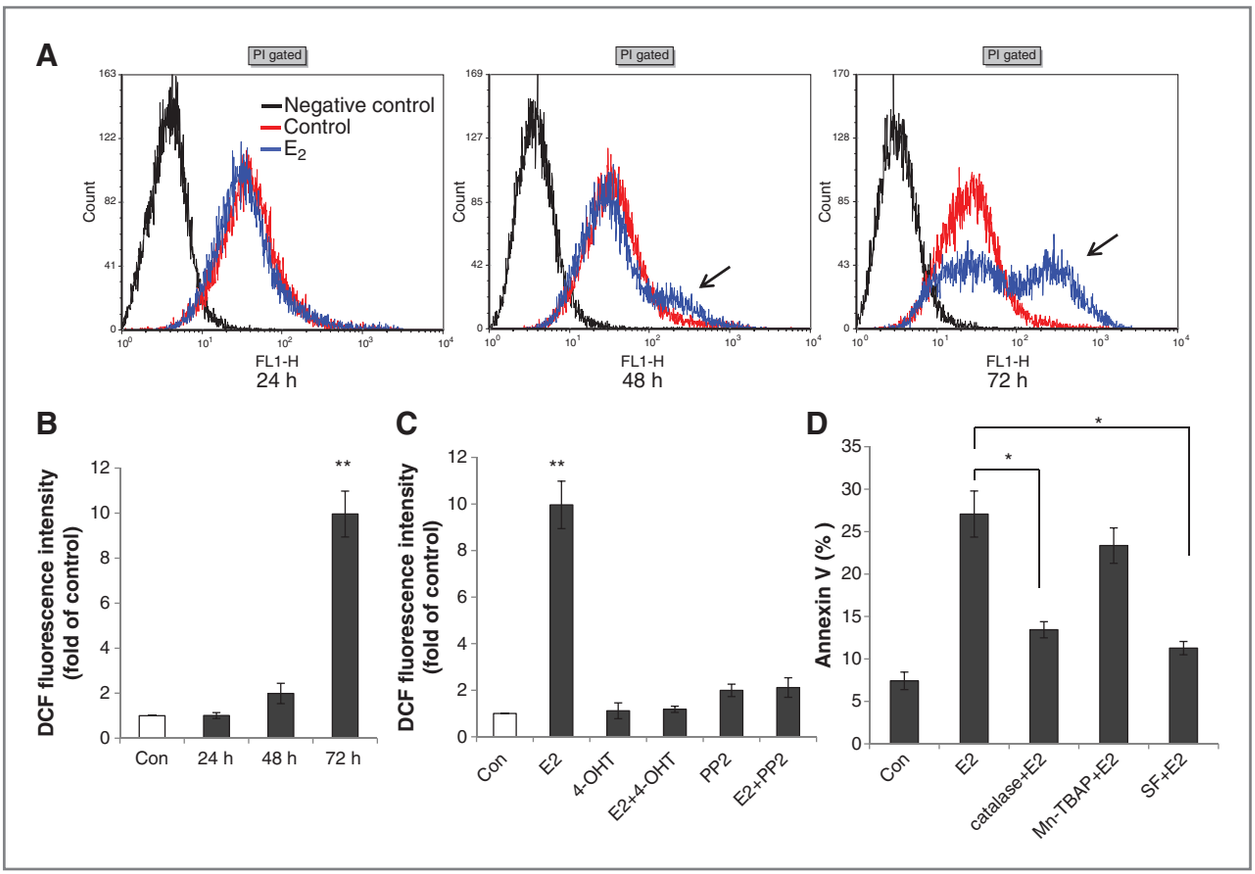

Figure 5. The c-Src inhibitor blocked estrogen-induced oxidative stress in MCF-7:5C cells. A, MCF-7:5C were treated with vehicle and E_2 for different durations. ROS was detected through flow cytometry. B, quantification of ROS production induced by E_2 was compared with control. **, $P < 0.001$, compared with control. C, MCF-7:5C cells were treated with different compounds as above. ROS production was detected through flow cytometry. **, $P < 0.001$, compared with control. D, MCF-7:5C cells were treated with vehicle (0.1% EtOH), E_2 (10^{-9} mol/L), catalase (5,000 U/mL) plus E_2 (10^{-9} mol/L), Mn-TBAP(5×10^{-5} mol/L) plus E_2 (10^{-9} mol/L), sodium formate (2×10^{-3} mol/L) plus E_2 (10^{-9} mol/L) for 72 hours. Apoptosis was detected through Annexin V binding assay. *, $P < 0.05$, compared with E_2-treated group.

in clinical studies (47). After a period of proliferative quiescence lasting a few months, the return of proliferation is similar to the relapses observed 12 to 18 months after primary hormonal therapy in patients. Multiple pathways are involved in the adaptive response to the pressure of E_2 deprivation (48). Although MCF-7 cells grown long-term have been shown to differ substantially in various properties depending upon the number of passages and geographic source of the cell lines, induction of apoptosis by physiologic concentrations of E_2 is the common characteristic of these *in vitro* model systems (20, 21). Nevertheless, how E_2 induces apoptosis is at present unclear. Our new observation (29) that a c-Src inhibitor paradoxically can block E_2-induced apoptosis naturally demands further study. We examined this aspect of c-Src pharmacology to describe fully this phenomenon and gain an insight into the convergence of ER and c-Src pathways for the modulations of an apoptotic trigger in breast cancer. Here, for the first time, we document that c-Src participates in the mediation of stress responses induced by E_2 to widely activate apoptosis-related genes involved in the intrinsic and the extrinsic apoptosis pathways.

The ER is the initial point for E_2 to induce apoptosis, as antiestrogens ICI 182,780 and 4-OHT completely block apoptosis triggered by E_2 (ref. 20 and Supplementary Fig. S3A). Contradictory to the traditional apoptosis mechanism caused by cytotoxic chemotherapy with cell-cycle arrest, E_2-induced apoptotic cells simultaneously undergo proliferation with an increased S-phase of cell cycle resulting in increased cell number despite p53 family members being upregulated (Figs. 1D and 4B). E_2 exerts a dual function on MCF-7:5C cells, with both initial proliferation and the apoptosis. In other words, the initial response of E_2 to stimulate growth is the upregulating of classical transcriptional activity by ER (Fig. 3B) without any detected apoptotic changes in the first 24 hours. Activation of apoptotic genes appeared after 48-hour treatment with E_2 (data not shown) and reached a peak by 72 hours (Fig. 4B–D). Consistently, characteristic apoptosis occurred at 72 hours (Fig. 2A). These data suggest that the higher rate of proliferation by E_2 might activate other pathways to trigger apoptosis. Our data show that E_2 caused endoplasmic reticulum stress, which activated the UPR within 24 hours (Fig. 6A). The initial aim of UPR is to restore normal function of the cell; however, if

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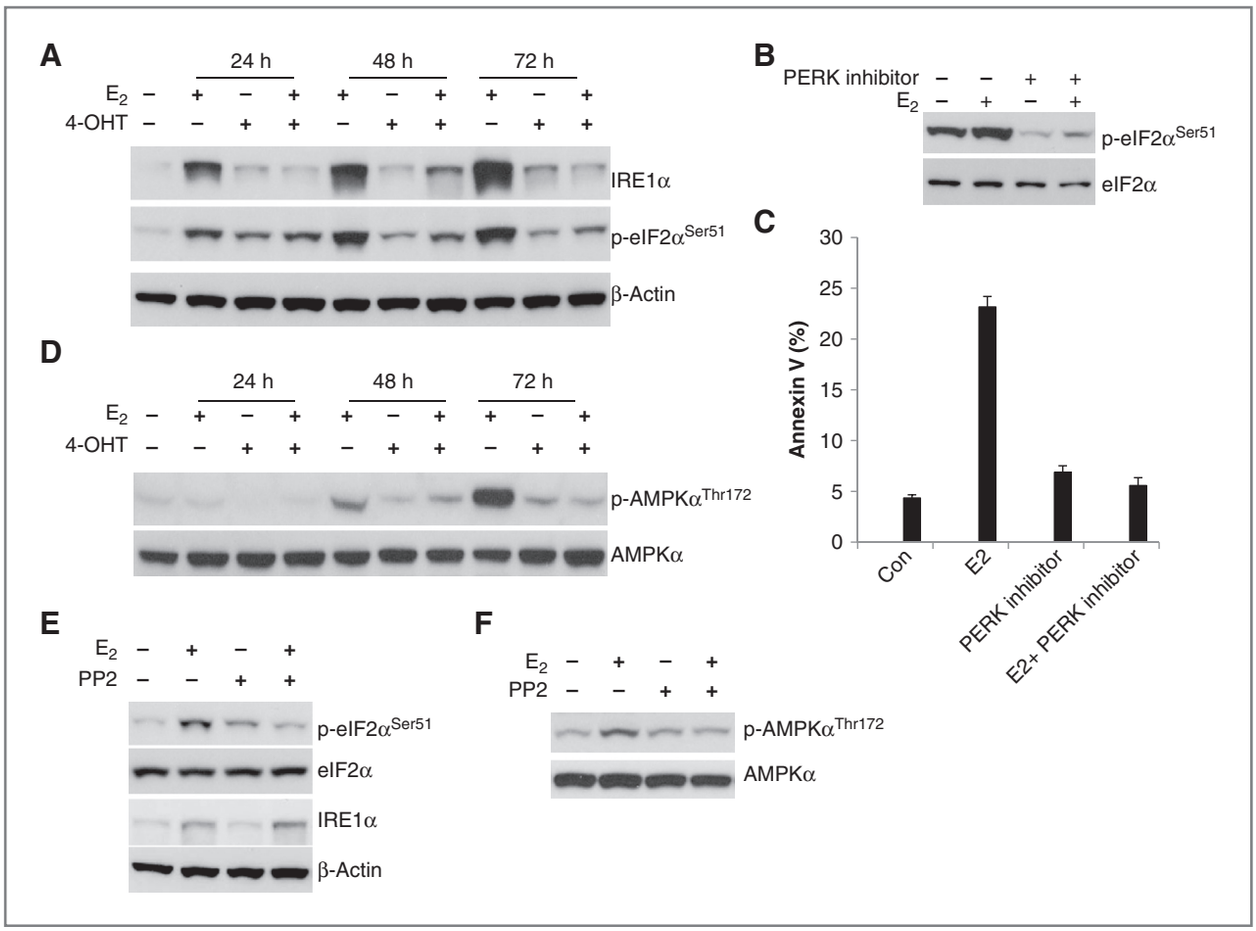

Figure 6. c-Src was involved in estrogen-induced endoplasmic reticulum stress in MCF-7:5C cells. A, MCF-7:5C were treated with E_2 (10^{-9} mol/L) or combined with 4-OHT (10^{-6} mol/L) for different durations. IRE1α and phosphorylated eIF2α were used as indicators of UPR activation. B, MCF-7:5C cells were treated with vehicle (0.1% dimethyl sulfoxide), E_2 (10^{-9} mol/L), PERK inhibitor (1×10^{-5} mol/L), E_2 (10^{-9} mol/L) plus PERK inhibitor (1×10^{-5} mol/L), respectively, for 24 hours. Phosphorylated eIF2α was examined as the downstream of PERK. Total eIF2α was determined for loading control. C, MCF-7:5C cells were treated with E_2 or combined with PERK inhibitor, respectively, for 72 hours. Apoptosis was detected through Annexin V binding assay. D, MCF-7:5C cells were treated with E_2 or combined with 4-OHT as above. Phosphorylated AMPK was examined by immunoblotting. Total AMPK was determined for loading control. E, MCF-7:5C cells were treated with E_2 or combined with PP2 for 24 hours. IRE1α and phosphorylated eIF2α were examined by immunoblotting. Total eIF2α and β-actin were determined for loading controls. F, MCF-7:5C cells were treated with E_2 or combined with PP2 for 48 hours. Phosphorylated AMPK and total AMPK were examined by immunoblotting.

the damage is too severe to repair, the UPR ultimately initiates cell death through activation of the apoptotic pathway (49).

c-Src functioned as an important downstream signal of ER in MCF-7:5C cells, which was activated by E_2 (Fig. 1B, Supplementary Fig. S1A–S1C) and showed multiple levels of association with ER (Figs. 1B and C, 2A, 3A, C, and D). An important finding in this study is that c-Src tyrosine kinase is critical for E_2 -induced apoptosis (Fig. 2A, C, and D). This, therefore, raised the question of the actual role played by c-Src in the process of apoptosis induced by E_2. c-Src mediated PI3K/AKT and MAPK growth pathways by E_2 (Fig. 1C). However, specific inhibitors of PI3K/Akt (LY294002) and MAPK (U0126) could inhibit cell growth but did not prevent E_2-induced apoptosis in MCF-7:5C cells (Supplementary Fig. S7), which imply that MAPK/Akt growth pathways are not directly involved in the apoptosis-induced by E_2. In MCF-7:5C cells, E_2 activated the nongenomic pathway after 10-minute treatment and the c-Src inhibitor blocked the nongenomic pathway (Supplementary Fig. S1A and S1B). Detectable elevation of c-Src phosphorylation appeared after 30-minute treatment with E_2 (Supplementary Fig. S1B). Consistent stimulation of c-Src appeared after 24-hour treatment and gradually increased when extending to 48 hours (Fig. 1B and Supplementary Fig. S1C). All of these data suggest that c-Src activation is a direct effect resulting from E_2. To further explore the function of the nongenomic pathway in the process of E_2-induced apoptosis, EDC was used to treat MCF-7:5C cell, which is very ineffective in stimulating transcription of endogenous E_2 target genes (38). The EDC (10^{-8} mol/L) activated the nongenomic pathway but without capacity to activate genomic pathway and did not induce apoptosis in MCF-7:5C cells (Supplementary Fig. S2). All of these results suggest that the nongenomic pathway does not play a critical role in the E_2-induced apoptosis. Interestingly, the EDC could continuously activate c-Src and Akt but without

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any effect on MAPK after 24-hour treatment (Supplementary Fig. S2E), which may be resulted from enhanced association between ERα and membrane growth factor receptor (48).

In addition, E_2 activated classical ERE activity but the c-Src inhibitor could not block the response (Fig. 3B). Furthermore, the c-Src inhibitor collaborated with E_2 to upregulate endogenous ER target genes pS2 and PR (Fig. 3C and D). All of these results imply that classical ER transcriptional pathways are not directly involved in E_2-induced apoptosis. Similarly, Zhang and colleagues reported that the inhibitory effects of E_2 on cell growth are independent of the classical ERE-regulated transcriptional genes (50). Our global gene array data suggest that E_2 signaling can occur through a nonclassical transcriptional pathway involving the interaction of ER with other transcription factors such as activator protein-1 (AP-1) and Sp1, which may regulate stress responses (25). In the present study, E_2 initiated UPR (Fig. 6A), increased ROS production (Fig. 5A), and widely activated apoptosis-related genes (Fig. 4A). The c-Src was involved in the stress responses and inhibition of c-Src decreased the expression of apoptosis-related genes induced by E_2, which are critical mechanisms for the blockade of c-Src to prevent E_2-induced apoptosis.

Overall, E_2 induces endoplasmic reticulum and mitochondrial stresses in MCF-7:5C cells, which subsequently upregulates apoptosis-related genes to activate intrinsic and extrinsic apoptotic pathways. Unexpectedly, c-Src tyrosine kinase plays a critical role in the stress response induced by E_2. These data clearly raise a concern about the ubiquitous use of c-Src inhibitors to treat patients with advanced aromatase inhibitor–resistant breast cancer, thereby undermining the beneficial effects of E_2-induced apoptosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The views and opinions of the author(s) do not reflect those of the U.S. Army or the Department of Defense.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Fan, F.A. Agboke, X. Zou, R.E. McDaniel, K. Creswell, J.W. Gray, V.C. Jordan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Fan, O.L. Griffith, P. Anur, K. Creswell, J. Katzenellenbogen, J.W. Gray, V.C. Jordan
Writing, review, and/or revision of the manuscript: P. Fan, O.L. Griffith, J. Katzenellenbogen, V.C. Jordan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): O.L. Griffith, R.E. McDaniel, V.C. Jordan
Study supervision: P. Fan, V.C. Jordan
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References

1. Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 2009;69:1243–54.
2. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007;72:7–25.
3. Sabnis G, Schayowitz A, Goloubeva O, Macedo L, Brodie A. Trastuzumab reverses letrozole resistance and amplifies the sensitivity of breast cancer cells to estrogen. Cancer Res 2009;69:1416–28.
4. Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 2007;25:5815–24.
5. Massarweh S, Osborne CK, Creighton CJ, Qin L, Tsimelzon A, Huang S, et al. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. Cancer Res 2008;68:826–33.
6. Sabnis G, Goloubeva O, Jelovac D, Schayowitz A, Brodie A. Inhibition of the phosphatidylinositol 3-kinase/Akt pathway improves response of long-term estrogen-deprived breast cancer xenografts to antiestrogens. Clin Cancer Res 2007;13:2751–7.
7. McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 2002;108:465–74.
8. Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000;6:2028–36.
9. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 2003;95:1597–608.
10. Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, et al. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*. J Natl Cancer Inst 2003;95:1586–97.
11. Ellis MJ, Gao F, Dehdashti F, Jeff DB, Marcom PK, Carey LA, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 2009;302:774–80.
12. Anderson GL, Chlebowski RT, Aragaki AK, Kuller LH, Manson JE, Gass M, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. Lancet Oncol 2012;13:476–86.
13. Obiorah I, Jordan VC. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. Menopause 2013;20:372–82.
14. Thomas SM, Brugge JS. Cellular functions regulated by Src family kinases. Ann Rev Cell Dev Biol 1997;13:513–609.
15. Pascoe D, Oursler MJ. The Src signaling pathway regulates osteoclast lysosomal enzyme secretion and is rapidly modulated by estrogen. J Bone Miner Res 2001;16:1028–36.
16. Jallal H, Valentino ML, Chen G, Boschelli F, Ali S, Rabbani SA. A Src/Abl Kinase inhibitor, SKI-606, blocks breast cancer invasion, growth, and metastasis *in vitro* and *in vivo*. Cancer Res 2007;67:1580–8.
17. Fan P, Wang J, Santen RJ, Yue W. Long-term treatment with tamoxifen facilitates translocation of estrogen receptor alpha out of the nucleus and enhances its interaction with EGFR in MCF-7 breast cancer cells. Cancer Res 2007;67:1352–60.

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18. Hiscox S, Jordan NJ, Smith C, James M, Morgan L, Taylor KM, et al. Dual targeting of SRC and ER prevents acquired antihormone resistance in breast cancer cells. Breast Cancer Res Treat 2009;115:57–67.
19. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309–12.
20. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 2005;97:1746–59.
21. Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 beta-estradiol. J Natl Cancer Inst 2001;93:1714–23.
22. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science 2004;305:626–9.
23. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. Cell 2005;120:483–95.
24. Ferri KF, Kroemer G. Organelle-specific initiation of cell death pathways. Nat Cell Biol 2001;3:E255–63.
25. Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, et al. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci USA 2011;108:18879–86.
26. Chakrabarti A, Chen AW, Varner JD. A review of the mammalian unfolded protein response. Biotechnol Bioeng 2011;108:2777–93.
27. Tsai YC, Weissman AM. The unfolded protein response, degradation from endoplasmic reticulum and cancer. Genes Cancer 2010;1:764–78.
28. Roberts CG, Gurisik E, Biden TJ, Sutherland RL, Butt AJ. Synergistic cytotoxicity between tamoxifen and the plant toxin persin in human breast cancer cells is dependent on Bim expression and mediated by modulation of ceramide metabolism. Mol Cancer Ther 2007;6:2777–85.
29. Fan P, McDaniel RE, Kim HR, Clagett D, Haddad B, Jordan VC. Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines. Eur J Cancer 2012;48:3488–98.
30. Tobaben S, Grohm J, Seiler A, Conrad M, Plesnila N, Culmsee C. Bid-mediated mitochondrial damage is a key mechanism in glutamate-induced oxidative stress and AIF-dependent cell death in immortalized HT-22 hippocampal neurons. Cell Death Differ 2011;18:282–92.
31. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-seq. Bioinformatics 2009;25:1105–11.
32. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, Baren MJ, et al. Transcript assembly and quantification by RNA-seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 2010;28:511–5.
33. Griffith M, Griffith OL, Mwenifumbo J, Goya R, Morrissy AS, Morin RD, et al. Alternative expression analysis by RNA sequencing. Nat Methods 2010;7:843–7.
34. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nat Protoc 2009;4:44–57.
35. Fan P, Yue W, Wang JP, Aiyar S, Li Y, Kim TH, et al. Mechanisms of resistance to structurally diverse antiestrogens differ under premenopausal and postmenopausal conditions: evidence from *in vitro* breast cancer cell models. Endocrinology 2009;150:2036–45.
36. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, et al. Nongenotropic, sex-onspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 2001;104:719–30.
37. Shupnik MA. Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. Oncogene 2004;23:7979–89.
38. Harrington WR, Kim SH, Funk CC, Madak-Erdogan Z, Schiff R, Katzenellenbogen JA, et al. Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. Mol Endocrinol 2006;20:491–502.
39. Chu I, Arnaout A, Loiseau S, Sun J, Seth A, McMahon C, et al. Src promotes estrogen-dependent estrogen receptor alpha proteolysis in human breast cancer. J Clin Invest 2007;117:2205–15.
40. Araujo JA, Zhang M, Yin F. Heme oxygenase-1, oxidation, inflammation, and atherosclerosis. Front Pharmacol 2012;3:119.
41. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, et al. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 2000;404:787–90.
42. Matsuda S, Umeda M, Uchida H, Kato H, Araki T. Alterations of oxidative stress markers and apoptosis markers in the striatum after transient focal cerebral ischemia in rats. J Neural Transm 2009;116:395–404.
43. Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell 2003;11:619–33.
44. Dagon Y, Avraham Y, Berry EM. AMPK activation regulates apoptosis, adipogenesis, and lipolysis by eIF2*a* in adipocytes. Biochem Biophys Res Commun 2006;340:43–7.
45. Birnbaum MJ. Activating AMP-activated protein kinase without AMP. Mol Cell 2005;19:289–90.
46. Sweeney EE, McDaniel RE, Maximov PY, Fan P, Jordan VC. Models and mechanisms of acquired antihormone resistance in breast cancer: significant clinical progress despite limitations. Horm Mol Biol Clin Investig 2012;9:143–63.
47. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 1992;90:77–86.
48. Santen RJ, Song RX, Zhang Z, Kumar R, Jeng MH, Masamura S, et al. Adaptive hypersensitivity to estrogen: Mechanisms and clinical relevance to aromatase inhibitor therapy in breast cancer treatment. J Steroid Biochem Mol Biol 2005;95:155–65.
49. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 2000;403:98–103.
50. Zhang Y, Zhao H, Asztalos S, Chisamore M, Sitabkhan Y, Tonetti DA. Estradiol-induced regression in T47D:A18/PKCalpha tumors requires the estrogen receptor and interaction with the extracellular matrix. Mol Cancer Res 2009;7:498–510.

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Prolactin-Induced Protein (PIP) Regulates Proliferation of Luminal A Type Breast Cancer Cells in an Estrogen-Independent Manner

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Abstract

Prolactin-induced Protein (PIP), an aspartyl protease unessential for normal mammalian cell function, is required for the proliferation and invasion of some breast cancer (BCa) cell types. Because PIP expression is particularly high in the Luminal A BCa subtype, we investigated the roles of PIP in the related T47D BCa cell line. Nucleic acid and antibody arrays were employed to screen effects of PIP silencing on global gene expression and activation of receptor tyrosine kinases (RTKs), respectively. Expression of PIP-stimulated genes, as defined in the T47D cell culture model, was well correlated with the expression of PIP itself across a cohort of 557 mRNA profiles of diverse BCa tumors, and bioinformatics analysis revealed cJUN and cMYC as major nodes in the PIP-dependent gene network. Among 71 RTKs tested, PIP silencing resulted in decreased phosphorylation of focal adhesion kinase (FAK), ephrin B3 (EphB3), FYN, and hemopoietic cell kinase (HCK). Ablation of PIP also abrogated serum-induced activation of the downstream serine/threonine kinases AKT, ERK1/2, and JNK1. Consistent with these results, PIP-depleted cells exhibited defects in adhesion to fibronectin, cytoskeletal stress fiber assembly and protein secretion. In addition, PIP silencing abrogated the mitogenic response of T47D BCa cells to estradiol (E2). The dependence of BCa cell proliferation was unrelated, however, to estrogen signaling because: 1) PIP silencing did not affect the transcriptional response of estrogen target genes to hormone treatment, and 2) PIP was required for the proliferation of tamoxifen-resistant BCa cells. Pharmacological inhibition of PIP may therefore serve the bases for both augmentation of existing therapies for hormone-dependent tumors and the development of novel therapeutic approaches for hormone-resistant BCa.

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Introduction

Prolactin-induced Protein (PIP), a.k.a. serum actin-binding protein (SABP) and gross cystic fluid protein (GCDFP)-15, is a ~15 KDa glycoprotein expressed by a majority of breast cancer (BCa) tumors [1]. Its expression is particularly high in the luminal A and androgen receptor (AR)-positive HER2-enriched breast cancer subtypes [2,3]. PIP is also biosynthesized and secreted by a number of normal apocrine cell types that produce milk, seminal fluid, tear, and saliva [1]. In addition to prolactin, PIP is induced by androgens, growth hormone and glucocorticoids [4,5]. In T47D BCa cells, 5α-dihydrotestosterone (DHT) at physiological concentrations was most potent inducer, increasing PIP expression by >12-fold [4,6,7]. Furthermore, immunohistochemical staining of BCa tumors suggested a strong correlation between the expression levels of PIP and the androgen receptor (AR), as well as between PIP and prostate-specific antigen (PSA), a classical AR-regulated gene [2]. Hormone stimulated expression of PIP requires Runx2, a pro-metastatic transcription factor. Co-recruitment of AR and Runx2 to an enhancer located ~11 Kb upstream of the PIP transcription start site [8] and the physical interaction between these two transcription factors [9], likely mediate synergistic stimulation of PIP expression. In turn, PIP formed a feed-forward loop by enhancing AR signaling [8]. Recently, an additional positive feedback loop was identified where PIP was required for the recruitment of CREB1 to the proximity of the PIP transcription start site [3].

Despite widespread expression, the function of PIP in both normal and cancer cells remains obscure. PIP deficient mice are essentially normal indicating that its function under physiological conditions is either non-essential or complimented by other protein/s. In contrast to normal cells, treatment of various human BCa cell lines with purified PIP enhanced their proliferation [10] and PIP silencing in both ERa-positive and ERa-negative BCa cell lines inhibited cell proliferation as well as invasion through an artificial extracellular matrix [3,8]. These studies indicate that PIP

acquires an essential function during cellular transformation. Potentially related to this function is its aspartyl protease activity, which was demonstrated using purified PIP and fibronectin as the substrate. The resultant fibronectin fragments bound integrin beta-1 receptors and activated signaling pathways related to BCa cell proliferation and invasion [3,11].

In pursuit of PIP-dependent signaling pathways that regulate BCa cell proliferation, we employed PIP knock down and high throughput mRNA profiling as well as antibody arrays to identify gene networks and receptor tyrosine kinases (RTKs) that execute PIP's function. The results suggest that PIP is required for the activation of specific RTKs, including FAK. Accordingly, we demonstrate a role of PIP in fibronectin adhesion and in cytoskeleton dynamics. Finally, we demonstrate requirement for PIP for the proliferation of tamoxifen-resistant BCa cells, suggesting that PIP may be targeted for the development of novel therapeutic approaches to treat BCa patients who do not respond to hormonal therapy.

Methods

Cell culture

ER-positive T47D and ZR-75 and ER-negative MDA-MB-453 cells were from American Type Culture Collection (ATCC). T47D cells were maintained in RPMI-1640, and MDA-MB-453 and ZR-75 cell lines in DMEM medium, both supplemented with 10% fetal bovine serum from Clontech, CA. Before hormone treatment cells were washed three times with PBS and maintained for 48 hours in phenol-red free growth medium supplemented with 10% charcoal-stripped serum (CSS). Tamoxifen resistant T47D cells ($T47D^{tamR}$) were derived earlier by long-term growth of cells in 1 μM 4- hydroxytamoxifen (4-OHT) [12,13]. The growth medium for $T47D^{tamR}$ cells was further supplemented with 1 μM 4-OHT, 10 mM non-essential amino acids, 200 mM L-glutamin, and 10 microgram/ml insulin.

Antibodies and Reagents

The anti-PIP antibody (ab 62363) was purchased from abcam Inc., Cambridge, MA. The antibodies for detecting total and phosphorylated form of AKT (9272, 9275), ERK1/2 (4695, 9101), and HGFR (3148, 13D11) were purchased from Cell Signaling Technology, Inc., Danvers, MA. Total JNK1/2 levels were detected by JNK antibody (sc-572) from Santa Cruz (Biotechnology Inc., Santa Cruz, CA. Levels of [$pThr^{183}$/Tyr^{185}]-JNK1/2 were assessed using phosphor-SAPK/JNK (4668) antibody from Cell Signaling Technology, Inc. The Anti-FAK antibody, clone 4.47 and PhosphoDetect™ Anti-FAK ($pTyr^{397}$) were purchased from Millipore, Billerica, MA. Antibodies detecting total Myc and [$pThr^{58}$/$pSer^{62}$]-Myc were from Cell Signaling Technology, Inc. The mouse monoclonal anti- β -tubulin antibody, developed by Dr. Charles Walsh, was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and The University of Iowa, Department of Biological Sciences, Iowa City, USA. The hormones 17β-estradiol (E2) and 4- OHT, and insulin were purchased from from Sigma, St Louis, MO. E2 and tamoxifen were used at a concentration of 30 nM and 1 microM, respectively. Equal volume of ethanol was used as vehicle control. The growth media RPMI-1640 and DMEM, as well as the supplemental non-essential amino acids and L-glutamine were purchased from Gibco, Grand Island, NY. Hygromycin B was purchased from Invitrogen, Carlsbad, CA, USA, and added to the growth medium at 50 μg/ml. Doxycycline from Calbiochem, La Jolla, CA was used at 250 ng/ml unless otherwise indicated. An equal volume of distilled water was used as vehicle control. Puromycin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma, St Louis, MO. Phalloidin powder for the visualization of actin stress fiber was purchased from Enzo Life Sciences, Inc., Farmingdale, NY. A stock solution was prepared by dissolving 0.1 mg of phalloidin powder in 1 ml of dimethyl sulfoxide (DMSO), and a working solution was prepared by a further 1:2000 dilution in PBS.

Plasmids

The dox-inducible lentiviral plasmids were based on the pSLIK (single lentivector for inducible knockdown) vector [23]. DNA sequences encoding shRNAs for PIP were designed using the RNAiCodex program (http://katahdin.cshl.org/html/scripts/resources.pl). Oligonucleotides used for cloning are listed in Table S1. The shRNA-coding oligonucleotides were initially cloned into the lentiviral entry vector pEN_TmiRc3 (ATCC® catalog: MBA-248), and the resulting plasmid was recombined using Gateway® LR Clonase® II enzyme mix (Invitrogen) with the pSLIK destination vector carrying a hygromycin resistance gene (ATCC® catalog: MBA-237). Constitutively expressing shRNA lentiviral plasmids targeting either a non-specific sequence or distinct PIP-specific sequences were purchased from Sigma (Table S1). The constitutively expressed shPIP/121 and shPIP/214 hairpin RNAs target the nucleotide sequences 121–140 and 214–230 within the PIP open reading frame (ORF), respectively. Dox-inducible shRNA targeted either nucleotides 263–283 of the PIP ORF or nucleotides 32–52 of the 3′UTR, the latter used in the co-culture assays only (Table S1).

RNA extraction, RT-qPCR, and analysis of global gene expression

RNA was extracted from cells using Aurum Total RNA kit from Bio-Rad Laboratories, Inc., Hercules, CA following the manufacturer's recommendations. For cDNA synthesis, 1 microgram of RNA was processed using qScript™ cDNA SuperMix as per the manufacturer's instructions (Quanta BioSciences, Inc., Gaithersburg, MD). The cDNA was diluted 10-fold with distilled water and subjected to real-time PCR amplification using Maxima® SYBR Green/Fluorescein qPCR Master Mix (2×) from Fermentas Inc., Glen Burnie, MD and CFX96™ RT-PCR system from Bio-Rad, Hercules, CA. The sequences of primers used for real-time PCR amplification are listed in Table S1. Gene expression profiling was performed using the BeadChip HumanHT-12 v4 Expression kit from Illumina®, which contains 47,231 gene-probes (Illumina® Inc., San Diego, CA). The raw signal intensities were imported and analyzed using the GenomeStudio® data software. After background subtraction and normalization, the signal intensity values were exported to the Partek® genomics expression analysis suite using "Partek's Report Plug-in" option in the GenomeStudio® software. Differentially expressed genes in the dox- *versus* vehicle-treated samples were identified using the "gene expression" workflow in the Partek® software. The differentially expressed probes were further investigated using the Ingenuity Pathways Analysis package (IPA™; http://www.ingenuity.com) to identify the association of differentially expressed genes with "disease and disorder" and "molecular and cellular functions" categories. Right-tailed Fisher's exact test as implemented in the IPA software was used to calculate a p-value for the probability of each network to be enriched for PIP-regulated genes due to chance alone. The microarray data has been deposited in the GEO database with the accession code GSE41894.

Table 1. Deduced Molecular and Cellular Functions of PIP in T47D/shPIP^{dox} cells.

Function	p-value	# Molecules
Cellular Growth and Proliferation	$1.3E^{-10}$ $-1.3E^{-02}$	98
Cell Cycle	$4.5E^{-08}$ $-1.3E^{-02}$	51
DNA Replication, Recombination, and Repair	$1.6E^{-07}$ $-1.3E^{-02}$	39

The 293 genes repressed by ≥1.5 fold after one day of dox-induced PIP knockdown were analyzed using the IPA software package for enriched Molecular and Cellular Functions.
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Lentivirus production

For packaging, the lentiviral expression plasmids were co-transfected by the calcium chloride method into HEK293T cells along with helper plasmids pMD.G1 and pCMVΔR8.91 [24,25]. Culture media containing viral particles were harvested after 48–72 hours and used for transduction of the indicated cells in the presence of 8 μg/ml Polybrene (Millipore Corp., MA, USA). The transduced cells were selected with either 50 μg/ml of Hygromycin or 3 microgram/mL of Puromycin.

Cell proliferation assay

Cell proliferation was assessed using either MTT- or luciferase-based assays. For MTT assays, cells were incubated at 37°C with 0.5 mg/mL of MTT dissolved in PBS for 2 hours. Cells were then lysed using DMSO and the development of color was quantified at 595 nM using Victor$_3$V^{TM} from PerkinElmer, Shelton, CT, USA. For luciferase assays cells were first transduced with lentiviral particles containing a constitutive green fluorescent protein (GFP)/luciferase cassette to facilitate their isolation using fluorescence activated cell sorting and to later assess their proliferation based on luciferase activity. Approximately 5,000 fluorescently-sorted cells were plated in a 24 well tissue culture plates in the presence of dox or an equal volume of water as vehicle control. Samples were harvested every 48 hours by lysing the cells in 200 microlitre of passive lysis buffer purchased from Promega, Madison, WI, and were stored at −80°C until quantification of luciferase activity using Victor$_3$V^{TM}.

Receptor Tyrosine Kinase Screen

We used the Human RTK Phosphorylation antibody array 1 kit (RayBiotech, Inc., Norcross, GA), which facilitates simultaneous detection of the phosphorylation status of 71 major receptor tyrosine kinases. T47D cells were transduced with lentiviruses encoding shRNA specific for either PIP (sh121) or a non-genomic DNA sequence (Table S1). Preparation of cell lysates as well as hybridization to the membrane containing the dedicated spots for 71 receptor tyrosine kinases and controls was conducted as per the manufacturer's recommendations. Briefly, 250 μg of cell lysate proteins was diluted in 1.2 ml blocking buffer and incubated for 2 hours at 4°C with gentle shaking. The membranes were washed and further incubated with biotin-conjugated antibodies that bind phosphotyrosine residues. To enable detection of phosphotyrosine-bound antibodies, the membranes were washed and incubated with biotinylated antiphosphotyrosine antibodies. Washed membranes were subjected to chemiluminescence-based imaging using the Western Lightning^{TM} Plus-ECL kit from PerkinElmer Inc, Waltham, MA and then exposed to Clear Blue X-ray Film from Bioland Scientific LLC, Paramount, CA.

Cell adhesion assay

The T47D/shNS/LUC, T47D/shPIP/121/LUC and T47D/shPIP/214/LUC cells encoding either non-specific (shNS) or PIP-specific (shPIP shPIP/121, shPIP/214) shRNAs, respectively, and a constitutively active luciferase gene were cultured for 2 days in the presence or absence of dox (250 ng/mL). Quadruplicates of about 10,000 cells were plated in a 24 well plate pre-coated for 1 hour at 37°C with 10 microgram/ml of either fibronectin or bovine serum albumin as background control (both from Sigma). After 1 hour of incubation, unbound cells were washed three times with PBS and the relative numbers of bound cells were assessed based on luciferase assays. The arginine-glycine-aspartate (GRGDSP) and arginine-glycine-glutamic acid (GRGESP) peptides were obtained from Anaspec, Inc., San Jose, CA.

Visualization of stress fibers by phalloidin staining

To visualize stress fibers, cells were grown on fibronectin-coated slides for 48 hours in the presence or absence of dox. Cells were washed three times with phosphate buffered saline and incubated for one hour in cell culture incubator in growth medium without serum. Cells were fixed with chilled 0.5% paraformaldehyde solution, permeabilized with chilled 0.1% Triton X-100 in PBS, and incubated in phalloidin solution for 1 hour at room temperature. Stress fibers were visualized using a Zeiss LSM 510 inverted confocal microscope and the Zeiss LSM Image Browser software (Carl Zeiss Inc., Jena, Germany) for image stacking.

Cell extract preparation and western blot analysis

Whole cell extracts were prepared by lysing 1×10^5–2×10^5 cells in 200 microlitre of lysis buffer [100 mM Tris (pH 7.4), 500 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% Nonidet® P-40] supplemented with Complete^{TM} protease inhibitor mix (Roche Diagnostics, Indianapolis, IN). Thirty microgram protein was mixed with an equal volume of 2× Laemmli buffer followed by SDS-PAGE. Electrophoresed proteins were transferred to Amersham Hybond^{TM}-P PVDF membranes (GE Healthcare, Piscataway, NJ), and detected using specific antibodies and the Western Lightning^{TM} Plus-ECL kit.

Results

PIP is preferentially expressed in breast cancer cells of the Luminal A subtype and is required for their proliferation independently of its secretion

PIP is expressed by a majority of BCa tumors and serves as a marker for disease progression [1]. Breast tumors are heterogeneous in nature with at least five well-recognized intrinsic molecular subtypes that differ in clinical progression and drug responsiveness: basal-like, HER2-enriched, luminal A, luminal B, and normal-like [14,15,16]. In order to select a relevant BCa cell line for this study, we assessed its expression with respect to tumor subtypes using mRNA profiles of 557 breast tumors compiled from the three microarray datasets GSE2034, GSE7390, and GSE11121 [8,17,18,19]. PIP mRNA was most highly expressed in the luminal A subtype, followed by HER2-enriched and normal-like tumors, with the least expression observed in basal-like tumors (Figure 1A). Consistent with these results, western blot analysis (Figure 1B) readily detected PIP expression in the T47D and MDA-MB-453 cell lines that represent the luminal A and HER2 subtypes, respectively [20]. In contrast, PIP expression was very low or absent in the ZR-75 and MDA-MB-231 cell lines (Figure 1B) that represent the luminal B and basal subtypes, respectively [21]). In subsequent experiments, we therefore

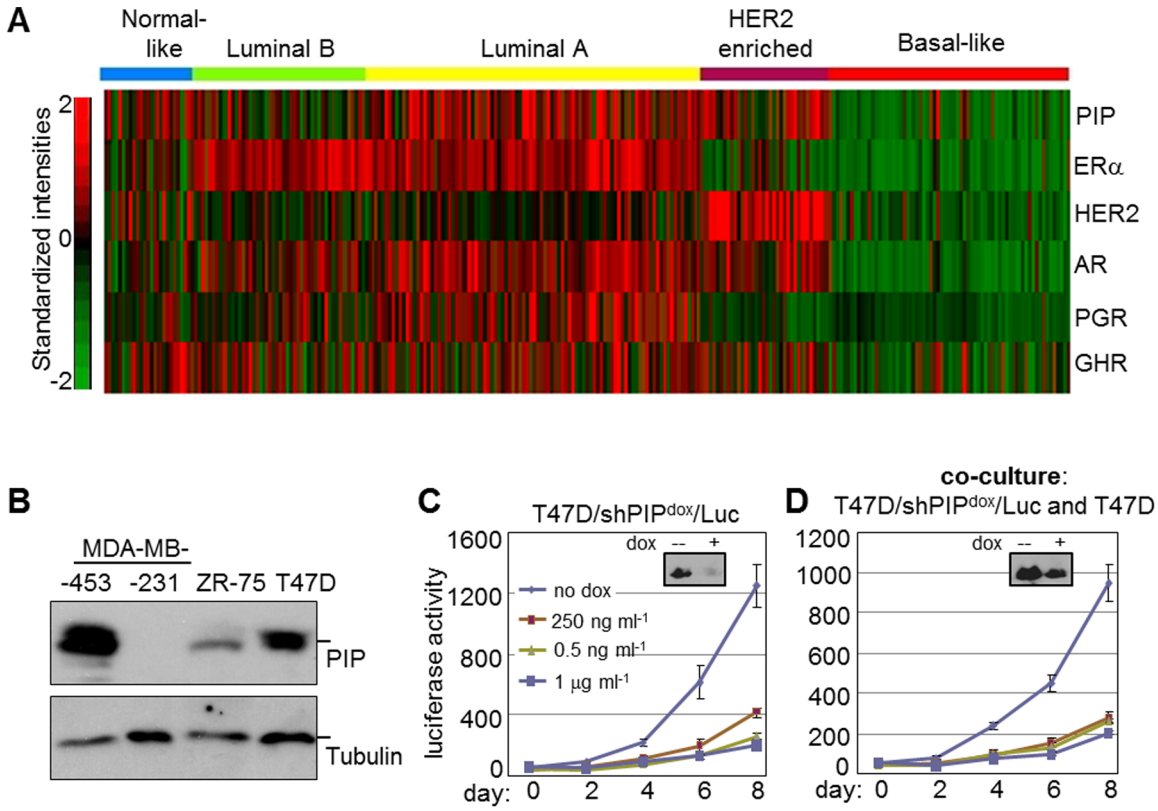

Figure 1. Relative expression of PIP in intrinsic breast cancer subtypes and requirement of intracellular PIP for cell proliferation. A, Heat map demonstrating the mRNA expression levels of PIP and various hormone receptors known to regulate its expression in a heterogeneous cohort of 557 BCa patients. The patient cohort was compiled from the publicly available GEO datasets GSE2034, GSE2603 and GSE12276 and was described previously [8]. The intrinsic subtype of each tumor was determined according to the PAM-50 algorithm [36]. Expression values for the indicated genes were derived from the signal intensities associated with the following affymetrix probesets: 206509_at (PIP), 205225_at (ESR1) 216836_s_at (HER2), 211110_s_at (AR), 208305_at (PGR) and 205498_at (GHR). **B**, Immunoblot analysis of PIP in the ER-alpha positive BCa cell lines MDA-MB-453, ZR-75 and T47D and in the ER–negative MDA-MB-231 BCa cell line. Tubulin was analyzed as control. **C–D**, Luciferase assays were performed at the indicated days after plating to assess the proliferation of T47D/PIP^{dox}/LUC cells cultured in serum-supplemented medium either alone (*C*) or together with naïve T47D cells (*D*). The co-cultures were started with roughly equal number (~5,000) of T47D/shPIP^{dox}/LUC and naïve T47D cells. Doxycycline (dox) was added at the time of plating at the indicated concentrations. Insets in *C* and *D* show PIP levels by immunoblotting after 4 days of treatment with either vehicle or 250 ng/mL dox.
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pursued the role of PIP in BCa cells by knocking it down in the luminal A-like T47D cell line.

We recently reported that PIP was required for the proliferation of breast cancer cells [8]. Although PIP is best known as a secreted protein, follow-up experiments showed that PIP-containing conditioned medium from naïve T47D cells did not rescue the growth arrest imposed by PIP knockdown (data not shown). This suggested that PIP was likely required for a critical intracellular cell cycle regulatory function(s). To directly address this notion, we measured the proliferation of T47D/shPIP^{dox}/LUC cells cultured either alone or together with naïve T47D cells (Figure 1C, D). The T47D/shPIP^{dox}/LUC cells are transduced with dox-inducible shRNA specifically targeting the PIP ORF [8]; Table S1). In addition, the cells are marked with a constitutively active luciferase gene, so that their growth can be monitored by following accumulation of luciferase activity per well over time. As shown in Figure 1C, dox treatment of the T47D/shPIP^{dox}/LUC cell cultures resulted in PIP silencing and subsequent dose-dependent inhibition of cell proliferation. In contrast to this simple culture system, when the T47D/shPIP^{dox}/LUC cells are co-cultured with naïve T47D cells (Figure 1D), the latter continue to secrete PIP even in the presence of dox, and could act in a paracrine manner to rescue proliferation of the dox-treated T47D/shPIP^{dox}/LUC cells. However, the dox-induced growth inhibition was equally apparent with and without co-cultured naive T47D cells, suggest that PIP was required for a novel intracellular function independent of its secretion.

Analysis of global gene expression indicates a role for PIP in stimulating a highly connected JUN/MYC-centered transcriptome

In pursuit of PIP-regulated gene networks mediating its function(s) in BCa cells, we initially investigated changes in global gene expression in response to dox-mediated knockdown of PIP in T47D/shPIP^{dox} cells. A time course experiment demonstrated that dox treatment knocked down PIP mRNA by 70% and 80% at the 24 h and 48 h time points, respectively (Figure 2A). We note that PIP mRNA expression was also responsive to serum, but the serum response and effects of the PIP shRNA appear unrelated (Figure S4 in File S1). Because dox did not significantly inhibit PIP expression at the earlier 6 h and 12 h time points, we reasoned that mRNA profiling of cells after 24 h and 48 h of dox treatment would disclose primary responses to PIP knockdown. Cells were treated and their mRNAs analyzed in biological triplicates (a total

of 12 samples) using Illumina's HumanHT-12 v4 BeadChips. Dox treatment differentially regulated the expression of 1,356 genes by ≥1.5-fold (690 repressed, 666 induced) at the 24 h or 48 h time points in a statistically significant manner ($p<0.04$; Table S2). RT-qPCR analysis of five randomly selected genes conformed to the microarray data (data not shown). Unsupervised hierarchical clustering of the most differentially regulated genes showing ≥2-fold effects at either 24 or 48 hours resulted in a clear separation between the dox-treated and control samples (Figure S1 in File S1). In general, the variation among the biological triplicates was small, and changes observed after 24 hours of treatment were maintained or intensified by 48 hours of treatment.

To examine the clinical relevance of our *in vitro* results, we interrogated publicly available RNA expression datasets for correlation between expression of *PIP* and that of genes that were down- or up-regulated by PIP silencing in the T47D cell culture model. We defined a PIP-activated metagene as the average normalized expression of genes that were most downregulated (≥2-fold; 41 genes, Figure S1 in File S1) after dox-mediated PIP knockdown in T47D/shPIP^{dox} cells. A PIP-repressed metagene was similarly defined as the average normalized expression of 41 genes most upregulated after dox-mediated PIP knockdown in T47D/shPIP^{dox} cells. A scatter plot of PIP expression *versus* that of the PIP-activated metagene across 557 BCa tumors revealed a highly significant positive correlation ($r^2 = 0.4$; $p<0.0001$), suggesting that in a clinical setting, PIP most likely regulates genes comprising the PIP-activated metagene as defined in the T47D/shPIP^{dox} culture system (Figure 2B). In contrast, there was no significant correlation across the BCa tumors between expression of *PIP* and that of the PIP-repressed metagene (Figure 2C), suggesting that changes in the expression of these genes may represent secondary *in vitro* effects of PIP knockdown that are irrelevant *in vivo*. We therefore went on to investigate the potential significance of the PIP-activated metagene.

We employed the Ingenuity Pathway Analysis (IPA^{TM}) software package to gain insight into the most likely biological roles of the PIP-activated metagene. The "IPA Pathways and Path Designer" platform visualizes connections between differentially regulated genes based on published articles that document functional interactions between genes. Analysis of the genes down-regulated by PIP knockdown revealed an intricate network with cMYC and cJUN, master transcriptional regulators of cell proliferation, forming two central hubs (Figure 2D). The high connectivity of the PIP-activated metagene contrasts the low connectivity between genes comprising the PIP-repressed metagene (Figure S2 in File S1), and suggests that the PIP-activated genes represent a network(s) with a well-established function(s). To identify molecular events that occur immediately after PIP knockdown we used a set of 293 genes that were down-regulated by ≥1.5-fold with high statistical significance ($p<0.04$) after 24 hours of dox treatment (Table S2). IPA analysis of this gene-set showed that "cellular growth and proliferation", "cell cycle" and "DNA replication, recombination, and repair" were the molecular and cellular functions most significantly associated with PIP ($p<1.3E^{-02}$; Table 1). In particular, the most PIP-responsive genes encoding nuclear proteins (Tables 2, S3) are clearly enriched for such with well established roles in promoting cell proliferation, including cMYC, cJUN, E2F2, MYB and MCM5 [22,23,24,25,26].

PIP is required for the activation of major receptor tyrosine kinases (RTKs) and their downstream kinase effectors AKT and ERK1/2

The PIP-regulated, highly connected gene network (Figure 2D) included mRNAs that encode not only transcription factors such as cMYC and cJUN, but also secreted ligands such as TGF-beta-3 and CXCL12, as well as secondary messengers such as Janus kinase 1 (JAK1) and Rho family GTPase 3 (RND3). Plausibly, PIP affected expression of genes encoding ligands, kinases and their regulatory proteins, ultimately switching on major signaling cascades that control cell proliferation. Because receptor tyrosine kinases (RTKs) are excellent candidates for such major switches, we screened T47D cells for RTKs that are inhibited upon PIP silencing. To this end, we employed the RayBio® human RTK phosphorylation antibody array-1 to analyze RTK activation in T47D cells expressing either a non-specific shRNA and one that specifically knocked down PIP (shPIP/121; Figure 3A). Differential hybridization to the RayBio® array allows simultaneous semi-quantitative analysis of phosphorylated tyrosine residues in 71 different receptor tyrosine kinases (RTKs). The results demonstrated that PIP knockdown was associated with dramatic decreases in the phosphorylation of focal adhesion-kinase (FAK), ephrin-B3 (EphB3), FYN, and hemopoietic cell kinase (HCK) (Figure 3B, C). We independently confirmed the results from the antibody-array by western blot analysis of cell extracts prepared from dox- and vehicle-treated T47D/shPIP^{dox} cells using specific antibodies that detect FAK phosphorylation at the Tyr^{397} residue. As shown in Figure 3D, PIP silencing significantly reduced FAK phosphorylation at the Tyr^{397} position. As control, western analysis with a pan FAK antibody showed no significant effect of PIP silencing on the total FAK protein level (Figure 3D). As additional control, PIP knockdown affected neither phospho- nor total HGFR levels (Figure 3D). Furthermore, western analyses of the same extracts showed that PIP silencing resulted in decreased cMYC levels, without a compensatory increase in cMYC phosphorylation at the Thr^{58} and Ser^{62} residues (Figure 3D). Thus, PIP is required for the activation of multiple RTKs as well as the downstream expression and activation of cMYC.

Activation of RTKs could account for the reliance of T47D cells on PIP for proliferation [27]. Because these RTKs signal through phosphorylation of downstream kinases such as AKT and ERK1/2, we studied the influence of PIP on these kinases by western analysis with specific phospho-antibodies. Constitutive knockdown of PIP resulted in strong inhibition of AKT and ERK1/2 phosphorylation without affecting their total protein levels (Figure 3E). These results suggest that PIP is required for the activation of signaling events through RTKs (FAK, EphB3, HCK, FYN), and downstream serine-threonine kinases (AKT, ERK1/2).

PIP plays a role in cell adhesion, cytoskeleton dynamics and protein secretion

Defects in the phosphorylation of FAK, FYN and the downstream AKT and ERK1/2 are likely related to the inhibition of cell cycle progression observed after PIP knockdown in T47D cells [27]. We next investigated whether these defects were also associated with changes in cytoskeleton dynamics. T47D/shPIP^{dox} cells were treated with dox for two days to silence PIP, and stress fiber assembly was then induced by one-hour serum starvation and visualized by phalloidin staining and confocal microscopy. As shown in Figure 4A, a network of stress fibers was assembled in the control cells, but much less in the dox-treated, PIP-depleted cells. Because the actin cytoskeleton plays pivotal roles in cancer-related cellular processes such as adhesion and vesicular transport, we tested the effects of PIP silencing on these processes. *In vitro* adhesion assays indicated that PIP knockdown inhibited adhesion of T47D cells to a fibronectin matrix by 36–39% as compared to control cells (Figure 4B). We then compared the culture supernatants from control *versus* PIP-depleted T47D cell cultures. T47D/shPIP^{dox} cells were grown in culture medium containing

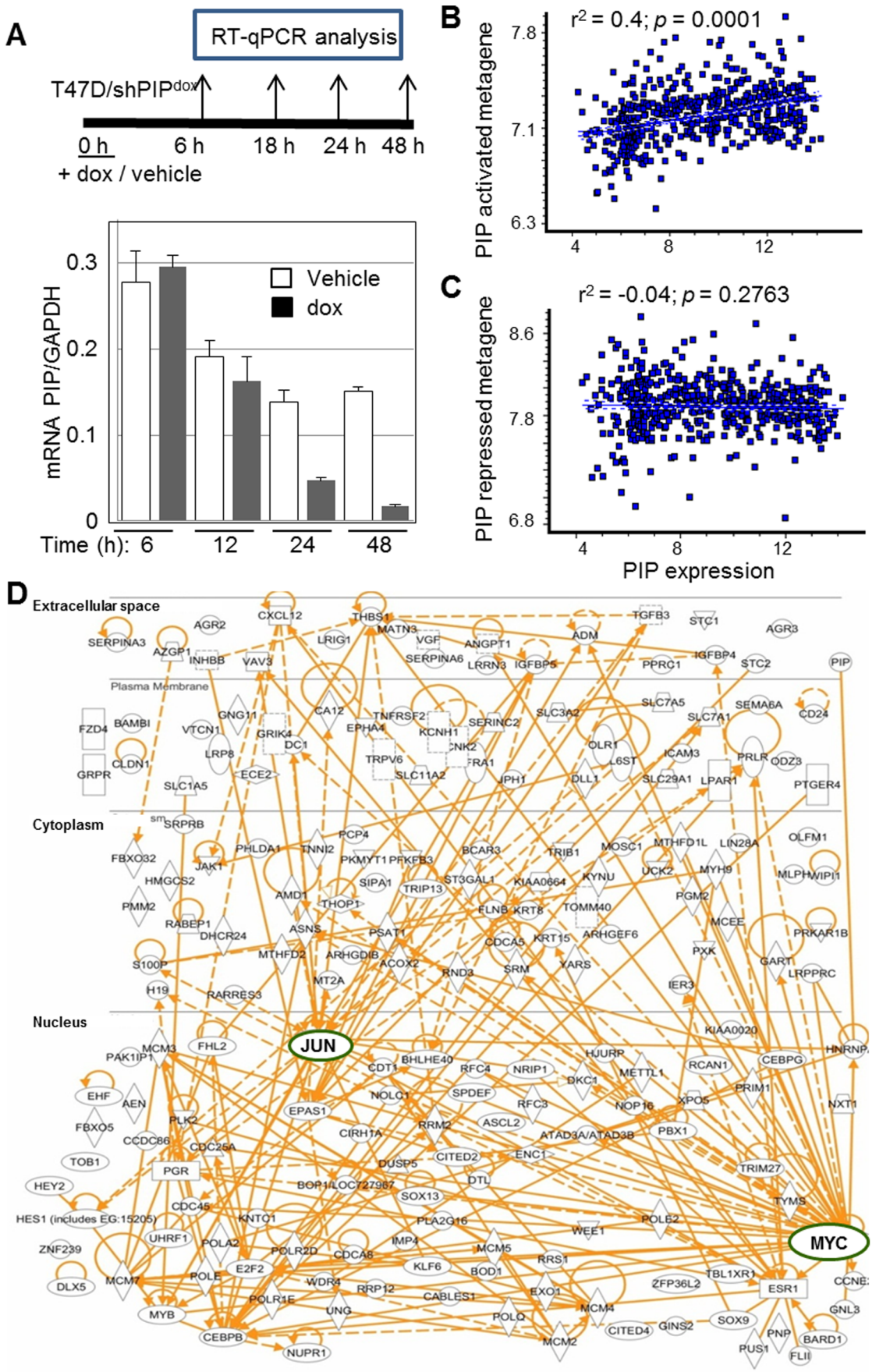
A
RT-qPCR analysis
T47D/shPIP^dox
0 h
6 h
18 h
24 h
48 h
+ dox / vehicle
mRNA PIP/GAPDH
Vehicle
dox
Time (h): 6
12
24
48
B
r² = 0.4; p = 0.0001
PIP activated metagene
C
r² = -0.04; p = 0.2763
PIP repressed metagene
PIP expression
D
Extracellular space
Plasma Membrane
Cytoplasm
Nucleus
JUN
MYC
ESR1

Figure 2. Identification of PIP-regulated genes in breast cancer. A, T47D/shPIP^{dox} cells were treated with dox (250 ng/ml) or vehicle, and RNA was extracted at four time points as schematically illustrated in the *upper panel*. RT-qPCR analysis of PIP with GAPDH as control (*bottom panel*) demonstrates effective PIP knockdown at the 24 and 48, but not at the 6 or 12-hour time points. **B–C,** Analysis of the clinical correlations in a cohort of 557 BCa tumors between PIP mRNA and either the PIP-activated (B) or the PIP-repressed metagenes (C), defined as the average normalized expression of genes that were either repressed (B) or stimulated (C) by ≥2-fold in the T47D/shPIP^{dox} cell culture model. **D**, 293 genes down-regulated by ≥1.5-fold one day after PIP knockdown were subjected to the pathway analysis tool from Ingenuity Systems (IPA^{TM}). Connections are shown among 200 genes for which matched entries were found. Each connection indicates at least one direct relationship found in the literature. Circles denote the highly connected cMYC and cJUN nodes.
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vehicle or dox for two days to achieve effective PIP knockdown, and the cells were then washed and further incubated for 24 hours in serum-free medium. SDS-PAGE and coomasie blue staining of proteins precipitated from the conditioned media clearly indicated absence of many proteins from the medium conditioned by the dox-treated as compared to the vehicle-treated T47D/shPIP^{dox} cells (Figure 4C). Thus, the loss of stress fiber formation in PIP-depleted T47D BCa cells appears to inhibit both cellular attachment and protein secretion.

Because FAK-mediated stress fiber formation is controlled at least in part by JNK1 [28], and because JNK1 regulates expression of cMYC [23,29], a major node in the PIP-activated genes network (Figure 2D), we studied the effect of PIP silencing on JNK1. T47D/shPIP^{dox} cells were maintained in serum-free medium for 24 hours in the presence or absence of dox, and JNK1 activation was assessed in cell extracts prepared 0, 5, 15, 30, 45, and 90 minutes after serum repletion. Western analysis with anti phospho-JNK1 antibodies showed that PIP knock-down diminished the phosphorylation of JNK1, but not the levels of total JNK1 or actin serving as controls (Figure 4D). Altogether, our data suggests that intracellular PIP regulates cytoskeleton dynamics, adhesion, secretion and proliferation of cancer cells via the phosphorylation of specific RTKs and downstream signaling pathways involving AKT, ERK1/2, JNK1 and cMYC.

Table 2. PIP-regulated genes encoding nuclear proteins.

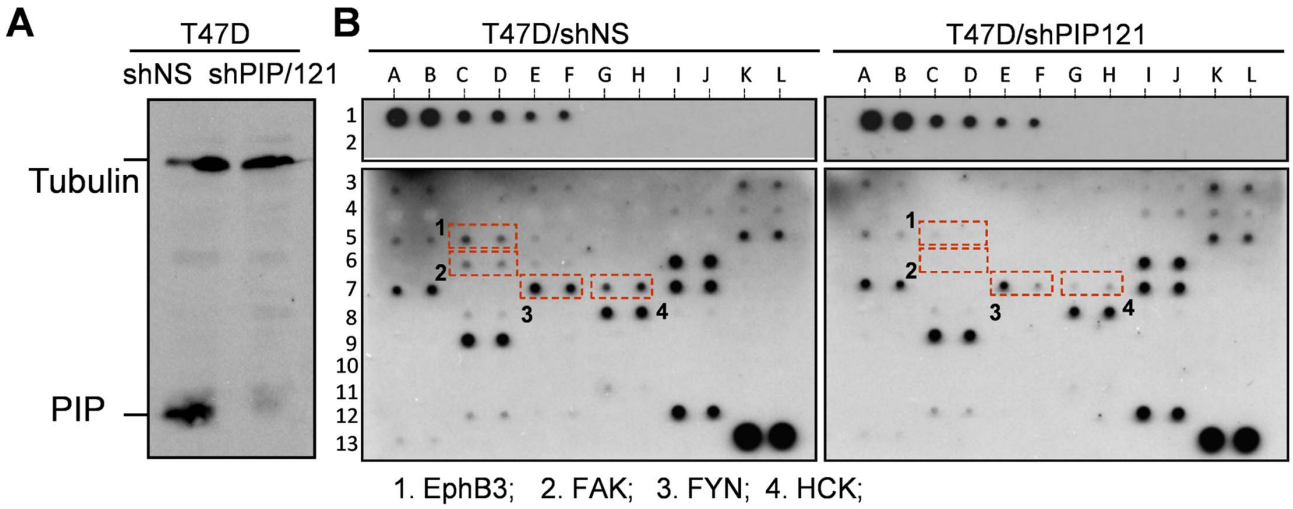
Gene ID	Entrez gene name	Fold repression
MCM5	Minichromosome maintenance complex component 5	2.3
RRM2	Ribonucleotide reductase M2	2.3
E2F2	E2F transcription factor 2	2.2
SPDEF	SAM pointed domain containing ets transcription factor	2.2
MYB	v-myb myeloblastosis viral oncogene homolog	2.2
CABLES1	Cdk5 and Abl enzyme substrate 1	2.1
UHRF1	Ubiquitin-like with PHD and ring finger domains 1	2.0
MYC	v-myc myelocytomatosis viral oncogene homolog	2.0
JUN	Jun proto-oncogene	2.0
SOX9	SRY (sex determining region Y)-box 9	1.9

PIP was silenced in T47D/shPIP^{dox} cells by dox treatment, and mRNA was globally profiled after 24 hours using BeadChip HumanHT-12 v4 Expression kit from Illumina®. Listed are the ten gens encoding nuclear proteins (as per Ingenuity Pathways Analysis package from IPA^{TM}), which were most repressed in the dox- versus vehicle-treated cells. The most repressed genes encoding extracellular, membrane, and cytoplasmic proteins are listed in Table S3.
doi:10.1371/journal.pone.0062361.t002

PIP is required for the proliferation of estrogen-dependent and tamoxifen-resistant T47D cells

As estrogens are major culprits in BCa initiation and progression, and because luminal A-type tumors, which frequently express PIP, are usually ER-positive (Figure 1A), we investigated the requirement for PIP in estrogen-driven BCa cell proliferation. Equal numbers of T47D/shPIP^{dox} cells were plated and grown in medium containing 10% CSS as well as dox and/or E2. MTT-based assays were performed every 48 hours to assess cell proliferation. As shown in Figure 5A, cells proliferation was slower in CSS relative to complete serum (compare to Figure 1C–D). As expected, E2 treatment resulted in a strong mitogenic effect, and importantly this effect was almost completely antagonized by PIP knockdown. PIP also inhibited the low proliferative activity observed in the absence of steroid hormones (Figure 5A). To test whether PIP was required for estrogen signaling in T47D cells, we investigated the effect of PIP knockdown on E2-stimulated transcription of the estrogen receptor (ER)- alpha target genes pS2, GREB1 and CXCL12 [30,31,32]. T47D/shPIP^{dox} cells were grown in culture medium supplemented with CSS and either dox or vehicle for 48 hours. E2 or vehicle was then added for 24 hours, and the transcript levels of the ER-alpha target genes were analyzed by RT-qPCR. As shown in Figure 5B PIP knockdown did not compromise E2-mediated stimulation of any of the ER target genes tested. Thus, T47D BCa cells require PIP for proliferation in order to facilitate activity of a mitogenic pathway(s) that does not necessarily depend on estrogen-driven transcription.

Because PIP is required for the proliferation of both ER-positive and ER-negative BCa cells (Figure 1C, Figure S3 in File S1; [3]), and its knockdown did not influence estrogen-driven transcription (Figure 5B), we hypothesized that PIP silencing would be required for the proliferation of hormonal therapy-resistant BCa cells. To test this hypothesis, we employed T47D^{tamR} cells, a T47D-derived cell line rendered resistant to tamoxifen by continuous growth in estrogen-deprived culture medium [12]. A T47D^{tamR}/shPIP^{dox} subline was engineered, which, like the T47D/shPIP^{dox} line, responds to dox treatment with expression of an shRNA that silences PIP expression. Western blot analysis indicated that T47D^{tamR} cells express PIP at comparable levels to those present in the parental T47D cells and that dox treatment reduced PIP expression in both of the engineered cell lines to barely detectable levels (Figure 5C). MTT-based assays were then conducted over time to compare the effects of PIP knockdown on T47D^{tamR}/shPIP^{dox} and T47D/shPIP^{dox} cell proliferation. As control, tamoxifen treatment alone strongly inhibited the proliferation of T47D/shPIP^{dox} but not T47D^{tamR}/shPIP^{dox} cells (Figure 5D, E). On the other hand, dox-induced PIP knockdown significantly inhibited the proliferation of both the T47D/shPIP^{dox} and the T47D^{tamR}/shPIP^{dox} cells and the extents of inhibition were comparable at each of the experimental time points. Because PIP is required for the proliferation of both hormonal therapy-sensitive and -resistant BCa cells, its pharmacological inhibition may prove beneficial for patients at multiple disease stages.



C

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS 1	POS 1	POS 2	POS 2	POS3	POS3	ABL1	ABL1	ACK1	ACK1	ALK	ALK
2	NEG	NEG	NEG	NEG	Axl	Axl	Blk	Blk	BMX	BMX	Btk	Btk
3	Csk	Csk	Dtk	Dtk	EGFR	EGFR	EphA1	EphA1	EphA2	EphA2	EphA3	EphA3
4	EphA4	EphA4	EphA5	EphA5	EphA6	EphA6	EphA7	EphA7	EphA8	EphA8	EphB1	EphB1
5	EphB2	EphB2	EphB3	EphB3	EphB4	EphB4	EphB6	EphB6	ErbB2	ErbB2	ErbB3	ErbB3
6	ErbB4	ErbB4	FAK	FAK	FER	FER	FGFR1	FGFR1	FGFR2	FGFR2	FGFR2 (α isoform)	FGFR2 (α isoform)
7	Fgr	Fgr	FRK	FRK	Fyn	Fyn	Hck	Hck	HGFR	HGFR	IGF-I R	IGF-I R
8	Insulin R	Insulin R	Itk	Itk	JAK1	JAK1	JAK2	JAK2	JAK3	JAK3	LCK	LCK
9	LTK	LTK	Lyn	Lyn	MATK	MATK	M-CSFR	M-CSFR	MUSK	MUSK	NGFR	NGFR
10	PDGFR-α	PDGFR-α	PDGFR-β	PDGFR-β	PYK2	PYK2	RET	RET	ROR1	ROR1	ROR2	ROR2
11	ROS	ROS	RYK	RYK	SCFR	SCFR	SRMS	SRMS	SYK	SYK	Tec	Tec
12	Tie-1	Tie-1	Tie-2	Tie-2	TNK1	TNK1	TRKB	TRKB	TXK	TXK	NEG	NEG
13	Tyk2	Tyk2	TYRO10	TYRO10	VEGFR2	VEGFR2	VEGFR3	VEGFR3	ZAP70	ZAP70	POS2	POS2

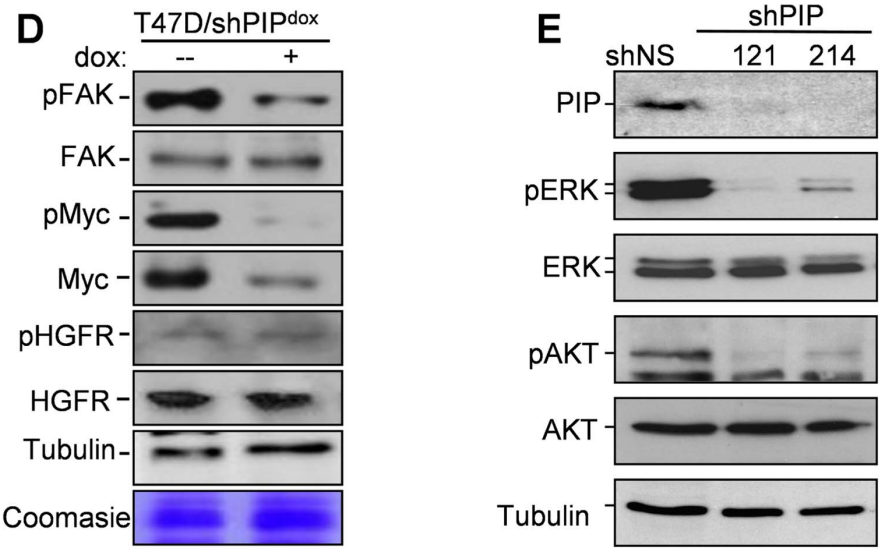


Figure 3. PIP silencing reduces phosphorylation of FAK, EphB3, FYN and HCK, and the downstream AKT and ERK1/2. A, Immunoblot analysis of PIP in T47D cells expressing shRNA against PIP (shPIP/121) or a non-genomic target (shNS). **B–C,** Receptor tyrosine kinase (RTK) phosphorylation screen (B), and a map of the 71 RTKs and controls spotted on the antibody array (C). Cell extracts from T47D/shPIP/121 and T47D/shNS cells were hybridized to the membranes and RTKs showing reduced phosphorylation in PIP-silenced cells are indicated by red boxes. **D,** Western blot analysis of T47D/shPIP^{dox} cells grown in serum-supplemented medium with dox or vehicle control. Antibodies were against [pTyr^{397}]-FAK and pan FAK, [pThr^{58}/pSer^{62}]-Myc and pan Myc, as well as [Tyr^{1003}]-HGFR and pan HGFR. Immunoblot of tubulin and a random coomasie blue-

stained band are shown as loading controls. **E,** Western blot analysis of total and phosphorylated forms of AKT and ERK1/2 in cell lysates prepared from T47D cells expressing shRNA specific for either PIP (shPIP/121, shPIP/214) or a non-genomic target (shNS).
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Discussion

Breast cancer (BCa) is a heterogeneous disease with multiple subtypes of tumors, some ER-alpha-positive (mostly luminal A, luminal B and normal-like), and others ER-alpha-negative (mostly HER2-enriched and basal-like). The estrogen and HER2 receptor-mediated pathways that primarily define these subtypes have been successfully targeted for treatment. However, frequent disease recurrence advocates the pursuit of new targets and

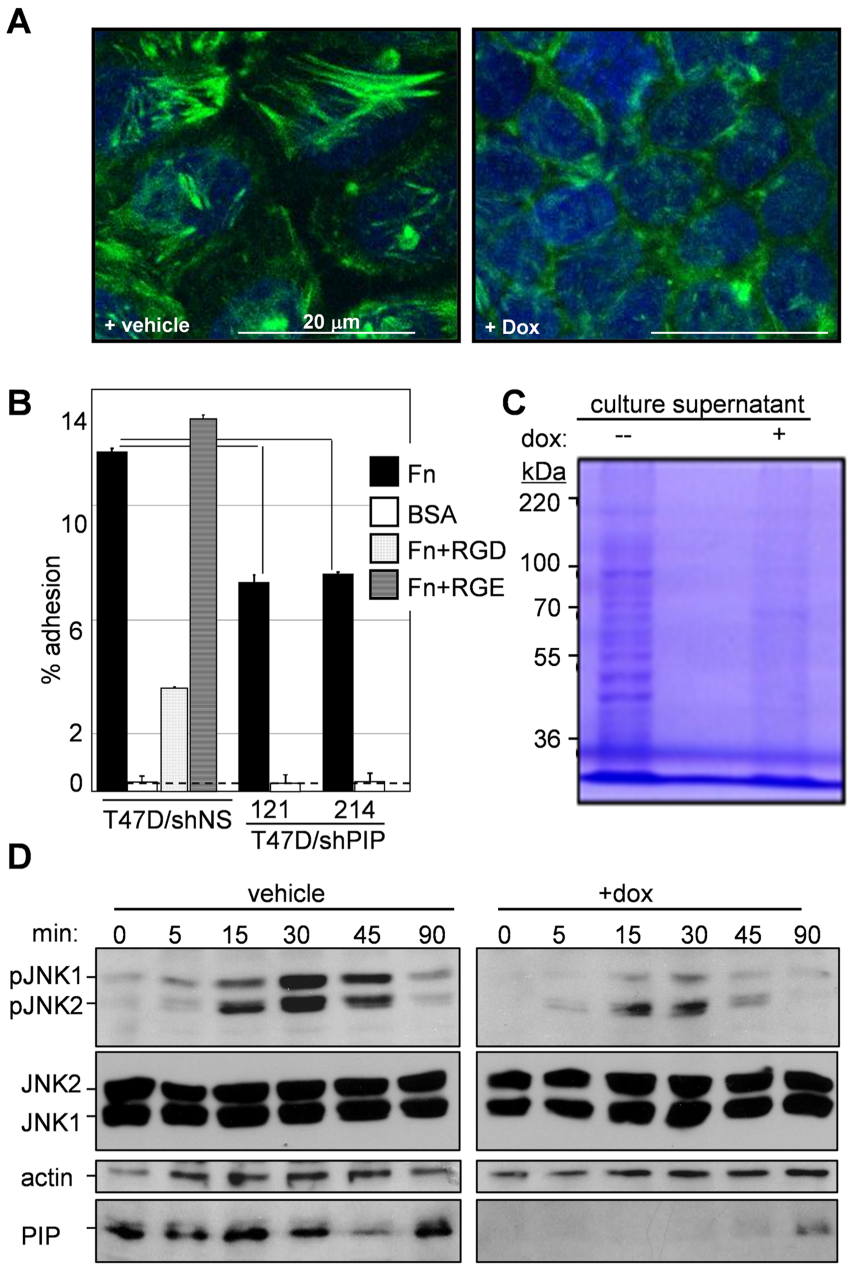

Figure 4. PIP is required for stress fiber assembly, cell adhesion, secretion, and JNK1 signaling in T47D BCa cells. A, Confocal images showing loss of phalloidin stained actin stress fibers in dox-treated *versus* vehicle-treated T47D/shPIP^{dox} cells. **B,** Adhesion assay showing inhibition of T47D cell adhesion to fibronectin after expression of PIP-specific (shPIP/121, shPIP/214) versus a non-specific (shNS) shRNA. Peptide competitors were used to assess specific (RGD) *versus* non-specific (RGE) binding to fibronectin. The dotted line indicates background binding to bovine serum albumin (BSA). **C,** Coomassie-stained SDS-polyacrylamide gel showing reduced levels of secreted proteins in the supernatant of dox- *versus* vehicle-treated T47D/shPIP^{dox} cell cultures. **D**, Western blot analysis of JNK1/2 phosphorylation in dox- *versus* vehicle-treated serum-starved T47D/shPIP^{dox} cells at the indicated time points after serum stimulation. Pan anti-JNK1/2 and tubulin antibodies were used as controls, and anti PIP antibodies were used to demonstrate effective dox-mediated PIP knockdown.
doi:10.1371/journal.pone.0062361.g004

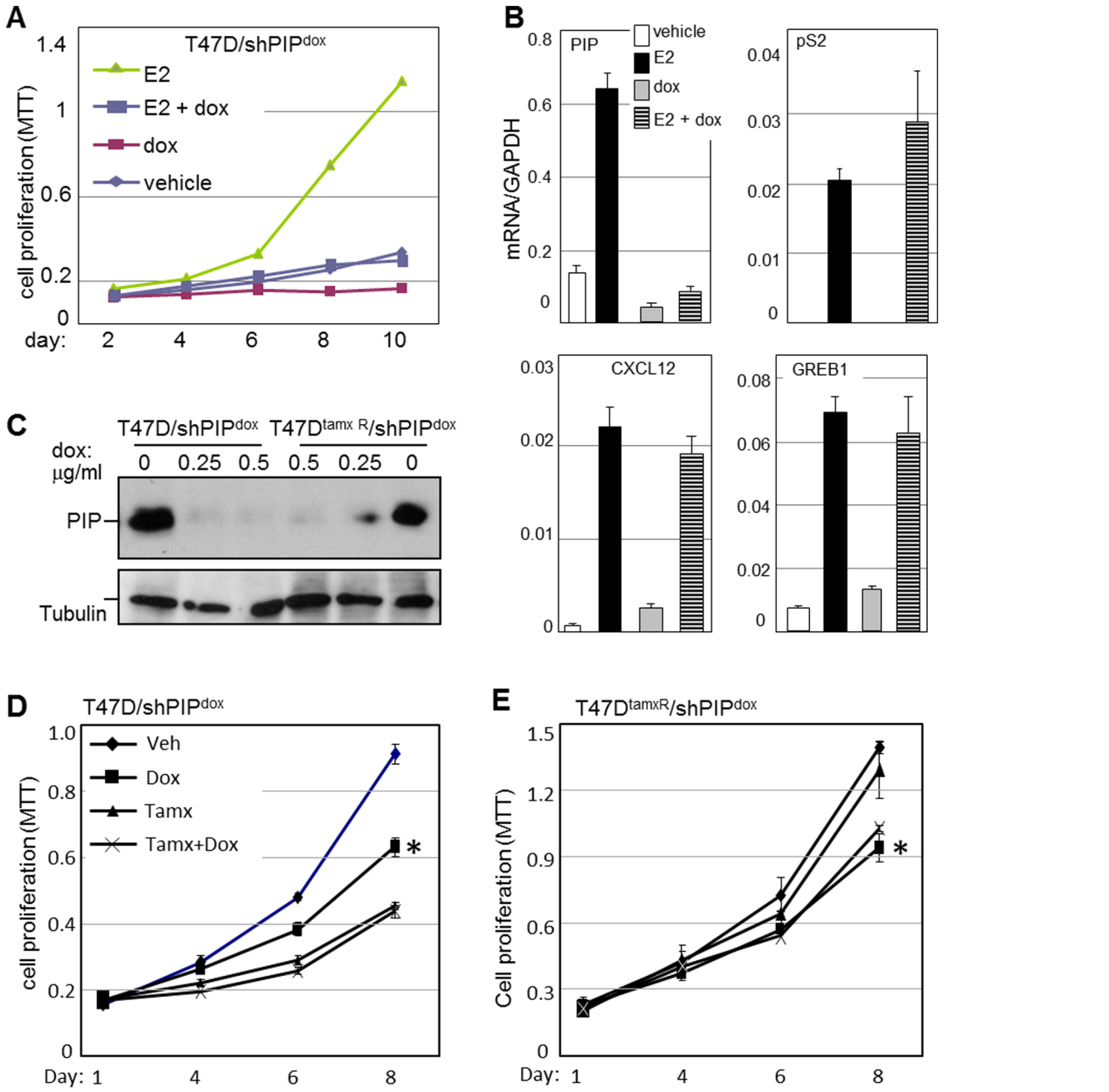

Figure 5. PIP is required for the proliferation of hormone-sensitive and –resistant breast cancer cells. A, The effect of PIP knockdown on E2-stimulated BCa cell proliferation was assessed in T47D/shPIP^{dox} cell cultures maintained in CSS-supplemented medium. Cells were treated with either E2 or vehicle in the presence or absence of dox, and MTT assays were performed every 48 hours for 10 days. **B,** RT-qPCR analysis of PIP and the estrogen-responsive genes pS2, CXCL12 and GREB1 in T47D/shPIP^{dox} cell cultures treated with E2 in the presence or absence of dox to silence PIP expression. Values are normalized per GAPDH expression, which itself did not significantly change by treatment with dox or E2. **C,** Immunoblot analysis showing the dox-induced PIP knockdown in the parental T47D and the tamoxifen-resistant T47D^{tamxR} cells, each engineered with a dox-inducible lentiviral vector encoding a small hairpin RNA that targets PIP (shPIP^{dox}). **D–E,** MTT-based proliferation assays conducted after 4, 6, and 8 days of treatment with tamoxifen and/or dox, showing that PIP silencing inhibited proliferation of both the tamoxifen-sensitive T47D and the tamoxifen-resistant T47D^{tamxR} cells. (Mean±SD; n = 3). *significantly different from vehicle-treated cells at the day-6 and day-8 experimental time points ($p<0.0004$).
doi:10.1371/journal.pone.0062361.g005

therapeutic modalities to be used either alone or together with those currently available in order to achieve long-term survival and cure. The present study suggests that PIP, a small polypeptide expressed by the majority BCa tumors, may serve as a target for the development of novel therapeutic approaches for BCa. Knockdown of PIP in ER-positive T47D cells dramatically reduced proliferation independent of estrogen-driven transcription. Of note, over-expression of PIP in either PIP-positive T47D or PIP-negative MDA-MB-231 cultures had no effect on cell proliferation (Figure S3B in File S1 and data not shown).

Unexpectedly, PIP supported cell proliferation via an intracellular mechanism because neither conditioned medium containing PIP nor co-culture with PIP-secreting cells rescued the proliferation defect caused by PIP silencing. These observations indicate a novel obligatory role for intracellular PIP in BCa cell proliferation. A screen of 71 receptor tyrosine kinases revealed that PIP silencing selectively reduced the tyrosine phosphorylation of FAK, FYN, EphB3, and HCK. Subsequent studies demonstrated defects in the activation of the downstream serine/threonine kinases AKT, ERK1/2, and JNK1. Furthermore, mRNA profiling followed by *in silico* analysis demonstrated an intricate PIP-controlled gene network with cMYC and cJUN, targets of JNK1 and ERK1/2, respectively, forming predominant regulatory nodes. Suggesting clinical significance of our *in vitro* data, expression of genes that were down-regulated upon PIP knockdown in T47D cell culture was significantly correlated with the levels of PIP mRNA across BCa tumors.

Even though PIP is most commonly expressed in ER-positive BCa tumors of the Luminal A-subtype, its requirement for cell proliferation is not unique to such cells. In fact, we have observed inhibition of cell proliferation upon PIP silencing in the ER-negative MDA-MB-453 cell line (Figure S3 in File S1), and this finding is consistent with the recent results of Naderi and Meyer in both MDA-MB-453 and HCC-1954 BCa cells [3]. Various mechanisms have been proposed to explain the requirement for PIP in the proliferation of various BCa cell subtypes. PIP was required for androgen signaling in the T47D BCa cell line [8], and its role in promoting MDA-MB-453 cell proliferation was related to activation of the HER2 receptor tyrosine kinase. Notably, HER2 is not over-expressed in the ER-positive T47D cells ([33]; Figure 3B) and our RTK screen suggested that other RTKs (FAK, FYN, EphB3, HCK) mediated the functions of PIP in these cells. We speculate that PIP plays a common fundamental function in various types of cancer cells, which utilize PIP to promote different pathways with crucial roles in the different cell types. Furthermore, promotion of BCa cell proliferation by various hormones and growth factors may require the stimulation of PIP. Indeed, such stimulation has been observed in response to estrogens (Figure 5B; [34,35]), androgens, prolactin and growth hormone [3,4,5,9]. The identity and flexible nature of a common PIP function operative as an obligatory component of various growth stimulatory networks remains to be explored. Thus, inhibition of PIP may serve as a therapeutic strategy to abrogate the adhesion and growth of BCa cells across multiple tumor subtypes. Furthermore, although PIP expression typifies ER-alpha positive BCa, future therapeutic approaches that target PIP-related mechanisms may be effective for the treatment of both ER-positive and –negative BCa cell lines (Figure S3A in File S1). The insensitivity of some BCa cell lines to PIP, however (Figure S3B in File S1), suggest that such future therapeutic approaches will not be universally efficacious.

Inhibition of PIP may prove effective for the treatment of BCa cells resistant to hormonal therapy, because it's silencing equally inhibited proliferation of the tamoxifen-resistant T47D^{tamR} cells and the naïve tamoxifen-sensitive T47D cells. The clinical significance of this observation is emphasized by reports that PIP expression is strongly enhanced upon tamoxifen treatment [34,35], and that drug-resistant BCa cells continue to express PIP (Figure 5C). Targeting PIP may therefore prove effective not only in early disease stages, but also in advanced tumors resistant to hormonal therapy.

In summary, our studies assign essential roles to PIP in both hormone-responsive and -unresponsive breast cancer cells, thereby extending the importance of PIP in the management of BCa from a mere clinical marker to a promising therapeutic target. Finally, the design of PIP inhibitors for the treatment of breast and other cancers will have to take into consideration our findings, suggesting a novel intracellular role that PIP plays in cancer cell proliferation independently of the roles that it plays as a secreted protein.

Supporting Information

File S1 Supplemental figures.
(PDF)

Table S1 Oligonucleotides used in this study.
(DOC)

Table S2 Dox regulated at D1 or D2.
(XLS)

Table S3 Day 1 1.5 fold repressed 293 genes.
(XLS)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: SKB BF. Performed the experiments: SKB NOC. Analyzed the data: SKB BF NOC DT. Contributed reagents/materials/analysis tools: VCJ. Wrote the paper: SKB BF DT VCJ.

References

1. Clark JW, Snell L, Shiu RP, Orr FW, Maitre N, et al. (1999) The potential role for prolactin-inducible protein (PIP) as a marker of human breast cancer micrometastasis. Br J Cancer 81: 1002–1008.
2. Frendo JL, Xiao G, Fuchs S, Franceschi RT, Karsenty G, et al. (1998) Functional hierarchy between two OSE2 elements in the control of osteocalcin gene expression in vivo. J Biol Chem 273: 30509–30516.
3. Naderi A, Meyer M (2012) Prolactin-induced protein mediates cell invasion and regulates integrin signaling in estrogen receptor-negative breast cancer. Breast Cancer Res 14: R111.
4. Murphy LC, Tsuyuki D, Myal Y, Shiu RP (1987) Isolation and sequencing of a cDNA clone for a prolactin-inducible protein (PIP). Regulation of PIP gene expression in the human breast cancer cell line, T-47D. J Biol Chem 262: 15236–15241.
5. Shiu RP, Iwasiow BM (1985) Prolactin-inducible proteins in human breast cancer cells. J Biol Chem 260: 11307–11313.
6. Chalbos D, Haagensen D, Parish T, Rochefort H (1987) Identification and androgen regulation of two proteins released by T47D human breast cancer cells. Cancer Res 47: 2787–2792.
7. Haagensen DE Jr, Dilley WG, Mazoujian G, Wells SA Jr (1990) Review of GCDFP-15. An apocrine marker protein. Ann N Y Acad Sci 586: 161–173.
8. Baniwal SK, Little GH, Chimge NO, Frenkel B Runx2 controls a feed-forward loop between androgen and prolactin-induced protein (PIP) in stimulating T47D cell proliferation. J Cell Physiol 227: 2276–2282.
9. Baniwal SK, Khalid O, Sir D, Buchanan G, Coetzee GA, et al. (2009) Repression of Runx2 by androgen receptor (AR) in osteoblasts and prostate cancer cells: AR binds Runx2 and abrogates its recruitment to DNA. Mol Endocrinol 23: 1203–1214.
10. Cassoni P, Sapino A, Haagensen DE, Naldoni C, Bussolati G (1995) Mitogenic effect of the 15-kDa gross cystic disease fluid protein (GCDFP-15) on breast-cancer cell lines and on immortal mammary cells. Int J Cancer 60: 216–220.

11. Caputo E, Manco G, Mandrich L, Guardiola J (2000) A novel aspartyl proteinase from apocrine epithelia and breast tumors. J Biol Chem 275: 7935–7941.
12. Murphy CS, Meisner LF, Wu SQ, Jordan VC (1989) Short- and long-term estrogen deprivation of T47D human breast cancer cells in culture. Eur J Cancer Clin Oncol 25: 1777–1788.
13. Park WC, Liu H, Macgregor Schafer J, Jordan VC (2005) Deregulation of estrogen induced telomerase activity in tamoxifen-resistant breast cancer cells. Int J Oncol 27: 1459–1466.
14. Network TCGA (2012) Comprehensive molecular portraits of human breast tumours. Nature 490: 61–70.
15. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, et al. (2000) Molecular portraits of human breast tumours. Nature 406: 747–752.
16. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, et al. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 98: 10869–10874.
17. Desmedt C, Piette F, Loi S, Wang Y, Lallemand F, et al. (2007) Strong time dependence of the 76-gene prognostic signature for node-negative breast cancer patients in the TRANSBIG multicenter independent validation series. Clin Cancer Res 13: 3207–3214.
18. Schmidt M, Bohm D, von Torne C, Steiner E, Puhl A, et al. (2008) The humoral immune system has a key prognostic impact in node-negative breast cancer. Cancer Res 68: 5405–5413.
19. Lee DM, Chang SJ, Park CG, Kim MW, Lim GH, et al. (2005) [Effects of tamoxifen on the voltage-dependent ionic currents in mouse colonic smooth muscle cells]. Korean J Gastroenterol 46: 388–395.
20. Holliday DL, Speirs V (2011) Choosing the right cell line for breast cancer research. Breast Cancer Res 13: 215.
21. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, et al. (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10: 515–527.
22. Bouchard C, Marquardt J, Bras A, Medema RH, Eilers M (2004) Myc-induced proliferation and transformation require Akt-mediated phosphorylation of FoxO proteins. Embo J 23: 2830–2840.
23. Boureux A, Furstoss O, Simon V, Roche S (2005) Abl tyrosine kinase regulates a Rac/JNK and a Rac/Nox pathway for DNA synthesis and Myc expression induced by growth factors. J Cell Sci 118: 3717–3726.
24. Graf T (1992) Myb: a transcriptional activator linking proliferation and differentiation in hematopoietic cells. Curr Opin Genet Dev 2: 249–255.
25. Treisman JE, Follette PJ, O'Farrell PH, Rubin GM (1995) Cell proliferation and DNA replication defects in a Drosophila MCM2 mutant. Genes Dev 9: 1709–1715.
26. Wu L, Timmers C, Maiti B, Saavedra HI, Sang L, et al. (2001) The E2F1-3 transcription factors are essential for cellular proliferation. Nature 414: 457–462.
27. Assoian RK, Schwartz MA (2001) Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression. Curr Opin Genet Dev 11: 48–53.
28. Liu S, Xu SW, Kennedy L, Pala D, Chen Y, et al. (2007) FAK is required for TGFbeta-induced JNK phosphorylation in fibroblasts: implications for acquisition of a matrix-remodeling phenotype. Mol Biol Cell 18: 2169–2178.
29. Hui L, Zatloukal K, Scheuch H, Stepniak E, Wagner EF (2008) Proliferation of human HCC cells and chemically induced mouse liver cancers requires JNK1-dependent p21 downregulation. J Clin Invest 118: 3943–3953.
30. Boudot A, Kerdivel G, Habauzit D, Eeckhoute J, Le Dily F, et al. (2011) Differential estrogen-regulation of CXCL12 chemokine receptors, CXCR4 and CXCR7, contributes to the growth effect of estrogens in breast cancer cells. PLoS One 6: e20898.
31. Ghosh MG, Thompson DA, Weigel RJ (2000) PDZK1 and GREB1 are estrogen-regulated genes expressed in hormone-responsive breast cancer. Cancer Res 60: 6367–6375.
32. Kim J, Petz LN, Ziegler YS, Wood JR, Potthoff SJ, et al. (2000) Regulation of the estrogen-responsive pS2 gene in MCF-7 human breast cancer cells. J Steroid Biochem Mol Biol 74: 157–168.
33. Konecny GE, Pegram MD, Venkatesan N, Finn R, Yang G, et al. (2006) Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. Cancer Res 66: 1630–1639.
34. Dejardin L, Gol-Winkler R, Collette J, Delvenne C, Carlisi I, et al. (1991) Stimulatory effect of oestradiol-17 beta and tamoxifen on gross cystic disease fluid protein 15,000 production and mRNA levels in T47D human breast cancer cells. J Mol Endocrinol 7: 105–112.
35. Haagensen DE, Stewart P, Dilley WG, Wells SA (1992) Secretion of breast gross cystic disease fluid proteins by T47D breast cancer cells in culture – modulation by steroid hormones. Breast Cancer Res Treat 23: 77–86.
36. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, et al. (2009) Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol 27: 1160–1167.

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Inhibition of c-Src blocks oestrogen-induced apoptosis and restores oestrogen-stimulated growth in long-term oestrogen-deprived breast cancer cells

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KEYWORDS
c-Src
Oestrogen
Apoptosis
Epithelial–mesenchymal transition
Breast cancer

Abstract ***Purpose:*** Our publications demonstrate that physiological concentrations of oestrogen (E_2) induce endoplasmic reticulum and oxidative stress which finally result in apoptosis in E_2-deprived breast cancer cells, MCF-7:5C. c-Src is involved in the process of E_2-induced stress. To mimic the clinical administration of c-Src inhibitors, we treated cells with either E_2, a c-Src inhibitor PP2, or the combination for 8 weeks to further explore the apoptotic potential of the c-Src inhibitor and E_2 on MCF-7:5C cells.

Methods: Protein levels of receptors and signalling pathways were examined by immunoblotting. Expression of mRNA was detected through real-time polymerase chain reaction (PCR). Cell cycles were analysed by flow cytometry.

Results: Long-term treatment with PP2 alone or E_2 alone decreased cell growth. In contrast, a combination of PP2 and E_2 blocked apoptosis and the resulting cell line (MCF-7:PF) was unique, as they grew vigorously in culture with physiological levels of E_2, which could be blocked by the pure antioestrogen ICI182,780. One major change was that PP2 collaborated with E_2 to increase the level of insulin-like growth factor-1 receptor beta (IGF-1Rβ). Blockade of IGF-1Rβ completely abolished E_2-stimulated growth in MCF-7:PF cells. Furthermore, combination treatment up-regulated transcription factors, Twist1 and Snail, and repressed E-cadherin expression which made MCF-7:PF cells display a characteristic phenotype of epithelial–mesenchymal transition (EMT).

Conclusions: These data illustrate the role of the c-Src inhibitor to block E_2-induced apoptosis and enhance E_2-stimulated growth. Caution must be exercised when considering c-Src inhibitors in clinical trials following the development of acquired resistance to aromatase inhibitors, especially in the presence of the patient's own oestrogen.

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1. Introduction

Aromatase inhibitors are the standard of care for the adjuvant treatment of postmenopausal patients with oestrogen receptor (ER) positive breast cancer [1]. Tamoxifen remains the adjuvant therapy of choice for ER-positive premenopausal patients [1]. Both 'antioestrogen' therapeutic strategies offer substantial benefits for enhancing patient survival [2]. However, long-term therapy also raises the spectre of antihormone resistance. Despite the paucity of human ER-positive cell lines to study molecular mechanisms of antihormone resistance [3], remarkable progress has occurred over the past 25 years that has not only replicated the clinical presentation of antihormone resistance, but also provided valuable clues to create novel second line therapies [4] like fulvestrant, the pure antioestrogen that destroys the tumour ER [5]. Laboratory findings with the MCF-7 breast cancer cell line grown in athymic mice first described tamoxifen-stimulated growth as a new mechanism of drug resistance to a therapeutic intervention [6]. However, it is the discovery that re-transplantation of tamoxifen-stimulated tumours into successive generations of athymic mice over 5 years results in the selection of a resistant tumour cell population that is killed by physiological levels of oestrogen (E_2) [7,8] that has resulted in the new biology of E_2-induced apoptosis [9]. Indeed, E_2-induced apoptosis has been used to explain the action of E_2 replacement therapy for postmenopausal women in their 60s having a lower incidence of breast cancer and mortality [10]. A period of E_2 deprivation (5–10 years) is necessary to select the cell population that will be vulnerable to E_2-induced apoptosis [11]. The same principle [7] has been suggested to explain the dramatic decrease in mortality observed in the decade after long-term adjuvant tamoxifen therapy is stopped [12]; the woman's own E_2 causes apoptosis in the vulnerable antihormone resistant breast cancer cells.

The description of the evolution of tamoxifen resistance *in vivo* to trigger rapid tumour regression with physiological concentrations of E_2 [7,8,13] was rapidly followed by similar reports *in vitro* with populations or selected clones of MCF-7 cells triggering apoptosis with physiological E_2 after long-term E_2 deprivation [14,15]. Thus E_2 deprivation produces the same selective pressure on MCF-7 cells as selective ER modulators (SERMs) [8,16] to create selective cellular populations vulnerable to E_2-induced apoptosis. All of these laboratory data with MCF-7 cells provide the scientific rationale for the subsequent finding that high dose (30 mg daily) or low dose (6 mg daily) E_2 produces a 30% clinical benefit rate in patients failing aromatase inhibitor therapy [17].

Overall, the new biology of E_2 action to trigger apoptosis translates appropriately to the responsiveness of human breast cancer in the clinical setting. As a result, we have used our cellular models to elucidate the molecular mechanisms that modulate E_2-induced apoptosis through inducing endoplasmic reticulum stress and oxidative stress [18,19]. Recently, we have found that the oncogene c-Src is activated in two long-term E_2-deprived breast cancer cell models [20] and is involved in the process of stress induced by E_2 [19]. Preclinical data in endocrine resistant models demonstrate that the crosstalk between ER and c-Src is an important resistance mechanism [21,22]. Blockade of c-Src signalling pathways is an attractive strategy to circumvent the resistance to antihormone therapy in breast cancer [23,24]. Here, we ask the question of what are the consequences of long-term physiological concentrations of E_2 in combination with the c-Src inhibitor on the shift of adaptive populations in E_2-deprived breast cancer cells?

To mimic the clinical administration of a c-Src inhibitor, we treated MCF-7:5C cells with different combinations in a long-term (8 weeks) study to further investigate the therapeutic potential of the combination of the c-Src inhibitor and E_2 on the growth of MCF-7:5C cells compared with either E_2 alone or PP2 alone. Contrary to our original hypothesis that the c-Src inhibitor would enhance the apoptotic effects of E_2, the c-Src inhibitor prevented E_2-induced apoptosis and allowed E_2 to stimulate growth. One major mechanistic change that reversed the E_2 response was that the c-Src inhibitor cooperated with E_2 to increase insulin-like growth factor-1 receptor beta (IGF-1Rβ) growth pathways, which was an important determinant for the signalling pathways of phosphatidylinositol-3 kinases/Akt and mitogen-activated protein kinase (MAPK). Furthermore, long-term combination treatment transcriptionally upregulated epithelial–mesenchymal transition (EMT) inducers, Twist1 and Snail, and disrupted E-cadherin mediated cell–cell adhesion. These data not only demonstrate the important role of c-Src in modulating E_2-induced apoptosis but also have implications for the poor performance with c-Src inhibitors in ER-positive antihormone resistant patients in clinical trials.

2. Materials and methods

2.1. Materials

Estradiol was purchased from Sigma–Aldrich (St. Louis, MO); ICI182,780 was from Tocris (Park Ellisville, MO). c-Src inhibitor PP2 and IGF-1Rβ inhibitor AG1024 were purchased from CalBiochem (San Diego, CA). Sources of antibodies for Western blot were as follows: ERα (sc-544), ERβ (sc-8974), progesterone receptor (PR) (sc-810) and IGF-1Rβ (sc-713) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Total MAPK (#9102), phosphorylated MAPK (#9101), total Akt (#9272), phosphorylated Akt (#9271),

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phosphorylated c-Src (#2101), E-cadherin (#3195), N-cadherin (#4061) and Snail (#3879) antibodies were from Cell Signaling Technology (Beverly, MA). Total c-Src (GD11) and Twist1 (3E11) antibodies were from Millipore (Temecula, CA). Fibrinogen antibody (HPA00190) was from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture conditions and establishment of MCF-7:PF cells

The ER-positive wild-type human breast cancer MCF-7 cells and long-term E_2-deprived cell lines were cultured as previously described [20]. In an attempt to investigate the therapeutic potential of combination E_2 and the c-Src inhibitor, PP2, MCF-7:5C cells were long-term treated with E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) using the same medium as for control MCF-7:5C cells. At the same time, MCF-7:5C cells were treated with E_2 alone (10^{-9} mol/L) and PP2 alone (5×10^{-6} mol/L) respectively which were set up as control groups to examine effects of E_2 and PP2 on MCF-7:5C cells. Medium was changed every 2–3 days, adding fresh compounds. Eight weeks later, the cells treated with E_2 plus PP2 that grew stably were named MCF-7:PF cells. The DNA fingerprinting patterns of MCF-7:PF and long-term E_2 deprived cell lines were consistent with the report by the American Type Culture Collection (ATCC) (Supplementary Fig. S1).

2.3. Cell proliferation assays

Cells were harvested after 7 days of treatment. The DNA content of the cells, a measure of proliferation, was determined as previously described [20], using a DNA fluorescence quantitation kit (Bio-Rad Laboratories, Hercules, CA).

2.4. Cell-cycle analysis

Briefly, differently treated cells were harvested and gradually fixed with 75% EtOH on ice. After being stained with propidium iodide (PI), cells were analysed using a FACSort flow cytometer (Becton Dickinson, San Jose, CA), and the data were analysed with ModFit software.

2.5. Immunoblotting

Proteins were extracted in cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with Protease Inhibitor Cocktail Set I and Phosphatase Inhibitor Cocktail Set II (Calbiochem, San Diego, CA). The immunoblotting was performed as previously described [20].

2.6. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA, isolated with an RNeasy Micro kit (Qiagen, Valencia, CA), was converted to first-strand cDNA using a kit from Applied Biosystems (Foster City, CA). Quantitative real-time PCR assays were done with the SYBR Green PCR Master Mixes (Applied Biosystems, Foster City, CA) and a 7900HT Fast Real-time PCR System (Applied Biosystems, Foster City, CA). All primers were synthesised in Integrated DNA Technologies (San Diego, CA). All the data were normalised by 36B4.

2.7. Boyden chamber migration assay

As described in reference [25], the transwell chambers (Corning Inc., Corning, NY) with eight micron pore size membrane were equilibrated overnight with media according to the manufacturer's recommendation. About 300,000 cells were added to the upper chamber. The lower chamber had 10% charcoal-stripped foetal bovine serum as chemoattractant. Cells were allowed to migrate for 24 h and, thereafter, non-migrated cells on the upper surface of the membrane were cleaned with a cotton swab. The migrated cells on the lower surface of the membrane were fixed in methanol and stained with a HEMA 3 staining set from Fisher Scientific. The cells were then counted in at least four microscopic fields at 10×10 magnification, and experiments were conducted three times.

2.8. Transient transfection reporter gene assays

Transient transfection assays were performed using a dual-luciferase system (Promega, Madison, WI). To determine ER and PR transcriptional activity, cells were transfected with an oestrogen response element (ERE)-regulated (pERE (5×) TA-ffLuc plus pTA-srLuc), or progesterone response element (PRE)-regulated (pPRE (5×) TA-ffLuc plus pTA-srLuc) dual-luciferase reporter gene sets [20]. The cells were treated for 24 h following the transfection. Then, the cells were harvested and processed for dual-luciferase reporter activity, in which the firefly luciferase activity was normalised by renilla luciferase activity.

2.9. Statistical analysis

All reported values are the means ± SE. Statistical comparisons were determined with two-tailed Student's t tests. Results were considered statistically significant if the P value was <0.05.

3. Results

3.1. The c-Src inhibitor completely blocked E_2-induced apoptosis in MCF-7:5C cells

We have found that non-receptor tyrosine kinase, c-Src, is activated in E_2-deprived breast cancer cell lines, MCF-7:5C and MCF-7:2A, and functions as an important transducer to mediate E_2-induced stress [19,20]. Here, to mimic the clinical administration of the c-Src inhibitor, we treated MCF-7:5C cells long-term (8 weeks) with a specific c-Src inhibitor, PP2, alone or in combination with E_2 to investigate the therapeutic potential in E_2-deprived MCF-7:5C cells. MCF-7:5C cells exhibited a cobblestone-like epithelial phenotype (Fig. 1A). PP2 alone treated cells appeared smaller and more contracted, with decreased cell spreading (Fig. 1A). Apoptotic impairment could be observed under the microscope in cells treated with E_2 alone (Fig. 1A). In contrast, combination E_2 and PP2 abolished the growth inhibitory actions by E_2 alone or PP2 alone and the resulting cell line (MCF-7:PF) grew vigorously, displaying a spindle-like morphology (Fig. 1A). Although E_2 significantly increases S phase after short-term treatment [19], further analysis of cell cycles showed that both the c-Src inhibitor and E_2 could clearly arrest cell cycles in G1 phase after long-term treatment which marks growth inhibition. However, combination PP2 and E_2 was unable to arrest cell cycles in G1 phase (Fig. 1B). These data confirmed that E_2-initiated apoptosis requires the c-Src tyrosine kinase pathway [19].

3.2. Inhibition of c-Src converted E_2 from inducing apoptosis to stimulating growth in MCF-7:PF cells

To further investigate the mechanisms underlying the c-Src inhibitor blocking the apoptosis-induced by E_2, the response to E_2 by differently long-term treated cells was first evaluated. Physiological levels of E_2 still caused growth inhibition in MCF-7:5C cells and long-term PP2 treated cells (Fig. 2A). Long-term treatment with E_2 initially caused massive apoptosis, but a small fraction of surviving cells subsequently re-grew. Although apoptotic morphology could be observed under the microscope at this time point (Fig. 1A), E_2 did not decrease cell number compared with control cells (Fig. 2A). It implied that a balance occurred between apoptosis and cell growth caused by E_2. In contrast, E_2 significantly stimulated cell growth in the resulting cell line (MCF-7:PF) generated from a combination treatment of PP2 and E_2 (Fig. 2A). This stimulation by E_2 could be completely blocked by the pure antioestrogen ICI182,780 which demonstrated that proliferation was mediated by ERα (Fig. 2B). Similarly, the c-Src inhibitor also converted the E_2 response from apoptosis to proliferation in

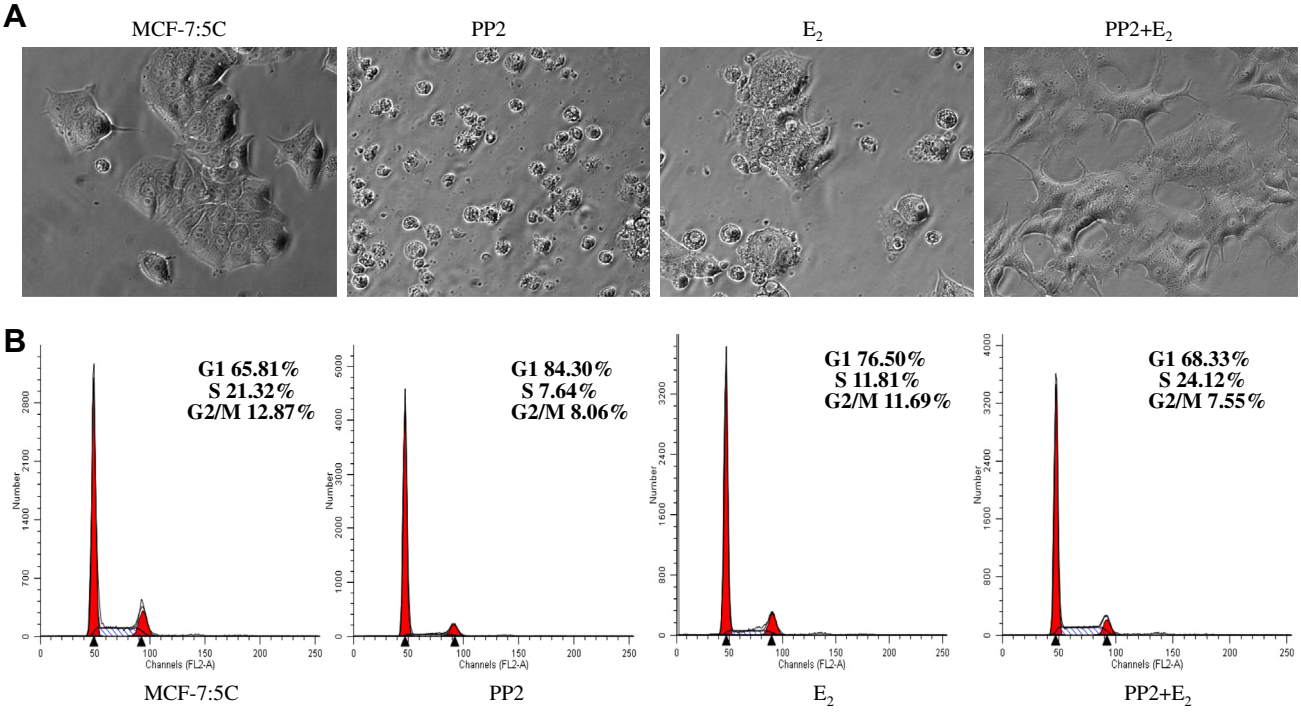

Fig. 1. The c-Src inhibitor completely blocked oestrogen (E_2)-induced apoptosis after long-term treatment. (A) The morphological changes after 8 weeks treatment with different combinations. MCF-7:5C cells were long-term treated with vehicle (0.1% EtOH), PP2 (5×10^{-6} mol/L), E_2 (10^{-9} mol/L) and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) in T_{25} flasks, respectively. Cells were photographed under bright field illumination at (×20) magnification (Zeiss). (B) Cell-cycle changes after different treatments. Cells treated in different combinations were harvested and gradually fixed with 75% EtOH on ice. After staining with propidium iodide (PI), cells were analysed through flow cytometry. All the data shown were representative of at least three separate experiments with similar results.

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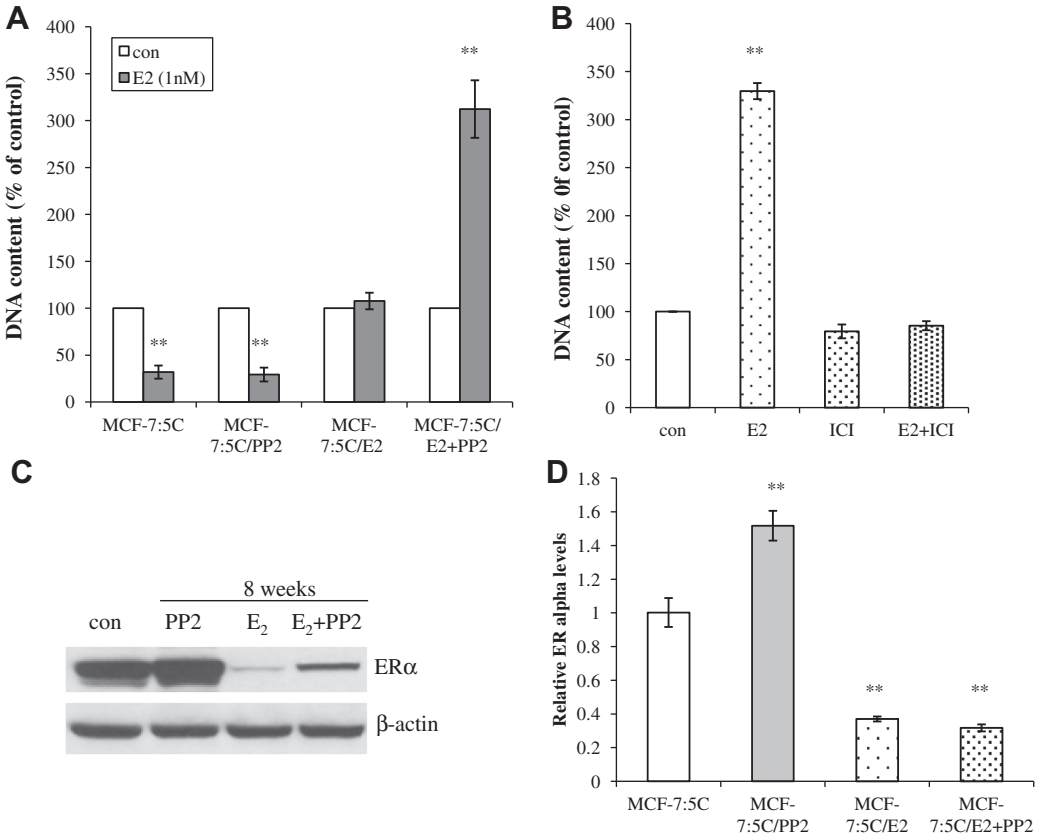

Fig. 2. The c-Src inhibitor converted oestrogen (E_2) responses from inducing apoptosis to stimulating growth. (A) Responses to E_2 in treated cells. Cells treated in different combinations were seeded in 24-well plates in triplicate. Cells were treated with vehicle (0.1% EtOH) or E_2 (10^{-9} mol/L) without any other compounds in the medium. The cells were harvested after 7 days treatment and total DNA was determined using a DNA fluorescence quantitation kit. $P < 0.001$, **compared with control. (B) E_2 proliferative effect was blocked by ICI182,780. MCF-7:PF cells were seeded in 24-well plates in triplicate. After one day, the cells were treated with vehicle (0.1% EtOH), E_2 (10^{-9} mol/L), ICI182,780 (10^{-6}mol/L) and E_2 (10^{-9}mol/L) plus ICI182,780 (10^{-6} mol/L) respectively. The cells were harvested and total DNA was determined as above. $P < 0.001$, **compared with control. (C) Changes of oestrogen receptor α (ERα) after long-term treatment. Cell lysates of different long-term treated cells were harvested. ERα was examined by immunoblotting. β-Actin was detected for loading control. (D) Changes of ERα mRNA levels. The RNA of different cells was harvested in TRIzol for real-time polymerase chain reaction (PCR) analysis. All the data shown were representative of at least three separate experiments with similar results.

another long-term E_2-deprived, late-apoptosis cell line MCF-7:2A ([18] and Supplementary Fig. S2). It is known that blocking c-Src tyrosine kinase increases ERα expression after 24 h treatment ([20] and Supplementary Fig. S3A). This elevated level of ERα was stably expressed after long-term PP2 treatment (Fig. 2C). As expected, cells treated with E_2 alone and MCF-7:PF cells expressed lower levels of ERα due to E_2 down-regulation (Fig. 2C). The ERα protein expression was not strictly consistent with mRNA levels (Fig. 2D). There was no change in ERβ expression after long-term treatment (Supplementary Fig. S3B). To further confirm that it was E_2 which down-regulated ERα, E_2 or/and PP2 were withdrawn from culture medium of MCF-7:PF cells at indicated time points. Withdrawal of E_2 but not PP2, ERα expression in MCF-7:PF cells recovered to similar levels as observed in MCF-7:5C cells (Supplementary Fig. S3C). This suggested that MCF-7:PF cells have functional ERα in response to E_2.

3.3. The c-Src inhibitor was additive with E_2 to elevate endogenous ER target genes in MCF-7:PF cells

Although ER expression levels were quite different (Fig. 2C), the oestrogen response element (ERE) activity was similar among differently treated cells (Fig. 3A). It was interesting to find that the c-Src inhibitor dramatically elevated an E_2-inducible gene pS2 mRNA (Fig. 3B), although the mechanisms were unclear. Moreover, the c-Src inhibitor was additive with E_2 to increase pS2 mRNA in MCF-7:PF cells (Fig. 3B). Another ER target gene, progesterone receptor (PR), was undetectable in MCF-7:5C cells compared with wild-type MCF-7 cells (Supplementary Fig. S4A). However, adding back E_2 into the medium recovered PR expression in cells treated with E_2 alone and MCF-7:PF cells (Fig. 3C). The c-Src inhibitor, PP2, by itself did not regulate PR expression (Fig. 3D). Nevertheless, it synergised with E_2 to up-regulate PR mRNA although

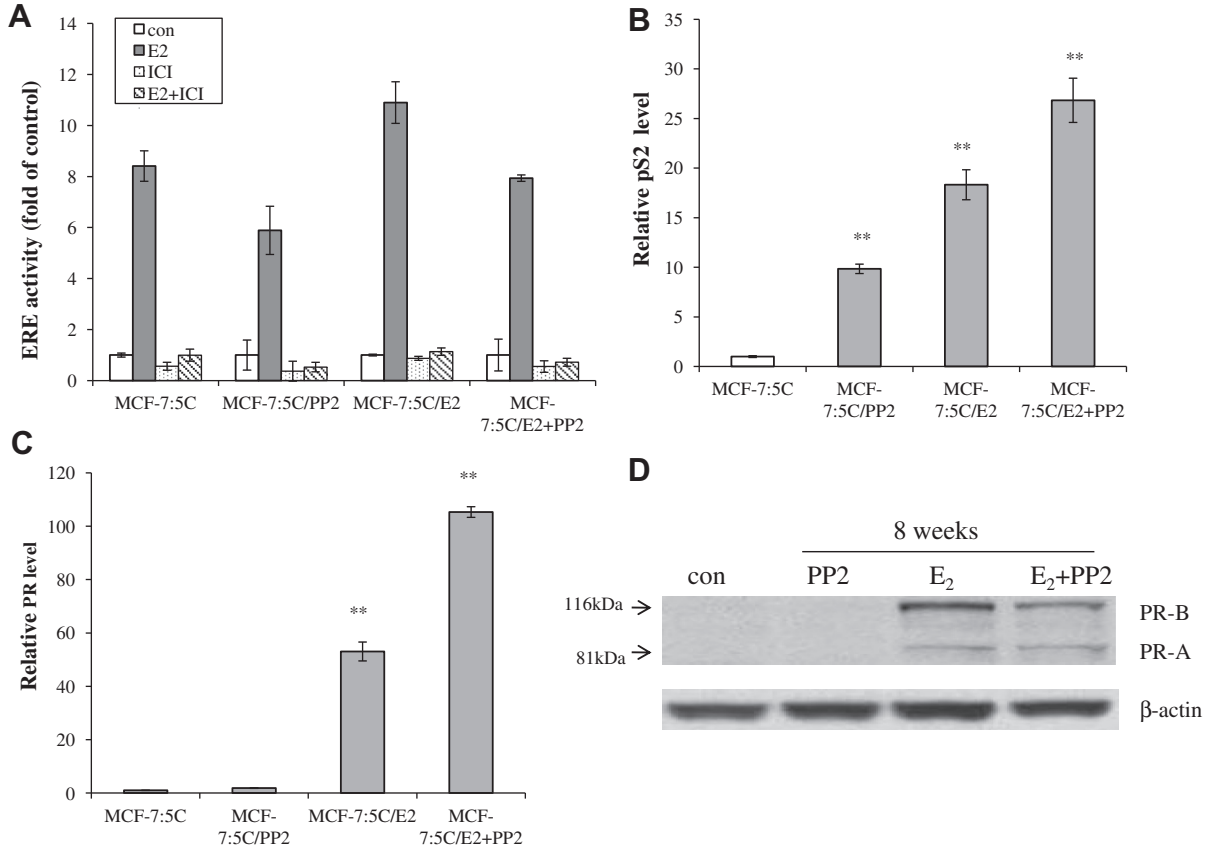

Fig. 3. The c-Src inhibitor collaborated with oestrogen (E_2) to up-regulate oestrogen receptor (ER) target genes. (A) Oestrogen response element (ERE) activity changes in different cells. Cells treated in different combinations were seeded in 24-well plates in triplicate and transfected with ERE firefly luciferase plasmid plus renilla luciferase plasmid as in Section 2. (B) The pS2 mRNA expression. Different combinations of treated cells were grown in six-well plates in triplicate. The RNA was harvested in TRIzol for real-time polymerase chain reaction (PCR) analysis. $P < 0.001$, **compared with control. (C) The progesterone receptor (PR) mRNA expressed levels. The RNA of different cells was harvested in TRIzol for real-time PCR analysis. $P < 0.001$, **compared with control. (D) PR protein changes after long-term treatment. Cell lysates were harvested from different treated cells. PR was examined by immunoblotting. β-Actin was detected for loading control. All the data shown were representative of at least three separate experiments with similar results.

without consistent high protein expression (Fig. 3D), implying the existence of a post-translational modification of PR in MCF-7:PF cells [26].

3.4. The c-Src inhibitor synergised with E_2 to elevate transcriptional activity of PR in MCF-7:PF cells

As shown in Fig. 3D, E_2 alone treated cells and MCF-7:PF cells had similar levels of PR protein. To further investigate the function of PR, progestin (R5020) was used to examine the activity of the progesterone response element (PRE). Interestingly, the progestin only activated PRE activity in MCF-7:PF cells which could be blocked by the anti-progestin RU486 (Fig. 4A). They also had different cell growth responses, progestin stimulated growth in MCF-7:PF cells, but not in E_2 alone treated cells (Fig. 4B). Activation of MAPK results in phosphorylation of PR on Ser294, affecting transcriptional function of PR [26]. In agreement with this report, we observed that phosphorylated MAPK and PR (Ser294) levels in MCF-7:PF cells were higher than that in E_2 alone treated cells (Fig. 4C). Inhibition of MAPK with U0126 effectively blocked the phosphorylation of PR on Ser294 in MCF-7:PF (Fig. 4D). Although anti-progestin RU486 blocked PRE activity induced by progestin in MCF-7:PF cells (Fig. 4A), it could not inhibit cell growth activated by progestin. RU486 itself significantly promoted MCF-7:PF cell growth (Supplementary Fig. S4B). This oestrogenic effect of RU486 [27] on MCF-7:PF cells could be blocked by ICI182,780 and was very similar to wild-type MCF-7 cells (Supplementary Fig. S4C and D). A specific siRNA was used to knock down PR that effectively inhibited MCF-7:PF cell growth (Fig. 4E). All of these results demonstrated that extracellular signal MAPK modifies PR and affects the transcriptional activity of PR.

3.5. The c-Src inhibitor collaborated with E_2 to enhance insulin-like growth factor-1 receptor beta (IGF-1Rβ) which drove growth pathways in MCF-7:PF cells

c-Src mediates the interaction between growth factor receptors and ER in breast cancer [22,28]. The c-Src inhibitor and E_2 could individually increase IGF-1Rβ

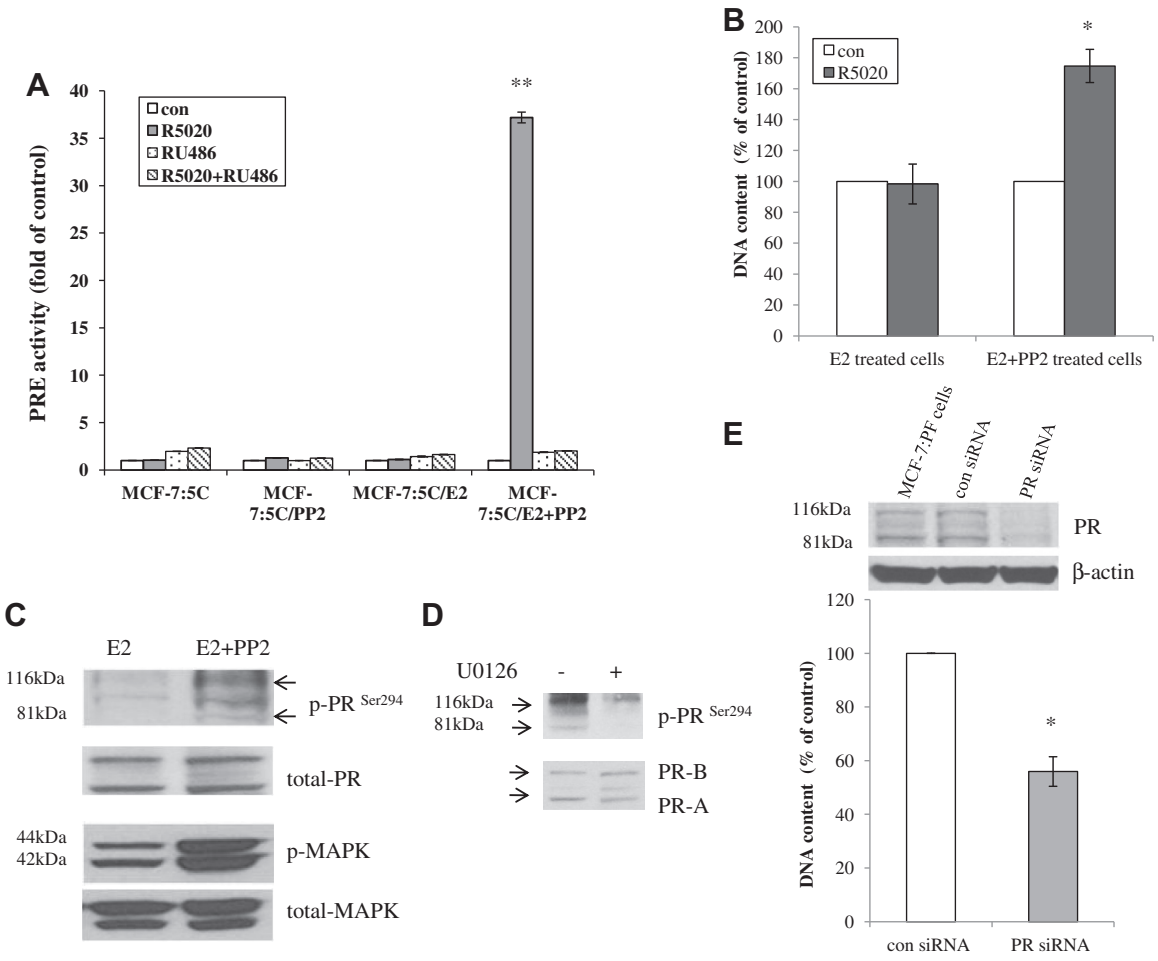

Fig. 4. The c-Src inhibitor synergised with oestrogen (E_2) to activate progesterone receptor (PR) transcriptional activity. (A) The progesterone response element (PRE) activity in treated cells. Cells treated in different combinations were transfected with PRE firefly luciferase plasmid plus renilla luciferase plasmid as in Section 2. The cells were treated with vehicle (0.1% EtOH), progestin (10^{-8} mol/L), RU486 (10^{-6} mol/L) and RU486 (10^{-6} mol/L) plus progestin (10^{-8} mol/L) in triplicate for 24 h. $P < 0.001$, **compared with control. (B) Different responses to progestin between cells treated with E_2 alone and MCF-7:PF cells. E_2 alone treated cells and MCF-7:PF cells were plated in 24-well plates in triplicate. Cells were treated with vehicle (0.1% EtOH) and progestin (10^{-8}mol/L) respectively. Cells were harvested after 7 days treatment and the total DNA was determined as above. $P < 0.05$, *compared with E_2 alone treated cells. (C) MCF-7:PF cells had higher phosphorylated PR than E_2 alone treated cells. Cell lysates of MCF-7:PF cells and E_2 alone treated cells were harvested. Phosphorylated PR and mitogen-activated protein kinase (MAPK) were examined by immunoblotting. Total PR and MAPK were used as loading controls. (D) The PR was phosphorylated by MAPK in MCF-7:PF cells. MCF-7:PF cells were treated with vehicle (0.1% dimethyl sulfoxide (DMSO)) and MAPK inhibitor U0126 (10^{-5} mol/L) for 48 h. Phosphorylated PR was examined by immunoblotting. Total PR was used as loading control. (E) Knockdown of PR by siRNA blocked cell growth. MCF-7:PF cells were transfected with control siRNA and specific PR target siRNA as manufacture's instruction. Cell lysates were harvested after 72 h to detect PR levels by immunoblotting. β-Actin was detected for loading control. As a parallel experiment, cells were harvested after 5 days transfection for DNA growth assay as above. $P < 0.05$, *compared with control siRNA.

expression after long-term treatment. Moreover, PP2 and E_2 were additive to elevate IGF-1Rβ in MCF-7:PF cells (Fig. 5A and B). To investigate the role of IGF-1Rβ in MCF-7:PF cells, a specific inhibitor of IGF-1Rβ, AG1024, was utilised to block receptor tyrosine kinase activity, which effectively abolished MAPK and Akt pathways (Fig. 5C) and inhibited cell growth (Fig. 5D). Importantly, AG1024 completely abolished E_2 stimulation in a concentration-dependent manner in MCF-7:PF cells (Fig. 5D and Supplementary Fig. S5A). These data supported the hypothesis that IGF-1Rβ is linked tightly with the ER function in MCF-7:PF cells.

3.6. The c-Src inhibitor disrupted E-cadherin-mediated cell–cell adhesion and collaborated with E_2 to increase epithelial–mesenchymal transition (EMT) in MCF-7:PF cells

Activation of c-Src kinase has been documented in E-cadherin-mediated cell–cell adhesion, which is thought to play an important role in cancer invasion and metastasis [29]. Therefore, we sought to examine changes of E-cadherin associated signals after long-term treatment with the c-Src inhibitor in MCF-7:5C cells. Contrary to the effects on wild-type MCF-7 cells [29], PP2 reduced E-cadherin but increased N-cadherin and fibrinogen in

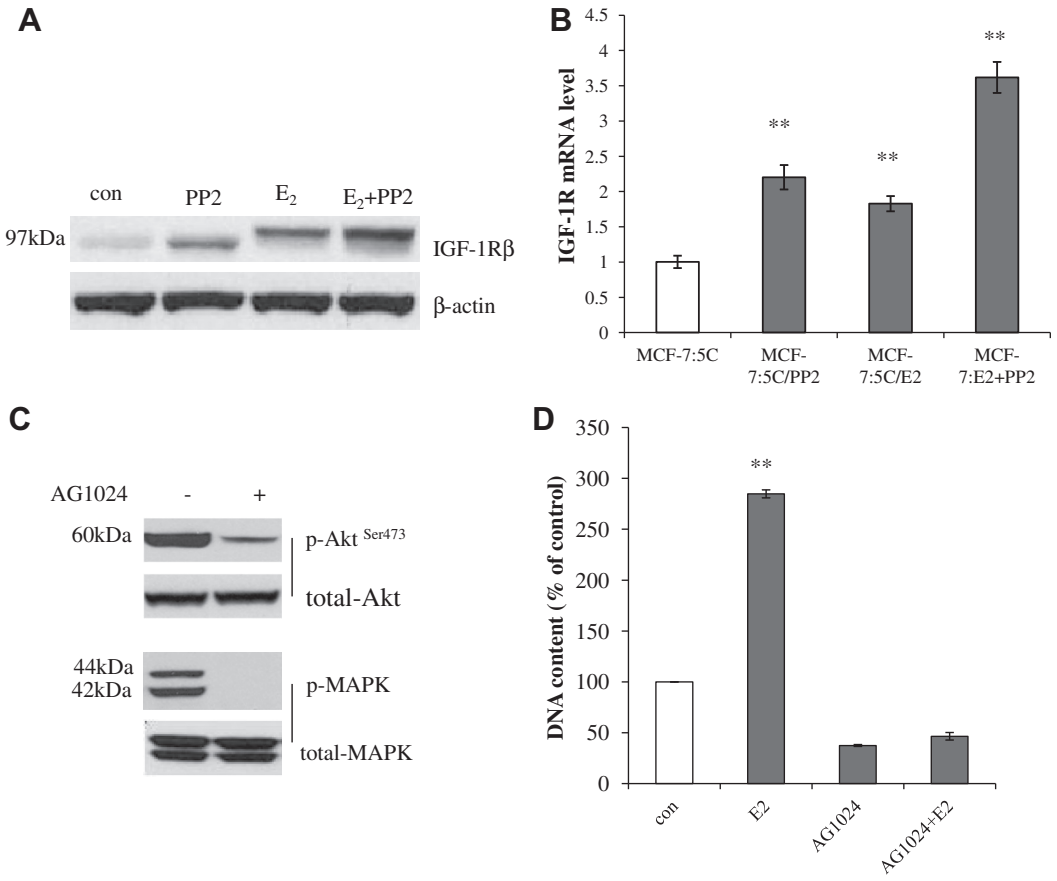

Fig. 5. The c-Src inhibitor collaborated with oestrogen (E_2) to elevate insulin-like growth factor-1 receptor beta (IGF-1Rβ). (A) IGF-1Rβ changes after long-term treatment. Cell lysates of cells treated in different combinations were harvested. IGF-1Rβ was examined by immunoblotting. β-Actin was detected for loading control. (B) IGF-1Rβ mRNA changes were consistent with protein levels. The RNA of differently long-term treated cells was harvested as above. $P < 0.001$, **compared with control. (C) Activation of Akt and mitogen-activated protein kinase (MAPK) pathways by IGF-1Rβ in MCF-7:PF cells. MCF-7:PF cells were treated with vehicle (0.1% dimethyl sulfoxide (DMSO)) and AG1024 (10^{-5}mol/L) for 48 h. Cell lysates were harvested. Phosphorylated Akt and MAPK were determined by immunoblotting. Total Akt and MAPK were examined for loading controls. (D) The IGF-1R inhibitor completely blocked E_2 stimulation in MCF-7:PF cells. MCF-7:PF cells were treated with vehicle (0.1% EtOH), E_2 (10^{-9}mol/L), AG1024 (10^{-5}mol/L) and E_2 (10^{-9} mol/L) plus AG1024 (10^{-5} mol/L) for 7 days. The cells were harvested and DNA content was determined as above. $P < 0.001$, **compared with control. All the data shown were representative of at least three separate experiments with similar results.

MCF-7:5C cells (Fig. 6A), which are characteristic features of EMT [29]. EMT is regulated by various signal transduction pathways including extracellular signal-regulated kinase (ERK) and Wnt [30]. The c-Src inhibitor effectively blocked c-Src phosphorylation in both PP2 alone treated cells and MCF-7:PF cells (Fig. 6B), whereas long-term E_2 treated MCF-7:5C cells still maintained the higher level of phosphorylated c-Src (Fig. 6B). Although the c-Src inhibitor blocks phosphorylated MAPK in the early stage [19,20], PP2 clearly increased MAPK but continuously blocked Akt after long-term treatment (Fig. 6B). Additionally, inducers of the EMT include several transcription factors such as Snail, Twist, as well as the secreted transforming growth factor beta (TGFβ) [31]. In our cell model, the c-Src inhibitor collaborated with E_2 to increase Snail and Twist1 in MCF-7:PF cells (Fig. 6C). Both PP2 alone and E_2 alone increased mRNA levels of TGFβ, but combination treatment decreased TGFβ by an unclear mechanism (Supplementary Fig. S6C). Nevertheless, MCF-7:PF cells had higher migratory capacities than MCF-7:5C cells, evaluated using a Boyden chamber migration assay (Fig. 6D and E). All of these results suggested that multiple EMT regulators are significantly modified after long-term combination treatment.

4. Discussion

Resistance to aromatase inhibitors is an important clinical problem. We have demonstrated in the laboratory that two long-term E_2-deprived MCF-7 breast cancer cell lines respond to physiological concentrations of E_2 by triggering apoptosis [18]. This laboratory observation has clinical relevance for the prevention or treatment of E_2-deprived diseases [10,17]; however, only 30% of patients receive clinical benefit [17]. This prompted us to investigate strategies to increase the therapeutic responsiveness in aromatase inhibitor-resistant breast

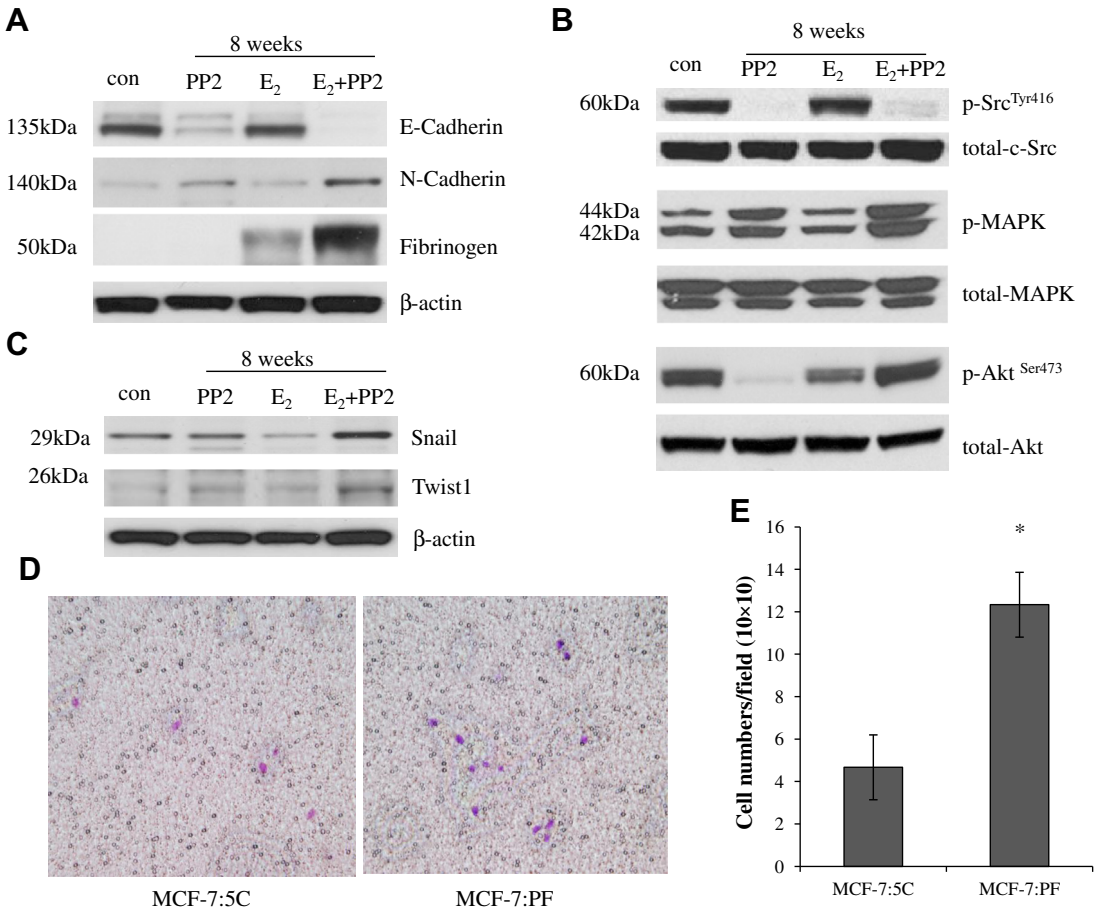

Fig. 6. The c-Src inhibitor collaborated with oestrogen (E_2) to promote epithelial–mesenchymal transition (EMT) and increase cell migration. (A) Changes of EMT biomarkers after different combinations treatment. Cell lysates of different treated cells were harvested. E-cadherin, N-cadherin and fibrinogen were examined by immunoblotting. β-Actin was detected for loading control. (B) Signalling pathway changes after different combinations treatment. Cell lysates of differently treated cells were harvested. Phosphorylated c-Src, mitogen-activated protein kinase (MAPK) and Akt were examined by immunoblotting. Total c-Src, MAPK and Akt were detected for loading controls. (C) Transcription factors, Twist1 and Snail, were up-regulated after long-term combination treatment. Cell lysates of different treated cells were harvested. Twist1 and Snail were examined by immunoblotting. β-Actin was detected for loading control. (D) Migratory capacities of MCF-7:PF cells, compared with MCF-7:5C cells. MCF-7:5C cells and MCF-7:PF cells were loaded in Boyden chambers as in Section 2. Images were taken under bright field illumination at (×10) magnification (Olympus). (E) MCF-7:PF cells had higher migratory capacities than MCF-7:5C cells. Migrated cells were stained as in Section 2. Cell numbers were counted in at least four microscopic fields at (10 × 10) magnification. $P < 0.05$, *compared with MCF-7:5C cells. All the data shown were representative of at least three separate experiments with similar results.

cancer. The oncogene c-Src is activated in E_2-deprived breast cancer cell lines [20]. Many observations highlight c-Src as an important therapeutic target to overcome endocrine resistance in breast cancer [21–24]. We chose an eight-week treatment period in the laboratory to mimic the clinical criteria to evaluate the efficacy of endocrine therapy. Unexpectedly, the c-Src inhibitor converted the E_2 response from inducing apoptosis to stimulating growth in two long-term E_2 deprived breast cancer cell lines (Fig. 2A and Supplementary Fig. S2). Most importantly, we found that the c-Src inhibitor enhanced the action of E_2 to up-regulate IGF-1Rβ which, in turn, promoted the MCF-7:PF cells to grow (Fig. 5A and D). Furthermore, the combination treatment enhanced embryonic transcription factors and repressed E-cadherin expression (Fig. 6A and C), a characteristic feature of EMT in the generation of invasive tumour cells.

We sought to find the mechanisms by which the c-Src inhibitor blocked the apoptosis-induced by E_2 after long-term combination treatment. Our recent observations show that the c-Src inhibitor effectively blocks oxidative stress and extrinsic apoptotic pathways induced by E_2 within 72 h [19] since these pathways are mediated by the c-Src tyrosine kinase. Paradoxically, physiological levels of E_2 still were able to induce apoptosis in long-term PP2 treated cells as in the original MCF-7:5C cells when the drug was washed out (Fig. 2A). We further found that c-Src phosphorylation was gradually recovered after withdrawal of PP2 from the medium (Supplementary Fig. S7A). Including PP2 in the medium could completely abolish E_2-induced

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apoptosis in MCF-7:5C/PP2 treated cells (Supplementary Fig. S7B). These data confirmed that c-Src phosphorylation is required for E_2-induced apoptosis [19,20] which is a critical initial protective response for cell survival. However, unlike 4-hydroxytamoxifen, the c-Src inhibitor cannot completely block apoptosis-induced by E_2 during 72 h of exposure [19]. This implies that other adaptation responses can potentially occur in cell populations after long-term combination treatment. It is well known that growth factor receptors crosstalk with c-Src and the ER pathways in breast cancer cells [22,28]. E_2 started to increase IGF-1Rβ levels after 4 h treatment which could be blocked by 4-hydroxytamoxifen in MCF-7:5C cells (Supplementary Fig. S5B and C), demonstrating an ER-dependent mechanism [32]. Blockade of c-Src further enhanced levels of IGF-1Rβ (Fig. 5A). Another important growth factor receptor, epidermal growth factor receptor (EGFR), was up-regulated by both the c-Src inhibitor and E_2 (Supplementary Fig. S6A). However, down-regulation of EGFR was found after combination treatment (Supplementary Fig. S6A). Importantly, blockade of EGFR had no inhibitory effects on MCF-7:PF cells, whereas inhibition of IGF-1R effectively blocked cell growth and completely abolished proliferation induced by E_2 (Fig. 5D and Supplementary Fig. S6B). These results highlighted the importance of phosphorylated IGF-1R in the mediation of E_2-stimulated growth. To determine whether autophosphorylation of IGF-1R by ligand IGF-1 plays an important role in the activation of receptor [33], we observed that IGF-1 levels were almost undetectable and neither E_2 nor PP2 up-regulated the IGF-1 expression in our cell models through real-time PCR (data not shown). It is also necessary to note that IGF-1R is required for the activation of Akt (Fig. 5C) which is also an important pathway to suppress apoptosis [34].

In addition to up-regulation of IGF-1Rβ, expression of PR was strictly regulated by E_2 (Fig. 3C and D). Interestingly, the function of PR was quite different between cells treated with E_2 alone and MCF-7:PF cells (Fig. 4A), even though they had similar PR protein levels (Fig. 3C). Our preliminary data suggested that this may be related to PR phosphorylation on Ser294 by extracellular signalling ERK/MAPK (Fig. 4B and C). Consistently, other groups have reported that PR transcriptional activity is regulated by a balance between the degree of PR phosphorylation and sumoylation which can dramatically alter genetic expression [26,35]. In this study, the c-Src inhibitor activated MAPK signal (Fig. 6B) which increased transcriptional activity of PR induced by E_2 and finally activated the response to progestin in MCF-7:PF cells (Fig. 4A and B).

We addressed the question of why the c-Src inhibitor increased the extracellular signalling ERK/MAPK in MCF-7:5C cells. Our recent publication [20] shows that the c-Src inhibitor exerts different effects on two basic growth pathways, Akt and MAPK, in different breast cancer cell lines. The c-Src inhibitor continuously inhibited Akt pathway (Fig. 6B) but transiently blocked MAPK in MCF-7:5C cells (Fig. 6B) [19,20]. The association of c-Src with the membrane cytoskeleton has been well documented [36]. Evidence implicates [37] a role for c-Src in the regulation of the formation of focal adhesions and the extracellular matrix to affect subsequent signalling pathways. In our study, the c-Src inhibitor disrupted E-cadherin-mediated cell–cell adhesion and made the cell gain mesenchymal cell markers such as N-cadherin and fibrinogen (Fig. 6A), a characterised feature of EMT. Deposition of fibrinogen into the extracellular matrix serves as a scaffold to support binding of growth factors to activate extracellular signalling ERK/MAPK (Fig. 6A and B) [38]. EMT, a complex reprogramming process of epithelial cells, plays an important role in tumour invasion and metastasis [39]. Current studies show that EMT is controlled by a group of embryonic transcriptional factors, such as Zeb-1/2, Twist1 and Snail, and each of these factors is capable of directly repressing E-cadherin expression (Fig. 6C) [39,40]. These results suggested that the antioestrogen resistant breast cancer cell is clearly reprogrammed with regard to the variations of those signalling pathways. Therefore, further studies are required to uncover the precise interaction among these EMT inducers that may hold promise for developing novel strategies to inhibit EMT and cancer metastasis.

In summary, this study suggested that physiological levels of E_2 (probably the patient's own E_2) is able to induce apoptosis in long-term E_2-deprived breast cancer. However, administration with a c-Src inhibitor will cause the tumour to grow after aromatase inhibitor resistance, with a variety of signalling networks regulated by the c-Src inhibitor to promote an aggressive phenotype (Supplementary Fig. S8). These data raise a concern regarding the ubiquitous use of c-Src inhibitors in advanced aromatase inhibitor-resistant breast cancer especially when combined with E_2.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2013.10.001.

References

[1] Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007;72:7–25.
[2] Dowsett M, Cuzick J, Ingle J, et al. Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 2010;28:509–18.
[3] Sweeney EE, McDaniel RE, Maximov PY, Fan P, Jordan VC. Models and mechanisms of acquired antihormone resistance in breast cancer: significant clinical progress despite limitations. Horm Mol Biol Clin Investig 2012;9:143–63.
[4] Gottardis MM, Jiang SY, Jeng MH, Jordan VC. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res 1989;49:4090–3.
[5] Ariazi EA, Lewis-Wambi JS, Gill SD, et al. Emerging principles for the development of resistance to antihormonal therapy: implications for the clinical utility of fulvestrant. J Steroid Biochem Mol Biol 2006;102:128–38.
[6] Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 1988;48:5183–7.
[7] Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 1993;127:23–33.
[8] Yao K, Lee ES, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000;6:2028–36.
[9] Lewis-Wambi JS, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? Breast Cancer Res 2009;11:206.
[10] Anderson GL, Chlebowski RT, Aragaki AK, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. Lancet Oncol 2012;13:476–86.
[11] Obiorah I, Jordan VC. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. Menopause 2013;20:372–82.
[12] Davies C, Pan H, Godwin J, et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. Lancet 2013;381:805–16.
[13] Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 2003;95:1597–608.
[14] Song RX, Mor G, Naftolin F, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 beta-estradiol. J Natl Cancer Inst 2001;93:1714–23.
[15] Lewis JS, Meeke K, Osipo C, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 2005;97:1746–59.
[16] Liu H, Lee ES, Gajdos C, et al. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 2003;95:1586–97.
[17] Ellis MJ, Gao F, Dehdashti F, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 2009;302:774–80.
[18] Ariazi EA, Cunliffe HE, Lewis-Wambi JS, et al. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci U S A 2011;108:18879–86.
[19] Fan P, Griffith OL, Agboke F, et al. C-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. Cancer Res 2013;73:4510–20.
[20] Fan P, McDaniel RE, Kim HR, Clagett D, Haddad B, Jordan VC. Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines. Eur J Cancer 2012;48:3488–98.
[21] Hiscox S, Morgan L, Green TP, Barrow D, Gee J, Nicholson RI. Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. Breast Cancer Res Treat 2006;97:263–74.
[22] Fan P, Wang J, Santen RJ, Yue W. Long-term treatment with tamoxifen facilitates translocation of estrogen receptor alpha out of the nucleus and enhances its interaction with EGFR in MCF-7 breast cancer cells. Cancer Res 2007;67:1352–60.
[23] Chen Y, Guggisberg N, Jorda M, et al. Combined Src and aromatase inhibition impairs human breast cancer growth in vivo and bypass pathways are activated in AZD0530-resistant tumors. Clin Cancer Res 2009;15:3396–405.
[24] Hiscox S, Jordan NJ, Smith C, et al. Dual targeting of SRC and ER prevents acquired antihormone resistance in breast cancer cells. Breast Cancer Res Treat 2009;115:57–67.
[25] Sengupta S, Schiff R, Katzenellenbogen BS. Post-transcriptional regulation of chemokine receptor CXCR4 by estrogen in HER2 overexpressing, estrogen receptor-positive breast cancer cells. Breast Cancer Res Treat 2009;117:243–51.
[26] Lange CA, Shen T, Horwitz KB. Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc Natl Acad Sci U S A 2000;97:1032–7.
[27] Jeng MH, Langan-Fahey SM, Jordan VC. Estrogenic actions of RU486 in hormone-responsive MCF-7 human breast cancer cells. Endocrinology 1993;132:2622–30.
[28] Bromann PA, Korkaya H, Courtneidge SA. The interplay between Src family kinases and receptor tyrosine kinases. Oncogene 2004;23:7957–68.
[29] Nam JS, Ino Y, Sakamoto M, Hirohashi S. Src family kinase inhibitor PP2 restores the E-cadherin/catenin cell adhesion system in human cancer cells and reduces cancer metastasis. Clin Cancer Res 2002;8:2430–6.

[30] Moustakas A, Heldin CH. Signaling networks guiding epithelial–mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci 2007;98:1512–20.
[31] Taube JH, Herschkowitz JI, Komurov K, et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. Proc Natl Acad Sci U S A 2010;107:15449–54.
[32] Genua M, Pandini G, Sisci D, et al. Role of cyclic AMP response element-binding protein in insulin-like growth factor-1 receptor up-regulation by sex steroids in prostate cancer cells. Cancer Res 2009;69:7270–7.
[33] Hernández-Sánchez C, Blakesley V, Kalebic T, Helman L, LeRoith D. The role of the tyrosine kinase domain of the insulin-like growth factor-I receptor in intracellular signaling, cellular proliferation, and tumorigenesis. J Biol Chem 1995;270:29176–81.
[34] Martin MJ, Melnyk N, Pollard M, et al. The insulin-like growth factor I receptor is required for Akt activation and suppression of anoikis in cells transformed by the ETV6-NTRK3 chimeric tyrosine kinase. Mol Cell Biol 2006;26:1754–69.
[35] Holmstrom S, Van Antwerp ME, Iniguez-Lluhi JA. Direct and distinguishable inhibitory roles for SUMO isoforms in the control of transcriptional synergy. Proc Natl Acad Sci U S A 2003;100:15758–63.
[36] Avizienyte E, Wyke AW, Jones RJ, et al. Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signaling. Nat Cell Biol 2002;4:632–8.
[37] Balzer EM, Whipple RA, Thompson K, et al. C-Src differentially regulates the functions of microtentacles and invadopodia. Oncogene 2010;29:6402–8.
[38] Rybarczyk BJ, Simpson-Haidaris PJ. Fibrinogen assembly, secretion, and deposition into extracellular matrix by MCF-7 human breast carcinoma cells. Cancer Res 2000;60:2033–9.
[39] Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer Res 2008;68:3645–54.
[40] Li CW, Xia W, Huo L, et al. Epithelial–mesenchymal transition induced by TNF-α requires NF-κB-mediated transcriptional upregulation of Twist1. Cancer Res 2012;72:1290–300.

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PRECLINICAL STUDY

Cyclin dependent kinase-9 mediated transcriptional de-regulation of cMYC as a critical determinant of endocrine-therapy resistance in breast cancers

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Abstract Endocrine therapy resistance in estrogen receptor alpha positive (ERα+) breast cancers remains a major obstacle for maintaining efficacy of targeted therapies. We investigated the significance and the mechanisms involved in cMYC over-expression in a MCF7 derived panel of ERα+ breast cancer cells which can proliferate in the absence of estrogen with different sensitivities to anti-hormone therapies. We show that all the resistant cell lines tested over-express cMYC as compared to parental MCF7 cells and its inhibition lead to the differential blocking of estrogen-independent proliferation in resistant cells. Further investigation of the resistant cell line, MCF7:5C, suggested transcriptional de-regulation of cMYC gene was responsible for its over-expression. Chromatin immunoprecipitation assay revealed markedly higher recruitment of phosphorylated serine-2 carboxy-terminal domain (CTD) of RNA polymerase-II at the proximal promoter of cMYC gene, which is responsible for transcriptional elongation of the cMYC RNA. The level of CDK9, a factor responsible for the phosphorylation of serine-2 of RNA polymerase II CTD, was found to be elevated in all the resistant cell lines. Pharmacological inhibition of CDK9 not only reduced the transcripts and the protein levels of cMYC in MCF7:5C cells but also selectively inhibited the estrogen-independent growth of all the resistant cell lines. This study describes the up-stream molecular events involved in the transcriptional over-expression of cMYC gene in breast cancer cells proliferating estrogen-independently and identifies CDK9 as a potential novel drug target for therapeutic intervention in endocrine-resistant breast cancers.

Keywords Aromatase inhibitor · Cyclin dependent kinase-9 · Breast cancer · Endocrine therapy resistance · cMYC

Abbreviations

AI	Aromatase inhibitor
ChIP	Chromatin-immuno precipitation assay
CTD	Carboxy-terminal domain
E2	17β-estradiol
ERα	Estrogen receptor alpha
RT-PCR	Real time polymerase chain reaction

Introduction

Resistance to endocrine therapies (tamoxifen and aromatase inhibitors) represents a major clinical concern for the survivorship of the estrogen receptor positive breast cancer patients [1–3]. The majority of hormone receptor positive advanced breast cancer patients report disease progression within 2–3 years of endocrine therapy treatment [4–6]. Recent clinical studies have found over-expression of the cMYC oncogene and the genes regulated by cMYC as one of the major predictor in the aromatase inhibitor resistant breast cancers [7–9] whereas its over-expression is sufficient to confer resistance to anti-estrogens [10]. Besides

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endocrine resistance, cMYC oncoprotein have been found to regulate the expression of "poor-outcome" signature genes responsible for metastasis [11]. Gain of cMYC is also associated with the progression of invasive ductal carcinoma from the ductal carcinoma in situ [12] and amplification of cMYC in breast cancer is significantly associated with risk of relapse and death [13]. It is therefore appropriate to study the underlying molecular mechanisms which contribute to estrogen independence and acquired resistance to identify novel therapeutic targets for the endocrine therapy resistant breast cancers.

Although targeting cMYC represents an obvious therapeutic opportunity to block the growth of the resistant breast cancer cells, this has not been successful due to the lack of a drug-able domain in its 'basic helix-loop-helix' structure [14]. Additionally, unacceptable toxicity is associated with cMYC inhibition, as the protein is critically involved in proliferation and regeneration of normal adult tissues [15, 16]. Other approaches such as synthetic lethality [17] and modulating chromatin-dependent signal transduction have been used to circumvent direct targeting of cMYC [18].

To determine the relevance and mechanism of cMYC over-expression in imparting estrogen-independence to the endocrine-resistant breast cancer cells, we used a panel of MCF7 ERα+ breast cancer cells which are known to proliferate in the absence of estrogen and exhibit different sensitivities to the anti-hormone therapies. The different MCF7 cell line derivatives used were MCF7:5C [19], MCF7:2A [20], MCF7/LCC1 [21], MCF7/LCC2 [22], and MCF7/LCC9 [23, 24]. All these cells mimic aromatase inhibitor resistance as they can grow in an estrogen-deprived condition. In addition, MCF7:5C and LCC2 cells are also resistant to anti-estrogens, 4-hydroxy-tamoxifen (4OHT) whereas LCC9 cells demonstrate resistance to 4OHT and fulvestrant. All these cell lines cells showed high expression of cMYC protein as compared to parent MCF7 cells and estrogen-independent growth of all the resistant cells was drastically inhibited by a cMYC inhibitor, 10058-F4 (F4). For focused studies, we chose MCF7:5C cells as we have extensive experience with this cell line and the LCC1, LCC2, and LCC9 cells showed modest estrogen stimulation of growth [21–23] despite being estrogen-independent. On the other hand MCF7:5C cells undergo apoptosis after estrogen treatment [25, 26]. This is a documented response clinically, following the development of anti-hormone resistance [27].

This study dissects the upstream molecular mechanism involved in the transcriptional over-expression of cMYC oncogene in the endocrine-therapy resistant cells, which imparts estrogen-independence. In addition, we present CDK9 as a potential target for therapeutic intervention which can suppress the deregulated transcriptional over-expression of cMYC leading to complete inhibition of estrogen-independent proliferation of the endocrine-resistant breast cancer cells.

Materials and methods

Cell culture and reagents

Cell culture media were purchased from Invitrogen Inc. (Grand Island, NY, USA) and fetal calf serum (FCS) was obtained from HyClone Laboratories (Logan, UT, USA). The ERα+ breast cancer cells MCF-7:WS8 (mentioned as MCF7) and estrogen-deprived MCF7:5C and MCF7:2A cells were derived from MCF7 cells obtained from the Dr. Dean Edwards, San Antonio, Texas as reported previously [19]. The MCF7/LCC1, LCC2, and LCC9 were obtained from the shared tissue culture facility of the Lombardi comprehensive cancer center. The cell lines were authenticated by DNA fingerprinting. All the cells except MCF7 cells were maintained in phenol red-free RPMI media (Invitrogen Inc., Grand Island, NY, USA) supplemented with 10 % charcoal dextran treated FCS, 6 ng/ml bovine insulin and penicillin and streptomycin. MCF7 cells were maintained in phenol red containing media with 10 % FCS. Three to four days prior to harvesting the MCF7 cells were cultivated in phenol red-free media containing 10 % charcoal dextran treated FCS. cMYC inhibitor, 10058-F4 was purchased from Sigma-Aldrich (St. Louis, MO, USA) and CDK9 inhibitor, CAN 508 (cat # 238811), was purchased from EMD Chemicals Inc. (San Diego, CA, USA). All the experiments were performed at least three times, in triplicate to confirm the results.

Cell growth assay

The cell growth assays were performed by measuring the total DNA per well in 24 well plates. Twenty to twenty five thousand cells were plated per well and treatment with indicated concentrations of compounds was started after 24 h, in triplicates. Media with specific treatments were changed every 48 h. The cells were harvested in hypotonic buffer solution followed by sonication after indicated time points. Total DNA was measured using a fluorescent dye (Hoechst 33258) in the DNA quantitation kit (Cat # 170-2480; Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions.

RNA isolation and real time PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNAeasy kit (Qiagen, Valencia, CA, USA) were used to isolate total RNA according to the manufacturer's

instructions. Real-time PCR was performed as previously described [28]. Briefly, cDNA was generated from RNA using High capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA, USA). Subsequently the cDNA was diluted and RT-PCR was performed using ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The change in expression of transcripts was determined as described previously using the ribosomal protein 36B4 mRNA as the internal control [28]. The primer sequences for the cMYC mRNA was 5′ GCCAGCTCTCCACACATCAG 3′ (forward); 5′ TCTTGGCAGCAGGATAGTCCTT 3′ (reverse).

Cell cycle analysis

The cells were treated with vehicle (0.1 % dimethyl sulfoxide), or cMYC inhibitor 10058-F4 at indicated concentrations and the cells were harvested and gradually fixed with 75 % EtOH on ice. Cells were stained with propidium iodide (PI), and analyzed using a fluorescence-activated cell sorter flow cytometer (Becton–Dickinson, San Jose, CA, USA), and the data analysis was performed by CellQuest software. All experiments were performed in triplicates and the graphs shown in the figures are representative of them.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously [28] with minor changes. Cells were cross-linked with paraformaldehyde and nuclei were isolated from cells which were re-suspended in SDS-lysis buffer followed by sonication and centrifugation. The supernatant were diluted 1:10 with ChIP dilution buffer. For the immuno-clearing and pull down of the immuno-complexes, protein A magnetic beads (Upstate cell signaling solutions, Temecula CA, USA) were linked to rabbit IgG raised against mouse IgM. This modification was essential to ensure effective pull-down by the anti-bodies against serine-2 phospho (Covance, Cat # MMS 129R; H5) and serine-5 phospho (Covance, Cat # MMS 134R; H14) RNA polymerase II. The beads bound to immuno-complexes were thereafter washed and precipitates were extracted twice using freshly made 1 % SDS and 0.1 M $NaHCO_3$ followed by de-crosslinking. The DNA fragments were purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA). RT-PCR was performed using 2 µl isolated DNA, using primers specific for cMYC proximal promoter. The primers used (forward: GAGCAGCAGAGAAAGGGAGA; reverse: CAGCCGAGCACTCTAGCTCT) recognizes a region ~150 bp upstream of transcription start site (TSS) of cMYC gene. The data is presented as percent input of starting chromatin input after subtracting the percent input pull down of the negative control (normal mouse IgM).

Western blotting

Whole cell protein lysates were isolated using RIPA buffer containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors I and II (EMD Chemicals Inc., San Diego, CA, USA). 15–20 µg of total protein was separated on the gels and transferred onto nitrocellulose membranes. The membranes were blocked with 5 % non-fat dry milk in tris-buffered saline and probed with primary and secondary antibodies. Specific bands were visualized using west-pico chemi-luminescence (Thermo-Fisher, Rockford, IL, USA). The antibodies used: cMYC (#5605), CDK9 (#2316), phospho-CDK9 (#2549), from Cell signaling Technologies (Danvers, MA); CTDP1 (#A301-172 A) Bethyl laboratories (Montgomery, TX, USA); beta-actin (#A5441; Sigma, St. Louis, MO). The bands were scanned and quantified using imageJ software (National Institutes of Health, Bethesda, MD, USA).

Relapse free survival (RFS) analysis

Kaplan–Meier plots for RFS analysis were generated using the on-line tool "kmplot.com" which has the annotated data set from various breast cancer studies and allows studying single gene association with RFS outcome of the patients using user defined parameters. To evaluate the effect of cMYC overexpression on RFS of endocrine-therapy versus chemotherapy treated breast cancer patients we compared the top 25 % patients expressing highest cMYC levels with the rest of the patient population. Two different plots were generated, one where the patients were treated with endocrine therapy (excludes chemotherapy) and the other with patients treated with chemotherapy (excludes endocrine-therapy). All other parameters were unchanged.

Statistics

Statistical significance of our data was assessed using the Student's *t* test wherever relevant. A p value of <0.05 was considered as statistically significant.

Results

Levels of cMYC and estrogen-independent growth of ERα+ endocrine resistant breast cancer cells

We found that all the endocrine-therapy resistant breast cancer cells used in this study, namely, MCF7:5C, MCF7:2A, MCF7/LCC1, MCF7/LCC2, and MCF7/LCC9 cells overexpress cMYC mRNA (Fig. 1a) and protein (Fig. 1b) as compared to parental MCF7 cells. All the

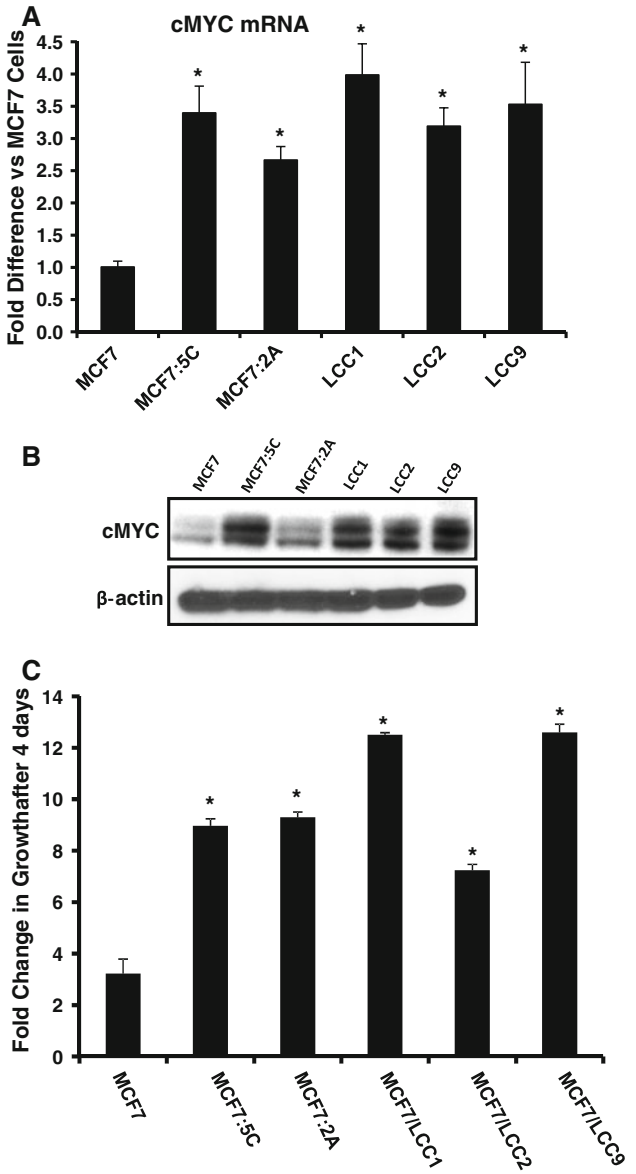

Fig. 1 Levels of cMYC and estrogen independent growth of endocrine therapy resistant breast cancer cells. **a** cMYC mRNA levels were measured in different MCF7 derivative endocrine therapy resistant cells using RT-PCR. Data is represented as fold difference in cMYC mRNA versus MCF7 cells. **b** Western blot of cMYC protein in MCF7 and. Beta actin was used as a loading control. **c** Estrogen independent growth of MCF7 and other endocrine therapy resistant breast cancer cells over a 4 day period. Untreated cells were grown, and total DNA was measured on day 4 after seeding. The data is represented as fold change in growth versus day '0'. (*$p < 0.05$ vs. MCF7 cells)

resistant cells showed ~3–4 fold higher growth as compared to the parental MCF7 cells (Fig. 1c) over a 4 day period. Cell cycle analysis of MCF7:5C cells revealed more than twofold higher "S" phase cells than in MCF7 cells (Supplementary Fig. S1b) and fivefold higher proliferation over a 6 day period (Supplementary Fig. S1a).

To determine if the high levels of cMYC mRNA was due to the elevated transcriptional activity or stability of the transcripts we performed a pulse chase assay and found that the cMYC mRNA had a similar rate of degradation in MCF7 and MCF7:5C cells (Supplementary Fig. 3).

Inhibition or depletion of cMYC blocks estrogen-independent proliferation of ERα+ endocrine resistant cells

We determined the functional role of cMYC over-expression in estrogen-independent growth of the endocrine-therapy resistant breast cancer cells by blocking the cMYC action using a pharmacological inhibitor 10058-F4 which has been shown to specifically inhibit actions of cMYC by blocking its interaction with MAX [29] and stabilizing the MYC monomer [30]. cMYC inhibition with 30 μM of 10058-F4 selectively inhibited 50–80 % of the estrogen-independent growth of all the resistant cells (Fig. 2a) whereas only 18 % growth inhibition was observed in MCF7 cells. Further experiments with MCF7:5C cells showed that 10058-F4 was selectively able to inhibit its growth in a dose-dependent manner as compared to MCF7 cells over a 4 day period (Fig. 2b). Cell cycle analysis confirmed that the decrease in proliferation resulted from a 57 % reduction in the 'S' phase cells of the MCF7:5C cells (Fig. 2c). In comparison, there was only 6 % decrease in the 'S' phase cells of the parental MCF7 cells. We also used the targeted approach to confirm the role of cMYC in MCF7:5C cells, by depleting cMYC levels using short interfering RNA (siRNA). Two different siRNA against cMYC depleted the levels of its protein in MCF7:5C cells which led to 50–75 % reduction in the number of 'S' phase cells (Fig. 2d) with a concurrent inhibition of cell growth over a period of 4 days (Supplementary Fig. S2a). Reduced phosphorylation of retinoblastoma protein (Supplementary Fig. S2b) was also evident in the cells depleted of cMYC protein.

cMYC gene expression correlates with RFS in endocrine therapy but not chemotherapy treated patients

The Kaplan–Meier plots were generated for cMYC gene association with RFS of early breast cancer patients who received endocrine-therapy or chemotherapy only as an adjuvant treatment. We used the on-line tool (www.kmplot.com) which has a combined data set from various annotated breast cancer studies and can be used to study the association of a single gene with patients outcome using various user defined parameters [31]. The top 25 % percent highest cMYC expressing patients (top quartile) were compared with the rest of the 75 %. Kaplan–Meier plots

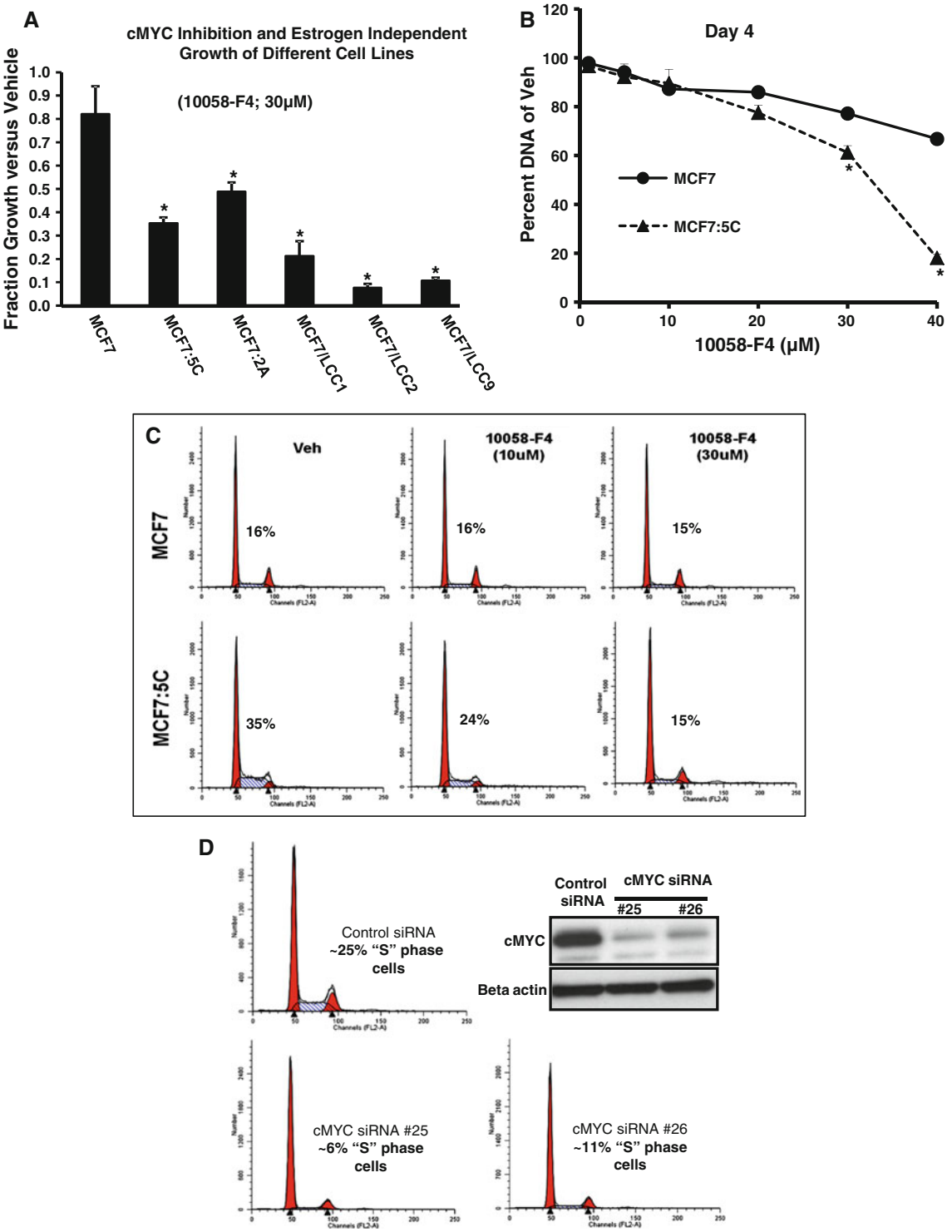

Fig. 2 Inhibition or depletion of cMYC blocks estrogen independent growth of endocrine therapy resistant breast cancer cells. **a** Total DNA was measured from the MCF7 and the resistant breast cancer cells after 4 days of treatment with 30 μM, cMYC inhibitor (10058-F4). (*$p < 0.05$ vs. MCF7 cells). **b** Total DNA was measured from the MCF7 and MCF7:5C cells after 4 days of treatment with cMYC inhibitor (10058-F4) with indicated concentration. (*$p < 0.05$ vs. MCF7 cells). **c** "S" phase cells were assessed using cell cycle analysis of MCF7 and MCF7:5C cells treated with indicated concentration of cMYC inhibitor for 24 h. The *numbers* on each graph represents the percentage of "S" phase cells. **d** Assessment of "S" phase cells using cell cycle analysis 48 h after siRNA mediated depletion of cMYC using two different siRNA (#25 and #26). The inset shows the western blot of cMYC protein levels after depletion of cMYC

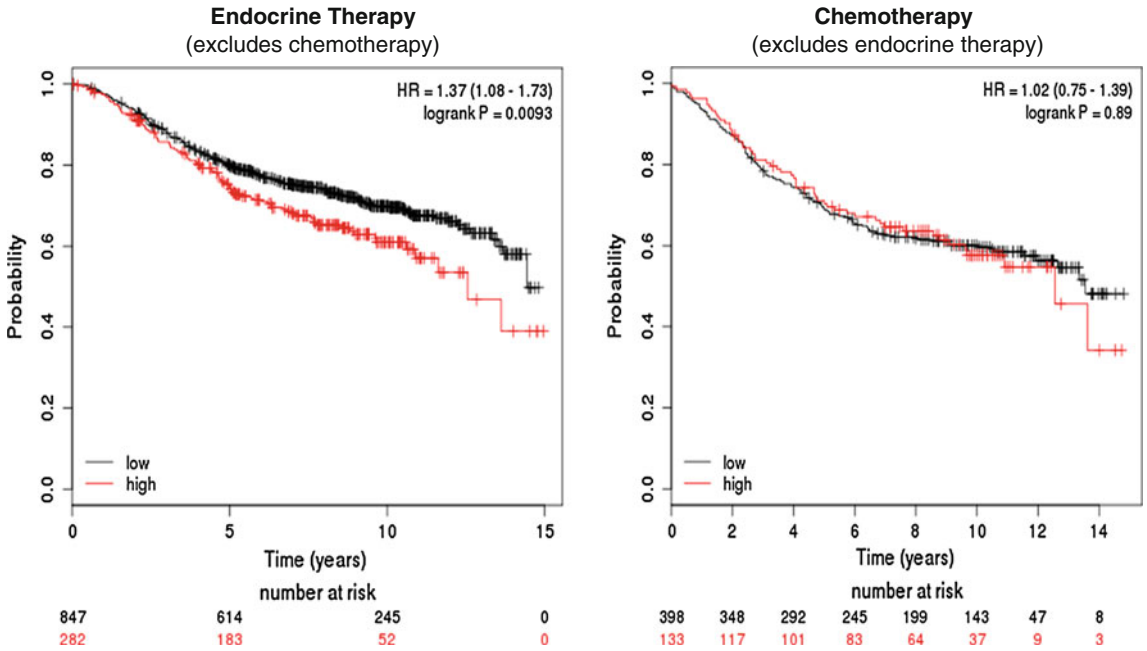

Fig. 3 cMYC gene expression correlates with relapse free survival (RFS) in endocrine therapy but not chemotherapy treated patients. The Kaplan–Meier plots show the association of cMYC gene expression and RFS in endocrine therapy or chemotherapy treated ERα+ breast cancer patients. The *top* 25 % percent highest expressing cMYC patients (*top quartile*; in *red*) were compared with the rest of the 75 % patient population (in *black*)

(Fig. 3) reveal that high levels of cMYC expression is associated with poor RFS (*p* value 0.0093) in 1129 patients treated with endocrine therapy only (Tamoxifen or AIs), whereas this association was not observed in the 531 patients (*p* value 0.89) treated with chemotherapy only.

Recruitment of phospho-serine-2 and phospho-serine-5 RNA polymerase II at the cMYC promoter in MCF7:5C and MCF7 Cells

To further determine the mechanism of steady-state transcriptional over-expression of the cMYC mRNA in MCF7:5C cells, we probed the proximal promoter of the cMYC gene (Fig. 4a) in terms of recruitment of phosphorylated serine-5 and phosphorylated serine-2 RNA polymerase II, which is responsible for the initiation and the elongation of the transcription of RNA, respectively. ChIP assay using phospho-specific RNA polymerase II antibodies revealed that in MCF7:5C cells the recruitment of serine-2 phosphorylated RNA polymerase II was more than threefold higher than parental MCF7 cells (Fig. 4b). However, no difference was observed in the recruitment of serine-5 phosphorylated RNA polymerase II at the cMYC promoter in MCF7:5C and MCF7 cells (Fig. 4c). We further confirmed that the total levels of phosphorylated serine-2 or serine-5 RNA polymerase was not different in MCF7:5C cells as compared to MCF7 cells (Fig. 4d).

Levels of cyclin dependent kinase 9 (CDK9) and its role in estrogen-independent growth of endocrine-therapy resistant cells

CDK9 is a major kinase which is responsible for the phosphorylation of serine-2 RNA polymerase II [32, 33] and the elongation of RNA transcripts [34]. We therefore examined the total CDK9 levels in the endocrine-therapy resistant cells and observed an over-expression in all the cells as compared to the MCF7 cells (Supplementary Fig. 4Sa). In MCF7:5C cells, the total as well as the phosphorylated CDK9 levels were elevated by 2.5 and 3.1 fold respectively (Fig. 5a). We also observed a slight increase in the levels of CTDP/FCP1 protein in MCF7:5C cells, which is known to dephosphorylate CDK9 [32] (Fig. 5a). Interestingly, FCP1 has also been reported to stimulate transcription elongation [35]. Next, we used a specific, potent, competitive inhibitor of CDK9, known as CAN 508 [36] to study the role of CDK9 in estrogen-independent growth of MCF7:5C cells and compared it with the parental MCF7 cells. A dose dependent effect was observed in MCF7:5C cells where 30 μM of CAN 508 compound completely inhibited its growth over a 6 day period (Fig. 5b). Furthermore, 30 μM of CAN 508 drastically blocked the growth of all endocrine-therapy resistant breast cancer cells used in this study (Supplementary Fig. 4Sb) whereas it had minimal growth inhibitory effect on the parental MCF7 cells.

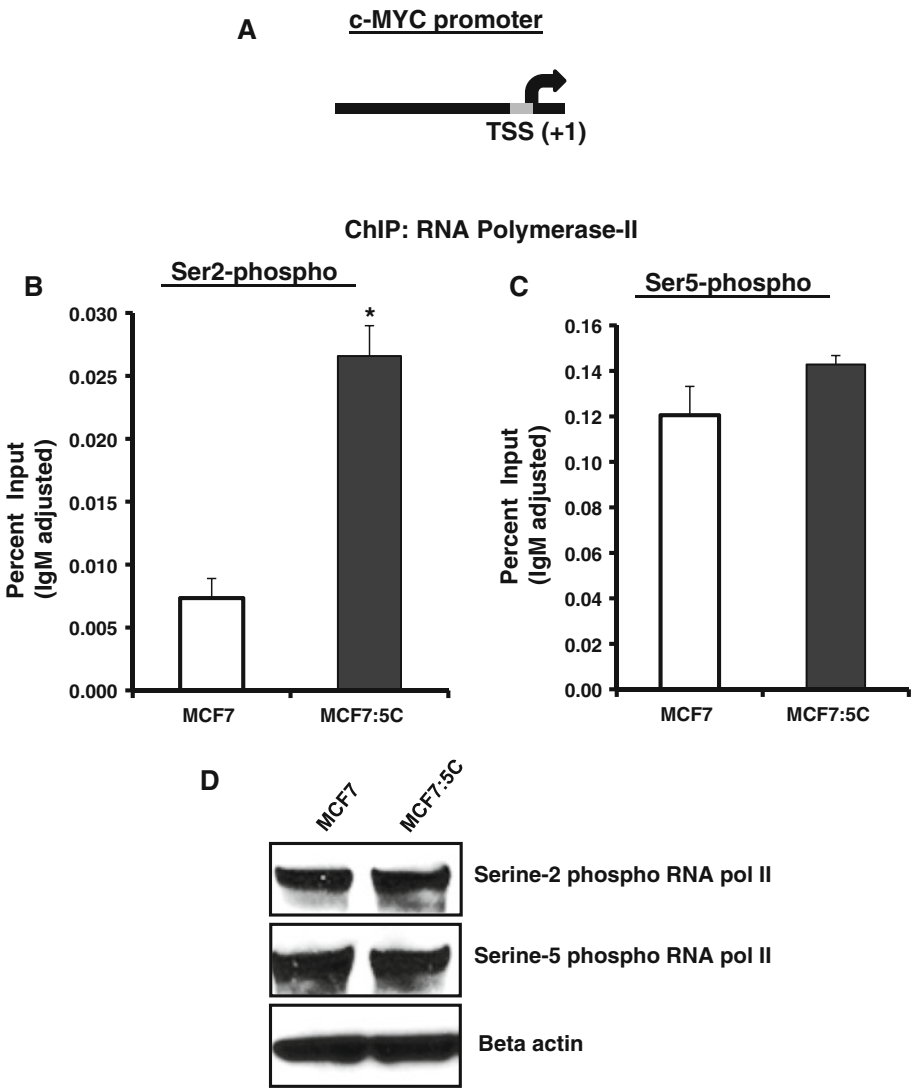

Fig. 4 Recruitment of serine-5 and serine-2 -phosphorylated RNA polymerase II at the cMYC promoter. **a** Schematic presentation of cMYC promoter showing the TSS. The *grey box* represents the region (~150 bp upstream of TSS) probed using real-time PCR following ChIP assay. b Recruitment of serine-2 phosphorylated RNA polymerase II and **c** serine-5 phosphorylated RNA polymerase II was assessed by ChIP assay followed by real-time PCR in MCF7: and MCF7:5C cells. Values are represented as percent input of the starting chromatin, adjusted for control IgM recruitment for each sample. (*$p < 0.05$ vs. MCF7 cells). **d** Total protein levels of serine-2 and serine-5 phosphorylated RNA polymerase II in MCF7 and MCF7:5C cells

CDK9 inhibition blocks transcription of cMYC RNA and levels of cMYC protein in MCF7:5C cells

Inhibition of CDK9 in MCF7:5C cells by using CAN 508, resulted in approximately 60 % decrease in cMYC mRNA within 1 h of treatment (Fig. 6a). This was followed by time dependent decline in cMYC protein levels (Fig. 6b). Concomitant inhibition of serine-2 phosphorylated RNA polymerase II CTD was also observed within an hour of treatment (Fig. 6b) indicating its role in cMYC transcription. As evident, serine-5 phosphorylation of RNA polymerase II CTD was not much altered within 4 h of CDK9 inhibition. Although later time points showed marked reduction in serine-5 phosphorylation, along with serine-2 phosphorylation which was most likely due to secondary effects of CDK9 inhibition. Inhibition of CDK9 also completely blocked the phosphorylation of retinoblastoma (Rb) protein within 12 h of treatment (Supplementary Fig. S5) in the MCF7:5C cells.

Discussion

Accumulative evidence indicates that cMYC overexpression and subsequent genes up-regulated in breast cancers are associated with resistance to AIs [8] and antiestrogens [7, 9]. This study establishes the role and mechanism of cMYC regulation in the estrogen-independent growth of ERα+, endocrine-resistant breast cancer cells. All the resistant cell models used in this study are MCF7-derived cell lines. Importantly, MCF7 cells retain the ERα protein after acquiring endocrine therapy resistance which mimics the clinical scenario as 80 % of the endocrine-therapy resistant breast cancer patients are ERα positive [37]. Interestingly, despite the limited availability of cell lines, significant translational advances have occurred [24]. Based on our results, we decipher a novel mechanism of transcriptional over-expression of cMYC in resistant breast cancer cells (Fig. 6c) which involves CDK9 mediated hyper-phosphorylation of serine-2 RNA polymerase-II

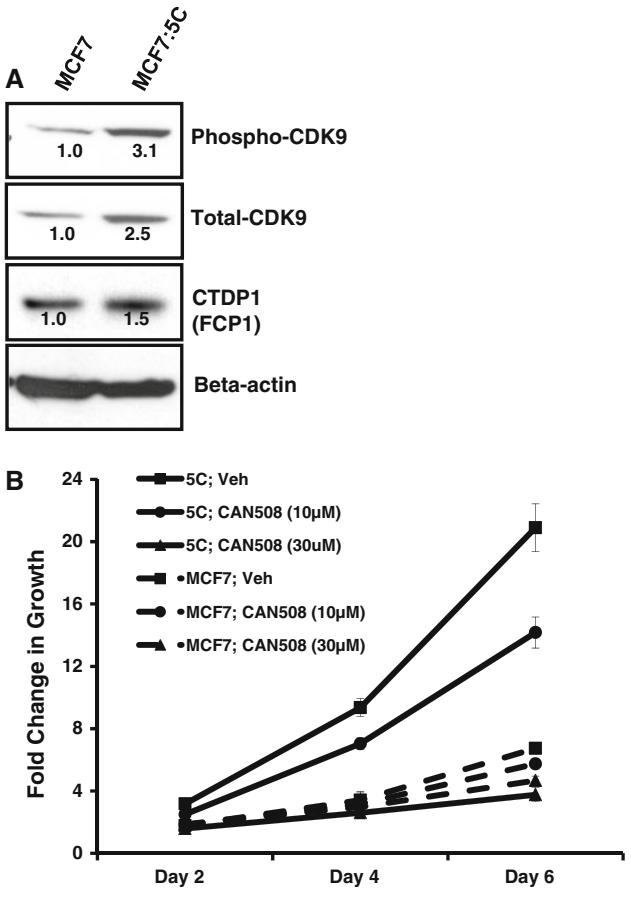

Fig. 5 Total CDK9 levels and effect of its inhibition on estrogen-independent growth. **a** Protein levels of phospho and total CDK9 and CTDP1 was assessed using western blotting in MCF7 and MCF7:5C cells. The numbers above each band correspond to the fold change in protein levels versus MCF7 cells adjusted for beta actin levels for each sample. **b** Total DNA was measured to assess the growth of MCF7 and MCF7:5C cells after 2, 4, and 6 days of treatment with indicated doses of the CDK9 inhibitor, CAN508

CTD at the promoter of cMYC gene. This, in turn, is responsible for the transcriptional elongation and overexpression of cMYC. Our analysis of the annotated breast cancer patient's database (Fig. 3) suggested that overexpression of cMYC correlates with the failure of endocrine therapy (but not chemotherapy) and eventual relapse of the disease.

Ectopic overexpression of cMYC in MCF7 cells is reported to be sufficient to confer resistance to endocrine therapy [7, 10]. We observed elevated cMYC levels in the ERα+, endocrine therapy–resistant breast cancer cells (Fig. 1a, b) which proliferated in the absence of estrogen. A previous study has also reported high cMYC levels in long-term estrogen deprived cells [38]. Inhibition of cMYC or its depletion blocked the proliferation of the cells (Fig. 2a, Supplementary Fig. S2a) demonstrating the critical role of cMYC overexpression in estrogen-independent growth of these resistant breast cancer cells. The reduction in 'S' phase cells (Fig. 2c, d) was achieved by de-phosphorylation of tumor suppressor retinoblastoma (Rb) protein (Supplementary Fig. S2b) which is known to arrest the cells in G1 phase of the cell-cycle [39].

Further, using a pulse chase assay, we ascertained that the high basal level of cMYC mRNA in the MCF7:5C cells was due to the high rate of transcription and not enhanced stability of the transcripts (Fig. S3). Since therapeutic targeting of cMYC is not feasible, we studied the upstream factors responsible for cMYC transcriptional over-expression from its natural proximal promoter in the MCF7:5C cells. Transcription of cMYC gene is regulated at the elongation step by promoter-proximal pausing of RNA polymerase II in eukaryotes [40, 41]. Importantly, cMYC is a well-defined estrogen-regulated gene [42] and the estrogen-induced growth of the hormone responsive breast cancer cells is contingent upon the expression of cMYC gene in these cells as majority of growth related genes which are estrogen regulated are cMYC target [43]. In MCF7 cells, studies have demonstrated [44] that the proximal promoter of the cMYC gene is pre-loaded with RNA polymerase II which is phosphorylated at serine 5 of its CTD, in the absence of estrogen. However, phosphorylation of serine-2 of CTD of RNA polymerase II is needed to overcome the elongation block of the transcripts which is achieved after estrogen stimulation. Our findings are consistent. In MCF7 cells, we observed high levels of serine-5 phosphorylation, and low serine-2 phosphorylation of RNA polymerase II CTD at the cMYC promoter under basal conditions (Fig. 4b, c). In contrast, under identical condition, the phosphorylation of serine-2 of CTD of RNA polymerase II is markedly elevated in MCF7:5C cells (Fig. 4a, b) which drives the higher transcriptional elongation of cMYC.

The kinase complex responsible for the phosphorylation of serine-2 of RNA polymerase II CTD and inducing transcriptional elongation is known as positive transcriptional elongation factor-b which is composed of CDK9 and cyclin T1 [45–47]. Our observation of higher levels of CDK9 in MCF7:5C cells (Fig. 5a) and in other resistant breast cancer cells (Supplementary Fig. S4a) strongly suggested that it is responsible for elevated serine-2 phosphorylation of RNA polymerase II CTD at the cMYC promoter of MCF7:5C cells. Indeed, using a pharmacological agent, CAN 508, which specifically inhibits CDK9 activity [36, 48], the growth of MCF7:5C cells (Fig. 5b) as well as other endocrine therapy resistant MCF7 derived ERα + breast cancer cells (Supplementary Fig. S4b) were selectively inhibited. This demonstrated that the estrogen-independent growth of the endocrine therapy resistant breast cancer cells was driven by CDK9. We further

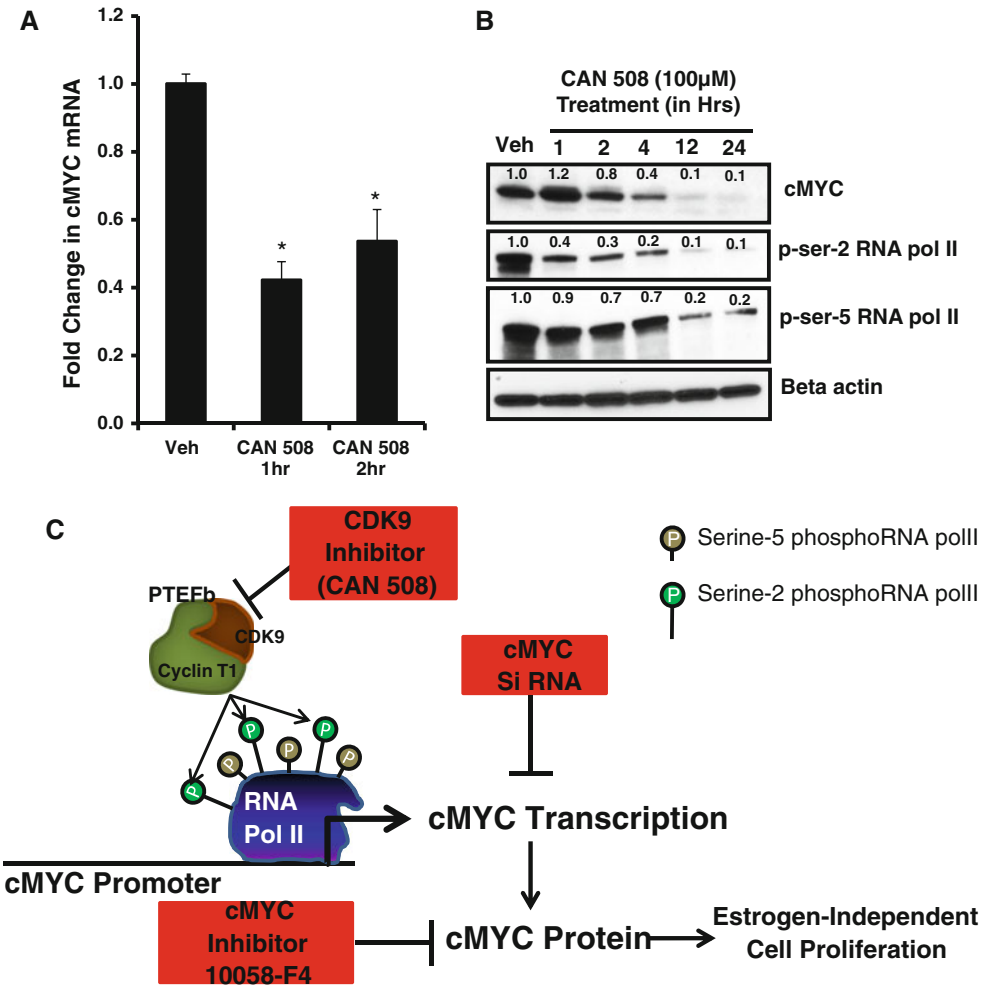

Fig. 6 Reduction of cMYC mRNA and protein by CDK9 inhibition and the proposed model of cMYC transcriptional regulation in MCF7:5C cells. **a** Levels of cMYC mRNA was measured by quantitative RT-PCR in MCF7:5C cells after one and 2 h of CDK9 inhibition by 100 μM of CAN508. (*$p < 0.05$ vs. vehicle (Veh) treatment). **b** Protein levels of cMYC, phospho-serine-2, and serine-5 RNA polymerase II after inhibition of CDK9 by 100 μM of CAN508 for indicated time points. The numbers above each band correspond to the fold change in protein levels versus vehicle (Veh) treatment adjusted for beta actin levels for each sample. **c** The cartoon depicts our findings on the CDK9 mediated cMYC transcriptional regulation and its role in estrogen-independent growth of the MCF7:5C cells

confirmed that inhibition of CDK9 led to the reduction of cMYC mRNA levels within 1 h of treatment in MCF7:5C cells followed by the protein level (Fig. 6a, b). The concurrent decrease in global serine-2 (but not serine-5) phosphorylation of RNA polymerase II CTD (Fig. 6b) suggested that CDK9 was responsible for cMYC transcriptional over-expression in the resistant cells. In addition, we confirmed that CDK9 inhibition reduced the level of phospho-Rb protein (Supplementary Fig. S5) in a similar manner as cMYC depletion in the MCF7:5C cells. This supports our hypothesis that the growth suppressive effect of CDK9 inhibition reduces cMYC levels in the endocrine-therapy resistant breast cancer cells. Furthermore, we found that CDK9 or cMYC inhibition was not deleterious to the immortalized human epithelial cells (MCF10A) (Supplementary Fig. S6) indicating that CDK9 can be a potential novel therapeutic target.

Since we did not detect any difference in the global level of serine-2 phosphorylated RNA polymerase II CTD between MCF7:5C and its parental MCF7 cells (Fig. 4d); further studies are required to establish the chromatin modifications at the cMYC promoter which ensue in the process of acquiring resistance. These changes are crucial as it allows the RNA polymerase II CTD to be hyper-permissive for serine-2 phosphorylation, thus ensuring elongation of the cMYC transcripts in the MCF7:5C cells. Intriguingly, recent reports have indicated that in hematologic malignancies bromo-domain containing protein 4 (BRD4), which has been known to recruit CDK9 and regulate serine-2 phosphorylation of RNA polymerase II [49, 50], is involved in cMYC overexpression [18, 51].

In this study, we have delineated the transcriptional mechanism of cMYC over-expression, endocrine-therapy resistant, ERα+ breast cancer cells, and propose that recruitment of hyper-phosphorylated serine-2 RNA polymerase II at the cMYC promoter which is mediated by CDK9, is responsible for the estrogen independent proliferation of these cells. We therefore suggest that there will be a potential clinical benefit by using CDK9 inhibitors in the treatment of endocrine therapy resistant breast cancers.

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Disclosures: None.

References

1. Sengupta S, Jordan VC (2008) Selective estrogen modulators as an anticancer tool: mechanisms of efficiency and resistance. Adv Exp Med Biol 630:206–219
2. Obiorah I, Jordan VC (2011) Progress in endocrine approaches to the treatment and prevention of breast cancer. Maturitas 70(4):315–321. doi:10.1016/j.maturitas.2011.09.006
3. Jordan VC, O'Malley BW (2007) Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 25(36):5815–5824. doi:10.1200/JCO.2007.11.3886
4. Chia S, Gradishar W, Mauriac L, Bines J, Amant F, Federico M, Fein L, Romieu G, Buzdar A, Robertson JF, Brufsky A, Possinger K, Rennie P, Sapunar F, Lowe E, Piccart M (2008) Double-blind, randomized placebo controlled trial of fulvestrant compared with exemestane after prior nonsteroidal aromatase inhibitor therapy in postmenopausal women with hormone receptor-positive, advanced breast cancer: results from EFECT. J Clin Oncol 26(10):1664–1670. doi:10.1200/JCO.2007.13.5822
5. Nabholtz JM, Buzdar A, Pollak M, Harwin W, Burton G, Mangalik A, Steinberg M, Webster A, von Euler M (2000) Anastrozole is superior to tamoxifen as first-line therapy for advanced breast cancer in postmenopausal women: results of a North American multicenter randomized trial. Arimidex Study Group. J Clin Oncol 18(22):3758–3767
6. Baselga J, Campone M, Piccart M, Burris HA 3rd, Rugo HS, Sahmoud T, Noguchi S, Gnant M, Pritchard KI, Lebrun F, Beck JT, Ito Y, Yardley D, Deleu I, Perez A, Bachelot T, Vittori L, Xu Z, Mukhopadhyay P, Lebwohl D, Hortobagyi GN (2012) Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med 366(6):520–529. doi:10.1056/NEJMoa1109653
7. McNeil CM, Sergio CM, Anderson LR, Inman CK, Eggleton SA, Murphy NC, Millar EK, Crea P, Kench JG, Alles MC, Gardiner-Garden M, Ormandy CJ, Butt AJ, Henshall SM, Musgrove EA, Sutherland RL (2006) c-Myc overexpression and endocrine resistance in breast cancer. J Steroid Biochem Mol Biol 102(1–5):147–155. doi:10.1016/j.jsbmb.2006.09.028
8. Ellis MJ, Ding L, Shen D, Luo J, Suman VJ, Wallis JW, Van Tine BA, Hoog J, Goiffon RJ, Goldstein TC, Ng S, Lin L, Crowder R, Snider J, Ballman K, Weber J, Chen K, Koboldt DC, Kandoth C, Schierding WS, McMichael JF, Miller CA, Lu C, Harris CC, McLellan MD, Wendl MC, DeSchryver K, Allred DC, Esserman L, Unzeitig G, Margenthaler J, Babiera GV, Marcom PK, Guenther JM, Leitch M, Hunt K, Olson J, Tao Y, Maher CA, Fulton LL, Fulton RS, Harrison M, Oberkfell B, Du F, Demeter R, Vickery TL, Elhammali A, Piwnica-Worms H, McDonald S, Watson M, Dooling DJ, Ota D, Chang LW, Bose R, Ley TJ, Piwnica-Worms D, Stuart JM, Wilson RK, Mardis ER (2012) Whole-genome analysis informs breast cancer response to aromatase inhibition. Nature 486(7403):353–360. doi:10.1038/nature11143
9. Miller TW, Balko JM, Ghazoui Z, Dunbier A, Anderson H, Dowsett M, Gonzalez-Angulo AM, Mills GB, Miller WR, Wu H, Shyr Y, Arteaga CL (2011) A gene expression signature from human breast cancer cells with acquired hormone independence identifies MYC as a mediator of antiestrogen resistance. Clin Cancer Res 17(7):2024–2034. doi:10.1158/1078-0432.CCR-10-2567
10. Venditti M, Iwasiow B, Orr FW, Shiu RP (2002) c-Myc gene expression alone is sufficient to confer resistance to antiestrogen in human breast cancer cells. Int J Cancer 99(1):35–42. doi:10.1002/ijc.10269
11. Wolfer A, Wittner BS, Irimia D, Flavin RJ, Lupien M, Gunawardane RN, Meyer CA, Lightcap ES, Tamayo P, Mesirov JP, Liu XS, Shioda T, Toner M, Loda M, Brown M, Brugge JS, Ramaswamy S (2010) MYC regulation of a "poor-prognosis" metastatic cancer cell state. Proc Natl Acad Sci U S A 107(8):3698–3703. doi:10.1073/pnas.0914203107
12. Heselmeyer-Haddad K, Berroa Garcia LY, Bradley A, Ortiz-Melendez C, Lee WJ, Christensen R, Prindiville SA, Calzone KA, Soballe PW, Hu Y, Chowdhury SA, Schwartz R, Schaffer AA, Ried T (2012) Single-cell genetic analysis of ductal carcinoma in situ and invasive breast cancer reveals enormous tumor heterogeneity yet conserved genomic imbalances and gain of myc during progression. Am J Pathol 181(5):1807–1822. doi:10.1016/j.ajpath.2012.07.012
13. Deming SL, Nass SJ, Dickson RB, Trock BJ (2000) C-myc amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. Br J Cancer 83(12):1688–1695. doi:10.1054/bjoc.2000.1522
14. Darnell JE Jr (2002) Transcription factors as targets for cancer therapy. Nat Rev Cancer 2(10):740–749. doi:10.1038/nrc906nrc906
15. Soucek L, Whitfield J, Martins CP, Finch AJ, Murphy DJ, Sodir NM, Karnezis AN, Swigart LB, Nasi S, Evan GI (2008) Modelling Myc inhibition as a cancer therapy. Nature 455(7213):679–683. doi:10.1038/nature07260
16. Trumpp A, Refaeli Y, Oskarsson T, Gasser S, Murphy M, Martin GR, Bishop JM (2001) c-Myc regulates mammalian body size by controlling cell number but not cell size. Nature 414(6865):768–773. doi:10.1038/414768a
17. Toyoshima M, Howie HL, Imakura M, Walsh RM, Annis JE, Chang AN, Frazier J, Chau BN, Loboda A, Linsley PS, Cleary MA, Park JR, Grandori C (2012) Functional genomics identifies therapeutic targets for MYC-driven cancer. Proc Natl Acad Sci USA 109(24):9545–9550. doi:10.1073/pnas.1121119109
18. Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, Kastritis E, Gilpatrick T, Paranal RM, Qi J, Chesi M, Schinzel AC, McKeown MR, Heffernan TP, Vakoc CR, Bergsagel PL, Ghobrial IM, Richardson PG, Young RA, Hahn WC, Anderson KC, Kung AL, Bradner JE, Mitsiades CS (2011) BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell 146(6):904–917. doi:10.1016/j.cell.2011.08.017
19. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 90(1):77–86
20. Pink JJ, Jiang SY, Fritsch M, Jordan VC (1995) An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kDa estrogen receptor-related protein. Cancer Res 55(12):2583–2590
21. Brunner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R (1993) Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. Cancer Res 53(2):283–290

22. Brunner N, Frandsen TL, Holst-Hansen C, Bei M, Thompson EW, Wakeling AE, Lippman ME, Clarke R (1993) MCF7/LCC2: a 4-hydroxytamoxifen resistant human breast cancer variant that retains sensitivity to the steroidal antiestrogen ICI 182,780. Cancer Res 53(14):3229–3232
23. Brunner N, Boysen B, Jirus S, Skaar TC, Holst-Hansen C, Lippman J, Frandsen T, Spang-Thomsen M, Fuqua SA, Clarke R (1997) MCF7/LCC9: an antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen. Cancer Res 57(16):3486–3493
24. Sweeney EE, McDaniel RE, Maximov PY, Fan P, Jordan VC (2012) Models and mechanisms of acquired antihormone resistance in breast cancer: significant clinical progress despite limitations. Horm Mol Biol Clin Investig 9(2):143–163. doi:10.1515/hmbci-2011-0004
25. Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, Tapia C, Kim HR, Yerrum S, Sharma CG, Nicolas E, Balagurunathan Y, Ross EA, Jordan VC (2011) Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci USA 108(47):18879–18886. doi:10.1073/pnas.1115188108
26. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, Jordan VC (2005) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97(23):1746–1759. doi:10.1093/jnci/dji400
27. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A, Jamalabadi-Majidi S, Crowder R, Siegel BA (2009) Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 302(7):774–780. doi:10.1001/jama.2009
28. Sengupta S, Obiorah I, Maximov P, Curpan R, Jordan VC (2013) Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. Br J Pharmacol 169(1):167–178. doi:10.1111/bph.12122
29. Yin X, Giap C, Lazo JS, Prochownik EV (2003) Low molecular weight inhibitors of Myc–Max interaction and function. Oncogene 22(40):6151–6159. doi:10.1038/sj.onc.1206641
30. Follis AV, Hammoudeh DI, Wang H, Prochownik EV, Metallo SJ (2008) Structural rationale for the coupled binding and unfolding of the c-Myc oncoprotein by small molecules. Chem Biol 15(11):1149–1155. doi:10.1016/j.chembiol.2008.09.011
31. Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, Szallasi Z (2010) An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat 123(3):725–731. doi:10.1007/s10549-009-0674-9
32. Saunders A, Core LJ, Lis JT (2006) Breaking barriers to transcription elongation. Nat Rev Mol Cell Biol 7(8):557–567. doi:10.1038/nrm1981
33. Shim EY, Walker AK, Shi Y, Blackwell TK (2002) CDK-9/cyclin T (P-TEFb) is required in two postinitiation pathways for transcription in the *C. elegans* embryo. Genes Dev 16(16):2135–2146. doi:10.1101/gad.999002
34. Larochelle S, Amat R, Glover-Cutter K, Sanso M, Zhang C, Allen JJ, Shokat KM, Bentley DL, Fisher RP (2012) Cyclin-dependent kinase control of the initiation-to-elongation switch of RNA polymerase II. Nat Struct Mol Biol 19(11):1108–1115. doi:10.1038/nsmb.2399
35. Sims RJ 3rd, Belotserkovskaya R, Reinberg D (2004) Elongation by RNA polymerase II: the short and long of it. Genes Dev 18(20):2437–2468. doi:10.1101/gad.1235904
36. Krystof V, Cankar P, Frysova I, Slouka J, Kontopidis G, Dzubak P, Hajduch M, Srovnal J, de Azevedo WF, Jr Orsag M, Orsag M Jr, Paprskarova M, Rolcik J, Latr A, Fischer PM, Strnad M (2006) 4-Arylazo-3,5-diamino-1H-pyrazole CDK inhibitors: SAR study, crystal structure in complex with CDK2, selectivity, and cellular effects. J Med Chem 49(22):6500–6509. doi:10.1021/jm0605740
37. Osborne CK, Schiff R (2011) Mechanisms of endocrine resistance in breast cancer. Annu Rev Med 62:233–247. doi:10.1146/annurev-med-070909-182917
38. Jeng MH, Shupnik MA, Bender TP, Westin EH, Bandyopadhyay D, Kumar R, Masamura S, Santen RJ (1998) Estrogen receptor expression and function in long-term estrogen-deprived human breast cancer cells. Endocrinology 139(10):4164–4174
39. Weinberg RA (1995) The retinoblastoma protein and cell cycle control. Cell 81(3):323–330
40. Bentley DL, Groudine M (1986) A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL60 cells. Nature 321(6071):702–706. doi:10.1038/321702a0
41. Krumm A, Meulia T, Brunvand M, Groudine M (1992) The block to transcriptional elongation within the human c-myc gene is determined in the promoter-proximal region. Genes Dev 6(11):2201–2213
42. Wang C, Mayer JA, Mazumdar A, Fertuck K, Kim H, Brown M, Brown PH (2011) Estrogen induces c-myc gene expression via an upstream enhancer activated by the estrogen receptor and the AP-1 transcription factor. Mol Endocrinol 25(9):1527–1538. doi:10.1210/me.2011-1037
43. Musgrove EA, Sergio CM, Loi S, Inman CK, Anderson LR, Alles MC, Pinese M, Caldon CE, Schutte J, Gardiner-Garden M, Ormandy CJ, McArthur G, Butt AJ, Sutherland RL (2008) Identification of functional networks of estrogen- and c-Myc-responsive genes and their relationship to response to tamoxifen therapy in breast cancer. PLoS ONE 3(8):e2987. doi:10.1371/journal.pone.0002987
44. Kininis M, Isaacs GD, Core LJ, Hah N, Kraus WL (2009) Postrecruitment regulation of RNA polymerase II directs rapid signaling responses at the promoters of estrogen target genes. Mol Cell Biol 29(5):1123–1133. doi:10.1128/MCB.00841-08
45. Nechaev S, Adelman K (2011) Pol II waiting in the starting gates: regulating the transition from transcription initiation into productive elongation. Biochim Biophys Acta 1809:34–45. doi:10.1016/j.bbagrm.2010.11.001
46. Buratowski S (2009) Progression through the RNA polymerase II CTD cycle. Mol Cell 36(4):541–546. doi:10.1016/j.molcel.2009.10.019
47. Peterlin BM, Price DH (2006) Controlling the elongation phase of transcription with P-TEFb. Mol Cell 23(3):297–305. doi:10.1016/j.molcel.2006.06.014
48. Baumli S, Hole AJ, Noble ME, Endicott JA (2012) The CDK9 C-helix exhibits conformational plasticity that may explain the selectivity of CAN508. ACS Chem Biol 7(5):811–816. doi:10.1021/cb2004516
49. Zhang W, Prakash C, Sum C, Gong Y, Li Y, Kwok JJ, Thiessen N, Pettersson S, Jones SJ, Knapp S, Yang H, Chin KC (2012) Bromodomain-containing protein 4 (BRD4) regulates RNA polymerase II serine 2 phosphorylation in human CD4+ T cells. J Biol Chem 287(51):43137–43155. doi:10.1074/jbc.M112.413047
50. Jang MK, Mochizuki K, Zhou M, Jeong HS, Brady JN, Ozato K (2005) The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-

dependent transcription. Mol Cell 19(4):523–534. doi:10.1016/j.molcel.2005.06.027

51. Zuber J, Shi J, Wang E, Rappaport AR, Herrmann H, Sison EA, Magoon D, Qi J, Blatt K, Wunderlich M, Taylor MJ, Johns C, Chicas A, Mulloy JC, Kogan SC, Brown P, Valent P, Bradner JE, Lowe SW, Vakoc CR (2011) RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. Nature 478(7370):524–528. doi:10.1038/nature10334

Mechanisms underlying differential response to estrogen-induced apoptosis in long-term estrogen-deprived breast cancer cells

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Abstract. Models of long-term estrogen-deprived breast cancer cells are utilized in the laboratory to mimic clinical aromatase inhibitor-resistant breast cancer and serve as a tool to discover new therapeutic strategies. The MCF-7:5C and MCF-7:2A subclones were generated through long-term estrogen deprivation of estrogen receptor (ER)-positive MCF-7 cells, and represent anti-hormone-resistant breast cancer. MCF-7:5C cells paradoxically undergo estrogen-induced apoptosis within seven days of estrogen (estradiol, E_2) treatment; MCF-7:2A cells also experience E_2-induced apoptosis but evade dramatic cell death until approximately 14 days of treatment. To discover and define the mechanisms by which MCF-7:2A cells survive two weeks of E_2 treatment, systematic experiments were performed in this study. The data suggest that MCF-7:2A cells employ stronger antioxidant defense mechanisms than do MCF-7:5C cells, and that oxidative stress is ultimately required for MCF-7:2A cells to die in response to E_2 treatment. Tumor necrosis factor (TNF) family member activation is also essential for E_2-induced apoptosis to occur in MCF-7:2A cells; upregulation of TNFα occurs simultaneously with oxidative stress activation. Although the unfolded protein response (UPR) signaling pattern is similar to that in MCF-7:5C cells, it is not sufficient to cause cell death in MCF-7:2A cells. Additionally, increased insulin-like growth factor receptor β (IGF-1Rβ) confers a mechanism of growth and anti-apoptotic advantage in MCF-7:2A cells.

Introduction

Aromatase inhibitor-resistant breast cancer cells are modeled *in vitro* by long-term E_2-deprived breast cancer cell lines. The MCF-7:WS8 cell line represents a clone of the estrogen receptor (ER)-positive cell line MCF-7 that is highly sensitive to E_2-stimulated growth (1). The MCF-7:5C and MCF-7:2A subclones are derived from the parental MCF-7 cell line through long-term E_2 deprivation (1-4). MCF-7:5C cells express wild-type ER at a higher level than the parental line, and are progesterone receptor (PR)-negative (3). These cells grow in the absence of E_2, and do not respond to 4-hydroxytamoxifen (4-OHT) (2,3). MCF-7:2A cells can induce expression of PR and express both wild-type (66 kDa) and mutant (77 kDa) ER (4,5). The mutant ER contains a repeat of exons 6 and 7 and cannot bind E_2 nor anti-estrogens; it is expressed 4- to 10-fold lower than the wild-type ER (6). The total ER level of MCF-7:2A cells is higher than in parental MCF-7 cells, and they also grow in E_2-free media. 4-OHT and pure anti-E_2 are able to block their growth (4,5).

In addition to the different responses to anti-E_2 observed in MCF-7:5C versus MCF-7:2A cells, they also have different apoptotic responses to E_2. The MCF-7:5C cells undergo apoptosis and die during the first week of E_2 treatment, whereas the MCF-7:2A cells die later, after two weeks of E_2 treatment (7). MCF-7:5C cell response to estrogens and anti-estrogens has been extensively studied in our lab; the data show that these cells undergo E_2-induced apoptosis through mechanisms associated with endoplasmic reticulum stress (ERS) and oxidative stress (8,9). Thus far, there has been less focus on the classification and mechanisms of the MCF-7:2A response.

Network enrichment analyses done using gene arrays in timecourse experiments show overexpression of apoptotic- and stress-related pathways in the MCF-7:5C cells after 24-96 h of E_2 treatment; however, these analyses show the MCF-7:2A cells expressing more genes associated with glutathione metabolism during this time period of E_2 exposure (Fig. 1). This suggests that the two cell lines respond to E_2 treatment using different signaling pathways. The MCF-7:5C cells respond by quickly inducing apoptosis, while the antioxidant pathway may be more relevant to the MCF-7:2A cells. Experiments were designed to interrogate the apoptotic, stress and antioxidant pathways in both cell lines to distinguish signaling mechanisms in response to E_2.

The concept of E_2-induced death is important because of its clinical relevance. A clinical study published in 2009 (10) compared two doses of E_2 for second-line treatment after breast cancer patients had failed aromatase inhibitor therapy. The authors showed that after long-term anti-hormone therapy, no response is lost with the lower dose of E_2; overall

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Key words: oxidative stress, estrogen deprivation, breast cancer, insulin-like growth factor receptor, glutathione

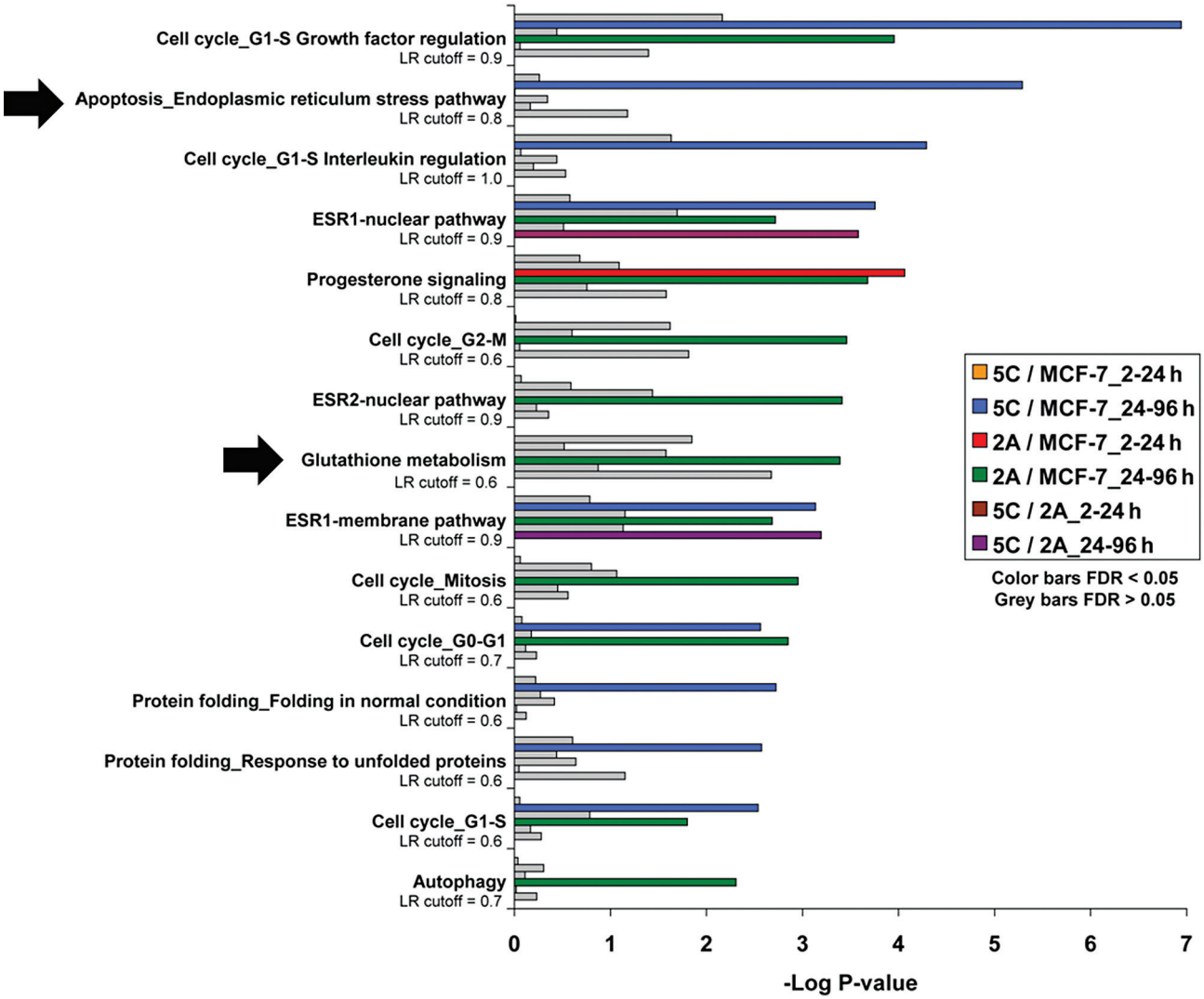

Figure 1. Network enrichment analysis for MCF-7:WS8, MCF-7:5C and MCF-7:2A cells. Global gene arrays were performed to compare activated gene networks associated with 1 nM E_2 treatment in the cell lines. Genes were analyzed after 2-24 and 24-96 h treatment.

about 30% of women responded to E_2 treatment. The goal of this study is to uncover the mechanisms preventing the other 70% of patients from responding, and perhaps find ways to circumvent their resistance. To this end, MCF-7:2A cells were used as a model for E_2-deprived breast tumors with the ability to evade E_2-induced apoptosis in the clinic.

Materials and methods

Cell culture. All cell lines were cultured in phenol red-free RPMI-1640 media supplemented with 10% charcoal-stripped fetal bovine serum (SFS). Media and treatments were replaced every three days. Estradiol (E_2) (Sigma-Aldrich, St. Louis, MO, USA), buthionine sulfoximine (BSO) (Sigma-Aldrich), and combinations were dissolved in ethanol and then in media. AG1024 (Calbiochem, San Diego, CA, USA) was dissolved in DMSO and then in media.

DNA assays. MCF-7:WS8, MCF-7:5C and MCF-7:2A cells were harvested after 7 or 14 days treatment with vehicle (0.1% ethanol), E_2 (10^{-9} mol/l, 1 nM), BSO (10^{-4} mol/l, 100 μM), or E_2 (1 nM) + BSO (100 μM). DNA content was measured as previously described (11).

Western blot analysis. Total MAPK (#9102), phosphorylated MAPK (#9101), total AKT (#9272), phosphorylated AKT (#4051L), total eIF2α (#9722S), phosphorylated eIF2α (#9721S), and IRE1α (#3294S) antibodies were all purchased from Cell Signaling Technology (Beverly, MA, USA). IGF-1Rβ antibody (sc-713) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). β-actin loading control antibody (A5441) was purchased from Sigma-Aldrich. Proteins were harvested from cells using cell lysis buffer (Cell Signaling Technology) supplemented with Protease Inhibitor Cocktail Set I and Phosphatase Inhibitor Cocktail Set II (Calbiochem). Bicinchoninic acid (BCA) assay was used to quantify total protein content (Rio-Rad Laboratories, Hercules, CA, USA). Protein (50 μg) was probed and visualized as previously described (11).

Cell cycle analysis. MCF-7:2A cells were cultured in dishes and treated with vehicle (0.1% ethanol) or E_2 (10^{-9} mol/l,

1 nM). Cells were harvested after 24 h, fixed in 75% ethanol on ice, stained with propidium iodide and sorted using FACS flow cytometry (Becton Dickinson, San Jose, CA, USA). Results were analyzed using CellQuest software.

RT-PCR. Cells were harvested using TRIzol, and RNA was isolated using RNeasy mini kit (Qiagen, Valencia, CA, USA). RNA was reverse transcribed to cDNA using a kit (Applied Biosystems, Foster City, CA). SYBR-Green (Applied Biosystems) was used for quantitative real-time polymerase chain reaction (RT-PCR) in a 7900HT Fast Real-Time PCR system (Applied Biosystems).

Glutathione assay. Cells were harvested and de-proteinized with 5% 5-sulfosalicylic acid solution (SSA) (Sigma-Aldrich). Total glutathione [reduced glutathione (GSH) plus glutathione disulfide (GSSG)] was measured spectroscopically at 412 nm using a Glutathione Assay Kit (CS0260, Sigma-Aldrich) and the manufacturer's instructions.

ROS assay. MCF-7:2A cells were harvested, stained with 10^{-6} mol/l (1 μM) CM-H2DCFDA (Invitrogen, Eugene, OR, USA), and analyzed for ROS fluorescence using flow cytometry.

Statistical analysis. Values reported are means ± standard deviation (SD). Significant differences were found by Student's t-test. P-values <0.05 were considered to indicate a statistically significant difference.

Results

MCF-7:2A initial response to E_2. The MCF-7:WS8, MCF-7:5C and MCF-7:2A cell lines respond differently to 10^{-9} mol/l (1 nM) E_2. In the presence of 1 nM E_2, MCF-7:WS8 cells are stimulated to proliferate over 7 days, whereas MCF-7:5C cells are killed by this time point (Fig. 2A). MCF-7:2A cell growth is unaffected by the presence of E_2 after one week, but their DNA is reduced by 50% after the second week of treatment (Fig. 2A). Interestingly, MCF-7:2A cells are initially stimulated to proliferate in response to E_2. After 24 h-treatment with 1 nM E_2, both the mitogen-activated protein kinase (MAPK) and serine/threonine protein kinase Akt (AKT) pathways are activated, as shown by an increase in phosphorylated MAPK (p-MAPK) and phosphorylated AKT (p-AKT) proteins, respectively (Fig. 2B). Further, MCF-7:2A cells treated with E_2 for 24 h show an increase in the percentage of dividing cells compared with vehicle treatment (34.78 versus 20.17%), illustrated by S-phase in cell cycle analysis (Fig. 2C).

MCF-7:5C and MCF-7:2A UPR. To determine whether the different biological effects observed in MCF-7:5C and MCF-7:2A cells is due to different patterns of the unfolded protein response (UPR), proteins associated with the UPR were measured over a 72 h timecourse. Two markers of the UPR, phosphorylated eIF2α (p-eIF2α) and IRE1α, were visualized by western blot analysis in MCF-7:5C and MCF-7:2A cells in the presence of vehicle and 1 nM E_2 (Fig. 3). p-eIF2α is directly downstream of protein kinase RNA-like endoplasmic reticulum kinase (PERK), a sensor which initiates UPR. Both cell lines show an increase in the protein expression of p-eIF2α and IRE1α by 72 h of E_2 treatment, indicating activated UPR. Though MCF-7:2A cells show a slightly higher basal p-eIF2α level, no differences in UPR activation can be seen between the two cell lines.

MCF-7:5C and MCF-7:2A estrogen-induced apoptosis. To determine whether MCF-7:2A cells experience apoptosis through the same mechanism as MCF-7:5C cells, RT-PCR was used to quantify mRNA levels of apoptosis-related genes. MCF-7:5C cells noticeably upregulate LTA (4.19±1.92 fold change), LTB (5.39±1.82), TNFα (9.40±3.86), and BCL2L11 (6.06±0.87) after 72 h of E_2 treatment, while MCF-7:2A cells show no major changes during this time period (Fig. 4A). MCF-7:2A cells were then treated with E_2 for a longer time period to measure apoptosis-related genes during the time when they appear to die. MCF-7:2A cells increase both TNFα (33.55±12.09 fold change) and BCL2L11 (3.71±0.35 fold change) after 12 days of 1 nM E_2 treatment (Fig. 4B). The upregulated apoptosis-related genes correspond to the time when cell death is most apparent in both cell lines, during week one in MCF-7:5C cells, and during week two in MCF-7:2A cells.

MCF-7:5C and MCF-7:2A oxidative stress. Heme oxygenase 1 (HMOX1) was used as an indicator to illustrate when MCF-7:5C and MCF-7:2A cells experience oxidative stress. After 72 h of 1 nM E_2 treatment, HMOX1 mRNA was increased 4.61-fold in MCF-7:5C cells (Fig. 5A), suggesting this cell line undergoes oxidative stress at this time point. MCF-7:2A cells did not generate an upregulation of HMOX1 mRNA until 12 days of 1 nM E_2 treatment when it increased 10.03-fold (Fig. 5B), suggesting an earlier protective mechanism inherent in these cells to prevent oxidative stress longer than MCF-7:5C cells.

Glutathione is a potent antioxidant and was quantified in MCF-7:5C and MCF-7:2A cells to illustrate a potential protective mechanism in MCF-7:2A cells against oxidative stress (Fig. 6A). In fact, MCF-7:2A cells have significantly more basal glutathione than do MCF-7:WS8 and MCF-7:5C cells (Fig. 6A). Buthionine sulfoximine (BSO) is a synthetic amino acid that blocks glutathione synthesis by inhibiting γ-glutamylcysteine synthetase. BSO (100 μM) dramatically decreases glutathione levels in both MCF-7:5C and MCF-7:2A cells (Fig. 6B). To ask the question of whether glutathione is protecting MCF-7:2A cells from oxidative stress and E_2-induced apoptosis, HMOX1 was measured following treatment with vehicle, 1 nM E_2 alone, 100 μM BSO alone, and 1 nM E_2 + 100 μM BSO after 24, 48 and 72 h (Fig. 6C). MCF-7:2A cells show increased HMOX1 mRNA at 72 h after treatment with 100 μM BSO and 1 nM E_2 + 100 μM BSO (3.57±0.36 and 2.60±0.70 fold change, respectively), suggesting a protective role of glutathione in these cells. Reactive oxygen species (ROS) increased 634% over vehicle in MCF-7:2A cells after 12 days of the combination treatment (Fig. 6D). Furthermore, 1 nM E_2 + 100 μM BSO treatment caused a significant decrease in DNA after 14 days treatment (Fig. 6E), suggesting that oxidative stress is a key factor in determining E_2-induced MCF-7:2A cell death.

MCF-7:5C and MCF-7:2A IGFR. Insulin-like growth factor receptor β (IGF-1Rβ) upregulation is another mechanism

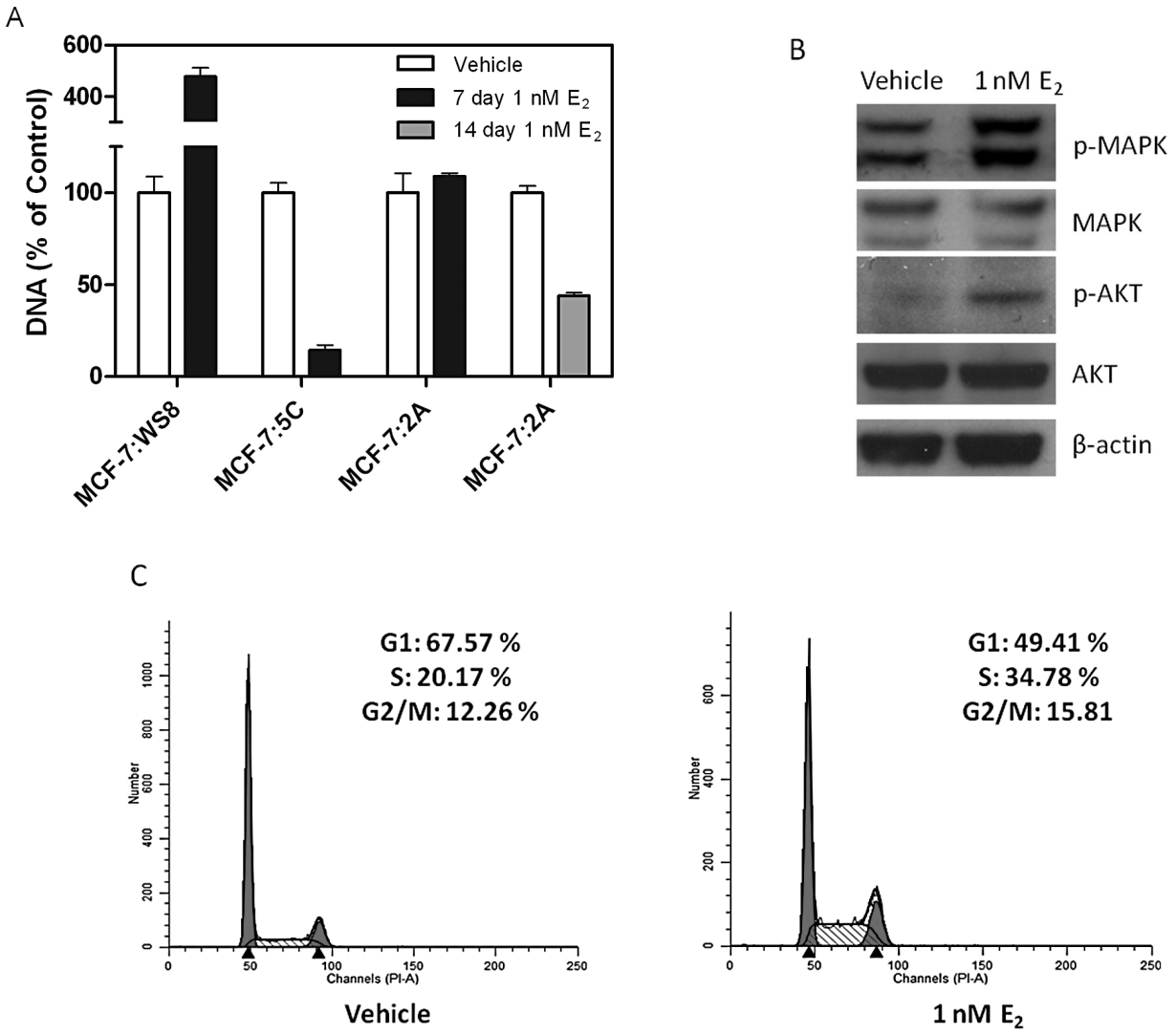

Figure 2. MCF-7:2A growth response to E_2. (A) DNA was measured from MCF-7:WS8, MCF-7:5C and MCF-7:2A cells after 7 or 14 days treatment with vehicle or 1 nM E_2. Values are normalized to vehicle-treated cells. Means represent samples in triplicate. (B) MAPK and AKT growth pathway protein levels were measured by western blot analysis after 24 h vehicle or 1 nM E_2 treatment. β-actin was used as a loading control. (C) Cell cycle analysis was performed after 24 h vehicle or 1 nM E_2 treatment.

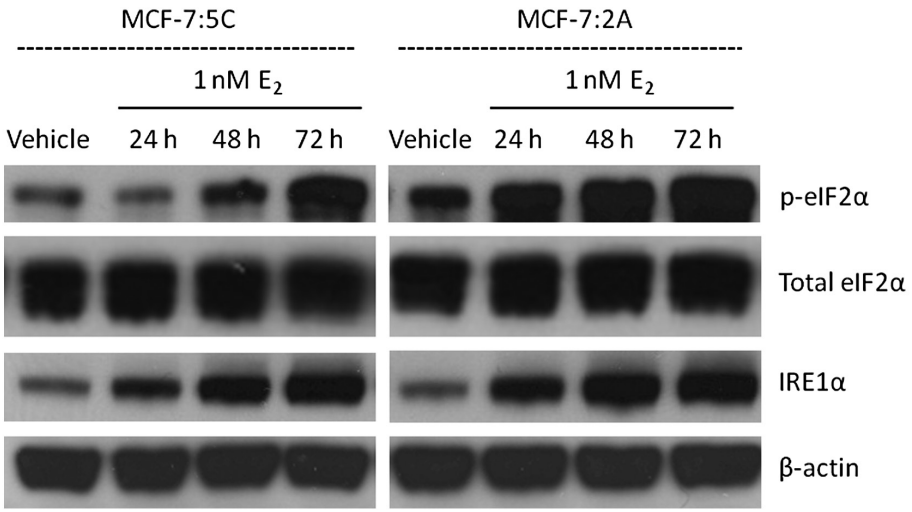

Figure 3. MCF-7:5C and MCF-7:2A UPR. Cell lines were probed for UPR-related proteins after treatment with vehicle or 1 nM E_2 for 24, 48 and 72 h. β-actin was used as a loading control.

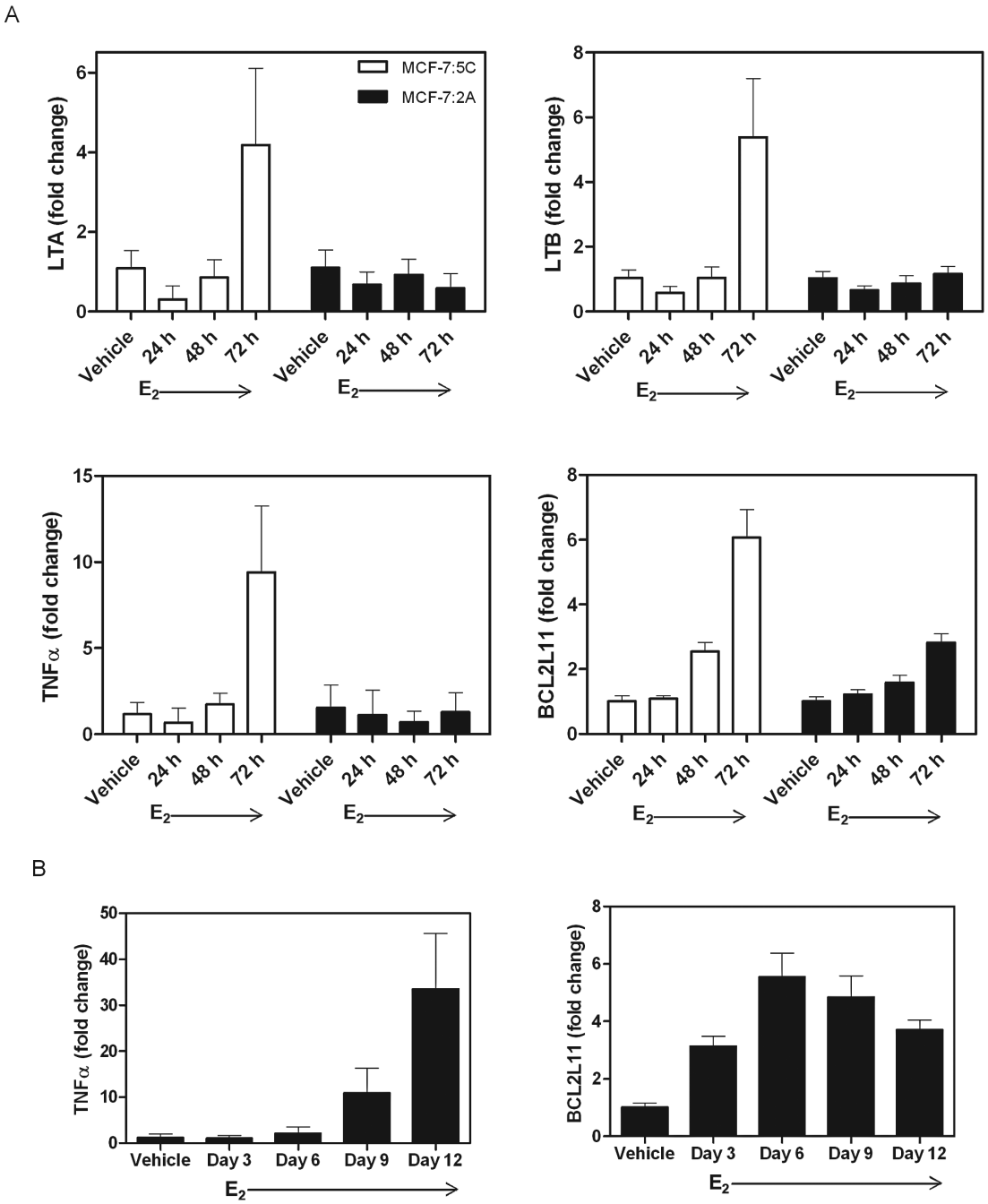

Figure 4. Apoptosis-related genes in MCF-7:5C (white bars) and MCF-7:2A (black bars) cells. (A) MCF-7:5C and MCF-7:2A cells were treated with vehicle or 1 nM E_2 for 24, 48 and 72 h. LTA, LTB, TNFα and BCL2L11 mRNA levels were measured using RT-PCR. 36B4 was used as an internal control. (B) MCF-7:2A cells were treated with vehicle or 1 nM E_2 for 3, 6, 9 and 12 days. TNFα and BCL2L11 mRNA levels were then measured using RT-PCR. 36B4 was used as an internal control. Means represent at 7 to 18 replicates.

through which MCF-7:2A cells could receive anti-apoptotic advantage over MCF-7:5C cells. MCF-7:2A cells exhibit 2.71-fold greater basal IGF-1Rβ mRNA than MCF-7:5C cells (Fig. 7A). This is consistent at the protein level as shown by western blot analysis, where MCF-7:2A cells exhibit more IGF-1Rβ protein expression than MCF-7:5C cells (Fig. 7B). When treated with an IGF-1Rβ inhibitor (10 μM AG1024) for 7 days, MCF-7:2A cells show significantly decreased DNA content when compared to vehicle and 1 nM E_2 treatments (Fig. 7C). Combination treatment of 1 nM E_2 + 10 μM AG1024 decreased DNA content significantly more than either treatment alone (Fig. 7C), suggesting an integral role of IGF-1Rβ in MCF-7:2A cells evading E_2-induced apoptosis. To interrogate this further, growth pathway proteins were measured in response to 10 μM AG1024 treatment. MAPK and AKT pathways are both blocked by the IGF-1Rβ inhibitor after 72 h as shown by decreased p-MAPK and p-AKT levels when compared to vehicle-treated MCF-7:2A cells (Fig. 7D).

Discussion

This study investigated the mechanisms through which MCF-7:2A cells evade E_2-induced apoptosis *in vitro* as a means to understand resistant breast cancer cells after

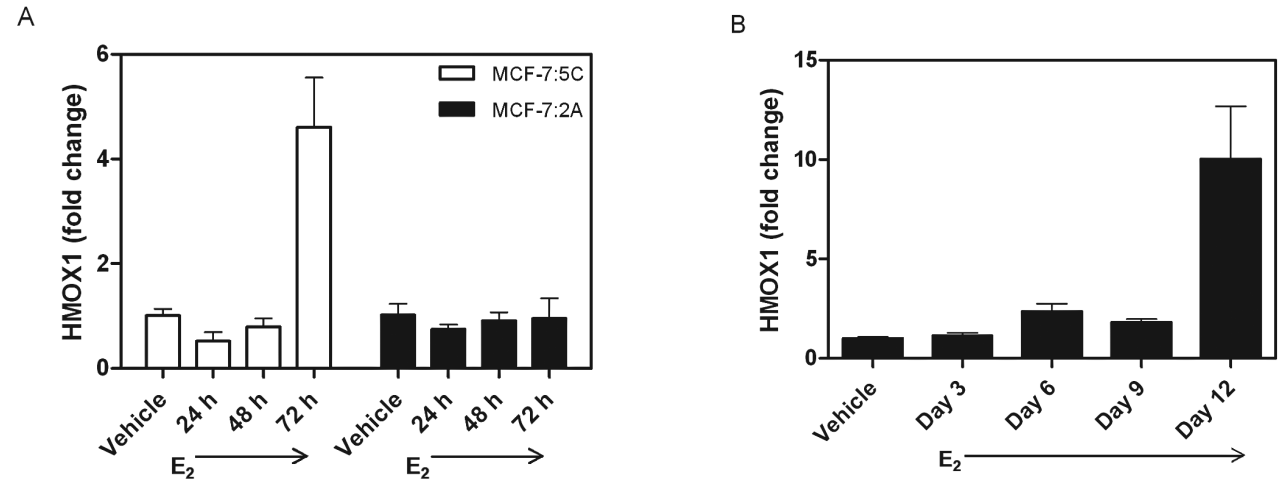

Figure 5. MCF-7:5C and MCF-7:2A HMOX1 regulation. (A) MCF-7:5C and MCF-7:2A cells were treated with vehicle or 1 nM E_2 for 24, 48 and 72 h; HMOX1 mRNA was measured using RT-PCR. 36B4 was used as an internal control. Mean represents 18 replicates. (B) MCF-7:2A cells were treated with vehicle or 1 nM E_2 for 3, 6, 9 and 12 days; HMOX1 mRNA was measured using RT-PCR. 36B4 was used as an internal control. Means represent at least 8 replicates.

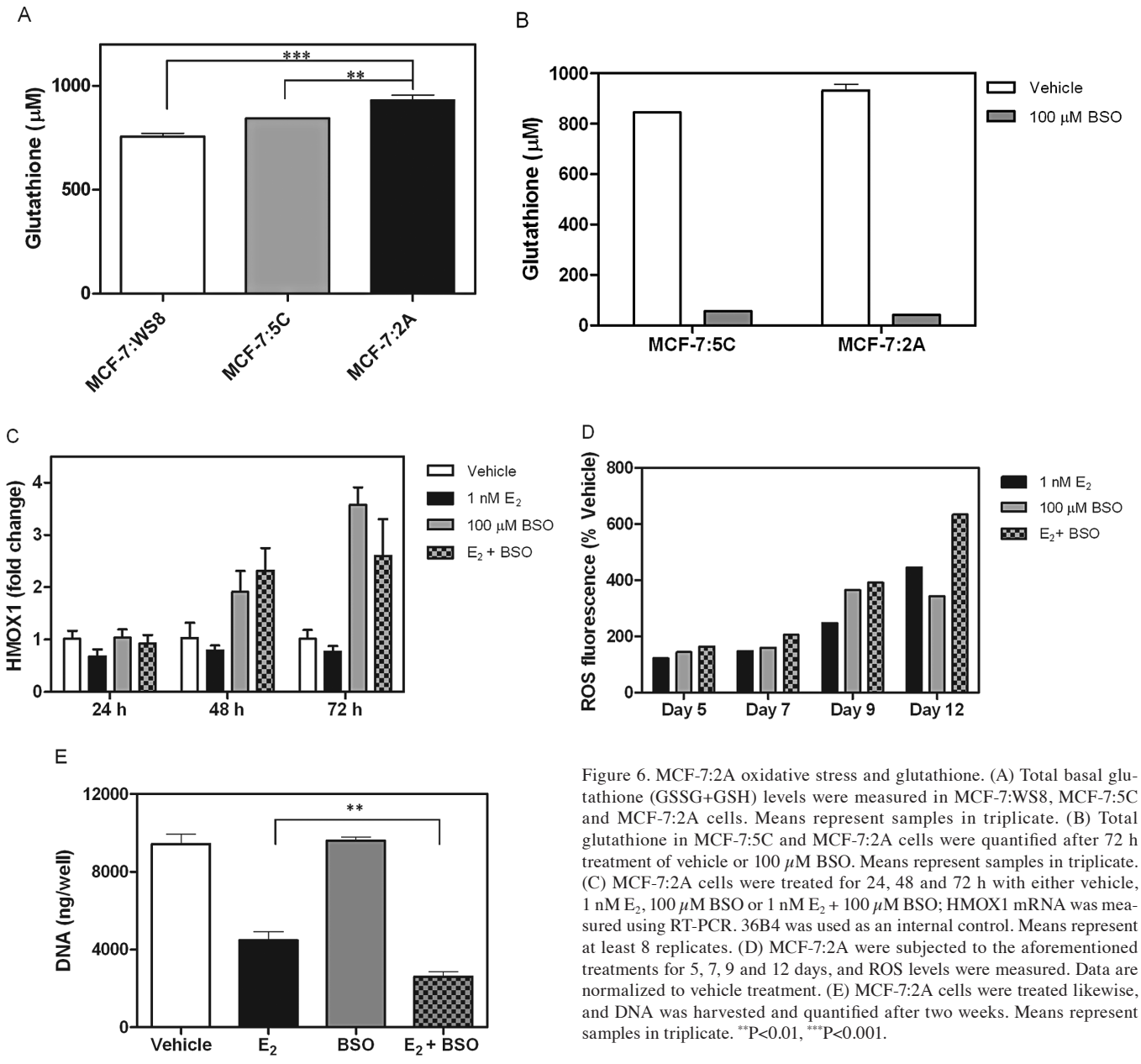

Figure 6. MCF-7:2A oxidative stress and glutathione. (A) Total basal glutathione (GSSG+GSH) levels were measured in MCF-7:WS8, MCF-7:5C and MCF-7:2A cells. Means represent samples in triplicate. (B) Total glutathione in MCF-7:5C and MCF-7:2A cells were quantified after 72 h treatment of vehicle or 100 μM BSO. Means represent samples in triplicate. (C) MCF-7:2A cells were treated for 24, 48 and 72 h with either vehicle, 1 nM E_2, 100 μM BSO or 1 nM E_2 + 100 μM BSO; HMOX1 mRNA was measured using RT-PCR. 36B4 was used as an internal control. Means represent at least 8 replicates. (D) MCF-7:2A were subjected to the aforementioned treatments for 5, 7, 9 and 12 days, and ROS levels were measured. Data are normalized to vehicle treatment. (E) MCF-7:2A cells were treated likewise, and DNA was harvested and quantified after two weeks. Means represent samples in triplicate. **P<0.01, ***P<0.001.

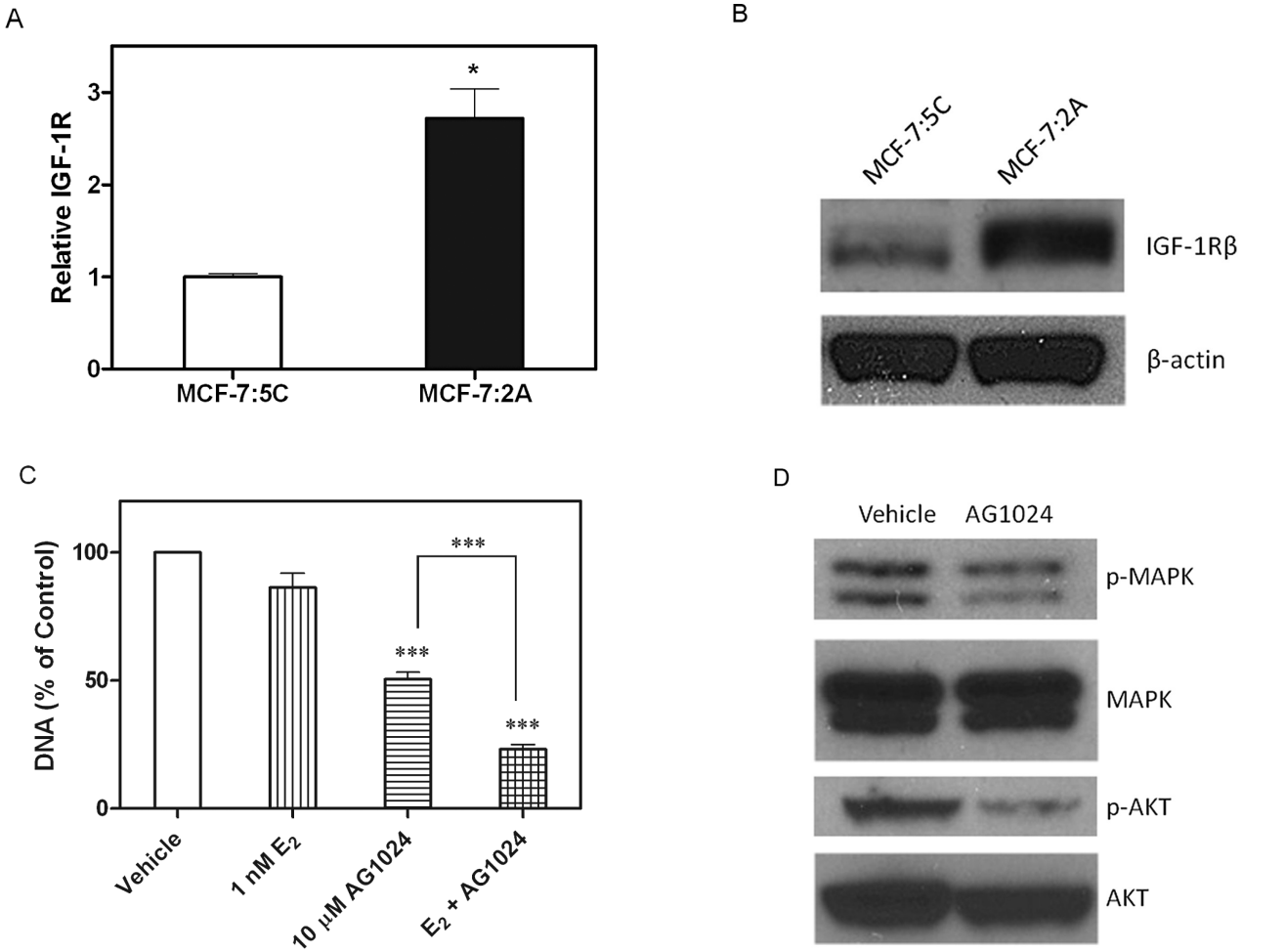

Figure 7. MCF-7:2A IGF-1Rβ. (A) Basal IGF-1Rβ mRNA was measured in MCF-7:5C cells and MCF-7:2A cells via RT-PCR. MCF-7:2A values are normalized to MCF-7:5C. 36B4 was used as an internal control. Means represent samples in triplicate. (B) Basal IGF-1Rβ protein levels were measured in MCF-7:5C and MCF-7:2A cells by western blot analysis. β-actin was used as a loading control. (C) MCF-7:2A cells were treated with vehicle, 1 nM E_2, 10 μM AG1024, or 1 nM E_2 + 10 μM AG1024. DNA was harvested and quantified after seven days. Means represent samples in triplicate. (D) MCF-7:2A cells were treated for 72 h with vehicle or 10 μM AG1024. Growth pathway protein levels were visualized via western blot analysis. Total MAPK and total AKT were used as loading controls. *P<0.05, ***P<0.001.

long-term anti-hormone therapy in the clinic. After failure on an aromatase inhibitor, approximately 30% of breast cancer patients will respond to treatment with E_2 (10); their nascent or remaining breast tumors will become cytostatic or disappear with physiological levels of E_2. Further, E_2 replacement therapy (ERT) has been shown to reduce the risk of breast cancer in hysterectomized post-menopausal women (12), perhaps due to E_2-deprived breast cancer cells undergoing E_2-induced apoptosis before resulting in clinically apparent disease. This study sought to discriminate between E_2-deprived breast tumors that will quickly respond to treatment with E_2 versus those that will respond more slowly and less dramatically. We modeled these different scenarios with MCF-7:5C and MCF-7:2A cell lines, respectively.

We have found that the UPR, associated with endoplasmic reticulum stress (ERS), is a fundamental element in E_2-induced MCF-7:5C cell apoptosis (8). In this setting, E_2 triggers UPR and rapidly causes apoptosis within one week of treatment. Two main sensors of the UPR, IRE1α and PERK are activated in both cell lines similarly. PERK activation is confirmed by elevated p-eIF2α, since eIF2α is phosphorylated by activated PERK. In MCF-7:2A cells, the same sensors are activated as in MCF-7:5C cells (Fig. 3), but significant cell death is not apparent at the same timepoint (Fig. 2A). Despite similar signaling patterns, the biological responses between the two cell lines differ. Our data suggested that another mechanism was preventing cell death after E_2-induced UPR in MCF-7:2A cells.

Oxidative stress is a critical pathway for MCF-7:2A cells to undergo E_2-induced apoptosis. MCF-7:2A cells inherently exhibit stronger survival and antioxidant mechanisms than MCF-7:5C cells (Figs. 4-6). This relationship is consistent with previously published data showing that MCF-7 cells with higher levels of glutathione peroxidase-1 (GSHPx-1) can survive better under oxidative stress conditions, such as hydrogen peroxide treatment (13), and that MCF-7 cells can increase antioxidant enzymes (i.e. manganese superoxide dismutase, MnSOD) to prevent TNF-mediated apoptosis (14). Activation of E_2-induced apoptosis in MCF-7:2A cells also seems to require TNF family member upregulation (Fig. 4A

Table I. Basal apoptosis gene expression in MCF-7:2A cells versus MCF-7:5C.

Gene symbol	Fold change
AIFM2	5.7601
AKT1	2.5203
ANXA1	57.2949
ANXA4	2.7965
APAF1	2.839
ATF5	2.5303
BAG1	2.7188
BCL2	**3.7598**
BCL2L1	3.0192
BDNF	5.8519
BIK	6.2803
BIRC7	33.6437
CARD9	2.7968
CASP7	2.5278
CD27	2.7439
CD5	3.884
CD70	8.1739
CRYAB	2.967
CUL3	3.2377
DAPK1	2.6145
DAPK2	6.023
EDAR	5.7874
ERCC3	2.7634
ERN2	5.1671
GRM4	6.4268
HTT	4.3186
HIP1	5.7736
HSPA1B	2.5548
HSPB1	7.5902
IGF1R	3.4421
IL1A	31.2667
INHA	2.5996
LGALS1	430.9062
MAL	3.0587
MALT1	3.2679
NLRC4	2.84
NOL3	2.9365
PLAGL1	3.3963
PLAGL2	3.0314
PPP1R13B	2.7465
PPP2R1B	4.5273
PRKCA	2.503
PRODH	3.8158
PTH	5.7472
PYCARD	3.1633
RARG	2.968
SEMA4D	2.9335
SFN	3.2245
SIPA1	3.777
SOCS2	4.3464
STK17B	3.8901
TBX5	3.3289
TNFRSF10D	2.5864
TNFRSF18	4.0067
TNFRSF19	76.9083
TNFRSF6B	2.7982
TNFRSF8	3.103
TNFSF14	4.5599
TP63	15.4118
TRAF2	2.5655
UNC13B	3.0047
VHL	3.1063
ZAK	2.8369

RT-PCR gene arrays of apoptosis-related genes were performed using MCF-7:5C and MCF-7:2A cells. Fold change represents gene expression of basal MCF-7:2A levels over basal MCF-7:5C levels. Only genes overexpressed in MCF-7:2A cells are shown. Particularly noteworthy in this study are BCL2 and BCL2L1.

and B). Oxidative stress occurs concurrently with upregulation of apoptosis-related genes in the TNF family. Whether increased TNFα causes oxidative stress or oxidative stress causes increased TNFα is not yet documented in this setting.

Additionally, B cell lymphoma 2 (BCL2) plays a role in preventing cell death caused by oxidative stress (15). In fact, MCF-7:2A cells exhibit 3.76-fold and 3.02-fold higher basal BCL2 and B cell lymphoma extra large (BCL-xL, BCL2L1) mRNA levels than MCF-7:5C cells, respectively (Table I), providing support for the idea of a stronger survival signal. Other data from our lab shows that MCF-7:2A cells exhibit 6.19-fold higher glutathione peroxidase 2 gene (GPX2) over MCF-7:5C cells (Table II), illustrating more evidence in favor of increased protection from E_2-induced oxidative stress and apoptosis in this context.

Increased IGFR promotes anti-hormone resistance in breast cancer, likely through growth factor receptor crosstalk and aberrant ER, MAPK, and AKT signal transduction pathway activation (16-18). Our data correlate with these findings in that higher IGF-1Rβ mRNA and protein expression confer a growth advantage and apoptotic resistance in MCF-7:2A cells despite treatment with E_2 (Fig. 7). This suggests an IGF-1Rβ signaling pathway that can circumvent normal ER signaling in long-term estrogen-deprived breast cancer cells. Studies using hepatocellular carcinoma cells (HCC) have demonstrated that IGF-1R overexpression can potentially cause increased glutathione transferase (GST) and protection from oxidative stress (19). Although

Table II. Top 10 overexpressed and underexpressed oxidative stress-related genes in MCF-7:2A versus MCF-7:5C.

Gene name	Gene symbol	Category	Fold change
Glutathione peroxidase 2	***GPX2***	**Glutathione peroxidases, oxidative stress responsive genes**	**6.19**
Keratin 1	*KRT1*	Oxidative stress responsive genes	2.71
Heme oxygenase 1	*HMOX1*	Oxidative stress responsive genes	2.66
Thioredoxin reductase 1	*TXNRD1*	Oxidative stress responsive genes, other antioxidants	2.24
Peroxiredoxin 1	*PRDX1*	Peroxiredoxins (TPx)	2.22
24-Dehydrocholesterol reductase	*DHCR24*	Oxidative stress responsive genes	2.21
Aldehyde oxidase	*AOX1*	Other genes involved in ROS metabolism	2.20
Forkhead box M1	*FOXM1*	Oxidative stress responsive genes	1.83
Thioredoxin	*TXN*	Oxidative stress responsive genes	1.71
Prostaglandin-endoperoxide synthase 1	*PTGS1*	Other peroxidases	1.71
Copper chaperone for superoxide dismutase	*CCS*	Other genes involved in superoxide metabolism	-1.65
Ring finger protein 7	*RNF7*	Oxidative stress responsive genes	-1.65
Neutrophil cytosolic factor 2	*NCF2*	Other genes involved in superoxide metabolism	-1.81
NADPH oxidase, EF-hand calcium binding domain 5	*NOX5*	Other genes involved in superoxide metabolism	-1.83
Scavenger receptor class A, member 3	*SCARA3*	Oxidative stress responsive genes	-1.98
Superoxide dismutase 3, extracellular	*SOD3*	Superoxide dismutases , other antioxidants	-2.55
Cytochrome b-245, beta polypeptide	*CYBB*	Other peroxidases	-3.19
Selenoprotein P, plasma, 1	*SEPP1*	Oxidative stress responsive genes	-4.94
Apolipoprotein E	*APOE*	Oxidative stress responsive genes, other antioxidants	-8.55
Chemokine (C-C motif) ligand 5	*CCL5*	Oxidative stress responsive genes	-50.23

Global gene expression analyses were performed, and oxidative stress-related genes were ranked by fold change of MCF-7:2A expression over MCF-7:5C expression. Notably, GPX2 shows the highest fold change.

this mechanism is shown in liver cancer cells, it may apply to our models of breast cancer as well. Perhaps the higher level of IGF-1Rβ in MCF-7:2A cells generates the increased glutathione levels necessary to escape cell death in the presence of E_2.

The evidence thus far shows that TNF family member gene expression, protection against oxidative stress, and growth factor signaling are major mechanisms underlying the different biological responses to E_2 seen in MCF-7:2A cells versus MCF-7:5C cells. Despite similar UPR signaling patterns, MCF-7:2A cells resist ERS-induced death longer and stronger than MCF-7:5C cells. Additional studies may provide further insight into the connection between IGF-1Rβ and glutathione in MCF-7:2A cells, and how this relationship functions in the presence and absence of a stressor such as E_2. In order to effectively treat breast cancer patients who have undergone exhaustive anti-hormone treatment, and to explain why ERT can prevent breast cancer in some post-menopausal women, the examination of breast cancer cell models of E_2 deprivation is proving invaluable. By understanding mechanisms that prevent apoptosis in these breast cancer cells, we can translate key findings into clinical practice.

Acknowledgements

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References

1. Sweeney EE, McDaniel RE, Maximov PY, Fan P and Jordan VC: Models and mechanisms of acquired resistance in breast cancer: Significant clinical progress despite limitations. Hom Mol Biol Clin Investig 9: 143-163, 2012.
2. Jiang SY, Wolf DM, Yingling JM, Chang C and Jordan VC: An estrogen receptor positive MCF-7 clone that is resistant to anti-estrogens and estradiol. Mol Cell Endocrinol 90: 77-86, 1992.
3. Lewis JS, Osipo C, Meeke K and Jordan VC: Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 94: 131-141, 2005.
4. Pink JJ, Jiang SY, Fritsch M and Jordan VC: An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. Cancer Res 55: 2583-2590, 1995.
5. Pink JJ and Jordan VC: Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. Cancer Res 56: 2321-2330, 1996.
6. Pink JJ, Wu SQ, Wolf DM, Bilimoria MM and Jordan VC: A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. Nucleic Acids Res 24: 962-969, 1996.
7. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Changel NS and Jordan VC: Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97: 1746-1759, 2005.
8. Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, Tapia C, Kim HR, Yerrum S, Sharma CG, Nicolas E, Balagurunathan Y, Ross EA and Jordan VC: Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci USA 108: 18879-18886, 2011.
9. Fan P, Griffith OL, Agboke FA, Anur P, Zou X, McDaniel RE, Creswell K, Kim SH, Katzenellenbogen JA, Gray JW and Jordan VC: c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. Cancer Res 73: 4510-4520, 2013.
10. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A, Jamalabadi-Majidi S, Crowder R and Siegel BA: Lower-dose vs high-dose oral estradiol therapy of hormone receptor positive, aromatase inhibitor resistant advanced breast cancer: A Phase 2 randomized study. JAMA 302: 774-780, 2009.
11. Fan P, McDaniel RM, Kim HR, Clagett D, Haddad B and Jordan VC: Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines. Eur J Cancer 48: 3488-3498, 2012.
12. Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, Bonds D, Brunner R, Brzyski R, Caan B, Chlebowski R, Curb D, Gass M, Hays J, Heiss G, Hendrix S, Howard BV, Hsia J, Hubbell A, Jackson R, Johnson KC, Judd H, Kotchen JM, Kuller L, LaCroix AZ, Lane D, Langer RD, Lasser N, Lewis CE, Manson J, Margolis K, Ockene J, O'Sullivan MJ, Phillips L, Prentice RL, Ritenbaugh C, Robbins J, Rossouw JE, Sarto G, Stefanick ML, Van Horn L, Wactawski-Wende J, Wallace R and Wassertheil-Smoller S; Women's Health Initiative Steering Committee: Effects of conjugated equine estrogen in post-menopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. J Am Med Assoc 291: 1701-1712, 2004.
13. Doroshow JH: Glutathione peroxidase and oxidative stress. Toxicol Lett 82-83: 395-398, 1995.
14. Siemankowski LM, Morreale J and Briehl MM: Antioxidant defenses in the TNF-treated MCF-7 cells: selective increase in MnSOD. Free Radic Biol Med 26: 919-924, 1999.
15. Lee YJ, Chen JC, Amoscato AA, Bennouna J, Spitz DR, Suntharalingam M and Rhee JG: Protective role of Bcl2 in metabolic oxidative stress-induced cell death. J Cell Sci 114: 677-684, 2001.
16. Brodie A, Macedo L and Sabnis G: Aromatase resistance mechanisms in model systems in vivo. J Steroid Biochem Mol Biol 118: 283-287, 2010.
17. Fox EM, Miller TW, Balko JM, Kuba MG, Sanchez V, Smith RA, Liu S, Gonzalez-Angulo AM, Mills GB, Ye F, Shyr Y, Manning HC, Buck E and Arteaga CL: A kinome-wide screen identifies the insulin/IGF-I receptor pathway as a mechanism of escape from hormone dependence in breast cancer. Cancer Res 71: 6773-6784, 2011.
18. Osborne CK and Schiff R: Mechanisms of endocrine resistance in breast cancer. Annu Rev Med 62: 233-247, 2011.
19. Lee JY, Han CY, Yang JW, Smith C, Kim SK, Lee EY, Kim SG and Kang KW: Induction of glutathione transferase in insulin-like growth factor type I receptor-overexpressed hepatoma cells. Mol Pharmacol 72: 1082-1093, 2007.

FULL PAPER

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Keywords: E2; oestrogen receptor; 4-hydroxy tamoxifen; RT–PCR; TNF; endoplasmic reticulum stress; paclitaxel; chemotherapy

Delayed triggering of oestrogen induced apoptosis that contrasts with rapid paclitaxel-induced breast cancer cell death

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Background: Oestrogen (E_2) induces apoptosis in long-term E_2-deprived MCF7 cells (MCF7:5C). Taxanes have been used extensively in the treatment of early and advanced breast cancer. We have interrogated the sequence of events that involve the apoptotic signalling pathway induced by E_2 in comparison with paclitaxel.

Methods: DNA quantification and cell cycle analysis were used to assess proliferation of cancer cells. Apoptosis was evaluated using annexin V and DNA staining methods. Regulation of apoptotic genes was determined by performing PCR-based arrays and RT–PCR.

Results: E_2-induced apoptosis is a delayed process, whereas paclitaxel immediately inhibits the growth and induces death of MCF7:5C cells. The cellular commitment for E_2-triggered apoptosis occur after 24 h. Activation of the intrinsic pathway was observed by 36 h of E_2 treatment with subsequent induction of the extrinsic apoptotic pathway by 48 h. Paclitaxel exclusively activated extramitochodrial apoptotic genes and caused rapid G2/M blockade by 12 h of treatment. By contrast, E_2 causes an initial proliferation with elevated S phase of cell cycles followed by apoptosis of the MCF7:5C cells. Most importantly, we are the first to document that E_2-induced apoptosis can be reversed after 24 h treatment.

Conclusions: These data indicate that E_2-induced apoptosis involves a novel, multidynamic process that is distinctly different from that of a classic cytotoxic chemotherapeutic drug used in breast cancer.

Endocrine therapy remains the standard of care in the treatment of oestrogen receptor (ER)-positive breast cancer (Jordan, 2009). Tamoxifen inhibits estradiol (E_2)-induced tumour growth; but continuous tamoxifen treatment of nude mice with transplantable ER-positive tumours results in tumour growth with either E_2 or tamoxifen (Osborne *et al*, 1987; Gottardis and Jordan, 1988). After 5 years of re-transplantation and tamoxifen treatment, these serially transplanted tamoxifen-stimulated tumours grow in response to tamoxifen, but paradoxically rapidly regress with physiological E_2 treatment (Yao *et al*, 2000). Development of acquired resistance to long-term (5 years) antihormonal therapy in breast cancer causes a reconfiguration of the tumour cells that now makes them vulnerable to physiological E_2-induced apoptosis. MCF7 breast cancer cells that are resistant to long-term oestrogen withdrawal undergo apoptosis in response to E_2 (Lewis *et al*, 2005a, b). Clinical trials (Lønning *et al*, 2001; Ellis *et al*, 2009) have evaluated this concept, and their results show that about 30% of patients with advanced breast cancer who have acquired resistant to anti-hormone therapy show an objective clinical response with oestrogen therapy. The Women Health Initiative trial (WHI, 1998), which compared conjugated equine oestrogen (CEE) therapy with placebo in hysterectomised postmenopausal women, noted a paradoxical decrease in incidence of breast cancer compared with combination of CEE and progestin (Rossouw *et al*, 2002; Chlebowski *et al*, 2013), and this observation was subsequently supported by results obtained from the Million

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Women Study (Beral *et al*, 2011). In neither clinical study was a molecular mechanism offered to explain the apparent anomaly that CEE alone does not induce a profound significant increase in breast cancer risk. However, reanalysis of the mature data from the Women Health Initiative CEE alone study (Anderson *et al*, 2012) now demonstrates a persistent and sustained decrease in the incidence and mortality of breast cancer in women who received E_2 alone therapy. We recently reported that constituents of CEE cause apoptosis in long-term E_2-deprived MCF7 cells (Obiorah and Jordan, 2013). Given that these laboratory observations translate to clinical benefit for patients, it is appropriate to investigate the molecular events that precede the induction of apoptosis by E_2.

Cancer chemotherapy induces rapid death of neoplastic cells (Kaufmann and Earnshaw, 2000; Makin and Dive, 2001), but E_2-induced apoptosis, in contrast, is a delayed event. Ariazi *et al* (2011) recently identified the total gene activation sequence that occurs over a 7-day period during E_2-induced apoptosis. Endoplasmic reticulum stress is induced by E_2 that activates unfolded protein response leading to upregulation of mitochondrial proapoptotic genes. Involvement of the extrinsic pathway in E_2-induced apoptosis have been implicated, but its exact role is not clearly defined (Song *et al*, 2001; Osipo *et al*, 2003). However, nothing is known on the effect of cytotoxic chemotherapy in the MCF7:5C cells. Paclitaxel, a member of the drug family, the taxanes, is a mitotic spindle inhibitor that prevents destabilization of microtubules (Jordan *et al*, 1993; Yvon *et al*, 1999). Taxanes are used extensively as part of combination therapy in metastatic breast cancer (Robert *et al*, 2011; Kelly *et al*, 2012), and are the gold standard in the adjuvant therapy of early breast cancer where they decrease risk of cancer recurrence and mortality(Ward *et al*, 2007; Gines *et al*, 2011).

The goal of this paper is to determine the critical trigger point for E_2-induced apoptosis. We have explored the differential gene expression as a prelude to determine the early molecular events in E_2-induced apoptosis in comparison with classic cytotoxic chemotherapy-induced apoptosis. Induction of mRNA levels of proapoptotic genes confirmed whether mitochondrial and tumour necrosis factor (TNF) apoptotic pathways were activated. We compared and contrasted the ability of E_2 and paclitaxel with arrest cell cycle to advance the molecular understanding of the new biology of E_2-induced apoptosis in therapy.

MATERIALS AND METHODS

Cell culture and reagents. Cell culture media were purchased from Invitrogen Inc. (Grand Island, NY, USA), and fetal calf serum was obtained from HyClone Laboratories (Logan, UT, USA). Compounds E_2, 4-hydroxytamoxifen (4OHT) and paclitaxel were obtained from Sigma (St Louis, MO, USA). MCF7:5C cells were derived from MCF7 cells obtained from the Dr Dean Edwards (San Antonio, TX, USA) as reported previously (Jiang *et al*, 1992). It was long-term cultured in E_2-deprived medium. MCF7 cells were maintained in RPMI media supplemented with 10% fetal calf serum, 6 ng ml^{-1} bovine insulin and penicillin and streptomycin. MCF7:5C cells were maintained in phenol-red-free RPMI media containing 10% charcoal dextran-treated fetal calf serum, 6 ng ml^{-1} bovine insulin and penicillin and streptomycin. The cells were treated with indicated compounds (with media changes every 48 h) for the specified time, and were subsequently harvested for tissue culture experiments.

Cell growth assay. The cell growth was monitored by measuring the total DNA content per well in 24-well plates.

Fifteen thousand cells were plated per well, and treatment with indicated concentrations of compounds was started after 24 h in triplicates. Media containing the specific treatments was changed every 48 h. On day 7, the cells were harvested and total DNA was assessed using a fluorescent DNA quantification kit (cat. no. 170-2480; Bio-Rad, Hercules, CA, USA) and was performed as previously described (Lewis *et al*, 2005a).

RNA isolation and real-time PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNAeasy kit according to the manufacturer's instructions. Real-time PCR was performed as previously described (Sengupta *et al*, 2010). The sequences for all primers were as follows: BCL2L11 (Bim) forward: 5′-TCGGACTGAGAAACGCAAG-3′; reverse: 5′-CTCGGTCACT CAGAACTTAC-3′. TNF forward: 5′-ACTTTGGAGTGATC GGCC-3′; reverse: 5′-GCTTGAGGGTTTGCTACAAC-3′. The change in expression of transcripts was determined as described previously and used the ribosomal protein 36B4 mRNA as the internal control (Sengupta *et al*, 2010).

Real-time profiler assay for apoptosis. RT–PCR profiler assay kits for apoptosis was used from a commercial vendor that used 384-well plates to profile the expression of 370 apoptosis-related human genes (Qiagen; SABiosciences Corp., Fredrick, MD, USA; cat. no. 330231 PAHS-3012E). All the procedures were followed as previously described(Sengupta *et al*, 2013). Briefly, MCF7:5C cells were treated with control or with indicated compounds (in triplicates), and total RNA was isolated using the method mentioned earlier. Two micrograms of total RNA was reverse transcribed and RT–PCR was performed using ABI 7900HT (Foster City, CA, USA). We created an apoptotic gene signature throughout these time points after comparing them with control treatment. This gene signature was generated by comparing the expression level of all the genes with vehicle treatment and selecting the genes that were at least 2.5-fold over- or under-expressed as compared with vehicle-treated cells at a statistical significance of *P*-value of 0.05. The fold change was calculated by ΔΔCt method and volcano plots were generated using the web-based tool, RT2 profile PCR array data analysis version 3.5 (Qiagen; SABiosciences Corp.).

Apoptosis assay. The concentration of paclitaxel was based on the publication by Gines *et al* (2011). The concentration of E_2 was based on the growth curve with different doses and our previous publication (Lewis *et al*, 2005a, b). MCF7:5C cells (1×10^6 cells per ml) were seeded in 100-mm dishes and cultured overnight in oestrogen-free RPMI 1640 medium containing 10% SFS. The next day, cells were treated with vehicle (0.1% ethanol) as control, E_2 (1 nM) for 48 and 72 h or with paclitaxel (1 μM) for 12 and 24 h, and then harvested in cold PBS (Invitrogen, Grand Island, NY, USA) and collected by centrifugation for 2 min at 500 **g**. Cells were then resuspended and stained simultaneously with either with FITC-labeled annexin V and propidium iodide (PI; Pharmingen, San Diego, CA, USA) or with DNA-binding dye, YO-PRO-1 and PI (Life Technologies, Grand Island, NY, USA). Apoptosis was verified based on loss of plasma membrane integrity. Viable cells excluded these dyes, whereas apoptotic cells allowed moderate staining. Cells were analysed using a fluorescence-activated cell sorter flow cytometer (Becton Dickinson, San Jose, CA, USA). Experiments were repeated three times with similar results.

Cell cycles analysis. MCF7:5C cells were cultured in dishes and were treated with vehicle (0.1% ethanol), E_2 (1 nM) and paclitaxel (1 μM). Cells were harvested and gradually fixed with 75% EtOH on ice. After staining with PI, cells were analysed using a fluorescence-activated cell sorter flow cytometer (Becton Dickinson), and the data were analysed with CellQuest software (BD Biosciences, San Jose, CA, USA).

Statistical analysis. All data were expressed as the mean of at least three determinations, unless otherwise stated. The differences between the treatment groups and the control group were determined by one-factor or two-way analysis of variance.

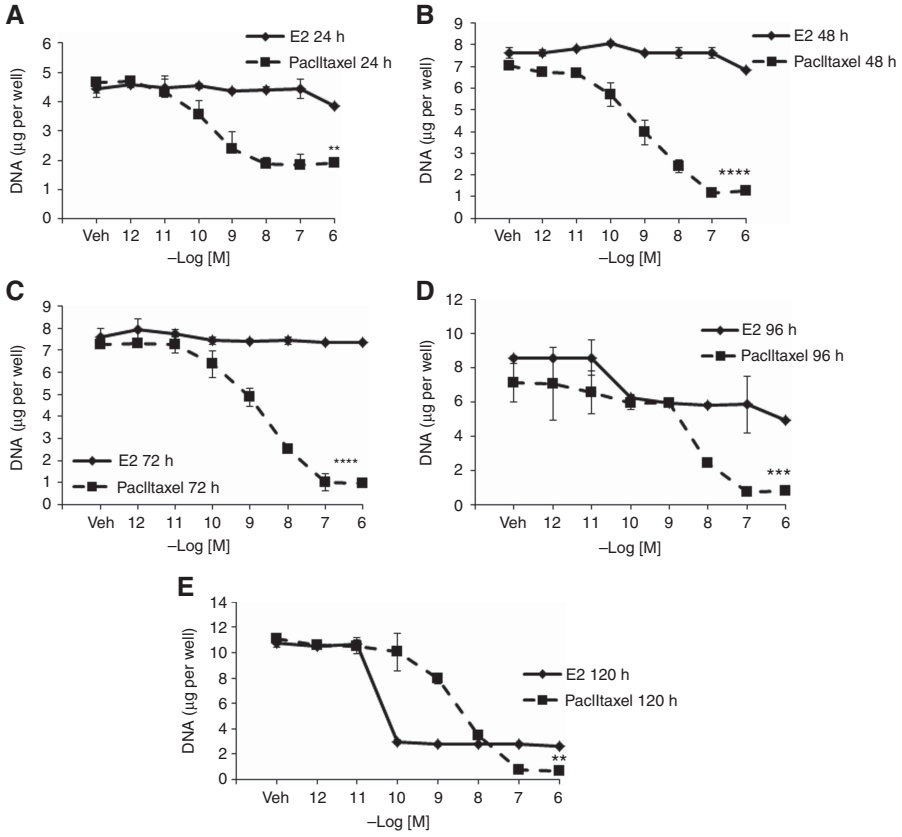


Figure 1. Effect of E_2 and paclitxel on the growth characteristics in the MCF7:5C cells. MCF5C cells were seeded in 24-well plate treated with the control vehicle (Veh) or E_2(♦) and paclitaxel (■) over a range of doses and cells were harvested after (**A**) 24 h, (**B**) 48 h, (**C**) 72 h, (**D**) 96 h and (**E**) 120 h. Data points shown are the average of three replicates ± s.d. (**$P<0.02$,***$P<0.0003$,****$P<0.0001$).

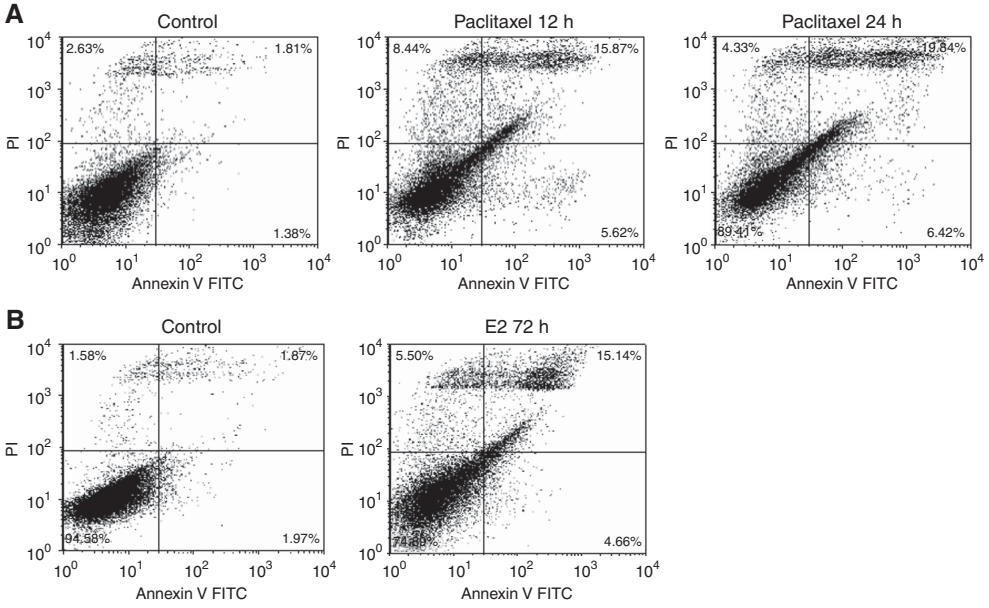


Figure 2. Differential apoptotic effects of E_2 and paclitaxel. MCF7:5C cells were treated with control or (**A**) paclitaxel (1 μM) for 12 and 24 h or (**B**) E_2 (1 nM) for 72 h, and then stained with annexin V-FITC and PI and analysed by flow cytometry. Viable cells (left lower quadrant) are annexin V-FITC− and PI−, early apoptotic cells (right lower quadrant) are annexin V-FITC+ and PI−, dead cells (left upper quadrant) are PI+ and late apoptotic cells (right upper quadrant) are annexin V-FITC+ and PI+. Increased staining for apoptosis is observed maximally in the right upper quadrant.

RESULTS

Cell growth and apoptotic effects of E_2 and paclitaxel on MCF7:5C cells. We sought to compare the antiproliferative activity between paclitaxel and E_2 in the MCF7:5C cell line and explore their potential to induce apoptosis. Paclitaxel induced rapid inhibition of growth in a concentration-dependent manner with maximum inhibition at 0.1 μM. Fifty percent growth inhibition was achieved in 24 h (Figure 1A), which increased to almost 100% after 48 h of treatment (Figure 1B). In contrast, E_2 achieved maximal growth inhibition at 0.1 nM, and did not quantitatively prevent cell proliferation until after 72 h (Figure 1C). Twenty-five percent of growth inhibition occurred

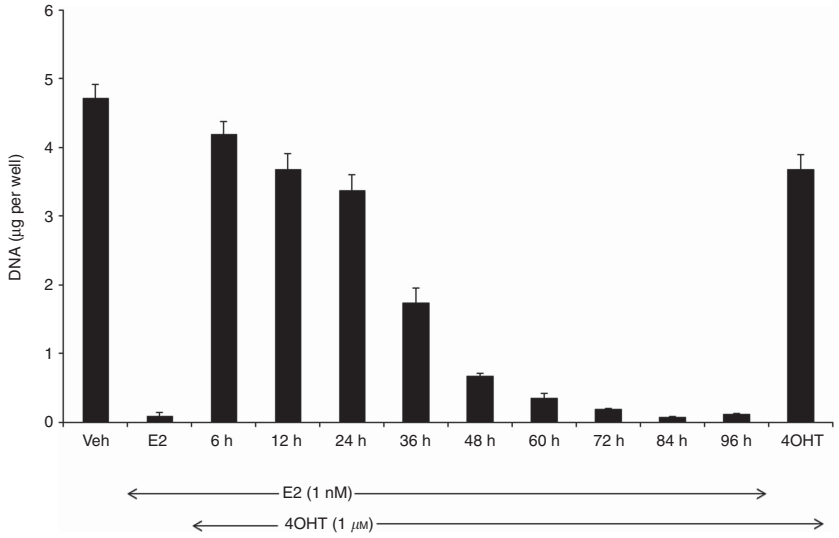


Figure 3. Deciphering the trigger point for E_2-induced apoptosis. Cells were treated with vehicle (Veh) or E_2 (1 nM) alone, and 1 μM 4OHT was added and used to block and reverse E_2 action at 6, 12, 24, 36, 48, 60, 72, 84 and 96 h. The cells were harvested after 7 days of treatment. The extent of apoptosis was determined by measuring the DNA content of the remaining cells in each well. The experiment was done in triplicates, and the data represent the mean of three independent experiments with 95% confidence intervals. The trigger point for E_2-mediated apoptosis was elucidated at the time when the apoptotic effects of E_2 could not be blocked by 4OHT.

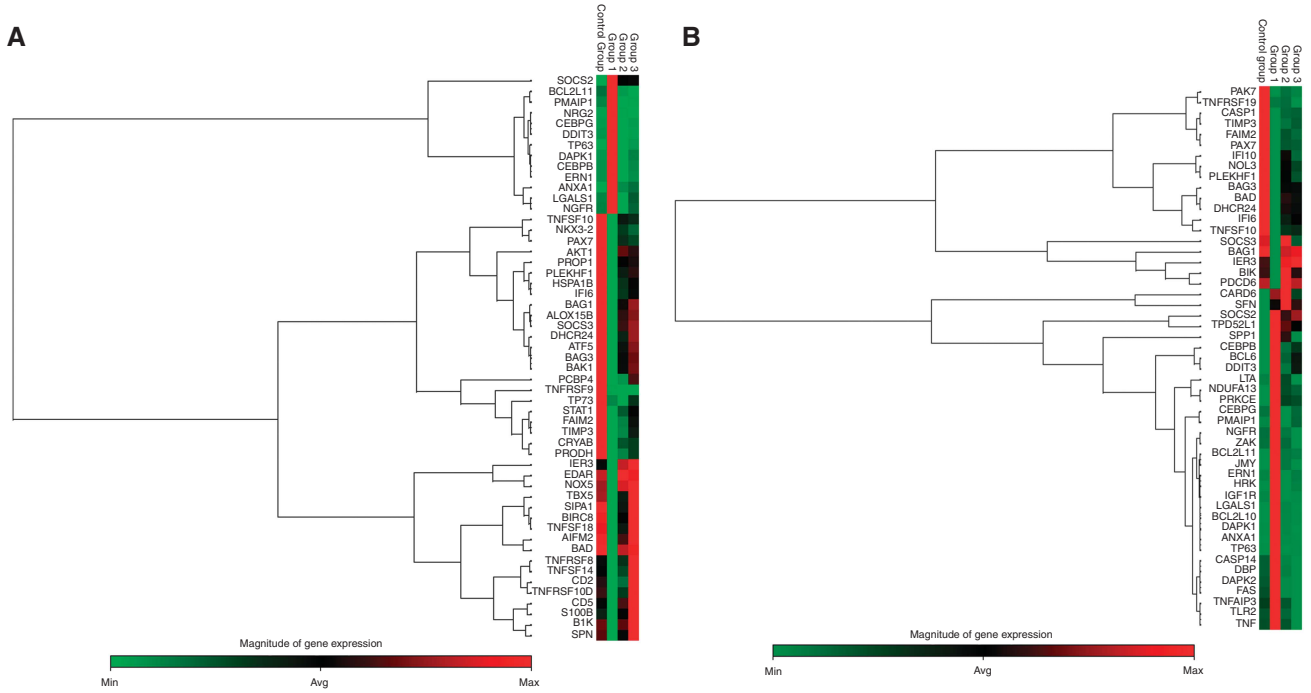
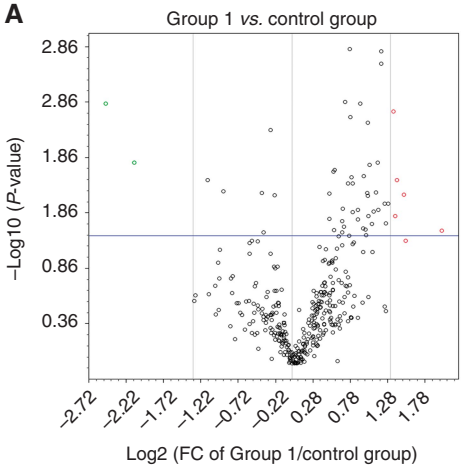


Figure 4. Heat map of E_2-mediated apoptotic genes that are differentially expressed by 36 and 48 h of treatment. Cells were parsed into groups of three replicates per treatment per time point, and then treated with either 0.1% ethanol (control group), 1 nM E_2 (group 1), 1 μM 4OHT (group 2), in the presence (group 3) or in the absence of E_2 over a period of 48 h. Total RNA was extracted and reverse transcribed as described in Materials and Methods section. Samples were loaded onto customised PCR array plates with primers for indicated apoptotic genes. Gene expression values were obtained and analysed in comparison with the controls at (**A**) 36 h and (**B**) 48 h. The maximum expressed level of any given gene is represented by red colour and minimum levels are represented as green colour.

at 96 h with E_2 treatment (Figure 1D) and this increased to 80% at the 120-h time point (Figure 1E). The decrease in cell number observed with E_2 and paclitaxel was further investigated to determine whether the growth inhibition was due to apoptosis. An increased apoptotic response (Figure 2A) was detected by increasing the percentage of annexin V staining from control 3.92–21.49% by paclitaxel after 12 h treatment, whereas an apoptotic effect was observed at 72 h with E_2 (Figure 2B). An apoptotic response was not detected after 24 h treatments with E_2 through annexin V staining (Supplementary Figure S1A). Experiments were repeated three times and a summary of results are represented in Supplementary Figure S1. Similar results were observed with a DNA-binding dye, YO-PRO-1 (Supplementary Figure S2).

Determination of the critical trigger point of estradiol-induced apoptosis. Although E_2 treatment induces apoptosis of MCF7:5C cells in a concentration-dependent manner, the cells are unresponsive to the anti-oestrogen, 4OHT. Rather 4OHT blocks E_2-mediated apoptosis (Maximov *et al*, 2011). To further investigate the delayed response to E_2-mediated apoptosis and to determine the critical trigger point for E_2-induced apoptosis, we used 4OHT to block and rescue the cells from the apoptotic effect of E_2. In this way, we established when the cells are committed to cell death. MCF7:5C cells were treated with 1 nM of E_2, and subsequently 1 μM of 4OHT was used to block the apoptotic effects of E2 at the indicated time points over a range of 96 h after the addition of E_2. Cells were then all collected for DNA assay on day 7. Apoptosis triggered by E_2 was competitively inhibited and rescued for up to 24 h, and thereafter it lost the ability to rescue cells committed to E_2-induced apoptosis (Figure 3). Between 24 and 36 h, the cells are committed to apoptosis despite the anti-oestrogenic action of 4OHT.These data suggest that the critical trigger for the commitment of the cell to the induction of apoptosis by E_2 lies between 24 and 36 h.

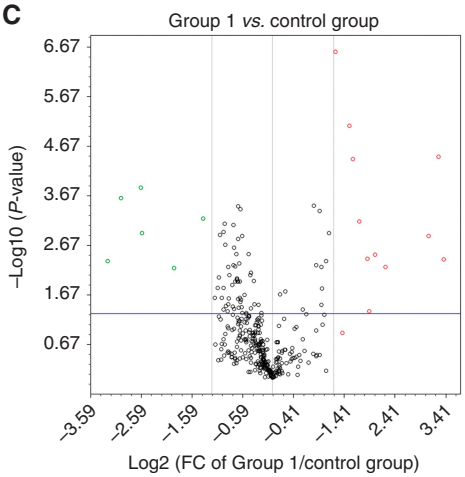
Differential gene expression of E_2-mediated apoptosis at the critical trigger point. To identify genes associated with E_2-induced apoptosis with a particular focus on the critical trigger time point, differential regulation of apoptotic gene expression in response to E_2 was interrogated in the MCF7:5C cells. At 24 h, as expected, significant evidence of apoptotic gene induction is not apparent, rather proapoptotic genes such as *BAD* and *BCL2L10,* and Caspases 1, 9 and 10 are differentially downregulated by E_2 (Supplementary Table S1). TNF-related genes, *TNFRSF8* and *TNFSF14*, are induced by both E_2 and 4OHT, and they do not have a definitive role in the TNF-mediated apoptosis but rather are involved in the T-cell response. Interestingly, at 36 h (Figure 4A), which represents the trigger point for apoptosis, E_2 induces



B

Gene symbol	Fold upregulation
HSPA1B	2.8724
SEMA4D	2.687
TNFAIP3	2.9231
TNFRSF10A	2.6083
TNFRSF10B	2.654
TNFRSF19	3.2439

Gene symbol	Fold downregulation
GDNF	−3.9205
LTA	−2.6799



D

Gene symbol	Fold upregulation
ALOX15B	4.4146
ANXA1	2.7694
CDKN1A	2.5468
DAPK1	12.2063
LTA	3.0582
LTB	5.5024
NME5	9.8936
NTF3	3.5263
PMAIP1	3.3451
SNCA	11.3361
FAS	4.756
TNF	3.8363
TNFAIP3	4.3023

Gene symbol	Fold downregulation
CARD11	−5.0698
CRYAB	−8.1087
GDNF	−3.2651
NDUFA13	−6.7381
TBX5	−2.7497
TNFSF18	−5.1435

Figure 5. Determination of apoptotic genes induced by a cytotoxic chemotherapy in MCF7:5C cells. MCF7: 5C cells were treated with either 0.1% ethanol (control), or 1 μM paclitaxel (group 1) for 12 and 24 h. Gene expression values were obtained and analysed in comparison with the controls, and volcano plots were generated at 12 h of treatment (**A**) and the expressed genes listed (**B**). Similarly, gene expression levels are analysed after 24 h of paclitaxel treatment (**C**) and genes are listed in **D**. The genes selected were at least 2.5-fold overexpressed or under-expressed as compared with vehicle at *P*-value = 0.05. Genes upregulated are represented in red and downregulated genes are represented in green.

proinflammatory genes such as *CEBPB, CEBPG* and *DAPK1*, and endoplasmic reticulum stress-related genes such as *DDIT3* and *ERN 1*. BCL2L11 (BIM), an important member of the mitochondrial pathway and an apoptosis activator, is also upregulated by E_2, suggesting an early involvement of the intrinsic pathway. Following 48 h of E_2 treatment (Figure 4B), the gene expression expands to involve the TNF-related genes such as *FAS, TNFRSF21* and *TNF*, and continued increased expression of endoplasmic reticulum stress and proinflammatory-related genes. In addition, p53 expression is increased at 48 h. PMAIP 1 (also known as NOXA), a Bcl-2 homology (BH3) only family and a p53-regulated gene is also upregulated by E_2. 4OHT acted as an anti-oestrogen and was able to block most of the effects of E_2. The identified apoptosis-related genes are listed in Supplementary Tables S1–S3.

Paclitaxel induces TNF family of apoptosis-related genes in MCF7:5C cells. We further investigated expressed genes activated by paclitaxel that may define a molecular mechanism. Based on the biological experiments shown above (Figures 1 and 2B), paclitaxel-induced apoptotsis happened after 12 h treatment and reached to a peak at 24 h. We mainly focused on detecting gene regulation by paclitaxel at these two time points. Paclitaxel selectively activated the TNF family of apoptosis-related genes. After an initial 12 h of treatment (Figure 5A and B), paclitaxel stimulated TNFRSF10A (TNF receptor superfamily, member 10a) and TNFRSF10B (TNF receptor superfamily, member 10b), which are known to be activated by the ligand TNF-related apoptosis inducing ligand (TNFSF10/TRAIL), and causes death through the extramitochondrial pathway.TNFRSF19 (TNF receptor superfamily, member 19) induces apoptosis in a caspase-independent manner. In addition, TNF proapoptotic genes, including *FAS* and *TNF*, and other TNF proinflammatory genes such as *LTA, LTB* and *TNFAIP3*, are activated by 24 h of treatment with paclitaxel (Figure 5C and D). Paclitaxel further induces NOXA and CDKN1A (p21) that are known to inhibit the activity of cyclin-CDK2 or -CDK4 complexes at the G1 phase. Although these two p53-regulated genes were upregulated by paclitaxel, p53 induction was not observed at 24 h. Unlike E_2, which increases BIM and TNF mRNA levels (Figure 6A and B), paclitaxel was only able to induce TNF expression (Figure 6C and D). These results highlight the differences in apoptosis-related genes induced by the two treatments.

Differential effect of paclitaxel in induction of G2 blockade in comparison with E_2. Paclitaxel prevents progression of mitosis and activates the mitotic checkpoint, paving a path for apoptosis. To elucidate whether the apoptotic effects of paclitaxel in comparison with E_2 were mediated through cell cycle arrest, we performed cell cycle analysis in MCF7:5C cells using flow cytometry. Our results reveal that paclitaxel treatment causes accumulation of cells in G_2/M phase with a concomitant reduction in the number of cells in G_1 and S phase (Figure 7) Cell cycle arrest in G_2/M phase was about threefold higher compared with control. In contrast, a G_1 or G_2 blockade was not observed with E_2 treatment. E_2 dramatically enhanced S phase at 12 h and rapidly increased to sevenfold by 48 h. This is consistent with our recent

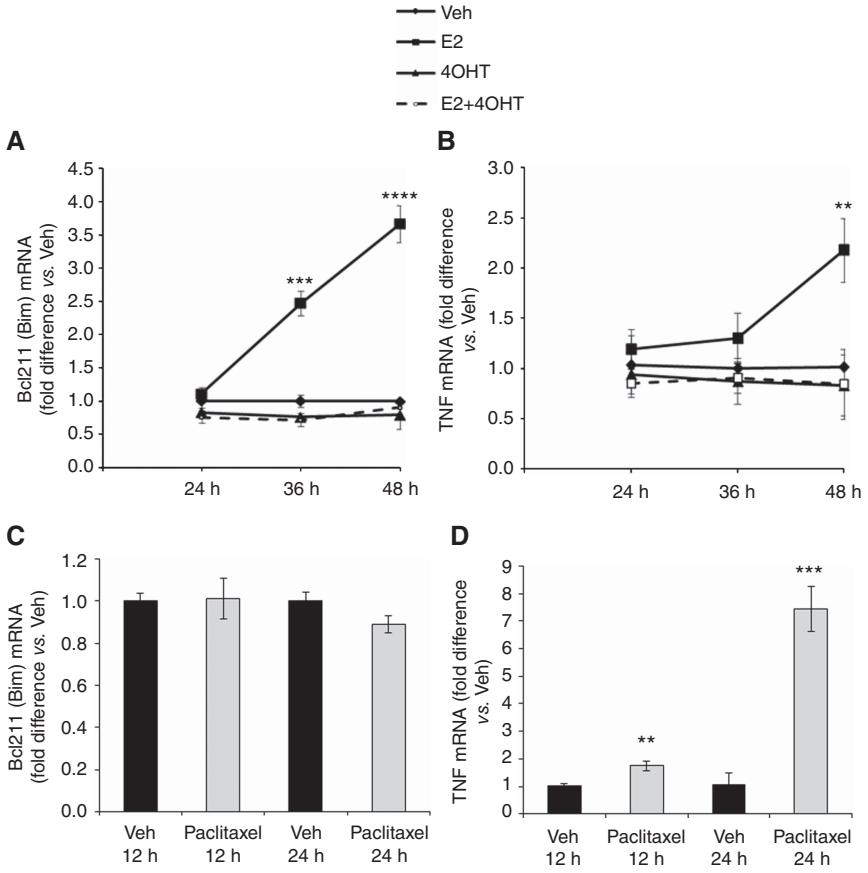


Figure 6. E_2 activates both mitochondrial and extrinsic pathway of apoptosis, whereas paclitaxel activates only the extrinsic pathway. MCF7:5C cells were treated with vehicle (Veh), 1 nM E2, 1 μM 4OHT or combination treatment of E_2 and 4OHT for 24, 36 and 48 h. Total RNA was reverse transcribed and assessed for (**A**) *BIM* and (**B**) *TNF* gene expression. Induction of (**C**) BIM and (**D**) TNF mRNA was determined in MCF7:5C cells treated with either Veh or 1 μM paclitaxel for 12 and 24 h using RT–PCR. PCR data values are presented as fold difference versus Veh-treated cells ± s.e.m. (**$P<0.02$, ***$P<0.0003$, ****$P<0.0001$).

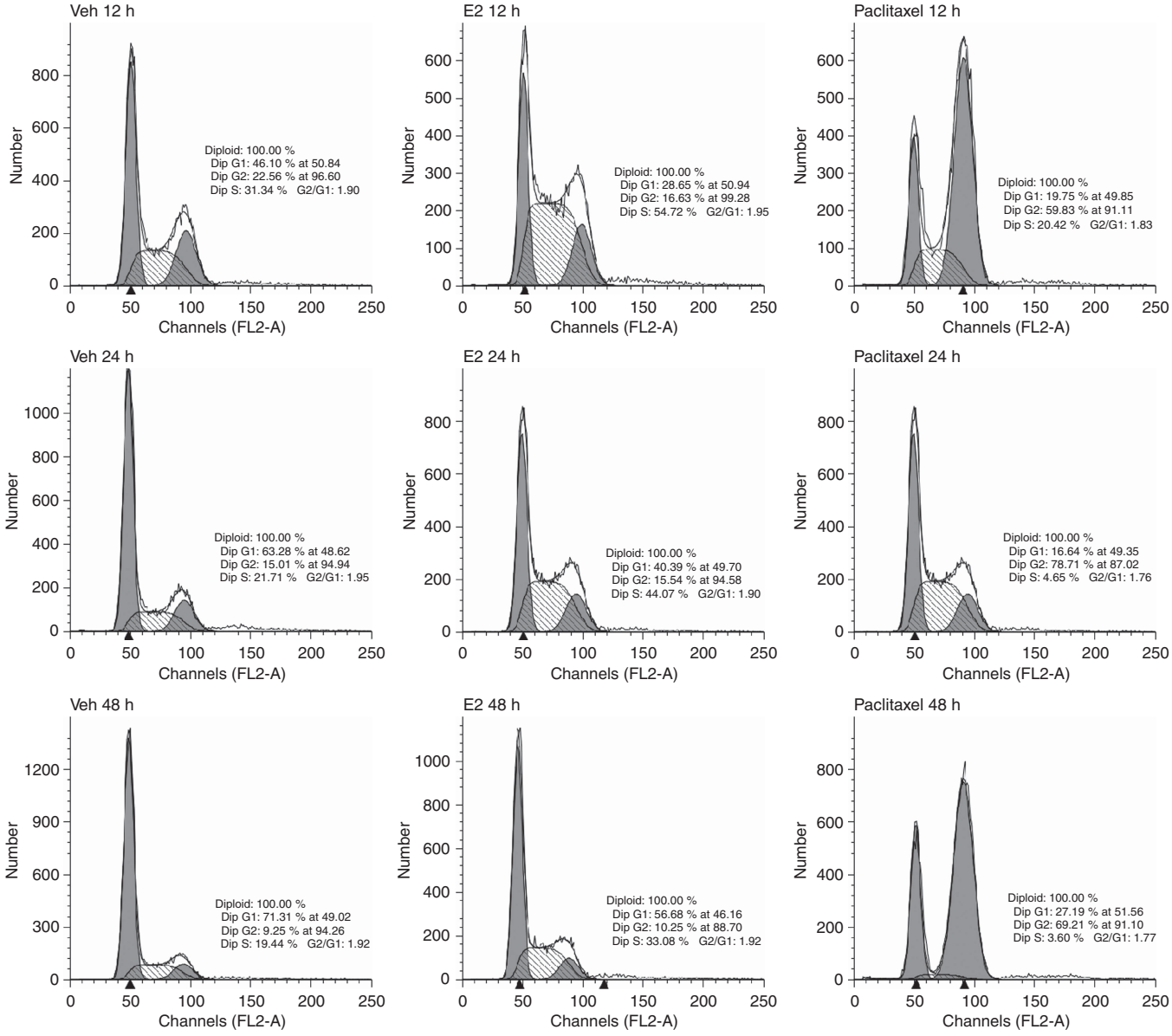


Figure 7. Cell cycle analysis of the effects of E_2 and paclitaxel in the MCF7:5C cells. Representative cell cycle profiles of MCF7:5C cells treated with either 0.1% ethanol (Veh), E_2 (1 nM) or paclitaxel (1 μM) for 12, 24 and 48 h. FL2-A represents the intensity of PI, and the *y*-axis represents the cell number.

publication (Fan *et al*, 2013) that E2 increased S phase after 72 h treatment. Based on these observations, we hypothesise that the apoptotic effects of paclitaxel in MCF7:5C cells results from a perturbation in the cell cycle check points, whereas E_2 induces cell proliferation finally resulting in apoptosis.

DISCUSSION

The molecular sequence of events resulting in either E_2-induced apoptosis or paclitaxel-induced apoptosis is completely different. E_2-induced apoptosis appears to be unique. Paclitaxel rapidly induces apoptosis of MCF7:5C cells, whereas E_2 shows a delayed process for the induction of apoptosis. Using 4OHT to block and rescue E_2-induced events necessary for an apoptotic response, we observed that the trigger for apoptosis occurs after 24 h and the cells become committed to apoptosis by and after 36 h. There is activation by E_2 of endoplasmic reticulum stress-related genes and proinflammatory genes at 36 h. Activation of the mitochondrial pathway was indicated by increased expression of BCL2L11, BIM, that continued to be upregulated at 48 h. Involvement of the extrinsic pathway was evidenced by induction of FAS, TNFRSF21 and TNF, and TNFAIP3 at 48 h. The TNF family genes are a group of cytokines that are involved in a number of processes including apoptosis (Micheau and Tschopp, 2003; Thorburn, 2004) and inflammation (De Paepe *et al*, 2012). The increased involvement of endoplasmic reticulum stress and inflammatory genes in E_2-induced apoptosis is not surprising because both pathways are known to intersect (Hu *et al*, 2006; Zhang and Kaufmann, 2008). Multiple genes induced by E_2 are NF-κB responsive that is a major regulator of inflammatory response (Baldwin, 1996; Dobrovolskaia and Kozlov, 2005). Upregulation of the observed genes provide a potential mechanism for E_2 to target a variety of inflammatory and apoptotic genes.

The importance of BIM and Bax have previously been noted and verified by selective increased expression of both proteins by E_2 (Lewis *et al*, 2005a). Involvement of the extrinsic signalling

pathway in E_2-induced apoptosis has been also observed. Osipo *et al* (2003) showed that E_2-induced regression of tamoxifen-stimulated breast cancer tumours by activating the death receptor Fas and inhibiting the antiapoptotic/prosurvival factors NF-κB and HER2/neu. In addition, the growth of raloxifene-resistant MCF7 cells *in vitro* and *in vivo* was inhibited by E_2 by increasing Fas expression and reduced NF-κB activity (Liu *et al*, 2003). However, unlike the present study, none of the previous studies investigated a time course of the intrinsic and extrinsic pathway in the MCF7:5C cells in E_2-induced apoptosis. Similar to our PCR array results, RNA sequencing of E_2-treated MCF7:5C cells revealed induction of multiple apoptosis-related genes (Fan *et al*, 2013); therefore, deletion of a single gene is unlikely to significantly affect E_2-mediated apoptosis in the MCF7:5C cells. E_2 induces apoptosis in osteoclasts within 24 h (Kameda *et al*, 1997) and is associated with upregulation of TGF-β and inhibition of E_2-treated cells with anti-TGF-β antibody inhibited E_2-induced apoptosis (Hughes *et al*, 1996). Therefore, this study show a unique sequential activation of endoplasmic reticulum stress, inflammatory-response genes as well as the intrinsic and extrinsic apoptosis-related genes in E_2-mediated apoptosis.

Paclitaxel, a cytotoxic chemotherapy extensively used in the treatment of breast cancer was used as a comparator to E_2 to demonstrate differences in the expression of apoptosis-related genes. Paclitaxel selectively induces the TNF proapoptotic genes, but BIM expression was not noted. On the other hand, paclitaxel kills the MCF7 cells by displacement of BIM from the BIM/BCL2 complex (Kutul and Lethai, 2010). Knockdown of BIM with siRNA significantly impairs the ability of paclitaxel to cause apoptosis in MCF7 cells (Kutul and Lethai, 2010; Ajabnoor *et al*, 2012). In contrast, another study(Czernick *et al*, 2009) showed that BIM was not required for paclitaxel-mediated apoptosis in MCF7 cells, and these apparent discrepancies could be because of differences that exist from MCF7 cell lines obtained from different sources. However, long-term deprivation of E_2 from the MCF7 cells may have induced changes in the microenvironment that may be responsible for the taxane to activate the TNF apoptosis-related genes. Flow cytometry studies show that E_2 causes both proliferation and apoptosis of the MCF7:5C cells, indicating that before the trigger for apoptosis occurs, the cells grow in response to E_2. Because cells continue to divide with elevated S phase of cell cycles, the reduction of cell number by E_2 do not become evident until after 4 days of treatment. In contrast, paclitaxel causes an immediate G2 blockade by 12 h that may explain the rapid reduction of cell number.

In conclusion, the initial target site of E_2 is ER. E_2 induces endoplasmic reticulum stress and mitochondrial apoptotic genes and a later recruitment of the TNF family of apoptotic genes, whereas paclitaxel induces a G2/M blockade and rapidly induces TNF apoptosis-related genes. The unique delayed aspect of E_2-induced apoptosis in antihormone-resistant breast cancer creates a new dimension in our opportunities to apply the knowledge for this targeted therapy of clinical significance (Ellis *et al*, 2009; Anderson *et al*, 2012; Obiorah and Jordan, 2013). This natural process of E_2-induced apoptosis may have significant applications in the further understanding of the cellular biology of cancer.

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REFERENCES

Ajabnoor GM, Crook T, Coley H (2012) Paclitaxel resistance is associated with switch from apoptotic to autophagic cell death in MCF-7 breast cancer cells. *Cell Death Dis* **3**(1): e260.

Anderson GL, Chlebowski RT, Aragaki AK, Kuller LH, Manson JE, Gass M, Bluhm E, Connelly S, Hubbell FA, Lane D, Martin L, Ockene J, Rohan T, Schenken R, Wactawski-Wende J (2012) Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *Lancet Oncol* **13**: 476–486.

Ariazi E, Cunliffe H, Lewis-Wambi JS, Slifker M, Willis A, Ramos P, Tapia C, Kim H, Yerrum S, Sharma C, Nicolas E, Balagurunathan Y, Ross EA, Jordan V (2011) Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *Proc Natl Acad Sci USA* **108**: 18879–18886.

Baldwin A (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* **14**: 649–683.

Beral V, Reeves G, Bull D, Green J (2011) Breast Cancer Risk in Relation to the Interval Between Menopause and Starting Hormone Therapy. *J Natl Cancer Inst* **103**: 296–305.

Chlebowski R, Manson J, Anderson G, Cauley J, Aragaki A, Stefanick M, Lane D, Johnson K, Wactawski-Wende J, Chen C, Qi L, Yasmeen S, Newcomb P, Prentice R (2013) Estrogen plus progestin and breast cancer incidence and mortality in the Women's Health Initiative Observational Study. *J Natl Cancer Inst* **105**: 526–535.

Czernick M, Rieger A, Goping I (2009) Bim is reversibly phosphorylated but plays a limited role in paclitaxel cytotoxicity of breast cancer cell lines. *Biochem Biophys Res Commun* **379**(1): 145–150.

De Paepe B, Creus KK, De Bleecker JL (2012) The tumor necrosis factor superfamily of cytokines in the inflammatory myopathies: potential targets for therapy. *Clin Dev Immunol* **369432**: 23–33.

Dobrovolskaia MA, Kozlov SV (2005) Inflammation and cancer: when NF-kappaB amalgamates the perilous partnership. *Curr Cancer Drug Targets* **5**: 325–344.

Ellis M, Gao F, Dehdashti F, Jeffe D, Marcom P, Carey L, Dickler M, Silverman P, Fleming P, Kommareddy A, Jamalabadi-Majidi S, Crowder R, Siegel B (2009) Lower-dose vs high-dose oral estradiol therapy of hormone receptor–positive, aromatase inhibitor–resistant advanced breast cancer: A phase 2 randomized study. *JAMA* **302**: 774–780.

Fan P, Griffith OL, Agboke FA, Anur P, Zou X, McDaniel RE, Creswell K, Kim SH, Katzenellenbogen JA, Gray JW, Jordan VC (2013) c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. *Cancer Res* **73**: 4510–4520.

Gines J, Sabater E, Martorell C, Grau M, Monroy M, Casado M (2011) Efficacy of taxanes as adjuvant treatment of breast cancer: a review and meta-analysis of randomised clinical trials. *Clin Transl Oncol* **13**: 485–498.

Gottardis MM, Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* **48**: 5183–5187.

Hu P, Han Z, Couvillon A, Kaufman R, Exton J (2006) Autocrine tumor necrosis factor a links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1α-mediated NF-κB activation and down-regulation of TRAF2 expression. *Mol Cell Biol* **26**: 3071–3084.

Hughes DE, Dai A, Tiffee JC, Li HH, Mundy GR, Boyce BF (1996) Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-beta. *Nat Med* **2**: 1132–1136.

Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* **90**: 77–86.

Jordan M, Toso R, Thrower D, Wilson L (1993) Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. *Proc Natl Acad Sci USA* **90**: 9552–9556.

Jordan VC (2009) A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. *Cancer Res* **69**: 1243–1254.

Kameda T, Mano H, Yuasa T, Mori Y, Miyazawa K, Shiokawa M, Nakamaru Y, Hiroi E, Hiura K, Kameda A, Yang NN, Hakeda Y, Kumegawa M (1997) Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. *J Exp Med* **186**: 489–495.

Kaufmann SH, Earnshaw WC (2000) Induction of apoptosis by cancer chemotherapy. *Exp Cell Res* **256**: 42–49.

Kelly C, Green M, Broglio K, Thomas E, Brewster A (2012) Phase III trial evaluating weekly paclitaxel versus docetaxel in combination with capecitabine in operable breast cancer. *J Clin Oncol* **30**: 930–935.

Kutul O, Lethai A (2010) Displacement of Bim by BmF and Puma rather than increase in Bim level mediates paclitaxel induced apoptosis in breast cancer cells. *Cell Death Differ* **17**(10): 1624–1635.

Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Jordan VC (2005a) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* **97**: 1746–1759.

Lewis JS, Osipo C, Meeke K, Jordan VC (2005b) Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* **94**: 131–141.

Liu H, Lee E-S, Gajdos C, Pearce ST, Chen B, Osipo C, Loweth J, McKian K, De Los Reyes A, Wing L, Jordan VC (2003) Apoptotic action of 17β-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. *J Natl Cancer Inst* **95**: 1586–1597.

Lønning P, Taylor P, Anker G, Iddon J, Wie L (2001) High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* **67**: 111–116.

Makin G, Dive C (2001) Apoptosis and cancer chemotherapy. *Trends Cell Biol* **11**: 22–26.

Maximov P, Sengupta S, Lewis-Wambi JS, Kim HR, Curpan RF, Jordan VC (2011) The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: a hypothesis. *Horm Mol Biol Clin Invest* **5**: 27–34.

Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* **114**: 181–190.

Obiorah I, Jordan VC (2013) 2012 NAMS/PFIZER- Wulf H. utian endowed lecture. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. *Menopause* **20**: 372–382.

Osborne CK, Coronado EB, Robinson J (1987) Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol* **23**: 1189–1196.

Osipo C, Gajdos C, Liu H, Chen B, Jordan VC (2003) Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* **95**: 1597–1608.

Robert N, Saleh M, Paul D, Generali D, Gressot L, Copur M, Brufsky A, Minton S, Giguere J, Smith J, Richards P, Gernhardt D, Huang X, Liau K, Kern K, Davis J (2011) Sunitinib plus paclitaxel versus bevacizumab plus paclitaxel for first-line treatment of patients with advanced breast cancer: a phase III, randomized, open-label trial. *Clin Breast Cancer* **11**: 82–92.

Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J (2002) Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *JAMA* **288**: 321–333.

Sengupta S, Obiorah I, Maximov PY, Curpan R, Jordan VC (2013) Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. *Br J Pharmacol* **169**: 167–178.

Sengupta S, Sharma C, Jordan VC (2010) Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells. *Horm Mol Biol Clin Invest* **2**: 235–243.

Song R, Mor G, Naftolin F, McPherson R, Song J, Zhang Z, Yue W, Wang J, Santen R (2001) Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17β-estradiol. *J Natl Cancer Inst* **93**: 1714–1723.

Thorburn A (2004) Death receptor-induced cell killing. *Cell Signal* **16**: 139–144.

Ward S, Simpson E, Davis S, Hind D, Rees A, Wilkinson A (2007) Taxanes for the adjuvant treatment of early breast cancer: systematic review and economic evaluation. *Health Technol Assess* **11**: 1–144.

WHI (1998) Design of the Women's Health Initiative Clinical Trial and Observational Study. *Controlled Clin Trials* **19**: 61–109.

Yao K, Lee E, Bentrem D, England G, Schafer J, O'Regan R, Jordan VC (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* **6**: 2028–2036.

Yvon AC, Wadsworth P, Jordan MA, Toso RJ, Thrower D, Wilson L (1999) Taxol suppresses dynamics of individual microtubules in living human tumor cells. *Mol Biol Cell* **10**: 947–959.

Zhang K, Kaufmann R (2008) From endoplasmic-reticulum stress to the inflammatory response. *Nature* **454**: 455–462.

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Defining the Conformation of the Estrogen Receptor Complex That Controls Estrogen-Induced Apoptosis in Breast Cancer[S]

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ABSTRACT

Development of acquired antihormone resistance exposes a vulnerability in breast cancer: estrogen-induced apoptosis. Triphenylethylenes (TPEs), which are structurally similar to 4-hydroxytamoxifen (4OHT), were used for mechanistic studies of estrogen-induced apoptosis. These TPEs all stimulate growth in MCF-7 cells, but unlike the planar estrogens they block estrogen-induced apoptosis in the long-term estrogen-deprived MCF7:5C cells. To define the conformation of the TPE:estrogen receptor (ER) complex, we employed a previously validated assay using the induction of transforming growth factor α (TGFα) mRNA in situ in MDA-MB 231 cells stably transfected with wild-type ER (MC2) or D351G ER mutant (JM6). The assays discriminate ligand fit in the ER based on the extremes of published crystallography of planar estrogens or TPE antiestrogens. We classified the conformation of planar estrogens or angular TPE complexes as "estrogen-like" or "antiestrogen-like" complexes, respectively. The TPE:ER complexes did not readily recruit the coactivator steroid receptor coactivator-3 (SRC3) or ER to the PS2 promoter in MCF-7 and MCF7:5C cells, and molecular modeling showed that they prefer to bind to the ER in an antagonistic fashion, i.e., helix 12 not sealing the ligand binding domain (LBD) effectively, and therefore reduce critical SRC3 binding. The fully activated ER complex with helix 12 sealing the LBD is suggested to be the appropriate trigger to initiate rapid estrogen-induced apoptosis.

Introduction

17β-Estradiol (E_2) is a key stimulus of growth for estrogen receptor (ER)–positive breast cancer. Endocrine therapy has been the gold standard of treatment in ER-positive breast cancer (Jordan, 2009), but acquired antihormone resistance to long-term antihormone therapy is a continuing clinical dilemma. Discovery of the evolution of acquired resistance exposed a vulnerability of cells by paradoxically triggering apoptosis with physiologic E_2 treatment (Jordan, 2004). Laboratory evidence demonstrates that E_2 is capable of inducing apoptosis in long-term estrogen-deprived MCF-7 cells (Lewis et al., 2005a,b). Similarly, tamoxifen-stimulated tumors (Osborne et al., 1987; Gottardis and Jordan, 1988) that develop in athymic nude mice in about 1 year will undergo regression after 5 years of tamoxifen if exposed to physiologic E_2 (Yao et al., 2000).

Clinical data support the use of estrogens in the treatment of ER-positive postmenopausal breast cancer. Synthetic high-dose estrogens induced regression of tumors in postmenopausal women with advanced breast cancer in the first ever reported cancer chemical therapy (chemotherapy)–mediated clinical study (Haddow et al., 1944). Clinical trials now exploit the concept for patients with metastatic breast cancer who develop resistance to endocrine therapy, which shows that estrogens induce a partial to complete response in about 30% of postmenopausal breast cancer patients who had previous exhaustive antihormone therapy (Lønning et al., 2001; Ellis et al., 2009). A reanalysis (Anderson et al., 2012) of the Women Health Initiative estrogen alone trial (Anderson et al., 2004), which compared conjugated equine estrogen therapy with placebo in hysterectomized postmenopausal women, showed a significant decrease in the incidence and mortality from breast cancer in these patients. The success of estrogen therapy in postmenopausal women depends on the menopausal

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ABBREVIATIONS: baze, bazedoxifene; ChIP, chromatin immunoprecipitation; DES, diethylstilbestrol; E_2, 17β-estradiol; endox, endoxifen; ER, estrogen receptor; EtOX, ethoxytriphenylethylene; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; ICI 182,780, 7α,17β-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol; LBD, ligand binding domain; 4OHT, 4-hydroxytamoxifen; 3OHTPE, trihydroxytriphenylethylene; ral, raloxifene; RT-PCR, real-time polymerase chain reaction; SERM, selective estrogen receptor modulator; SRC3, steroid receptor coactivator-3; TPE, triphenylethylene; TGFα, transforming growth factor α.

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status of the patients. Women who are more than 5 years postmenopausal, i.e., long-term estrogen deprived, have better tumor remission rate as well as prevention from breast cancer (Obiorah and Jordan, 2013).

The ER is the key signal transduction pathway for breast cancer growth and apoptosis based on studies with competitive inhibition of E_2 action with antiestrogen (Lewis et al., 2005a; Maximov et al., 2011). The question to be addressed is how a series of estrogens with planar or angular structures can reprogram the estrogen–ER complex to be either a survival signal in breast cancer or to trigger apoptosis. We previously classified estrogens (Jordan et al., 2001) based on reported data on the crystallization of the ligand binding domain (LBD) of the ER with estrogens (E_2, diethylstilbestrol) and antiestrogens (4-hydroxytamoxifen [4OHT] and raloxifene) (Brzozowski et al., 1997; Shiau et al., 1998) (see Fig. 1). The planar estrogens are sealed within the LBD by helix 12, thus activating the AF2 domain, which leads to coactivator binding and subsequent interaction of AF1 and AF2 (Tzukerman et al., 1994) to initiate growth and protein synthesis. In contrast, the bulky side chain of nonsteroidal antiestrogen causes displacement of helix 12 and prevents coactivator binding to the AF2 resulting in antiestrogenic action. Tamoxifen, a substituted triphenylethylene (TPE) derivative possesses estrogen-like activity (Harper and Walpole, 1966; Levenson et al., 1998; MacGregor and Jordan, 1998). We previously discovered that the surface amino acid D351 within the LBD is critical for the estrogenic actions of 4OHT (MacGregor Schafer et al., 2000; Jordan et al., 2001). Unlike raloxifene, which is less estrogenic and possesses an antiestrogen side chain that shields and neutralizes D351, the side chain in 4OHT is too short (Liu et al., 2002).

To interrogate the relationship of structure of an estrogenic ligand to program the conformation of the ER complex, we synthesized a range of estrogenic TPEs (Maximov et al., 2010), which are structurally similar to 4OHT. We and others hypothesize that the structure of the ligand governs the external surface of the ER complex with either planar estrogens or the TPEs (McDonnell et al., 1995; Jordan et al., 2001). As a result of the ligand shape, the estrogens can program the conformation of the estrogen–ER complex to modulate rapid or delayed apoptosis. The growth response of the ER-positive breast cancer cells is very sensitive to a wide range of estrogenic ligands. This is to ensure cancer cell survival in austere estrogen environments. This may not be true for estrogen-induced apoptosis, and the ligand shape may be required to be more specific to trigger cell death. The estrogen-deprived cancer cell is protected.

We investigated the actions of clinically relevant planar estrogens (E_2, diethylstilbestrol, equilin, estrone, and equilenin), antiestrogens (4OHT, endoxifen, raloxifene, and bazedoxifene), and model TPEs (bisphenol, trihydroxytriphenylethylene, and ethoxytriphenylethylene) on growth in MCF-7 cells and apoptosis in MCF7:5C cells. To understand the biologic activity of the TPE:ER, we employed a validated ER engineered assay using induction of the mRNA for the transforming growth factor α (TGFα) gene in situ in MDA-MB231 cells stably transfected with

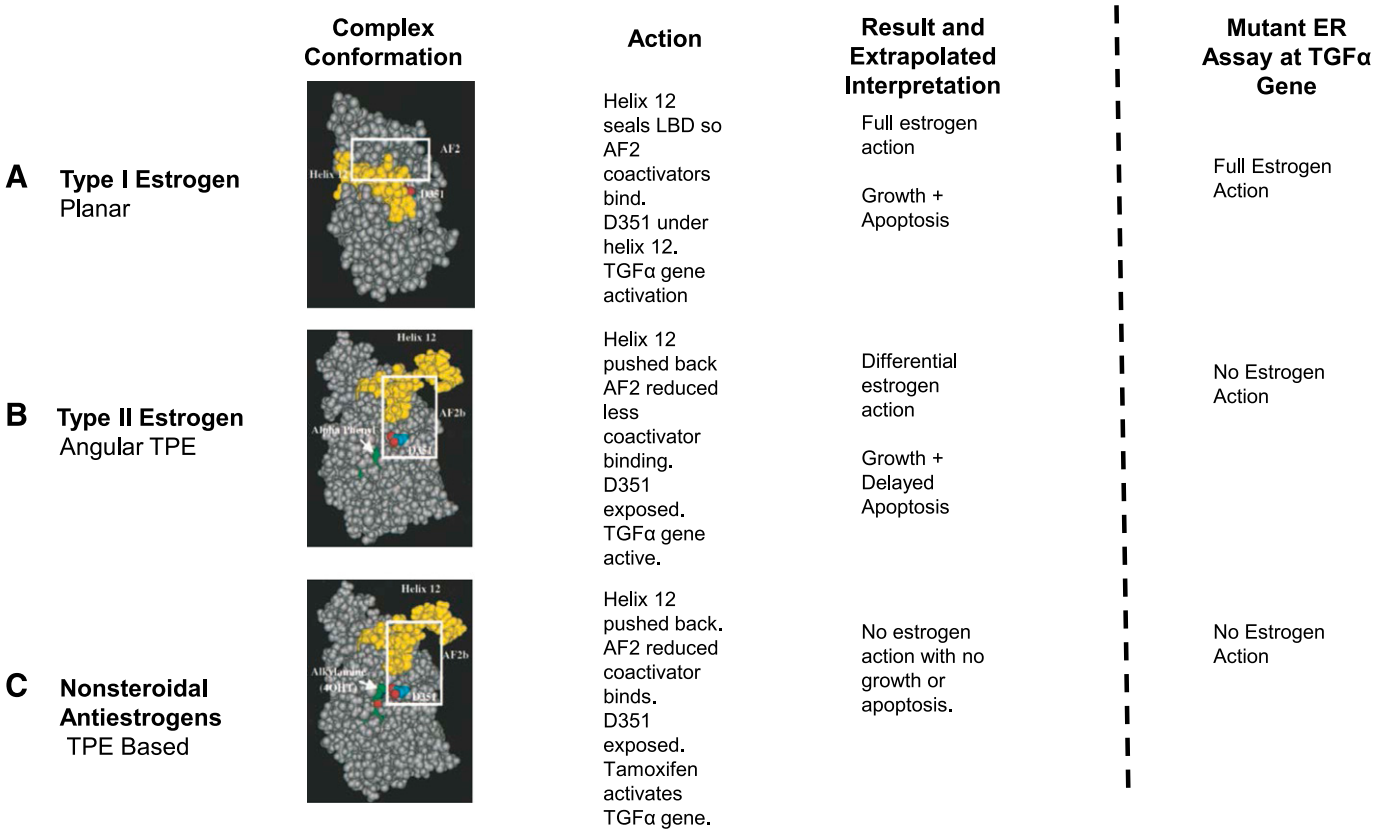

Fig. 1. Functional test: Putative conformations of the complex with ligand in LBD for Type II estrogen to be "antiestrogenic" with regard to helix 12 positioning. The assay discriminates between ligands (A), which allow helix 12 to seal the LBD or not (B and C). Sealing of helix 12 over the LBD is important for the ability of the ligands to trigger apoptosis.

wild-type ER or mutant D351G:ER (Jordan et al., 2001) (Fig. 1). We classified the structure of the ligands based on their ability to initiate TGFα mRNA synthesis through the ER complex. The biologic assay predicts two extremes of the ligand ER complex based on known X-ray crystallography (Brzozowski et al., 1997; Shiau et al., 1998): an "estrogen-like" shape and an "antiestrogen-like" shape. We find that the TPE:ER complex is antiestrogen-like, which explains the delayed apoptosis in MCF7:5C cells compared with the estrogenic complex formed by the planar estrogens.

Materials and Methods

Cell Culture and Reagents. Cell culture media were purchased from Invitrogen (Grand Island, NY) and fetal calf serum (FCS) was obtained from HyClone Laboratories (Logan, UT). Compounds E_2, diethylstilbesterol (DES), equilin, estrone, equilenin, ICI 182,780 (7α,17β-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol), and 4OHT were obtained from Sigma-Aldrich (St. Louis, MO), and chemical structures are as previously described (Brzozowski et al., 1997; Shiau et al., 1998; Sawicki et al., 1999; Howell et al., 2000; Wang et al., 2009). Raloxifene (ral) was a gift from Eli Lilly (Indianapolis, IN), and bazedoxifene (baze) was synthesized as previously described (Lewis-Wambi et al., 2011). The TPEs were synthesized as previously described (Maximov et al., 2010). MCF7:5C were derived from MCF-7 cells obtained from the Drs. Bill McGuire and Dean Edwards (San Antonio, TX), as reported previously (Jiang et al., 1992; Lewis et al., 2005b). MC2 and JM6 were obtained as previously described (MacGregor Schafer et al., 2000). MCF7:WS8 cells were derived from the original MCF-7 wild-type and were maintained in RPMI media supplemented with 10% FCS, 6 ng/ml bovine insulin, and penicillin and streptomycin. These have been maintained for >20 years. The MCF-7 cells were cultivated in phenol red-free media containing 10% charcoal dextran-treated FCS for 3 days prior to the start of the experiment. MCF7:5C cells were maintained in phenol-red free RPMI media containing 10% charcoal dextran treated FCS, 6 ng/ml bovine insulin, and penicillin and streptomycin. MC2 and JM6 cells were maintained in phenol red-free minimal essential medium supplemented with 5% 33 dextran-coated, charcoal-treated calf serum, 2 mM glutamine, 6 ng/ml bovine insulin, 100 units/ml penicillin/100 mg/ml streptomycin, nonessential amino acids, and 500 mg/ml G418. The cells were treated with indicated compounds for the specified time and were subsequently harvested for tissue culture experiments.

Cell Growth Assay. The cell growth was monitored by measuring the total DNA content per well in 24-well plates. Fifteen thousand cells were plated per well, and treatment with indicated concentrations of compounds was started after 24 hours, in triplicates. Media containing the specific treatments were changed every 48 hours. On day 7, the cells were harvested using hypotonic buffer solution and were subsequently sonicated. The DNA content was assessed using a fluorescent DNA quantitation kit (Cat # 170-2480; Bio-Rad, Hercules, CA) and was performed as previously described (Lewis et al., 2005a).

Annexin V Analysis of Apoptosis. The annexin V–fluorescein isothiocyanate (FITC)–labeled Apoptosis Detection Kit I (Pharmingen, San Diego, CA) was used to detect and quantify apoptosis by flow cytometry according to the manufacturer's instructions. In brief, MCF7:5C cells (1×10^6 cells/ml) were seeded in 100-mm dishes and cultured overnight in estrogen-free RPMI 1640 medium containing 10% stripped fetal serum. The next day, cells were treated with <0.1% ethanol vehicle (control), estradiol (1 nM), TPEs (1 μM), or selective estrogen receptor modulators (SERMs) (1 μM) for 72 hours and then harvested in cold phosphate-buffered saline (Invitrogen) and collected by centrifugation for 10 minutes at 500*g*. Cells were then resuspended and stained simultaneously with FITC-labeled annexin V and propidium iodide. Cells were analyzed using a fluorescence-activated cell sorter flow cytometer (Becton Dickinson, San Jose, CA).

RNA Isolation and Real-Time Polymerase Chain Reaction. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and RNAeasy kit according to the manufacturer's instructions. Real-time polymerase chain reaction (RT-PCR) was performed as previously described (Sengupta et al., 2010). Briefly, high-capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA) was used to generate cDNA using 1 μg of total RNA in a total volume of 20 μl. The cDNA was subsequently diluted to 500 μl, and RT-PCR was performed using ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). In each well, a 20-μl reaction volume included 10 μl SYBR green PCR master mix (Applied Biosystems), 125 nM each of forward and reverse primers, and 5 μl of diluted cDNA. RT-PCR was performed using specific primers as previously described (Sengupta et al., 2013), and the change in expression of transcripts was determined and the ribosomal protein 36B4 mRNA was used as the internal control.

Transforming Growth Factor Assay. Three hundred thousand MDA-MB231 cells stably transfected with wild-type ER (MC2) or mutant D351ER were seeded in six-well plates and treated with either vehicle control or various concentrations of planar estrogens, triphenylethylenes, or antiestrogens after 24 hours. After 24 hours, the cells were harvested for mRNA, and RT-PCR was performed to quantify TGFα mRNA levels as mentioned previously. The assay elucidates the putative conformation of ligand–receptor complex in relation to apoptotic-inducing action of the ligands (Fig. 1).

Immunoblotting. Proteins were extracted in cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with Protease Inhibitor Cocktail (Roche, Indianapolis, IN) and Phosphatase Inhibitor Cocktail Set I and Set II (Calbiochem, San Diego, CA). Total protein content of the lysate was determined by a standard bicinchoninic acid assay using the reagent from Bio-Rad. Twenty-five micrograms of total protein was separated on 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with primary antibodies followed by incubation with secondary antibody conjugated with horseradish peroxidase and reaction with Western Lighting plus-ECL enhanced chemiluminescent substrate (PerkinElmer, Waltham, MA). Protein bands were visualized by exposing the membrane to X-ray film.

Chromatin Immunoprecipitation Assay. Chromatin immunoprecipitation (ChIP) assay was performed as described previously (Maximov et al., 2011). Briefly, cells were treated with indicated compounds for 45 minutes and cross-linked using 1.25% paraformaldehyde for 15 minutes; cross-linking was subsequently stopped with 2 M glycine. Cells were collected, followed by nuclei isolation by centrifugation. Isolated nuclei were resuspended in SDS-lysis buffer followed by sonication and centrifugation at 14,000*g* for 20 minutes at 4°C. The supernatants were diluted 1:10 with ChIP dilution buffer. Normal rabbit IgG and Magna ChIP protein A magnetic bead (Upstate Cell Signaling Solutions, Temecula, CA) were used to immunoclear the supernatant followed by immunoprecipitation with antibodies against ERα (1:1 mixture of cat# sc-543 and sc-7207; Santa Cruz Biotechnology Inc., Dallas, TX) and steroid receptor coactivator-3 (SRC3) (cat# 13066; Santa Cruz Biotechnology, Inc.). Immunocomplexes were pulled down using protein A magnetic beads and a magnet. The beads bound to immunocomplexes were washed using different buffers as described (Maximov et al., 2011). Precipitates were finally extracted twice using freshly made 1% SDS and 0.1 M $NaHCO_3$ followed by decross-linking. The DNA fragments were purified using QiaQuick PCR purification kit (Qiagen, Valencia, CA). RT-PCR was performed using 2 μl isolated DNA, using primers specific for PS2 promoter (Maximov et al., 2011). The data are presented as percent input of starting chromatin input after subtracting the percent input pull down of the negative control (normal rabbit IgG).

Molecular Modeling. The molecular modeling study was performed using the available X-ray crystallographic structures of ERα in the agonist and antagonist conformations. The three-dimensional

coordinates of ERα cocrystallized with E_2 (1GWR) and 4OHT (3ERT) were extracted from RCSB Protein Data Bank (Data Supplements) (Berman et al., 2000). The ligand was prepared for docking using the LigPrep utility (LigPrep 2.5; Schrodinger, LLC, Portland, OR). Protein preparation workflow (Schrodinger, LLC) was employed to prepare the proteins for molecular docking. The residues well known to be important for biologic activity, D351 and E353, were kept charged in both receptors, the free rotation of hydroxyl group for T347 was allowed, and H524 residue was protonated at the epsilon nitrogen atom based on the available literature data. Glide software (Glide 5.7; Schrodinger, LLC) was used for molecular docking, and the best docking poses were selected based on the composite score, Emodel, which accounts not only for the binding affinity but also for the energetic terms, such as ligand strain energy and interaction energy

Results

Growth Effects of Estrogens and Antiestrogens in MCF-7 Cells. To study the biologic activity of the planar estrogens (Fig. 2A), which include E_2, DES, equilin, estrone, and equilenin), and triphenylethylenes (Fig. 2B), namely, ethoxytriphenylethylene (EtOX), trihydroxytriphenylethylene (3OHTPE), and bisphenol, we tested their ability to induce cell proliferation in wild-type MCF-7cells. As controls we used SERMs: 4OHT, endoxifen (endox), ral, and baze (Fig. 2C), which are known antiestrogens. MCF-7 cells were grown in estrogen-free media for 3 days and treated with various concentrations of the indicated compounds, and their effects were compared with E_2. All planar estrogens (Fig. 3A) were able to induce cell proliferation in a concentration-dependent manner to the same maximum level as E_2. DES, equilin, and estrone induced cell proliferation with maximum stimulation occurring at 0.1 nM, whereas equilenin reached maximal stimulation at 1 nM compared with 0.01 nM for E_2. Similarly, the triphenylethylenes tested were able to induce cell growth to the same maximum level as E_2, although their agonistic potency was less than E_2 (Fig. 3B). Bisphenol, EtOX, and 3OHTPE all induced cell proliferation in a concentration-dependent manner with maximum stimulation at 1–10 nM compared with 0.01 nM for E_2. Nonetheless, the TPEs all were potent estrogen agonists in this assay. On the other hand, as expected, the SERMs, 4OHT, endox, ral, and baze (Fig. 3C), which are antiestrogens, did not induce cell growth.

Effects of Planar Estrogens, TPEs, and SERMS on Apoptosis in MCF7:5C Cells. We tested whether TPEs and SERMS were able to induce apoptosis in long-term

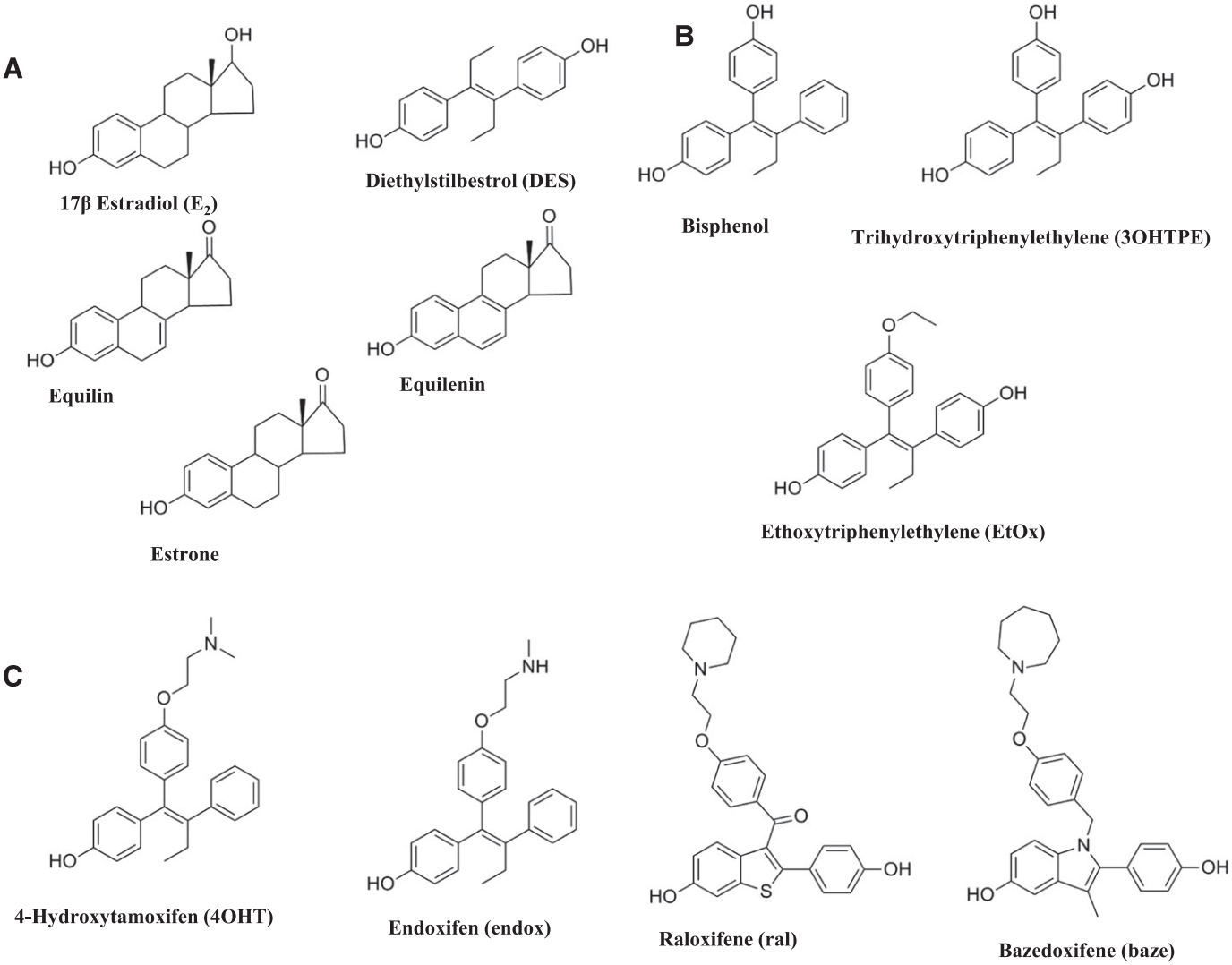

Fig. 2. Chemical structures of the compounds used in the experiments: planar estrogens (A), triphenylethylenes (B), and selective estrogen receptor modulators (C).

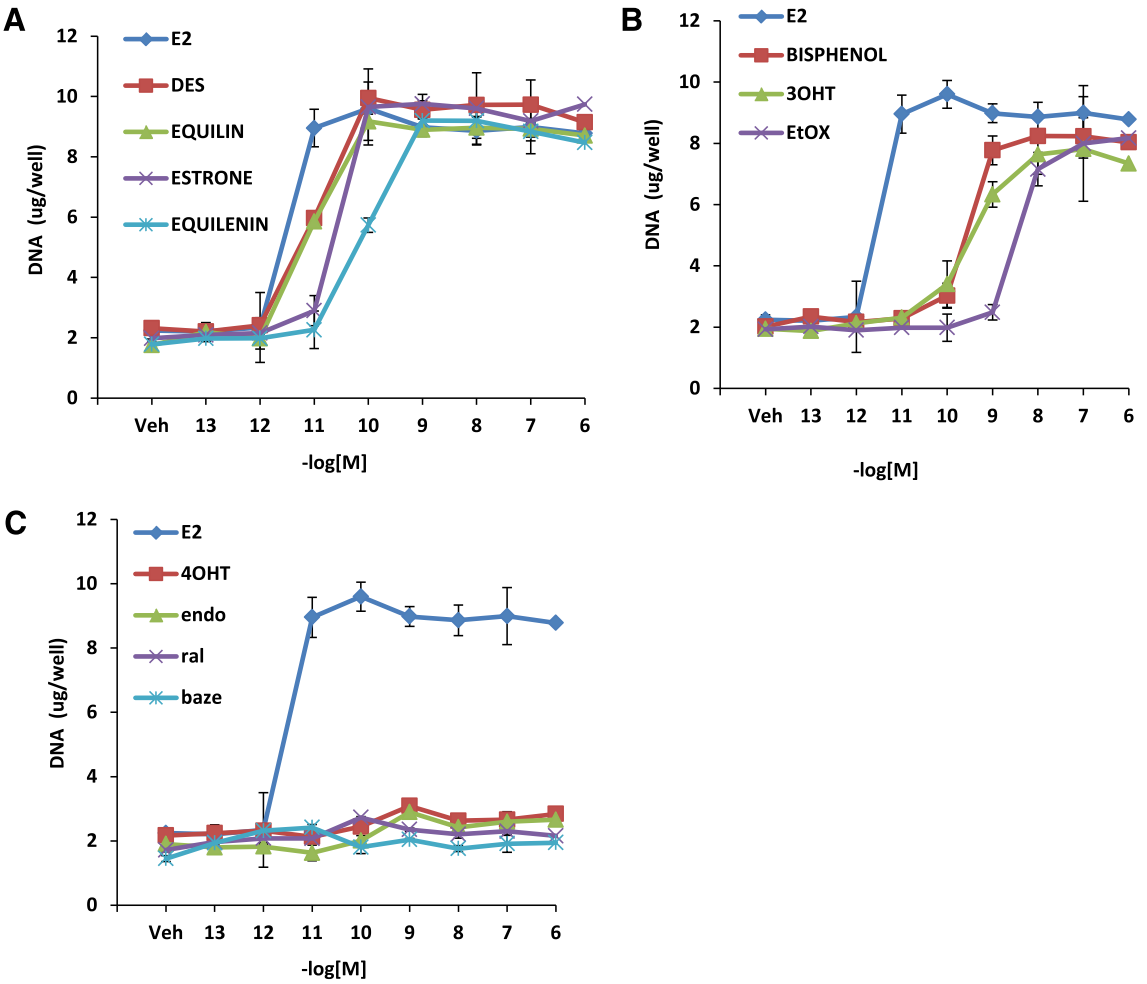

Fig. 3. Growth characteristics of planar estrogens and triphenylethylenes in MCF7:WS8 cells. MCF7:WS8 cells were seeded in 24-well plate and treated with planar estrogens (A) over a range of doses for 7 days. Cell growth was assessed as DNA content in each well. Induction of cell growth by triphenylethylenes (B) and SERMs (C) was assessed in comparison with E_2. Each data point is average ± S.D. of three replicates.

estrogen-deprived MCF7:5C breast cancer cells as effectively as E_2. All planar estrogens were able to cause growth inhibition as effectively as E_2 (Fig. 4A). All the planar estrogens achieved maximal growth inhibition in the range of 1 nM compared with E_2, which achieved maximal growth at 0.1 nM. To confirm that the decrease in cell proliferation was due to apoptosis, MCF7:5C cells were treated with ethanol vehicle (control), E_2 (1 nM) or DES (1 nM), equilin (1 nM), estrone (1 nM), and equilenin (1 nM) for 72 hours, and annexin V–FITC and propidium iodide fluorescence was determined by flow cytometry. In the control-treated group, only 5.9% stained positive for apoptosis, whereas, in the E_2-treated group (Supplemental Fig. 1A), cells that stained positive for apoptosis increased by 3-fold. Interestingly, the estrogenic triphenylethylenes did not inhibit the growth of MCF7:5C cells even at higher concentrations (Fig. 4B) at the end of a 7-day assay. Compared with E_2, bisphenol, 3OHTPE, and EtOX did not show any effective apoptosis even at micromolar concentration (Supplemental Fig. 1B) and were comparable to that of the SERMs (Fig. 4C). Furthermore, the TPEs were able to block E_2-mediated apoptosis in a similar manner to the SERMs (Fig. 4, D–E). However, the TPEs were able to induce apoptosis after 14 days of treatment (Fig. 4F), whereas the SERMs still did not induce apoptosis in the MCF7:5C cells (Supplemental Fig. 1C).

Regulation of TGFα Gene by Planar and Nonplanar Estrogens in MDA:MB-231 Cells Stably Transfected with Wild-Type ERα or D351G Mutant ERα. The TGFα gene is induced by 4OHT as effectively as E_2 in MDA:MB-231 cells stably transfected with wild-type ERα (MC2 cells). In contrast, in MDA:MB-231 cells stably transfected with a mutant D351ER (JM6 cells), 4OHT fails to induce expression of the TGFα gene, but E_2 retains its ability to induce the TGFα gene. We determined if the TPEs (3OHTPE, EtOX, and bisphenol) and the planar estrogens (DES, equilin, estrone, and equilenin) resembled E_2 or 4OHT in inducing the TGFα gene expression by using the assay system summarized in Fig. 1. As expected, all the planar estrogens were able to induce TGFα gene expression in a concentration-dependent manner in both wild-type ERα (MC2) (Fig. 5A) and D351G mutant ERα (JM6) cells (Fig. 5D). On the other hand, the TPEs and tamoxifen metabolites 4OHT and endox were able to induce TGFα gene expression in MC2 cells (Fig. 5B) in a concentration-dependent manner, whereas ral and baze do not activate the TGFα gene in this cell line (Fig. 5C). By contrast, the TPEs 4OHT and endox distinctly failed to induce TGFα gene expression (Fig. 5E) in JM6 cells, which express the D351G

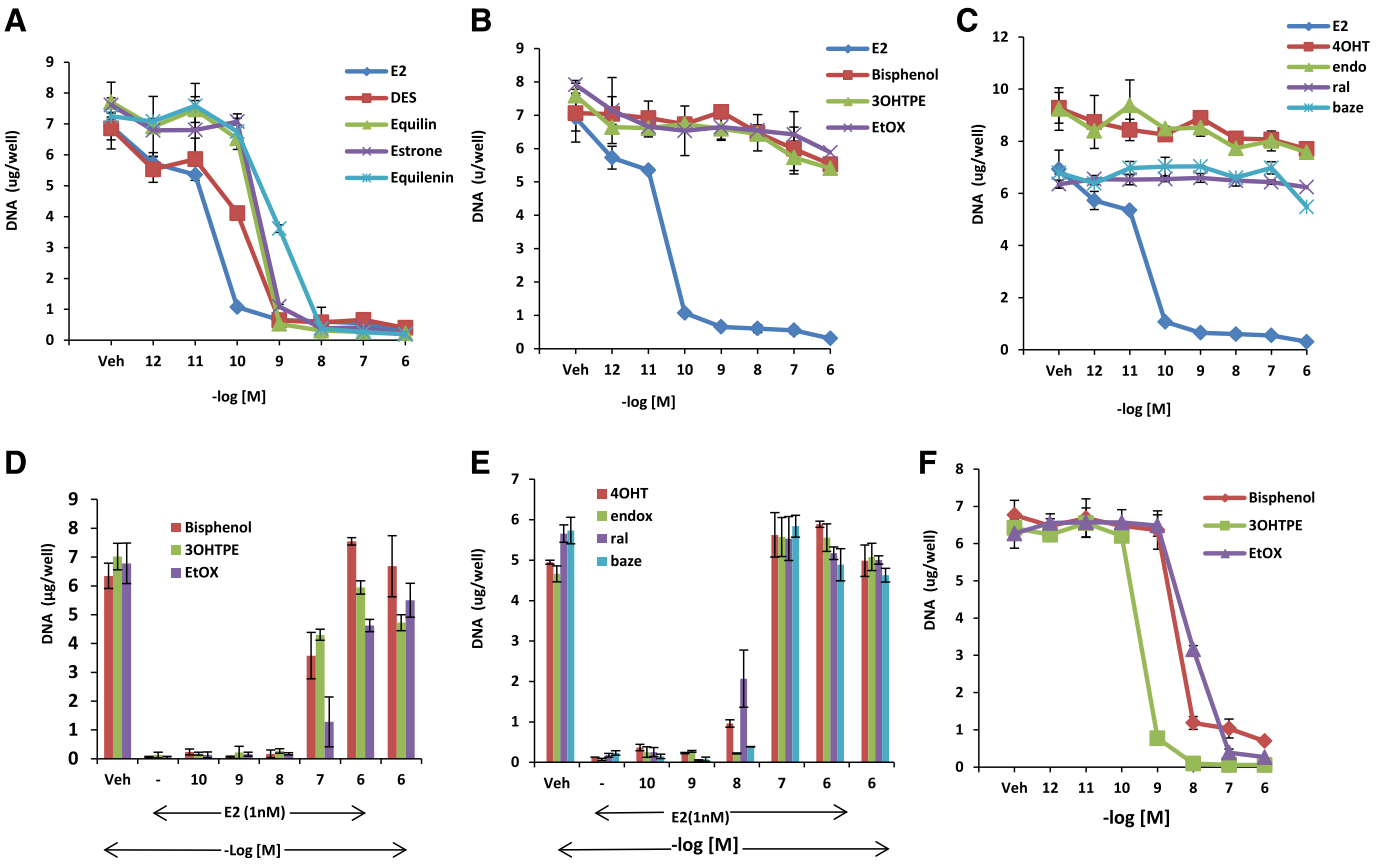

Fig. 4. Differential effect of planar estrogens and triphenylethylenes in MCF7:5C cells. Dose dependent effect of planar estrogens (A), triphenylethylenes (B), and SERMs (C) on apoptosis of MCF7:5C cells treated for 7 days as indicated. Cells were treated with 1 nM E_2 in presence of increasing concentration of indicated TPEs (D) and SERMs (E). (F) Effect of TPE in MCF7:5C cells after 14 days of treatment. Each data point is average ± S.D. of three replicates.

mutant form of the ERα, rather they block E_2-mediated TGFα induction (Fig. 5F). Similarly, ral and baze are antiestrogenic in the mutant stable transfectant. These findings indicate that the TPEs possess antiestrogenic properties and bind with ERα in a manner that is distinctly different from the planar estrogens but strikingly resembles 4OHT and endox.

Recruitment of ERα and SRC3 at the Proximal Promoter of PS2 Gene after Treatment with Triphenylethylenes. To further understand the ER-mediated mechanism involved in the regulation of the model estrogen responsive gene PS2 by the TPEs in MCF7:WS8 and MCF7:5C cells, we determined the recruitment of the ERα and SRC-3 protein at the proximal promoter of PS2 gene, which has a classic estrogen responsive element (Fig. 6A), using ChIP assay after 45 minutes of treatment with TPEs (1 μM) and compared it with E2 (1 nM) and 4OHT (1 μM). The whole assay was repeated two more times with similar results occurring in each cell line (Supplemental Figs. 4 and 5). In MCF7:WS8 cells, E_2 was able to recruit very high level of ERα at the PS2 promoter (Fig. 6B), where more than 8% of input PS2 promoter region was occupied by ERα. On the other hand TPEs were ~50% as efficient as E_2 treatment in terms of recruiting ERα, whereas a very low level (~20% of E_2) of ERα recruitment was observed after 4OHT treatment. Recruitment of the coactivator SRC3, which is critical in inducing the estrogen responsive gene, was not observed at all after 4OHT treatment at the PS2 promoter. All the TPEs tested recruited only about 15–20% of SRC3 compared with E_2 treatment, which showed 0.9% of input PS2 promoter region was occupied by SRC3 protein. Interestingly, in MCF7:5C cells treated with E_2, around 5% of input PS2 promoter region was occupied by ERα (Fig. 6C). In MCF7:5C, cells treated with TPEs had 50% less ERα occupancy, and ~80% less SRC3 occupancy was observed compared with E_2 treatment in MCF7:5C cells, whereas no SRC3 recruitment was observed after 4OHT treatment. These ChIP data concur with the PS2 mRNA induction level in MCF7:WS8 and MCF7:5C cells with their respective treatments (Supplemental Fig. 2, A and B).

Induction of ERα Expression by Planar and Nonplanar Estrogens. To test whether the structure the compounds create with the ER affects the ERα expression levels, 4 breast cancer cell lines, which include MCF7:WS8, MCF7:5C, MC2, and JM6 cells, were treated with planar estrogens (1 nM), TPEs (1 μM), and SERMs (1 μM) for 24 hours, and ERα levels were determined by Western blotting. ICI was included as a positive control. All planar estrogens and ICI caused decrease in the ERα protein levels in MDA-MB231 cells stably transfected with either wild-type ER (MC2) (Fig. 7A) or with the mutant receptor (JM6) (Fig. 7B). On the other hand, the TPEs do not decrease the ERα protein levels in the MC2 cells, whereas 4OHT and endox cause accumulation of the receptor, whereas ral and baze cause moderate downregulation of the ER. In the JM6 cells, all TPEs and SERMs did not dramatically affect the ERα protein expression. As expected, all planar estrogens and ICI cause a decrease of ERα protein levels in MCF7:WS8 (Fig. 7C) and

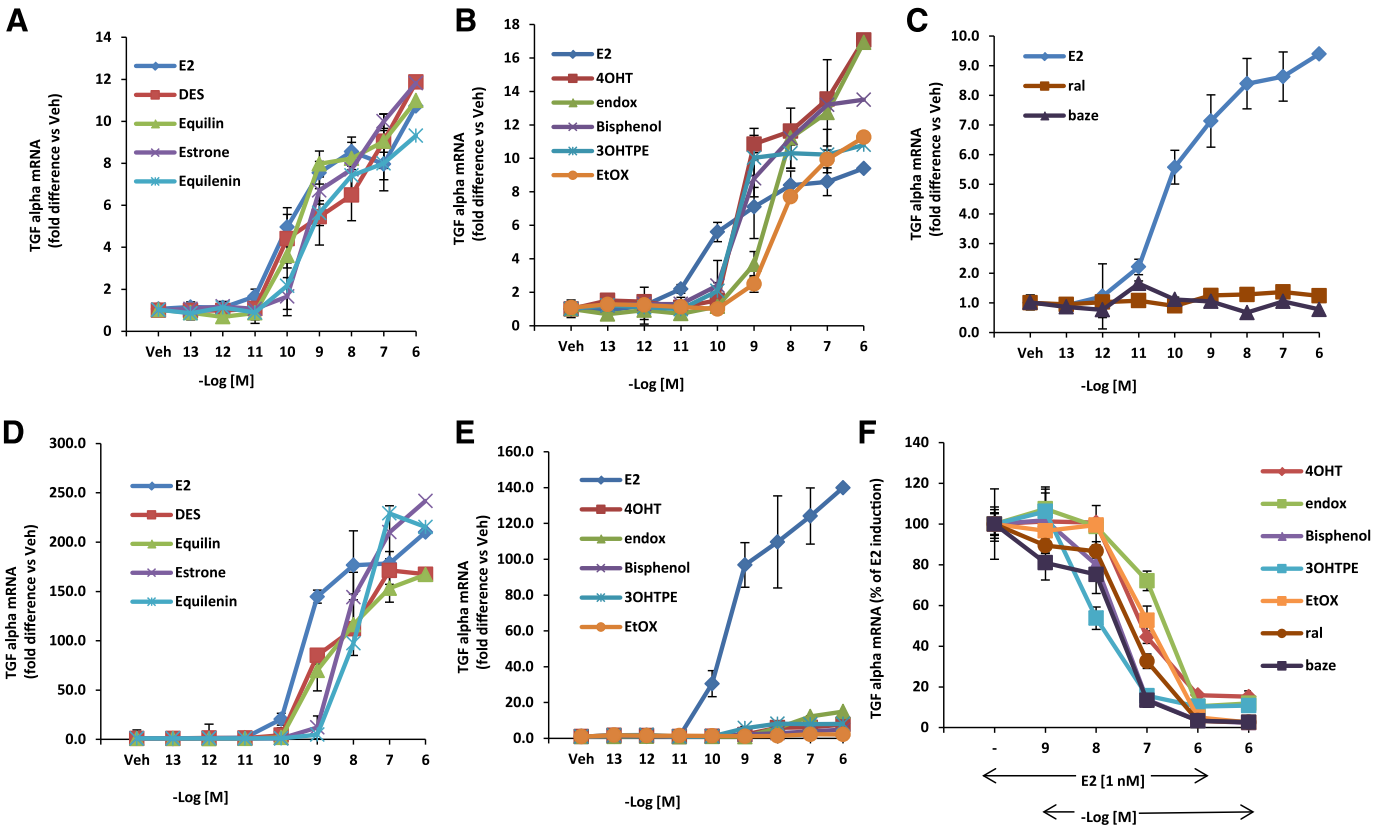

Fig. 5. The concentration-dependent action of test compounds using wild-type (MC2) and mutant D351G ER (JM6) stable transfectants. MC2 cells were treated with planar estrogens (A) and the TPEs 4OHT and endox (B) for 24 hours at indicated concentrations, and expression of TGFα RNA was measured using quantitative real-time PCR. (C) MC2 cells treated with ral and baze in a dose-responsive manner. JM6 cells were treated with planar estrogens (D) and E_2 (E), TPEs, active metabolites of tamoxifen (4OHT and endox) for 24 hours with various concentrations and expression of TGFα RNA was measured using quantitative real-time PCR. (F) JM6 cells were treated with 1 nM E_2 alone or in presence of increasing concentration of indicated TPEs and SERMs. A–E are represented as fold difference versus vehicle-treated cells. Each data point is average ± S.D. of three replicates.

MCF7:5C (Fig. 7D) cells, whereas the tamoxifen metabolites caused increase in ERα protein expression. Interestingly the TPEs cause moderate decrease of ERα in MCF7:WS8 and MF7:5C cells compared with E_2, and the reduction is more dramatic in the MCF7:5C cells. In contrast to the tamoxifen metabolites, ral and baze also cause a reduction in the protein levels of ERα in both MCF-7–derived breast cancer cell lines. ERα protein levels of all breast cancer cell lines used in the study are compared in Supplemental Fig. 3.

Binding of Bisphenol to the LBD of ERα. Next, the binding mode of the TPEs was investigated by the molecular docking of bisphenol to the LBD of ERα. Thus, the flexible docking of bisphenol into the LBD of the receptor cocrystallized with E_2 and 4OHT (Fig. 8, A and B) was performed. The superimposition of the top-ranked docking pose of the ligand onto the E_2 cocrystallized with ERα, the agonist conformation of the receptor, shows some incompatibility (Fig. 8C). Hence the resulting model revealed sterical clashes between bisphenol and "Leu crown," mostly with the side chains of Leu525 and Leu540. Because of this steric hindrance, it is most unlikely for bisphenol to bind in a conformation of ERα that is similar to that of E_2. On the other hand, when bisphenol is docked into the binding site of 4OHT cocrystallized with ERα (Fig. 8D), the binding mode is similar to that of 4OHT. Namely, the same alignment of the ligand in the binding pocket is noticed, having the propensity to form the same hydrophobic contacts with the amino acids lining the binding cavity and to recapitulate the complex H-bond network involving E353, R394, and a highly ordered water molecule. Taken together, these data show that bisphenol and extrapolating TPEs would most likely bind to the ERα in the antagonist conformation of the receptor.

Discussion

Estrogens are potent mitogens for the proliferation of breast cancer cells. In contrast, planar estrogens (class 1) can induce apoptosis of long-term estrogen-deprived MCF-7 cells (MCF7:5C) in a paradoxical manner. 4OHT has no effect in the MCF:5C cells but rather blocks E_2-mediated apoptosis (Maximov et al., 2011). TPEs, which are structurally similar to 4OHT, possess estrogenic properties in the MCF-7 cells at comparable concentrations to the planar estrogens. The TPEs (class II angular estrogens) do not rapidly trigger estrogen-induced apoptosis in MCF7:5C cells, but block class 1 planar estrogen-induced apoptosis. However, prolonged treatment with the TPEs leads to an eventual induction of apoptosis in the MCF7:5C cells, whereas the cells continue to be resistant to the actions of the SERMs, which are known antiestrogens (Supplemental Fig. 1C). As a result of these aforementioned findings, we initially proposed a hypothesis (Maximov et al., 2011) that the TPE–ER complex mimics an antiestrogen-ER complex and this may be responsible for the delay of apoptosis by the TPEs. We addressed the hypothesis in four ways: utilizing our validated functional assay to classify estrogens

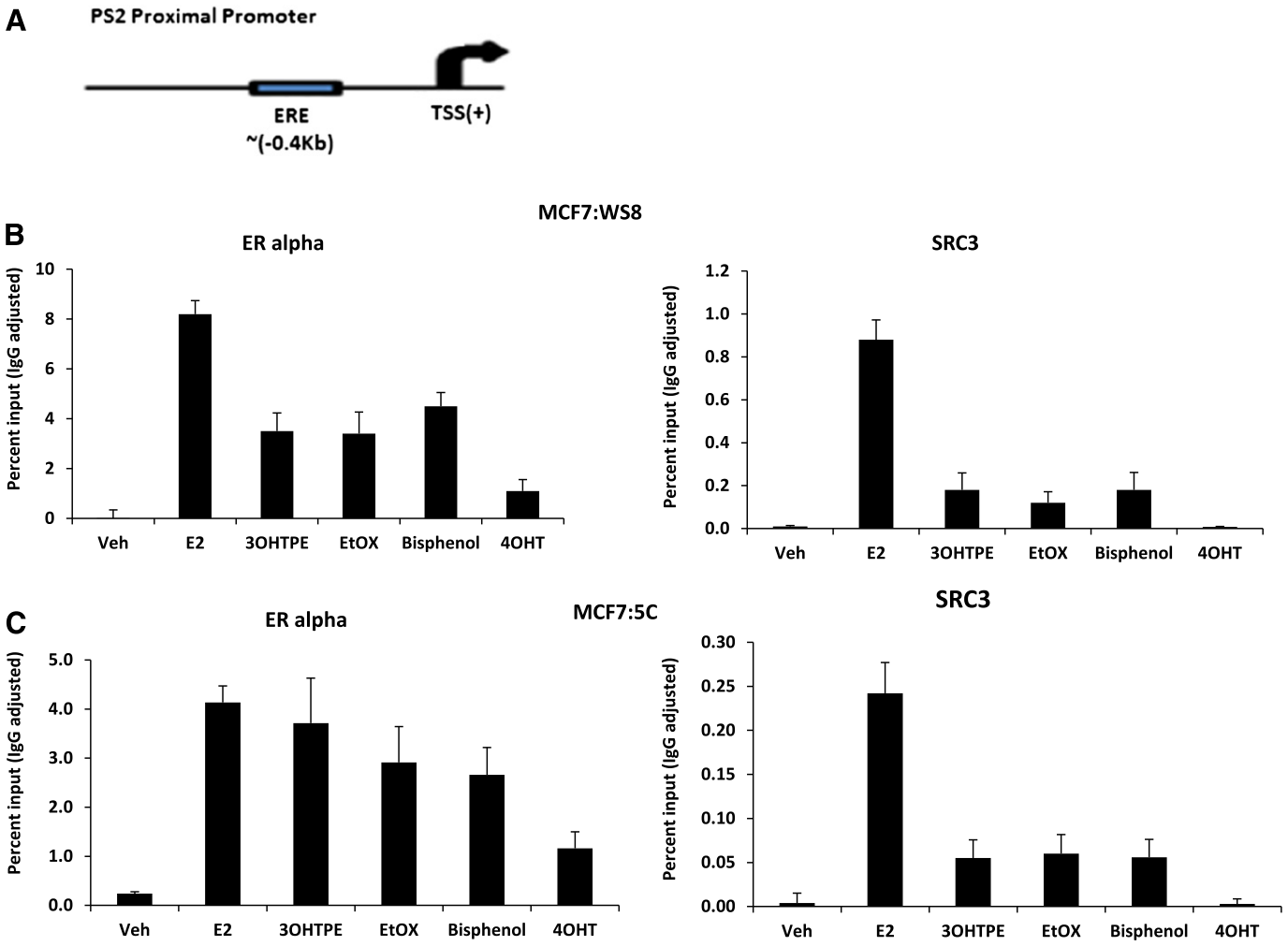

Fig. 6. Recruitment of ERα and SRC3 (AIB1) at PS2 proximal promoter region containing estrogen responsive element (ERE) in MCF7:WS8 and MCF7:5C cells. (A) Depiction of PS2 proximal promoter region and the ERE region relative to transcription start site (TSS). (B) MCF7:WS8 cells treated for 45 minutes with E_2 (1 nM), 3OHTPE (1 μM), EtOX (1 μM), bisphenol (1 μM), and 4OHT (1 μM), and ChIP assay was performed as described in *Materials and Methods*. (C) MCF7:5C cells were treated as mentioned above, and ChIP assay was performed under identical conditions. Data are represented as percent input of the starting chromatin used for the ChIP. Veh, vehicle.

using the induction of the TGFα gene (Jordan et al., 2001) (Fig. 1), binding of ER and recruitment of SRC3 to the promoter region of a model estrogen response gene (PS2) (Fig. 6), ligand-bound ER accumulation, or reduction and putative ER docking experiments (Fig. 8).

We previously demonstrated the critical importance of D351 in modulating the SERM:ER complex (Levenson et al., 2001) for the estrogen-like actions of the 4OHT by removing the exposed surface charge by engineering a mutant ER D351G, which causes a conversion of the 4OHT:ER from being estrogenic to completely antiestrogenic at the TGFα gene (Levenson et al., 1998; MacGregor Schafer et al., 2000). The anchoring role of D351 in the activation of the helix 12–mutated ER has recently (Merenbakh-Lamin et al., 2013; Toy et al., 2013) been illustrated in tissue from metastatic breast cancer resistant to antihormones. Mutations of Y537 in helix 12 are shown to anchor to D351 to accomplish sealing of the unoccupied LBD by helix 12. This provides evidence of the clinical relevance of our assay system.

To determine whether the conformation of the ER complex determines the triggering of apoptosis in long-term estrogen-deprived ER-positive breast cancer cells, MCF7:5C, we employed (Jordan et al., 2001) an assay using induction of the mRNA for the TGFα gene in situ in MDA-MB-231 cells stably transfected with cDNA wild-type (MC2) or D351G ER (JM6). As expected, all planar estrogens cause activation of the TGFα gene in the MC2 and JM6 cells. The planar estrogens are not affected by the mutation on D351, because upon binding to the ER, they are sealed within the LBD by helix 12, allowing for coactivator binding on the surface of helix 12 (AF-1) and gene activation. The TPEs induce TGFα gene at comparable concentrations to the tamoxifen metabolites, 4OHT and endox in the MC2 cells (Fig. 5B), but lose this estrogen-like action in the JM6 cells (Fig. 5E) and block E_2 induction of TGFα (Fig. 5F). The results of the TGF assay imply that TPEs adopt a 4OHT-like conformation with the ER with helix 12 pushed back and D351 exposed. By inference, the "antiestrogenic conformation" of the TPE:ER complex is responsible for the initial inhibition of E_2-induced apoptosis. The short aminoethoxy side chain of the tamoxifen metabolites (MacGregor Schafer et al., 2000) and the absence of this side chain in the TPEs prevent adequate shielding of the

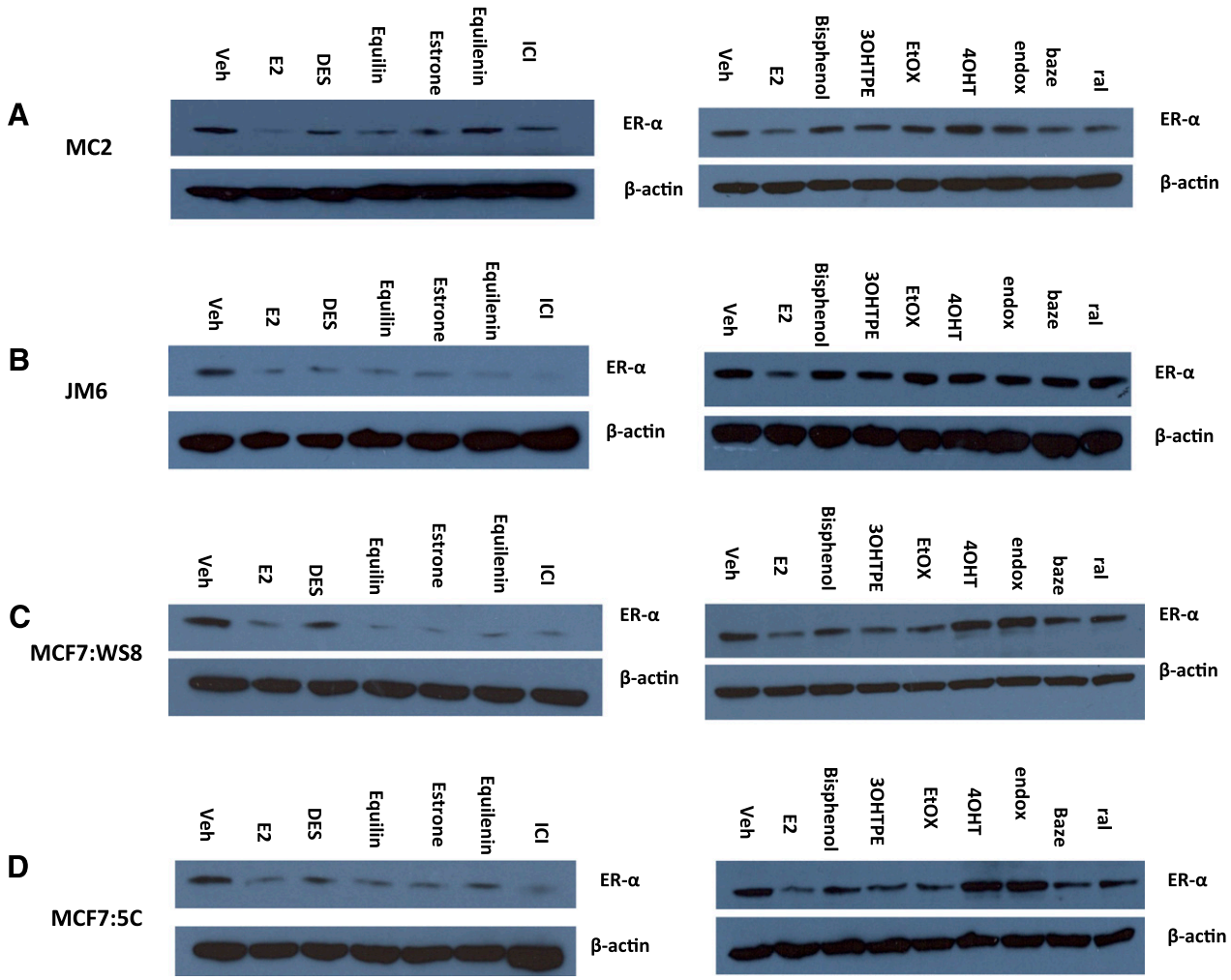

Fig. 7. Differential regulation of the ERα protein by planar and nonplanar estrogens. MC2 (A), JM6 (B), MCF7:WS8 (C), MCF7:5C (D) cells were treated with E_2 (1 nM), DES (1 nM), equilin (1 nM), estrone (1 nM), equilenin (1 nM), 3OHTPE (1 μM), EtOX (1 μM), bisphenol (1 μM), 4OHT (1 μM), endox (1 μM), baze (1 μM), ral (1 μM), and cell lysates were analyzed by Western blotting by anti-ERα antibody. Blot was reprobed by anti-actin antibody.

charged D351, whereas the antiestrogenic side chain of ral and baze provides effective interaction and neutralization of this charge (Liu et al., 2002) (Fig. 5C). Thus, this prevents the induction of the TGFα gene by ral and baze.

SRC3 has been shown to be extremely important in estradiol-induced growth in breast cancer cells (Font de Mora and Brown, 2000; List et al., 2001; Lahusen et al., 2009). Additionally, SRC3 knockdown was found to reduce apoptosis induced by E_2 in MCF7:5C cells (Hu et al., 2011). Using ChIP assays we show that TPEs are able to recruit ERα but less efficiently compared with E_2, and this was further observed with SRC3 (Fig. 6). The ER:TPE complex binds to the promoter with about 50% of E_2, but SRC3 binding is $<$25% of E_2. This suggests that treatment with TPEs influences the conformation of the liganded–ERα complex such that efficiency of ERα binding to estrogen responsive element region is moderately inhibited, whereas binding of SRC3 is severely inhibited compared with E_2, which is a planar estrogen. This may also explain why bisphenol is a partial agonist at the prolactin gene and exhibits antiestrogen properties (Jordan and Lieberman, 1984; Jordan et al., 1984). Of notable importance, the magnitude of SRC3 recruitment by the TPEs is far less in MCF7:5C cells (Fig. 6C) compared with MCF7:WS8 cells (Fig. 6B) and may play a crucial role in manifesting the functional role of the TPEs in these cells. This observation may contribute to the robust cell replication in MCF-7 with TPEs but delayed apoptosis in MCF7:5C.

Estradiol induces downregulation of the ER in breast cancer cells (Borras et al., 1994, 1996; Reid et al., 2003), and this process is inhibited by 4OHT, thereby causing accumulation of ERα (Wijayaratne et al., 1999). Similarly in all our cell lines, the planar estrogens all downregulate the ER, whereas tamoxifen metabolites 4OHT and endox do not (Fig. 7). The Western blot analysis shows that the TPEs do not readily decrease ERα protein levels when compared with the planar estrogens. This illustrates the fact that the TPE:ER complex appears to be "antiestrogen-like" compared with 4OHT and endox (Fig. 7). However, in the MCF7:5C cells, their ability to downregulate ERα protein levels is more apparent. The ER complex resembles the vehicle (control) rather than the extremes of E_2 or 4OHT. Ral and baze also cause moderate decrease in ERα levels, which concurs with previous studies done on these compounds (Lewis-Wambi et al., 2011). Bourgoin-Voillard et al. (2010) determined that class II ligands such as bisphenol had less tendency to promote recruitment of coactivators containing LxxLL motif,

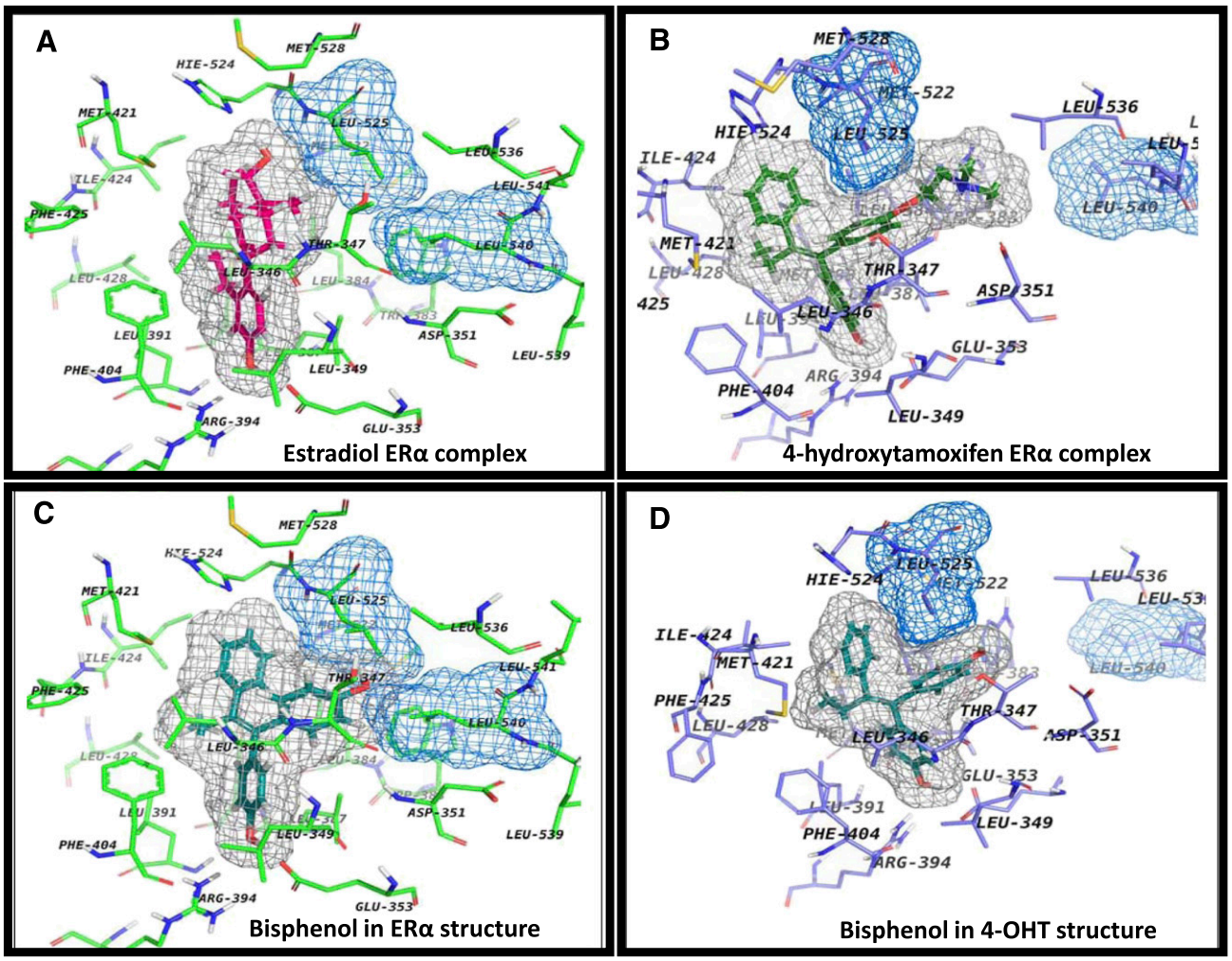

Fig. 8. The binding site of ERα with different ligands. The ligands are depicted with their corresponding grid molecular surfaces colored in gray. Also, Leu525 and Leu540 are depicted as grid molecular surfaces colored in blue. (A) Agonist conformation of ERα with E_2 (magenta; PDB ID 1GWR). (B) Antagonist conformation of ERα with 4OHT (green; PDB ID 3ERT). (C) Docking of bisphenol in agonist conformation (cyan; PDB ID 1GWR). (D) Docking of bisphenol in antagonist conformation (cyan: PDB ID 3ERT).

and this appeared to be a requirement for the downregulation of the ER in MCF-7 cells. Bourgoin-Voillard et al. (2010) also illustrated the accumulation of the bisphenol:ER complex in MCF-7 cells using immunocytochemistry.

The molecular modeling data (Fig. 8) provide evidence that the TPEs bind to the ERα in a manner similar to that observed with 4OHT using X-ray crystallography. The bulky phenyl ring of the TPEs prevent helix 12 from sealing the LBD and will result in an initial steric hindrance when attempting to bind in the E_2–ERα conformation, resulting in their blockade of E_2-induced apoptosis. However, continuous treatment of the MCF7:5C with the TPEs for 14 days results in induction of apoptosis similar to the planar estrogens. This suggests that the antiestrogenic conformation the TPEs create with the ER prevents immediate coactivator binding, causing a delay in the trigger for apoptosis, but this delay disappears with prolonged treatment. This conclusion correlates with the Haddow clinical study (Haddow et al., 1944), where postmenopausal women with advanced breast cancer were treated with TPE-like estrogens, leading to an about 30% response rate during breast cancer therapy. The planar estrogens form a compact estrogen–ER complex with excellent SRC3 binding and recruitment, and it appears that this event is necessary to induce apoptosis in the MCF7:5C cells. On the other hand, angular TPEs form an antiestrogen-like ER complex with less SRC3 binding and recruitment, thereby leading to delayed apoptosis, whereas the SERMS do not recruit SRC3 so this results in no apoptosis.

In conclusion, we have advanced the hypothesis that TPE–ER conformation is initially similar to that of tamoxifen metabolites 4OHT and endox, and our molecular classification assay indicates that helix 12 is pushed back in the TPE–ER complex. The antiestrogenic conformation of the TPE–ER complex appears to be responsible for the initial blocking of apoptosis and reduction in coactivator recruitment observed with the TPEs in the MCF7:5C cells. It is important to stress that the evidence we present suggests that the TPE:ER complex conformation may in fact be in between the extreme structures of E_2:ER and 4OHT:ER ligand binding domain (Brzozowski et al., 1997; Shiau et al., 1998). Because prolonged treatment with TPEs causes triggering of ER-mediated apoptosis similar to that of the planar estrogens but 4OHT does not, an intermediate conformation of the TPE:ER complex may be responsible for these observations.

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Authorship Contributions

Participated in research design: Obiorah, Sengupta, Jordan.

Conducted experiments: Obiorah, Sengupta, Curpan.

Performed data analysis: Obiorah, Sengupta, Curpan, Jordan.

Wrote or contributed to the writing of the manuscript: Obiorah, Curpan, Jordan.

References

Anderson GL, Chlebowski RT, Aragaki AK, Kuller LH, Manson JE, Gass M, Bluhm E, Connelly S, Hubbell FA, and Lane D et al. (2012) Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *Lancet Oncol* **13**:476–486.

Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, Bonds D, Brunner R, Brzyski R, and Caan B et al.; Women's Health Initiative Steering Committee (2004) Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *JAMA* **291**:1701–1712.

Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, and Bourne PE (2000) The Protein Data Bank. *Nucleic Acids Res* **28**:235–242.

Borrás M, Hardy L, Lempereur F, el Khissiin AH, Legros N, Gol-Winkler R, and Leclercq G (1994) Estradiol-induced down-regulation of estrogen receptor. Effect of various modulators of protein synthesis and expression. *J Steroid Biochem Mol Biol* **48**:325–336.

Borrás M, Laios I, el Khissiin A, Seo HS, Lempereur F, Legros N, and Leclercq G (1996) Estrogenic and antiestrogenic regulation of the half-life of covalently labeled estrogen receptor in MCF-7 breast cancer cells. *J Steroid Biochem Mol Biol* **57**: 203–213.

Bourgoin-Voillard S, Gallo D, Laïos I, Cleeren A, Bali LE, Jacquot Y, Nonclercq D, Laurent G, Tabet J-C, and Leclercq G (2010) Capacity of type I and II ligands to confer to estrogen receptor alpha an appropriate conformation for the recruitment of coactivators containing a LxxLL motif-Relationship with the regulation of receptor level and ERE-dependent transcription in MCF-7 cells. *Biochem Pharmacol* **79**:746–757.

Brzozowski AM, Pike ACW, Dauter Z, Hubbard RE, Bonn T, Engström O, Ohman L, Greene GL, Gustafsson J-A, and Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389**:753–758.

Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, and Kommareddy A et al. (2009) Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *JAMA* **302**:774–780.

Font de Mora J and Brown M (2000) AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Mol Cell Biol* **20**:5041–5047.

Gottardis MM and Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* **48**:5183–5187.

Haddow A, Watkinson JM, Paterson E, and Koller PC (1944) Influence of synthetic oestrogens upon advanced malignant disease. *BMJ* **2**:393–398.

Harper MJ and Walpole AL (1966) Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. *Nature* **212**:87.

Howell A, Osborne CK, Morris C, and Wakeling AE (2000) ICI 182,780 (Faslodex): development of a novel, "pure" antiestrogen. *Cancer* **89**:817–825.

Hu ZZ, Kagan BL, Ariazi EA, Rosenthal DS, Zhang L, Li JV, Huang H, Wu C, Jordan VC, and Riegel AT et al. (2011) Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. *PLoS ONE* **6**:e20410.

Jiang SY, Wolf DM, Yingling JM, Chang C, and Jordan VC (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* **90**:77–86.

Jordan VC (2004) Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* **5**:207–213.

Jordan VC (2009) A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. *Cancer Res* **69**:1243–1254.

Jordan VC and Lieberman ME (1984) Estrogen-stimulated prolactin synthesis in vitro. Classification of agonist, partial agonist, and antagonist actions based on structure. *Mol Pharmacol* **26**:279–285.

Jordan VC, Lieberman ME, Cormier E, Koch R, Bagley JR, and Ruenitz PC (1984) Structural requirements for the pharmacological activity of nonsteroidal antiestrogens in vitro. *Mol Pharmacol* **26**:272–278.

Jordan VC, Schafer JM, Levenson AS, Liu H, Pease KM, Simons LA, and Zapf JW (2001) Molecular classification of estrogens. *Cancer Res* **61**:6619–6623.

Lahusen T, Henke RT, Kagan BL, Wellstein A, and Riegel AT (2009) The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer. *Breast Cancer Res Treat* **116**:225–237.

Levenson AS, MacGregor Schafer JI, Bentrem DJ, Pease KM, and Jordan VC (2001) Control of the estrogen-like actions of the tamoxifen-estrogen receptor complex by the surface amino acid at position 351. *J Steroid Biochem Mol Biol* **76**:61–70.

Levenson AS, Tonetti DA, and Jordan VC (1998) The oestrogen-like effect of 4-hydroxytamoxifen on induction of transforming growth factor alpha mRNA in MDA-MB-231 breast cancer cells stably expressing the oestrogen receptor. *Br J Cancer* **77**:1812–1819.

Lewis-Wambi JS, Kim H, Curpan R, Grigg R, Sarker MA, and Jordan VC (2011) The selective estrogen receptor modulator bazedoxifene inhibits hormone-independent breast cancer cell growth and down-regulates estrogen receptor α and cyclin D1. *Mol Pharmacol* **80**:610–620.

Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, and Jordan VC (2005a) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* **97**: 1746–1759.

Lewis JS, Osipo C, Meeke K, and Jordan VC (2005b) Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* **94**:131–141.

List H-J, Lauritsen KJ, Reiter R, Powers C, Wellstein A, and Riegel AT (2001) Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells. *J Biol Chem* **276**:23763–23768.

Liu H, Park W-C, Bentrem DJ, McKian KP, Reyes AdeL, Loweth JA, Schafer JM, Zapf JW, and Jordan VC (2002) Structure-function relationships of the raloxifene-estrogen receptor-α complex for regulating transforming growth factor-α expression in breast cancer cells. *J Biol Chem* **277**:9189–9198.

Lønning PE, Taylor PD, Anker G, Iddon J, Wie L, Jørgensen LM, Mella O, and Howell A (2001) High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* **67**: 111–116.

MacGregor JI and Jordan VC (1998) Basic guide to the mechanisms of antiestrogen action. *Pharmacol Rev* **50**:151–196.

MacGregor Schafer J, Liu H, Bentrem DJ, Zapf JW, and Jordan VC (2000) Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. *Cancer Res* **60**:5097–5105.

Maximov P, Sengupta S, Lewis-Wambi JS, Kim HR, Curpan RF, and Jordan VC (2011) The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: a hypothesis. *Horm Mol Biol Clin Investig* **5**:27–34.

Maximov PY, Myers CB, Curpan RF, Lewis-Wambi JS, and Jordan VC (2010) Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. *J Med Chem* **53**:3273–3283.

McDonnell DP, Clemm DL, Hermann T, Goldman ME, and Pike JW (1995) Analysis of estrogen receptor function in vitro reveals three distinct classes of antiestrogens. *Mol Endocrinol* **9**:659–669.

Merenbakh-Lamin K, Ben-Baruch N, Yeheskel A, Dvir A, Soussan-Gutman L, Jeselsohn R, Yelensky R, Brown M, Miller VA, and Sarid D et al. (2013) D538G mutation in estrogen receptor-α: a novel mechanism for acquired endocrine resistance in breast cancer. *Cancer Res* **73**:6856–6864.

Obiorah I and Jordan VC (2013) 2012 NAMS/PFIZER-Wulf H. Utian endowed lecture. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. *Menopause* **20**:372–382.

Osborne CK, Coronado EB, and Robinson JP (1987) Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol* **23**:1189–1196.

Reid G, Hübner MR, Métivier R, Brand H, Denger S, Manu D, Beaudouin J, Ellenberg J, and Gannon F (2003) Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. *Mol Cell* **11**:695–707.

Sawicki MW, Erman M, Puranen T, Vihko P, and Ghosh D (1999) Structure of the ternary complex of human 17β-hydroxysteroid dehydrogenase type 1 with 3-hydroxyestra-1,3,5,7-tetraen-17-one (equilin) and NADP+. *Proc Natl Acad Sci USA* **96**:840–845.

Sengupta S, Obiorah I, Maximov PY, Curpan R, and Jordan VC (2013) Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. *Br J Pharmacol* **169**:167–178.

Sengupta S, Sharma CG, and Jordan VC (2010) Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells. *Horm Mol Biol Clin Investig* **2**:235–243.

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, and Greene GL (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**:927–937.

Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, Li Z, Gala K, Fanning S, and King TA et al. (2013) ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nat Genet* **45**:1439–1445.

Tzukerman MT, Esty A, Santiso-Mere D, Danielian P, Parker MG, Stein RB, Pike JW, and McDonnell DP (1994) Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol Endocrinol* **8**:21–30.

Wang Z, Wijewickrama GT, Peng KW, Dietz BM, Yuan L, van Breemen RB, Bolton JL, and Thatcher GR (2009) Estrogen receptor alpha enhances the rate of oxidative DNA damage by targeting an equine estrogen catechol metabolite to the nucleus. *J Biol Chem* **284**:8633–8642.

Wijayaratne AL, Nagel SC, Paige LA, Christensen DJ, Norris JD, Fowlkes DM, and McDonnell DP (1999) Comparative analyses of mechanistic differences among antiestrogens. *Endocrinology* **140**:5828–5840.

Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, and Jordan VC (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* **6**:2028–2036.

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Title

Differences in the Rate of Oestrogen-induced Apoptosis in Breast Cancer by Estradiol and the Triphenylethylene Bisphenol.

I E Obiorah and V C Jordan

Running title: Modulation of triphenylethylene induced apoptosis

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BACKGROUND AND PURPOSE

Triphenylethylene (TPE)-like compounds were the first agents to be used in the treatment of metastatic breast cancer in postmenopausal women. Though structurally related to the antioestrogen, 4-hydroxytamoxifen, TPEs possess oestrogenic properties in fully oestrogenised breast cancer cells but do not induce apoptosis with short term treatment in long term oestrogen deprived breast cancer cells. This study determined the differential effects of bisphenol (BP), a TPE has on growth and apoptosis based on the modulation of the shape of the ligand-oestrogen receptor complex.

EXPERIMENTAL APPROACH

Apoptotic flow cytometric studies were used to evaluate apoptosis over time. Proliferation of the breast cancer cells were assessed using DNA quantification and cell cycle analysis. RT-PCR was performed to quantify mRNA levels of apoptotic genes. Regulation of cell cycle and apoptotic genes was determined using PCR-based arrays.

KEY RESULTS

Bisphenol induced cell cycle genes similar to those induced by 17ϐ oestradiol (E_2). Unlike the trigger by E_2 that occurs after 24h the trigger of apoptosis for BP occurs at 4 days with quantifiable apoptotic changes noted at 6 days. A prolonged induction of endoplasmic reticulum

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stress and inflammatory stress response genes was observed with subsequent activation of apoptosis related genes in the second week of treatment with BP.

CONCLUSIONS AND IMPLICATIONS

The BP: ERŬ complex induces delayed biological effects on the growth and apoptosis of breast cancer cells. Both the shape of the complex and duration of treatment control the initiation of apoptosis.

Abbreviations

BP, bisphenol, E_2, 17ƀ oestradiol; ER, oestrogen receptor; ERS, endoplasmic reticulum stress; IS, inflammatory stress RT-PCR, real-time polymerase chain reaction; TPE, triphenylethylene; UPR, unfolded protein response; 4OHT, 4-hydroxytamoxifen.

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INTRODUCTION

Apoptosis triggered by physiological oestrogen levels in antihormone resistant oestrogen receptor (ER) positive breast cancer (Wolf and Jordan, 1993, Yao et al., 2000, Song et al., 2001, Lewis et al., 2005a), is a well-documented laboratory phenomenon, which has clinical significance. Low dose oestradiol causes 30% clinical benefit for the treatment of aromatase resistant breast cancer(Ellis et al., 2009) and treatment of postmenopausal women in their 60ǿs with conjugated equine oestrogen causes a decrease in breast cancer incidence and mortality(Anderson et al., 2012) in oestrogen deprived cancer in women who are 15 years postmenopausal. It is proposed that the key to triggering oestrogen-induced apoptosis is the selection of vulnerable ER positive tumour cell populations that evolve and eventually dominate the tumour during long oestrogen deprivation or antihormone therapy (Jordan, 2004, Jordan, 2008, Jordan et al., 2011, Obiorah and Jordan, 2013). However unlike the immediate and catastrophic initiation of apoptosis by paclitaxel, there is a delay with commitment of the cell for 24h with E_2 that completes the process by 96h(Obiorah et al., 2014a). Oestrogen induced apoptosis is heralded by endoplasmic reticulum stress (ERS) and an unfolded protein response (UPR) (Ariazi et al., 2011, Fan et al., 2013).

Oestrogen can be classified into planar(class 1) and angular (class II) ligands that create different shapes when complexed with the ERŬ(Jordan et al., 2001). A class 1 oestrogen such as E_2 causes cell replication and apoptosis because the ligand is sealed by helix 12 in the ligand

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binding domain of the ERŬ complex. By contrast, a class II angular oestrogen such as bisphenol (BP) (Figure S1) causes cell replication but cannot cause oestrogen induced apoptosis in a short term 7 day *in vitro* assay (Maximov et al., 2010, Sengupta et al., 2013). In fact, BP blocks oestradiol-induced apoptosis in a manner similar to 4-hydroxytamoxifen(4OHT) (Sengupta et al., 2013). It appears that BP can adopt the conformation of the 4OHT-ERŬ complex (Bourgoin-Voillard et al., 2010). However, these laboratory data are paradoxical as they do not conform to the known antitumour effects on class 1 and II oestrogens in the successful treatment of metastatic breast cancer in postmenopausal women(Haddow et al., 1944).

High dose oestrogen therapy was the first ñchemical therapyò to be used successfully to treat any cancer. Haddow (Haddow et al., 1944) demonstrated, after preliminary laboratory studies, that high doses of two structurally different oestrogens diethylstiboestrol (class 1) and triphenylchlorethylene (class II), were both effective in producing a 30% response rate in post-menopausal women with metastatic breast cancer. Haddow (Haddow, 1970)also noted that responses were more likely in breast cancer if the patient was more than 5 years post menopause. Today, it is recognized that oestrogen deprivation caused by menopause creates a selection pressure for breast tumour cells that results in the outgrowth of cellular populations more likely to die with oestrogens than grow (Obiorah and Jordan, 2013).

We have addressed the paradox that an angular class II oestrogen, BP, can act as an inhibitor of oestrogen-induced apoptosis by adopting an ñantiestrogenic conformationò for the BP:ERŬ complex(Bourgoin-Voillard et al., 2010, Sengupta et al., 2013), but related triphenylethylenes are effective antitumour agents in patients (Haddow et al., 1944). We have found that the trigger for oestrogen-induced apoptosis is dependent not only on the shape of the oestrogen-ERŬ complex, but also on the duration of oestrogen exposure.

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METHODS

Cell Culture and Reagents

Cell culture media were purchased from Invitrogen Inc. (Grand Island, NY) and foetal calf serum (FCS) was obtained from HyClone Laboratories (Logan, UT). Compounds E_2 , 4OHT, ICI 182,780 were obtained from Sigma, St. Louis, MO, and BP(Figure S1) was obtained as previously described (Maximov et al., 2010) Caspase 4 inhibitor with the peptide sequence z-LEVD-fmk (z, benzyloxycarbonyl;LEVD -Leu-Glu(OMe)-Val-Asp(OMe),fmk, fluoromethyl ketone) was from Biovision. Drug/molecular target nomenclature used conforms to the required guide to receptors and channels(Alexander et al., 2011). MCF7:5C were derived from MCF7 cells obtained from the Drs. Dean Edwards and Bill McGuire, San Antonio, Texas as reported previously (Lewis et al., 2005b). MCF7 cells were maintained in RPMI media supplemented with 10% FCS, 6 ng/ml bovine insulin and penicillin and streptomycin. They have been maintained for more than 20 years. Prior to starting experiments, MCF-7 cells were cultured in phenol-red free RPMI media containing 10% charcoal dextran treated FCS, 6ng/ml bovine insulin and penicillin and streptomycin. MCF7:5C cells were maintained in phenol-red free RPMI media containing 10% charcoal dextran treated FCS, 6ng/ml bovine insulin and penicillin and streptomycin. The cells were treated with indicated compounds (with media changes every 48 hrs) for the specified time and were subsequently harvested for tissue culture experiments.

Cell growth assay

The cell growth was monitored by measuring the total DNA content per well in 24 well plates. Fifteen thousand cells were plated per well and treatment with either the indicated concentrations of the compounds or the vehicle control (0.1% ethanol) were started after 24 hours, in triplicates. Media containing the specific treatments were changed every 48 hours. The cells were harvested

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and total DNA was assessed using a fluorescent DNA quantification kit (Cat # 170-2480; Bio-Rad,Hercules, CA, USA) and was performed as previously described(Lewis et al., 2005a).All timings for determining select genes by RT PCR, Real time profiler assays or apoptosis assays were based on results from cell growth assays.

RNA isolation and real time PCR

Total RNA was isolated using RNAeasy kit (Qiagen) and was converted to first strand using a kit from Applied Biosystems. Quantitative real-time PCR assays were done with the SYBR Green PCR Master Mixes (Applied Biosystems) and a 7900HT Fast Real-time PCR System (Applied Biosystems). Real-time PCR was performed as previously described (Sengupta et al., 2010). The sequences for all primers are as follows: BCL2L11 (Bim) Forward: TCGGACTGAGAAACGCAAG; Reverse: CTCGGTCACTCAGAACTTAC. TNF Forward: ACTTTGGAGTGATCGGCC. Reverse: GCTTGAGGGTTTGCTACAAC. FAS Forward AAGCTCTTTCACTTCGGAGG; Reverse GGGCATTAACACTTTTGGACG. FADD Forward CCTGGTACAAGAGGTTCAGC; Reverse CTGTGTAGATGCCTGTGGTC. Caspase 4 Forward CCATAGAACGACTGTCCATGAC Reverse GCTGTACTAATGAAGGTGCTCC. LTA Forward TCTTCTTTGGAGCCTTCGC Reverse AGACTTGAGCTGTTGGAATGG. The change in expression of transcripts was determined as described previously and used the ribosomal protein 36B4 mRNA as the internal control(Sengupta et al., 2010).

Real time profiler assay

RT-PCR profiler assay kits for apoptosis and cell cycle was used from a commercial vendor which uses 384 well plates (Qiagen; SABiosciences Corp, Fredrick, MD; Cat# PAHS-3012E). to profile the expression of 370 apoptosis related human genes and 4 x 96 well plates to profile expression of 84 cell cycle related genes (Qiagen;SABiosciences Corp, Fredrick, MD; Cat#

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PAHS-020). All the procedures were followed as previously described(Sengupta et al., 2013). Briefly, cells were treated with indicated compounds (in triplicates) for the indicated time points. To identify cell cycle or apoptosis related genes, total RNA was isolated using the method mentioned earlier. Two micrograms of total RNA was reverse transcribed and RT PCR was performed using ABI 7900HT. We created a gene signature throughout the indicated time points after comparing them with vehicle treatment. This gene signature was generated by comparing the expression level of all the genes with vehicle treatment and selecting the genes which were at least 2.5 fold over-expressed or under-expressed as compared to vehicle treated cells at a statistical significance of p value of 0.05. The fold change was calculated by $\Delta\Delta$Ct method (Qiagen; SABiosciences Corp, Fredrick, MD).

Apoptosis assay

Apoptosis was verified in MCF7:5C cells in response to BP based on loss of plasma membrane integrity. This verification was determined by flow cytometric analysis of cells stained with either FITC labeled annexin v or DNA binding dye, YO-PR0-1. Viable cells excluded these dyes, whereas apoptotic cells allowed moderate staining. In brief, MCF-7:5C cells were treated with 0.1% ethanol vehicle (control) or BP (1 εM) for 6 days and then harvested in cold PBS (Invitrogen) and resuspended in 1X binding buffer and stained simultaneously with FITC-labeled annexin V and propidium iodide (PI) (Life technologies, (Grand Island, NY). The experiment was repeated and the cells were stained with YO-PRO-1 and PI (Pharmingen, San Diego, CA). Cells were analyzed using a fluorescence- activated cell sorter (FACS) flow cytometer (Becton Dickinson, San Jose, CA).Percent of apoptosis is calculated as the addition of the right upper and lower quadrants.

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Cell cycles analysis.

MCF7:5C cells were cultured in dishes and were treated with vehicle control (0.1% ethanol), E_2 (1 nM) and BP (1 εM) for 24h 48h 72h and 96h respectively. Cells were harvested and gradually fixed with 75% EtOH on ice. After staining with propidium iodide (PI), cells were analyzed using a fluorescence-activated cell sorter (FACS) flow cytometer (Becton Dickinson, San Jose, CA), and the data were analyzed with ModFit software.

Statistical analysis

All data are expressed as the mean of at least three determinations, unless otherwise stated. The differences between the treatment groups and the control group were determined by two-sample *t* test or one-factor or two way analysis of variance using the Graph Pad prism software.

RESULTS

Differential expression of cell cycle genes induced by bisphenol and 17β oestradiol

We have previously shown that BP, a triphenylethylene can induce the growth of MCF7 breast cancer cells as effectively as E_2 (Maximov et al., 2010, Sengupta et al., 2013). To identify cell cycle genes associated with BP induced cell growth, MCF7 cells were treated with 1ÕM BP for 6h, 12h and 24h and compared to 1nM E_2 and 1μM 4OHT as positive and negative regulators of cell replication respectively. The antiestrogen, 4OHT was used to block the stimulatory effects of BP and E_2. We used RT-PCR array kits that contain 4 x 96 well plates to profile the expression of 84 genes key to cell cycle regulation. At 6h, E_2 induces several genes such as cyclin D1 (CCND1), CDK5R1, HERC5, CHEK2 and RBBP8 (Figure 1A). Bisphenol and 4OHT only induced HERC5. Interestingly CCND1 was downregulated by BP at this time point. There was increased expression of cell cycle related genes by E_2 at 12h (Figure 1B), which further increased by almost 2 fold at 24h (Figure 1C). Similarly, BP induced 60% and 50% of the cell cycle related

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genes that were up-regulated by E_2 at 12h and 24h respectively. The rest of the cell cycle related genes induced by BP show an obvious trend of overexpression when compared to the control. Similarly, all cell cycle genes downregulated by BP are equally decreased by E_2 treatment. List of genes induced by E_2 and BP are presented in table S1. Furthermore, E_2 and BP decrease retinoblastoma protein (RB1) mRNA levels in a time dependent manner (Figure S2). Unlike the estrogens, 4OHT did not activate the cell cycle related genes but rather blocked the effects of E_2 and BP. These results demonstrate that BP induces similar cell cycle related genes as E_2, although not as effectively.

Effect of bisphenol on apoptosis in MCF7:5C cells

The planar type 1 oestrogen, E_2 induces apoptosis in long term oestrogen deprived MCF7 (MCF7:5C) cells. On the other hand, the angular oestrogen BP does not initially induce apoptosis in MCF7:5C cells and blocks E_2 induced apoptosis in a similar manner as does 4OHT(Sengupta et al., 2013). To evaluate the long term effects of BP, we treated MCF7:5C cells with 1 ÕM BP, 1nM E_2 and 0.1% ethanol vehicle (control). Growth of the cells was inhibited by E_2 after 3 days of treatment and the effect became maximal by 6 days of treatment (Figure 2A). On the other hand, BP increases the growth of the cells up to 6 days of treatment (Figure 2A) but causes 100% inhibition of growth by 9 days of treatment (Figure 2B). The inhibition of growth observed with BP was further investigated for apoptosis using flow cytometry. Following 6 days of treatment, BP caused a 7 fold increase in the percent of cells (4.15% vs. 27.61%) undergoing apoptosis compared to the control (Figure 2C) using Annexin V staining. A similar effect was observed using a DNA binding stain, YO-PRO-1 (Figure S3).

Determination of the point of commitment for BP induced apoptosis

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Next, we investigated the delayed response of BP, MCF7:5C cells were treated with BP [1ÕM] and 4OHT [1ÕM] was used to block the antiproliferative and apoptotic effects of BP at daily intervals over a range of 9 days. Cells were harvested after 13 days of treatment and total DNA was quantified using a fluorescent DNA quantification kit. Apoptosis induced by BP was blocked by daily additions of 4OHT for up to 3 days and afterwards the cells became committed to apoptosis mediated by BP (Figure 3). 4OHT alone caused a small decrease in DNA similar to that observed at day 1,2,3. After day 3, an irreversible decline occurred with BP that was not rescued. The day 4 value was about 50% of the control or 4OHT alone values. Cells could not be rescued from BP induced apoptosis by 4OHT after 4 days of treatment suggesting that the cell commitment trigger for apoptosis has occurred. The experiment was repeated with ICI 182,780 as the estrogen antagonist and similar results were obtained (Figure S4). It is important to emphasize that each of the two ñrescueò experiments adds antiestrogens 4OHT or ICI 182,780 at specific days after BP and measures cellular DA at 13 days. MCF7:5C cells are both committed to apoptosis after day 3 with either antiestrogen (Fig 3, Fig S4).

Apoptosis related genes induced by bisphenol

To determine the early events preceding BP induced apoptosis, the induction of apoptosis related genes were investigated in MCF7:5C cells treated with BP [1ÕM], 0.1% ethanol vehicle (control), 1ÕM 4OHT and BP in combination with 4OHT (in triplicates) for 3, 4, 5 days. We used 384 well RT-PCR profiler plates to monitor expression of 370 apoptosis related human genes (see Methods). Comparative analysis showed that significant evidence of apoptotic gene induction did not occur until after 3 days of treatment. At 4 days (Figure 4A) BP induces ERS related genes; DDIT3 and inflammatory stress (IS) response genes such as CEBPB, IFI6, IFI16 and DAPK1 At 5 days of treatment (Figure 4B) ,there is continued increase in the up-regulation

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of ERS and IS associated genes including LTA and caspase 4, an inflammatory caspase. Levels of LTA and caspase 4 mRNA were elucidated using RT-PCR (Figure S5). The apoptosis related genes detected using the PCR arrays are listed in Tables S2-3. Bim/BCL2L11 is important for E_2 induced apoptosis. Its activation by E_2 occurs by 36h of treatment(Obiorah et al., 2014a) and E_2 subsequently induces the TNF family of proapoptosis related genes. The induction of these genes by BP was investigated by extending the duration of treatment for 7, 8 and 9 days mRNA levels of BCL2L11 and TNF were quantified by RT-PCR. Upregulation of Bim/BCL2L11 (Figure 5A), TNF (Figure 5B), FAS (Figure 5C) and FADD (Figure 5D) was observed by 8 days of treatment with continued increase of all genes at 9 days of treatment with BP. These data indicate that there is a prolonged induction of ERS and IS associated genes by 4 days of treatment with subsequent up-regulation of mitochondrial and TNF related apoptosis genes.

Differential effect of bisphenol on cell cycle

Since the BP induced apoptosis is not apparent until the second week of treatment, we evaluated the effect of BP on the regulation of the cell cycle. MCF7:5C cells were treated with either vehicle control (0.1% ethanol), 1nM E_2 or 1ÕM BP for 24h, 48h and 96h and performed cell cycle analysis using flow cytometry (Figure 6). As suspected, BP and E_2 cause a consistent increase in the S phase when compared to the control. Although, the trigger for apoptosis occurred for E_2 and BP at 36h (Obiorah et al., 2014a) and 96h (Fig 3 and Fig S4) respectively, no checkpoint blockade was noted after treatment with either compound and contrasts dramatically with early cell cycle arrest at G2/M with paclitaxel (Obiorah et al., 2014a).

Functional importance of caspase 4 in bisphenol induced apoptosis

Caspase 4, an inflammatory caspase, is upregulated in the MCF7:5C cells by 5 days of treatment with BP. To determine the role of caspase 4 in BP induced apoptosis, cells were treated with

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control or BP (1μM) and the effects of caspase 4 was blocked by caspase 4 inhibitor-z-LEVD-fmk (10μM). Growth inhibited by BP was reversed by z-LEVD-fmk (Figure 7A). Proliferation was determined after 12 days of exposure to BP and quantified by DNA mass per well. Apoptosis induced after 6 days of exposure to BP was completely reversed by z-LEVD-fmk (Figure 7B). Thus, the blockade of BP induced apoptosis by caspase 4 inhibitor-z-LEVD-fmk indicates that caspase 4 plays an important role for the induction of apoptosis by BP.

DISCUSSION AND CONCLUSIONS

The aim of our study is to elucidate the growth and induction of apoptosis by BP in fully oestrogenised and long term oestrogen deprived breast cancer cells. The ERŬ in breast cancer cells can either initiate replication or trigger apoptosis based on the context of cell selection in estrogen replete or deprived environments (Lewis-Wambi and Jordan, 2009). Originally, oestrogens including, E_2 and TPE derivatives were discovered using a bioassay of the induction of vaginal cornification in ovarectomised mice. Replication and cornification of vaginal cells in the mouse was the early appropriate method of establishing the structure-function relationships of an oestrogenic TPE molecule(Robson and Schonberg, 1937). Initial structure-function studies *in vitro* established an ERŬ mediated mechanism for E_2 stimulate prolactin (an oestrogen responsive gene) synthesis in rat pituitary cells(Lieberman et al., 1983). However, BP and other TPE derivatives were found to act as partial agonists with antioestrogenic properties at the prolactin gene *in vitro* (Jordan and Lieberman, 1984a, Jordan et al., 1984b). Structure-function relationship studies to modulate prolactin synthesis by extending the length of the ñantioestrogenic side chainò created an antioestrogen that blocked oestrogen stimulated prolactin synthesis (Jordan et al., 1984b, Jordan et al., 1986). These are the basic early facts of the pharmacological function of the oestrogen-ERŬ complex that now allows us to interpret our

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current findings on the modulation of apoptosis.

Our results show that BP induces cell cycle regulated genes that are similar to those activated by E_2 in MCF-7 cells. This correlates with the ability of BP to induce replication of MCF-7 cells in a comparable manner as E_2(Maximov et al., 2010). On the other hand, 4OHT, which possesses a bulky alkylaminoethoxy side chain(Shiau et al., 1998), failed to induce cell cycle regulated genes in a time dependent manner but rather blocks E_2 and BP mediated activation of cell cycle genes, therefore confirming its role as an antioestrogen. Although BP possesses a bulky phenyl substituent, it does not have an akylaminoethoxy side chain. Molecular modeling studies suggest that the phenyl component of TPEs prevent the complete sealing of the ligand binding domain of the ERŬ by helix 12(Maximov et al., 2010, Sengupta et al., 2013). The reduced number of gene changes noted with BP treatment compared with E_2 (Figure 1) may be caused by differences in the structure of the ligand-ERŬ complex, thus resulting in a reduction in the full oestrogenic potential of BP induced replication. Additionally, BP unlike E_2 does not readily induce apoptosis in long term oestrogen deprived MCF-7 cells but rather appear to possess early antioestrogenic properties (Sengupta et al., 2013). Using cell proliferation assays, BP induces growth of MCF7:5C cells in the first week of treatment. In contrast, growth inhibition occurs after the third day of treatment with E_2. Inhibition of growth in oestrogen deprived MCF7:5C cells with BP is seen after 8 days of treatment. Similarly, apoptotic effects of BP are observed following 6 days of BP treatment using flow cytometry studies (Figure 2, S3). Previous studies have shown that MCF7:5C cells are resistant to the actions of 4OHT, which has the ability to reverse and block E_2 mediated apoptosis(Maximov et al., 2011). Using 4OHT or ICI 182,780 to block and rescue the cells from BP induced apoptosis suggests that the trigger for apoptosis occurs with BP after 4 days of treatment. There was no evidence of cell cycle arrest

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with either E_2 or BP (Fig 6) prior to apoptosis. This contrasts dramatically with our previous publication of E_2 and the rapid G2 blockade triggered by paclitaxel prior to apoptosis , no checkpoint blockade was noted after treatment with either compound and contrasts dramatically with early cell cycle arrest at G2/M with paclitaxel (Obiorah et al., 2014a).

The apoptosis related genes clearly demonstrate that the majority of genes that are upregulated by BP at 4 days of treatment are ERS and IS response genes. DDIT3 also known as CHOP or GADD153 is a key ERS protein associated with cell death(Oyadomari and Mori, 2003, Kim et al., 2006) , whereas CEBPB, which is known to induce proinflammatory cytokines such as IL6(Akira et al., 1990), is activated by ERS and is important for nuclear transport of DDIT3(Ron and Habener, 1992, Styner et al., 2012). There is a continued induction of similar proapoptotic genes at 5 days of treatment including caspase 4, an inflammatory caspase that predominantly localizes to the endoplasmic reticulum and undergoes cleavage and induces effector caspases in response to ERS (Hitomi et al., 2004, Bian et al., 2009). Upregulation of Bim, FAS, TNF and FADD mRNA are observed by 7 days of treatment with BP. Microarray analysis indicate ERS-mediated apoptosis as the top scoring pathway of apoptosis induced by E_2 in MCF7:5C cells(Ariazi et al., 2011) Oestradiol induces ERS and IS response genes by 36h of treatment and apoptotic genes such as Bim and TNF are activated by 48h of treatment(Obiorah et al., 2014a). A similar trend is observed with BP, however there is a prolonged ERS and IS with subsequent induction of caspase 4 at 5 days of treatment and mitochondrial and extramitochondrial apoptotic genes at 7 days of treatment. After 48h treatment with BP, there is no induction of apoptotic genes (Sengupta et al., 2013) but an increase in growth (Figures 2A,6) and the cells can be rescued from apoptosis with antioestrogens (Figure 3).

The initial resistance to trigger apoptosis may also result from the antioestrogenic

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conformation BP creates with the ERŬ. Angular TPEs such as BP have a reduced tendency to promote recruitment of coactivators containing LxxLL motif(Bourgoin-Voillard et al., 2010). We have previously shown that BP recruits the ERŬ and SRC3 to the PS2 promoter ERE less efficiently when compared to planar estrogens(Sengupta et al., 2013, Obiorah et al., 2014b) thus indicating that complete sealing of helix 12 of the LBD and interaction of coactivators with the TPE-ERŬ complex is necessary for the rapid activation of apoptosis observed with planar estrogens(Maximov et al., 2011). Depletion of SRC3 in the MCF7:5C cells and MCF-7 cells leads loss of E_2 induced apoptosis(Hu et al., 2011) and growth (List et al., 2001, Lahusen et al., 2009) respectively.

Since caspase 4 is specifically activated by ERS(Hitomi et al., 2004) and it was induced by 2 fold with E_2 within 24h(Ariazi et al., 2011) but by 2 fold by BP within 96h, a specific caspase 4 inhibitor (Hitomi et al., 2004) was used to block activation of caspase 4 in BP treated cells and this resulted in reversal of BP inhibited growth and apoptosis (Figure 7). We previously reported that E_2 induced apoptosis can be blocked by a caspase 4 inhibitor (Ariazi et al., 2011). Together, these results suggest that BP activates IS and ERS related genes which interact with resultant induction of caspase 4 between 4 and 5 days of treatment and subsequent activation of mitochondrial and extramitochodrial related apoptotic genes in the second week of treatment. This delayed sequence for BP contrast with early activation by E_2 (Ariazi et al., 2011, Obiorah et al., 2014a).

In summary, we have used cell based assays and gene profiling studies to demonstrate the biological response of the TPE, BP on both growth and apoptosis. TPEs were among the first chemical therapy used in the treatment of

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advanced breast cancer in postmenopausal women(Haddow et al., 1944). These data support the apoptotic mechanism of TPEs in early clinical practice.

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List of Authorship Contributions

Participated in research design: I E Obiorah and V C Jordan.

Conducted experiments: I E Obiorah

Performed data analysis: I E Obiorah and V C Jordan.

Wrote or contributed to the writing of the manuscript: I E Obiorah and V C Jordan.

Statement of conflicts of interest

None

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REFERENCES

Akira S, Isshiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y et al(1990) A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. Embo J **9**:1897-1906.

Alexander SPH, Mathie A, Peters JA (2011) Guide to Receptors and Channels (GRAC), 5th edition. Br J Pharmacol 164:S1-S2.

Anderson GL, Chlebowski RT, Aragaki AK, Kuller LH, Manson JE, Gass M et al (2012) Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *Lancet Oncol* **13**:476-486.

Ariazi E, Cunliffe H, Lewis-Wambi JS, Slifker M, Willis A, Ramos P et al (2011) Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci USA **108**:18879-18886.

Bian Z-M, Elner SG, Elner VM (2009) Dual involvement of caspase-4 in Inflammatory and ER stress-induced apoptotic responses in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* **50**:6006-6014.

Bourgoin-Voillard S, Gallo D, Laµos I, Cleeren A, Bali LE, Jacquot Y et al (2010) Capacity of type 1 and 11 ligands to confer to estrogen receptor alpha an appropriate conformation for the recruitment of coactivators containing a LxxLL motifð Relationship with the regulation of

This article is protected by copyright. All rights reserved.

receptor level and ERE-dependent transcription in MCF-7 cells. *Biochem Pharmacol* **79**:746-757.

Ellis M, Gao F, Dehdashti F, Jeffe D, Marcom P, Carey L et al (2009) Lower-dose vs high-dose oral estradiol therapy of hormone receptorï positive, aromatase inhibitorï resistant advanced breast cancer: A phase 2 randomized study. JAMA **302**:774-780.

Fan P, Griffith OL, Agboke FA, Anur P, Zou X, McDaniel RE et al (2013) c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. *Cancer Res* **73**:4510-4520.

Haddow A (1970) David A Karnosky Memorial lecture: Thoughts on chemical therapy. *Cancer Res* **26**:737-754.

Haddow A, Watkinson JM, Patterson E (1944) Influence of synthetic oestrogens upon advanced malignant disease *BMJ* **2**:393-398.

Hitomi J, Katayama T, Eguchi Y, Kudo T, Taniguchi M, Koyama Y et al (2004) Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Aß-induced cell death. *J Cell Biol* **165**:347-356.

Hu ZZ, Kagan BL, Ariazi EA, Rosenthal DS, Zhang L, Li JV, et al (2011) Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells *PLoS One* **6**:e20410.

Jordan VC (2004) Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer cell* **5**:207-213.

Jordan VC (2008) The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer - survival or death? *J Clin Oncol* **26**:3073 - 3082.

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

Jordan VC, Koch R, Mittal S, Schneider MR (1986) Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. *Br J Pharmacol* **87**:217-223.

Jordan VC, Lieberman ME (1984a) Estrogen-stimulated prolactin synthesis in vitro. Classification of agonist, partial agonist, and antagonist actions based on structure. *Mol Pharmacol* **26**:279-285.

Jordan VC, Lieberman ME, Cormier E, Koch R, Bagley JR, Ruenitz PC (1984b) Structural requirements for the pharmacological activity of nonsteroidal antiestrogens in vitro. *Mol Pharmacol* **26**:272-278.

Jordan VC, Obiorah I, Fan P, Kim HR, Ariazi E, Cunliffe H et al (2011) The St. Gallen Prize Lecture 2011: evolution of long-term adjuvant anti-hormone therapy: consequences and opportunities. *Breast* **Suppl 3:**S1-11.

Jordan VC, Schafer JM, Levenson AS, Liu H, Pease KM, Simons LA et al (2001) Molecular classification of estrogens. *Cancer Res* **61**:6619-6623.

Kim S-J, Zhang Z, Hitomi E, Lee Y-C, Mukherjee AB (2006) Endoplasmic reticulum stress-induced caspase-4 activation mediates apoptosis and neurodegeneration in INCL. Hum Mol Genet **15**:1826-1834.

Lahusen T, Henke RT, Kagan BL, Wellstein A, Riegel AT (2009) The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer. *Breast Cancer ResTreat* **116**:225-237.

Lewis-Wambi J, Jordan VC (2009) Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? *Breast Cancer Res* **11**:206.

This article is protected by copyright. All rights reserved.

Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T et al 2005a) Intrinsic mechanism of estradiol-Induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* **97**:1746-1759.

Lewis JS, Osipo C, Meeke K, Jordan VC (2005b) Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* **94**:131-141.

Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. *J Biol Chem* **258**:4741-4745.

List H-J, Lauritsen KJ, Reiter R, Powers C, Wellstein A, Riegel AT (2001) Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 Is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells. *J Biol Chem* **276**:23763-23768.

Maximov P, Sengupta S, Lewis-Wambi JS, Kim HR, Curpan RF, Jordan VC (2011) The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: a hypothesis. *Horm Mol Biol Clin Investig* **5**:27-34.

Maximov PY, Myers CB, Curpan RF, Lewis-Wambi JS, Jordan VC (2010) Structureī function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. *J Med Chem* **53**:3273-3283.

Obiorah I, Jordan VC (2013) 2012 NAMS/PFIZER- Wulf H. Utian endowed lecture. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. *Menopause* **20**:372-382.

Obiorah I, Sengupta S, Curpan R, Jordan VC (2014b) Defining the conformation of the estrogen receptor complex that controls estrogen induced apoptosis in breast cancer. *Mol Pharmacol* **85**:789-99.

This article is protected by copyright. All rights reserved.

Accepted Article

Obiorah I, Surojeet S, Fan P, Jordan VC (2014a) Delayed triggering of estrogen induced apoptosis that contrasts with rapid paclitaxel induced breast cancer cell death. . *B J Cancer* **110**:1488-96.

Oyadomari S, Mori M (2003) Roles of CHOP//GADD153 in endoplasmic reticulum stress. *Cell Death Differ* **11**:381-389.

Robson JM, Schonberg A (1937) Oestrous reactions, including mating, produced by triphenylethylene *Nature (Lond)* **140**:196

Ron D, Habener JF (1992) CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev* **6**:439-453.

Sengupta S, Obiorah I, Maximov PY, Curpan R, Jordan VC (2013) Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. *B J Pharmacol* **169**:167-178.

Sengupta S, Sharma CG, Jordan VC (2010) Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells. *Horm Mol Biol Clin Investig* **2**:235-243.

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA et al (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**:927-937.

Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z et al (2001) Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *J Natl Cancer Inst* **93**:1714 - 1723.

This article is protected by copyright. All rights reserved.

Accepted Article

Styner M, Meyer MB, Galior K, Case N, Xie Z, Sen B et al (2012) Mechanical strain downregulates C/EBPbeta in MSC and decreases endoplasmic reticulum stress. *PLoS One* **7**:12.

Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res* **127**:23 - 33.

Yao K, Lee E, Bentrem D, England G, Schafer J, O'Regan R et al (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* **6**:2028-2036.

FIGURE LEGENDS

Figure 1. Heat map of the time course pattern of E_2 and BP-regulated expression of cell cycle genes. MCF-7 breast cancer cells were treated with either control, E_2 (1nM), BP (1ÕM) or 4OHT (1ÕM) over a period of 24h and 4OHT was used to block the effects of E_2 and BP. Genes which are at least 2.5 fold over-expressed (red) or under-expressed (green) as compared to control at p value of 0.05 at **(A)** 6h, **(B)** 12h and **(C)** 24h are presented. Cell cycle genes induced by E2 and BP are indicated in black.

Figure 2. Effect of BP in the growth and apoptosis of MCF7:5C breast cancer cells. **(A)** Cells were seeded in triplicates and treated with either control, E_2 (1nM) or BP (1ÕM) and the cells were harvested daily for 6 days. **(B)** Treatment with BP versus the control was extended for 13 days and the DNA content of the remaining cells in each well was quantified. The data represent the mean of three independent experiments. **(C)** MCF7:5C cells were treated with

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control or BP (1ÕM) for 6 days and then stained with annexin v-FITC and propidium iodide and analysed by flow cytometry. Viable cells (left lower quadrant) are annexin v-FITC ī and PI ī , early apoptotic cells (right lower quadrant) are annexin v-FITC + and PI ī , dead cells (left upper quadrant) are PI + and late apoptotic cells (right upper quadrant) are annexin v-FITC + and PI +. Increased late apoptotic effect is observed in the right upper quadrant.

Figure 3. Determination of the trigger point for BP induced apoptosis. MCF7:5C cells were treated with BP (1ÕM) alone and 1ÕM 4OHT was added and used to block and reverse BP action daily over a period of 9 days. The cells were harvested after 14 days of treatment. The DNA content of the remaining cells was quantified using a fluorescent DNA quantification kit. The point of trigger for apoptosis induced by BP is determined by the time when the apoptotic effects of BP could not be blocked by 4OHT.

Figure 4. Determination of apoptotic genes differentially expressed by BP treatment in MCF7:5C cells. MCF7: 5C cells were treated with vehicle (control), 1ÕM BP, 1 ÕM 4OHT, in the presence or absence of BP over a period of 5days. Gene expression values were obtained and analyzed in comparison to the controls and heat maps were generated at **(A)** 96h and **(B)** 120h of treatment and the expressed genes listed. The selected genes were at least 2.5 fold over-expressed (red) or under-expressed (green) as compared to control at p value of 0.05.

Figure 5. Induction of apoptotic genes by BP. BP induces apoptotic genes after 7 days of treatment. MCF7:5C cells were treated with Vehicle (Veh), BP (1 µM), 4OHT, 1ÕM or combination treatment of BP and 4OHT for 7-9 days. Total RNA was isolated and reverse transcribed and **(A)** BIM and **(B)** TNF **(C)** FAS and **(D)** FADD mRNA levels was determined

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using RT-PCR. PCR data values are presented as fold difference versus vehicle treated cells Ñ SEM. [* P< 0.05, ** p<0.005, *** p<0.0001, ****p<0.0005]

Figure 6. Diverse effects of BP and E2 on cell cycle progression. Distribution of the cells through the cell-cycle phases was analyzed by flow cytometry in cells treated with E_2 (1nM), BP (1 µM), or control for 24 h, 48h and 96h. The percentage of the cells in each fraction is calculated using the ModFit software. The y axis represents the number of cells and FL2-A represents the intensity of propidium iodide.

Figure7. Caspase 4 is important for BP induced apoptosis. MCF7:5C cells were treated with control(0.1% ethanol) or BP (1ÕM) or caspase 4 (casp4) inhibitor with or without BP for either **(A)** 12 days and assessed for cell proliferation or **(B)** for 6 days and evaluated for apoptosis. Apoptosis and inhibition of growth of cells were blocked by caspase 4 inhibitor z-LEVD-fmk (10 µM).

Supporting information

Figure S1. Chemical structure of 17β Bisphenol (BP).

Figure S2. Inhibition of (retinoblastoma protein) RB1 by E_2 and BP. MCF-7 cells were treated with either Veh (0.1% ethanol), E_2 (1nM) or BP (1ÕM) for 4h, 8h, 12h and 24h. 4OHT (1ÕM) and combination of 4OHT with either E_2 or BP were used as negative controls. Retinoblastoma protein (RB) mRNA levels were determined using RT-PCR.

Figure S3. Apoptotic effect of BP in MCF7:5C cells. MCF7:5C cells were treated with control (0.1% ethanol) or BP (1ÕM) for 5days and then stained with nucleic acid dye YO-PRO-1 and propidium iodide and analysed by flow cytometry. Viable cells (left lower quadrant) are YO-PRO-1 ī and PI ī , early apoptotic cells (right lower quadrant) are YO-PRO-1 + and PI ī , dead

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cells (left upper quadrant) are PI + and late apoptotic cells (right upper quadrant) are YO-PRO-1 + and PI +. Increased late apoptotic effect is observed in the right upper and lower quadrants.

Figure S4. **Determination of the critical trigger of apoptosis.** Cells were treated with BP (1ÕM) alone and ICI 182 780 (1ÕM) was added at the indicated time points and used to block and reverse BP action over a period of 9 days. The cells were harvested after 14 days of treatment. The DNA content of the remaining cells was quantified using a fluorescent DNA quantification kit.

Figure S5. mRNA levels of caspase 4 and LTA. Cells were treated with vehicle (0.1% ethanol), BP (1ÕM) and 4OHT (1ÕM) in the presence or absence of BP for 72h, 96h and 120h. Cells were harvested and caspase 4 and LTA mRNA levels were determined using RT-PCR.

Table S1 List of cell cycle regulated genes induced by E_2 (1nM), BP (1ÕM) and 4OHT (1ÕM) combination treatments of 4OHT and E2 and 4OHT and BP in MCF-7 cells after 24 h of treatment versus control.

Table S2 List of apoptosis regulated genes induced by BP (1ÕM) and 4OHT (1ÕM) in the presence or absence of BP in MCF7: 5C cells after 96 h of treatment versus control.

Table S3 List of apoptosis regulated genes induced by BP (1ÕM) and 4OHT (1ÕM) in the presence or absence of BP in MCF7: 5C cells after 120 h of treatment versus control.

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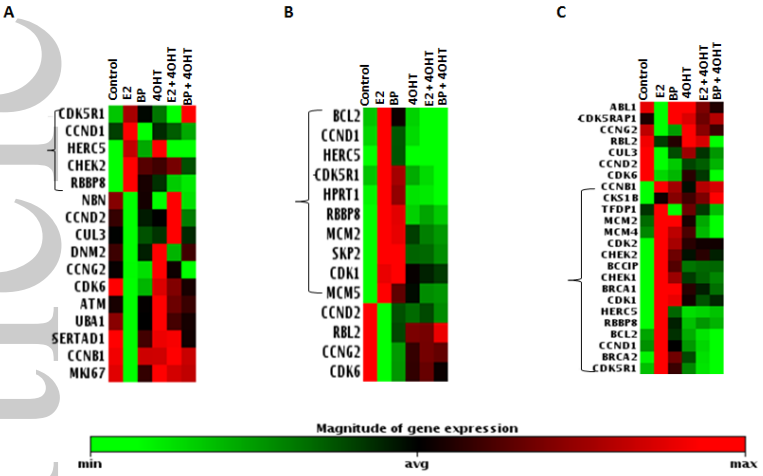

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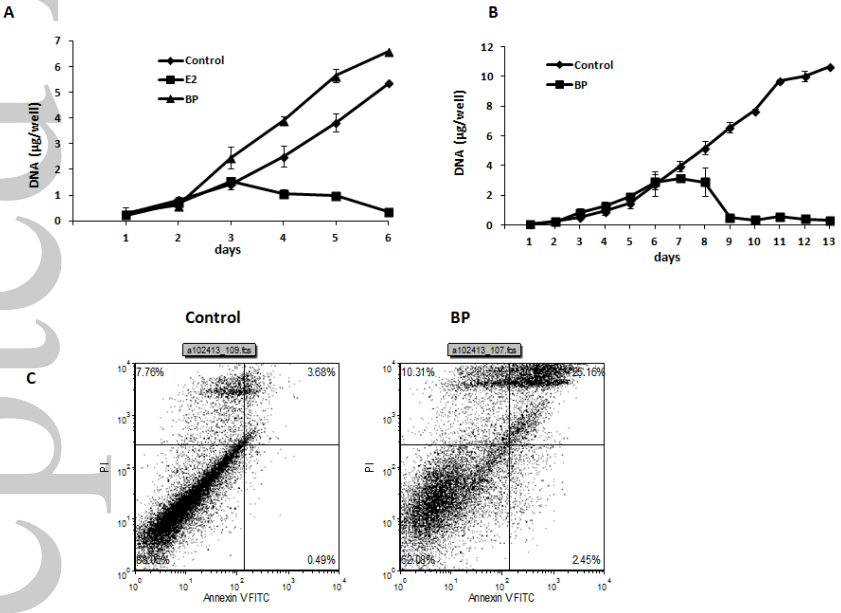

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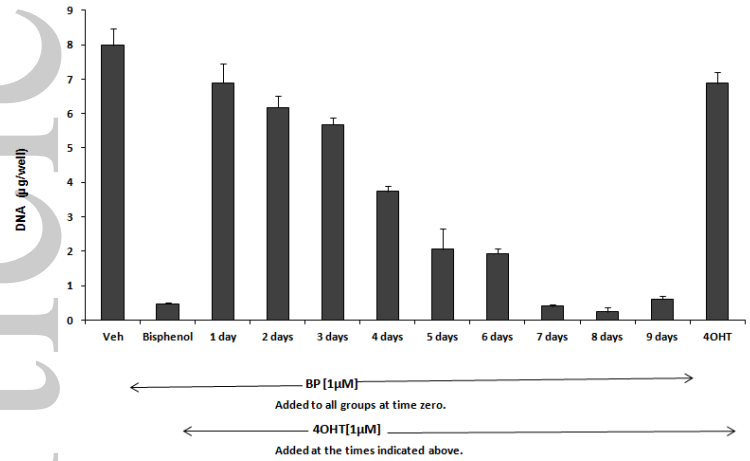

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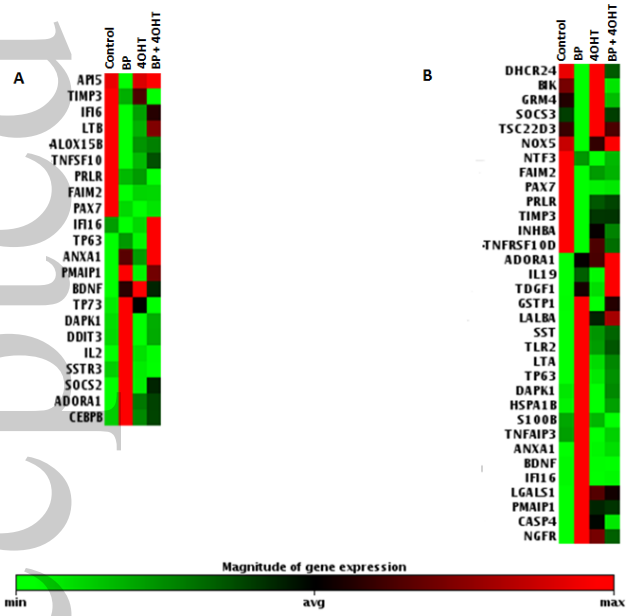

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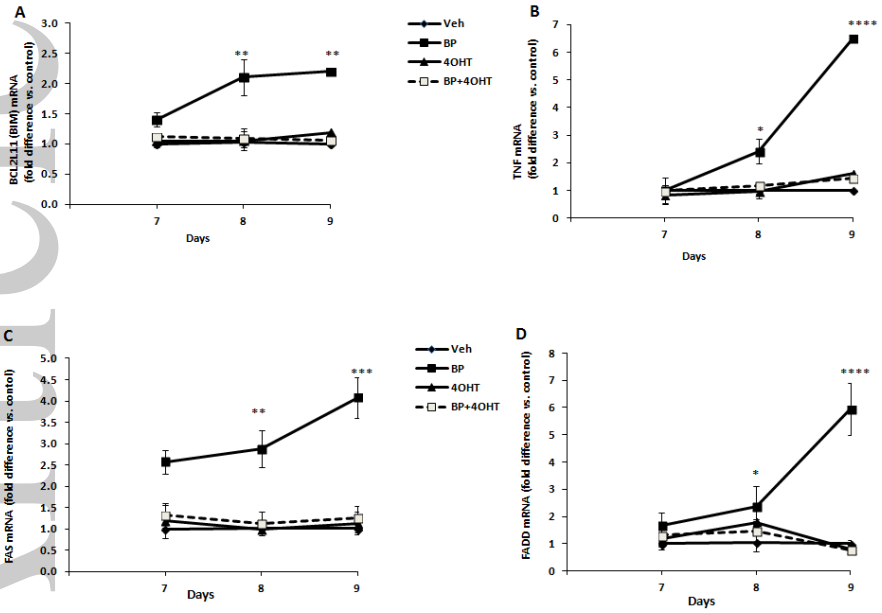

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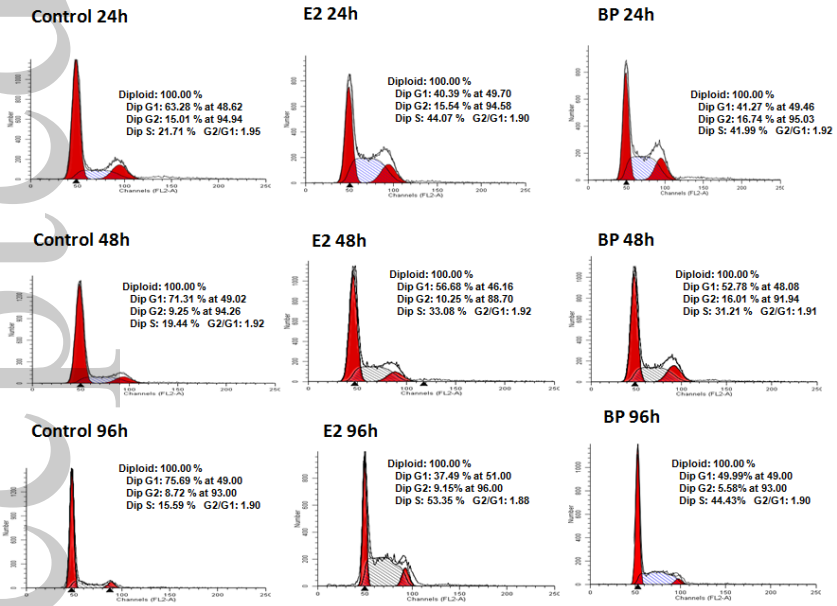

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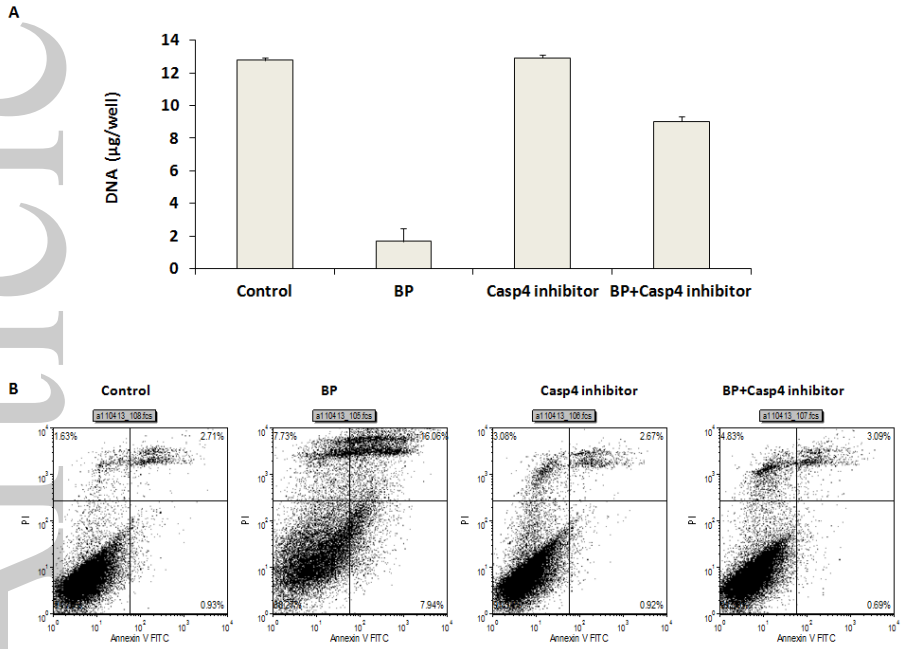

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Influence of the Length and Positioning of the Antiestrogenic Side Chain of Endoxifen and 4-Hydroxytamoxifen on Gene Activation and Growth of Estrogen Receptor Positive Cancer Cells

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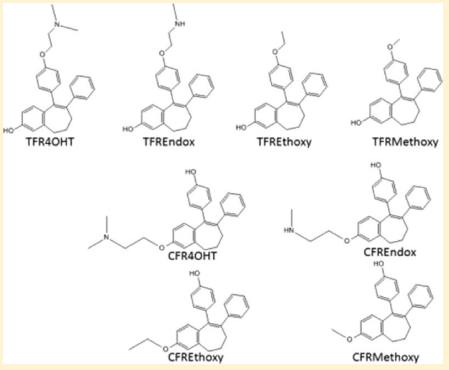
Supporting Information

ABSTRACT: Tamoxifen has biologically active metabolites: 4-hydroxytamoxifen (4OHT) and endoxifen. The *E*-isomers are not stable in solution as *Z*-isomerization occurs. We have synthesized fixed ring (FR) analogues of 4OHT and endoxifen as well as FR *E* and *Z* isomers with methoxy and ethoxy side chains. Pharmacologic properties were documented in the MCF-7 cell line, and prolactin synthesis was assessed in GH3 rat pituitary tumor cells. The FR *Z*-isomers of 4OHT and endoxifen were equivalent to 4OHT and endoxifen. Other test compounds used possessed partial estrogenic activity. The *E*-isomers of FR 4OHT and endoxifen had no estrogenic activity at therapeutic serum concentrations. None of the newly synthesized compounds were able to down-regulate ER levels. Molecular modeling demonstrated that some compounds would each create a best fit with a novel agonist conformation of the ER. The results demonstrate modulation by the ER complex of cell replication or gene transcription in cancer.



INTRODUCTION

Tamoxifen remains an important, lifesaving medicine for the adjuvant treatment of early stage breast cancer.[1−3] It is listed as an essential medicine in oncology by the World Health Organization and is available to prevent breast cancer in high risk women in both the United States and United Kingdom. The continued use of tamoxifen has profound effect on public health worldwide. For these reasons, it is appropriate to study the molecular pharmacology of tamoxifen and its metabolites and analogues. Indeed, the fact that tamoxifen will most likely be administered for 10 or more years for the treatment of breast cancer,[3] and there is a role for long-term tamoxifen treatment in the prevention of breast cancer in healthy women,[4] reinforces the value of understanding the molecular pharmacology of the medicine.

Tamoxifen ((*Z*)-1-(*p*-*β*-dimethylaminoethoxyphenyl-1,2-diphenylbut-1-ene) is the antiestrogenic *Z*-isomer of an estrogenic substituted triphenylethylene[5] that is converted at the 4-position[6] to two hydroxylated metabolites 4-hydroxytamoxifen (4OHT)[7] and 4-hydroxy-*N*-desmethyltamoxifen (endoxifen),[8,9] both of which have high binding affinity for the estrogen receptor (ER) found in estrogen target tissues or hormone-dependent tumors.[7,10,11] The metabolites have similar pharmacology and activate or depress a similar gene profile in vitro.[11−13] An interesting aspect of tamoxifen and its isomers is that the *E*-isomer (ICI 47 699) of tamoxifen (ICI 46 474) is an estrogen in vivo[5] and in vitro.[14] By contrast, the *E*-isomer of 4OHT is unstable and isomerizes to a mixture of *E*- and *Z*-isomers, displaying antiestrogenic activity both in vivo[15] and in vitro.[14] Subsequent studies examined fixed ring (FR) derivatives of the *E*- and *Z*-isomers of 4OHT[16] using a previously reported synthetic pathway.[17] The *E*-isomer was a weak antiestrogen.

We synthesized a series of FR analogues of the alkylaminoethoxy side chain of 4OHT to link molecular modeling with cell replication in breast cancer (MCF-7:WS8) and prolactin synthesis in the rat pituitary gland cancer cell line GH3. We took these approaches to study structure−function relationships: reducing the antiestrogenic side chain of 4OHT and comparing results with bisphenol (BPTPE) and trihydroxytriphenylethylene (3OHTPE),[18] comparing *E* and *Z* FR isomers and finally the length and bulk of the antiestrogenic side chain of *E*-isomer of FR4OHT (EFR4OHT). Select ER-responsive genes (pS2, GREB1, and PgR) were measured following 48 h of incubation of all test compounds with MCF-7:WS8 cells as well as ER levels determined by Western blotting. Also we evaluated the impact of therapeutic concentrations of *E*-isomers of FR4OHT and FR endoxifen (FREndox) alone or in combination with therapeutic levels of

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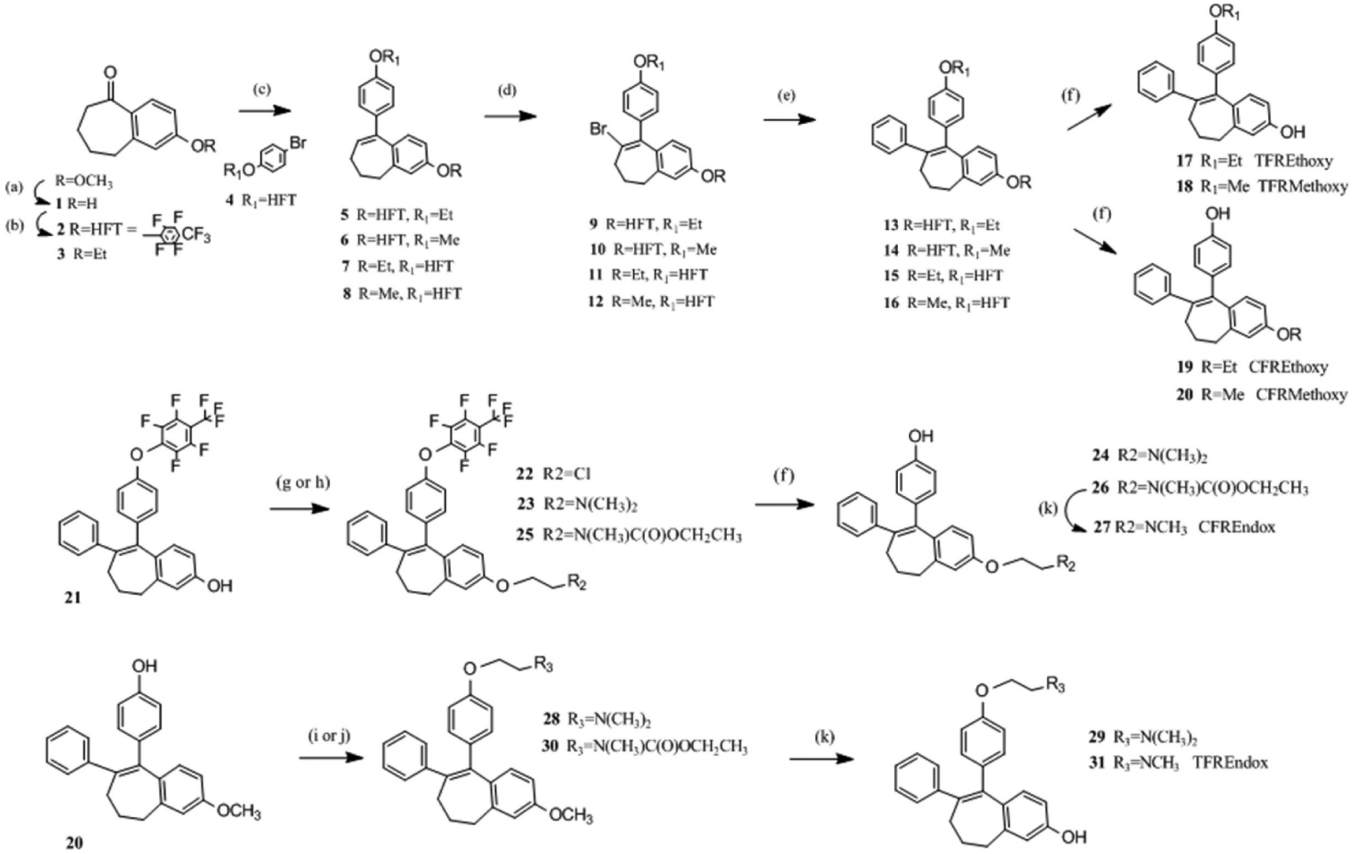
Scheme 1. Synthesis of the Isomerically Stable Fixed Ring (FR) Isomers of Methoxy, Ethoxy Substituted Triphenylethylene Derivatives and Fixed Ring 4OHT and Endox



Z-isomers on the growth of MCF-7:WS8 breast cancer cell line to estimate therapeutic relevance during breast cancer treatment with tamoxifen for tumor cell growth control by putative estrogenic metabolites.

RESULTS

Chemistry. Five novel FR4OHT analogues (ZFRMethoxy, ZFREthoxy, EFREthoxy, ZFREndox, and EFREndox) were synthesized in a multistep sequence involving a Gringard reaction of a protected *p*-bromophenol with a substituted benzosuberone. Subsequent modifications provided two key intermediates that have a methoxy or heptafluorotolyl (HFT) protecting group on either of the phenolic groups. This versatile scaffold was important for the synthesis of the *E*- and *Z*-isomers of FRMethoxy, FREthoxy, FR4OHT, and FREndox compounds. Both isomers of FR4OHT (**24** and **29** in Scheme 1) and CFRMethoxy (**20**) were synthesized according to McCague et al.,[17] while compounds 3OHTPE and BPTPE were synthesized according to Maximov et al.[18]

***Z* Fixed Ring Methoxy. *E* and *Z* Fixed Ring Ethoxy Analogues (ZFRMethoxy and ZFR/EFREthoxy).** 2-Methoxyheptenone (benzosuberone) was demethylated to **1** (Scheme 1) by refluxing with aluminum chloride in toluene.[19,20] Phenol **1** was protected with octafluorotoluene using phase transfer reaction conditions to **2** or converted to the ethoxy analogue **3** using ethyl iodide and potassium carbonate in acetone. Both compounds were treated with the Grignard reagent of a protected *p*-bromophenol that resulted in the formation of the ethoxycycloheptene **5** and the methoxy analogue **6**.[17] For compounds **7** and **8**, 4-bromophenyl 2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenyl ether **4** was obtained by the method of Jarman and McCague.[21] This compound was converted to the Grignard reagent and reacted with suberone **3** which yielded **7** or reacted with 2-methoxybenzosuberone which led to **8**. Bromine was introduced at the 8-position using pyridine hydrobromide perbromide (**9**−**12**) that was subsequently replaced with a phenyl moiety upon treatment with phenylzinc chloride and a palladium catalyst yielding compounds **13**−**16**. These key intermediates were selectively deprotected to provide either *E*- or *Z*-isomer of FRMethoxy (**18** and**20**) and FREthoxy (**17** and **19**) tamoxifen analogues.

***E* Fixed Ring Endoxifen (EFREndox).** The synthesis of EFREndox **27** was first attempted by selective demethlyation of EFR4OHT **24** using 1-chloroethyl chloroformate both with and without magnesium oxide,[22] as well as vinyl chloroformate[23] with no formation of product detected by LC/MS. In addition, demethylation using ruthenium chloride in methanol followed by treatment with hydrogen peroxide was also tried without success.[24] Also, the attempted direct methyl amination of chloroethoxybenzocycloheptene **22** by heating with 33% methylamine in ethanol failed. *Z*-isomer of 4OHT (model compound) was converted to its *N*-oxide by stirring with 30% hydrogen peroxide in acetone but did not demethylate using selenium oxide.[25] Alternatively, we investigated several methods for attaching the protected ethylamine side chain directly onto phenol **21**. Methods included reaction of **21** with ethyl (2-bromoethyl)(methyl)carbamate[22] by heating with cesium carbonate in DMF, heating with sodium hydride in DMF, and using phase transfer reaction conditions. All produced **25** in various yields with the last method giving the best overall

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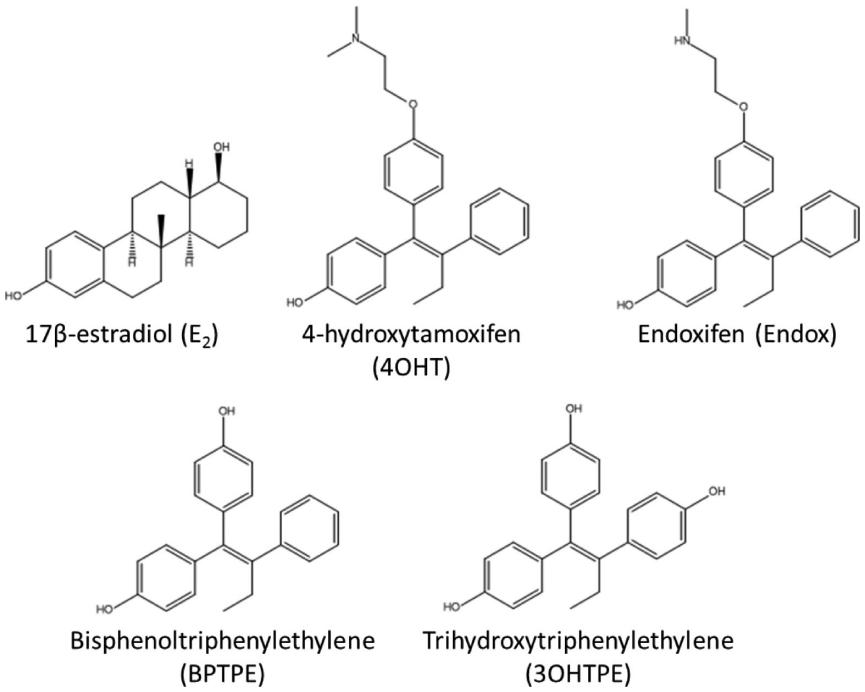

Figure 1. Structures of compounds used in the current study that were purchased (E_2, 4OHT, and Endox) or not synthesized (BPTPE and 3OHTPE).[18]

yield. In the next step, the heptylfluorotolyl protecting group was removed using sodium methoxide in DMF to **26**, followed by removal of the carbamate with pyridine HCl to **27**. A faster route to EFREndox **27** could be achieved by starting with **14**, where both protecting groups would be removed concurrently.

***Z* Fixed Ring Endoxifen (ZFREndox).** The synthesis of TFREndox **31** was attempted with selective demethlyation of the *Z* tamoxifen **29** using vinyl chloroformate[23] with no product formation. The ZFREndox compound **30** was synthesized by heating compound **20** with ethyl (2-bromoethyl)(methyl)carbamate[23] and cesium carbonate in DMF, but the reaction was not as efficient as using ethyl (2-hydroxyethyl)(methyl)carbamate,[23] TPP, and DIAD in THF. Both the methoxide and carbamate protecting groups of **30** were removed simultaneously by heating with pyrdine·HCl to give ZFREndox **31**.

Pharmacology. To assess estrogenic and antiestrogenic properties of the test compounds, we employed a DNA quantification assay with the ER positive human breast cancer cell line MCF-7:WS8 as described in Materials and Methods and have compared the results with the test compounds with previously described angular estrogens BPTPE and 3OHTPE.[18] Estradiol (E_2) induced growth of cells (Figure 2A) in a concentration-dependent manner with maximal stimulation starting at a concentration of 10^{-11} M. All of the test compounds are partial agonists and do not reach the same level of growth induction as with E_2. It is therefore not appropriate to calculate EC_{50} against E_2. However, they do cluster by their levels of growth induction. Compounds BPTPE, ZFRMethoxy, ZFREthoxy, and EFR4OHT induce the same levels of growth of MCF-7:WS8 cells at a concentration of 10^{-6} M with no statistical difference ($P < 0.05$). Thus, we estimated the potency of these compounds by comparing their EC_{50} concentrations (Figure 1). The results demonstrate that BPTPE is a much more potent partial agonist in MCF-7:WS8 cells (EC_{50} of 1.5×10^{-11} M) than other test compounds in this group (Figure 1). The ZFRMethoxy and ZFREthoxy compounds with the shortest side chains have EC_{50} of 3×10^{-10} M, while EFR4OHT compound has the highest EC_{50} in this group of compounds of 1.5×10^{-8} M (Figure 1). The next group of compounds (EFRMethoxy, EFREthoxy, and EFREndox) induce cell growth a little higher but statistically more significantly than the previous group ($P < 0.05$), so their EC_{50} concentrations can be estimated between these compounds (Figure 1). EFRMethoxy compound has an EC_{50} of 4×10^{-9} M, while EFREthoxy has EC_{50} of 2.7×10^{-9} M and EFREndox has EC_{50} of 2×10^{-8} M. The ZFR4OHT and ZFREndoxifen, like the structurally similar *Z*-4OHT and endoxifen, have no estrogenic properties over the whole concentration range of 10^{-12}–10^{-6} M (Figure 1) ($P > 0.05$ for all concentration points when compared to each of their respective vehicle controls). Estrogenic properties on the growth of MCF-7:WS8 cells of 3OHTPE were previously described[18] and are not shown here. The EC_{50} of 1.5×10^{-10} M is similar to that of BPTPE.

To test the antiestrogenic properties of test compounds, we employed the same DNA based growth assay with combination treatments with 10^{-10} M E_2. The *Z*-isomers of the FR4OHT and FREndox produce an equivalent antiestrogenic effect (average IC_{50} of 3×10^{-9} M in MCF7:WS8 cells) inhibiting 10^{-10} M E_2 completely ($P > 0.05$ at 10^{-6} M points when compared to vehicle control) like 4OHT and endoxifen (Figure 1B). ZFRMethoxy, ZFREthoxy, EFRMethoxy, EFREthoxy, EFR4OHT, ECFREndox, BPTPE, and 3OHTPE compounds all have very weak antiestrogenic properties (Figure 2B), inhibiting E_2-stimulated cell growth by about 20% at top concentration ($P < 0.05$ compared to control); however, the ZFREthoxy compound seems to have a little more antiestrogenic properties than the rest of the group by about 20% ($P < 0.05$ at 10−6 M), and EFREndox inhibits only by about 10% compared to vehicle control ($P < 0.05$). All this is consistent with the intrinsic activity of test compounds alone (Figure 2A).

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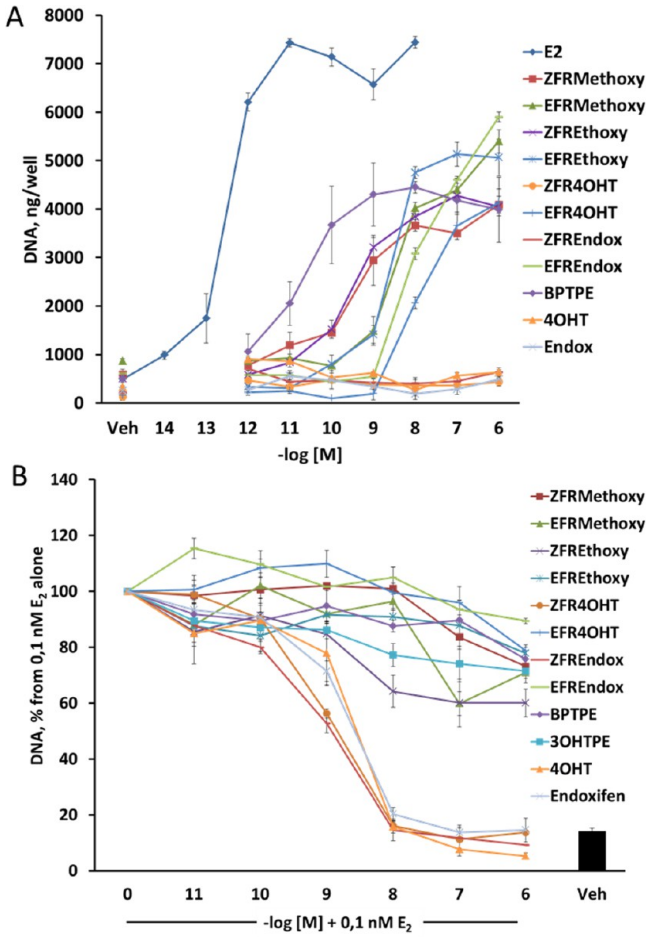

Figure 2. Assessment of estrogenic/antiestrogenic properties of the test compounds in MCF-7:WS8 ER-positive human breast cancer cell lines: (A) treatments of the MCF-7:WS8 cells with compounds alone; (B) treatments of MCF-7:WS8 cells with compounds in combination with 10^{-10} M E_2. All DNA content was normalized to a corresponding 10^{-10} M E_2 control of each of the experiments.

MCF-7:WS8 cells were treated with therapeutic concentrations of *E*- and *Z*-isomers of FR4OHT and endoxifen found in postmenopausal breast cancer patients treated with tamoxifen.[26] Results show that pharmacological concentrations of tested *E*-isomers alone or in combination with *Z*-isomers were not able to induce significant cell growth ($P > 0.05$ compared to control), compared to cell proliferation induced by postmenopausal levels of estrogens (E_1/E_2) found in postmenopausal women taking tamoxifen (Figure 3) ($P < 0.05$ compared to control). The concentrations of estrogens corresponding to average levels of estrogens in postmenopausal women were 7.8×10^{-11} M for E_1 and 4.7×10^{-11} M for E_2 and were obtained from pervious publications.[27,28] The levels for the test compounds corresponding to mean therapeutic levels of tamoxifen metabolites in breast cancer patients taking tamoxifen were the following: ZFR4OHT, 5.81×10^{-9} M; ZFREndox, 29.1×10^{-9} M; EFR4OHT, 0.56×10^{-9} M; EFREndox, 1.17×10^{-9} M.[26]

Real-Time PCR. To assess the pharmacological properties the test compounds on estrogen responsive genes, we used real-time polymerase chain reaction (RT-PCR) in the ER positive rat pituitary tumor cell line GH3 to assess the modulation of the prolactin gene (Prl) and also in estrogen-responsive genes pS2, progesterone receptor (PgR), and GREB1 in MCF7:WS8

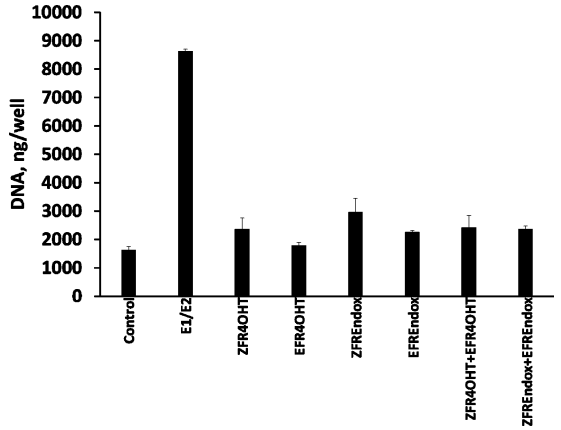

Figure 3. Assessment of estrogenic properties of different stable isomers of tamoxifen's metabolites 4OHT and endoxifen in MCF-7:WS8 at average therapeutic concentrations.[26] The levels for the tested compounds corresponding to mean therapeutic levels of tamoxifen metabolites were the following: ZFR4OHT, 5.81×10^{-9} M; ZFREndox, 29.1×10^{-9} M; EFR4OHT, 5.6×10^{-9} M; EFREndox, 1.17×10^{-9} M.

cells. All cells were first estrogen starved and then processed as described in Materials and Methods. Results of the Prl gene expression analysis show that the Prl gene in rat GH3 cells has elevated expression of mRNA in response to E_2 in a concentration-dependent manner (Figure 4A) with maximal stimulation at 10^{-9} M ($P < 0.05$ compared to control). All of the test compounds had shallow partial agonist dose–response curves (Figure 4A). As a result of the inability of test compounds to induce maximal Prl gene actions higher than 40% of E_2, it is inappropriate to estimate EC_{50}. In combination with 1 nM E_2 all test compounds exhibited antiestrogenic properties; however, only ZFR4OHT, ZFREndox, and 4OHT were able to completely inhibit 1 nM E_2-induced Prl gene up-regulation to control levels at their top concentration of 10^{-6} M ($P > 0.05$) (Figure 4B). All other test compounds inhibited the effects of 1 nM E_2 and the levels of the intrinsic activity of compounds alone (Figure 4B).

RT-PCR of estrogen regulated genes pS2, GREB1, and PgR in MCF-7:WS8 cells treated with test compounds show a differential effect based on the structure of the ligands. Estradiol (10^{-10} M) induced expression of all test genes compared to vehicle control (Figure 5) after 48 h of treatment ($P < 0.05$ for all genes). Treatments with 3OHTPE and BPTPE produced a partial estrogenic effect on all genes ($P < 0.05$ when comparing to E_2 treatment or vehicle control) and no significant difference between each other ($P > 0.05$) in any of the genes. Treatments with isomers of FRMethoxy and FREthoxy compounds produced partial estrogenic effects in all estrogen-responsive genes ($P < 0.05$ when compared to vehicle control); however, *E*-isomers were able to produce a higher induction of expression in all studied genes compared with corresponding *Z*-isomers ($P < 0.05$). ZFR4OHT, ZFREndox, 4OHT, and Endox produced no significant effect on mRNA synthesis in pS2 and GREB1 genes ($P > 0.05$ when compared to vehicle control) and were similar to each other ($P > 0.05$) but did induce 3- to 4-fold increase in PgR mRNA levels (Figure 5C) compared to vehicle control ($P < 0.05$). EFR4OHT and EFREndox compounds were able to induce expression of all genes investigated (Figure 5), significantly higher than their *Z*-isomers ($P < 0.05$). Higher than therapeutic concentrations of

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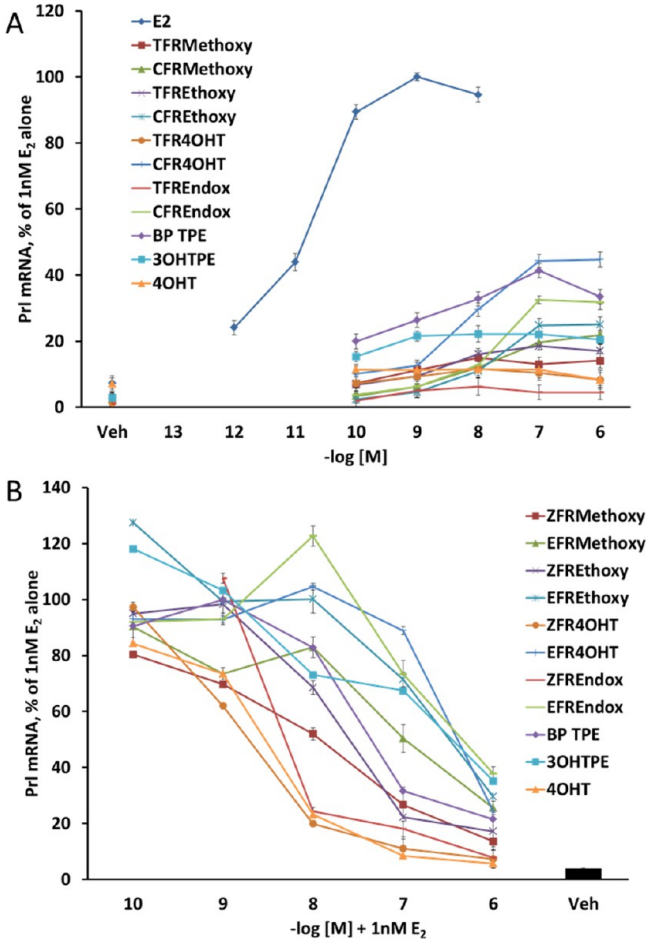

Figure 4. Assessment of estrogenic/antiestrogenic properties of the test compounds on inducing prolactin (Prl) gene's mRNA expression in GH3 rat pituitary tumor cells. (A) Treatments of the GH3 cells with compounds alone. The fold change of the mRNA was first calculated using the ΔΔCt method. The corresponding 10^{-10} M E_2 control values were considered as 100%, and all other treatments were calculated accordingly. (B) Treatments of the GH3 cells with compounds in combination with 10^{-9} M E_2. The fold change of the mRNA was first calculated using the ΔΔCt method. The corresponding 10^{-10} M E_2 control values were considered as 100%, and all other treatments were calculated accordingly.

test compounds, in particular isomers of FR4OHT and FREndox, were chosen to demonstrate their ability to regulate estrogen responsive genes at concentrations consistent with their inhibitory effects on the estrogen-induced cell proliferation (Figure 2B).

Immunoblotting. Immunoblotting was performed to assess the impact of the test compounds on the regulation of the ERα protein levels in MCF-7:WS8 cells. We starved the cells in the same way as estrogen starvation for cell proliferation assays. After 24 h of treatment with compounds, cells were harvested and processed as described in Materials and Methods. Results showed that 1 nM E_2 reduces the level of ERα by about 60% as measured by densitometry. In contrast, 4OHT and endoxifen and their ZFR analogues all caused an up-regulation of the ERα protein. The estrogen-like *E*-isomers of FR4OHT and FREndox did not induce the down-regulation of the protein. Fulvestrant (ICI), which degrades ERα, was used as a positive control and was able to down-regulate the ERα by more than 90%. Interestingly, compounds with shorter side chains like FRMethoxy and FREthoxy *E* and *Z* isomers and BPTPE and 3OHTPE were not able to induce any degradation of the ERα like E_2, despite their estrogenic properties in these cells, and actually up-regulated the protein levels (Figure 6).

Molecular Modeling. To study the binding mode of FR derivatives of endoxifen and 4OHT in the ER binding pocket, flexible docking simulations were carried out against both conformations of ER ligand-binding domain (LBD), agonist (PDB codes 1GWR (ER LBD cocrystallized with E_2),[29] 3ERD (ER LBD cocrystallized with DES),[30] 3Q97 (ER LBD cocrystallized with ethoxytriphenylethylene isomers),[31] and antagonist (PDB codes 3ERT (ER LBD cocrystallized with 4OHT),[30] 1UOM (ER LBD cocrystallized with 2-phenyl-1-[4-(2-piperidin-1-ylethoxy)phenyl]-1,2,3,4-tetrahydroisoquinolin-6-ol, PTI),[32] 2OUZ (ER LBD cocrystallized with lasofoxifene)[33]). The X-ray structures to be used for docking were selected based on the shape similarity between the investigated compounds and cocrystallized ligands of ER LBD complexes from PDB. In the following, the most relevant results obtained in docking simulations run against antagonist conformation 3ERT (Figure 7A), and two agonist conformations 1GWR (Figure 7 B) and 3Q97 (Figure 7C) are discussed. We have selected this antagonist structure because the native ligand shows the highest structural similarity with the investigated compounds. The cocrystallized ligands were docked to their native experimental structures to evaluate the docking method efficiency. The best ranked docking poses of the native ligands recapitulate the binding mode of the ligand to the active site of the experimental structures, and the same interactions with the amino acids lining the binding pocket were found (Supporting Information Figures S1, S2, and S3).

The predicted binding mode of the ZFR4OHT and ZFREndox to the antagonist conformation of ER 3ERT is similar to that of 4OHT (Figure 7A). In these models the ligands are accommodated well in the binding pocket, the complex H-bond network involving amino acids Asp351, Glu353, and Arg394 is recapitulated, and similar hydrophobic interactions are encountered (Figure 7D). Conversely, the EFR4OHT and EFREndox are docked to the 3ERT binding site in a completely different alignment but forming the H-bonds with Asp351, Glu353, and Arg394 (Figure 7E). Although the *E*-isomers form the H-bond network, they do not fit the binding pocket of ER antagonist conformation as well as the *Z*-isomers, as can be seen from the docking scores (Table 1), especially the values for Emodel. *E*-Isomers do not fill the binding pocket and are not involved in hydrophobic interactions with the important amino acids of the binding site like the *Z*-isomers and 4OHT. These remarks are supported by the van der Waals (vdW) parameter which accounts for hydrophobic interactions and shows favorable values for *Z*-isomers (Table 1). This binding alignment has been recapitulated in docking experiments performed for other experimental structures of ER LBD in antagonist conformation, 1UOZ and 2OUM (data not shown). These results show that it is highly probable for the *E*-isomers to be accommodated in a different conformation of ER LBD. Docking runs performed at the agonist conformations of ER (the receptor cocrystallized to E_2, PDB entry 1GWR (Figure 7B), and to DES, PDB entry 3ERD) have led to conflicting results; thus, no valid docking pose could be found. For this reason other experimental structures of ER in the agonist conformation were selected from PDB, based on the 3D similarity between the cocrystallized ligands and *E*-isomers. The structure showing the highest

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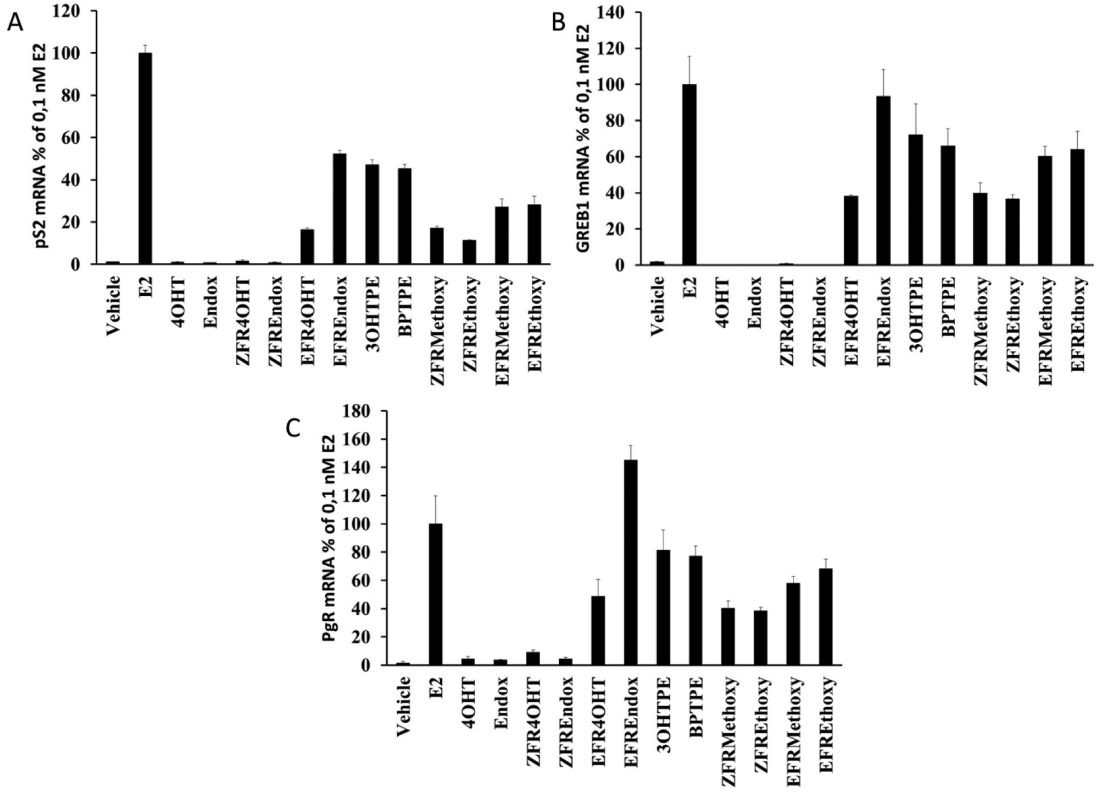

Figure 5. Assessment of estrogenic/antiestrogenic properties of the test compounds on inducing estrogen-responsive gene's mRNA expression in MCF-7:WS8 breast cancer cell line: (A) pS2 gene; (B) GREB1 gene; (C) PgR gene. Treatment with E_2 was made at 10^{-10} M. All of the other test compounds were treated at 10^{-6} M. The fold change of the mRNA was first calculated using the ΔΔCt method. Corresponding 10^{-10} M E_2 control values were considered as 100%, and all other treatments were calculated accordingly.

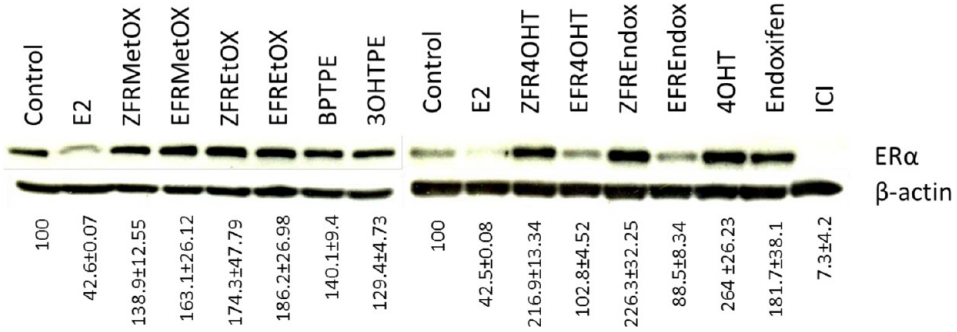

Figure 6. Immunoblotting results for test compounds after a 24 h treatment of MCF-7:WS8 breast cancer cells. Percent of control was calculated by comparison with the actin band. Immunoblotting experiments were repeated three times.

shape similarity between the native ligand and *E*-isomers was selected, namely, PDB entry 3Q97 (Figure 7C). Interestingly, this experimental structure contains two isomers corresponding to *E*- and *Z*-isomers of a triphenylethylene derivative, cocrystallized with ER LBD. The binding pocket of 3Q97 (Figure 7C) is wider and larger than the ones of 1GWR or 3ERD, and it can accommodate the *E*-isomers. The top ranked docking poses of EFR4OHT and EFREndox are shown in Figure 7F, and it can be seen that they fit in the binding pocket. The *Z*-isomers were ranked with lower docking scores and were docked in an orientation similar to that from the antagonist conformation of ER. It can be concluded from these findings that the predicted binding mode of *Z*-isomers is similar to that of 4OHT and other antagonists of ER, showing higher values of the docking scores when compared with *E*-isomers docked to antagonist conformation of ER LBD. The former compounds do not fit into the encapsulated binding pocket of ER, corresponding to agonist conformation of the receptor, even if some degree of flexibility has been allowed to the receptor. It is highly probable for *E*-isomers to induce conformational changes to the active site of ER upon binding which would be reflected in the repositioning of helix 12 to a conformation related to that of the experimental structure 3Q97.

The *Z*- and *E*-isomers of FRMethoxy and FREthoxy compounds were also docked to the experimental structures of ER LBD in the agonist (PDB entries 1GWR and 3Q97) and antagonist (PDB entry 3ERT) conformations. Analysis of docking results shows *Z*-isomers being better accommodated in the agonist conformation of ER than the *E*-isomers (Figure 8B and Figure 8C). The Emodel and docking scores have higher values for *Z*-isomers (Table 2). Few details indicate that it is possible for these isomers to bind to a conformation of ER

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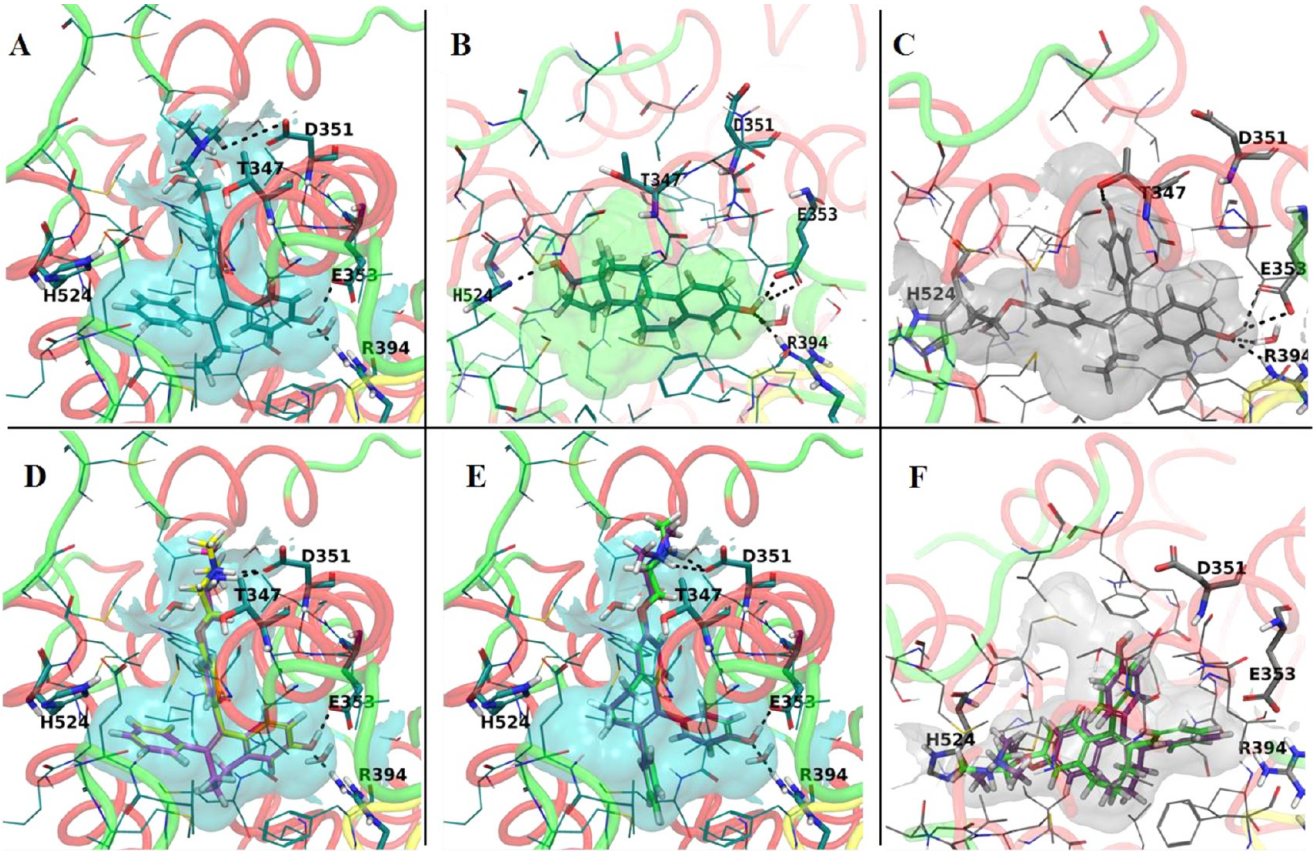

Figure 7. Representations of the experimental structures binding pockets used for modeling: (A) antagonist conformation of ER LBD cocrystallized with 4OHT (PDB code 3ERT); (B) agonist conformation of ER LBD cocrystallized with E_2 (PDB code 1GWR); (C) the agonist conformation of ER LBD cocrystallized with a *E*-isomer of ethoxytriphenylethylene (PDB code 3Q97). The best docking poses of the *Z*-isomers to the LBD of ER (antagonist conformation; PDB code 3ERT) were (D) ZFREndox (yellow) and ZFR4OHT (magenta). *E* isomers of fixed ring 4OHT and endoxifen do not fit very well into the antagonist conformation (3ERT): (E) EFREndox (green) and EFR4OHT (purple). The best docking poses of the *Z*-isomers to the LBD of ER (agonist conformation, PDB code 3Q97) were (F) EFREndox (green) and EFR4OHT (purple).

Table 1. Docking Results for X-ray Structure 3ERT[a]

compd	GScore	H bond	vdW	Coul	Emodel	CvdW
ZFREndox	−14.22	−1.5	−48.6	−15.4	−92.6	−64
ZFR4OHT	−13.22	−1.5	−50	−13.4	−92.6	−63.4
EFREndox	−10.65	−1.6	0.6	−7.9	38.6	−7.3
EFR4OHT	−10.59	−1.9	1.3	−9.4	37.8	−8.1

[a]CvdW = Coul + vdW is the non-bonded interaction energy between the ligand and the receptor. Emodel is a specific combination of GScore. GlideScore (GScore in kcal/mol) is given by GScore = $a \times$ vdW + $b \times$ Coul + Lipo + Hbond + Metal + Rewards + RotB + Site, where vdW = van der Waals interaction energy, Coul = Coulomb interaction energy, Lipo = lipophilic contact plus phobic attractive term; Hbond = hydrogen-bonding term; Metal = metal-binding term (usually a reward); Rewards = various reward or penalty terms; RotB = penalty for freezing rotatable bonds; Site = polar interactions in the active site. The coefficients of vdW and Coul are a = 0.050, b = 0.150 for Glide 5.0 (the contribution from the Coulomb term is capped at −4 kcal/mol).

similar to that of 3Q97. Thus, in the agonist structure 1GWR the alkoxy substituent is involved in clashes with the side chains of Leu525 and Leu540 of helix12 while the fused rings system of the ZFREthoxy derivative is involved in clashes with Ile424 and Leu428 (Figure 8B). Thus, the best ranked docking poses of ZFRMethoxy and ZFREthoxy derivatives in the binding site of 3Q97 are free of these unfavorable contacts while a larger number of favorable interactions are formed with other hydrophobic amino acids of the binding site (Figure 8C). The binding site of the antagonist conformation, 3ERT, is larger and exposed to the solvent, and although the top ranked docking poses of *Z*-isomers form the H-bond network, the favorable hydrophobic contacts with Leu525 and Leu540 are missing (Figure 8A). As a result, it can be concluded that it is highly probable for *Z*-isomers to bind to a conformation of ER similar to the experimental structure 3Q97. Regarding the *E*-isomers, the binding mode most frequently predicted by the docking poses is similar for the antagonist conformation 3ERT (Figure 8D) and agonist conformation 1GWR (Figure 8E) with the methoxy and ethoxy substituents pointing toward the region of the binding pocket lined by amino acids Glu353 and Arg394. However, in this alignment clashes are encountered with these. Conversely, the top ranked docking poses at 3Q97 binding pocket show the alkoxy substituents oriented toward His524 in the opposite region of site and no H-bonds are formed (Figure 8F). Also, no clashes have been noticed with other amino acids of the binding site.

DISCUSSION AND CONCLUSIONS

The goal of this investigation is to link estrogenic/antiestrogenic ligand structures of tamoxifen metabolites with the well documented estradiol responses of cell replication or an estrogen target gene activation in cancer and apply biological end points to molecular modeling of the ER complex. This

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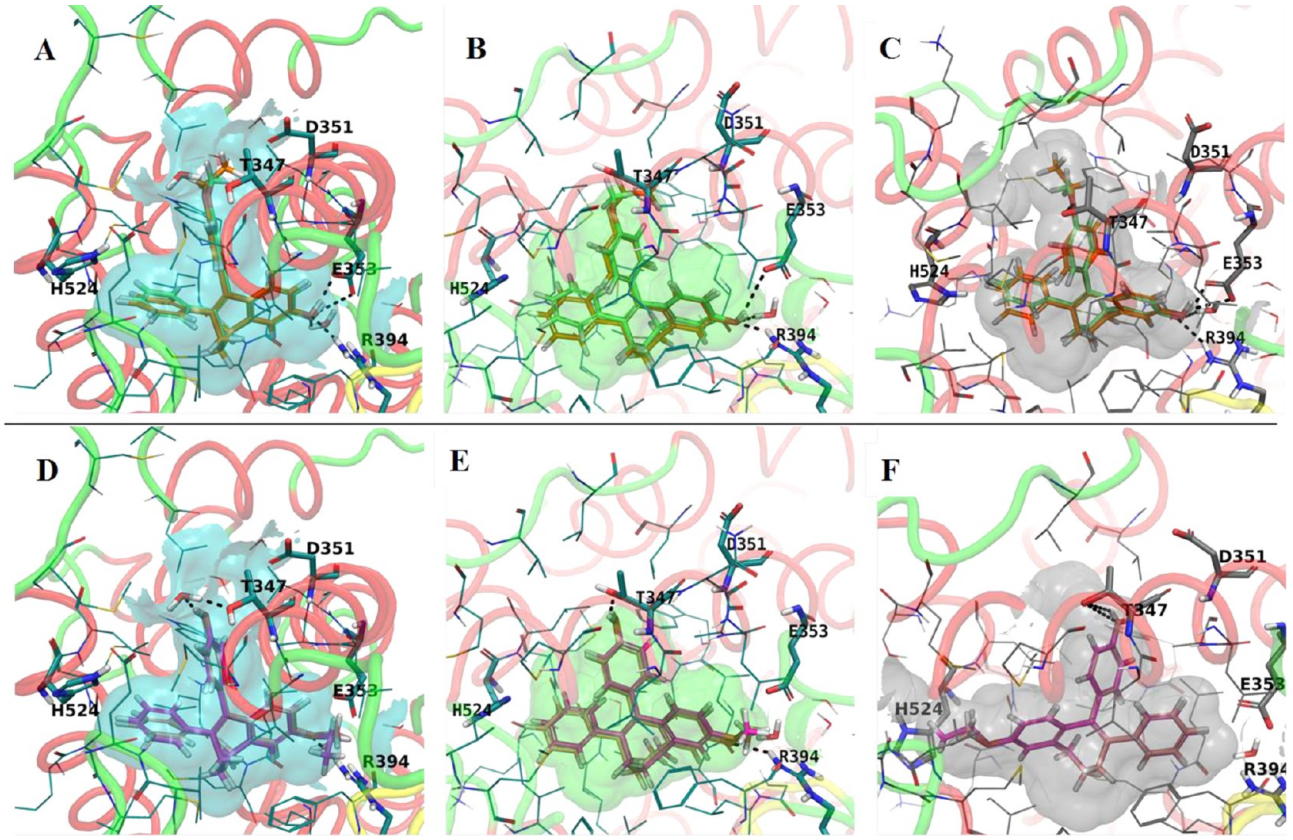

Figure 8. Representations of the experimental structures binding pockets used for modeling: (A) best docking poses of the *Z*-isomers to the LBD of ER (antagonist conformation, PDB code 3ERT) of ZFRMethoxy (green) and ZFREthoxy (orange); (B) agonist conformation of ER LBD cocrystallized with E_2 (PDB code 1GWR) of ZFRMethoxy (green) and ZFREthoxy (orange); (C) agonist conformation of ER LBD cocrystallized with a *E*-isomer of ethoxytriphenylethylene (PDB code 3Q97) of ZFRMethoxy (green) and ZFREthoxy (orange); (D) best docking poses of the *Z*-isomers to the LBD of ER (antagonist conformation, PDB code 3ERT) of EFRMethoxy (light pink) and EFREthoxy (magenta); (E) agonist conformation of ER LBD cocrystallized with E_2 (PDB code 1GWR) of EFRMethoxy (light pink) and EFREthoxy (magenta); (F) agonist conformation of ER LBD cocrystallized with a *E*-isomer of ethoxytriphenylethylene (PDB code 3Q97) of EFRMethoxy (light pink) and EFREthoxy (magenta).

Table 2. Docking Scores for X-ray Structure 3Q97 (Binding Site Cocrystallized with *Z*-Isomer of Ethoxytriphenylethylene)

compd	GScore	H bond	vdW	Coul	Emodel	CvdW	Intern
BPTPE	−11.96	−1.5	−44.7	−5.9	−85.4	−50.6	2.1
3OHTPE	−12.76	−2.2	−37	−14.7	−86.4	−51.7	2.9
ZFRMethoxy	−12.76	−1.5	−48.2	−4.8	−89.4	−53	3.6
ZFREthoxy	−12.35	−1.3	−39.5	−9.9	−90.7	−49.4	3
EFRMethoxy	−10.8	−1	−37.1	−2.4	−67.6	−39.5	0
EFREthoxy	−10.41	−0.7	−30.1	0.7	−44.2	−29.4	6.9

study has its origins with original published reports[16,17,34] of the synthesis and evaluation of *E* and *Z* isomers of FR4OHT. We now extend earlier work with this study of *E* and *Z* ER endoxifen, investigate new *Z* and *E* FRMethoxy and FREthoxy derivatives of triphenylethylene (TPE), and compare our results with the angular estrogens BPTPE and 3OHTPE.[18] The biological end points used were cell replication in MCF-7:WS8 cells and the estrogen-regulated prolactin gene (Prl) in rat pituitary gland tumor GH3 cell line.

There are several important new findings with the structure−function relationship of new FR compounds. The length and positioning of the side chain of the new *Z* and *E* FR compounds govern estrogen-induced cell replication of MCF-7:WS8 cells (Figure 2A). The natural estrogen E_2 is extremely active as a full agonist over the range 10^{-14}−10^{-8} M; however, each *Z* FR derivative is a partial agonist, so comparative EC_{50} calculations are not appropriate. Nevertheless, BPTPE is a potent partial agonist (50% max of E_2 curve) over the range 10^{-12}−10^{-9} M. The *Z* FRMethoxy and FREthoxy partial agonist curve is displaced a log to the right, and the EFRMethoxy and EFREthoxy is displaced further. The *E* FR isomers of 4OHT and endoxifen are both low potency estrogens, and this is consistent with their lower ligand-binding activity of the ER.[14] Only the nonsteroidal antiestrogens 4OHT and endoxifen and their ZFR derivatives were antiestrogenic on cell proliferation. By contrast, all compounds were antiestrogenic (Figure 4B) at 1 μM in the GH3 rat pituitary prolactin assay, i.e., down to the level of the partial agonist activity of each compound (Figure 4A). The inability of substituted angular estrogens to be unable to initiate prolactin gene synthesis fully but stimulate mouse vaginal cornification (which classifies them as estrogens) has been noted previously.[35−37]

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The partial gene regulation (pS2, GREB1, and PgR) is also noted with BPTPE and 3OHTPE as well as the *E* and *Z* FRMethoxy and FRMEthoxy TPEs. It is interesting to note that at 1 μM EFREndox is particularly active in triggering pS2, GREB1, and PgR (Figure 5), so the ability of the *E* isomers of FR4OHT and Endox were tested at therapeutic concentrations[26] to determine whether estrogen-induced cell replication could occur during therapy. None was noted (Figure 3).

Additionally, results from RT-PCR of the estrogen-responsive genes in MCF-7:WS8 cells show that the *E*-isomers are inducing higher expression of pS2, GREB1, and PgR genes mRNAs, and also Prl gene mRNA in rat GH3 cells. This contrasts with the *Z*-isomers. Considering all the results, it is possible to conclude that the *E*-isomers of the biologically active tamoxifen metabolites 4OHT and endoxifen have estrogenic properties in human breast cancer cells, but this is not of biological significance during therapy with tamoxifen.

The most important general observation was the sensitivity of all the different TPE structures to trigger cell replication (Figure 2A). This supersensitivity is clearly required for cancers to survive through relentless cell replication. Antiestrogenic activity blocking replication requires a correctly positioned alkylaminoethoxy side chain.[38] By contrast, estrogen-regulated protein synthesis is much less successful with test compounds and the resulting complex is clearly less promiscuous, tending to create a biologically inert "antiestrogenic complex" (Figure 4B).

It is interesting to note that the accumulation of ER determined by Western blotting for all compounds is independent of estrogenic or antiestrogenic activity. The turnover of ER complexes is regulated by ubiquitinilation and proteosomal degradation,[39] but it is clearly the shape of the ligand and the resulting conformation of the complex that determine accumulation or destruction. The shape of the ligand is critical; a planar class I (estradiol) ligand causes reduction of ER, whereas nonsteroidal antiestrogens such as 4OHT and endoxifen[40] cause the ER complex to accumulate. The same is true of angular TPEs[40] which are also all of the new FR compounds investigated here that bind to the ER. By contrast, fulvestrant (ICI 182,780) causes the rapid destruction of ER.[41] A previous study by Wu et al.[42] demonstrated that endoxifen also caused rapid destruction of ER, but this was not observed in this study. We used endoxifen obtained from the Mayo Clinic and the *Z* FR endoxifen, both of which had the same accumulation of the ER.

Molecular modeling demonstrates that most likely the positioning of the *E*-isomers in the ligand-binding cavity of the ER is different because of repositioned side chains, potentially reducing the affinity to the receptor. However, this structural change also alters the pharmacological properties of the *E*-isomers, as they are more estrogenic rather than antiestrogenic. The molecular modeling shows that the *E*-isomers fit better into the ER conformation when the receptor is bound to a structurally similar *E*-isomer of ethoxytriphenylethylene where X-ray crystallography (PDB entry 3Q97) shows that the H12 of the LBD is actually closed, which resembles the conformation induced by estrogens.[30] This is also confirmed by the Western blotting results for the ER protein levels, which show that the *Z*-isomers of FR4OHT and endoxifen, being antiestrogens, are inducing up-regulation of the ER protein levels; however, the *E*-isomers are not inducing the same up-regulation, indicating their different properties (Figure 6). However, that is not the case with fixed-ring compounds with shorter side chains. In contrast, *Z*-isomers of FR 4OHT and endoxifen fit better into the antagonist conformation of the ER LBD.[30] Compounds with shorter side chain fit better into the conformation of the ER LDB that accommodates their *E*-isomers, resulting in the H12 being closed. This results in estrogenic activity.

In summary, a well-defined series of compounds has been classified and characterized for cell growth and estrogen target protein synthesis. The important finding is that replication in the ER-positive breast cancer cell is extremely sensitive to stimulation by a broad range of synthetic estrogens. This supersensitivity to growth stimuli is the major survival mechanism of cancer. It is a simple principle based on growth to survive from any source through the ER signal transduction pathway. This promiscuous pathway is only stopped when the antiestrogenic side chain of antiestrogens interacts with Asp351 and Helix 12 is prevented from closing.[43] By contrast, the transcription of RNA for estrogen target genes such as prolactin is highly selective with these new compounds synthesized in this study. The compounds tend to become antiestrogenic (Figure 6) possibly because the conformation of the ER complex cannot recruit all necessary transcription factors. The conformation of the complex is critical. However, it is also important to appreciate that X-ray crystallography of complex 3Q97, which appears to be estrogen-like, only gives a glimpse at that one moment of time of low energy crystallization. We anticipate that progressive changes occur over time as the estrogen ER complex adapts to the changing environment within the cell. Biological end points are correlated with the receptor docking of a new intermediate form of the ER ligand-binding domain (PDB entry 3Q97). These data will be used in the future to decipher and to advance the understanding of the molecular mechanisms of estrogen-induced apoptosis.[44]

MATERIALS AND METHODS

Chemistry. The general schemes of synthesis are described in Scheme 1.

General Procedures. Unless stated otherwise, reactions were performed in heat-dried glassware under a positive pressure of nitrogen using solvents that were distilled from or stored over calcium hydride, $LiAlH_4$, or molecular sieves. Commercial grade reagents and solvents were used without further purification except as stated. Thin layer chromatography (TLC) was performed on precoated silica gel 60 F_{254} plates and visualized by UV light (254). Flash column chromatography was performed on hand packed silica gel (230−400 mesh 60A) columns using the dry loading method. Automated column chromatography purifications were done using a Teledyne ISCO apparatus (CombiFlash Rf) with prepacked silica gel columns (4−40 g). ^{1}H NMR was recorded on a Bruker Avance 300 MHz instrument. Chemical shifts were quoted in parts per million, and coupling constants were reported in hertz. ^{13}C NMR was recorded at 75 MHz. HPLC−MS analyses and purifications were performed on a Waters HPLC system consisting of a model 2545 binary gradient pump, 2424 ELS detector, 2487 dual UV detector (254 and 365 nm), and a model 3100 single quadrupole mass spectrometer detector with electrospray and chemical ionization. Deltapak-C18 15 μm 300A reverse phase columns were used for analyses (3.9 mm × 30 cm) and preparative (30 mm × 30 cm) separations. The mobile phase was either a mixture of MeOH/H_2O or CH_3CN (0.05% FA)/H_2O (0.05% FA) with a flow rate of 0.8 on the analytical side or 20 mL/min for preparative scale. Gradient system for analytical (15 m) and preparative (30 m) was a 5−95% linear gradient. For preparative runs, fractions were collected by hand using UV and MS detectors. High resolution MS results were obtained using an Acquity UPLC (ultraperformance liquid chromatography)−QTOF-MS (quadrupole time of flight mass spectrometry)

Premiere system (Waters Corporation, USA). All final compounds were tested with a purity of more than 95% as analyzed by LC/MS.

Synthesis. *Z*-Fixed Ring Ethoxy (ZFREthoxy). 2-Hydroxy-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-5-one (1). 2-Methoxyheptenone was demethylated according to the procedure of Lal et al. (adapted from Kahn et al.).[20] The product was extracted with chloroform, resulting in a quantitative yield of **1**. TLC (6% MeOH, 94% chloroform) R_f = 0.28. LC/MS t_R = 13.10, (M + H$^+$) 177. ^{1}H NMR ($CDCl_3$): δ = 1.79−190 (m, 4H); 2.74 (m, 2H); 2.90 (m, 2H); 6.68 (d, 1H, J = 2.4); 6.75 (dd, 1H, J = 2.4 and 8.4); 7.75 (d, 1H, J = 8.4).

2-(4-(2,3,5,6-Tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7,8,9-tetrahydro-5H-benzocyclohepten-5-one (2). Octafluorotoluene (807 mg, 484 μL, 3.42 mmol) and 2-hydroxy-6,7,8,9-tetrahydro-5*H* -benzocyclohepten-5-one (**1**) (587 mg, 3.33 mmol) were dissolved in dichloromethane (15 mL) and 1 N NaOH (15 mL). Then tetrabutylammonium hydrogen sulfate (572 mg) was added and the solution was stirred overnight at room temperature. The organic layer was separated, and the aqueous layer was extracted with dichloromethane (2 × 50 mL). The combined organic layers were washed with water and dried in vacuo. The product was purified from tetrabutylammonium hydrogen sulfate by flash column chromatography over silica (2.3 cm × 5 cm on 2.3 cm × 20 cm) and eluted with 400 mL of chloroform. Fractions (25 mL) 2−6 were combined to give **2** (923 mg, 71% yield). TLC (6% MeOH, 94% chloroform) R_f = 0.88. LC/MS t_R = 19.37, (M + H$^+$) 393. ^{1}H NMR ($CDCl_3$): δ = 1.81−195 (m, 4H); 2.74 (m, 2H); 2.94 (m, 2H); 6.83 (d, 1H, J = 2.4); 6.88 (dd, 1H, J = 2.4 and 8.4); 7.78 (d, 1H, J = 8.4).

4-Bromophenyl 2,3,5,6-Tetrafluoro-4-(trifluoromethyl)-phenyl Ether (4). Octafluorotoluene (30 g, 0.127 mol) and 4-bromophenol (21 g, 0.121 mol) were dissolved in dichloromethane (100 mL) and 1 N NaOH (100 mL). Then tetrabutylammonium hydrogen sulfate (10 g) was added and the solution was stirred at room temperature for 4 h. The dichloromethane layer was separated and the aqueous layer extracted with dichloromethane (2 × 50 mL). The combined organic layers were dried under reduced pressure. The residue was triturated with hexanes, and the insoluble tetrabutylammonium sulfate was filtered. The filtrate was evaporated under reduced pressure to give **4** (46.41 g, 98% yield). TLC (5% dichloromethane, 95% hexanes) R_f = 0.63. ^{1}H NMR ($CDCl_3$): δ = 6.87 (d, 2H, J = 8.7); 7.44 (d, 2H, J = 8.7).

3-(2,3,5,6-Tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7-dihydro-9-(4-ethoxyphenyl)-5*H*-benzocycloheptene (5). Bromophenetole (507 μL, 710 mg, 3.53 mmol) was dissolved in ether (20 mL) with stirring. Then magnesium turnings (125 mg) were added, followed by dropwise addition of 1,2-dibromoethane (142 μL) in diethyl ether (10 mL) over 1 h. Once the Grignard reagent had formed, 2-(4-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7,8,9-tetrahydro-5*H* -benzocyclohepten-5-one (**2**) (923 mg in 10 mL ether) was added, and the mixture was stirred overnight at room temperature. The next day, it was poured into 0.1 N hydrochloric acid solution (30 mL) and extracted with ether (3 × 50 mL). The ether was removed by evaporation under reduced pressure, and the residue was dissolved in ethanol (20 mL). Concentrated hydrochloric acid (5 mL) was added, and the solution was refluxed for 2 h. It was cooled, poured into water (50 mL), and extracted with ether (3 × 50 mL). The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (3.0 cm × 3.0 cm on 3.0 cm × 36.0 cm) over silica. The product was eluted with 250 mL of 100% hexanes, followed by 1 L of 10% dichloromethane, 95% hexanes. Fractions (25 mL) 37−70 contained the product and were combined and evaporated in vacuo to give **5** (608 mg, 52% yield). TLC (20% dichloromethane, 80% hexanes) R_f = 0.29. LC/MS t_R = 22.45, (M + H$^+$) 497. ^{1}H NMR ($CDCl_3$): δ = 1.41 (t, 3H, J = 6.9); 1.96 (m, 2H, J = 7.2); 2.16 (t, 2H, J = 7.2); 2.62 (t, 2H, J = 6.9); 4.03 (q, 2H, J = 6.9); 6.36 (t, 1H, J = 7.3); 6.75−7.24 (m, 7H).

3-(2,3,5,6-Tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7-dihydro-8-bromo-9-(4-ethoxyphenyl)-5*H*-benzocycloheptene (9). 3-(2,3,5,6-Tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7-dihydro-9-(4-ethoxyphenyl)-5*H*-benzocycloheptene (**5**) (608 mg, 1.224 mmol) and pyridine hydrobromide perbromide (428 mg) were stirred in dichoromethane (15 mL) at room temperature for 4 h. The orange solution was washed with 0.1 M HCl solution (25 mL) which contained sodium sulfite (20 mg), followed by water. Next the solvent was evaporated under reduced pressure to **9** (689 mg, 98%). TLC (10% toluene, 90% hexanes) R_f = 0.31. LC/MS t_R = 22.39, (M + H$^+$) 575. ^{1}H NMR ($CDCl_3$): δ = 1.42 (t, 3H, J = 6.9); 2.31 (m, 2H, J = 6.9); 2.58 (t, 2H, J = 6.9); 2.76 (t, 2H, J = 6.9); 4.05 (q, 2H, J = 6.9); 6.71−6.86 (m, 5H); 7.13 (d, 2H, J = 7.8).

3-(2,3,5,6-Tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7-dihydro-8-phenyl-9-(4-ethoxyphenyl)-5*H*-benzocycloheptene (13). Anhydrous zinc chloride (433 mg) was dissolved in THF (15 mL) with stirring. Phenyllithium in di-*n*-butyl ether (1.8 mL of 1.8 M solution) and THF (10 mL) were added dropwise over 15 min to the zinc chloride solution while it was cooled in an ice bath. After the solution was allowed to warm to room temperature, 3-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7-dihydro-8-bromo-9-(4-ethoxyphenyl)-5*H*-benzocycloheptene (**9**) (584 mg, 1.015 mmol) in THF (10 mL) was added dropwise followed by $Pd(PPh_3)_4$ (10 mg). The mixture was refluxed for 6 h and left to stir at room temperature overnight. The reaction mixture was poured into water (50 mL) and extracted with diethyl ether (3 × 50 mL). The combined ether extracts were dried in vacuo. Purification was performed with flash column chromatography over silica (2.3 cm × 4 cm on 2.3 cm × 20 cm). The column was equilibrated with 200 mL of 100% hexanes, and the product was eluted with 750 mL of 5% toluene, 95% hexanes. Fractions (25 mL) 23−41 were combined and evaporated in vacuo to give white solid **13** (372 mg, 64% yield). TLC (20% toluene, 80% hexanes) R_f = 0.33. LC/MS t_R = 23.52, (M + H$^+$) 573. ^{1}H NMR ($CDCl_3$): δ = 1.28 (t, 3H, J = 6.9); 2.12 (m, 2H); 2.30 (t, 2H, J = 6.9); 2.71 (t, 2H, J = 6.9); 3.85 (q, 2H, J = 6.9); 6.51−6.57 (m, 2H); 6.66−6.82 (m, 5H); 7.08 (m, 5H).

3-Ethoxy-6,7-dihydro-8-phenyl-9-(4-hydroxyphenyl)-5*H*-benzocycloheptene (17). 3-(2,3,5,6-Tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7-dihydro-8-phenyl-9-(4-ethoxyphenyl)-5*H*-benzocycloheptene (**13**) (372 mg, 0.640 mmol) and sodium ethoxide (400 mg) in DMF (5 mL) were heated to 40 °C for 2 h with stirring. The brown solution was poured into saturated sodium bicarbonate solution (50 mL) and extracted with ether (3 × 50 mL). The combined ether layers were evaporated in vacuo. Purification was performed with silica column chromatography (2.3 cm × 3 cm on 2.3 cm × 21 cm) equilibrated with 200 mL of 100% hexanes. The product was eluted with 1.75 L of 50% dichloromethane, 50% hexanes. Fractions (25 mL) 34−68 were combined and evaporated in vacuo to give white solid **17** (203 mg, 89% yield). It was recrystallized in dichloromethane/hexanes (171 mg, mp 242−243 °C). TLC (50% dichloromethane, 50% hexanes) R_f = 0.13. LC/MS t_R = 17.40, (M + H$^+$) 357. ^{1}H NMR ($CDCl_3$): δ = 1.38 (t, 3H, J = 6.9); 2.16 (m, 2H, J = 7.2); 2.39 (t, 2H, J = 7.2); 2.76 (t, 2H, J = 7.2); 3.94 (q, 2H, J = 6.9); 6.59−6.63 (m, 3H); 6.74−6.82 (m, 4H); 7.15 (m, 5H). HRMS calculated for $C_{25}H_{24}O_2$ (M + H)$^+$ 357.1855; found 357.1859.

***Z*-Fixed Ring Methoxy (ZFRMethoxy). 3-(2,3,5,6-Tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7-dihydro-9-(4-methoxyphenyl)-5*H*-benzocycloheptene(6).** 2-(4-(2,3,5,6-Tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-5-one (**2**) (6.198 g, 15.8 mmol) was dissolved in ether (20 mL) with stirring. Then 4-methoxyphenylmagnesium bromide (0.5 M solution in THF, 47.5 mL, 23.7 mmol) was added dropwise at room temperature, and it was stirred overnight. The next day, the orange solution was heated to reflux for 12 h. Then it was poured into 0.1 N hydrochloric acid solution (100 mL) and extracted with ether (3 × 100 mL). The ether was removed by evaporation under reduced pressure, and the residue was dissolved in ethanol (200 mL). Concentrated hydrochloric acid (5 mL) was added, and the solution was refluxed for 2 h. The solution turned from orange to green, forming a sticky tan precipitate. It was cooled and poured into water (200 mL). The product was extracted with ether (3 × 100 mL), and the combined ether layers were evaporated under reduced pressure (9.57 g). It was purified by column chromatography over silica on the CombiFlash Rf instrument. Compounds were eluted with ethyl

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acetate/hexanes gradient on a 40 g gold silica column. The sample was injected onto the column using a dry method with 50 g of silica. Fractions (25 mL) 3−10 were combined and evaporated in vacuo to give **6** (3.60 g, 47% yield). TLC (10% ethyl acetate, 90% hexanes) R_f = 0.69. LC/MS t_R = 19.27, (M + H$^+$) 483. ^{1}H NMR ($CDCl_3$): δ = 1.97 (m, 2H, J = 7.5); 2.17 (t, 2H, J = 7.2); 2.63 (t, 2H, J = 7.2); 3.81 (s, 3H); 6.37 (t, 1H, J = 7.5); 6.78−7.02 (m, 5H); 7.05−7.25 (m, 2H).

3-(2,3,5,6-Tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7-dihydro-8-bromo-9-(4-methoxyphenyl)-5*H*-benzocycloheptene (10). 3-(2,3,5,6-Tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7-dihydro-9-(4-methoxyphenyl)-5*H*-benzocycloheptene (**6**) (3.6 g, 8.4 mmol) and pyridine hydrobromide perbromide (2.94 g) were stirred in dichorormethane (50 mL) at room temperature for 20 h. The orange solution was washed with 0.1 M HCl solution (50 mL) which contained sodium sulfite (200 mg), followed by water. It was evaporated under reduced pressure and purified by column chromatography over silica on the CombiFlash Rf instrument. Compounds were eluted with ethyl acetate/hexanes gradient on a 40 g gold silica column. The sample was injected onto the column using a dry method with 25 g of silica. Fractions (25 mL) 5−16 were combined and evaporated in vacuo to give **10** (3.374g, 72% yield). TLC (10% ethyl acetate, 90% hexanes) R_f = 0.64. LC/MS t_R = 19.47, (M + H$^+$) 561. ^{1}H NMR ($CDCl_3$): δ = 2.30 (m, 2H, J = 7.2); 2.60 (t, 2H, J = 7.2); 2.74 (t, 2H, J = 7.2); 3.81 (s, 3H); 6.69−6.96 (m, 5H); 7.16 (d, 2H, J = 8.7).

3-(2,3,5,6-Tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7-dihydro-8-phenyl-9-(4-ethoxyphenyl)-5*H*-benzocycloheptene (14). Anhydrous zinc chloride (2.4 mg) was dissolved in THF (50 mL) with stirring. Then 1.8 M solution of phenyllithium in di-*n*-butyl ether (9.8 mL) in THF (10 mL) was added dropwise over 30 min to the zinc chloride solution while it was cooled in an ice bath below 0 °C. After allowing the mixture to warm to room temperature, 3-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7-dihydro-8-bromo-9-(4-methoxyphenyl)-5*H*-benzocycloheptene (**10**) (3.374 g, 6.01 mmol) in THF (10 mL) was added dropwise followed by $Pd(PPh_3)_4$ (57 mg). The reaction was refluxed for 3 h and then left to stir overnight at room temperature. The reaction mixture was poured into water (50 mL) and extracted with diethyl ether (3 × 50 mL). The combined ether extracts were evaporated under reduced pressure. The residue was purified by column chromatography over silica on the CombiFlash Rf instrument. Compounds were eluted with ethyl acetate/hexanes gradient on the 40 g gold silica column. Flow rate was 25 mL/min. The sample was injected onto the column using a dry method with 30 g of silica. Fractions (25 mL) 9−26 were combined and evaporated under reduced pressure to give **14** (2.90 g, 86% yield). TLC (5% EtOAc, 95% hexanes) R_f = 0.40. LC/MS t_R = 19.95, (M + H$^+$) 559. ^{1}H NMR ($CDCl_3$): δ = 2.21 (m, 2H, J = 7.2); 2.41 (t, 2H, J = 7.2); 2.81 (t, 2H, J = 7.2); 3.76 (s, 3H); 6.63−6.94 (m, 7H); 7.13−7.21 (m, 5H).

3-Methoxy-6,7-dihydro-8-phenyl-9-(4-hydroxyphenyl)-5*H*-benzocycloheptene (18). 3-(2,3,5,6-Tetrafluoro-4-(trifluoromethyl)phenoxy)-6,7-dihydro-8-phenyl-9-(4-ethoxyphenyl)-5*H*-benzocycloheptene (**14**) (2.90 g, 5.19 mmol) and sodium methoxide (3.6 g) in DMF (25 mL) were heated to 35 °C for 3 h. The orange solution was poured into saturated sodium bicarbonate solution (100 mL) and extracted with ether (3 × 100 mL). The combined ether layers were evaporated in vacuo, and the residue was purified by column chromatography over silica on the CombiFlash Rf instrument. Compounds were eluted with ethyl acetate/hexanes gradient of 0−100% ethyl acetate over 35 min on a 40 g gold silica column. Flow rate was 25 mL/min. The sample was injected onto the column using a dry method with 20 g of silica. Fractions (25 mL) 8−10 were combined and evaporated in vacuo to give **18** (300 mg, 17% yield). TLC (5% EtOAc, 95% hexanes) R_f = 0.30. LC/MS t_R = 15.67, (M + H$^+$) 343. ^{1}H NMR ($CDCl_3$): δ = 2.18 (m, 2H, J = 7.2); 2.41 (t, 2H, J = 7.2); 2.78 (t, 2H, J = 7.2); 3.75 (s, 2H); 6.62−6.89 (m, 7H); 7.15 (m, 5H). HRMS calculated for $C_{24}H_{22}O_2$ (M + H)$^+$ 343.1698; found 343.1700.

***E*-Fixed Ring Ethoxy (EFREthoxy). 2-Ethoxy-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-5-one (7-Ethoxy-1-benzosuberone) (3).** 2-Hydroxy-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-5-one (**1**) (2.171 g, 12.32 mmol) and anhydrous potassium carbonate (5.102 g, 15.21 mmol) were dissolved in acetone (50 mL). Then iodoethane (5.43 mL) was added and it was stirred at room temperature overnight. The reaction mixture was evaporated in vacuo and purified by flash chromatography (2.3 cm × 4 cm on 2.3 cm × 23 cm) over silica. The column was equilibrated with hexanes (200 mL), and the product was eluted in chloroform. Fractions (25 mL) containing product were combined and evaporated in vacuo to **3** (1.72 g, 68% yield). LC/MS t_R = 13.35, (M + H$^+$) 205. ^{1}H NMR ($CDCl_3$): δ = 1.32 (t, 3H, J = 7.2); 1.81−195 (m, 4H); 2.74 (m, 2H); 2.92 (m, 2H); 6.87 (m, 2H); 3.93 (q, 2H, J = 6.9); 7.77 (d, 1H, J = 8.4); 7.75 (d, 1H, J = 8.4).

3-Ethoxy-6,7-dihydro-9-(4-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)phenyl)-5*H*-benzocycloheptene (7). 4-Bromophenyl 2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenyl ether (6.244 g, 16.05 mmol) was dissolved in ether (50 mL) with stirring. Then magnesium turnings (500 mg) were added followed by dropwise addition of 1,2-dibromoethane (0.564 mL) in ether (5 mL) over 30 min. After the Grignard reagent formed, the mixture was heated for 1 h. Next, 2-ethoxy-6,7,8,9-tetrahydro-5*H* -benzocyclohepten-5-one (**3**) (1.72 g, 8.42 mmol) in ether (30 mL) was added and the solution was stirred overnight at room temperature. The next day, it was heated for 10 h. LC/MS analysis confirmed the formation of the intermediate tertiary alcohol. The reaction mixture was poured into 0.1 N hydrochloric acid solution (50 mL) and extracted with ether (3 × 50 mL). The ether was removed by evaporation under reduced pressure, and the residue was dissolved in ethanol (50 mL). Concentrated hydrochloric acid (10 mL) was added, and the solution was refluxed for 2 h. After cooling, it was poured into water (100 mL), extracted with ether (3 × 50 mL), and evaporated under reduced pressure. The product was purified by silica column chromatography (4.0 cm × 1.0 cm on 4.0 cm × 23.0 cm). The column was equilibrated with 500 mL of 100% hexanes, and the product was eluted with 2 L of 5% dichloromethane, 95% hexanes. Fractions (250 mL) 10−31 contained the product and were combined and evaporated in vacuo to give **7** (1.802 g, 43% yield). LC/MS t_R = 19.67, (M + H$^+$) 497. ^{1}H NMR ($CDCl_3$): δ = 1.31 (t, 3H, J = 7.2); 1.84 (m, 2H); 2.05 (t, 2H; J = 7.2); 2.51 (t, 2H; J = 6.9); 3.92 (q, 2H, J = 6.9); 6.21 (t, 1H, J = 8.4); 6.71−6.81 (m, 5H); 7.13 (d, 2H, J = 8.4).

3-Ethoxy-6,7-dihydro-8-bromo-9-(4-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)phenyl)-5*H*-benzocycloheptene (11). 3-Ethoxy-6,7-dihydro-9-(4-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)phenyl)-5*H*-benzocycloheptene (**7**) (1.802 g, 3.63 mmol) was dissolved in dichoromethane (20 mL) with stirring. Then pyridine hydrobromide perbromide (1.27 g) was added and the solution was stirred at room temperature for 4 h. The orange solution was washed with 0.1 M HCl solution (25 mL) containing sodium sulfite (0.1 g), followed by water. It was dried by evaporation under reduced pressure to give **11** (1.798 g, 86% yield). LC/MS t_R = 19.77, (M + H$^+$) 575. ^{1}H NMR ($CDCl_3$): δ = 1.38 (t, 3H, J = 7.2); 2.28 (m, 2H); 2.58 (t, 2H; J = 6.9); 2.73 (t, 2H; J = 6.9); 3.99 (q, 2H, J = 7.2); 6.61−6.79 (m, 3H); 6.94 (d, 2H, J = 8.4); 7.24 (d, 2H, J = 8.4).

3-Ethoxy-6,7-dihydro-8-phenyl-9-(4-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)phenyl)-5*H*-benzocycloheptene (15). Anydrous zinc chloride (1.28 g) was added dropwise over 15 min to the zinc chloride solution while it was cooled in an ice bath. After it was allowed to warm to room temperature, 3-ethoxy-6,7-dihydro-8-bromo-9-(4-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)phenyl)-5*H*-benzocycloheptene (**11**) (1.798 g, 3.15 mmol) in THF (10 mL) was added dropwise followed by $Pd(PPh_3)_4$ (50 mg). It was heated to reflux for 3 h. The orange solution was poured into water (40 mL) and extracted with diethyl ether (3 × 40 mL). The combined ether extracts were dried over sodium sulfate, filtered, and evaporated under reduced pressure. The product was purified on a silica gel column (3 cm × 3 cm on 3 cm × 30 cm) that was eluted with 250 mL of hexanes, 500 mL of 5% dichloromethane, 95% hexanes, and 1.5 L of 10% dichloromethane 90% hexanes. Fractions (25 mL) 41−78 were combined and evaporated in vacuo to give **15** (1.523 g). TLC (30% dichloromethane, 70% hexanes) R_f = 0.47. LC/MS t_R = 20.35, (M + H$^+$) 573. ^{1}H NMR ($CDCl_3$): δ = 1.42 (t, 3H, J = 7.2); 2.19 (m, 2H);

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2.40 (t, 2H; J = 6.9); 2.77 (t, 2H; J = 6.9); 4.05 (q, 2H, J = 7.2); 6.58–6.96 (m, 7H); 7.12–7.19 (m, 5H).

3-Ethoxy-6,7-dihydro-8-phenyl-9-(4-hydroxyphenyl)-5*H*-benzocycloheptene (19). 3-Ethoxy-6,7-dihydro-8-phenyl-9-(4-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)phenyl)-5*H*-benzocycloheptene (**15**) (414 mg, 1.01 mmol) and sodium methoxide (1.6 g) were dissolved in DMF (25 mL) with stirring. The mixture was stirred at room temperature for 4 h. The solution was poured into saturated sodium bicarbonate solution (50 mL), extracted with ether (3 × 40 mL), and dried in vacuo. Purification was performed using silica gel column chromatography (3.0 cm × 3 cm on 3.0 cm × 25 cm), equilibrating with 200 mL of 100% hexanes. The product was eluted with 500 mL of 25% dichloromethane, 75% hexanes; 500 mL of 50% dichloromethane, 50% hexanes; and 1 L of 75% dichloromethane, 25% hexanes. Fractions (25 mL) 16–22 were combined and evaporated in vacuo to give white solid **19** (240 mg, 67% yield). TLC (75% dichloromethane, 25% hexanes) R_f = 0.17. LC/MS t_R = 17.40, $(M + H^+)$ 357. ^{1}H NMR ($CDCl_3$): δ = 1.43 (t, 3H, J = 6.9); 2.18 (m, 2H); 2.38 (t, 2H, J = 7.2); 2.77 (t, 2H, J = 7.2); 4.05 (q, 2H, J = 6.9); 6.52 (d, 2H, J = 8.7); 6.72–6.81 (m, 5H); 7.01–7.17 (m, 5H). ^{13}C NMR ($CDCl_3$): δ = 14.8; 32.4; 33.6; 34.2; 63.3; 111.6; 114.2; 114.6; 125.8; 127.7; 129.4; 130.2; 132.6; 134.8; 135.6; 137.8; 138.2; 142.9; 143.7; 154.2; 157.5. HRMS calculated for $C_{25}H_{24}O_2$ $(M + H)^+$ 357.1855; found 357.1859.

***E*-Fixed Ring Endoxifen (EFREndox). 6,7-Dihydro-8-phenyl-9-(4-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)phenyl)-5*H*-benzocycloheptene-3-ol (21).** 3-Methoxy-6,7-dihydro-8-phenyl-9-(4-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)phenyl)-5*H*-benzocycloheptene (**16**) (929 mg, 1.663 mmol) was suspended in 33% HBr in acetic acid solution (20 mL) in a flask fitted with a condenser and drying tube. It was refluxed for 6 h and analyzed by LC/MS which determined the reaction was incomplete. Additional 48% HBr in water (4 mL) and AcOH (4 mL) were added, and the mixture was refluxed for an additional 4 h. The orange reaction mixture was poured into water (100 mL), and 1 N sodium hydroxide was added until the solution was basic to pH paper. Then saturated sodium bicarbonate (50 mL) was added, and the product was extracted with ether (2 × 100 mL). The combined ether layers were washed with water, dried over sodium sulfate, and evaporated under reduced pressure to give **21** (905 mg, 100% yield). LC/MS (MeOH) t_R = 21.00, $(M - H^+)$ 543. ^{1}H NMR ($CDCl_3$): δ = 2.18 (m, 2H); 2.41 (t, 2H, J = 6.9); 2.77 (t, 2H, J = 6.9); 6.61–6.97 (m, 7H); 7.15 (m, 5H).

Ethyl Methyl-(2-((8-phenyl-9-(4-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)phenyl)-6,7-dihydro-5*H*-benzocyclohepten-3-yl)oxy)ethyl)carbamate (25). 6,7-Dihydro-8-phenyl-9-(4-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)phenyl)-5*H*-benzocyclohepten-3-ol (21) (259 mg, 0.476 mmol), ethyl (2-bromoethyl)-(methyl)carbamate (600 μL), 1.5 N sodium hydroxide (2 mL), dichloromethane (2 mL), and tetrabutylammonium hydrogen sulfate (600 mg) were stirred at room temperature for 16 h. The aqueous layer was extracted with chloroform (3 × 50 mL), and the combined organic layers were dried in vacuo. The residue was purified by preparative HPLC using a CH_3CN/H_2O gradient. The sample was injected in THF (2 mL). Fractions 36–40 min were collected and dried in vacuo to give 25 (35 mg, 11% yield). LC/MS (CH_3CN) t_R = 20.25, $(M + H^+)$ 674. ^{1}H NMR ($CDCl_3$): δ = 1.22 (m, 3H); 2.18 (m, 2H); 2.38 (m, 2H); 2.77 (m, 2H); 3.04 (s, 3H); 3.63 (t, 2H, J = 4.8); 4.11 (m, 4H); 6.68–6.89 (m, 7H); 7.13 (m, 5H).

Ethyl (2-((9-(4-Hydroxyphenyl)-8-phenyl-6,7-dihydro-5*H*-benzocycloehepten-3-yl)oxy)ethyl)(methyl)carbamate (26). Ethyl methyl(2-((8-phenyl-9-(4-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenoxy)phenyl)-6,7-dihydro-5*H* -benzocyclohept-en-3-yl)oxy)ethyl)carbamate (**25**) (120 mg, 178 mmol) and sodium methoxide (200 mg) in DMF (10 mL) were heated to 80 °C for 6 h with stirring. After cooling, the reaction mixture was evaporated under reduced pressure. It was purified by flash column chromatography over silica (4 g gold silica column) on the CombiFlash Rf instrument. The gradient was 0–50% ethyl acetate in hexanes over 30 min. Flow rate was 10 mL/min. The sample was injected onto the column using the solid loading option (10 g of silica). Product was collected in fractions (25 mL) 3–6 and dried in vacuo to give **26** (61 mg, 75% yield). LC/MS (CH_3CN) t_R = 16.32, $(M + H^+)$ 458. ^{1}H NMR ($CDCl_3$): δ = 1.24 (m, 3H); 2.20 (m, 2H); 2.36 (m, 2H); 2.77 (m, 2H); 3.06 (s, 3H); 3.67 (m, 2H); 3.89 (m, 2H); 4.15 (m, 2H); 6.56–7.26 (m, 12H).

4-(3-(2-(Methylamino)ethoxy)-8-phenyl-6,7-dihydro-5*H*-benzocyclohepten-9-yl)phenol (27). Ethyl (2-((9-(4-hydroxyphenyl)-8-phenyl-6,7-dihydro-5*H*-benzocyclohepten-3-yl)oxy)ethyl)-(methyl)carbamate (**26**) (61 mg, 0.133 mmol) and pyridine HCl (200 mg) were heated to 150 °C in an oil bath with stirring for 3 h. The black residue was purified by preparative HPLC with a CH_3CN/H_2O gradient. Sample was injected in 2 mL of MeOH. Fraction at 13–20 min was collected and dried in vacuo to give **25** (21 mg, 41% yield). LC/MS (CH_3CN) t_R = 12.82, $(M + H^+)$ 386. ^{1}H NMR (MeOD): δ = 2.11 (m, 2H); 2.33 (t, 2H, J = 6.9); 2.78 (m, 2H); 2.81 (s, 3H); 3.40 (m, 2H); 4.27 (m, 2H); 6.43–6.95 (m, 7H); 7.23 (m, 5H). HRMS calculated for $C_{26}H_{27}NO_2$ $(M + H)^+$ 386.2120; found 386.2122.

***Z*-Fixed Ring Endoxifen (ZFREndox). Ethyl (2-(4-(3-Methoxy-8-phenyl-6,7-dihydro-5*H*-benzocyclohepten-9-yl)phenoxy)-ethyl)(methyl)carbamate (30).** 3-Methoxy-6,7-dihydro-8-phenyl-9-hydroxyphenol-5*H*-benzocycloheptene (**20**) (208 mg, 0.607 mmol), 2-hydroxyethylmethylcarbamate (114 mg, 0.774 mmol) and triphenylphosphine (164 mg, 1.544 mmol) were stirred in tetrahydrofuran (20 mL). The reaction vial was cooled to below 0 °C, and diisopropyl azodicarboxylate (500 μL) was added dropwise over 5 min. The reaction mixture was stirred at room temperature for 3 days. The solution changed from yellow to orange during this time and was dried in vacuo. The compound was purified by preparative HPLC with a gradient of 5–75% in 30 min, 75% hold until 45 min, 75%–100% at 60 min in $MeOH/H_2O$ system. The sample was injected in 3 mL of MeOH. Fraction at 58–64 min was collected and evaporated in vacuo to give **30** (111 mg, 39% yield). LC/MS (CH_3CN) t_R = 19.12, $(M + H^+)$ 472. ^{1}H NMR ($CDCl_3$): δ = 1.26 (m, 3H); 2.14 (m, 4H); 2.38 (t, 2H, J = 6.9); 2.78 (t, 2H, 6.9); 2.99 (s, 3H); 3.58 (t, 2H); 3.82 (s, 3H); 4.11 (q, 2H, J = 7.2); 6.57–6.83 (m, 7H); 7.14 (m, 5H).

9-(4-(2-(Methylamino)ethoxy)phenyl)-8-phenyl-6,7-dihydro-5*H*-benzocyclohepten-3-ol (31). Ethyl (2-(4-(3-methoxy-8-phenyl-6,7-dihydro-5*H*-benzocyclohepten-9-yl)phenoxy)ethyl)(methyl)-carbamate (**30**) (156 mg, 0.331 mmol) and pyridine hydrochloride (600 mg) were heated in an oil bath to 180 °C with stirring for 3 h. The black solid was dissolved in methanol and purified by preparative HPLC using a CH_3CN/H_2O gradient. Sample was injected in 2 mL of MeOH. Fraction at 15–21 min was collected and dried in vacuo (61 mg). This was purified further by flash column chromatography over silica (2.3 cm × 1.0 cm on 2.3 cm × 23 cm). It was equilibrated with 200 mL of dichloromethane and eluted with 400 mL of 10% MeOH, 90% CH_2Cl_2, followed by 600 mL of 15% MeOH, 85% CH_2Cl_2. Fractions (25 mL) 15–36 were combined and evaporated in vacuo to give **31** (52 mg, 41% yield). TLC (15% MeOH, 85% dichloromethane) R_f = 0.31. LC/MS (CH_3CN) t_R = 15.42, $(M + H^+)$ 386. ^{1}H NMR (MeOH): δ = 2.12 (m, 2H); 2.35 (t, 2H, J = 6.9); 2.61 (t, 2H, 6.9); 2.74 (s, 3H); 3.18 (t, 2H, J = 5.1); 4.09 (t, 2H, J = 5.1); 6.56–6.83 (m, 7H); 7.11 (m, 5H). HRMS calculated for $C_{26}H_{27}NO_2$ $(M + H)^+$ 386.2120; found 386.2114.

Cell Culture. The ER positive MCF-7:WS8 and GH3 cell lines were used in this study. The human ER positive breast cancer cells MCF-7:WS8 are hypersensitive to estrogens and were cloned from wild type MCF-7 cells and were maintained in phenol-red RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin at 100 U/mL, streptomycin at 100 μg/mL, 1× nonessential amino acids (all from Life Technologies, Carlsbad, CA), and bovine insulin at 6 ng/mL (Sigma-Aldrich, St. Louis, MO). Rat pituitary GH3 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin at 100 U/mL, streptomycin at 100 μg/mL, 1× nonessential amino acids, and bovine insulin at 6 ng/mL. All cells were cultured in T185 flasks (Nalge Nunc International, Rochester, NY) and passaged twice a week. All cell lines were grown in 5% CO_2 at 37 °C.

Pharmacological Evaluation. All the biological properties of the synthesized compounds were tested by assessing the cell proliferation

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of the ER positive MCF-7:WS8 cells. Before the start of the experiment cells were estrogen starved by splitting them into RPMI 1640 medium without phenol red, and containing 10% charcoal stripped fetal serum (estrogen free), for 3 days. Cells were seeded into 24-well plates at a density of 10 000 cells per well. Next day after seeding (day 1) cells were treated with serial dilutions of the tested drugs in estrogen-free medium. The medium was changed every 2 days for a total of 7 days. All concentration points were performed in triplicate. On the last day the cells were harvested by medium aspiration and washed in cold PBS (Life Technologies, Carlsbad, CA) once and analyzed with fluorescent DNA quantification kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions, and samples were read in a Mirthas LB540 fluorimiter/luminometer (Berthold Technologies, Oak Ridge, TN) in black wall 96-well plates (Nalge Nunc International, Rochester, NY).

Real-Time PCR. Real-time polymerase chain reaction (RT-PCR) was performed on all cells after a 3-day starvation in estrogen free medium. Cells were seeded the day prior to treatment in six-well plates at a density of 300 000 cells per well. Cells were treated with all treatments for 48 h, after which they were harvested in Trizol reagent (Invitrogen, Carlsbad, CA) and then frozen at −80 °C. RNA was isolated using RNeasy mini kit (Quiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was synthesized using high capacity cDNA reverse transcription kit (Applied Bioscience, Carlsbad, CA) according to the manufacturer's instructions and using 1 μg of purified RNA. Synthesized cDNA was diluted in nuclease-free water and used for RT-PCR. For RT-PCR a Power SYBR green PCR master mix was used (Applied Bioscience, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR was run using a 7900HT fast real time PCR system thermocycler (Applied Bioscience, Carlsbad, CA). Primers sequences that were used for human pS2 cDNA amplification are 5′-CATCGACGTCCCTCCAGAAGA-3′ sense and 5′-CTCTGGGACTAATCACCGTGCTG-3′ anti-sense; human progesterone receptor (PgR), 5′-CGTGCCTATCCTGCCTCTCAA-3′ sense and 5′-CCGCCGTCGTAACTTTCGT-3′ anti-sense; human GREB1 gene, 5′-CAAAGAATAACCTGTTGGCCCTGC-3′ sense and 5′-GACATGCCTGCGCTCTCATACTTA-3′ anti-sense; the reference gene 36B4, 5′-GTGTCCGACAATGGCAGCAT-3′ sense and 5′-GACACCCTCCAGGAAGCGA-3′ anti-sense. All primers were obtained from Integrated DNA Technologies Inc. (IDT, Coralville, IA) and were tested by plotting dissociation curves which gave single peaks for all primer pairs. The fold changes of the mRNA after treatments to vehicle controls were calculated using ΔΔCt method and then normalized, including standard deviations, to each of the corresponding E_2 control values for each of the experiments.

Immunoblotting. MCF-7:WS8 cells were seeded on 10 cm Petri dishes at a density of 3 million cells per plate after being estrogen starved in phenol red-free RPMI 1640 medium for 3 days. The cells were treated for 24 h with the tested compounds, and the cells were subsequently washed with cold PBS (Life Technologies, Carlsbad, CA) and were lysed using 1× lysis buffer (Cell Signaling Technology Inc., Danvers, MA), which contained 1× Complete Mini protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and 1× phosphatase inhibitors (Calbiochem, Gibbstown, NJ). The cells were lysed for 60 min on ice and subsequently centrifuged at 12 000 rpm for 20 min. Supernatants were transferred in fresh tubes and stored at −20 °C. The concentration of proteins in the lysates were measured using a Pierce BCA protein assay (Thermo Scientific, Rockfold, IL) according to the manufacturer's instructions. An amount of 20 μg of each protein sample, diluted in a NuPAGE loading dye (Life technologies, Carlsbad, CA), was loaded and separated on NuPAGE 4−12% Bis-Tris gel (Life technologies, Carlsbad, CA). After electrophoresis the samples were transferred onto Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ), which were subsequently blocked with blocking solution with TBS-T (Tris-Bis saline with Tween 20:50 nM Tris-HCl, pH 7.5, 150 nM NaCl, 0.1% Tween-20), containing 5% skim milk for 1 h at room temperature. The membranes were subsequently probed with primary antibodies anti-ERα, (Santa Cruz Biotechnology, Santa Cruz, CA) and with anti-β-actin (Sigma-Aldrich, St. Louis, MO) diluted in blocking buffer at ratios recommended by the supplier at 4 °C overnight. The membranes were washed three times for 10 min with TBS-T buffer and subsequently incubated with the appropriate horseradish peroxidase (HRP) linked secondary antibodies (anti-mouse or anti-rabbit from Cell Signaling Technology Inc., Danvers, MA) diluted in blocking buffer for 1 h at room temperature. The membranes were washed again as described above with TBS-T buffer, and the signal was visualized using ECL Western blotting detection reagents (PerkinElmer, Waltham, MA). All results were replicated in three independent experiments, and each result was analyzed by densitometry using Image J imaging software (NIH). Pixel intensities of all lanes were normalized to their corresponding β-actin lanes with background intensity subtracted and were normalized to vehicle control as 100%.

Molecular Modeling. Ligand Preparation. The three-dimensional structures of the ligands to be docked were generated and prepared for docking using the LigPrep utility (*LigPrep*, version 2.5; Schrödinger, LLC: New York, NY, 2011). In this stage a series of treatments are applied to the structures. For example, conversions are performed and then corrections are applied to the structures, ionization states (pH 7 ± 0.4) and tautomers are generated, and finally the geometries are optimized using OPLS_2005 force field.

Proteins Selection and Preparation. The experimental X-ray structures of ERα LBD to be used for docking were selected from Protein Databank[45] based on the three-dimensional shape similarity between the compounds to be docked and cocrystallized ligands extracted from the receptor−ligand complexes. The three-dimensional shape similarity was computed using the ROCS utility of Openeye. As query data set, the ligands of interest were used while the screening library was compiled from the ligands extracted from all the available crystal structures of human ERα deposited in PDB. Shape Tanimoto parameter was used for scoring with a cutoff value of 0.8, and four ligands met this criterion. The 3D coordinates of the corresponding ERα complexes were extracted from PDB entries 3ERT,[30] 1UOM,[32] 2OUZ[33] (antagonist conformations of the receptor) and 3Q97[31] (agonist conformation). For comparison reasons, the other two experimental structures of the agonist conformation of ERα were extracted, PDB entries 1GWR (ERα cocrystallized with E2)[29] and 3ERD (the receptor cocrystallized with diethylstilbestrol, DES).[30]

Subsequently, the structures were prepared for docking using the Protein Preparation Workflow (Schrödinger, LLC, New York, NY, 2011) accessible from within the Maestro program (*Maestro*, version 9.2; Schrödinger, LLC: New York, NY, 2011). Shortly, the hydrogens were properly added to the complexes, water molecules beyond 5 Å from a heteroatom were deleted, bond corrections were applied to the cocrystallized ligands, and the orientation of hydroxyl groups, Asn, Gln, and the protonation state of His were optimized to maximize hydrogen bonds formation. All Asp, Glu, Arg, and Lys residues were left in their charged state. In the final stage a restrained minimization on the ligand−protein complexes was carried out with the OPLS_2001 force field and the default value for rmsd of 0.30 Å for non-hydrogen atoms was used. Docking simulations were performed with Glide software (*Glide*, version 5.7; Schrödinger, LLC: New York, NY, 2011), a grid-based docking method that can be run rigid or fully flexible for the ligand.[46,47] To some extent, a degree of flexibility was allowed to the X-ray structures of ERα in agonist conformation by scaling down the van der Waals radii of nonpolar atoms with a scale factor of 0.8 and allowing the free rotation of hydroxyl groups. The van der Waals radii of ligands nonpolar atoms were kept to the default value of the scaling factor of 0.8. The receptor grids were generated using the prepared proteins, with the docking grids centered on the center of the bound ligand for each receptor. The binding sites were enclosed in a grid box of 10 $Å^3$ with default parameters and without constrains. The generated ligand poses were evaluated with Schrodinger's proprietary version of ChemScore empirical scoring function, GlideScore.[47] This algorithm recognizes favorable hydrophobic, hydrogen-bonding, and metal-ligation interactions like ChemScore but adds a steric-clash term and buried polar terms to penalize electrostatic discrepancies. However, the composite energy scoring function, Emodel, was used to select the best-docked pose for each

ligand.[47,48] This energy function is a combination of the ligand−receptor molecular mechanics interaction energy, the binding affinity predicted by GlideScore, and the ligand strain energy (for flexible docking). For each receptor and docking run five poses were retrieved and the best ones were selected based on the Emodel score.

Reagents and Supplies. Estradiol (E_2), 4-hydroxytamoxifen (4OHT), endoxifen (*Z*-isomer), bovine insulin, and mouse anti-β-actin antibodies were all obtained from Sigma-Aldrich, St. Louis, MO. Fetal bovine serum (FBS), 2 mM glutamine, penicillin at 100 U/mL, streptomycin at 100 μg/mL, 1× nonessential amino acids, RPMI 1640 with phenol red and without media, DMEM media with and without phenol red, PBS buffer, Trizol reagent, NuPAGE loading dye, and NuPAGE 4−12% Bis-Tris gel were all obtained from Life Technologies, Carlsbad, CA. Fluorescent DNA quantification kit was obtained from Bio-Rad, Hercules, CA. RNeasy Mini isolation kits were obtained from Quiagen, Valencia, CA. High capacity cDNA reverse transcription kit and Power SYBR green PCR master mix were obtained from Applied Bioscience, Carlsbad, CA. All primers were obtained from Integrated DNA Technologies Inc., Coralville, IA. The 1× lysis buffer and anti-mouse and anti-rabbit horseradish peroxidase (HRP) linked secondary antibodies were purchased from Cell Signalling Technology Inc., Danvers, MA. The 1× Complete Mini protease inhibitor cocktail were from Roche Diagnostics, Indianapolis, IN. The 1× phosphatase inhibitors were from Calbiochem, Gibbstown, NJ. Pierce BCA protein assay was obtained from Thermo Scientific, Rockfold, IL. Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes were from Amersham Biosciences, Piscataway, NJ. Primary rabbit anti-ERα antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. ECL Western blotting detection reagents were from PerkinElmer, Waltham, MA.

Statistical Analysis. Statistical analysis of the data was performed for each of the repeated experiments separately using standard *t* test, paired and two-tailed in Microsoft Excel. *P* values less than 0.05 were considered significant.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures showing structure and docking poses. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

FR, fixed ring; ER, estrogen receptor; pS2, trefoil factor 1 gene, aka TFF1; GREB1, growth regulation by estrogen in breast cancer 1 gene; PgR, progesterone receptor gene; Prl, prolactin gene; TPP, triphenylphosphine; DIAD, diisopropyl azodicarboxylate; RT-PCR, real time polymerase chain reaction; E_2, 17β-estradiol; vdW, van der Waals parameter

■ REFERENCES

(1) Maximov, P. Y.; McDaniel, R. E.; Jordan, V. C. *Tamoxifen: Pioneering Medicine in Breast Cancer*; Springer: Basel, Switzerland, 2013.

(2) Davies, C.; Godwin, J.; Gray, R.; Clarke, M.; Cutter, D.; Darby, S.; McGale, P.; Pan, H. C.; Taylor, C.; Wang, Y. C.; Dowsett, M.; Ingle, J.; Peto, R. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* **2011**, *378*, 771−784.

(3) Davies, C.; Pan, H.; Godwin, J.; Gray, R.; Arriagada, R.; Raina, V.; Abraham, M.; Medeiros Alencar, V. H.; Badran, A.; Bonfill, X.; Bradbury, J.; Clarke, M.; Collins, R.; Davis, S. R.; Delmestri, A.; Forbes, J. F.; Haddad, P.; Hou, M. F.; Inbar, M.; Khaled, H.; Kielanowska, J.; Kwan, W. H.; Mathew, B. S.; Mittra, I.; Muller, B.; Nicolucci, A.; Peralta, O.; Pernas, F.; Petruzelka, L.; Pienkowski, T.; Radhika, R.; Rajan, B.; Rubach, M. T.; Tort, S.; Urrutia, G.; Valentini, M.; Wang, Y.; Peto, R. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. *Lancet* **2013**, *381*, 805−816.

(4) Fisher, B.; Costantino, J. P.; Wickerham, D. L.; Cecchini, R. S.; Cronin, W. M.; Robidoux, A.; Bevers, T. B.; Kavanah, M. T.; Atkins, J. N.; Margolese, R. G.; Runowicz, C. D.; James, J. M.; Ford, L. G.; Wolmark, N. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J. Natl. Cancer Inst.* **2005**, *97*, 1652−1662.

(5) Harper, M. J.; Walpole, A. L. Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. *Nature* **1966**, *212*, 87.

(6) Allen, K. E.; Clark, E. R.; Jordan, V. C. Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure−activity relationships. *Br. J. Pharmacol.* **1980**, *71*, 83−91.

(7) Jordan, V. C.; Collins, M. M.; Rowsby, L.; Prestwich, G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J. Endocrinol.* **1977**, *75*, 305−316.

(8) Lien, E. A.; Solheim, E.; Kvinnsland, S.; Ueland, P. M. Identification of 4-hydroxy-*N*-desmethyltamoxifen as a metabolite of tamoxifen in human bile. *Cancer Res.* **1988**, *48*, 2304−2308.

(9) Lien, E. A.; Solheim, E.; Lea, O. A.; Lundgren, S.; Kvinnsland, S.; Ueland, P. M. Distribution of 4-hydroxy-*N*-desmethyltamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. *Cancer Res.* **1989**, *49*, 2175−2183.

(10) Borgna, J. L.; Rochefort, H. Hydroxylated metabolites of tamoxifen are formed in vivo and bound to estrogen receptor in target tissues. *J. Biol. Chem.* **1981**, *256*, 859−868.

(11) Johnson, M. D.; Zuo, H.; Lee, K. H.; Trebley, J. P.; Rae, J. M.; Weatherman, R. V.; Desta, Z.; Flockhart, D. A.; Skaar, T. C. Pharmacological characterization of 4-hydroxy-*N*-desmethyl tamoxifen, a novel active metabolite of tamoxifen. *Breast Cancer Res. Treat.* **2004**, *85*, 151−159.

(12) Lim, Y. C.; Desta, Z.; Flockhart, D. A.; Skaar, T. C. Endoxifen (4-hydroxy-*N*-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen. *Cancer Chemother. Pharmacol.* **2005**, *55*, 471−478.

(13) Lim, Y. C.; Li, L.; Desta, Z.; Zhao, Q.; Rae, J. M.; Flockhart, D. A.; Skaar, T. C. Endoxifen, a secondary metabolite of tamoxifen, and 4-OH-tamoxifen induce similar changes in global gene expression patterns in MCF-7 breast cancer cells. *J. Pharmacol. Exp. Ther.* **2006**, *318*, 503−512.

(14) Lieberman, M. E.; Gorski, J.; Jordan, V. C. An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. *J. Biol. Chem.* **1983**, *258*, 4741−4745.

(15) Jordan, V. C.; Haldemann, B.; Allen, K. E. Geometric isomers of substituted triphenylethylenes and antiestrogen action. *Endocrinology* **1981**, *108*, 1353−1361.

(16) Jordan, V. C.; Koch, R.; Langan, S.; McCague, R. Ligand interaction at the estrogen receptor to program antiestrogen action: a study with nonsteroidal compounds in vitro. *Endocrinology* **1988**, *122*, 1449−1454.

(17) McCague, R.; Leclercq, G.; Jordan, V. C. Nonisomerizable analogues of (*Z*)- and (*E*)-4-hydroxytamoxifen. Synthesis and endocrinological properties of substituted diphenylbenzocycloheptenes. *J. Med. Chem.* **1988**, *31*, 1285−1290.
(18) Maximov, P. Y.; Myers, C. B.; Curpan, R. F.; Lewis-Wambi, J. S.; Jordan, V. C. Structure−function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. *J. Med. Chem.* **2010**, *53*, 3273−3283.
(19) Lal, B.; Khanna, J. M.; Anand, N. Phenethylamine in a rigid framework. 2,3-Substituted cis- and trans-6-amino-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-5-ols. *J. Med. Chem.* **1972**, *15*, 23−27.
(20) Kahn, A. M.; Proctor, G. R.; Rees, L. Novel aromatic systems. Part IV. Synthesis and dehydrogenation of 4′-hydroxy-1,2-benzocycloheptatrienes. *J. Chem. Soc.* **1966**, 990−994.
(21) Jarman, M.; McCague, R. Heptafluoro-*p*-tolyl and tetrafluoro-4-pyridyl as novel and selective protecting groups for phenolic and alcoholic functions: synthesis and cleavage of perfluoroaryl ethers of steroids. *J. Chem. Res., Synop.* **1985**, 114−115.
(22) Fauq, A. H.; Maharvi, G. M.; Sinha, D. A convenient synthesis of (*Z*)-4-hydroxy-*N*-desmethyltamoxifen (endoxifen). *Bioorg. Med. Chem. Lett.* **2010**, *20*, 3036−3038.
(23) Stearns, V.; Johnson, M. D.; Rae, J. M.; Morocho, A.; Novielli, A.; Bhargava, P.; Hayes, D. F.; Desta, Z.; Flockhart, D. A. Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. *J. Natl. Cancer Inst.* **2003**, *95*, 1758−1764.
(24) Murahashi, S. I.; Naota, T.; Miyaguchi, N.; Nakato, T. Ruthenium-catalyzed oxidation of tertiary-amines with hydrogen-peroxide in the presence of methanol. *Tetrahedron Lett.* **1992**, *33*, 6991−6994.
(25) Lee, H. W.; Ahn, J. B.; Lee, J. H.; Kang, S. K.; Ahn, S. K.; Ha, D. C. Selective N-demethylation of tertiary aminofumagillols with selenium dioxide via a non-classical Polonovski type reaction. *Heterocycles* **2006**, *68*, 915−932.
(26) Murdter, T. E.; Schroth, W.; Bacchus-Gerybadze, L.; Winter, S.; Heinkele, G.; Simon, W.; Fasching, P. A.; Fehm, T.; Eichelbaum, M.; Schwab, M.; Brauch, H. Activity levels of tamoxifen metabolites at the estrogen receptor and the impact of genetic polymorphisms of phase I and II enzymes on their concentration levels in plasma. *Clin. Pharmacol. Ther.* **2011**, *89*, 708−717.
(27) Jordan, V. C.; Fritz, N. F.; Tormey, D. C. Endocrine effects of adjuvant chemotherapy and long-term tamoxifen administration on node-positive patients with breast cancer. *Cancer Res.* **1987**, *47*, 624−630.
(28) Ravdin, P. M.; Fritz, N. F.; Tormey, D. C.; Jordan, V. C. Endocrine status of premenopausal node-positive breast cancer patients following adjuvant chemotherapy and long-term tamoxifen. *Cancer Res.* **1988**, *48*, 1026−1029.
(29) Warnmark, A.; Treuter, E.; Gustafsson, J. A.; Hubbard, R. E.; Brzozowski, A. M.; Pike, A. C. Interaction of transcriptional intermediary factor 2 nuclear receptor box peptides with the coactivator binding site of estrogen receptor alpha. *J. Biol. Chem.* **2002**, *277*, 21862−21868.
(30) Shiau, A. K.; Barstad, D.; Loria, P. M.; Cheng, L.; Kushner, P. J.; Agard, D. A.; Greene, G. L. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **1998**, *95*, 927−937.
(31) Rajan, S. S.; Kim, Y.; Vanek, K.; Joachimiak, A.; Jordan, C.; Greene, G. L., Crystal structure of human estrogen receptor alpha LBD in complex with GRIP peptide and two ismoers of ethoxy triphenylethylene. Manuscript to be published.
(32) Renaud, J.; Bischoff, S. F.; Buhl, T.; Floersheim, P.; Fournier, B.; Halleux, C.; Kallen, J.; Keller, H.; Schlaeppi, J. M.; Stark, W. Estrogen receptor modulators: identification and structure−activity relationships of potent ERalpha-selective tetrahydroisoquinoline ligands. *J. Med. Chem.* **2003**, *46*, 2945−2957.
(33) Vajdos, F. F.; Hoth, L. R.; Geoghegan, K. F.; Simons, S. P.; LeMotte, P. K.; Danley, D. E.; Ammirati, M. J.; Pandit, J. The 2.0 Å crystal structure of the ERalpha ligand-binding domain complexed with lasofoxifene. *Protein Sci.* **2007**, *16*, 897−905.
(34) Murphy, C. S.; Langan-Fahey, S. M.; McCague, R.; Jordan, V. C. Structure−function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. *Mol. Pharmacol.* **1990**, *38*, 737−743.
(35) Jordan, V. C.; Lieberman, M. E. Estrogen-stimulated prolactin synthesis in vitro. Classification of agonist, partial agonist, and antagonist actions based on structure. *Mol. Pharmacol.* **1984**, *26*, 279−285.
(36) Jordan, V. C.; Lieberman, M. E.; Cormier, E.; Koch, R.; Bagley, J. R.; Ruenitz, P. C. Structural requirements for the pharmacological activity of nonsteroidal antiestrogens in vitro. *Mol. Pharmacol.* **1984**, *26*, 272−278.
(37) Jordan, V. C.; Koch, R.; Mittal, S.; Schneider, M. R. Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. *Br. J. Pharmacol.* **1986**, *87*, 217−223.
(38) Murphy, C. S.; Parker, C. J.; McCague, R.; Jordan, V. C. Structure−activity relationships of nonisomerizable derivatives of tamoxifen: importance of hydroxyl group and side chain positioning for biological activity. *Mol. Pharmacol.* **1991**, *39*, 421−428.
(39) Lonard, D. M.; Nawaz, Z.; Smith, C. L.; O'Malley, B. W. The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. *Mol. Cell* **2000**, *5*, 939−948.
(40) Obiorah, I. E.; Sengupta, S.; Curpan, R.; Jordan, V. C. Defining the conformation of the estrogen receptor complex that controls estrogen induced apoptosis in breast cancer. *Mol. Pharmacol.* **2014**, *85*, 789−799.
(41) Nicholson, R. I.; Gee, J. M.; Manning, D. L.; Wakeling, A. E.; Montano, M. M.; Katzenellenbogen, B. S. Responses to pure antiestrogens (ICI 164384, ICI 182780) in estrogen-sensitive and -resistant experimental and clinical breast cancer. *Ann. N.Y. Acad. Sci.* **1995**, *761*, 148−163.
(42) Wu, X.; Hawse, J. R.; Subramaniam, M.; Goetz, M. P.; Ingle, J. N.; Spelsberg, T. C. The tamoxifen metabolite, endoxifen, is a potent antiestrogen that targets estrogen receptor alpha for degradation in breast cancer cells. *Cancer Res.* **2009**, *69*, 1722−1727.
(43) Liu, H.; Lee, E. S.; Deb Los Reyes, A.; Zapf, J. W.; Jordan, V. C. Silencing and reactivation of the selective estrogen receptor modulator−estrogen receptor alpha complex. *Cancer Res.* **2001**, *61*, 3632−3639.
(44) Jordan, V. C. The 38th David A. Karnofsky Lecture: The paradoxical actions of estrogen in breast cancer—survival or death? *J. Clin. Oncol.* **2008**, *26*, 3073−3082.
(45) Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. The Protein Data Bank. *Nucleic Acids Res.* **2000**, *28*, 235−242.
(46) Friesner, R. A.; Banks, J. L.; Murphy, R. B.; Halgren, T. A.; Klicic, J. J.; Mainz, D. T.; Repasky, M. P.; Knoll, E. H.; Shelley, M.; Perry, J. K.; Shaw, D. E.; Francis, P.; Shenkin, P. S. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J. Med. Chem.* **2004**, *47*, 1739−1749.
(47) Halgren, T. A.; Murphy, R. B.; Friesner, R. A.; Beard, H. S.; Frye, L. L.; Pollard, W. T.; Banks, J. L. Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening. *J. Med. Chem.* **2004**, *47*, 1750−1759.
(48) Friesner, R. A.; Murphy, R. B.; Repasky, M. P.; Frye, L. L.; Greenwood, J. R.; Halgren, T. A.; Sanschagrin, P. C.; Mainz, D. T. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J. Med. Chem.* **2006**, *49*, 6177−6196.

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Cancer Prevention Research

Breast cancer cell apoptosis with phytoestrogens is dependent on an estrogen deprived state

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Title

Breast Cancer Cell Apoptosis with Phytoestrogens is dependent on an estrogen deprived state

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ABSTRACT

Phytoestrogens have been investigated as natural alternatives to hormone replacement therapy and their potential as chemopreventive agents. We investigated the effects equol, genistein and coumestrol on cell growth in fully estrogenized MCF-7 cells, simulating the perimenopausal state, and long term estrogen deprived MCF7:5C cells which simulate the postmenopausal state of a woman after years of estrogen deprivation and compared the effects to that of steroidal estrogens: 17β estradiol (E_2) and equilin present in conjugated equine estrogen. Steroidal and phytoestrogens induce proliferation of MCF-7 cells at physiologic concentrations but inhibit the growth and induce apoptosis of MCF7:5C cells. Although steroidal and phytoestrogens induce estrogen responsive genes, their anti-proliferative and apoptotic effects are mediated through the estrogen receptor. Knockdown of ERα using siRNA blocks all estrogen induced apoptosis and growth inhibition. Phytoestrogens induce endoplasmic reticulum stress and inflammatory response stress related genes in a comparable manner as the steroidal estrogens. Inhibition of inflammation using dexamethasone blocked both steroidal and phytoestrogen induced apoptosis and growth inhibition as well as their ability to induce apoptotic genes. Together, this suggests that phytoestrogens can potentially be used as chemopreventive agents in older postmenopausal women but caution should be exercised when used in conjunction with steroidal anti-inflammatory agents due to their anti-apoptotic effects.

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INTRODUCTION

Acquired resistance to anti-hormone therapy occurs despite the successful use of endocrine treatment to improve survival in breast cancer patients. Early laboratory models show that re-transplantation of tamoxifen resistant tumors into ovarectomized athymic mice led to tumor growth in response to tamoxifen and estradiol(E_2)(1, 2). Continued retransplantation of the tamoxifen stimulated tumors in nude mice for up to 5years resulted to a rapid regression of the tumors in response to E_2 (3). This correlates with the finding that E_2 induces apoptosis in long term estrogen deprived MCF-7 breast cancer cells (4, 5). The use of estrogens has been beneficial in the treatment of metastatic breast cancer in postmenopausal women with acquired resistance to endocrine therapy. A clinical study (6) found that high dose diethylstilbestrol induced an objective response in 30 percent of postmenopausal breast cancer patients who had previous exhaustive antihormone therapy. Ellis and colleagues (7) showed that postmenopausal women with aromatase inhibitors resistant metastatic breast cancer, had a 29% clinical benefit with low dose estrogen (6mg daily) but the same clinical benefit but more side effects with high dose estrogen (30mg daily). Additional clinical evidence for the antitumor action of low dose estrogen comes from the Women Health Initiative (WHI) trial which compared conjugated equine estrogen (CEE) therapy with placebo in hysterectomised postmenopausal women which show a persistent decrease in the incidence and mortality of breast cancer in women who received estrogen alone therapy(8, 9). Studies in vitro show that constituents of CEE cause apoptosis in long term estrogen deprived MCF7 cells (10). The clinical and laboratory studies suggest that the ability of estrogen therapy to treat or prevent tumors is most apparent in the postmenopausal state of a woman and how long they have been physiologically deprived of estrogen(10).

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Phytoestrogens are plant derived polyphenolic compounds that are structurally similar to E_2. Phytoestrogens consists of isoflavones (genistein, diadzein), coumestans (coumestrol), the lignans (enterolactone, enterodiol) and stilbenes (resveratrol).Isoflavones are principally found in soy based products which are staple foods in many Asian countries and are becoming increasing popular in western countries. An inverse relationship found between soy consumption in Asian countries and decreased breast cancer risk has sparked a sustained interest in the use of phytoestrogens in breast cancer prevention. However, the clear beneficial effects of these estrogens remain controversial. Several meta-analysis(11-13) that assessed soy exposure and breast cancer risk revealed that studies conducted in Asian countries showed a significant trend of a reduced risk with increased soy intake in both pre- and postmenopausal Asian women. On the other hand no association was observed between soy consumption and breast cancer risk in low soy consuming western populations(11, 13), suggesting that consumption of soy products in amounts taken in the Asian population may have protective benefits. Evaluation of the breast cancer protective effects of isoflavones stratified by menopausal status is still undefined. Trock and colleagues(14) reported in their meta-analysis, a stronger association between soy exposure and breast cancer risk in premenopausal women. However, the analyses included studies with incomplete measurements, potential confounders and lack of a dose response that make the findings inconclusive. On the other hand, another study reported that adult or adolescent soy consumption was associated with reduced risk of premenopausal breast cancer(15) and no significant associations were reported for the risk of postmenopausal breast cancer. Furthermore, there is increased evidence that the chemo-protective effects of isoflavones are dependent on early exposure. High soy consumption during adolescence is associated with reduced risk of adult breast cancer (15-17). This concurs with the findings in animal model experiments, where

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prepubertal exposure to genistein causes mammary gland differentiation, thereby resulting in increased breast cancer prevention(18, 19). The effect of phytoestrogens in breast cancer cells have been extensively studied. At low pharmacological concentrations, phytoestrogens stimulate the growth of estrogen receptor positive breast cancer cells (20-22). In contrast at high concentrations (>5μM), these plant derived estrogens inhibit the growth of the cancer cells (21, 23, 24). Ingestion of soy isoflavones in healthy premenopausal women resulted in increased breast tissue proliferation (25),epithelial hyperplasia (26) and a weak estrogenic response in inducing estrogen regulated markers (27). On the other hand in postmenopausal women, soy supplementation resulted in either a protective effect(28) or no effect (29-31) on breast cancer risk. Only one of the postmenopausal studies(31) consisted of healthy subjects, the rest included breast cancer patients. Fink et al (32) reported a decreased all-cause mortality in pre- and postmenopausal breast cancer patients who had a high intake of isoflavones, whereas a reduced breast cancer mortality was observed in postmenopausal women. However the DietCompLyf study (33) which investigated associations between phytoestrogens and breast cancer recurrence and survival found no significant associations between pre-diagnosis phytoestrogen intake and reduced breast cancer risk. Interestingly Shu and colleagues (34) found that soy food consumption was significantly associated with decreased risk of death and recurrence in breast cancer patients.

In this study we have evaluated the apoptotic and potential chemopreventive effects of phytoestrogens using a unique cell model that simulates a postmenopausal cellular environment. Genistein, coumestrol and equol, a gastrointestinal metabolite of diadzein are used in comparison to E_2 and equilin a constituent of conjugated equine estrogen (CEE) in hormone replacement therapy (HRT) to determine their proliferative and apoptotic potential using fully estrogenised

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and an estrogen deprived breast cancer cells respectively. Here, we test the hypothesis that the phytoestrogens have biologic effects similar to that of E_2 and CEE in breast cancer prevention and this may have clinical implications for the strategic use of phytoestrogens as alternatives to HRT in postmenopausal populations.

MATERIALS AND METHODS

Cell Culture and Reagents

Cell culture media were purchased from Invitrogen Inc. (Grand Island, NY) and fetal calf serum (FCS) was obtained from HyClone Laboratories (Logan, UT). Compounds E_2 , equilin, equol,genistein and coumestrol (Supplementary Fig. S1), ICI 182,780 and 4-hydroxytamoxifen (4OHT) (were obtained from Sigma, St. Louis, MO). Dexamethasone was obtained from Tocris Biosciences, Bristol, UK. MCF7:5C were derived from MCF7 cells obtained from the Dr. Dean Edwards, San Antonio, Texas as reported previously (35). MCF7:WS8 cells were derived from MCF-7 cells as previously described(35) and maintained in RPMI media supplemented with 10% FCS, 6 ng/ml bovine insulin and penicillin and streptomycin. The MCF7:WS8 and the MCF7:5C cells have been fully characterized and experiments were done as previously reported (36). The expected growth/apoptotic responses to E2, biomarker statuses of estrogen receptor-α (ERα), PgR, and HER2, and ER-regulated transcriptional activity were confirmed in both cell lines. Protein levels of ERα, PgR, and HER2 were characterized by semiquantitative immunoblot analysis and ER transcriptional activity was evaluated using an estrogen responsive element (ERE) -regulated dual luciferase reporter gene system(36). The last characterization was reported in (37) and the DNA fingerprinting patterns of the cell lines were consistent with the report by the American Type Culture Collection. MCF7 cells are cultured in phenol-red free RPMI media containing 10% charcoal dextran treated FCS, 6ng/ml bovine insulin and penicillin and

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streptomycin for three days prior to starting experiments. MCF7:5C cells were maintained in phenol-red free RPMI media containing 10% dextran coated charcoal-treated FCS, 6ng/ml bovine insulin and penicillin and streptomycin. The cells were treated with indicated compounds (with media changes every 48 h) for the specified time and were subsequently harvested for tissue culture experiments.

Cell growth assay

The cell growth was monitored by measuring the total DNA content per well in 24 well plates. Fifteen thousand cells were plated per well and treatment with indicated concentrations of compounds was started after 24h, in triplicates. Media containing the specific treatments were changed every 48h. On day 7 the cells were harvested and total DNA was assessed using a fluorescent DNA quantification kit (Cat # 170-2480; Bio-Rad,Hercules, CA, USA) and was performed as previously described (38).

RNA isolation and real time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and RNAeasy kit according to the manufacturer's instructions. Real-time PCR was performed as previously described (39). The sequences for all primers are documented in Supplementary Table S1. The change in expression of transcripts was determined as described previously and used the ribosomal protein 36B4 mRNA as the internal control (39).

Apoptosis assay

In brief, MCF-7:5C cells were seeded in 100-mm dishes and cultured overnight in estrogen-free RPMI 1640 medium containing 10% SFS. The next day, cells were treated with <0.1% ethanol (control), E_2 (1 nM) equilin (1 nM), equol (1 μM), genistein(1 μM) and coumestrol(1 μM) for 72h and the cells were detached using accutase (Life Technologies, Carlsbad, California), a

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marine-origin enzyme with proteolytic and collagenolytic activity, in 1:3 dilution using PBS (Invitrogen, Grand Island, NY) as the diluent. The cells were collected by centrifugation for 2 min at 500 × *g*. Cells were then resuspended and stained simultaneously with either FITC-labeled annexin V and propidium iodide (PI) (Pharmingen, San Diego, CA) or DNA binding dye, YO-PRO-1 and PI (Life technologies, (Grand Island, NY). Apoptosis was verified based on loss of plasma membrane integrity. Viable cells excluded these dyes, whereas apoptotic cells allowed moderate staining. Cells were analyzed using a fluorescence- activated cell sorter (FACS) flow cytometer (Becton Dickinson, San Jose, CA). The percentage of apoptosis was calculated by adding the percentage of cells stained with either annexin V alone (early apoptosis) in the right lower quadrant and those stained with both PI and annexin V (late apoptosis) in the right upper quadrant. Experiments are repeated three times with similar results.

Cell cycles analysis

MCF7:5C cells were cultured in dishes and were treated with with <0.1% ethanol (control), E_2 (1 nM) equilin (1 nM), equol (1 μM), genistein(1 μM) and coumestrol(1 μM) for the indicated times. Cells were harvested and gradually fixed with 75% EtOH on ice. After staining with PI, cells were analyzed using a fluorescence-activated cell sorter (FACS) flow cytometer (Becton Dickinson, San Jose, CA), and the data were analysed with Modfit software (Topsham, ME).

Immunoblotting

Proteins were extracted in cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with Protease Inhibitor Cocktail (Roche, Indianapolis, IN) and Phosphatase Inhibitor Cocktail Set I and Set II (Calbiochem, San Diego, CA). Total protein content of the lysate was determined by a standard bicinchoninic acid assay using the reagent from Bio-Rad

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Laboratories (Hercules, CA). Twenty five micrograms of total protein was separated on 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with primary antibodies followed by incubation with secondary antibody conjugated with HRP and reaction with Western Lighting™ plus-ECL enhanced chemiluminescent substrate (PerkinElmer Inc., Waltham MA). ERα and β antibody was from Santa Cruz Biotechnology (Dallas, TX). Phosphorylated eIF2α, total eIF2α, IRE1α and β actin antibodies were from Cell Signaling Technology (Danvers,MA). Protein bands were visualised by exposing the membrane to X-ray film.

Small Interfering RNA Transfection

For transient transfections, MCF7:5C cells were seeded at a density of 50-70% in 6 well plates in estrogen free RPMI media containing 10% SFS. The following day, cells were transfected with 100 nM small interfering RNAs (siRNAs) for ER α (Dharmacon Pittsburgh PA, SMART pool: ON-TARGETplus ESR1 siRNA product number L-003401-00-0005) and ERβ (Dharmacon, SMART pool: ON-TARGETplus ESR2 siRNA product number L-003402-00-0005) using DharmaFECT transfection reagent (Dharmacon Pittsburgh PA, product number T-2001-03), according to the manufacturer's recommended protocol. Non target siRNA was purchased from Dharmacon and was used as a control (Silencer negative control siRNA, product number D-001810-01-20). The cells were harvested 72h posttransfection and analyzed by western blot (as described above).Transfected cells were also treated with vehicle, steroidal estrogens or phytoestrogens for either an additional 72h, or 6 days and apoptotic cells and DNA content were measured using annexinV staining and DNA quantification assays respectively (as described above).

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Statistical analysis

All data are expressed as the mean of at least three determinations, unless otherwise stated. The differences between the treatment groups and the control group were determined by one-factor analysis of variance (ANOVA with Tukey's post test and two-way ANOVA with Bonferroni post test using GraphPad Prism, version 5.00 (GraphPad Software Inc., La Jolla, CA). Results were considered statistically significant if the $P < 0.05$.

RESULTS

Effect of phytoestrogens on breast cancer cells

Based on the controversy surrounding breast cancer risk and the use of phytoestrogens, we decided to determine the biological properties of the genistein, equol and coumestrol in comparison to E_2 and equilin in two different models of breast cancer cell models. Estrogens have been shown to regulate the growth of ER positive MCF-7 breast cancer cells. First, we tested the ability of test compound to induce proliferation in MCF7:WS8 cells which are estrogen responsive breast cancer cells grown in fully estrogenised medium. MCF-7:WS8 cells were grown in estrogen free media for 3 days and treated with various concentrations of genistein, coumestrol and equol and their effects were compared to E_2 and equilin (Fig. 1A). The phytoestrogens, equol [EC_{50}: 1.72 x 10^{-9}], genistein [EC_{50}: 1.08 x 10^{-8}] and coumestrol[EC_{50}: 3.07 x 10^{-9}], all stimulated cell growth in a concentration related manner with maximum stimulation occurring at 0.1μM, whereas E_2 [EC_{50}: 3.11x 10^{-12}] and equilin [EC_{50}: 1.01 x 10^{-11}] maximally induced cell growth at 10 pM and 0.1 nM respectively.

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Growth inhibition was observed with the phytoestrogens at 10μM with genistein (10^{-5}M vs 10^{-7}M; P<0.05). Next we investigated the growth properties of the genistein, equol and coumestrol in long term estrogen deprived MCF7:5C cells in comparison to E_2 and equilin (Fig. 1B). Genistein[IC_{50}: 2.77 x 10^{-8}], equol[IC_{50}: 4.67 x 10^{-8}] and coumestrol[IC_{50}: 2.34 x 10^{-8}] drastically inhibited the growth of the MCF7:5C cells at higher concentrations compared to E_2. Maximum growth inhibition was observed with all phytoestrogens at 0.1μM. E_2 [IC_{50}: 2.06 x 10^{-11}] achieved maximum growth inhibition at 0.1nM, while equilin [IC_{50}: 2.32 x 10^{-10}] reached maximum growth inhibition at 1nM after 7 days of treatment.

Phytoestrogens induce apoptosis in a long term estrogen deprived breast cancer cell line

Based on the fact that the decrease in cell growth observed with the steroidal estrogens is due to apoptosis(10), we investigated whether the anti-proliferative effects of the phytoestrogens was also due to an increase in apoptosis. MCF7:5C cells were treated with E_2 (1nM), equilin (1nM), genistein(1μM), equol(1μM) and coumestrol(1μM) for 72h and stained with annexinV-FITC and PI fluorescence and cells were analyzed using the flow cytometry. In the control treated group, only 6.8% of cells stained for apoptosis, whereas E_2(24.56%), equilin(17.49%), genistein (14.79%), equol (14.89%) and coumestrol (17.83%) all show increased apoptotic staining compared to the control treated cells (Fig. 2A). A similar effect was noted using a DNA binding stain, YO-PRO-1 (Supplementary Fig. S2). E_2, equilin and all phytoestrogens induced apoptotic genes; *BCL2L11/BIM, TNF, FAS* and *FADD* (Fig. 2B-C) after 48h of treatment. Induction of these genes is consistent with the apoptotic status determined using the flow cytometry. Although evidence of apoptosis occurs with the phytoestrogens by 48h, a consistent increase in the S phase when compared to the control was observed with all estrogens(Supplementary Fig. S3). In contrast to other reports(23, 40) which indicate that genistein causes a G2/M arrest, no

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checkpoint blockade was noted after treatment with all compounds, indicating that the initial response of the cells to estrogens is growth, then apoptosis in MCF7:5C cells.

Phytoestrogens possess estrogenic properties mediated through the estrogen receptor in the MCF7:5C cells

We explored the ability of phytoestrogens to regulate estrogen response genes in comparison to E_2 and equilin. Genistein, equol and coumestrol were all able to induce *TFF1/PS2* and *GREB1* (Fig. 3A). Phytoestrogens have been shown to induce apoptosis through an estrogen receptor (ER) independent mechanism(21, 41). To evaluate the involvement of ER in the effects of the phytoestrogens, we investigated their anti-proliferative effects in the presence of 4-hydroxytamoxifen (4OHT) (Fig. 3B) and ICI 182 780 (Supplementary Fig. S4). The combination of various concentrations of 4OHT or ICI 182 780 with E_2, equilin and each phytoestrogens blocked estrogen induced apoptosis suggesting that the phytoestrogens mediate apoptosis via the ER. We sought to examine the effects of genistein, equol and coumestrol on the ER. Following treatment of MCF7:5C cells with E_2, equilin and the phytoestrogens for 24h, ERα levels were determined by western blotting. All phytoestrogens caused a decrease in the ERα protein levels in a comparable manner as E_2 and equilin (Fig. 3C). Similarly the same effect was noted with all estrogens on the ERα mRNA levels (Fig. 3D). Interestingly, E_2, equilin, genistein, equol and coumestrol, all have no effect on the ERβ protein and mRNA levels suggesting different regulatory effects the phytoestrogens may have on the ERα and ERβ . A relative ratio of ERα to ERβ in MCF7:5C cells is shown in (Supplementary Fig. S5).

ERα is important for steroidal and phytoestrogen induced apoptosis and growth inhibition

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To determine whether ER α or β is required for the antiproliferative and apoptotic effects of the estrogens, MCF7:5C cells were transfected with either ERα or ERβ siRNA or nontarget siRNA(control) for 72h. Knockdown of ERα and ER β protein level was determined by western blot (Fig. 4A, D). RNA- interference mediated inhibition of ER α abolished both steroidal and phytoestrogen induced apoptosis (Fig. 4B) and growth inhibition (Fig. 4C) compared with cells transfected with the control siRNA. Interestingly, loss of ERβ using siRNA did not prevent the ability of the steroidal or phytoestrogens to either induce apoptosis (Fig.4E) or inhibit the growth (Fig.4F) of the MCF7:5C cells. Taken together, this indicates that ERα is the initial site for the indicated estrogens to cause growth inhibition and apoptosis in the MCF7:5C cells.

Phytoestrogens induce endoplasmic reticulum stress and inflammatory stress response genes

Microarray analysis indicates that endoplasmic reticulum stress (ERS) and inflammatory response genes are top scoring pathways associated with E_2 induced apoptosis(36). To investigate whether phytoestrogens induce ERS genes, we used RT-PCR to quantitate mRNA levels. After 48h of treatment, genistein, equol, coumestrol and equilin and E2 all induce *DDIT3*(also known as CHOP), a marker of ERS associated with cell death, and inositol requiring protein 1 alpha (*IRE1α*) , an unfolded protein response sensor which is activated to relieve stress(Fig. 5A). Significant induction of *IRE1α* and phospho-eukaryotic translation initiation factor-2α (p-eIF2α), another UPR sensor, protein levels occur by 24h (Fig. 5B). Next we determined whether genistein, coumestrol and equol induce proinflammatory response genes using RT-PCR. At 48h, E_2, equilin and all phytoestrogens activate caspase 4, an inflammatory caspase; *CEBP β* which is known to bind to IL-1 response element in *IL6* and a downstream target of ERS; *IL6*, a proinflammatory cytokine; lymphotoxin beta (*LTB*),an inducer of

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inflammation response, (Fig. 5C-D). This indicates that the phytoestrogens activate similar genes involved in the apoptotic pathway of E_2.

Inflammation is required for phytoestrogen mediated apoptosis

Next we investigated the importance of inflammatory response in phytoestrogen mediated apoptosis. Dexamethasone, a synthetic glucocorticoid with potent anti-inflammatory properties was used to inhibit inflammation in the MCF7:5C cells. Cells were treated with 1nM E_2 or equilin or 1μM phytoestrogens and various concentrations of dexamethasone were added to block the biological effects of the compounds. Although dexamethasone has an inhibitory effect in the MCF7:5C cells, it was able to reverse the steroidal estrogen or phytoestrogen inhibited growth(Fig. 6A). Similarly, flow cytometry studies revealed that 1μM dexamethasone reversed the apoptotic effects mediated by E_2, equilin, genistein, equol and coumestrol (Fig. 6B). To determine that inflammatory stress response was inhibited by dexamethasone, MCF7:5C cells were treated with the indicated estrogens for 48h and total RNA was extracted and reverse transcribed. Dexamethasone inhibited the ability of all estrogens to induce caspase 4, *CEBP β*, *BIM* and *TNF* (Fig. 6C-D). Together, this suggests that inflammation is important for both steroidal and phytoestrogen mediated apoptosis.

DISCUSSION

Phytoestrogen consumption is associated with a decrease in the incidence of breast cancer in the Asian population probably due to early exposure to a high soy diet. This correlates to animal studies which suggest that it is due to mammary cell differentiation and a decrease in terminal

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end buds which are sites of early tumor proliferation(42, 43). Phytoestrogens increase cell growth of ER positive breast cancer cells but induce apoptosis at high concentrations in these cells. Although studies (11, 28) may support use of phytoestrogens in postmenopausal women, their full chemopreventive properties is yet to be clearly defined. E_2 and CEE induce apoptosis in long term estrogen deprived breast cancer cells. Therefore we addressed the question of whether low concentrations of phytoestrogens will induce apoptosis in MCF7:5C cells which simulate a postmenopausal state that is dependent on the duration of estrogen deprivation following menopause. Genistein, equol and coumestrol all increase cell growth in MCF7:WS8,(which simulate the premenopausal or perimenopausal state) after 3 days of estrogen deprivation at low concentrations. These cells have adapted to an estrogen rich environment and will grow with a natural resupply of estrogens provided with exogenous phytoestrogens treatment. This correlates with the results of Andrade and colleagues (44)who show that long-term consumption of low GEN doses (≤500 ppm) promotes MCF-7 tumor growth in vivo. However at low concentrations <1μM, all phytoestrogens inhibit cell growth. In contrast the phytoestrogens, although less potent than E_2 and equilin, induce apoptosis in MCF-7 cells that have undergone long term estrogen deprivation. Therefore a potential use of phytoestrogens at physiologic concentrations will be in an estrogen deprived environment which is induced either by natural withdrawal of estrogens caused by menopause or by treatment with exhaustive anti-estrogen therapy for breast cancer with aromatase inhibitors or tamoxifen.

Studies (45-47)suggest that phytoestrogens possess anti-estrogenic properties which may be responsible for their chemopreventive effects. Here we show that the phytoestrogens do in fact induce estrogen responsive genes just like steroidal estrogens in the estrogen deprived MCF7:5C cells and that their growth inhibition and apoptosis are mediated through the ER. In

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contrast, it has been reported that genistein mediates apoptosis through an ER independent mechanism in the MCF-7 cells (41, 45) and the ability of phytoestrogens to induce apoptosis is observed maximally in the presence of E_2. It is important to note however that apoptosis was mediated by the phytoestrogens only at high concentrations in these studies (41, 45). As another potential mechanism of apoptosis, phytoestrogens show increased binding affinity to ERβ(48) which is thought to be responsible for its growth inhibitory properties. In our study, loss of ERβ did not affect the anti-proliferative and apoptotic properties of the steroidal and phytoestrogens. However, we determined that knockdown of ERα prevents both steroidal and phytoestrogen mediated growth inhibition and apoptosis suggesting that ER α signaling is required for their biological actions.

Genistein, equol and coumestrol induce ERS and inflammatory stress response, intrinsic and extrinsic apoptosis related genes which correlates with results of differential gene expression in response to E_2 interrogated using agilent based microarray analysis(36). Activated ERS genes indicate that E_2 prevents protein folding leading to accumulation of unfolded proteins and widespread inhibition of protein translation and crosstalk with inflammatory response genes and subsequent induction of cell death. Inhibition of *PERK/EIF2AK3*, a key ERS sensor of UPR and inducer of pEIF2α (49) prevents E_2 mediated apoptosis(50). *PERK* is also known to induce apoptosis by sustaining levels of *DDIT3*(51),another major ERS gene involved in apoptosis, which is known to dimerize with *CEBPβ* under stress conditions(52, 53). Ablation of *CEBPβ* using siRNA decreases expression of *DDIT3* (53) suggesting a crosstalk between ERS and inflammatory stress response. Similarly, inhibition of caspase 4, an inflammatory response gene and a downstream target of ERS, using caspase 4 inhibitor-z-LEVD-fmk also blocks E_2 induced apoptosis. To show that inflammation is important in phytoestrogen induced apoptosis,

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dexamethasone was used to block inflammation globally, resulting in inhibition of all estrogen induced apoptosis and their ability to induce inflammatory response and apoptosis related genes. Therefore the clinical implication is that caution should be exercised in the use of steroidal anti-inflammatory agents in conjunction with these phytoestrogens, which could prevent the full chemopreventive benefits.

Successful use of estrogens to treat or prevent tumors is dependent on the timing of estrogen withdrawal. Estrogen therapy was the first chemical used in the treatment of advanced breast cancer in postmenopausal women and this therapy resulted in the regression of 30% of tumors in the first reported clinical trial(54). It was noted that "the beneficial responses were three times more frequent in women over the age of 60 years than in those under that age; that estrogens may, on the contrary, accelerate the course of mammary cancer in younger women, and that their therapeutic use should be restricted to cases 5 years beyond the menopause"(55). Stoll and colleagues(56) noted that objective remission rate from estrogen treatment in 407 patients with advanced breast cancer was higher in women more than 5 years postmenopausal(35%) when compared to women who were less than 5 years postmenopausal(9%). In more recent clinical studies, about thirty percent of patients with advanced breast cancer who have been exposed to exhaustive antihormone therapy show an objective clinical response with estrogen therapy(6, 7). CEEs reduced the incidence and mortality from breast cancer but this is probably because the majority of these women were over 65 years(9). Furthermore, 10 years adjuvant tamoxifen therapy produced a further reduction in recurrence and mortality from breast cancer when compared to 5 years of tamoxifen therapy(57) suggesting that it was the woman's own estrogen that destroys the appropriately sensitive tamoxifen resistant micrometastasis once long term tamoxifen is stopped(58).

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In conclusion, it is important to note that in order to obtain the full breast cancer chemopreventive benefits of phytoestrogens, it is necessary to begin up to five years following menopause. Commencing soy consumption during perimenopause may cause growth of nascent ER positive breast tumors which may increase breast cancer risk, whereas phytoestrogen therapy 5 years after menopause will most likely induce apoptotic cell death and enhanced patient survival.

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

REFERENCES

1. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 Tumors in athymic mice after long-term antiestrogen administration. Cancer Res 1988; 48:5183-5187.
2. Osborne CK, Coronado EB, Robinson J. Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. Eur J Cancer Clin Oncol 1987; 23:1189-1196.
3. Yao K, Lee E, Bentrem D, England G, Schafer J, O'Regan R et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000; 6:2028-2036.
4. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 2005; 97:1746-1759.
5. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 2005; 94:131-141.
6. Lønning P, Taylor P, Anker G, Iddon J, Wie L. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat 2001; 67:111-116.
7. Ellis M, Gao F, Dehdashti F, Jeffe D, Marcom P, Carey L et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor–positive, aromatase inhibitor–resistant advanced breast cancer: A phase 2 randomized study. JAMA 2009; 302:774-780.
8. Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H et al. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. JAMA 2004; 291:1701-1712.
9. Anderson G, Chlebowski R, Aragaki A, Kuller L, Manson J, Gass M et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. Lancet Oncol 2012; 13:476-486.
10. Obiorah I, Jordan VC. 2012 NAMS/PFIZER- Wulf H. utian endowed lecture. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. Menopause 2013; 20:372-382.
11. Wu AH, Yu MC, Tseng CC, Pike MC. Epidemiology of soy exposures and breast cancer risk. Br J Cancer 2008; 98:9-14.
12. Nagata C, Mizoue T, Tanaka K, Tsuji I, Tamakoshi A, Matsuo K et al. Soy Intake and Breast Cancer Risk: An Evaluation Based on a Systematic Review of Epidemiologic Evidence Among the Japanese Population. Japanese Journal of Clinical Oncology 2014; 44:282-295.
13. Chen M, Rao Y, Zheng Y, Wei S, Li Y, Guo T et al. Association between Soy Isoflavone Intake and Breast Cancer Risk for Pre- and Post-Menopausal Women: A Meta-Analysis of Epidemiological Studies. PLoS One 2014; 9.
14. Trock BJ, Hilakivi-Clarke L, Clarke R. Meta-analysis of soy intake and breast cancer risk. J Natl Cancer Inst 2006; 98:459-471.

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

15. Lee SA, Shu XO, Li H, Yang G, Cai H, Wen W et al. Adolescent and adult soy food intake and breast cancer risk: results from the Shanghai Women's Health Study. Am J Clin Nutr 2009; 89:1920-1926.
16. Thanos J, Cotterchio M, Boucher BA, Kreiger N, Thompson LU. Adolescent dietary phytoestrogen intake and breast cancer risk (Canada). Cancer Causes Control 2006; 17:1253-1261.
17. Wu AH, Wan P, Hankin J, Tseng CC, Yu MC, Pike MC. Adolescent and adult soy intake and risk of breast cancer in Asian-Americans. Carcinogenesis 2002; 23:1491-1496.
18. Whitsett TG, Jr., Lamartiniere CA. Genistein and resveratrol: mammary cancer chemoprevention and mechanisms of action in the rat. Expert Rev Anticancer Ther 2006; 6:1699-1706.
19. Murrill WB, Brown NM, Zhang JX, Manzolillo PA, Barnes S, Lamartiniere CA. Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. Carcinogenesis 1996; 17:1451-1457.
20. Hsieh C-Y, Santell RC, Haslam SZ, Helferich WG. Estrogenic Effects of Genistein on the Growth of Estrogen Receptor-positive Human Breast Cancer (MCF-7) Cells in Vitro and in Vivo. Cancer Res 1998; 58:3833-3838.
21. Le Bail J-C, Champavier Y, Chulia A-J, Habrioux G. Effects of phytoestrogens on aromatase, 3β and 17β-hydroxysteroid dehydrogenase activities and human breast cancer cells. Life Sci 2000; 66:1281-1291.
22. Zava DT, Duwe G. Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro. Nutr Cancer 1997; 27:31-40.
23. Pagliacci MC, Smacchia M, Migliorati G, Grignani F, Riccardi C, Nicoletti I. Growth-inhibitory effects of the natural phyto-oestrogen genistein in MCF-7 human breast cancer cells. Eur J Cancer 1994; 30:1675-1682.
24. Shao ZM, Shen ZZ, Fontana JA, Barsky SH. Genistein's "ER-dependent and independent" actions are mediated through ER pathways in ER-positive breast carcinoma cell lines. Anticancer Res 2000; 20:2409-2416.
25. McMichael-Phillips DF, Harding C, Morton M, Roberts SA, Howell A, Potten CS et al. Effects of soy-protein supplementation on epithelial proliferation in the histologically normal human breast. Am J Clin Nutr 1998; 68:1431S-1435S.
26. Petrakis NL, Barnes S, King EB, Lowenstein J, Wiencke J, Lee MM et al. Stimulatory influence of soy protein isolate on breast secretion in pre- and postmenopausal women. Cancer Epidemiol Bio Prev 1996; 5:785-794.
27. Hargreaves DF, Potten CS, Harding C, Shaw LE, Morton MS, Roberts SA et al. Two-Week Dietary Soy Supplementation Has an Estrogenic Effect on Normal Premenopausal Breast. J Clin Endocrinol Metabol 1999; 84:4017-4024.
28. Thompson LU, Chen JM, Li T, Strasser-Weippl K, Goss PE. Dietary flaxseed alters tumor biological markers in postmenopausal breast cancer. Clin Cancer Res 2005; 11:3828-3835.
29. Sartippour MR, Rao JY, Apple S, Wu D, Henning S, Wang H et al. A Pilot Clinical Study of Short-Term Isoflavone Supplements in Breast Cancer Patients. Nutr Cancer 2004; 49:59-65.
30. Palomares MR, Hopper L, Goldstein L, Lehman CD, Storer BE, Gralow JR. Effect of soy isoflavones on breast proliferation in postmenopausal breast cancer survivors. Breast Cancer Res Treat 2004; 88 4002.

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Author Manuscript Published OnlineFirst on June 3, 2014; DOI: 10.1158/1940-6207.CAPR-14-0061
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

31. Cheng G, Wilczek B, Warner M, Gustafsson J, Landgren B. Isoflavone treatment for acute menopausal symptoms. Menopause 2007; 14:468–473.
32. Fink BN, Steck SE, Wolff MS, Britton JA, Kabat GC, Gaudet MM et al. Dietary flavonoid intake and breast cancer survival among women on Long Island. Cancer Epidemiol Biomarkers Prev 2007; 16:2285-2292.
33. Swann R, Perkins KA, Velentzis LS, Ciria C, Dutton SJ, Mulligan AA et al. The DietCompLyf study: A prospective cohort study of breast cancer survival and phytoestrogen consumption. Maturitas 2013; 75:232-240.
34. Shu XO, Zheng Y, Cai H, Gu K, Chen Z, Zheng W et al. Soy food intake and breast cancer survival. JAMA 2009; 302:2437-2443.
35. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 1992; 90:77-86.
36. Ariazi E, Cunliffe H, Lewis-Wambi JS, Slifker M, Willis A, Ramos P et al. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci USA 2011; 108:18879-18886.
37. Fan P, Agboke FA, McDaniel RE, Sweeney EE, Zou X, Creswell K et al. Inhibition of c-Src blocks oestrogen-induced apoptosis and restores oestrogen-stimulated growth in long-term oestrogen-deprived breast cancer cells. Eur J Cancer 2014; 50:457-468.
38. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T et al. Intrinsic Mechanism of Estradiol-Induced Apoptosis in Breast Cancer Cells Resistant to Estrogen Deprivation. J Natl Cancer Inst 2005a; 97:1746-1759.
39. Sengupta S, Sharma C, Jordan VC. Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells. Horm Mol Biol Clin Investig 2010; 2:235-243.
40. Traganos F, Ardelt B, Halko N, Bruno S, Darzynkiewicz Z. Effects of genistein on the growth and cell cycle progression of normal human lymphocytes and human leukemic MOLT-4 and HL-60 cells. Cancer Res 1992; 52:6200-6208.
41. Sakamoto T, Horiguchi H, Oguma E, Kayama F. Effects of diverse dietary phytoestrogens on cell growth, cell cycle and apoptosis in estrogen-receptor-positive breast cancer cells. J Nutr Biochem 2010; 21:856-864.
42. Fritz WA, Coward L, Wang J, Lamartiniere CA. Dietary genistein: perinatal mammary cancer prevention, bioavailability and toxicity testing in the rat. Carcinogenesis 1998; 19:2151-2158.
43. Lamartiniere CA, Wang J, Smith-Johnson M, Eltoum IE. Daidzein: bioavailability, potential for reproductive toxicity, and breast cancer chemoprevention in female rats. Toxicol Sci 2002; 65:228-238.
44. Andrade JE, Ju YH, Baker C, Doerge DR, Helferich WG. Long-term exposure to dietary sources of genistein induces estrogen-independence in the human breast cancer (MCF-7) xenograft model. Mol Nutr Food Res 2014; 24:201300780.
45. Peterson G, Barnes S. Genistein inhibits both estrogen and growth factor-stimulated proliferation of human breast cancer cells. Cell Growth Differ 1996; 7:1345-1351.
46. Peterson G. Evaluation of the Biochemical Targets of Genistein in Tumor Cells. J Nutr 1995; 125:784S-789S.

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Author Manuscript Published OnlineFirst on June 3, 2014; DOI: 10.1158/1940-6207.CAPR-14-0061
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

47. Diel P, Geis R-B, Caldarelli A, Schmidt S, Leschowsky UL, Voss A et al. The differential ability of the phytoestrogen genistein and of estradiol to induce uterine weight and proliferation in the rat is associated with a substance specific modulation of uterine gene expression. Molecular and Cellular Endocrinology 2004; 221:21-32.
48. Kostelac D, Rechkemmer G, Briviba K. Phytoestrogens modulate binding response of estrogen receptors alpha and beta to the estrogen response element. J Agric Food Chem 2003; 51:7632-7635.
49. Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D. Perk Is Essential for Translational Regulation and Cell Survival during the Unfolded Protein Response. Mol cell 2000; 5:897-904.
50. Fan P, Griffith OL, Agboke F, Anur P, Zou X, McDaniel RE et al. c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. Cancer Res 2013; 73:4510-4520.
51. Verfaillie T, Rubio N, Garg AD, Bultynck G, Rizzuto R, Decuypere JP et al. PERK is required at the ER-mitochondrial contact sites to convey apoptosis after ROS-based ER stress. Cell Death Differ 2012; 19:1880-1891.
52. Ron D, Habener JF. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes Dev 1992; 6:439-453.
53. Styner M, Meyer MB, Galior K, Case N, Xie Z, Sen B et al. Mechanical strain downregulates C/EBPbeta in MSC and decreases endoplasmic reticulum stress. PLoS One 2012; 7:12.
54. Haddow A, Watkinson JM, Patterson E. Influence of synthetic oestrogens upon advanced malignant disease BMJ 1944; 2:393-398.
55. Haddow A, David A. Karnosky Memorial lecture: Thoughts on chemical therapy. Cancer Res 1970; 26:737-754.
56. Stoll B: Palliation by castration or by hormone administration. In Stoll B, ed. Breast Cancer Management Early and Late. London, UK: William Herman Medical Books Ltd; 1977;133-146.
57. Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. Lancet 2013; 381:805-816.
58. Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 1993; 127:23 - 33.

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FIGURE LEGENDS

Figure 1.

Growth characteristics of 17β-estradiol, equilin and phytoestrogens in breast cancer cells

(A) MCF7:WS8 cells were seeded in 24-well plate and treated with steroidal and phytoestrogens over a range of doses for seven days. Cell growth was assessed as DNA content in each well. **(B)** Inhibition of cell growth in MCF7:5C cells by genistein, equol and coumestrol was assessed in comparison to E_2 and equilin. Each data point is average +/- SD of three replicates. [* $P< 0.05$].

Figure 2.

Induction of apoptosis by phytoestrogens and steroidal estrogens

(A) MCF7:5C cells were treated with 0.1% ethanol vehicle (control), or 1nM E_2, 1nM equilin or phytoestrogens (1μM) for 72h and then stained with annexin v-FITC and propidium iodide and analysed by flow cytometry. Increased apoptotic effect is observed in the right upper and lower quadrant. E_2, equilin and phytoestrogens increase **(B)** *BIM, TNF* **(C)** *FAS* and *FADD* mRNA levels. PCR data values are presented as fold difference versus vehicle treated cells ± SEM. [* $P< 0.05$]

Figure 3.

Steroidal and phytoestrogens act as agonists via an estrogen receptor dependent mechanism

(A) MCF7:5C cells were treated with 0.1% ethanol vehicle (control), 1nM E_2, 1nM equilin or phytoestrogens (1μM). Total RNA was isolated after 24h and reverse transcribed and PS2, GREB1, progesterone receptor (PgR) mRNA levels was obtained using RT-PCR. (B) Various

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concentrations of 4-hydroxytamoxifen (4OHT) block steroidal estrogen- or phytoestrogen mediated growth inhibition (C) MCF-7:5C cells were treated with vehicle (control) and steroidal and phyto-estrogens for 24 hours. ERα and ERβ protein was detected by immunoblotting. (D) ERα and ERβ mRNA was quantified with real time PCR (RT-PCR). *, $P < 0.05$, compared with control.

Figure 4

ERα is required for estrogen induced growth inhibition and apoptosis

MCF7:5C cells were transfected with either non target RNA (consi) or siRNA of ERα for 72 h. (A) ERα was detected by immunoblotting. Then, cells were treated with either control (0.1% EtOH), 1nM steroidal estrogens or 1μM phytoestrogens for (B) 72h and apoptosis was determined using annexin V binding assay.(C) Growth inhibition in the transfected cells was assessed after 6 days of treatment with indicated compounds using DNA quantification assay. [* P< 0.05]

Figure 5

Endoplasmic reticulum stress and inflammatory stress response are involved phytoestrogen induced apoptosis

(A) The indicated estrogens induce endoplasmic reticulum stress related genes, *DDTT3* and *IRE1α*. (B) MCF-7:5C were treated with E_2 (1nM), equilin (1nM) or phytoestrogen (1μM) for 24h. IRE1α and phosphorylated eIF2α were used as indicators of UPR activation and their protein expression were examined by immunoblotting. Total eIF2α and β-actin were determined for loading controls. Indicators of inflammatory stress response (C) caspase4, *CEBP β*, (D) *IL6* and *LTB* were activated by E_2, equilin and phytoestrogens. [* P< 0.05]

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Figure 6

Inflammation is important for phytoestrogen mediated apoptosis

(A) Cells were treated with the indicated estrogens in presence of increasing concentration of dexamethasone(dexa). (B) Dexamethasone completely reverses E_2, equilin and all phytoestrogen induced apoptosis. Apoptosis was assessed using the flow cytometry. Dexamethasone blocked the induction of **(C)** *CEBP β,*caspase 4 **(D)** *BIM,* and *TNF* by E_2, equilin and phytoestrogens. [* $P< 0.05$]

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Figure 1

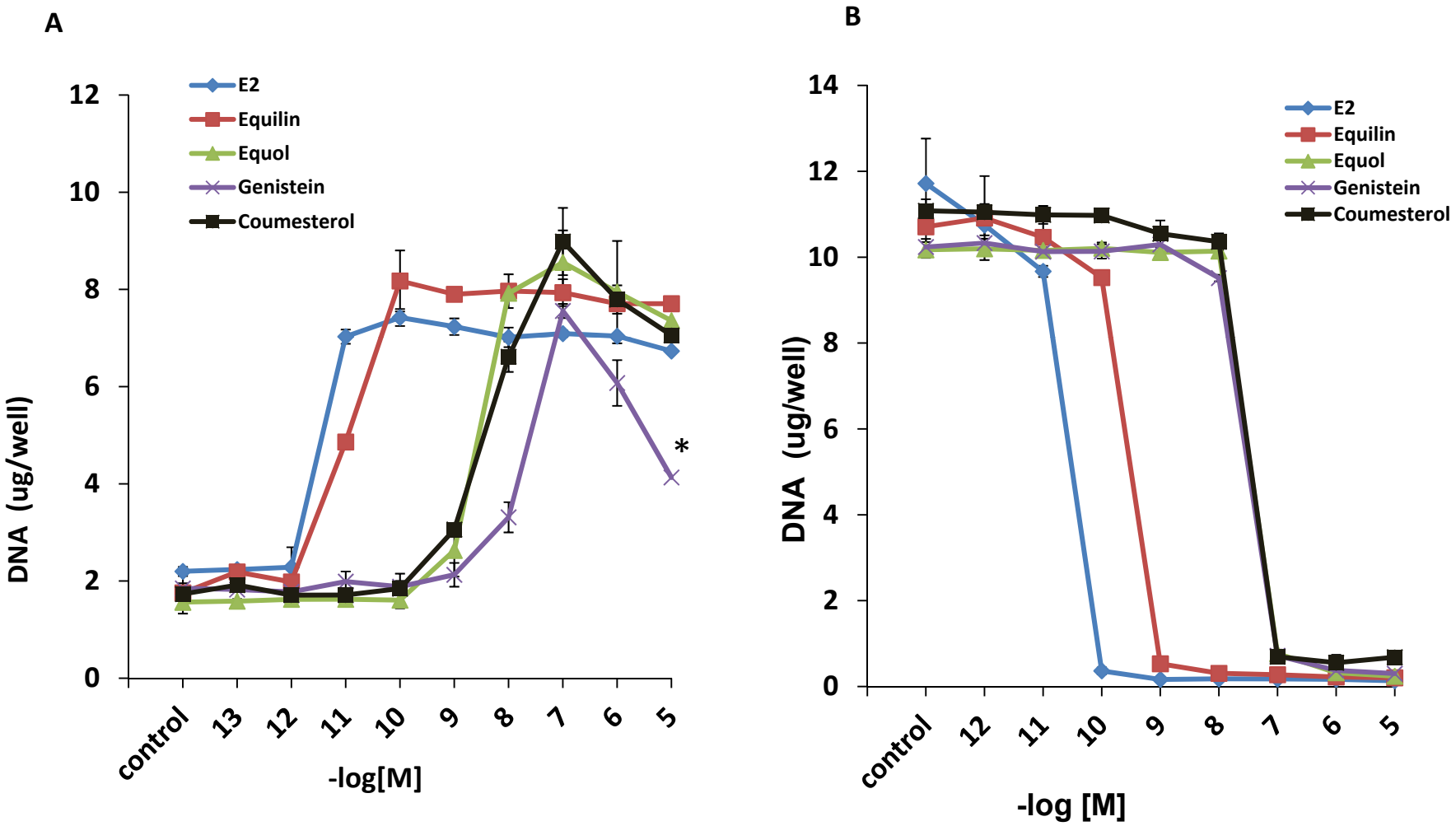

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Figure 2

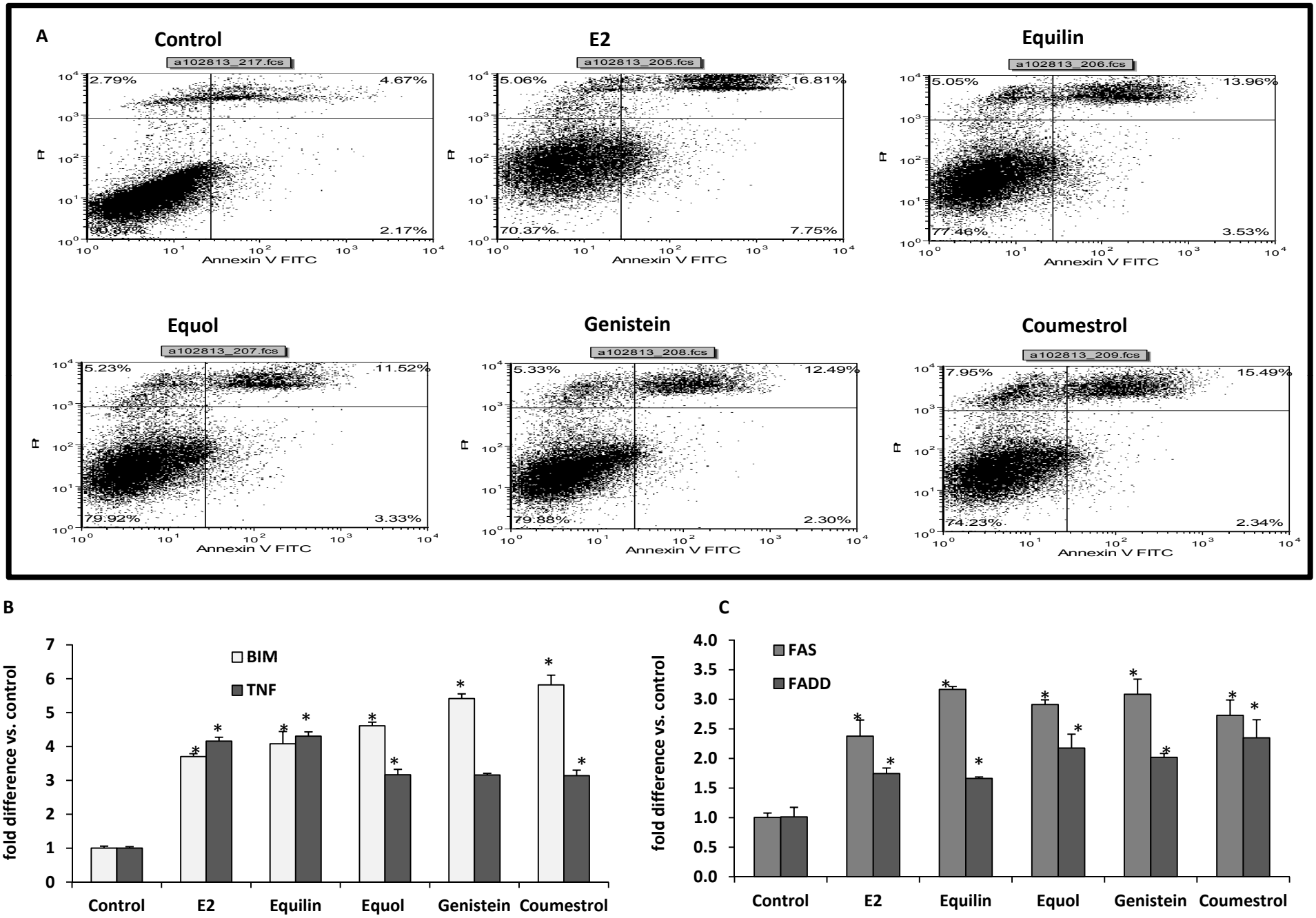
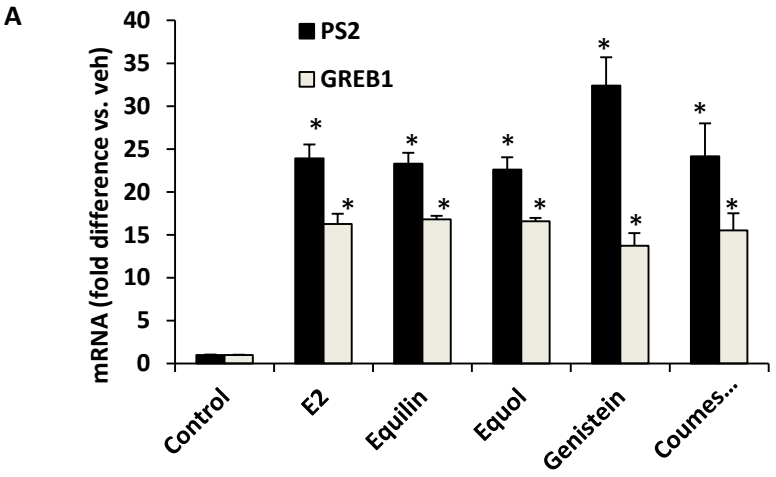
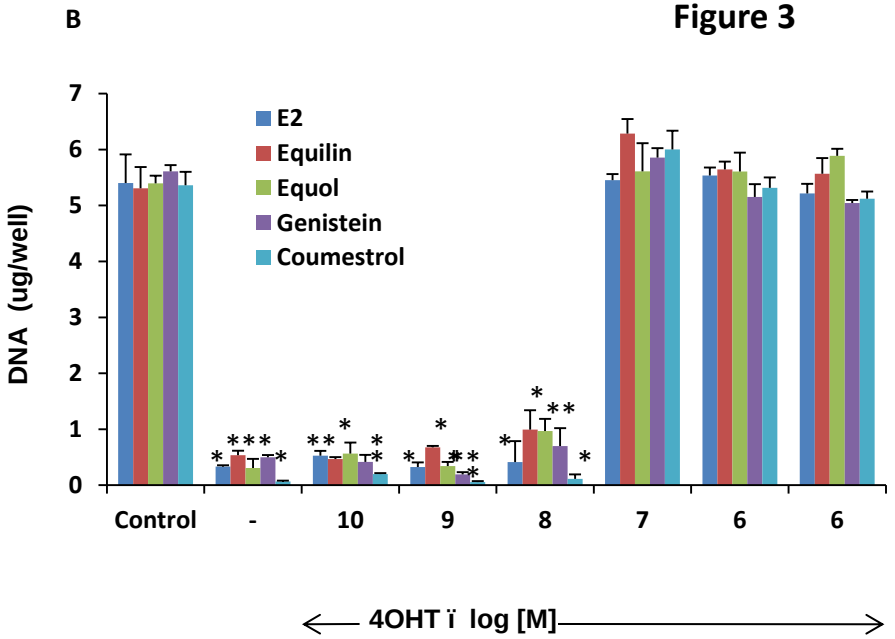
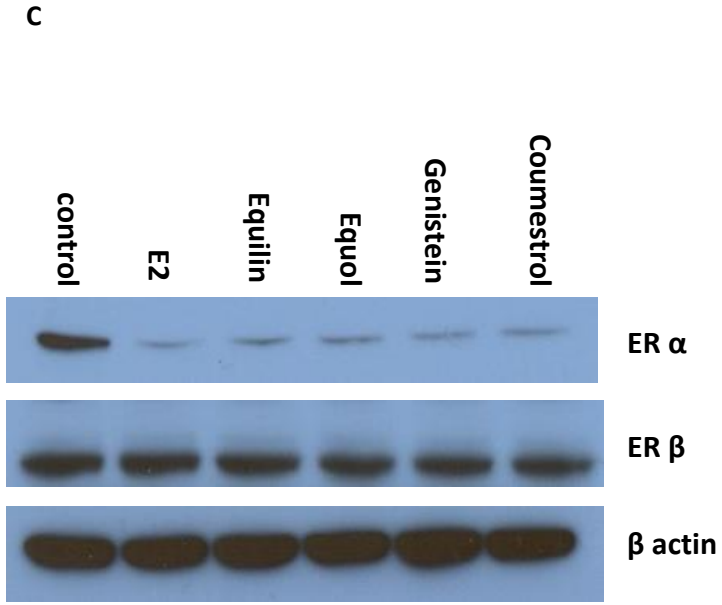


Figure 3







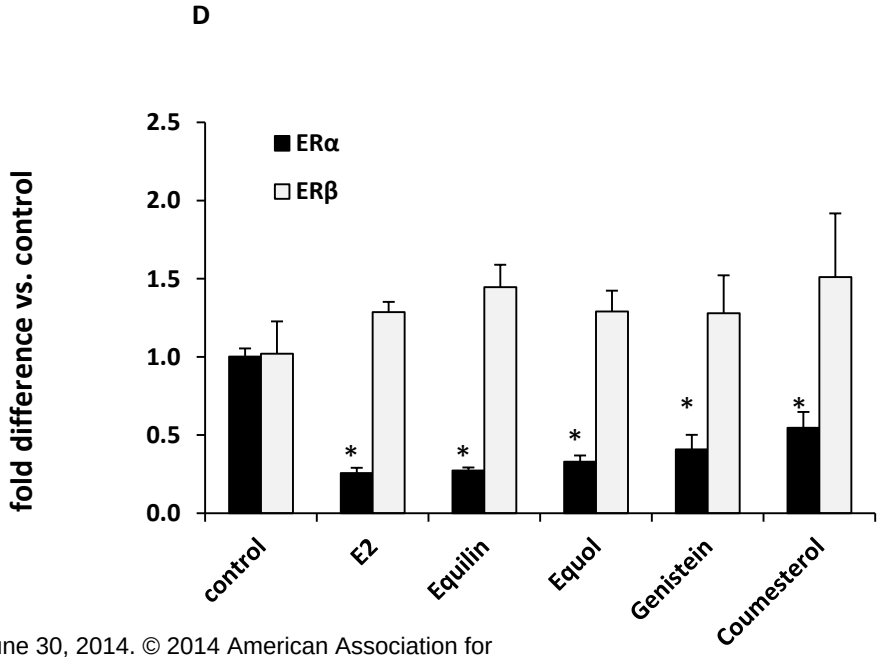


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Figure 4

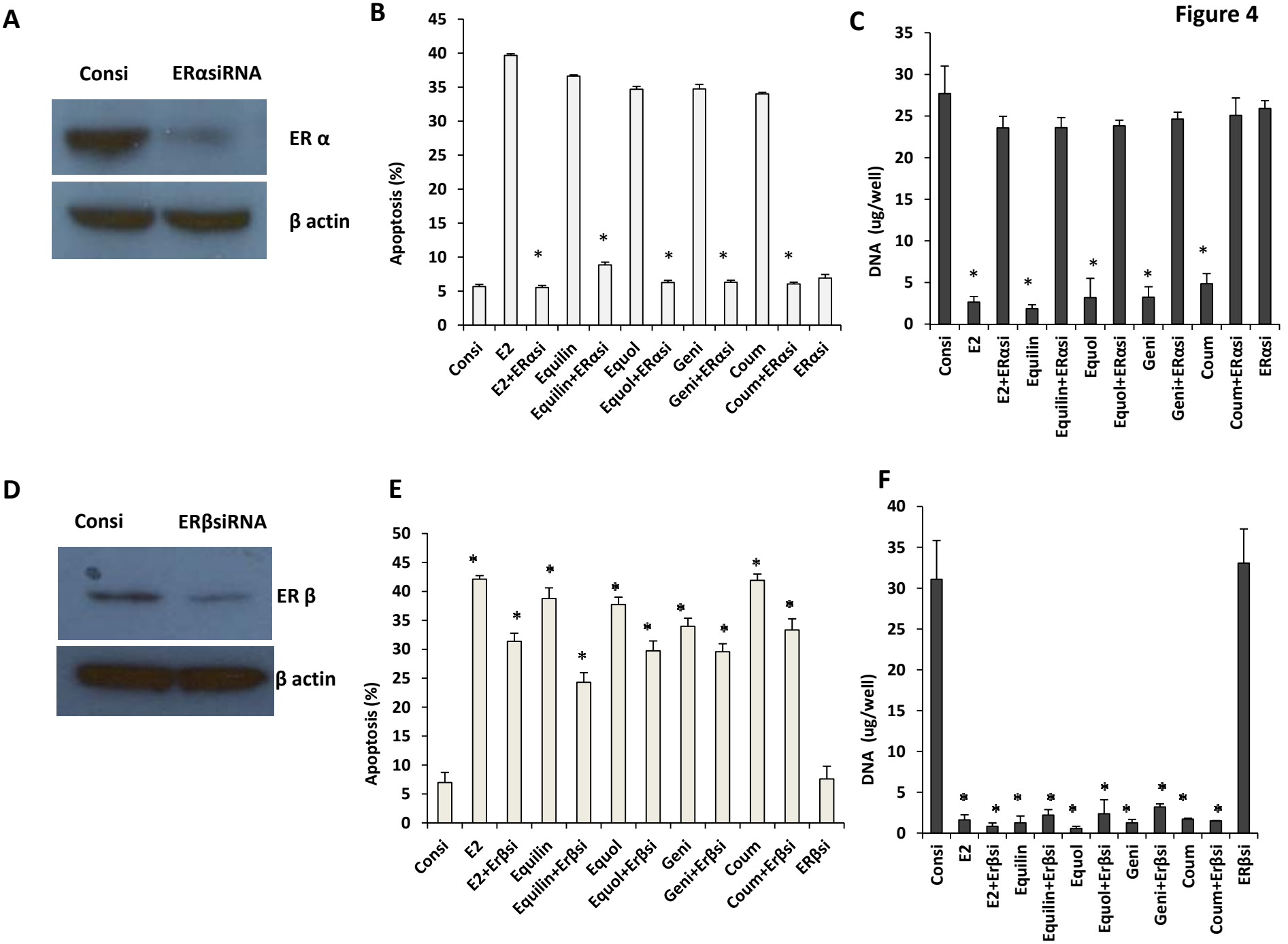
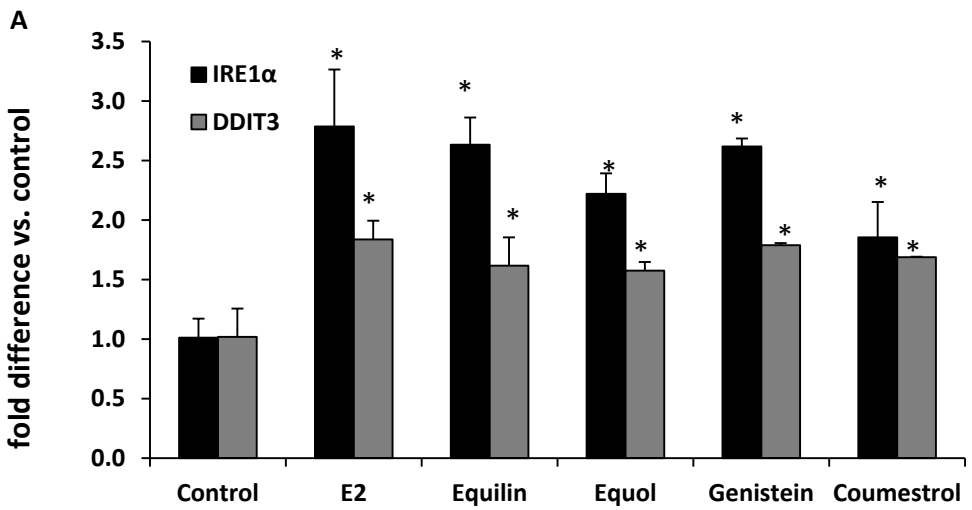
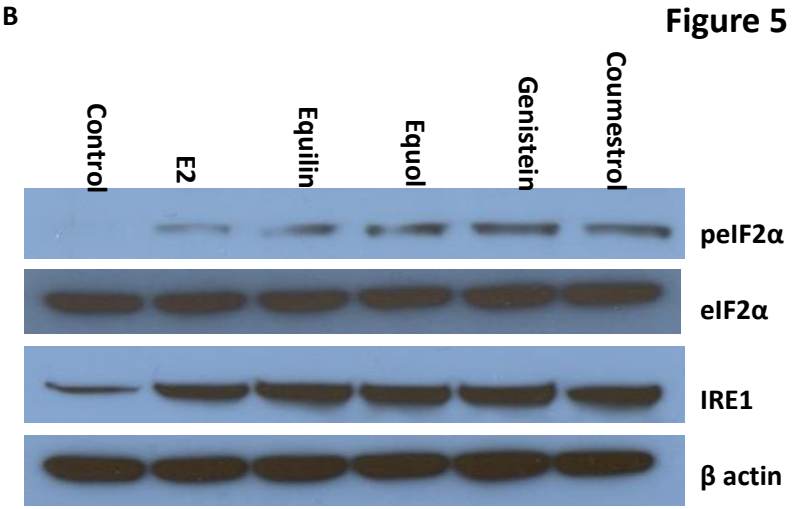
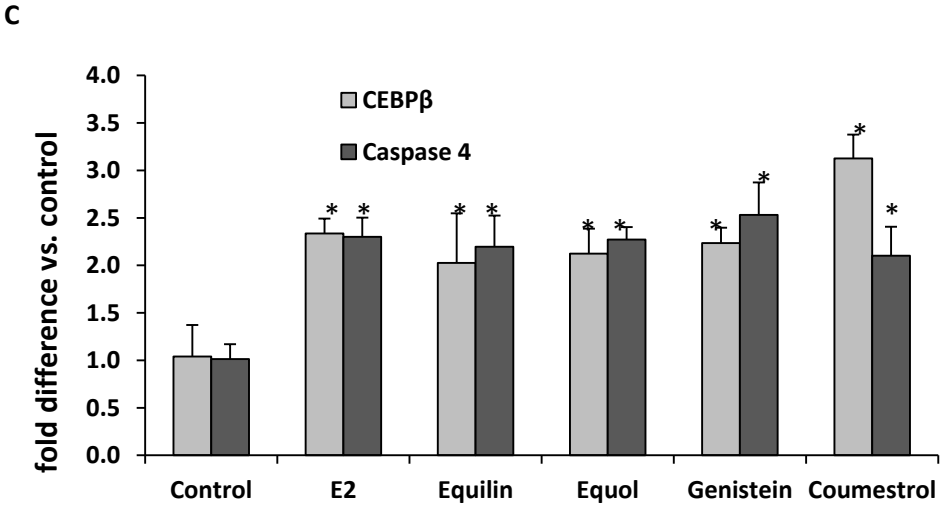


Figure 5







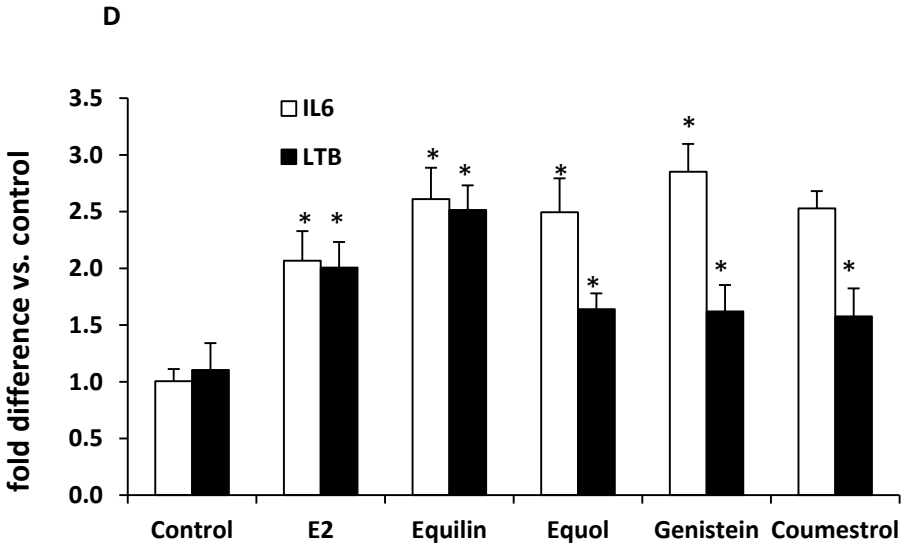


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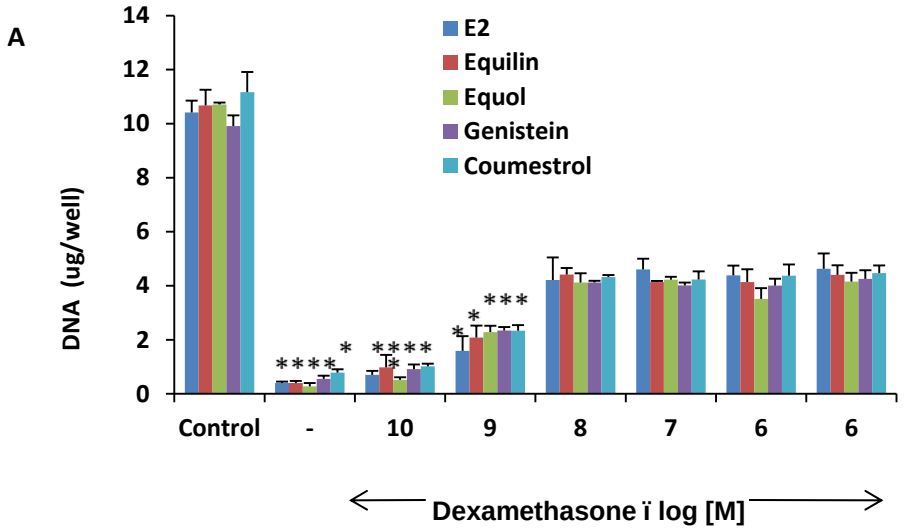
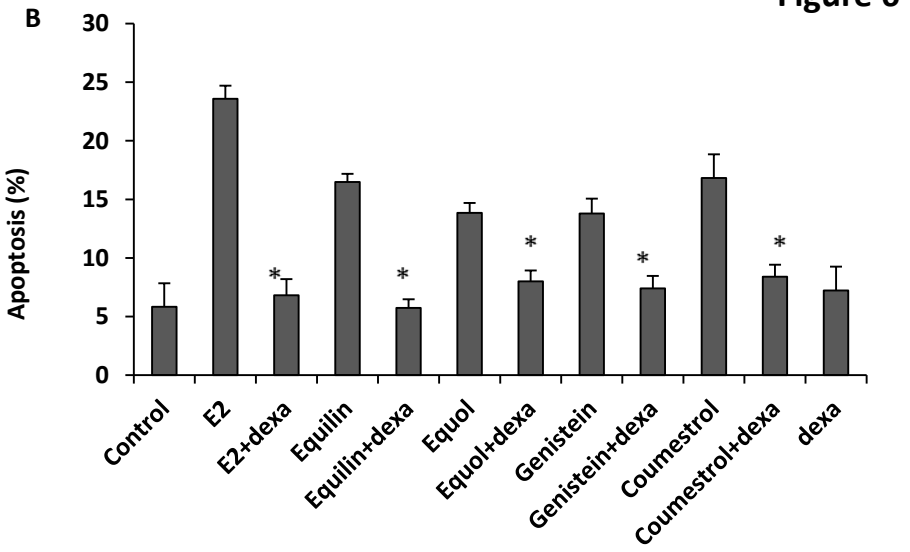
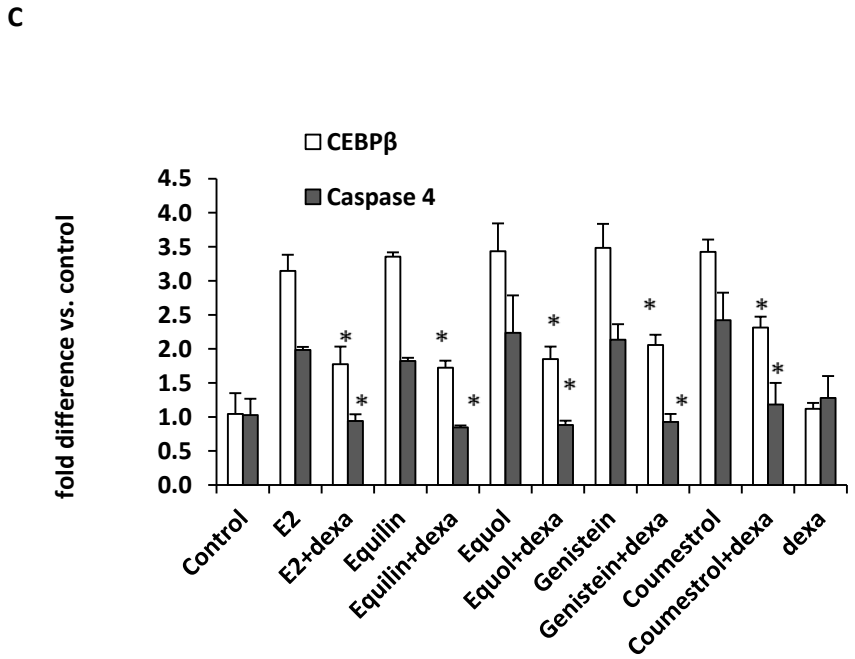
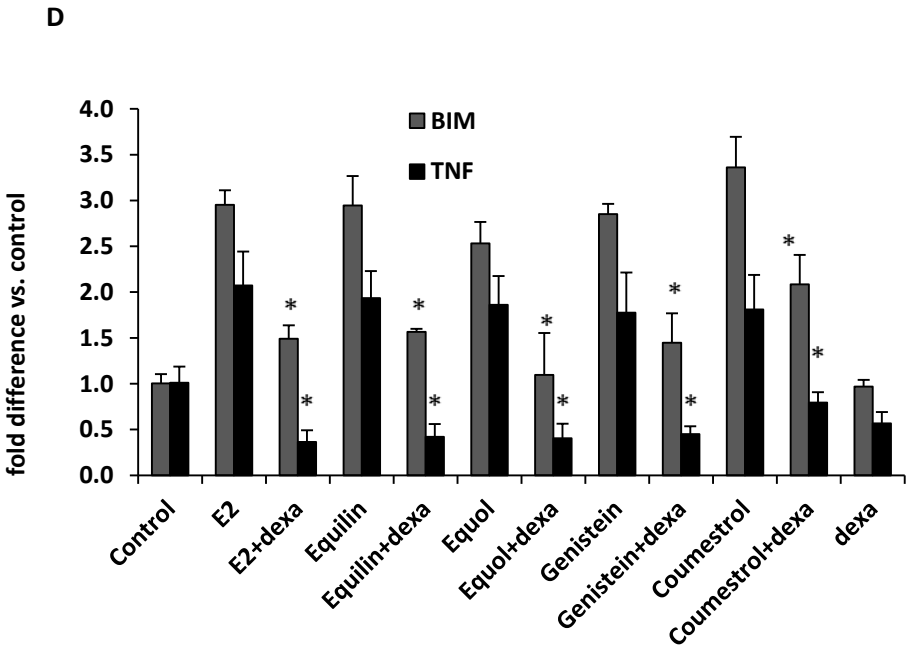
Figure 6









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Pharmacological relevance of endoxifen in a laboratory simulation of breast cancer in postmenopausal patients

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Background: Tamoxifen is metabolically activated via a CYP2D6 enzyme system to the more potent hydroxylated derivatives 4-hydroxytamoxifen and endoxifen. This study addresses the pharmacological importance of endoxifen by simulating clinical scenarios in vitro.

Methods: Clinical levels of tamoxifen metabolites in postmenopausal breast cancer patients previously genotyped for CYP2D6 were used in vitro along with clinical estrogen levels (estrone and estradiol) in postmenopausal patients determined in previous studies. The biological effects on cell growth were evaluated in a panel of ER positive breast cancer cell lines via cell proliferation assays and real-time PCR. Data were analyzed with 1 and 2-way ANOVA and Student t-test. All statistical tests were two-sided.

Results: Postmenopausal levels of estrogens induced proliferation of all test breast cancer cell lines (mean fold induction ± SD vs. vehicle control: MCF-7, 11±1.74 ($P<0.001$); T47D, 7.52±0.72 ($P<0.001$); BT474, 1.75±0.23 ($P<0.001$); ZR-75-1, 5.5±1.95 ($P=0.001$). Tamoxifen and primary metabolites completely inhibited cell growth regardless of the CYP2D6 genotype in all cell lines (mean fold induction ± SD vs. vehicle control: MCF-7, 1.57±0.38 ($P=0.54$); T47D, 1.17±0.23 ($P=0.79$); BT474, 0.96±0.2 ($P=0.98$); ZR-75-1, 0.86±0.67 ($P=0.99$). Interestingly, tamoxifen and its primary metabolites were not able to fully inhibit the estrogen-stimulated expression of estrogen-responsive genes in MCF-7 cells ($P<0.05$ for all genes), but addition of endoxifen was able to produce additional antiestrogenic effect on these genes.

Conclusions: The results indicate that tamoxifen and other metabolites, excluding endoxifen, completely inhibit estrogen stimulated growth in all cells lines, but additional antiestrogenic action from endoxifen is necessary for complete blockade of estrogen stimulated genes.

Endoxifen is of supportive importance for the therapeutic effect of tamoxifen in a postmenopausal setting.

Introduction

Tamoxifen, a nonsteroidal antiestrogen, is a pioneering therapy for the treatment of breast cancer targeted to the tumor estrogen receptor (ER) (1). The laboratory strategy (2) of targeting tumor ER with long-term antihormone therapy accurately translated to clinical practice with enhanced control of disease recurrence following long term adjuvant therapy in both pre- and postmenopausal patients. This targeted adjuvant treatment strategy ultimately resulted in a 30% decrease in mortality (3-6). Indeed, long term (5 years) adjuvant tamoxifen therapy contributes substantially to national statistics with the reduction in death rate from breast cancer during the past decade (7).

Early findings demonstrated that tamoxifen is metabolically activated to 4-hydroxytamoxifen (Fig. 1) that has a hundred fold increase in affinity for the ER (8, 9). However, high affinity of a ligand for the ER is an advantage but not a requirement for antiestrogenic activity (10, 11). Indeed, studies comparing the antitumor activity of tamoxifen and 4-hydroxytamoxifen (4OHT) *in vivo* demonstrated that tamoxifen was the superior agent for clinical development (10). 4-Hydroxytamoxifen is cleared from the body faster than tamoxifen while the parent drug tamoxifen accumulates (12). Nevertheless, the subsequent identification of 4-hydroxy-N-desmethyltamoxifen (13-15) (later called endoxifen) (Fig. 1) and the demonstration of a reduction of endoxifen production in women taking Selective Serotonin Reuptake Inhibitors (SSRI) (for reduction of hot flashes), which block the enzymatic activity of CYP2D6 (16), suggested that this important drug interaction reduces tamoxifen's efficacy. Subsequently, a hypothesis was developed and connected with genotypic aberrations in CYP2D6 alleles (17). The hypothesis depends on the concept that different polymorphisms of CYP2D6 can result in altered enzymatic activity of the cytochrome P450 2D6, which results in different rates of tamoxifen metabolism and thus different levels of tamoxifen metabolites N-desmethyltamoxifen (NDMTAM) and endoxifen.

Some clinical studies have shown association between the CYP2D6 genotypes and the clinical outcomes (18-20), and some have not found any association (21-23). Numerous papers using retrospective clinical data presented convincing cases for or against the hypothesis. However, the low incidence of poor metabolizers (PM) (24), and the known poor compliance rate (25) of long term anti-hormone therapies make the hypothesis difficult to validate in retrospective patient samples.

In this article we address the role of the CYP2D6 genotype during tamoxifen treatment of breast cancer in a laboratory simulation. We have characterized a panel of 4 representative ER positive breast cancer cell lines: MCF-7, T47D, BT474, and ZR-75-1, and carefully calibrated their concentration responsiveness to both estradiol (E_2) and estrone (E_1). We selected concentrations of total estrogen (E_1/E_2) corresponding to the previously reported circulating levels in postmenopausal breast cancer patients (26, 27). We address two hypotheses 1) that tamoxifen is a prodrug that needs to be metabolically activated to NDMTAM and 4OHT, and 2) that circulating concentrations of tamoxifen and its two metabolites NDMTAM and 4OHT are sufficient to block estrogen stimulated breast cancer cell growth and gene expression in a postmenopausal patient simulation. Our chosen concentrations of tamoxifen and metabolites have been determined recently for postmenopausal patients genotyped for CYP2D6 as EM (Extensive Metabolizers), IM (Intermediate Metabolizers), and PM (28). The experiment was repeated with all cell lines to address the second hypothesis with and without relevant concentrations of endoxifen for each genotype. Overall, metabolic activation to endoxifen appears to play a supportive role in blocking estrogen induced cell replication in breast cancer cell lines in a simulated model of postmenopausal breast cancer. Conversion to endoxifen is perhaps consolidating the long-term benefits of antiestrogen therapy but is not essential for the immediate antiestrogenic antitumor actions of tamoxifen in the postmenopausal setting.

Methods

Cells and Culture Conditions

The ER-positive human breast cancer cell lines MCF7, T47D, BT474 and ZR-75-1 were used in this study. All cells were obtained from American Type Culture Collection (ATCC) and were maintained in phenol-red RPMI 1640 medium. All cultures were grown in 5% CO_2 at 37°C. All cell lines were verified using DNA fingerprinting analysis performed at the Tissue Culture shared resource at the Lombardi Comprehensive Cancer Center (29).

Cell proliferation assays.

All cells were cultured in estrogen-free medium (phenol red-free RPMI 1640 media supplemented with 10% charcoal-stripped FBS) for 3 days before beginning the proliferation assay. The proliferation assays were performed after a 7 day treatment as previously described (30) using a fluorescent DNA quantification kit and calf thymus DNA for the standard curve. To study the pharmacological importance of endoxifen we simulated the postmenopausal estrogen setting for patients in each of our cell lines and used the actual clinical concentrations of tamoxifen and its metabolites found in patients previously genotyped for CYP2D6 and treated with tamoxifen (Supplementary Table 1, available online). To simulate the clinical setting in vitro we selected a panel of ER positive human breast cancer cell lines: MCF-7, T47D, BT474 and ZR-75-1; all were previously shown to be ER positive (31) by Western blotting and estrogen-responsive for growth. Experiments, each consisting of 3 replicates, were performed at least 3 times.

Real-time PCR

Real-time PCR was performed as previously described (32). Primers sequences that were used for human pS2 cDNA amplification are: 5'-CATCGACGTCCCTCCAGAAGA-3' sense, and 5'-CTCTGGGACTAATCACCGTGCTG-3' anti-sense; human GREB1 gene: 5'-CAAAGAATAACCTGTTGGCCCTGC-3'sense,5'-GACATGCCTGCGCTCTCATACTTA-

3’ anti-sense; human progesterone receptor (PgR): 5’-CGTGCCTATCCTGCCTCTCAA-3’ sense, 5’-CCGCCGTCGTAACTTTCGT-3’ anti-sense; the reference gene 36B4: 5’-GTGTTCGACAATGGCAGCAT-3’ sense, 5’-GACACCCTCCAGGAAGCGA-3’ anti-sense. All primers were obtained from Integrated DNA Technologies Inc. (Coralville, IA). Experiments, each consisting of 3 replicates, were performed at least 3 times.

Immunoblotting

Immunoblotting was performed as previously described (30).The membranes were probed with rabbit polyclonal anti-ERα antibody at 1:500 dilution, (Santa Cruz Biotechnology, Santa Cruz, CA) and with mouse monoclonal anti-β-actin antibody at 1:40,000 dilution (Sigma-Aldrich, St. Louis, MO) antibodies as recommended by the supplier. All protein levels were analyzed by densitometry using ImageJ software (NIH). Experiments, each consisting of 3 replicates, were performed at least 3 times.

Molecular Modelling

The experimental X-ray structures of ERα LBD to be used for docking were selected from Protein Databank (33) based on the three-dimensional shape similarity between the compounds to be docked and co-crystallized ligands extracted from the receptor - ligand complex similarity. The three-dimensional shape was computed using the ROCS utility of Openeye. The ligands of interest were used as the query dataset while the screening library was compiled from the ligands extracted from all of the available crystal structures of human ERα deposited in PDB. ShapeTanimoto parameter was used for scoring with a cutoff value of 0.8 and 4 ligands met this criterion. The 3D coordinates of the corresponding ERα complexes were extracted from PDB entries 3ERT (34). Subsequently, the structures were prepared for docking using the Protein Preparation Workflow (Schrödinger, LLC, New York, NY, 2011) accessible from within the Maestro program (Maestro, version 9.2; Schrödinger, LLC, New York, NY, 2011). Briefly, the hydrogens were properly added to the complexes, water

molecules beyond 5Å from a heteroatom were deleted, bond corrections were applied to the co-crystallized ligands, the orientation of hydroxyl groups, Asn, Gln and the protonation state of His were optimized to maximize hydrogen bond formation. All Asp, Glu, Arg and Lys residues were left in their charged state. In the final stage a restrained minimization on the ligand-protein complexes was carried out with the OPLS_2001 force field and the default value for RMSD of 0.30Å for non-hydrogen atoms was used. Docking simulations were performed with Glide software (Glide, version 5.7; Schrödinger, LLC, New York, NY, 2011), a grid-based docking method which can be run rigid or fully flexible for the ligand (35, 36). To some extent, a degree of flexibility was allowed to the X-ray structures of ERα in agonist conformation by scaling down the van der Waals radii of non-polar atoms with a scale factor of 0.8 and allowing the free rotation of hydroxyl groups.

Statistical Analysis

To test the interactions between treatment and genotype in the proliferation assays, we used ANOVA with a balanced 2-factor design. When a statistically significant interaction was present, we used one way ANOVA to investigate simple effects of the independent factors. A P-value less than 0.05 was considered significant. All computations were carried out using R, Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria). For testing the significance of treatment in RT-PCR experiments the Student's t-test was used. All statistical tests were two-sided.

Results

Effects of estrogens, tamoxifen and metabolites, and endoxifen on cell proliferation

Concentration response curves for all cell lines with increasing concentrations of either E_1 or E_2 are shown in Figure 2. The estrogen levels in postmenopausal patients treated with tamoxifen alone that were used are based on the calculated levels of E_1 and E_2 in patient plasma reported in the literature (26, 27). The recalculated concentrations used for all assays were $7.8x10^{-11}$M for E_1 (which corresponds to the average 21 pg/mL in patient plasma) and $4.7x10^{-11}$M for E_2 (which corresponds to the average 13 pg/mL in patient plasma); these concentrations combined were used in subsequent treatments of the cells to simulate the postmenopausal setting. Estrogen levels were also consistent with plasma levels reported by other groups (37, 38) for postmenopausal patients. To simulate the clinical setting of postmenopausal patients treated with tamoxifen, we used concentrations of tamoxifen and all its metabolites previously published for a clinical study of different CYP2D6 genotypes (28) (Supplementary Table 1). The metabolic activation of tamoxifen to endoxifen by different CYP2D6 genotypes occurs at different rates, resulting in different plasma levels of endoxifen, NDMTAM and tamoxifen itself. 4OHT remains consistently low. Using the published concentrations of tamoxifen and its metabolites, along with the calculated concentrations of estrogens, we treated all cell lines and assessed the biological effect on growth using a 7 day proliferation assay.

All cell lines (Figure. 3) were responsive to treatment with the estrogen combination (E_1/E_2) alone (mean fold induction ± SD vs. vehicle control: MCF-7, 11±1.74, $P<0.001$, Figure 3A; T47D, 7.52±0.72, $P<0.001$, Figure 3B; BT474, 1.75±0.23, $P<0.001$, Figure 3C; ZR-75-1, 5.5±1.95, $P=0.001$, Figure 3D; all P by 1-way ANOVA).

The addition of tamoxifen plus primary metabolites NDMTAM and 4OHT (TPM) to E_1/E_2 (Figure 3) completely inhibited estrogen stimulated cell growth (mean fold induction ±

SD vs. vehicle control: MCF-7, 1.57±0.38, P=0.54, Figure 3A; T47D, 1.17±0.23, P=0.79, Figure 3B; BT474,, 0.96±0.2, P=0. 98, Figure 3C; ZR-75-1, 0.86±0.67, P=0.99, Figure 3D, all P by 1-way ANOVA).

Addition of endoxifen to the TPM and E_1/E_2 treatment (Figure 3A-D, col. 6) did not produce a further antiestrogenic effect in any of the genotype groups (P=0.82 for MCF-7, P=0.95 for T47D, P=0.95 for BT474, and P=0.99 for ZR-75-1, 1-way ANOVA compared to TPM + E_1/E_2). Assessment of endoxifen's pharmacological impact when added to the TPM + E_1/E_2 treatment in all tested cell lines using a 2-way ANOVA approach did not reveal any statistically significant antiestrogenic effect enhancement in any of the genotype categories, with all P values higher than 0.05.

To assess the importance of the active metabolite 4OHT, we performed proliferation assays in our panel of cells after treatment with postmenopausal concentrations of estrogens and estrogens in combination with tamoxifen and NDMTAM, and estrogens in combination with tamoxifen and its primary metabolites. The results demonstrate that treatment with tamoxifen plus the primary metabolite NDMTAM at concentrations corresponding to patients with EM genotype (Supplementary Table 1) inhibited the estrogen stimulated growth of MCF-7 cells by 39.62±2.26% (P=0.02 Student t-test) Figure 4A, col 3 ; however, addition of 4OHT in the EM genotype concentration (Supplementary Table 1) completely inhibited the proliferation action of estrogens (94.72±0.06% average inhibition vs. E1/E2 treatment (P=0.15 vs. vehicle control, Student t-test) (Figure 4A).

Additionally, we assessed the biological effect of tamoxifen metabolites in a postmenopausal setting in hypothetical osteoporotic women. To simulate an osteoporotic scenario, we used estrogen concentrations10-fold lower than the average concentrations used in our study, which coincides with estrogen plasma levels in postmenopausal patients from the clinical study of osteoporotic women determined in the results for the analysis of the

Multiple Outcomes of Raloxifene Evaluation study (39). The results demonstrated that treatment of the MCF-7 breast cancer cell line with a combination of tamoxifen and primary metabolites without endoxifen completely inhibit estrogen action (Supplementary Figure 1, available online).

Endoxifen is necessary to inhibit estrogen-stimulated genes completely

To assess the estrogenic and antiestrogenic effect on the transcriptional activity of ER and estrogen-responsive gene expression in MCF-7 cells. We focused on the contribution to the overall effect of estrogens and the antiestrogenic effect of either or both of the active metabolites 4OHT and endoxifen. We performed RT-PCR as described in the Methods section and used primers for, GREB1, pS2, and PgR estrogen-responsive genes. Results for all genes were similar. We demonstrated that GREB1 (Figure 4B), a regulator of hormone response in breast cancer (40), was activated 333.42±58.18 fold by estrogens compared to vehicle control (P=0.005, Student t-test). Addition of tamoxifen and NDMTAM reduced the estrogenic effect by 73±5.3% of E_1/E_2 treatment, P=0.015 compared to E_1/E_2 treatment alone but was statistically significantly different from vehicle control (P=0.007). Addition of 4OHT in EM concentration reduced the fold change in GREB1 mRNA levels even more when compared to the tamoxifen and NDMTAM combination treatment (down to 90±1.08% of E_1/E_2 treatment, P=0.022). Addition of endoxifen to tamoxifen and its primary metabolite mix (TPM) completely inhibited the estrogenic effect, bringing the fold change down to control levels (P=0.34). Additionally, we studied the effects of these treatments on other estrogen-responsive genes, such as pS2 (Figure 4C) and PgR (Figure 4D). Estrogens induced both genes; however, in both cases, addition of tamoxifen and NDMTAM to the E_1/E_2 mix elevated the average mRNA expression of these genes; however, this was not statistically

significant (P=0.26 for pS2 and P=0.24 for PgR). Addition of 4OHT to tamoxifen and NDMTAM in both genes reduced mRNA expression (78.9±1.34%, P=0.006 for pS2 and 91±2.4%, P= 0.04 for PgR compared to estrogen treatment). Addition of endoxifen (EN) to tamoxifen and primary metabolites (TPM) further inhibited the estrogen-stimulated expression of the mRNAs but not statistically significantly vs. TPM treatment (P=0.23 for pS2 and P=0.48 for PgR).

Effect of tamoxifen and metabolites on ERα protein stability

We assessed the effect of tamoxifen and metabolites on ERα protein stability after 24 hours of treatment in MCF-7 cells. Our results indicated that MCF-7 cells in a postmenopausal estrogen (E_1/E_2) environment reduced the levels of ERα protein by approximately 15% as measured by densitometry compared to vehicle control (Figure 5A, lane 2). Tamoxifen plus primary metabolites (TPM) at EM concentrations stabilized the levels of ERα by approximately 27% when compared to vehicle control as measured by densitometry (Figure 5A, lane 3). In combination with E_1/E_2, TPM reversed the effect of estrogens on ERα, consistent with the antiestrogenic effect of tamoxifen (Figure 5A, lane 4) and stabilized the levels of the ERα protein by about 37% when compared to vehicle control. We compared the effects of endoxifen from different sources (Dr Margarete Fischer-Bosch-Institute of Clinical Pharmacology,Stuttgart, Germany; Indiana Medical School; and the Mayo Clinic) in concentrations reported for patients with an EM genotype (Fig. 5A). Results showed clear differences in the stabilization of the ERα protein, but we did not find any reduction in protein levels (Fig. 5A). 4-Hydroxytamoxifen clearly stabilized the ERα protein levels at EM genotype concentrations by approximately 36% when compared to vehicle control. TPM and endoxifen prevented the estrogen-induced degradation of the ERα protein. ICI 182780 (fulvestrant) was used as a positive control for ER degradation at a 1μM concentration.

We assessed the effects of 4OHT and endoxifen on ERα protein stability in MCF-7 cells at high concentrations of 1μM with 1nM E_2 after 24 hours of treatment (Fig. 5B). Treatment with 1 nM E_2 treatment resulted in downregulation of the ERα protein by 50% compared to vehicle control as measured by densitometry. Treatments with 1 μM 4OHT alone and 1 μM endoxifen alone each stabilized the ERα protein by 100% and 90% respectively when compared to vehicle control as measured by densitometry. A combination of 1 nM E_2, with either 1 μM 4OHT or endoxifen reversed the estrogen-induced degradation of ERα (ERα protein stabilization by 92% with 4OHT and 54% with endoxifen when compared to vehicle control as measured by densitometry), showing the consistent antiestrogenic regulation of ERα protein stability with endoxifen.

Endoxifen induces ER ligand-binding domain pose similar to 4OHT based on molecular modelling.

The superimposition of the top ranked induced fit docking pose of endoxifen onto the ERα-4OHT complex shows a predicted binding alignment of endoxifen similar to that of 4OHT. The same H-bond network is recapitulated, which engages contacts between the phenolic hydroxyl group of endoxifen and E353, R394 and a crystallized water molecule. The core structures of both ligands overlap almost perfectly, the major difference being in the aminoalkyl side chain. The positioning of the flexible methylaminoethyl group of endoxifen and the side chain of D351 is shifted when compared to the 4OHT complex. Thus, the carboxylate group of D351 is moved towards the aminomethyl group, lying 2.6 Å away, compared to 3.8 Å in the case of the 4OHT complex and being involved in a salt bridge formation. This indicates that the conformation of the ER in complex with endoxifen is very similar to the known conformation of the ER-4OHT complex.

Discussion

To our knowledge, this study is the first to employ the actual circulating concentrations of tamoxifen and its metabolites measured in tamoxifen treated postmenopausal patients and used to block estrogen action from clinically derived plasma levels using a panel of human breast cancer cell lines. We found that tamoxifen and its primary metabolites in concentrations found in three CYP2D6 genotypes (EM, IM and PM) are sufficient to inhibit estrogen-induced replication in the postmenopausal setting. We also established the importance of 4OHT as the active primary metabolite in the antiestrogenic action of tamoxifen in the postmenopausal setting. The estrogenic steroids would be anticipated to accumulate in the cell by binding to the ER. However, at premenopausal levels of estrogens, we show an association between the antiestrogenic effect and the levels of endoxifen corresponding to different CYP2D6 genotypes(41).

Estrogens activate the ER and induce its transcriptional activity via interaction with the estrogen responsive gene promoters. Estrogen receptor protein turnover is required (42) to maintain the continuous transcription of mRNA. This turnover is achieved by proteosomal degradation of the ER protein. Binding of antiestrogens, in particular 4OHT, blocks the ER and promotes stabilization of ER protein (43). In contrast Wu et al (44) reported that endoxifen targets ERα protein for degradation in MCF-7 and T47D cells at higher concentrations (100 and 1000 nM) (44). However, these concentrations are not comparable to circulating endoxifen concentrations in patients. The same authors used (44) circulating EM and PM genotype concentrations of endoxifen with circulating concentrations of tamoxifen and its primary metabolites in MCF-7 cells and demonstrated ERα degradation only at EM levels of endoxifen. Endoxifen is biologically very similar to 4OHT in breast cancer cells (45). According to the molecular model of 4OHT action (34) (Fig. 5C), the structurally similar endoxifen should bind to the ER and induce a similar conformation of the ER. The

fact that the lone pair of electrons on the free nitrogen of endoxifen now interacts with the Asp351 means that it is not available to interact with the appropriate amino acid at Helix 12. This means that it is less likely to create a closed Helix 12 and the instability will result in destruction of accumulated ERα compared with 4OHT (Fig. 5A and 5B). This contrasts with the selective estrogen receptor modulators raloxifene and bazedoxifene that completely shield Asp351 and prevent Helix 12 closure. This is why the unstable complex is degraded (46, 47). Fulvestrant, which completely disrupts the complex, is the extreme case of complex destruction (48).

Wu et al. (49) proposed a link between endoxifen-mediated destruction of ERα protein and levels of ERβ in the cells. However, the artificial overexpression of ERβ in cells may not accurately depict the natural mechanism of ERα degradation in tumors. MCF-7 cells have very low levels of ERβ expression compared with ERα. Breast tumors have very low expression of ERβ protein or mRNA (50-52) compared with ERα.

Tamoxifen and its metabolites regulate the transcriptional activity of estrogen-responsive genes. GREB1 plays a substantial role in breast cancer cell hormone dependent proliferation (40). We demonstrate that tamoxifen and NDMTAM partially inhibit GREB1 estrogen induced mRNA synthesis; however, addition of 4OHT and endoxifen statistically significantly enhances the antiestrogenic activity of the tamoxifen metabolite pool (Figure 4B). These results show that endoxifen, unlike the short-term cell growth end point, actually plays a substantial role in inhibiting the estrogen-mediated activation of responsive genes. We further examined the role of endoxifen in the estrogen regulation of pS2 and PgR gene expression (Fig. 4C and 4D). Although these genes are not important for proliferation, they illustrate the diversity of responsiveness to tamoxifen and its metabolites. The addition of tamoxifen and NDMTAM elevated both pS2 and PgR, an effect that has been noted previously (53, 54). Adding the other tamoxifen metabolite 4OHT partially decreased gene

expression of pS2 and PgR, but endoxifen suppressed gene expression further. It may be that these observations are relevant during prolonged adjuvant therapy (6) as incomplete suppression of gene function may lead to estrogen-stimulated proliferation, development of resistance, and, ultimately, recurrence. These conclusions are supported by a recent report by Hawse et al (55).

This study also had some limitations. Although these data *in vitro* model the antiestrogen environment in postmenopausal patients taking tamoxifen in the short term, there are other dimensions to consider for therapeutic efficacy during adjuvant therapy. Tamoxifen and metabolites are competitive inhibitors of estrogen-induced cell proliferation (56). Therefore, circulating and tumor cell estrogen levels are critical for successful antiestrogen therapy. We have demonstrated (41) that the efficacy of the metabolite mix for tamoxifen, and the role of endoxifen, is critically dependent upon the circulating estrogen levels. The studies which titrate endoxifen in a parallel premenopausal model, i.e, much higher circulating levels of estrone and estradiol, show that only by titrating down estrogen levels, can the positive role of endoxifen be defined (41).

There is no laboratory model that can simulate the action of a decade of adjuvant therapy. It is now emerging that tamoxifen and metabolites are not simply acting just as inhibitors of estrogen action but there is a dynamic change in breast cancer cell population over years as selection pressure creates acquired resistance in micrometastases. The principle has recently been illustrated by the extended cell culture of aromatase inhibitor resistant breast cancer cells incubated in a cytostatic environment for 8 weeks. Cell populations change dramatically (57). For the future, we propose to monitor the evolution of breast cancer cell populations under the selection pressure of appropriate antiestrogenic therapy for years. This will stimulate the evolution of cell populations during adjuvant therapy that become vulnerable to a woman's own estrogen to trigger estrogen induced apoptosis. This approach was originally

used with tamoxifen to describe the evolution of acquired resistance of tumors in the athymic mouse model over years of treatment (58). This cytostatic mechanism of tamoxifen and its metabolites needs time, compliance and high endoxifen levels to create effective selection pressure in different estrogen environments over years. It is these interlocking factors that expose the vulnerability of breast cancer to estrogen-induced apoptosis that results in a decrease in mortality in the decade after tamoxifen is stopped (59). However, to extrapolate appropriately to clinical care it is important to appreciate the requirement to validate methodologies to determine the CYP2D6 genotyping as there is a profound loss of heterozygosity of the gene in breast cancer tissue. This quality control issue in genotyping was emphasized recently (60).In conclusion, based on the experimental data in vitro, in a panel of breast cancer cell lines in a postmenopausal setting, the presence of endoxifen in any concentration is secondary for blocking growth but necessary to block estrogen-regulated genes completely

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References

1. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2003; 2:205-213.
2. Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 2008; 44:30-38.
3. EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. . *Lancet* 1998; 351:1451-1467.
4. Davies C, Godwin J, Gray R et al. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* 2011; 378:771-784.
5. EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005; 365:1687-1717.
6. Davies C, Pan H, Godwin J et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. *Lancet* 2013; 381:805-816.
7. Berry DA, Inoue L, Shen Y et al. Modeling the impact of treatment and screening on U.S. breast cancer mortality: a Bayesian approach. *J Natl Cancer Inst Monogr* 2006:30-36.
8. Jordan VC, Collins MM, Rowsby L et al. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* 1977; 75:305-316.
9. Jordan VC, Dix CJ, Naylor KE et al. Nonsteroidal antiestrogens: their biological effects and potential mechanisms of action. *J Toxicol Environ Health* 1978; 4:363-390.
10. Allen KE, Clark ER, Jordan VC. Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. *Br J Pharmacol* 1980; 71:83-91.
11. Lieberman ME, Jordan VC, Fritsch M et al. Direct and reversible inhibition of estradiol-stimulated prolactin synthesis by antiestrogens in vitro. *J Biol Chem* 1983; 258:4734-4740.
12. Furr BJ, Jordan VC. The pharmacology and clinical uses of tamoxifen. *Pharmacol Ther* 1984; 25:127-205.
13. Mauvais-Javis P, Baudot N, Castaigne D et al. trans-4-Hydroxytamoxifen concentration and metabolism after local percutaneous administration to human breast. *Cancer Res* 1986; 46:1521-1525.
14. Lien EA, Solheim E, Kvinnsland S et al. Identification of 4-hydroxy-N-desmethyltamoxifen as a metabolite of tamoxifen in human bile. *Cancer Res* 1988; 48:2304-2308.
15. Lien EA, Solheim E, Lea OA et al. Distribution of 4-hydroxy-N-desmethyltamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. *Cancer Res* 1989; 49:2175-2183.
16. Stearns V, Johnson MD, Rae JM et al. Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. *J Natl Cancer Inst* 2003; 95:1758-1764.
17. Jin Y, Desta Z, Stearns V et al. CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. *J Natl Cancer Inst* 2005; 97:30-39.
18. Schroth W, Goetz MP, Hamann U et al. Association between CYP2D6 polymorphisms and outcomes among women with early stage breast cancer treated with tamoxifen. *JAMA* 2009; 302:1429-1436.
19. Lammers LA, Mathijssen RH, van Gelder T et al. The impact of CYP2D6-predicted phenotype on tamoxifen treatment outcome in patients with metastatic breast cancer. *Br J Cancer* 2010; 103:765-771.
20. Madlensky L, Natarajan L, Tchu S et al. Tamoxifen metabolite concentrations, CYP2D6 genotype, and breast cancer outcomes. *Clin Pharmacol Ther* 2011; 89:718-725.
21. Lash TL, Cronin-Fenton D, Ahern TP et al. CYP2D6 inhibition and breast cancer recurrence in a population-based study in Denmark. *J Natl Cancer Inst* 2011; 103:489-500.

22. Regan MM, Leyland-Jones B, Bouzyk M et al. CYP2D6 Genotype and Tamoxifen Response in Postmenopausal Women with Endocrine-Responsive Breast Cancer: The Breast International Group 1-98 Trial. *J Natl Cancer Inst* 2012; 104:441-451.
23. Rae JM, Drury S, Hayes DF et al. CYP2D6 and UGT2B7 Genotype and Risk of Recurrence in Tamoxifen-Treated Breast Cancer Patients. *J Natl Cancer Inst* 2012; 104:452-460.
24. Zanger UM, Fischer J, Raimundo S et al. Comprehensive analysis of the genetic factors determining expression and function of hepatic CYP2D6. *Pharmacogenetics* 2001; 11:573-585.
25. Jordan VC, Obiorah I, Fan P et al. The St. Gallen Prize Lecture 2011: evolution of long-term adjuvant anti-hormone therapy: consequences and opportunities. *Breast* 2011; 20 Suppl 3:S1-11.
26. Jordan VC, Fritz NF, Tormey DC. Endocrine effects of adjuvant chemotherapy and long-term tamoxifen administration on node-positive patients with breast cancer. *Cancer Res* 1987; 47:624-630.
27. Ravdin PM, Fritz NF, Tormey DC et al. Endocrine status of premenopausal node-positive breast cancer patients following adjuvant chemotherapy and long-term tamoxifen. *Cancer Res* 1988; 48:1026-1029.
28. Murdter TE, Schroth W, Bacchus-Gerybadze L et al. Activity levels of tamoxifen metabolites at the estrogen receptor and the impact of genetic polymorphisms of phase I and II enzymes on their concentration levels in plasma. *Clin Pharmacol Ther* 2011; 89:708-717.
29. Balaburski GM, Dardes RC, Johnson M et al. Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. *Int J Oncol* 2010; 37:387-398.
30. Maximov P, Sengupta S, Lewis-Wambi JS et al. The Conformation of the Estrogen Receptor Directs Estrogen-Induced Apoptosis in Breast Cancer: A Hypothesis. *Horm Mol Biol Clin Investig* 2011; 5:27-34.
31. Sweeney EE, McDaniel RE, Maximov PY et al. Models and Mechanisms of Acquired Antihormone Resistance in Breast Cancer: Significant Clinical Progress Despite Limitations. *Horm Mol Biol Clin Investig* 2012; 9:143-163.
32. Sengupta S, Sharma CG, Jordan VC. Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells. *Horm Mol Biol Clin Investig* 2010; 2:235-243.
33. Berman HM, Westbrook J, Feng Z et al. The Protein Data Bank. *Nucleic Acids Res* 2000; 28:235-242.
34. Shiau AK, Barstad D, Loria PM et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 1998; 95:927-937.
35. Friesner RA, Banks JL, Murphy RB et al. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J Med Chem* 2004; 47:1739-1749.
36. Halgren TA, Murphy RB, Friesner RA et al. Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening. *J Med Chem* 2004; 47:1750-1759.
37. Cauley JA, Gutai JP, Kuller LH et al. The epidemiology of serum sex hormones in postmenopausal women. *Am J Epidemiol* 1989; 129:1120-1131.
38. Hankinson SE, Willett WC, Manson JE et al. Plasma sex steroid hormone levels and risk of breast cancer in postmenopausal women. *J Natl Cancer Inst* 1998; 90:1292-1299.
39. Cummings SR, Duong T, Kenyon E et al. Serum estradiol level and risk of breast cancer during treatment with raloxifene. *JAMA* 2002; 287:216-220.
40. Rae JM, Johnson MD, Scheys JO et al. GREB 1 is a critical regulator of hormone dependent breast cancer growth. *Breast Cancer Res Treat* 2005; 92:141-149.
41. Maximov PY, McDaniel RE, Fernandes DJ et al. Simulation with cells in vitro of tamoxifen treatment in premenopausal breast cancer patients with different CYP2D6 genotypes. *Br J Pharmacol* 2014; in press.

42. Lonard DM, Nawaz Z, Smith CL et al. The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. *Mol Cell* 2000; 5:939-948.
43. Wijayaratne AL, McDonnell DP. The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. *J Biol Chem* 2001; 276:35684-35692.
44. Wu X, Hawse JR, Subramaniam M et al. The tamoxifen metabolite, endoxifen, is a potent antiestrogen that targets estrogen receptor alpha for degradation in breast cancer cells. *Cancer Res* 2009; 69:1722-1727.
45. Johnson MD, Zuo H, Lee KH et al. Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. *Breast Cancer Res Treat* 2004; 85:151-159.
46. Levenson AS, Jordan VC. The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. *Cancer Res* 1998; 58:1872-1875.
47. Lewis-Wambi JS, Kim H, Curpan R et al. The selective estrogen receptor modulator bazedoxifene inhibits hormone-independent breast cancer cell growth and down-regulates estrogen receptor alpha and cyclin D1. *Mol Pharmacol* 2011; 80:610-620.
48. Pike AC, Brzozowski AM, Walton J et al. Structural insights into the mode of action of a pure antiestrogen. *Structure* 2001; 9:145-153.
49. Wu X, Subramaniam M, Grygo SB et al. Estrogen receptor-beta sensitizes breast cancer cells to the anti-estrogenic actions of endoxifen. *Breast Cancer Res* 2011; 13:R27.
50. Roger P, Sahla ME, Makela S et al. Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. *Cancer Res* 2001; 61:2537-2541.
51. Iwao K, Miyoshi Y, Egawa C et al. Quantitative analysis of estrogen receptor-alpha and -beta messenger RNA expression in breast carcinoma by real-time polymerase chain reaction. *Cancer* 2000; 89:1732-1738.
52. Shaaban AM, O'Neill PA, Davies MP et al. Declining estrogen receptor-beta expression defines malignant progression of human breast neoplasia. *Am J Surg Pathol* 2003; 27:1502-1512.
53. Horwitz KB, Koseki Y, McGuire WL. Estrogen control of progesterone receptor in human breast cancer: role of estradiol and antiestrogen. *Endocrinology* 1978; 103:1742-1751.
54. May FE, Johnson MD, Wiseman LR et al. Regulation of progesterone receptor mRNA by oestradiol and antioestrogens in breast cancer cell lines. *J Steroid Biochem* 1989; 33:1035-1041.
55. Hawse JR, Subramaniam M, Cicek M et al. Endoxifen's molecular mechanisms of action are concentration dependent and different than that of other anti-estrogens. *PLoS One* 2013; 8:e54613.
56. Jordan VC. Biochemical pharmacology of antiestrogen action. *Pharmacol Rev* 1984; 36:245-276.
57. Fan P, Agboke FA, McDaniel RE et al. Inhibition of c-Src blocks oestrogen-induced apoptosis and restores oestrogen-stimulated growth in long-term oestrogen-deprived breast cancer cells. *Eur J Cancer* 2014; 50:457-468.
58. Yao K, Lee ES, Bentrem DJ et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000; 6:2028-2036.
59. Jordan VC. Linking estrogen-induced apoptosis with decreases in mortality following long-term adjuvant tamoxifen therapy. *J Natl Cancer Inst* 2014; in press.
60. Brauch H, Schwab M. Prediction of tamoxifen outcome by genetic variation of CYP2D6 in post-menopausal women with early breast cancer. *Br J Clin Pharmacol* 2014; 77:695-703.

Figure legends

Figure 1. The chemical structures of all metabolites of tamoxifen in this study and their metabolism pathways and metabolizing cytochrome P450 (CYP) enzymes with relative binding affinities (RBA) for the estrogen receptor (ER). Tamoxifen is metabolically activated to 4-hydroxytamoxifen (4OHT) and endoxifen (N-desmethyl-4-hydroxytamoxifen) by the CYP2D6 enzyme system. Tamoxifen is also metabolized into N-desmethyltamoxifen (NDMTAM), which has a similar RBA for the ER as tamoxifen.

Figure 2. Dose response cell-growth curves to estrone (E_1) and estradiol (E_2) in **A)** MCF-7, **B)** T47D, **C)** BT474, and **D)** ZR-75-1 cell lines. Data represent means and error bars are SD from 3 independent experiments with 3 replicates.

Figure 3. Biological response to clinical levels of metabolites of tamoxifen with and without endoxifen, and in presence of postmenopausal levels of estrogens in the media in **A)** MCF-7, **B)** T47D, **C)** BT474, and **D)** ZR-75-1 cell lines. All cells lines were treated for 7 days with the indicated treatments after a 3 day starvation in the estrogen free media. The biological effect was assessed by DNA quantification assay. Treatments were made as follows in all cell lines (panels A-D): 1- Vehicle control, 2- postmenopausal concentration of E1/E2, 3- tamoxifen plus primary metabolites (NDMTAM and 4OHT) (TPM), 4- E1/E2 plus TPM, 5- TPM plus endoxifen (EN), 6- E1/E2 plus TPM plus EN. Data represent means and error bars are SD from 3 independent experiments with 3 replicates. P values from 1-way ANOVA. All statistical tests were two-sided.

Figure 4. The pharmacological importance of 4-hydroxytamoxifen (4OHT) in MCF-7 cells in the postmenopausal setting. **A)** The proliferation assay after treatment of MCF-7 cells with tamoxifen plus primary metabolites (TPM) at EM concentrations to assess the importance of 4OHT. **)** RT-PCR results for GREB1 gene after treating MCF-7 cells with EM concentrations

of TPM to assess the importance of 4OHT and endoxifen in regulating estrogen responsive gene expression. RT-PCR results for the same treatments for **C)** pS2 and **D)** PgR. Treatments were made as follows in all cell lines (panels A-D): 1- Vehicle control, 2- postmenopausal concentration of E1/E2, 3- E1/E2 plus tamoxifen and NDMTAM, 4- E1/E2 with tamoxifen plus primary metabolites NDMETAM and 4OHT (TPM), 5- E1/E2 plus TPM plus endoxifen (EN). Data represent means and error bars are SDfrom 3 independent experiments with 3 replicates. P values from Student t test. All statistical tests were two-sided.

Figure 5. Effect of tamoxifen and metabolites on ERα protein stability and molecular model of 4OHT action. **A)** Protein levels of ERα in MCF-7 cells after 24 hour treatment with EM concentrations of tamoxifen metabolites with and without endoxifen. The levels of ERα protein after treatment with tamoxifen metabolites are similar to upregulation after treatment with 4-hydroxytamoxifen (4OHT) at extensive metabolizer (EM) concentration (Supplementary Table 1). **B)** Protein levels of ERα in MCF-7 cells after treatment with 1nM E_2, and tamoxifen primary metabolites 4OHT and endoxifen at 1μM individually and in combination. **C)** Endoxifen is docked within the same pocket that binds 4OHT in the antagonist conformation of ERα ligand binding domain.

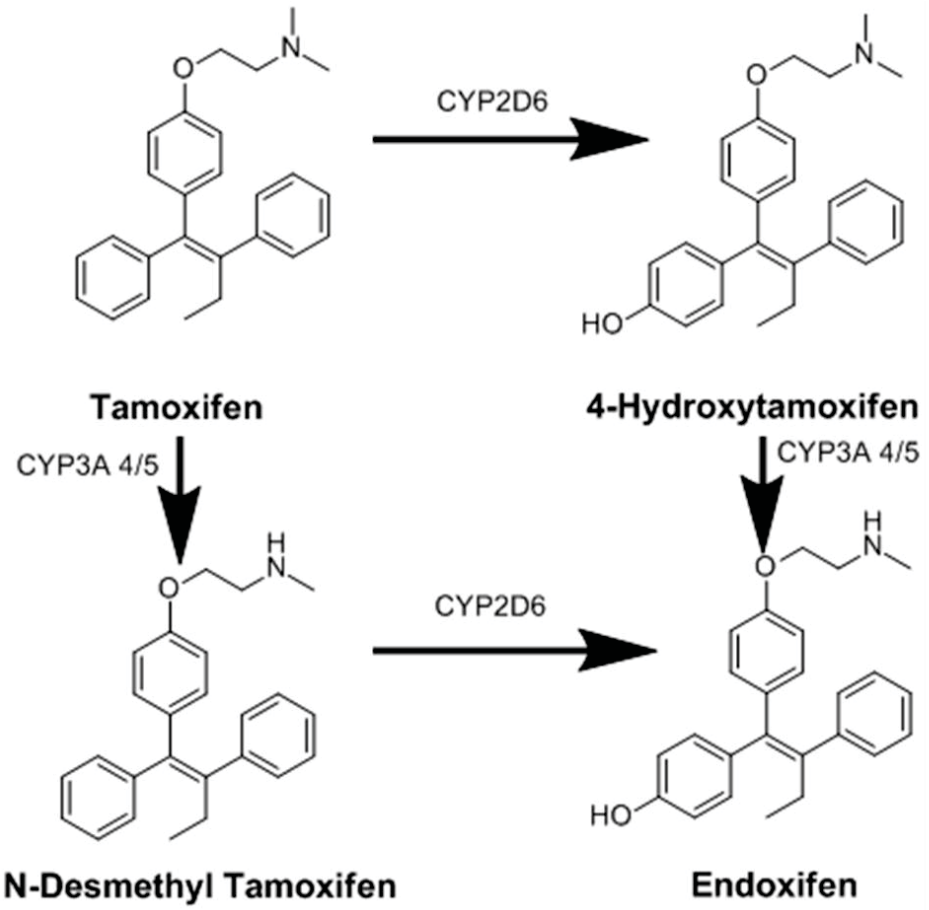
CYP2D6
Tamoxifen
4-Hydroxytamoxifen
CYP3A 4/5
CYP3A 4/5
CYP2D6
N-Desmethyl Tamoxifen
Endoxifen
RBA (ER)
Tamoxifen – 2.8
NDMTAM – 2.4
4OHT – 181
Endoxifen - 181

Figure 1

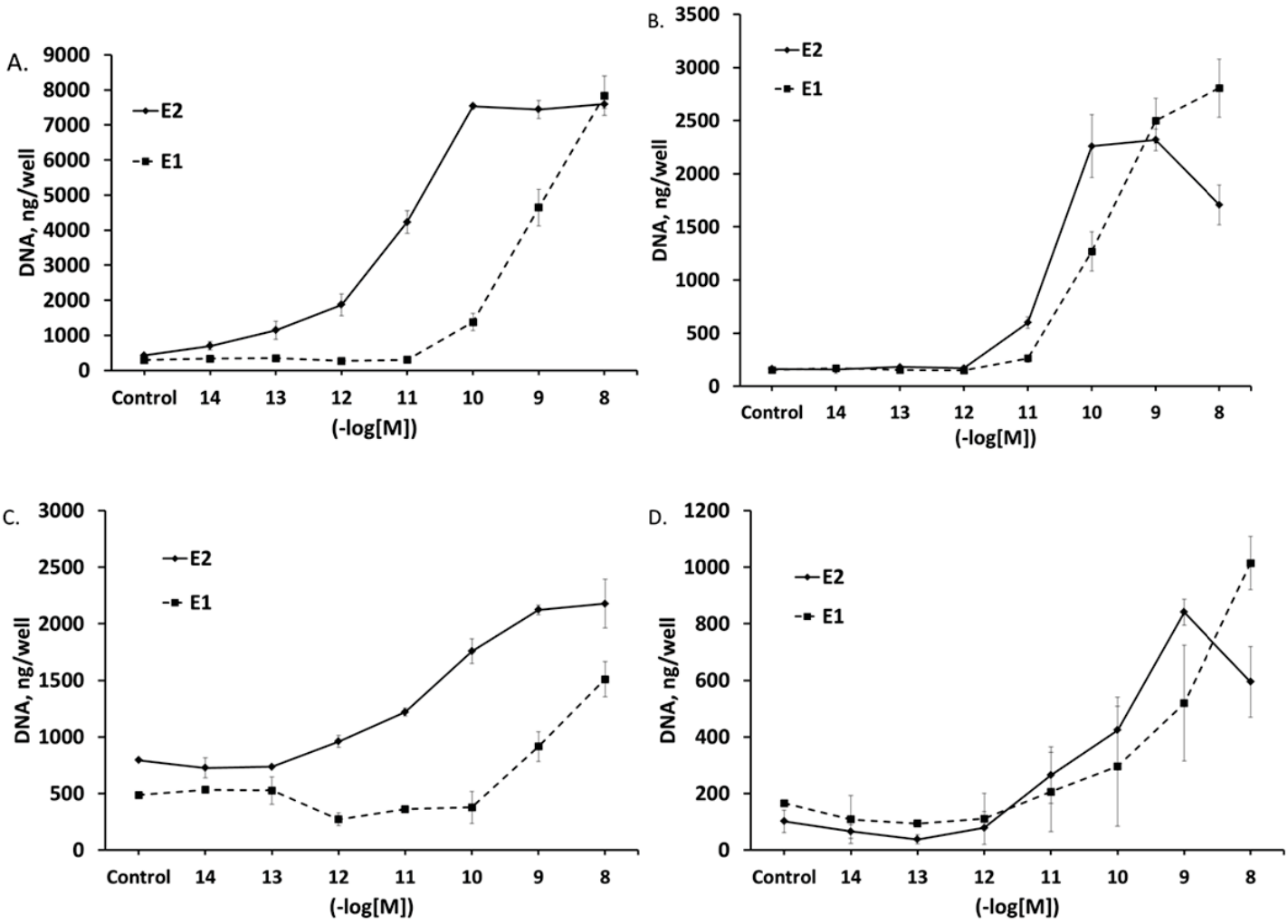

Figure 2

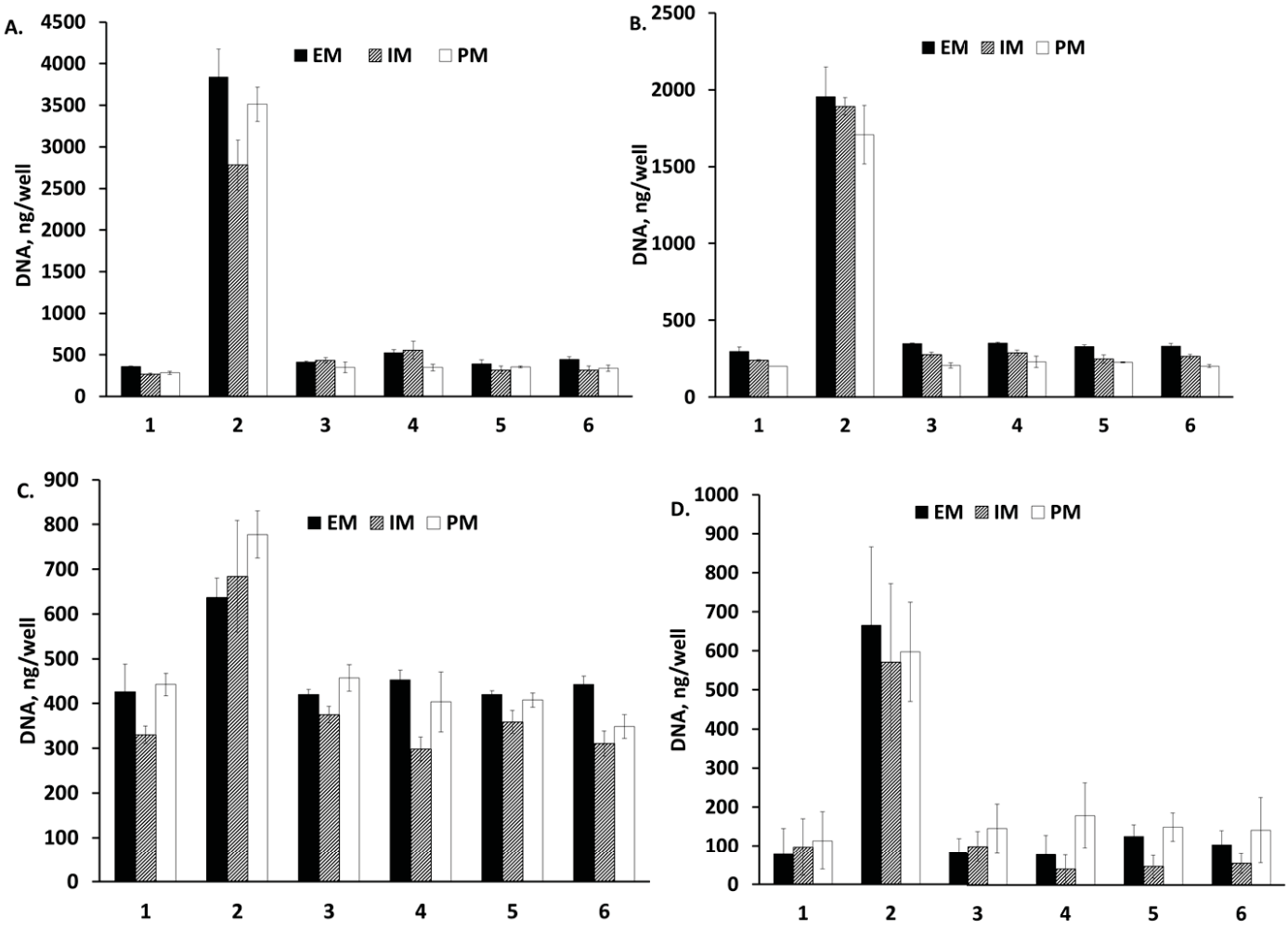
A.
B.
C.
D.
EM
IM
PM
DNA, ng/well
1. Vehicle; 2. E1/E2; 3. TPM; 4. E1/E2+TPM; 5. TPM+EN; 6 E1/E2+TPM+EN

Figure 3

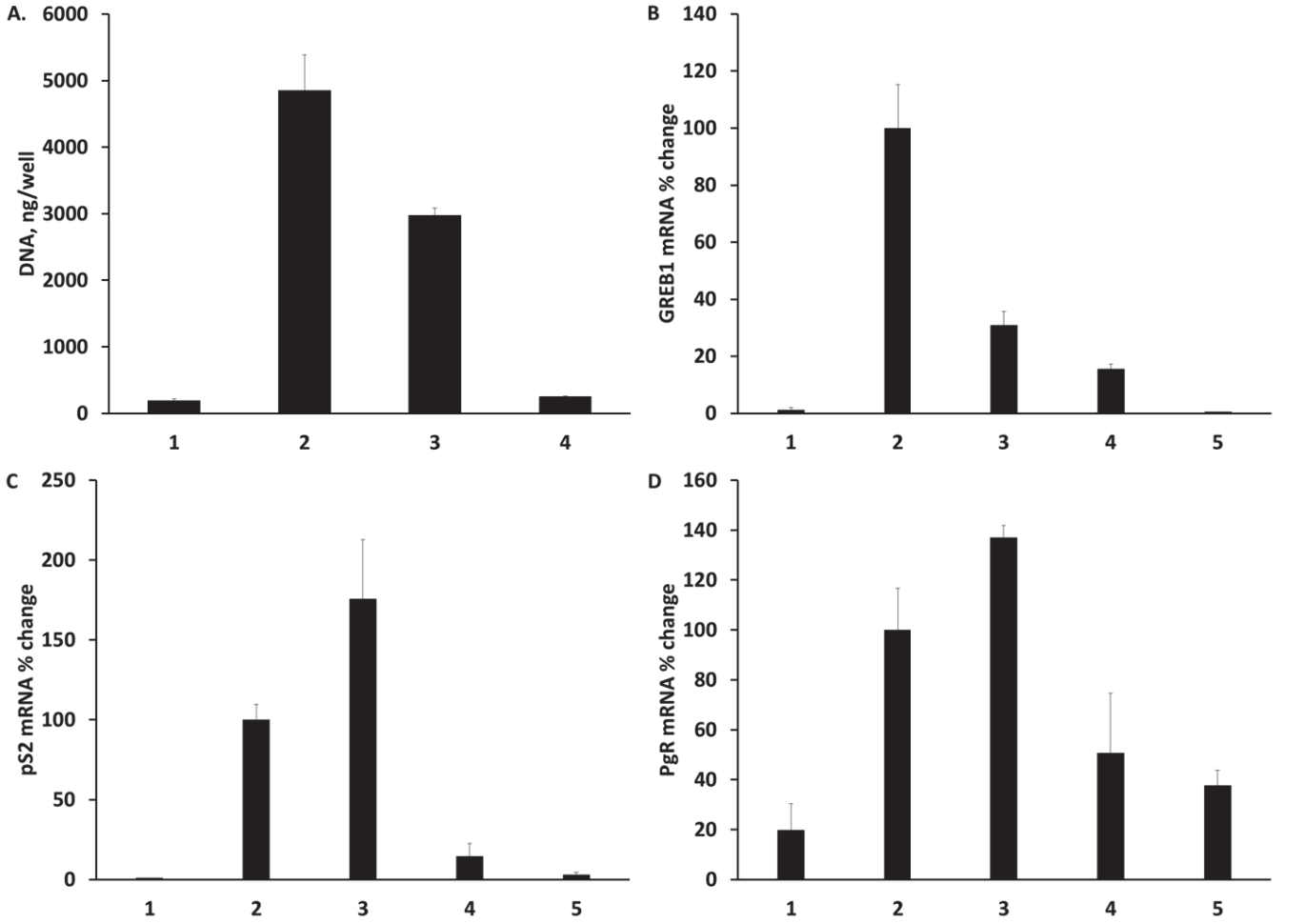
A.
DNA, ng/well
6000
5000
4000
3000
2000
1000
0
1
2
3
4
B
GREB1 mRNA % change
140
120
100
80
60
40
20
0
1
2
3
4
5
C
pS2 mRNA % change
250
200
150
100
50
0
1
2
3
4
5
D
PgR mRNA % change
160
140
120
100
80
60
40
20
0
1
2
3
4
5
1. Vehicle; 2. E1/E2; 3. E1/E2+TAM+NDMTAM; 4. E1/E2+TPM; 5. E1/E2+TPM+EN

Figure 4

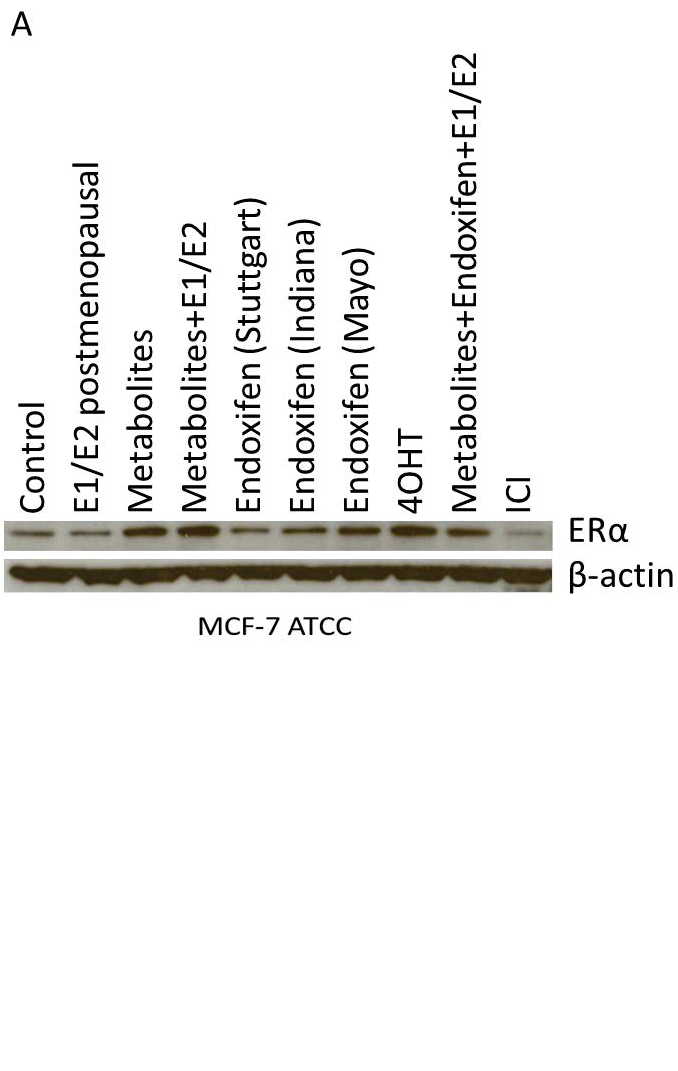
A
Control
E1/E2 postmenopausal
Metabolites
Metabolites+E1/E2
Endoxifen (Stuttgart)
Endoxifen (Indiana)
Endoxifen (Mayo)
4OHT
Metabolites+Endoxifen+E1/E2
ICI
ERα
β-actin
MCF-7 ATCC
B
Control
E2 1nM
4OHT 1uM
Endoxifen 1uM
E2+4OHT
E2+Endoxifen
ERα
β-actin
MCF-7
C
LEU-536
LEU-540
ASP-351
THR-347
LEU-525
LEU-349
GLU-353
ARG-394
LEU-384
LEU-391

Figure 5

Simulation with cells in vitro of tamoxifen treatment in premenopausal breast cancer patients with different CYP2D6 genotypes.

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Cross-cutting area:	Cancer, Pharmacogenomics/pharmacogenetics
Additional area(s):	Ligands, Nuclear hormone receptors

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For Peer Review

Professor J.C. (Ian) McGrath,
Editor-in-Chief of the
British Journal of Pharmacology

25 June 2014

Dear Professor McGrath,

Enclosed is our revised manuscript entitled "Simulation with cells in vitro of tamoxifen treatment in premenopausal breast cancer patients with different CYP2D6 genotypes" by Philipp Y. Maximov, Russell E. McDaniel, Daphne J. Fernandes, Valeriy R. Korostyshevskiy, Puspanjali Bhatta, Thomas E. Muerdter, David A. Flockhart, V. Craig Jordan; manuscript ID 014-BJP-0462-RP.R2

We have addressed all of the reviewers' comments and have made amendments as advised. The changes in the manuscript are indicated with red font in the text. We also enclose detailed responses to the reviewers in a separate document.

We hope the revised manuscript will be now accepted for publication.

Yours Sincerely,

Responses to the reviewer's comments for manuscript entitled "Simulation with cells in vitro of tamoxifen treatment in premenopausal breast cancer patients with different CYP2D6 genotypes" by Philipp Y. Maximov, Russell E. McDaniel, Daphne J. Fernandes, Valeriy R. Korostyshevskiy, Puspanjali Bhatta, Thomas E. Muerdter, David A. Flockhart, V. Craig Jordan; manuscript ID 014-BJP-0462-RP.R2

Reviewer 1

We have reviewed the manuscript for any errors and language usage. We have made a couple of minor corrections and have made all the modifications suggested by the reviewer. Specifically:

Comment: P4 L8 (from the bottom): please rephrase "... using TAM and metabolite concentrations mimicking its circulating levels ..."

Response: We have replaced the phrase on P4 to "…using TAM and metabolite concentrations mimicking their circulating levels…"

Comment: P6 L5: please replace:"treatments" by "substances" or equivalent

Response: Replaced word "treatments" on P6 L5 to "test compounds"

Comment: P10 L16-21: Please condense the two phrases in one, without repetitions.

Response: Condensed the sentences on P10 L16-21 and made more clear, now reads: "We used the circulating concentrations of TAM and its metabolites provided by Flockhart (Table 2) based on a study (Irvin et al., 2011) in breast cancer patients treated with TAM. These patients were genotyped for CYP2D6 as IM and PM and treated with both standard dose of TAM (20 mg/day) and 40 mg/day (Irvin et al., 2011)."

Comment: P11 L7: Reference out of context.

Response: We removed the reference that was out of context, thank you for pointing that out.

Comment: Finally, the Discussion section should be shortened and should be focused in the results provided in the manuscript. Authors could present the inefficiency of animal models in a more succinct manner, without repetitions and references out of the context of the present investigation.

Comment: P15 L1-10: These phrases can be omitted as they produce confusion and don't act anything to the results reported to this study.

Response: Omitted sentences that were not directly relevant to results discussions on P15 L1-10 and also additionally, as per reviewers request, have omitted a similar number of sentences on P15 L11 of the revised manuscript to simplify the results discussions.

For Peer Review

Reviewer 2

Comment: Unfortunately authors again overlooked the "paradoxal drop in the TAM concentration in IM group in Table 2". THIS TYPO HAS NOT YET BEEN CORRECTED in the revised version. This is not just a non-significant spelling mistake: this is 100nM error in reporting a key parameter of the experiment! Please make sure that typo in table 2 is corrected at the proof-reading stage. Doubt in used concentrations may cast doubt on the whole paper, which would be unfair because the paper is interesting.

Also authors may wish to double check all other tables during the proof-reading. For instance, ratios of respective endoxifen concentrations between Table 2 and Supplementary Table 1 are not constant (though the deviations are reasonably small).

Response: Thank you very much for pointing out the error in Table 2. We profusely apologize for the mistake and have corrected the concentration. Additionally we now provide more accurate endoxifen and 4OHT concentrations in the table as per reviewer's request.

For Peer Review

Title

Simulation with cells in vitro of tamoxifen treatment in premenopausal breast cancer patients with different CYP2D6 genotypes.

Authors' names and affiliations

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For Peer Review

Summary

Background and Purpose TAM is a prodrug that is metabolically activated by 4-hydroxylation to the potent primary metabolite 4-hydroxytamoxifen (4OHT) or via another primary metabolite N-desmethytamoxifen (NDMTAM) to a biologically active secondary metabolite endoxifen through a cytochrome P450 variant 2D6 system (CYP2D6). We have determined the antioestrogenic efficacy of TAM and its metabolites, including endoxifen, at concentrations corresponding to serum levels measured in breast cancer patients with various CYP2D6 genotypes, simulating TAM treatment. This could provide new data to understand TAM action further and the relevance of endoxifen.

Experimental Approach The biological effects on cell growth and oestrogen-responsive gene modulation were evaluated in a panel of ER positive breast cancer cell lines. Actual clinical levels of TAM metabolites in breast cancer patients were used *in vitro* along with actual levels of oestrogens observed in premenopausal patients taking tamoxifen.

Key Results TAM and its primary metabolites (4OHT and NDMTAM) are only able to inhibit oestrogen action partially. Addition of endoxifen in concentrations corresponding to different CYP2D6 genotypes, demonstrates a correlation between the antioestrogenic effect and the concentrations of endoxifen. Results show that the addition of endoxifen in concentrations corresponding with the Extensive Metabolizer genotype is able to inhibit the oestrogen actions further. By contrast, addition of lower concentrations of endoxifen (Intermediate and Poor metabolizers), the antioestrogenic effect was not as prominent or absent.

Conclusions and Implications Endoxifen may be a clinically relevant metabolite in premenopausal patients as it provides additional antioestrogenic actions during TAM treatment.

Keywords Breast cancer, tamoxifen, endoxifen, CYP2D6

For Peer Review

Introduction

The development of antioestrogenic strategies (Jordan & Brodie, 2007) for the adjuvant treatment of oestrogen receptor (ER) positive breast cancer has revolutionized the prospects for patients survival (Davies *et al.*, 2011; Dowsett *et al.*, 2010). There are two targeted approaches to antioestrogenic therapy: tamoxifen (TAM) and its metabolites block the tumour ER and prevent oestrogen stimulated growth whereas an aromatase inhibitor (AI) blocks the small but relevant background production of oestrogen in postmenopausal patients. The AIs are now considered to be the adjuvant treatment of choice for postmenopausal breast cancer patients; however, TAM remains the antihormone adjuvant therapy of choice for premenopausal patients with an ER positive node positive or negative breast cancer. Longer therapy (up to 5 years) is currently recommended as standard therapy as shorter therapy (< 5years) does not as efficiently control recurrence or enhance survival (Davies *et al.*, 2011). Recent data demonstrate that 10 years of adjuvant TAM is superior to 5 years of adjuvant TAM (Davies *et al.*, 2013).

TAM itself is a prodrug that is metabolized by cytochrome P450 isoforms into potent metabolites (Allen *et al.*, 1980) (Figure 1). Two biologically relevant metabolites of TAM are 4-hydroxytamoxifen (4OHT) and 4-hydroxy-N-desmethyltamoxifen (endoxifen) formed from the primary metabolite N-desmethyltamoxifen (NDMTAM). Hydroxylation of TAM or NDMTAM at the 4 position increases the compound's affinity for the ER a 100 fold when compared with TAM (Johnson *et al.*, 2004; Jordan *et al.*, 1977), or the major primary metabolite of TAM N-desmethyltamoxifen (NDMTAM). The CYP2D6 enzyme was first implicated in the hydroxylation of TAM in human liver in 1997 (Dehal & Kupfer, 1997). Subsequently it was found that Selective Serotonin Reuptake Inhibitors (SSRIs) used to treat hot flashes in breast cancer patients taking TAM blocked CYP2D6 and reduced endoxifen levels (Stearns *et al.*, 2003). The CYP2D6 enzyme was subsequently identified as responsible

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for endoxifen synthesis (Desta *et al.*, 2004). A later hypothesis connected aberrant CYP2D6 genotypes with clinical outcome of TAM therapy (Goetz *et al.*, 2005) but this hypothesis remains controversial and the relevance in postmenopausal patients remains unresolved (Schroth *et al.*, 2009). Attempts to improve TAM's effectiveness unequivocally by selecting out poor metabolizers (PM) of TAM based on an absent CYP2D6 genotype have been unsuccessful in postmenopausal patients (Rae *et al.*, 2012; Regan *et al.*, 2012); although an overview meta-analysis of studies demonstrates a weak association with 5 years of TAM treatment (Province *et al.*, 2014).

Since TAM is the standard of care for premenopausal ER-positive breast cancer patients, we now address the hypothesis that the conversion of TAM to endoxifen is of value for the antitumour actions of TAM in the average oestrogen environment during the menstrual cycle, *ie*: oestrone (E_1) plus oestradiol (E_2), observed in premenopausal patients taking adjuvant TAM as monotherapy. There are no extensive clinical studies that address this issue. The complicating factor with TAM therapy in premenopausal women is the increase in circulating oestrogen caused by an antioestrogenic blockade of the feedback loop in the hypothalamic pituitary axis (Jordan *et al.*, 1991). Our study is the first to illustrate the potential pharmacological impact of TAM and its metabolites including endoxifen on ER-positive breast cancer cells *in vitro*, using TAM and metabolites concentrations mimicking their circulating levels in breast cancer patients that were CYP2D6 genotyped (Extensive Metabolizers (EM), Intermediate Metabolizers (IM), and Poor Metabolizers (PM)), which were provided by Muerdter and Flockhart from previous studies (Irvin *et al.*, 2011; Murdter *et al.*, 2011). We have evaluated the proposal that doubling the dose of TAM (40 mg/daily) could be employed to treat patients (Irvin *et al.*, 2011), thereby increasing the overall mix of "antioestrogenic metabolites" to block the replication of breast cancer. Lastly, we addressed the effectiveness of TAM and its metabolites to control oestrogenic events in breast cancer

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cells exposed to perimenopausal levels of oestrogens i.e.: women with intermitted and low oestrogen levels but not a cessation of ovarian function at menopause. In general, endoxifen appears to be a clinically relevant metabolite and necessary to control breast cancer cell growth in a high oestrogen environment.

Methods

Cells and Culture Conditions

A panel of ER-positive human breast cancer cell lines MCF7, T47D, BT474 and ZR-75-1 were used in this study. All cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in phenol-red RPMI 1640 medium, containing 10% foetal bovine serum (FBS) (HyClone Laboratories , Logan, UT, USA), 2mM glutamine, penicillin at 100 units/mL, streptomycin at 100 µg/mL, 1 X non-essential amino acids (all from Life Technologies, Grand Island, NY, USA), and bovine insulin at 6 ng/mL (Sigma-Aldrich, St. Louis, MO, USA). All cells were cultured in T185 culture flasks (Thermo Scientific, Pittsburgh, PA, USA) and passaged twice a week in 1:3 ratio. All cultures were grown in 5% CO_2 at 37°C.

Reagents for treatments

Oestrone (E_1), 17β-oestradiol (E_2), TAM, 4OHT, NDMTAM were all purchased from Sigma-Aldrich, St. Louis, MO, USA. Endoxifen was a generous gift from Dr. James Ingle (Mayo Clinic, Rochester, MN) and was used for Western blot experiments. Endoxifen was also synthesized by Muerdter and used for all other experiments in this study. All compounds were dissolved in ethanol and were stored at -20˚C and protected from light.

Cell proliferation assays

All cells were cultured in oestrogen-free medium (phenol red-free RPMI 1640 media supplemented with 10% charcoal-stripped FBS (SFS)) for 3 days before beginning the proliferation assay. On day 0 of the experiment cells were seeded in oestrogen-free RPMI

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media containing 10% SFS at a density of 10,000 cells per well respectively in 24-well cell culture plates (Corning, Tewksbury, MA, USA). After 24h cells were treated with combinations of oestrogens, TAM and its metabolites in different concentrations (Tables 1-3) prepared in oestrogen-free RPMI. All treatments were performed in triplicate. The medium containing the test compounds was changed on days 4 and 7, and the experiment was stopped on day 8. Cells were washed with cold PBS (Life Technologies, Grand Island, NY, USA) at least twice and analyzed with Fluorescent DNA quantification kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions, and samples were read on a Mithras LB540 fluorometer/luminometer (Berthold Technologies, Oak Ridge, TN, USA) in black wall 96-well plates (Thermo Scientific, Pittsburgh, PA, USA).

Real-time PCR

MCF-7 cells were cultured in oestrogen-free medium for 3 days before seeding and treatment. Cells were seeded after oestrogen starvation in 6-well cell culture plates (Corning, Tewksbury, MA, USA) at density of 300,000 cells per well. Cells were treated with test compounds for 48 hours. Total RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY, USA) and an RNeasy RNA purification kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed by first synthesizing cDNA by reverse transcribing 1 µg of total RNA using a high-capacity cDNA reverse transcription kit (Life Technologies, Grand Island, NY, USA) as per the manufacturer's instructions and subsequently diluted to 500 µL with nuclease-free water. The real-time PCR was performed in a 20 µL reaction which included 1x SYBR green PCR master mix (Life Technologies, Grand Island, NY, USA), 125 nM each of forward and reverse primers and 5 µL of diluted cDNA using an ABI Prism 7900 HT Sequence Detection System (Life Technologies, Grand Island, NY, USA). The fold change in expression of transcripts was calculated using the ΔΔCt method, with the ribosomal protein RPLP0 mRNA

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as the internal control. Primers sequences that were used for human TFF1 cDNA amplification are: 5'-CATCGACGTCCCTCCAGAAGA-3' sense, and 5'-CTCTGGGACTAATCACCGTGCTG-3' anti-sense; human GREB1 gene: 5'-CAAAGAATAACCTGTTGGCCCTGC-3'sense,5'-GACATGCCTGCGCTCTCATACTTA-3' anti-sense; the reference gene RPLP0: 5'-GTGTTCGACAATGGCAGCAT-3' sense, 5'-GACACCCTCCAGGAAGCGA-3' anti-sense. All primers were obtained from Integrated DNA Technologies Inc. (IDT, Coralville, IA, USA).

Immunoblotting

The cells were oestrogen starved in oestrogen free media for 3 days before seeding. Cell were seeded on 10cm Petri dishes (Corning, Tewksbury, MA, USA) at a density of 3 million cells per plate and were incubated overnight. The cells were treated for 24 hrs with the test compounds. Subsequently cells were washed with cold PBS (Life Techologies, Grand Island, NY) twice and were lysed using a RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA), that contained 1x Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN, USA) and 1x phosphatase inhibitors (Calbiochem, Gibbstown, NJ, USA). The cells were lysed for 60 minutes on rotation at 4˚C and then centrifuged at 12,000rpm for 20 minutes. Supernatants were transferred into fresh tubes and stored on ice. The concentrations of proteins were measured via BCA assay (Pierce, Rockford, IL, USA). 20 µg of each protein sample diluted in a NuPAGE LDS loading dye were loaded and separated on NuPAGE 4–12% Bis-Tris Gel (Life Techologies, Grand Island, NY, USA). After the electrophoresis the samples were transferred onto Hybond-ECL Nitrocellulose Membranes (Amersham Biosciences, Piscataway, NJ, USA), which were then blocked using 5% skim milk in TBS-T (50nM Tris-HCl pH 7.5, 150nM NaCl, 0.1% Tween-20) for 1 hour at room temperature. The membranes were subsequently probed with primary antibodies anti-ERα (clone G-20) (Santa Cruz Biotechnology, Santa Cruz, CA), and with anti-β-actin (clone AC-

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15) (Sigma-Aldrich, St. Louis, MO, USA) diluted in blocking buffer at ratios recommended by the suppliers at 4°C overnight. The membranes were washed three times (ten minutes each) the next day with the TBS-T buffer and subsequently incubated with the appropriate HRP-linked secondary antibodies (anti-mouse or anti-rabbit from Cell Signaling Technology, Danvers, MA, USA) diluted in blocking buffer for 1 hour at room temperature. The membranes were washed again as described above with TBS-T buffer and the signal was visualized using Western Lightning Plus-ECL Reagents (Perkin Elmer, Waltham, MA, USA). The immunoblot was analyzed by densitometry using Image J software (NIH) measuring the pixel intensity of the lanes, normalized to their corresponding β-actin lane pixel intensity and then normalized to vehicle control as 100 percent.

Statistical Analysis

To test the effects and possible interactions between treatment and genotype in the proliferation assays, we used ANOVA with a balanced 2-factor design, followed by Tukey pairwise comparison of treatments and genotypes. A P-value less than 0.05 was considered significant. To test the effects of treatment alone, including control, we used one way ANOVA, followed by Tukey pairwise comparisons of treatment doses. To test the effects and possible interactions of treatment and dose, we used ANOVA with a balanced 2-factor design. All computations were carried out using R, Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria). For testing the significance of treatment in RT-PCR experiments Student's t-test was used.

Nomenclature

All drug/molecular nomenclature conforms to British Journal of Pharmacology Guide to Receptors and Channels (Alexander *et al.*, 2011).

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Results

Proliferation Assays

To assess the biological effect of the different treatments on the panel of ER positive breast cancer cell lines (MCF-7, T47D, ZR-75-1, BT474), we used a DNA quantification based assay as described in the Methods section. To simulate the premenopausal environment we used oestrone (E_1) and oestradiol (E_2) concentrations at average circulating levels measured in premenopausal women taking TAM (Jordan *et al.*, 1991). The calculated concentrations for E_1 and E_2 were 4nM and 2nM respectively for luteal phase, which corresponds to the average levels of oestrogens throughout the 30-day menstrual cycle in patients taking TAM. The concentrations of TAM and its metabolites grouped by CYP2D6 genotypes were acquired during a previously published study (Murdter *et al.*, 2011) (Table 1). Oestrogens were able to stimulate the growth of all cells lines ($P< 0.05$ by 1-way ANOVA with Tukey pairwise comparisons) (Figure 2) and the addition of TAM (labeled as T in the figures) and the combined primary metabolites 4OHT and NDMTAM (labeled as M in the figures) were able to only partially but significantly inhibit the oestrogen action in all the cell lines ($P< 0.05$ by 1-way ANOVA with Tukey pairwise comparisons) (Figure 2). Endoxifen (labeled as E in the figures) in EM concentration in combination with TAM, 4OHT and NDMTAM was able to further inhibit oestrogen action and reduce proliferation further ($P< 0.05$ by 1-way ANOVA with Tukey pairwise comparisons) (Figure 2). Addition of endoxifen at the IM concentration produced less of an antioestrogenic effect when compared to EM concentration but still significant in MCF-7, T47D and BT474 cells with $P< 0.05$ (2-way ANOVA), but not in ZR-75-1 cell lines when compared to treatment with no endoxifen in that genotype group with $P>0.05$ with 2-way ANOVA (Figure 2). Lastly, endoxifen added at the PM concentration did not result in statistically significant reduction of the oestrogenic

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effect on cell growth in any of the tested cell lines (P> 0.05 by 2-way ANOVA for all cell lines) (Figure 2).

It should be noted that the addition of endoxifen to TAM and its primary metabolites was not able to completely inhibit the effects of oestrogens to vehicle control levels in any of the cell lines, in any of the genotype groups (P<0.05 by 1-way ANOVA with Tukey pairwise comparisons), except ZR-75-1 cells in EM and IM genotype groups only (P>0.05 by 2-way ANOVA) (Figure 2). This is most obvious in the most oestrogen-responsive cell line MCF-7. There is a significant difference in cell numbers between vehicle and endoxifen with TAM and primary metabolites treatment (P< 0.05 by 1-way ANOVA with Tukey pairwise comparisons) (Figure 2). We decided to focus in our further experiments on the MCF-7 cell line, in particular, since it is the most oestrogen-responsive and most difficult to block growth.

We assessed the hypothesis that increasing the administered dose of TAM from the standard 20 mg/day to a higher dose (40 mg/day) would be beneficial by subsequently increasing endoxifen levels for patients with IM and PM genotypes and further inhibit the oestrogenic effect. We used the circulating concentrations of TAM and its metabolites provided by Flockhart (Table 2) based on a study (Irvin *et al.*, 2011) in breast cancer patients treated with TAM. These patients were genotyped for CYP2D6 as IM and PM and treated with both standard dose of TAM (20 mg/day) and 40 mg/day (Irvin *et al.*, 2011). Treatments of MCF-7 cells with concentrations for 20 mg/day of TAM and its metabolites in the study (Irvin *et al.*, 2011) showed that the EM concentration of endoxifen was able to further inhibit the oestrogenic action in cells than TAM with 4OHT and NDMTAM alone (P< 0.05 by 1-way ANOVA with Tukey pairwise comparisons) (Figure 3A). The IM concentration of endoxifen was less potent than EM concentration; however, this was still able to enhance the antioestrogenic effect of TAM and mixture of its primary metabolites (P< 0.05 by 1-way

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ANOVA with Tukey pairwise comparisons) (Figure 3A). The PM concentration of endoxifen was not able to produce any additional antioestrogenic effect in these cells (P> 0.05 by 1-way ANOVA with Tukey pairwise comparisons) (Figure 3A). To further test the antioestrogenic potency TAM and its metabolites during treatment with increased dose of 40 mg/day we have used corresponding concentrations shown in Table 2. The 2-way ANOVA analysis shows that there are no statistically significant interactions between the treatment and dose for the antioestrogenic efficacy of endoxifen and TAM with primary metabolites at concentrations corresponding to 40 mg/day when compared to 20 mg/day concentrations in both IM and PM genotype scenarios in MCF-7 cells (P>0.05) (Figure 3B). Though it should be noted, that in both genotype scenarios increased concentrations corresponding to 40mg/day dose did reduce cell proliferation compared to 20 mg/day.

Using the ratios of increase for concentrations of each metabolite in both IM and PM genotypes with 40 mg/day dose from 20 mg/day, provided by Flockhart (Irvin *et al.*, 2011), we have calculated what would the metabolite concentrations be at 40mg/day using the 20 mg/day results obtained by Muerdter (Murdter *et al.*, 2011). The resulting concentrations are presented in Table 3 and were used in the treatments of MCF-7 cells. The 2-way ANOVA analysis of the results again showed that there are no significant interactions between treatment and dose, without taking genotype as a factor (P> 0.05) (Figure 3C), meaning that the antioestrogenic efficacy of TAM and its metabolites in concentrations corresponding to 40 mg/day and 20 mg/day of TAM with primary metabolites and endoxifen in both IM and PM concentrations had no significant biological improvement on inhibiting the effects of average premenopausal concentrations of oestrogens in MCF-7 cells.

To assess why the antioestrogens were not able to fully inhibit the growth in the MCF-7 cell line, we have used premenopausal levels of oestrogens in combination with TAM and its primary metabolites at EM concentrations (Table 1) with increasing concentration of

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endoxifen (Figure 4). The results demonstrate that addition of increasing concentrations of endoxifen is able to inhibit the proliferation effects of oestrogens. The inhibition results in this experiment are consistent with the inhibition results in previous experiments in MCF-7 cells (Figs. 2 & 3). However, to fully inhibit oestrogen-stimulated growth a much higher concentration of endoxifen is needed, which is outside the range of concentrations observed in patients (Table 1 and 2) (Figure 4, solid line). We have also tested the antioestrogenic properties of increasing concentrations of endoxifen alone against constant premenopausal levels of oestrogens. The results show that endoxifen alone (Figure 4, dashed line) is not an effective antioestrogen as in combination with TAM and its primary metabolites (Figure 4, solid line).

Since complete inhibition of oestrogen action in MCF-7 cells occurred only with very high concentrations of endoxifen, we have also assessed the impact of the different levels of TAM metabolites in perimenopausal women (49-55 years). We have defined the level as perimenopausal when TAM and its primary metabolites inhibit oestrogen-induced growth of MCF-7 cells by 50%. We have used constant TAM and primary metabolites at EM concentrations (Murdter *et al.*, 2011) (table 1) and serially diluted the average premenopausal concentrations of oestrogens down 32 fold (Figure 5A). The concentration of oestrogens that was inhibited by TAM and primary metabolites by 50% was 4 times lower the average premenopausal levels of oestrogens (Figure 5A), however, this still produced same level of cell proliferation as in previous experiments (Figure 5B). Addition of endoxifen at concentrations corresponding to EM, IM and PM genotypes to that perimenopausal level of oestrogens showed that the antioestrogens almost completely inhibit cell proliferation with no differences in between genotype groups (Figure5B).

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Real-time PCR

To assess the antioestrogenic properties of endoxifen and TAM with primary metabolites on regulating oestrogen responsive genes in MCF-7 we employed real-time PCR using primers for GREB1 and TFF1 cDNAs. The cells were treated for 48 hours. The results show that average premenopausal levels of oestrogens are able to induce both of the selected oestrogen responsive genes (Figure 6) ($P< 0.05$ by Student's t-test). Results demonstrate that TAM and it primary metabolites in EM concentrations are not able to significantly reduce the oestrogen-induced RNA production (Figure 6A) ($P> 0.05$ by Student's t-test), the same result was observed for TFF1 gene (Figure 6B) ($P> 0.05$ by Student's t-test). However, addition of endoxifen in EM concentration was able to significantly reduce the oestrogen-induced GREB1 and TFF1 mRNA expression by an average of 50% ($P< 0.05$ by Student's t-test for both genes) (Figure 6).

Immunoblotting

To investigate the impact of TAM and its metabolites in an average premenopausal environment on ERα protein we have performed treatments in MCF-7 cells for 24 hours and investigated total cell lysates by Western blotting. The results show that the E_1/E_2 treatment is able to reduce the levels of ERα protein (Figure 7). Addition of TAM and its primary metabolites (T+M) and EM concentrations partially reversed the oestrogen action, however addition of endoxifen at EM concentration to the antiestrogenic mix (T+M+E), not just completely reversed the oestrogen action, but also increased the ERα protein levels, enhancing the antioestrogenic effect. The pure antiestrogen fulvestrant (ICI) was used as a positive control for ERα protein degradation.

Discussion and Conclusions

Tamoxifen and its metabolites have always been classified (Jordan, 1984) as competitive inhibitors of oestrogen action at the ER. Overall, our findings confirm this

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classification in a simulation of the antioestrogenic therapeutic environment of women with functioning ovaries being treated with tamoxifen to control the growth of ER-positive breast cancer. We demonstrate the competitive and reversible relationship between tamoxifen and its metabolites with major oestrogens (E_1/E_2) measured in premenopausal women taking tamoxifen. We successfully demonstrate the positive relationship between the serum concentrations of endoxifen and the PM, IM and EM genotypes that control the successful conversion of NDMTAM to its active metabolite endoxifen (Fig. 1). In practical terms tamoxifen is not really a classical pro-drug. Tamoxifen does not need to be metabolically activated, it is an advantage but not a requirement for antioestrogenic efficacy (Allen *et al.*, 1980). The medicine accumulates in patients and achieves steady state within four weeks and both TAM and NDMTAM, which are capable of blocking oestrogen action, have a circulating half-life of 7 and 14 days respectively (Furr & Jordan, 1984). Clinical and laboratory data concerning the role and actions of endoxifen remain inconsistent and contradictory so it is perhaps appropriate to place our observations into perspective with other reports and models.

The traditional approach to study different antioestrogens to prevent tumour growth would be to employ the athymic mouse animal model that is used ubiquitously (Wardell *et al.*, 2013). However, this approach cannot be employed to address the current question of specific mixes of human metabolites of tamoxifen linked to genotypes. Published results are instructive because of known differences in metabolism, excretion and human/animal differences in pharmacokinetics. Oestrogen administration is required for athymic mice to sustain MCF-7 tumour growth (Soule & McGrath, 1980) as there is a hypothalamopituitary lesion that prevents oestrous cycles from occurring (Weinstein, 1978). A silastic sustained oestradiol release capsule may be used and the circulating oestrogen levels to stimulate tumour cell growth track well from patients, to the athymic animal model to cell culture, but

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this is not true for the xenobiotic tamoxifen and its metabolites in humans (Langan-Fahey *et al.*, 1990; Robinson *et al.*, 1991; Robinson *et al.*, 1989).

Published human circulating levels at steady state of tamoxifen and metabolites during adjuvant therapy (20mg/daily- 465±54days) are: TAM 108±23 ng/ml, 4OHT 2.6±0.5 ng/ml and NDMTAM 238±58 mg/ml with endoxifen detected only in 6 out of 10 samples (Robinson *et al.*, 1989). These are almost exactly the concentrations of TAM and primary metabolites used by Hawse et al (Hawse *et al.*, 2013) for the 24 hour gene array studies *in vitro* (competing against 10 nM E_2), that are used to show new unique differences for actions of endoxifen. By contrast, reported levels of tamoxifen and metabolites are completely different in athymic mice treated to inhibit oestrogen-stimulated growth (Robinson *et al.*, 1989). There is clear evidence that the antitumour actions of a xenobiotic tamoxifen in an animal tumour model, does not correlate with human serum levels therefore the present cell culture approach is the appropriate experimental strategy to simulate the premenopausal patient. Another approach *in vivo* is to administer endoxifen alone. Gong (Gong *et al.*, 2013) calculate the precise concentration of endoxifen (53 nM) to prevent tumour growth of ER-positive MCF-7 tumours following a dose ranging study of 0.1-100 mg of endoxifen/kg orally alone. However, this approach is focused on the possible use of endoxifen as a therapy alone (Goetz *et al.*, 2013).

If the ER complex is the key element in oestrogen regulated breast cancer cell growth it is particularly interesting that Wu et al (Wu *et al.*, 2009) report that endoxifen alone causes the rapid destruction of the ER complex. The pure antiestrogen fulvestrant is classified as an ER downregulator as the fulvestrant-ER complex adopts an alien conformation targeting it for rapid ubiquitination and proteosomal lysis. Fulvestrant alone was used as a positive control for ER downregulation in the current study (Fig. 7). Endoxifen reversed the downregulation of ER noted with E_1/E_2 and caused an accumulation of ER when compared to

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treatment with TAM and its primary metabolites without endoxifen (Fig. 7). We recently report that endoxifen alone does not produce down regulation of ER (Maximov *et al.*, 2014). The Wu et al (Wu *et al.*, 2009) also noted a profound downregulation of ER complex with endoxifen in T47D cells, but these cells have a unique form of ER regulation; oestrogen is necessary to increase ER synthesis so it would be anticipated that a potent nonsteroid antiestrogen such as endoxifen would switch off ER synthesis (Pink & Jordan, 1996).

Two further studies support the data presented in Fig. 7 for the accumulation of ER with endoxifen in MCF-7 cells. Firstly, the same pattern of ER complex accumulation (Fig. 7) is noted with ER and endoxifen alone in multiple cellular context and compared with 4OHT (Obiorah *et al.*, 2014). Secondly, there is the possibility of different proportions of geometric isomers of endoxifen occur in different preparations. If the isomers have different oestrogenic/antioestrogenic pharmacology at the ER then this could lead to complex biochemical changes in structure and function. This is an explanation offered by Hawse et al (Hawse *et al.*, 2013) to explain why Lim and colleagues (Lim *et al.*, 2006) note no significant changes in gene array profile between 4OHT and endoxifen; the Lim endoxifen (Lim *et al.*, 2006) was apparently a 3:1 mixture of Z and E isomers but the Hawse endoxifen was 98% Z isomer. In our studies we used the endoxifen provided by the same group at the Mayo Clinic, MN. To address the pharmacology of the isomers the E and Z isomers of endoxifen and 4OHT we synthesized the individual isomers as fixed ring derivatives. We examined each compound for oestrogenic/antioestrogenic activity in MCF-7 cells and the regulation of prolactin gene expression in GH3 rat pituitary tumour cells (Maximov *et al.*, 2014). The E fixed ring isomers are weak oestrogens with antioestrogenic properties whereas the Z fixed ring isomers are antioestrogens. However, the Z isomers of 4OHT and endoxifen cause accumulation of the ER complex just like the commercially available Z endoxifen or 4OHT and the E isomers, though weak oestrogens, do not down regulate ER like oestradiol. It is the

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shape of the oestrogen and the conformation that cause ER complex degradation not the fact it is an oestrogen. Although a mixture of endoxifen isomers as noted clinically from patient serum by Muerdter (Murdter *et al.*, 2011) the proportion found is predominantly antioestrogenic on breast cancer cell growth (Maximov *et al.*, 2014). Molecular modelling demonstrate that Z endoxifen and 4OHT have a very similar fit in the ER complex whereas the SERMs raloxifene and bazedoxifene with their larger side chain rings can cause down regulation of ER (Wardell *et al.*, 2013) (but not as dramatic as the pure antiestrogen fulvestrant (Nicholson *et al.*, 1995; Osborne *et al.*, 1995)), and have the capacity to completely neutralize and shield amino acid D351 thereby preventing helix 12 from closing.

We have created a simulation of genotypes of tamoxifen metabolism *in vitro* because it is not possible to replicate these genotypes *in vivo* in tumours being grown in athymic mice or rats used in other antitumour studies (Robinson *et al.*, 1991; Robinson *et al.*, 1989). Nevertheless, many important lessons are taught by the development of a precise database *in vitro* because they are instructive to interpret current results with endoxifen *in vivo*. The models *in vitro* create an understanding of the circulating ratios of tamoxifen metabolites that can effectively control precise levels of oestrogen stimulated ER positive tumour growth. There is, however, one final caveat. Tamoxifen is equally efficacious as a therapy in pre- and postmenopausal patients despite large increases in circulating oestrogen in premenopausal patients (EBCTCG, 2005). However, the simulation using reported concentrations of oestrogens and optimal reported mixtures of TAM and metabolites do not completely block oestrogen-induced replication or gene activation except in a "perimenopausal" scenario (Fig. 5). Nevertheless, the TAM + the mix of antioestrogenic metabolites do predictably reverse the down regulation of ER with oestrogens alone (Fig. 7). This is yet another dimension of complexity when addressing the pharmacology of tamoxifen in the laboratory. We are ignorant about the actual concentrations of tamoxifen and metabolites in the tumour cell and

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these may be log concentrations higher than circulating levels once steady state is achieved. Thus circulating levels of tamoxifen, a lipophilic and highly protein bound drug may only be a rough guide to reality at the receptor. The animal studies already in the literature already teach us that lesson.

Based on the results of cell growth assays, gene expression regulation, and ER protein level regulation, we can conclude that endoxifen plays a major antioestrogenic role. At concentrations corresponding to different genotypes of CYP2D6 endoxifen in combination with TAM and its metabolites can cause inhibition of oestrogen-induced growth of breast cancer cells at higher concentrations, but not in concentrations corresponding to PM genotype of CYP2D6, and also can block the action of oestrogens on oestrogen-responsive genes and ER protein. These results are indicative that higher concentrations of endoxifen are important for producing more antioestrogenic effects during adjuvant therapy.

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List of author Contributions

Maximov PY experimental design and execution, manuscript preparation, McDaniel RE technical support, Fernandes DJ technical support, Korostyshevskiy VR statistical analysis, Bhatta P technical support, Muerdter TE provided clinical concentrations of tamoxifen and metabolites, Flockhart DA provided clinical concentrations of tamoxifen and metabolites, and Jordan VC experimental design and project leader.

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Conflict of Interest

None

Abbreviations

ER oestrogen receptor

4OHT 4-hydroxytamoxifen

NDMTAM N-desmethyltamoxifen

TAM tamoxifen

CYP2D6 cytochrome P450 2D6 variant

E_1 oestrone

E_2 oestradiol

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References

Alexander SP, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th edition. *Br J Pharmacol* **164 Suppl 1:** S1-324.

Allen KE, Clark ER, Jordan VC (1980). Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. *Br J Pharmacol* **71**(1)**:** 83-91.

Davies C, Godwin J, Gray R, Clarke M, Cutter D, Darby S*, et al.* (2011). Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* **378**(9793)**:** 771-784.

Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V*, et al.* (2013). Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. *Lancet* **381**(9869)**:** 805-816.

Dehal SS, Kupfer D (1997). CYP2D6 catalyzes tamoxifen 4-hydroxylation in human liver. *Cancer Res* **57**(16)**:** 3402-3406.

Desta Z, Ward BA, Soukhova NV, Flockhart DA (2004). Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: prominent roles for CYP3A and CYP2D6. *J Pharmacol Exp Ther* **310**(3)**:** 1062-1075.

Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, Bliss J*, et al.* (2010). Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. *J Clin Oncol* **28**(3)**:** 509-518.

EBCTCG (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* **365**(9472)**:** 1687-1717.

Furr BJ, Jordan VC (1984). The pharmacology and clinical uses of tamoxifen. *Pharmacol Ther* **25**(2)**:** 127-205.

Goetz MP, Rae JM, Suman VJ, Safgren SL, Ames MM, Visscher DW*, et al.* (2005). Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. *J Clin Oncol* **23**(36)**:** 9312-9318.

Goetz MP, Suman VA, Reid JR, Northfelt DW, Mahr MA, Dockter T*, et al.* (2013). A first-in-human phase I study of the tamoxifen (TAM) metabolite, Z-endoxifen hydrochloride (Z-

For Peer Review

Endx) in women with aromatase inhibitor (AI) refractory metastatic breast cancer (MBC) (NCT01327781). Abstract PD3-4. In: *San Antonio Breast Cancer Symposium*. San Antonio, TX.

Gong IY, Teft WA, Ly J, Chen YH, Alicke B, Kim RB*, et al.* (2013). Determination of clinically therapeutic endoxifen concentrations based on efficacy from human MCF7 breast cancer xenografts. *Breast Cancer Res Treat* **139**(1)**:** 61-69.

Hawse JR, Subramaniam M, Cicek M, Wu X, Gingery A, Grygo SB*, et al.* (2013). Endoxifen's molecular mechanisms of action are concentration dependent and different than that of other anti-estrogens. *PLoS One* **8**(1)**:** e54613.

Irvin WJ, Jr., Walko CM, Weck KE, Ibrahim JG, Chiu WK, Dees EC*, et al.* (2011). Genotype-guided tamoxifen dosing increases active metabolite exposure in women with reduced CYP2D6 metabolism: a multicenter study. *J Clin Oncol* **29**(24)**:** 3232-3239.

Johnson MD, Zuo H, Lee KH, Trebley JP, Rae JM, Weatherman RV*, et al.* (2004). Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. *Breast Cancer Res Treat* **85**(2)**:** 151-159.

Jordan VC (1984). Biochemical pharmacology of antiestrogen action. *Pharmacol Rev* **36**(4)**:** 245-276.

Jordan VC, Brodie AM (2007). Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* **72**(1)**:** 7-25.

Jordan VC, Collins MM, Rowsby L, Prestwich G (1977). A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* **75**(2)**:** 305-316.

Jordan VC, Fritz NF, Langan-Fahey S, Thompson M, Tormey DC (1991). Alteration of endocrine parameters in premenopausal women with breast cancer during long-term adjuvant therapy with tamoxifen as the single agent. *J Natl Cancer Inst* **83**(20)**:** 1488-1491.

Langan-Fahey SM, Tormey DC, Jordan VC (1990). Tamoxifen metabolites in patients on long-term adjuvant therapy for breast cancer. *Eur J Cancer* **26**(8)**:** 883-888.

Lim YC, Li L, Desta Z, Zhao Q, Rae JM, Flockhart DA*, et al.* (2006). Endoxifen, a secondary metabolite of tamoxifen, and 4-OH-tamoxifen induce similar changes in global gene expression patterns in MCF-7 breast cancer cells. *J Pharmacol Exp Ther* **318**(2)**:** 503-512.

For Peer Review

Maximov PY, Fernandes DJ, McDaniel RE, Myers CB, Curpan RF, Jordan VC (2014). Influence of the length and positioning of the antiestrogenic side chain of endoxifen and 4-hydroxytamoxifen on gene activation and growth of estrogen receptor positive cancer cells. *J Med Chem* **epub ahead of print**.

Murdter TE, Schroth W, Bacchus-Gerybadze L, Winter S, Heinkele G, Simon W*, et al.* (2011). Activity levels of tamoxifen metabolites at the estrogen receptor and the impact of genetic polymorphisms of phase I and II enzymes on their concentration levels in plasma. *Clin Pharmacol Ther* **89**(5)**:** 708-717.

Nicholson RI, Gee JM, Manning DL, Wakeling AE, Montano MM, Katzenellenbogen BS (1995). Responses to pure antiestrogens (ICI 164384, ICI 182780) in estrogen-sensitive and -resistant experimental and clinical breast cancer. *Ann N Y Acad Sci* **761:** 148-163.

Obiorah IE, Sengupta S, Curpan R, Jordan VC (2014). Defining the conformation of the estrogen receptor complex that controls estrogen induced apoptosis in breast cancer. *Mol Pharmacol* **epub ahead of print**.

Osborne CK, Coronado-Heinsohn EB, Hilsenbeck SG, McCue BL, Wakeling AE, McClelland RA*, et al.* (1995). Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. *J Natl Cancer Inst* **87**(10)**:** 746-750.

Pink JJ, Jordan VC (1996). Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* **56**(10)**:** 2321-2330.

Province MA, Goetz MP, Brauch H, Flockhart DA, Hebert JM, Whaley R*, et al.* (2014). CYP2D6 genotype and adjuvant tamoxifen: meta-analysis of heterogeneous study populations. *Clin Pharmacol Ther* **95**(2)**:** 216-227.

Rae JM, Drury S, Hayes DF, Stearns V, Thibert JN, Haynes BP*, et al.* (2012). CYP2D6 and UGT2B7 genotype and risk of recurrence in tamoxifen-treated breast cancer patients. *J Natl Cancer Inst* **104**(6)**:** 452-460.

Regan MM, Leyland-Jones B, Bouzyk M, Pagani O, Tang W, Kammler R*, et al.* (2012). CYP2D6 genotype and tamoxifen response in postmenopausal women with endocrine-responsive breast cancer: The Breast International Group 1-98 Trial. *J Natl Cancer Inst* **104**(6)**:** 441-451.

Robinson SP, Langan-Fahey SM, Johnson DA, Jordan VC (1991). Metabolites, pharmacodynamics, and pharmacokinetics of tamoxifen in rats and mice compared to the breast cancer patient. *Drug Metab Dispos* **19**(1)**:** 36-43.

For Peer Review

Robinson SP, Langan-Fahey SM, Jordan VC (1989). Implications of tamoxifen metabolism in the athymic mouse for the study of antitumor effects upon human breast cancer xenografts. *Eur J Cancer Clin Oncol* **25**(12)**:** 1769-1776.

Schroth W, Goetz MP, Hamann U, Fasching PA, Schmidt M, Winter S*, et al.* (2009). Association between CYP2D6 polymorphisms and outcomes among women with early stage breast cancer treated with tamoxifen. *JAMA* **302**(13)**:** 1429-1436.

Soule HD, McGrath CM (1980). Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice. *Cancer Lett* **10**(2)**:** 177-189.

Stearns V, Johnson MD, Rae JM, Morocho A, Novielli A, Bhargava P*, et al.* (2003). Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. *J Natl Cancer Inst* **95**(23)**:** 1758-1764.

Wardell SE, Nelson ER, Chao CA, McDonnell DP (2013). Bazedoxifene exhibits antiestrogenic activity in animal models of tamoxifen-resistant breast cancer: implications for treatment of advanced disease. *Clin Cancer Res* **19**(9)**:** 2420-2431.

Weinstein Y (1978). Impairment of the hypothalamo-pituitary-ovarian axis of the athymic "nude" mouse. *Mech Ageing Dev* **8**(1)**:** 63-68.

Wu X, Hawse JR, Subramaniam M, Goetz MP, Ingle JN, Spelsberg TC (2009). The tamoxifen metabolite, endoxifen, is a potent antiestrogen that targets estrogen receptor alpha for degradation in breast cancer cells. *Cancer Res* **69**(5)**:** 1722-1727.

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Figure Legends:

Figure 1. Metabolism of tamoxifen (TAM) by isoforms of cytochrome P450 to N-desmethyltamoxifen (NDMTAM) and hydroxylated metabolites 4-hydroxytamoxifen (4OHT) and 4-hydroxy-N-desmthyltamoxifen (endoxifen) with high affinity for the ER. CYP2D6 plays a major role in the metabolism of NDMTAM into endoxifen.

Figure 2. Results of ER-positive breast cancer cell proliferation assays: A) MCF-7, B) T47D, C) ZR-75-1 and D) BT474. Treatments were made as follows: **Veh**- Vehicle control, **E1/E2 premen**- premenopausal oestrogens (E_1 4nM, E_2 2 nM), **E1/E2 premen+T+M**- oestrogens at premenopausal levels with TAM (T) and its primary metabolites (NDMTAM and 4OHT) (M) at different CYP2D6 genotype concentrations (Table 1), **E1/E2 premen+T+M+E**- oestrogens at premenopausal levels, TAM (T) , primary metabolites (M) and endoxifen (E) at different CYP2D6 genotype concentrations (Table 1). Asterisk indicates statistically significant change in treatment from addition of endoxifen.

Figure 3. (A) Cell proliferation assessment in MCF-7 cell line using premenopausal levels of oestrogens (E_1/E_2) and antioestrogens TAM and its primary metabolites (NDMTAM and 4OHT) alone or in combination with endoxifen corresponding to concentrations obtained by Flockhart in breast cancer patients with Extensive Metabolizers (EM), Intermediate Metabolizers (IM) and Poor Metabolizers (PM) CYP2D6 genotype (Irvin *et al.*, 2011). Treatments were made as follows: **Veh**- Vehicle control, **E1/E2**- the premenopausal average oestrogen concentrations, **E1/E2+T+M**- oestrogens with TAM (T) and its primary metabolites (NDMTAM and 4OHT) (M) (Table 2), **E1/E2+T+M+E**- oestrogens, TAM, primary metabolites and endoxifen (E) (Table 2). Asterisk indicates statistically significant change in treatment from addition of endoxifen. (B) Cell proliferation assessment in MCF-7 cells after treatment with premenopausal levels of oestrogens (E_1/E_2) and with TAM (T) and

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its primary metabolites (M) without or with endoxifen (E) corresponding to IM and PM genotypes during treatment with 20mg daily or 40 mg daily; measured by Flockhart (Irvin *et al.*, 2011) (Table 2). Treatments were made as follows: **Veh**- Vehicle control, **E1/E2**- the premenopausal average oestrogen concentrations, **E1/E2+T+M 20mg**- premenopausal oestrogens with TAM and its primary metabolites (NDMTAM and 4OHT) corresponding to 20mg/day treatments corresponding to IM and PM genotypes (Table 2), **E1/E2+T+M+E 20mg**- oestrogens, TAM, primary metabolites and endoxifen corresponding to 20mg/day treatment corresponding to IM and PM genotypes (Table 2), **E1/E2+T+M 40mg**- oestrogens with TAM and its primary metabolites corresponding to 40mg/day treatments (Table 2), **E1/E2+T+M+E 40mg**- oestrogens, TAM, primary metabolites and endoxifen corresponding to 40mg/day treatment (Table 2). (C) Cell proliferation assessment in MCF-7 cells after treatment with premenopausal levels of oestrogens (E1/E2) and with TAM (T) and its primary metabolites (M) with or without endoxifen (E) corresponding to IM and PM genotypes during treatment with 20 mg/day measured by Murdter (Murdter *et al.*, 2011) and calculated 40 mg/day concentrations based on the metabolite concentration increase ratios from concentrations provided by Flockhart (Irvin *et al.*, 2011). Treatments were made as follows: **Veh** - Vehicle control, **E1/E2**- the premenopausal average oestrogen concentrations, **E1/E2+T+M 20mg**- premenopausal oestrogens with TAM (T) and its primary metabolites (NDMTAM and 4OHT) (M) corresponding to 20mg/day treatments corresponding to IM and PM genotypes (Table 1), **E1/E2+T+M+E 20mg** - oestrogens, TAM (T), primary metabolites (M) and endoxifen (E) corresponding to 20mg/day treatment corresponding to IM and PM genotypes (Table 1), **E1/E2+T+M 40mg**- oestrogens with TAM (T) and its primary metabolites (M) corresponding to 40mg/day treatments (Table 3), **E1/E2+T+M+E 40mg**- oestrogens, TAM (T), primary metabolites (M) and endoxifen (E) corresponding to 40mg/day treatment (Table 3).

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Figure 4. Assessment of MCF-7 cell line growth after treatment with increasing concentrations of endoxifen alone (broken line) or in combination with TAM and its primary metabolites (NDMTAM and 4OHT) at EM levels (solid line) (Murdter *et al.*, 2011) (Table 1). Single asterisk indicates concentrations provided by Muerdter; double asterisk indicates concentrations provided by Flockhart.

Figure 5. (A) Determination of the average putative "perimenopausal" concentrations of oestrogens. MCF-7 cells were treated with TAM (T) and primary metabolites (NDMTAM and 4OHT) (M) at EM genotype concentrations (Table 1) in combination with titrated premenopausal concentrations of oestrogens (E_1/E_2). Asterisk indicates statistically significant change in treatment from addition of premenopausal levels of oestrogens alone when compared to vehicle control. (B) Cell proliferation assay in MCF-7 cells showing the impact of different levels of endoxifen corresponding to different CYP2D6 genotypes in "perimenopausal women". Treatments were made as follows: **Veh-** Vehicle control, **E1/E2 perimen-** calculated perimenopausal oestrogens (E_1 1nM, E_2 0.5 nM), **E1/E2 perimen+T+M-** oestrogens at "perimenopausal" levels with TAM (T) and its primary metabolites (NDMTAM and 4OHT) (M) at different CYP2D6 genotype concentrations (Table 1), **E1/E2 perimen+T+M+E-** oestrogens at "perimenopausal" levels, TAM (T) , primary metabolites (M) and endoxifen (E) at different CYP2D6 genotype concentrations (Table 1). Asterisk indicates statistically significant change in treatment from addition of endoxifen or addition of oestrogens when compared to vehicle control.

Figure 6. Pharmacological impact of TAM and its metabolites with or without endoxifen at concentrations corresponding to EM genotype on oestrogen-responsive gene expression. GREB1 (A), and TFF1 (B) genes mRNA expression measurement by real-time PCR were chosen. The results show that endoxifen is crucial for inhibition of premenopausal oestrogen-stimulated gene expression (GREB1 and TFF1) by at least 50%. Treatments were made as

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follows: **Veh**- Vehicle control, **E1/E2**- the premenopausal average oestrogen concentrations, **E1/E2+T+M**- oestrogens with TAM (T) and its primary metabolites (NDMTAM and 4OHT) (M) at EM genotype concentrations (Table 1), **E1/E2+T+M+E**- oestrogens, TAM (T), primary metabolites (M) and endoxifen (E) at EM genotype concentrations (Table 1). Asterisk indicates statistically significant change in treatment from addition of endoxifen.

Figure 7. Western blotting in MCF-7 cells after 24 hour treatment to show the regulation of ERα protein level regulation. Treatments were made as follows: **Veh**- Vehicle control, **E1/E2**- the premenopausal average oestrogen concentrations, **E1/E2+T+M**- oestrogens with TAM (T) and its primary metabolites (NDMTAM and 4OHT) (M) at EM genotype concentrations (Table 1), **E1/E2+T+M+E**- oestrogens, TAM (T), primary metabolites (M) and endoxifen (E) at EM genotype concentrations (Table 1). Fulvestrant (ICI) was used used as a positive control for ERα protein degradation.

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Table legends:

Table 1. TAM and its metabolites circulating levels measured in breast cancer patients who were genotyped for CYP2D6 and categorized into Extensive (EM), Intermediate (IM) and Poor (PM) Metabolizers. Concentrations were provided by Muerdter and were acquired during a previous study (Murdter *et al.*, 2011).

Table 2.TAM and its metabolites circulating levels measured in breast cancer patients who were genotyped for CYP2D6 and categorized in EM, IM and PM categories (Irvin *et al.*, 2011). TAM dosage during treatment in patients in IM and PM categories was increased from 20 mg/day to 40 mg/day. Concentrations were provided by Flockhart and were acquired during a previous study (Irvin *et al.*, 2011).

Table 3. Calculated concentrations of circulating TAM and its metabolites in breast cancer patients with IM and PM genotype based on concentrations provided by Dr. Murdter. Ratios for concentrations increase during 20 mg to 40 mg daily TAM dosage increase for IM and PM genotype provided by Flockhart were applied to IM and PM genotype concentrations from Dr. Muerdter.

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Table 1

Drug and Metabolite	Extensive metabolizers	Intermediate metabolizers	Poor metabolizers
TAM	383 nM	413 nM	459 nM
NDMTAM	558 nM	776 nM	952 nM
4OHT	6.3 nM	5.3 nM	5.1 nM
Endoxifen	35.6 nM	24.7 nM	9.0 nM

Concentrations provided by Muerdter and were acquired during a previous study (Muerdter et al, 2011).

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Table 2

Drug and Metabolite	Extensive metabolizers	Intermediate metabolizers		Poor metabolizers	
		20 mg/day	40 mg/day	20 mg/day	40 mg/day
TAM	228.7 nM	270.9 nM	342.7 nM	265.7 nM	425.3 nM
NDMTAM	409.6 nM	573.1 nM	763.4 nM	748.2 nM	1198.3 nM
4OHT	4 nM	3 nM	3.8 nM	3.1 nM	3.9 nM
Endoxifen	78.4 nM	49.7 nM	58.6 nM	11.3 nM	34.6 nM

Concentrations were provided by Flockhart and were acquired during previously published study (Irvin et al, 2011). Original concentrations were measured in ng/ml units and are available in Supplementary Table 1.

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Table 3

Drug and Metabolite	Intermediate metabolizers	Poor metabolizers
TAM	520 nM	706 nM
NDMTAM	1132 nM	1580 nM
4OHT	6.7 nM	6.3 nM
Endoxifen	28.9 nM	27 nM

Concentration increase ratios from clinical concentrations provided by Flockhart (Irvin et al, 2011) were applied to concentrations provided by Muerdter (Muerdter et al, 2011) for IM and PM patients to obtain hypothetical concentrations for TAM dose increase to 40mg/day.

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Supplementary Table 1

Drug and Metabolite	Extensive metabolizers	Intermediate metabolizers		Poor metabolizers	
		20 mg/day	40 mg/day	20 mg/day	40 mg/day
TAM	85.15 ng/mL	97.04ng/mL	122.79ng/mL	98.9 ng/mL	152.37ng/mL
NDMTAM	146.76 ng/mL	205.33ng/mL	273.5ng/mL	268.07ng/mL	429.55ng/mL
4OHT	1.57 ng/mL	1.2ng/mL	1.49ng/mL	1.22ng/mL	1.58ng/mL
Endoxifen	34.3 ng/mL	18.5 ng/mL	21.8 ng/mL	4.2 ng/mL	12.9 ng/mL

Concentrations were provided by Flockhart and were acquired during previously published study (Irvin et al, 2011).

Supplementary Table 1. Clinical concentrations of TAM and its metabolites measured in breast cancer patients by Flockhart. Concentrations were obtained during a previous study (Irvin et al, 2011).

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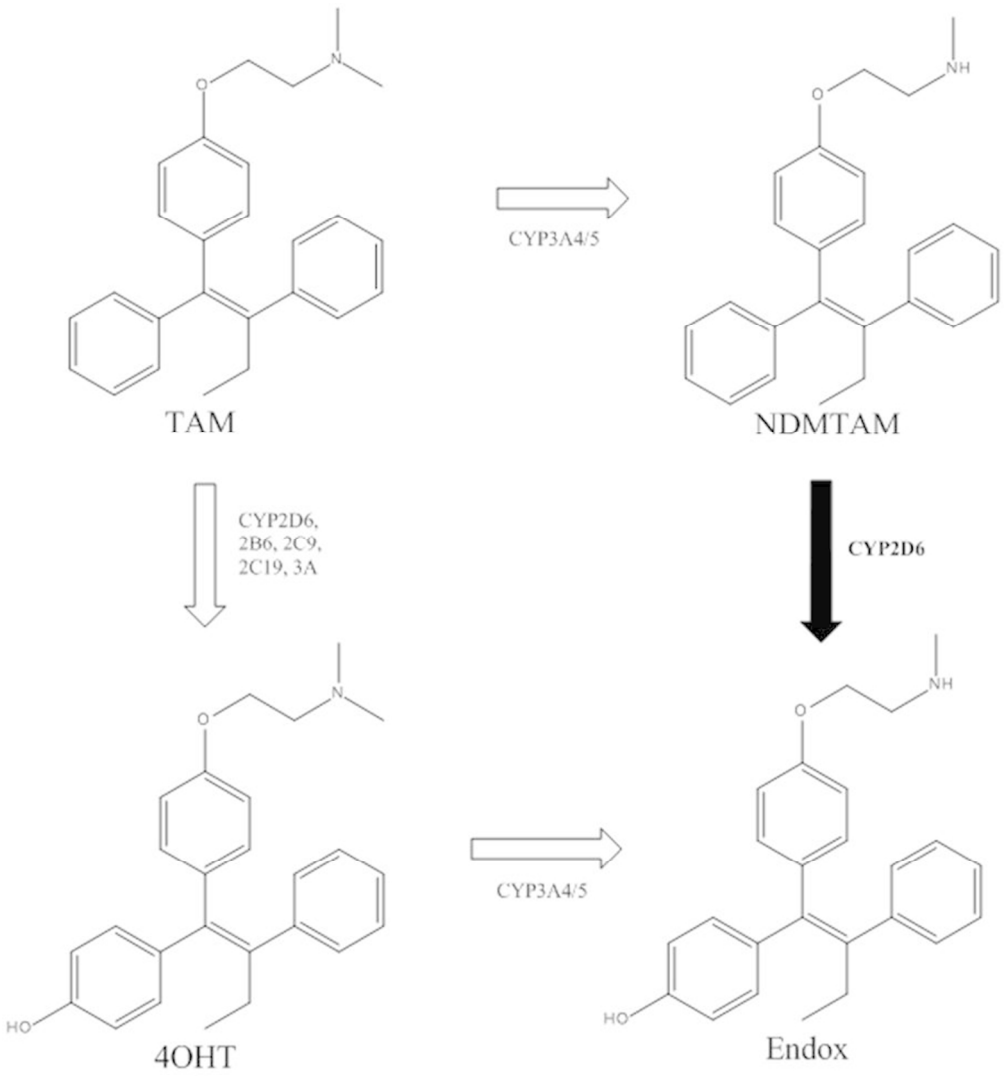

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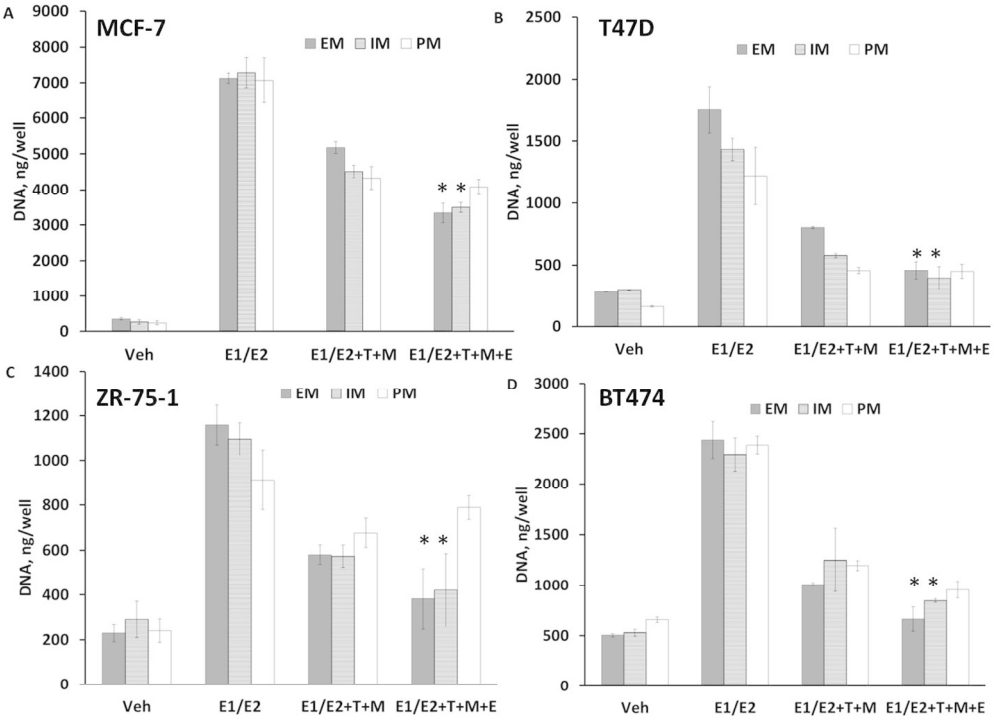

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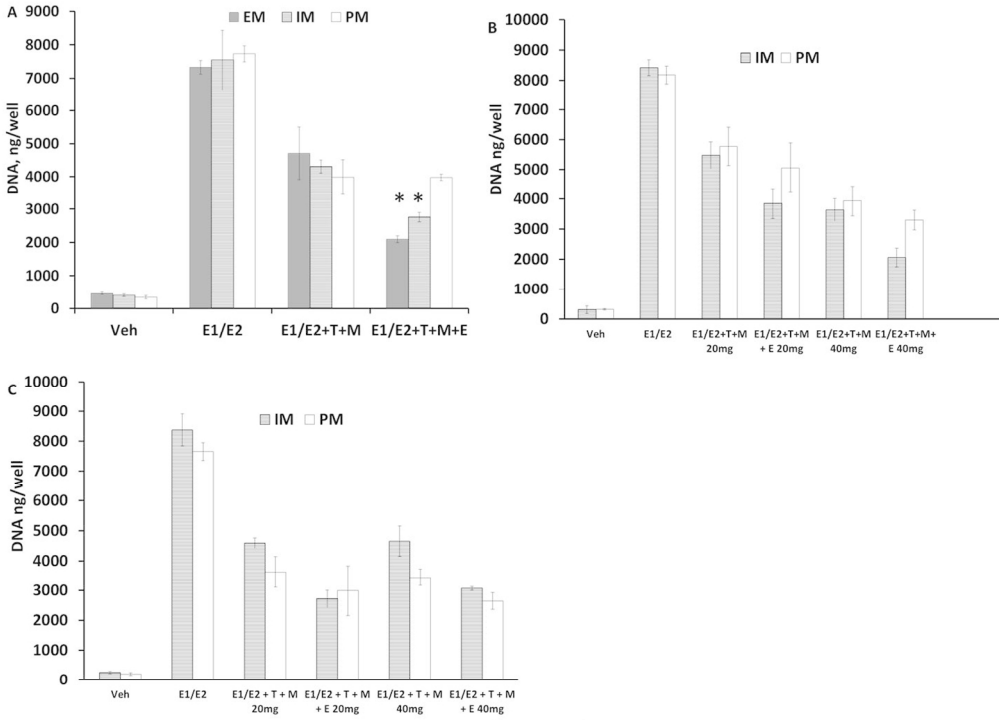

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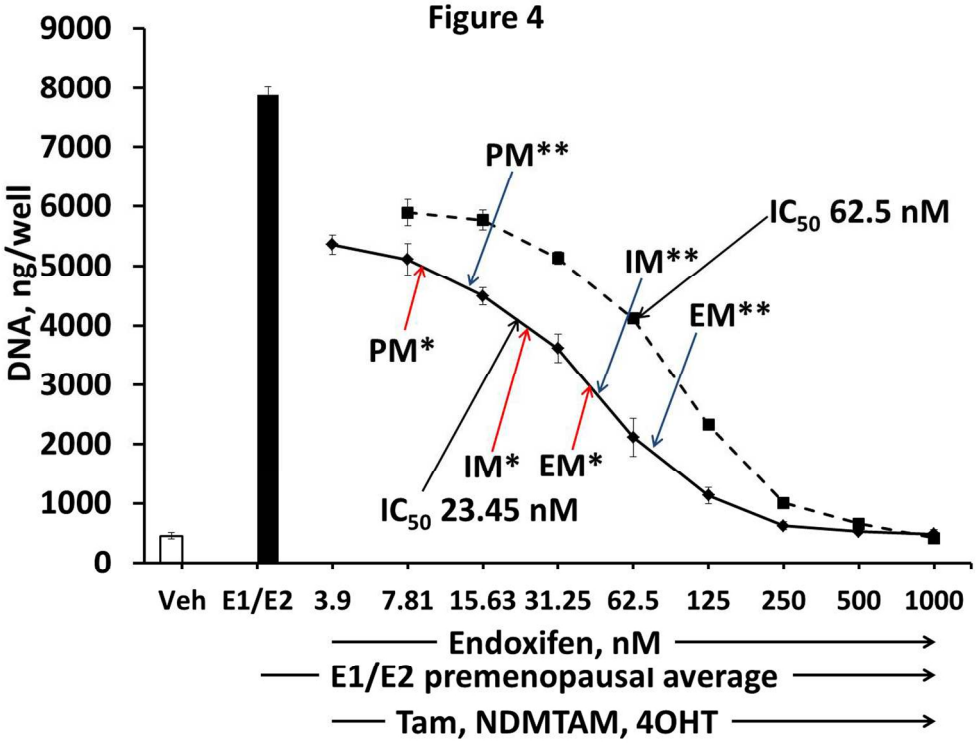

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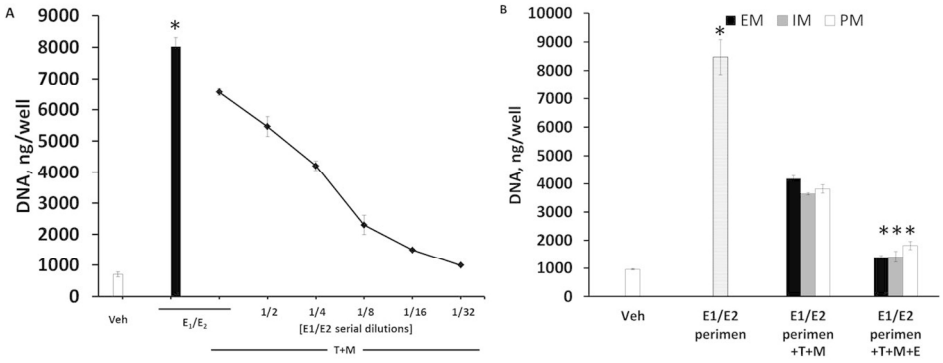

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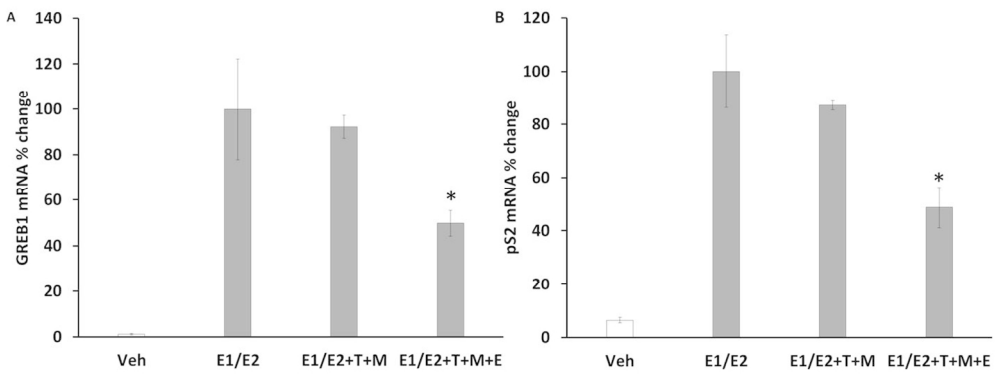

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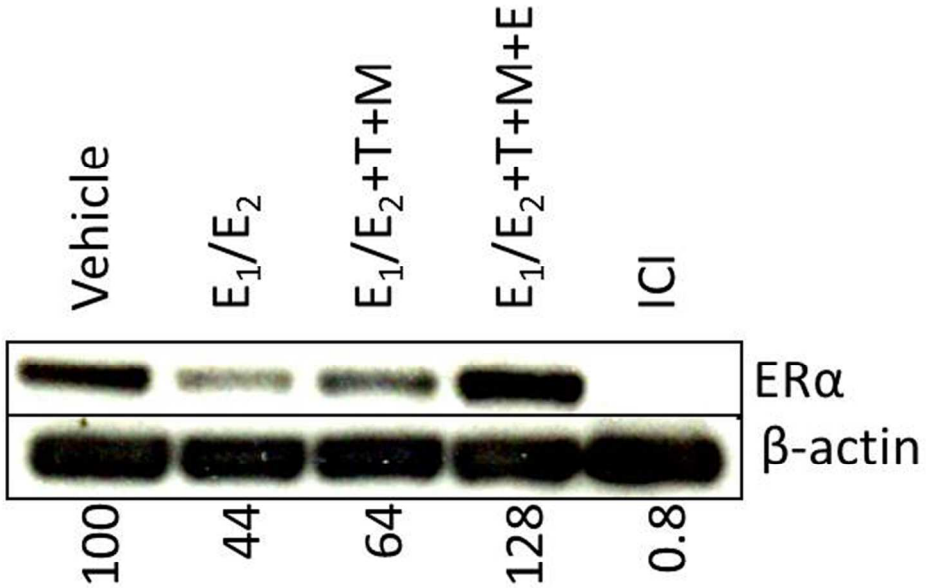

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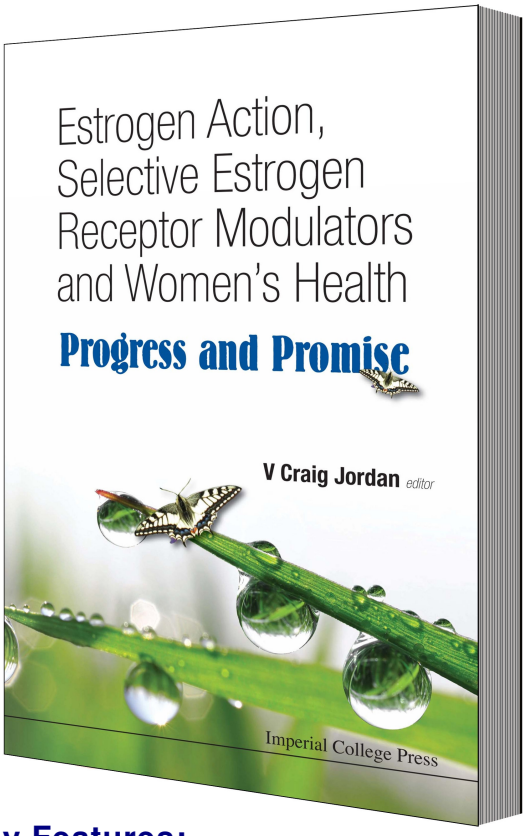

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About the Author & Editor:

Professor V. Craig Jordan was educated at Leeds University, UK (BSc 1969, PhD 1973, DSc 1985, Hon DM 2001) and is referred to by the media and by his professional colleagues as the "Father of Tamoxifen". He defined the scientific strategy for reinventing a failed contraceptive, ICI 46,474, to become the gold standard for the long-term, adjuvant treatment for breast cancer and a pioneering medicine for the prevention of breast cancer. He also was the first to recognize the new drug group (of which tamoxifen is a member) Selective Estrogen Receptor Modulators (SERMs) that switch on and switch off estrogen target sites throughout a woman's body. This knowledge advanced the clinical development of raloxifene for the prevention of both osteoporosis and breast cancer. His recent research on estrogen-induced apoptosis in breast cancer is the result of a 20-year study of the vulnerability of breast cancer during the evolution of acquired resistance to anti-hormone therapy.

As a result of his contributions to medical science and therapeutics, Professor Jordan has received numerous International Awards, including: the Bristol-Myers Squibb Award and Medal for Distinguished Achievement in Cancer Research (2001), the American Cancer Society Medal of Honor (Basic Research Award)(2002), the Charles F. Kettering Prize of the General Motors Cancer Research Foundation (2003), the David A. Karnofsky Award from the American Society for Clinical Oncology (2008) and the St. Gallen International Clinical Breast Cancer Prize (2011). In 2002, Her Majesty Queen Elizabeth II appointed Professor Jordan as Officer of the Most Excellent Order of the British Empire for his contributions to International Breast Cancer Research.

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Philipp Y. Maximov • Russell E. McDaniel •
V. Craig Jordan

Tamoxifen

Pioneering Medicine in Breast Cancer

Springer

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Thanks to all the "Tamoxifen Teams" who translated ideas into life saved over the past 35 years

With the indispensable assistance of Fadeke Agboke, Puspanjali Bhatta, and Amy Botello

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Foreword

I joined the Clinical Research Department of ICI Americas (ICI) in Wilmington, Delaware, in 1973, after competing in the World Championships for Rowing in Moscow, Russia, as a member of the first US women's rowing team. I mention this competition because as I was part of a team who was pioneering the international competition of women's crew, I was among the team at ICI who was pioneering the support and development of "targeted therapies," the first being tamoxifen. The operative word here is *team*. Having previously worked at the National Cancer Institute supporting the Breast Cancer Task Force, I was considered the most qualified individual at the time in the newly formed ICI to plan and organize the clinical investigation of the antiestrogen ICI46,474 in the United States!

I remember asking my director how long it takes to have a drug approved. He told me about 8 years; as a competitor, and not understanding all the aspects of pharmaceutical drug development, I said to myself, "We will do it *four years*." As it is known, the Food and Drug Administration (FDA) approved the labeling for tamoxifen on December 31, 1977, just 4 years and 5 months from the day I was hired. Thinking back over those early years, I recall a number of my colleagues as dedicated individuals who understood the importance of developing tamoxifen—Beverly Bach, Fran Ehrlich, David Sofi, and Bruce Decker—working in clinical research, regulatory affairs, market research, and marketing. Eventually, dozens of staff were all on the mission as a *team* to make tamoxifen available as quickly as possible to those patients who were most likely to benefit.

As you will read throughout this book, the early clinical development of tamoxifen was driven by clinical investigators and scientists in the United States, Canada, and Europe, who devoted their lives to the treatment of patients with breast cancer, such as Pierre Band, Harvey Lerner, and Lucien Israel. In fact, it was Harvey Lerner who demonstrated to Stuart Pharmaceuticals the urgency of continuing to develop this agent when the financial forecast was not compelling.

As you will read, the story of ICI46,474 began with its discovery in the fertility control program at ICI Pharmaceuticals, Alderley Park, Cheshire. It was an excellent morning-after pill in rats, but in fact stimulated ovulation in subfertile women. Although marketed in the United Kingdom for the induction of ovulation, the

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agent's main focus in America was to treat breast cancer. A few small clinical studies of ICI46,474 conducted in Europe had reported modest activity in metastatic breast cancer (Cole et al. *British Journal of Cancer*, 1971;25:270–275 and Ward *British Medical Journal*, 1973;5844:13–14).

In the early 1970s, US clinical trial cooperative groups were focusing on the use of combination cytotoxic chemotherapy with the goal of curing breast cancer. Endocrine therapy was largely viewed as palliative; so there was little possibility that this antiestrogen would make much of an impact in the treatment of metastatic breast cancer or provide reasonable financial returns for investment in clinical studies. Then, in 1973, I met Craig Jordan, one of the few people in the world with a background in, and understanding of, the pharmacology of nonsteroidal antiestrogens. I arranged with my management to provide Craig with an unrestricted research grant at the Worcester Foundation and visited him to discuss the progress as he reinvented the strategic therapeutic use of ICI46,474 to become the drug tamoxifen that we know today. Craig's laboratory studies supported the exclusive use of tamoxifen to treat estrogen receptor (ER)-positive tumors. We used his results, prior to their publication, in our "investigators brochure."

I suggested that Craig become our scientific advisor for tamoxifen and arranged for him to meet the senior leadership of the Eastern Cooperative Oncology Group (ECOG): Doug Tormey, head of the ECOG Breast Committee, and Paul Carbone, chairman of ECOG. ICI Americas continued supporting his research, and in the laboratory, Craig discovered the strategy used today, that of long-term adjuvant tamoxifen therapy specifically targeting ER-positive breast tumors.

Looking at "the good, the bad, and the ugly" of tamoxifen, Craig's laboratory raised the question of whether the agent would increase the incidence of endometrial cancer. It did. This led to the recruitment of gynecologists to the breast cancer patient's care team, an extremely valuable advance at the end of the 1980s, as tamoxifen was about to be tested as a chemopreventive agent in high-risk women.

On a personal note, Craig and I had numerous adventures over the years, coincident with various clinical trial meetings. Here, I relate a story that demonstrates his philosophy of honoring commitment. In 1979, Craig was to be the opening speaker at the tamoxifen meeting in Sorrento, Italy. He was working in Bern, Switzerland, and was scheduled to fly down on an Alitalia flight from Zurich to Naples on the evening before his talk. Craig had to leave Zurich on the last flight that evening, as he had a site visit at the Institute in Bern earlier in the day. Then disaster struck. I learned that Alitalia was to go on strike that evening and urged him to leave Bern at lunch time, if there was to be any hope of his presenting at the meeting. Craig declared, "But I have a room full of site visitors from America—not possible," followed by, "Don't worry, I will be there." After my call, Craig immediately contacted his technician Brigitte Haldemann to drive him through the night over the 730 miles to Sorrento. With an hour to spare and after a shower, he presented his talk.

To this day, tamoxifen remains in the news. The Adjuvant Tamoxifen Longer Against Shorter (ATLAS) trial shows that 10 years of adjuvant tamoxifen is superior to 5 years of tamoxifen (Davies C et al., *Lancet*, 2012; epub 12/12/

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2012). The therapeutic strategy is again being tested successfully, but the benefit in decreasing mortality occurs in the second decade after stopping longer-duration tamoxifen. This phenomenon (Wolf D, and Jordan VC, *Recent Results in Cancer Research*, 1993;127:23–33) led to the new biology of estrogen-induced apoptosis.

What happened to chemoprevention? Tamoxifen became the first agent to be approved by the Food and Drug Administration for reduction of breast cancer incidence in high-risk premenopausal and postmenopausal women. In January 2013, the National Institute for Health and Clinical Excellence (NICE) recommended tamoxifen be made available through the National Health Service in the United Kingdom for the chemoprevention of breast cancer.

This book tells the humanistic story of the development of tamoxifen. It is a tribute of gratitude to the tens of thousands of women and men who participated in clinical trials throughout the development of tamoxifen, which is now a therapeutic agent for the prevention as well as the treatment of minimal through advanced stages of breast cancer, depending on the patient's hormonal receptor status. It is also an acknowledgment of hundreds of clinical oncology health teams working to advance our understanding of the biology of breast cancer as well as thousands of clinicians caring for those with breast cancer.

I am amazed and so grateful that so many millions of lives have been extended and many more have benefited from the research and therapeutic strategies retold in this book. I am personally grateful to have played a role, minimal as it was and is, in the development of tamoxifen.

West Conshohocken, PA, USA

Lois Trench-Hines
Founder and Chief Executive Officer
Meniscus Limited

Pictured from *left* to *right*, George Hines, Lois Trench-Hines, Alexandra Jordan-Noel, and V. Craig Jordan. Photographed at a celebration at the Swiss Ambassador's Residence in Washington, DC, to celebrate the award of the St. Gallen Prize for Outstanding Accomplishments in the Adjuvant Treatment of Breast Cancer in 2011

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Preface

The story of tamoxifen is unique. This pioneering medicine was not conceived as part of a major development plan in the pharmaceutical industry to create a blockbuster, but rather tamoxifen (ICI46,474) was an orphan product that had failed its first indication as a "morning-after pill." Breast cancer was a consideration, but the company terminated clinical development in 1972. The resurrection of the medicine then occurred and, after a period of dismissal by the clinical community in the mid-1970s, successes went from strength to strength.

The success of the product depended upon individuals being in the right place at the right time and a "gentleman's agreement" between industry (ICI Pharmaceuticals Division now AstraZeneca) and academia (Worcester Foundation and the Leeds University) to create a new strategy for the treatment and prevention of breast cancer. The gestation period for that strategy was the whole of the 1970s [1–4]. The principles conceived of targeting the tumor estrogen receptor (ER) and using long-term adjuvant endocrine therapy translated effectively to clinical trials that demonstrated dramatic and lasting reduction in mortality [5]. It is estimated that the hundreds of thousands, perhaps millions, of women are alive today because of the successful translation of research conducted in the 1970s.

Additionally, laboratory research on the prevention of mammary carcinogenesis [2, 3] in animals would translate to successful clinical trials [6–8] with tamoxifen being the first medicine to be approved by the Food and Drug Administration (FDA) for the reduction of the incidence of breast cancer in pre- and postmenopausal women at high risk. Tamoxifen was the first medicine to be approved to reduce the risk for any cancer.

Without the economic success of tamoxifen, there would have been no incentive to develop the aromatase inhibitors for the adjuvant treatment of ER-positive breast cancer in postmenopausal patients. Without the study of the "good, the bad, and the ugly" of the tamoxifen, there would be no selective ER modulators (SERMs). The chance finding that tamoxifen and also a failed breast cancer drug keoxifene (to be renamed 5 or 6 years later as raloxifene) would maintain bone density in ovariectomized rats [9] opened the door to the suggestion that

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> Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. [10]

Today, raloxifene is approved by the FDA for the prevention and treatment of osteoporosis in postmenopausal women and for the prevention of breast cancer in high-risk postmenopausal women [11]. However, tamoxifen became the pioneering SERM that switched on or switched off estrogen target sites around a woman's body. This new drug group also led to the idea of now being able to treat diseases via any member of the nuclear hormone receptor superfamily. Specificity would be enhanced and side effects reduced.

This monograph documents the milestones achieved during the curious twists and turns in the development of tamoxifen over the past 40 years. The story starts with the systemic synthesis of nonsteroidal estrogens that through serendipity suddenly gave us the nonsteroidal antiestrogens. The discovery by Leonard Lerner AU5 in the 1950s of MER25 (or ethamoxytriphetol) and subsequently clomiphene [10] and the finding that they were antifertility agents in rats [10] aroused the interest of the pharmaceutical industry to develop "morning-after pills." Nonsteroidal antiestrogens, however, were excellent contraceptives in rats but actually induced ovulation in subfertile women. Interest in nonsteroidal antiestrogens waned.

Cancer treatment was a consideration because of the known link between estrogen and the growth of some metastatic breast cancers. However, again there was no real enthusiasm from the pharmaceutical industry. Tamoxifen, after an unlikely start in the 1960s, advanced alone during the 1970s to become the "gold standard" for the antihormone treatment and prevention of breast cancer fro the next 20 years. Despite all the "ups and downs" of the story, tamoxifen remains a cheap and effective lifesaving drug around the world. Indeed, the concept first described by our studies in the 1970s that "longer was better" as the treatment strategy for adjuvant therapy with tamoxifen for patients with ER-positive breast cancer continues to go from strength to strength in clinical trial. Ten years AU6 of adjuvant therapy is now known to be superior to 5 years of adjuvant therapy, but the profound decrease in mortality occurs during the decade after stopping tamoxifen at 10 years [12]. Again, there is a prediction we made in the 1990s that tamoxifen causes the evolution of drug resistance in the undetected micrometastases that exposes a vulnerability to estrogen-induced apoptosis in the tumor cells [13].

Lois Trench-Hanes generously accepted my invitation to contribute our Foreword. She was there at the beginning of tamoxifen in America and was the one who AU7 recruited me, on Arthur Walpole's recommendation, to advance the science and to support clinical development. We had many adventures over the years but her attitude of "get the job done" was essential to the start of this milestone. She was a force to be reckoned with, that through her willingness to see the project succeed for AU8 her company by establishing the correct clinical contacts not only propelled tamoxifen forward but helped my career development. She and her husband George are

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lifelong friends and Lois is a godmother to my youngest daughter Alexandra (see pictures in Lois's Foreword).

This monograph has been assembled by my Tamoxifen Team (VCJ) at the Lombardi Comprehensive Cancer Center at Georgetown University, Washington, DC. It is intended to illustrate and document the real journey traveled by this milestone in medicine.

V. Craig Jordan
Russell E. McDaniel
Philipp Y. Maximov

References

1. Jordan VC, Koerner S (1975) Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 11:205–206
2. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
3. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
4. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44:30–38
5. Davies C, Godwin J, Gray R et al (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378:771–784
6. Fisher B, Costantino JP, Wickerham DL et al (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90:1371–1388
7. Fisher B, Costantino JP, Wickerham DL et al (2005) Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97:1652–1662
8. Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M (2007) Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. J Natl Cancer Inst 99:283–290
9. Jordan VC, Phelps E, Lindgren JU (1987) Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10:31–35
10. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189
11. Vogel VG, Costantino JP, Wickerham DL et al (2006) Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 295:2727–2741

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12. Davies C, Pan H, Godwin J et al (2013) Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. Lancet 381(9869):805–816
13. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127:23–33

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Acknowledgments

"We are in it for life"™
Tamoxifen Team
Georgetown University

Dr. Jordan wishes to thank all of his "Tamoxifen Teams," who for the past four decades have converted ideas into lives saved. He also wishes to thank the Department of Defense Breast Program under award number W81XWH-06-1-0590 Center of Excellence; subcontract under the SU2C (AACR) grant number SU2C-AACR-DT0409; the Susan G Komen for the Cure Foundation under award number SAC100009; and the Lombardi Comprehensive Cancer Center Support Grant (CCSG) Core Grant NIH P30 CA051008 for his current research funding. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

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About the Authors

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V. Craig Jordan, OBE, Ph.D., D.Sc., FMedSci, member of the National Academy of Sciences, is known as the "father of tamoxifen." He was educated in England, obtaining his Ph.D. in Pharmacology (1973) studying a group of failed antifertility agents called nonsteroidal antiestrogens. There was no interest in drug development, but his work in academia blossomed into tamoxifen. Over a 40-year career, he researched all aspects of antiestrogens and then SERMs using structure-function relationships to investigate molecular mechanisms, developed new models, studied metabolism, developed the first realistic models of SERM resistance in vivo, and translated all of his concepts into clinical trials. He was there for the birth of tamoxifen as he is credited for reinventing a "failed morning-after contraceptive" to become the "gold standard" for the treatment of breast cancer. During his work, Jordan has held professorships at Wisconsin (1985–1993), Northwestern (1993–2004) (also the Diana Princess of Wales Professor), the Fox Chase Cancer Center (2004–2009) (also the Alfred Knudson Professor), and currently Georgetown Lombardi Cancer Center where he is the scientific director. He has contributed more than 600 scientific articles with more than 23,000 citations. His work on SERMs has been recognized with the ACS Medal of Honor, the BMS Award, the Kettering Prize, the Karnofsky Award (ASCO), the Landon Award (AACR), and the St. Gallen Prize. He is a member of the National Academy of Sciences and the Academy of Medical Sciences (UK) and is one of the 90 honorary fellows of the Royal Society of Medicine worldwide, and he received the Order of the British Empire (OBE) for services to International Breast Cancer Research in 2002. The chapters described in this book are all written by Dr. Jordan as he contributed personally to every aspect of tamoxifen application in therapeutics and all aspects of tamoxifen's pharmacology. He discovered SERMs and the new biology of estrogen-induced apoptosis.

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Each chapter is a personal journey with a few decades of discovery that forever changed women's health.

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Philipp Y. Maximov, M.D., Ph.D., graduated from the Russian National Research Medical University named after N. I. Pirogov (RNRMU) in Moscow, Russia, specializing in medical biochemistry in 2006, and completed his postgraduate program in 2010, receiving the Candidate of Medical Sciences degree (equivalent to Ph.D. in the USA) in medical biochemistry and molecular biology with a thesis titled "Structure-functional relationship of triphenylethylene estrogens and the estrogen receptor alpha in human breast cancer cells" under the mentorship of Dr. V. Craig Jordan, OBE, Ph.D., D.Sc. Dr. Maximov during his last year of medical school was chosen to be one of the top students in his class to represent his university in an exchange program between Fox Chase Cancer Center (FCCC) in Philadelphia, PA. From 2005 to 2010, Dr. Maximov was a graduate student at FCCC and, from 2006, was a Ph.D. student in Dr. Jordan's laboratory. After his Ph.D. thesis defense in Moscow, Dr. Maximov rejoined Dr. Jordan's lab as a Susan G. Komen for the Cure International Postdoctoral Fellow at Georgetown University in 2011. Dr. Maximov is a member of the American Association for Cancer Research (AACR) and the Royal Medical Society in London, UK.

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Russell E. McDaniel received his BS in Biochemistry in 2007 from Temple University in Philadelphia, PA. He served in the Peace Corps for 2 years, teaching high school chemistry in Mozambique, before joining Dr. Jordan's laboratory. At present, he is pursuing a master's degree in biotechnology at Georgetown.

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Abstract	The application of synthetic organic chemistry to establish the simplest basic structure of estrogenic compounds was a major triumph for medicinal chemistry in the 1930s. Two groups of compounds were discovered: the hydroxylated stilbenes with high potency and rapid excretion and the triphenylethylenes with high lipophilicity, metabolic activation, and a very long duration of action. A study of structure-function relationships in laboratory animals would result in the use of high-dose estrogen treatment for metastatic breast cancer in postmenopausal patients in the 1940s. The triphenylethylene-based antiestrogens would evolve into the nonsteroidal antiestrogens that in the 1960s were predicted to be potential postcoital contraceptives in women based on compelling rodent studies. This application did not succeed and enthusiasm for clinical development waned.	

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Chapter 1
Discovery and Pharmacology of Nonsteroidal Estrogens and Antiestrogens

Abstract The application of synthetic organic chemistry to establish the simplest basic structure of estrogenic compounds was a major triumph for medicinal chemistry in the 1930s. Two groups of compounds were discovered: the hydroxylated stilbenes with high potency and rapid excretion and the triphenylethylenes with high lipophilicity, metabolic activation, and a very long duration of action. A study of structure-function relationships in laboratory animals would result in the use of high-dose estrogen treatment for metastatic breast cancer in postmenopausal patients in the 1940s. The triphenylethylene-based antiestrogens would evolve into the nonsteroidal antiestrogens that in the 1960s were predicted to be potential postcoital contraceptives in women based on compelling rodent studies. This application did not succeed and enthusiasm for clinical development waned.

Introduction

It is now more than 75 years since the first compound, with known chemical structure, was shown to produce estrogenic effects in animals [1] (Fig. 1.1, compound 1). Since that time, thousands of compounds have been screened for estrogenic activity. During the past 50 years, the early events involved in the molecular mechanism of action of estrogens in their target tissues (e.g., vagina, uterus, pituitary gland, or breast), via the estrogen receptor (ER), have been described [2–4]. In this opening chapter, we will describe how the structure-function relationships of nonsteroidal estrogens set the stage for the serendipitous discoveries of nonsteroidal antiestrogens and the selective estrogen receptor modulators (SERMs). The story, with its twists and turns, is more about people and the exploitation of opportunities by individuals than a plan implicated in the drug development department of any pharmaceutical company.

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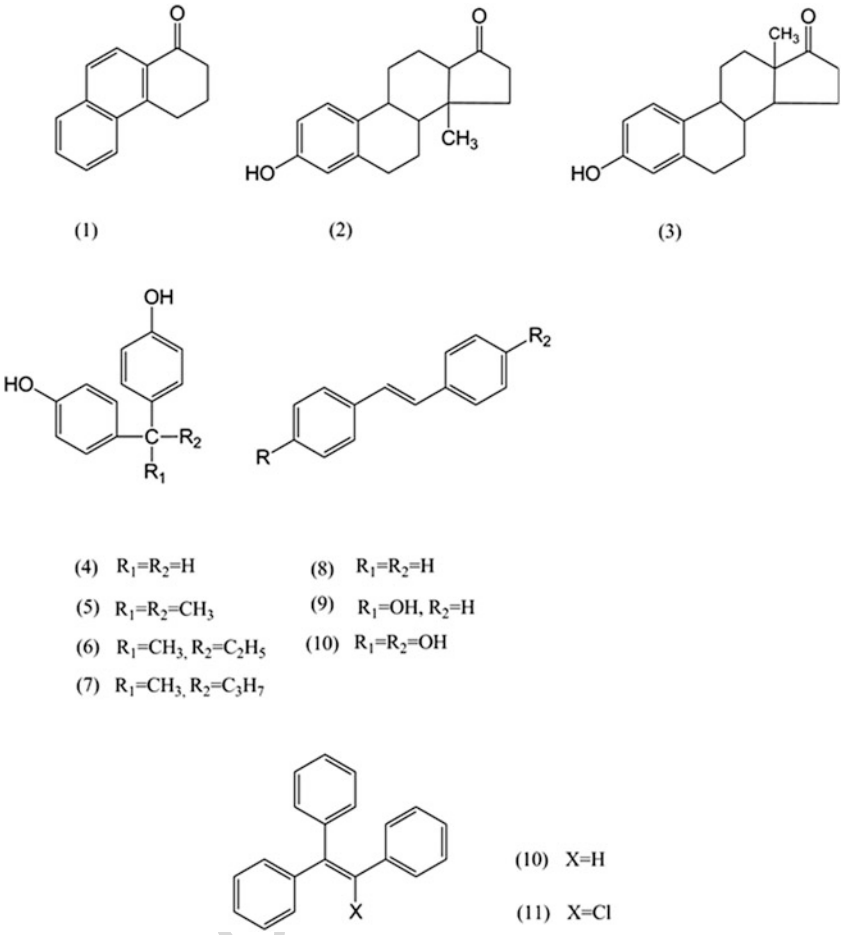

Fig. 1.1 Formulae of compounds found, in the 1930s, to have estrogenic activity in vivo. Compound 2 was believed to be the molecular structure of ketohydroxyestrin (estrone). This is now known to be incorrect and compound 3 is estrone

Testing Methods for Estrogen

To discover new knowledge about the control of fertility by hormones, animal models are required to detect target tissue-specific effects of test compounds. The Allen-Doisy test [5] depends upon the induction of vaginal cornification in castrate animals 60–80 h after the subcutaneous administration of estrogens. A colony of animals is ovariectomized and used for assays 2 weeks later. To maintain the sensitivity of the colony and retard atrophy of the uterus and vagina [6], the animals are primed with 1 μg estradiol (SC) every 6 weeks. The animals are not used for 2 weeks following either priming or experimental use. However, it is often wise to

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screen the animals by the vaginal smear technique to check for incomplete ovariectomy or test compounds with prolonged biological activity. This technique accurately identified a "principle" that Allen and Doisy called estrogen in ovarian follicular fluid [5].

Direct administration of estrogens into the vagina increases the sensitivity of the Allen-Doisy test and cornification occurs earlier since the response is not dependent upon distribution and metabolism [7, 8]. Emmens [7–10] assayed and evaluated the structural derivatives of stilbene and triphenylethylene by both intravaginal and systemic Allen-Doisy tests. This early work accurately established the relative potency of the test compounds.

Martin and Claringbold [11] developed the intravaginal assay to study the early events of estrogen stimulation by using the increase in vaginal mitoses and vaginal epithelium thickening as measures of the estrogenic response. Martin [12] further showed that the reduction of 2,3,5-triphenyltetrazolium chloride to formazan in epithelial cells of the vagina following the local application of estrogens could form the basis of a sensitive assay procedure for early estrogenic events.

The increase in uterine weight of young castrate rats was used to determine systemic estrogen and activity by Bülbring and Burn [13]. The preparation of castrate animals has been found to be an unnecessary step, and immature rats or mice are usually used [14, 15]. Estrogens induce a rapid early imbibition of water by the uterus, and this effect was used in the 6-h assay of estrogens by Astwood [16]. However, this technique cannot distinguish between full estrogens and partial agonists and also suffers from differences in the release of test compounds from the injection site which will ultimately affect the time course of the uterine response. Most assays utilize a 3-day injection technique to stimulate full uterine growth [17].

Potential estrogenic activity can be inferred for a compound by its ability to inhibit the binding of [^{3}H]estradiol to its target tissues in vivo [18, 19]. However, many nonsteroidal antiestrogens produce the same effect [20, 21] so this effect cannot be assumed to predict biological activity. Similarly, the ability of a compound to inhibit the binding of [^{3}H]estradiol to ERs in vitro suggests a potential mechanism of action via the ER but again this alone cannot predict biological activity, i.e., agonist or antagonist actions [22]. Armed with the bioassay technique in vivo, a host of compounds were screened during the 1930–1960s to find potential novel agents for clinical applications.

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Structure-Activity Relationships of Estrogens

The pioneering studies by Sir Charles Dodds laid the foundation for all the subsequent research on the structure-activity relationships of nonsteroidal estrogens. The 1930s saw a remarkable expansion of knowledge that culminated in the description of the optimal structural requirements in a simple molecule to produce estrogen action. The first compound of known structure (1-keto-1,2,3,4-tertrahydrophenanthrene) (Fig. 1.1, compound 1) to be found to have estrogenic

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activity [1] was tested because of its structural similarity to the presumed structure of ketohydroxyestrin (Fig. 1.1, compound 1). As it turned out, the structure of the natural steroid (estrone) was incorrect (Fig. 1.1, compound 2), but this did not matter; the fact that nonsteroidal compounds can exhibit estrogenic properties was established. A phenanthrene nucleus was later found to be unnecessary for estrogenic activity [23]. Simple bisphenolic compounds are active (Fig. 1.1, compounds 4–7) and, as will be seen later in this chapter, this is a recurrent feature of many nonsteroidal estrogens. The finding that hydroxystilbenes (Fig. 1.1, compounds 8–10) possess potent estrogenic activity provided a valuable clue that stimulated a systematic investigation of analogs to optimize the potency. At this time, an interesting side issue occurred that deserves comment, as it illustrates how parallel research endeavors can eventually reach the same conclusions. Anol, a simple phenol derived from anethole (Fig. 1.2), was reported to possess extremely potent estrogenic activity with 1 μg capable of inducing estrus in all rats [24]. These results were not confirmed with different preparations of anol [25, 26], but it was found that dimerization of anol to dianol (Fig. 1.2) can occur and this impurity, which was known to have potent estrogenic [27] properties, was the compound responsible for the controversy [27]. At this time, Dodds reported [28–30] that diethyl substitution at the ethylenic bond of stilbestrol (Fig. 1.2) produces an extremely potent estrogen [31]; other substitutions produce less active compounds [28, 32]. The structural similarity between diethylstilbestrol and estradiol (the formula was established by 1938) was noted, but an attempt to mimic the rigid steroid structure by the synthesis of dihydroxyhexahydrochrysene (Fig. 1.2) resulted in a drop in estrogenic potency. Dihydroxyhexahydroxchrysene is approximately 1/2,000 as potent as diethylstilbestrol [23]. AU1

There was considerable interest in the development of a long-acting synthetic estrogen because of the potential for clinical application. The duration of action of diethylstilbestrol can be increased dramatically by esterification of the phenolic groups [28]. A 10-μg dose of diethylstilbestrol dipropionate can produce estrus for more than 50 days, while the phenol at the same dose is active for only 5 days. The simple hydrocarbon triphenylethylene (Fig. 1.1, compound 11) is a weakly active estrogen [33], but 10 mg can produce vaginal cornification in mice for up to 9 weeks. Replacement of the free ethylenic hydrogen with chlorine (Fig. 1.1, compound 12) increases the potency and duration of action by subcutaneous administration [34], but when administered orally, triphenylchloroethylene has a similar duration or action as diethylstilbestrol or estradiol benzoate. In the search for orally active agents, Robson and Schonberg [35] showed that DBE (Fig. 1.2) was very effective by the oral route. The long duration of action is related to depot formation in body fat [36], but DBE did not reach clinical trial. The related compound trianisylchloroethylene (TACE) became available clinically as a long-acting estrogen (Fig. 1.2). TACE is stored in body fat for prolonged periods [37–39]. It was around the mid-1940s and early 1950s that the discovery that high-dose synthetic estrogens could cause the regression of about 30 % of metastatic breast cancers in postmenopausal women became the standard of care for the treatment of breast cancer [40, 41]. This is interesting not only because this was the

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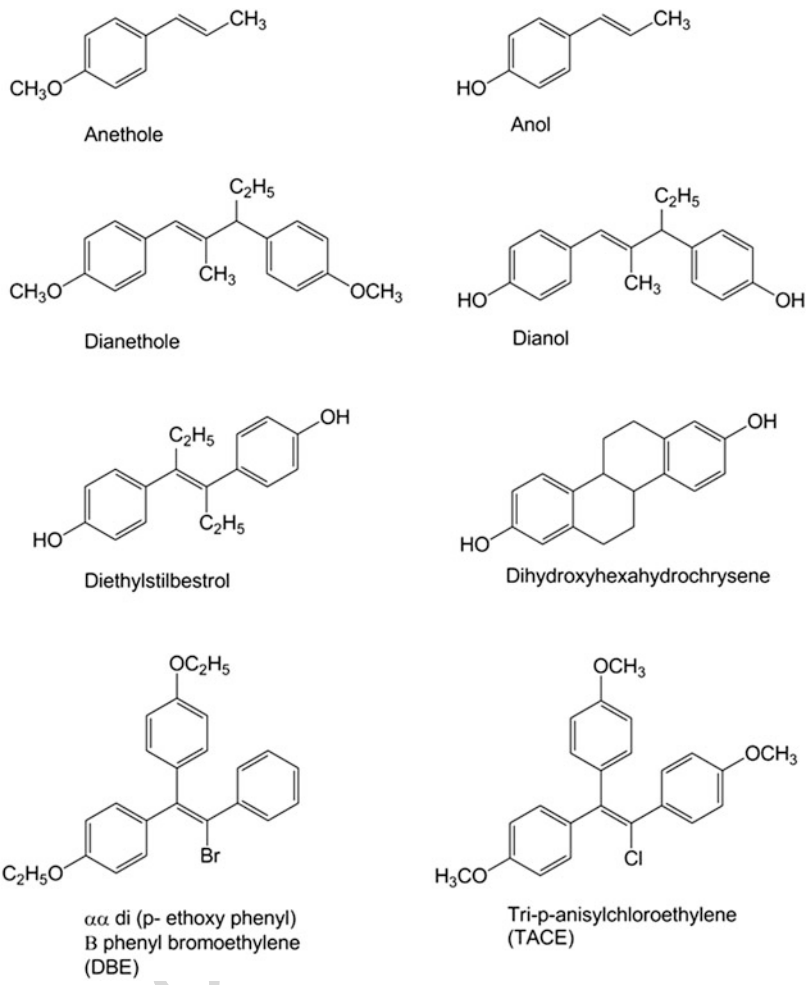

Fig. 1.2 Formulae of nonsteroidal compounds with estrogenic (or suspected) activity in vivo

first time a chemical therapy was shown to cause regression of cancer but also the compounds that Haddow used were made and provided by chemists at Imperial Chemical Industries (ICI). High-dose synthetic estrogen therapy was to remain the standard of care for the palliative treatment of breast cancer until the late 1970s early 1980s when another synthetic estrogen derivative, also produced by chemists at ICI pharmaceutical division. ICI 46,474 (later to be known as tamoxifen) would revolutionize breast cancer treatment and prevention. This is the story of this book.

The first 25 years established many of the important structural features that govern the potency and duration of action of estrogens. This is a remarkable feat of structure-functional relationships without knowledge of the ER target. We will now briefly consider the evolving subcellular mechanism of estrogen action in its target

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tissues before describing the structure-activity relationships and pharmacological properties of the nonsteroidal antiestrogens.

Estrogen Action

The reason for the target site specificity of the estrogens remained obscure until the synthesis of tritium-labeled compounds with high specific activity. The synthesis of [^{3}H]hexestrol (reduction of diethylstilbestrol with tritium and a palladium catalyst) by Glascock working with Sir Charles Dodds [42] and the subsequent observation that there was binding of hexestrol in the uterus, vagina, mammary glands, and pituitary gland of immature female goats and sheep [43] provided the first evidence for the target tissue localization of estrogens. The subsequent applications of [^{3}H] hexestrol to determine hormone responsiveness in metastatic breast cancer was a big step in our antiestrogen story [44]. The subsequent fundamental study by Jensen and Jacobson [2] of the distribution and binding of [^{3}H]estradiol in the immature rat demonstrated that estradiol selectively binds to, and is retained by, the uterus, vagina, and pituitary gland. These systematic studies suggested there is a specific receptor for estradiol in its target tissues. The biochemical identification of an estrogen-binding protein in the immature rat uterus and the observation that [^{3}H] estradiol becomes located in the receptor nucleus of the cell provided a model to describe the initiation of estrogen-stimulated events. The early evidence for an ER system has been described [3, 4]. Simply stated, the estrogen dissociates from plasma proteins and readily diffuses into the cell. Initially it was thought that the cytoplasmic ER binds the ligand and the resulting receptor complex is activated before translocation to the nucleus. Interaction with nuclear acceptors (now referred to as promoter regions of estrogen-responsive gene) results in the activation of RNA and DNA polymerases to initiate subsequent protein synthesis and cell proliferation, respectively. There were, however, an increasing number of observations that were inconsistent with the classical two-step hypothesis. These reports have been reviewed [45]. Two innovative approaches to the question of the actual subcellular localization of unoccupied ER deserve comment. These methods, which did not require cellular disruption, settle the issue of where the unoccupied receptors resided in the cell. Therefore, if it was, in fact, cell disruption that causes the unoccupied receptor to “fall out of the nucleus” but ER complexes are “stuck” in the nucleus, this would explain the early translocation model. Indeed, a series of studies with weakly binding antiestrogens injected into the immature rat arrived at the same conclusion [46]. Monoclonal antibodies raised to the ER were used as tags for immunohistochemical studies. The antibody is linked to a peroxidase enzyme system to visualize the receptor, which appears to be located exclusively in the nuclear compartment, even in the absence of estrogen [47]. The other approach was to enucleate ER-containing GH3 rat pituitary tumor cells with cytochalasin B. Unoccupied receptors are observed in nucleoplasts rather than cytoplasts [48]. Similar studies were subsequently published using estrogen-free culture of

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ER-positive MCF-7 breast cancer cells [49]. Although it was possible that these studies were generating artifactual results, the simplified model of estrogen action, i.e., unoccupied ER is a nuclear protein, is now considered to represent subcellular events in vivo.

Nonsteroidal Antiestrogens

The finding by Lerner and coworkers [50] that the compound ethamoxytriphetol (MER 25, Fig. 1.3) is an inhibitor of estrogen action provided a new tool for laboratory research and clinical investigation. It is of considerable interest that MER 25 had been synthesized as part of a cardiovascular pharmacology program and only found its way to endocrine testing at Dr. Lerner's request. Lerner had spotted that MER 25 looked like the nonsteroidal triphenylethylenes so he wanted to test it for estrogenic properties. There were none but he discovered the first nonsteroidal antiestrogen. MER 25 was subsequently found to have antifertility properties in the rat [51–53], so clinical use as an oral contraceptive seemed logical. Preliminary clinical trials with MER 25 were scientifically successful [54–56]; however, the clinical studies were discontinued because of low potency and toxic side effects. In the search for new compounds, a structural derivative of triphenylethylene, clomiphene (also called chloramiphene or MRL 41; in Fig. 1.3, the generic isomers enclomiphene and zuclomiphene are shown. Clomiphene is a mixture of isomers) was found to be a potent antifertility agent in rats [57, 58] and it became the forerunner of many structurally similar compounds that were synthesized and tested as potential postcoital antifertility agents [59–64]. The spectrum of compounds was reviewed by Emmens [65].

Structure-Activity Relationships in the Rat

There are no published reports specifically documenting the structure-activity relationships of MER 25. Apart from one triphenylethane MRL 37, with a hydrogen substituted for MER 25's alcoholic hydroxyl [53, 66], most interest has focused on compounds related to triphenylethylene. The original antiestrogens can be classified into two major groups: substituted triphenylethylenes and bicyclic antiestrogens.

Substituted Triphenylethylenes

Early studies with clomiphene used a mixture of geometric isomers [53, 57, 58]. The *cis* and *trans* isomers were separated [67] and each has been reported to

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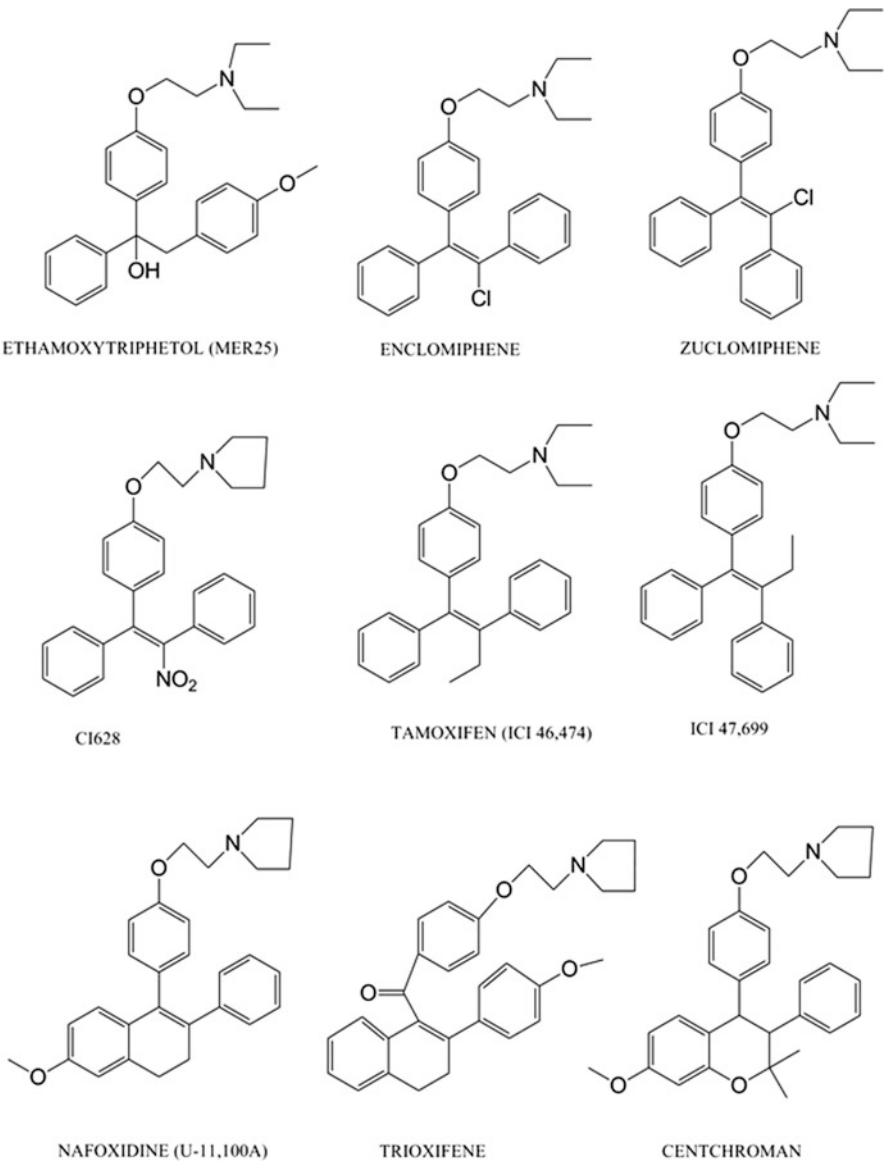

Fig. 1.3 The formulae of nonsteroidal antiestrogens mentioned in the text. Zuclomiphene and ICI 47,699 are the estrogenic geometric isomers of the antiestrogenic enclomiphene and ICI 46,474 (tamoxifen)

possess different biologic activities [68–70]; however, some controversy surrounded the designation of the isomers in relation to their observed biologic properties. They were originally labeled as geometric isomers! It is now clear that the *trans* isomer enclomiphene (originally named isomer B or *cis* clomiphene) has

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antiestrogenic properties in the rat, whereas the *cis* isomer zuclomiphene (originally named isomer A or *trans* clomiphene) is estrogenic (Fig. 1.3). Comparison of the isomers of tamoxifen and enclomiphene in the uterine weight test demonstrated only minor differences in their dose-response curves [71]. Parenthetically, in 1972, during the examination of my Ph.D. entitled "A study of the oestrogenic and anti-oestrogenic activities of some substituted triphenylethylenes and triphenylethanes," I was asked by my external examiner Dr. Arthur Walpole, head of the fertility program of ICI Pharmaceutical Division, why the biological properties of the geometric isomers of clomiphene and tamoxifen were opposite? I replied it was obviously the influence of the chlorine in clomiphene, never considering that the geometric isomers of clomiphene were misidentified. Walpole knew that!

The fundamental importance of the geometric shape of a molecule for antiestrogenic activity was realized after the report by Harper and Walpole [72] of the contrasting biological properties of the *cis* and *trans* isomers of substituted triphenylethylenes. Tamoxifen (ICI 46,474) and its *cis* isomer ICI 47,699 (Fig. 1.3) have been identified by nuclear magnetic resonance [73] and the structure of ICI 47,699 confirmed as the *cis* isomer by X-ray crystallography [74]. The simultaneous administration of tamoxifen with estradiol to immature rats prevents the increases in uterine wet weight or vaginal cornification observed with estradiol alone. In contrast, ICI 47,699 is only estrogenic in conventional tests [75]; however, very high doses have been shown to inhibit estradiol action in the uterus [71].

p-Methoxy-substituted derivatives of tamoxifen have been synthesized and tested [76] but this type of structural modification does not increase antiestrogenic activity.

CI628 (CN-55, 945–27) (Fig. 1.3) is an estrogen antagonist in the rat [77]. The isomeric mixture was used only briefly for the experimental treatment of advanced breast cancer; however, there is a considerable literature on the use of CI628 in studies with the human breast cancer ER in vitro [78]. It is an antitumor agent in the rat mammary carcinoma model [79]. There is no information on the biological properties of the separated geometric isomers; both appear to be antiestrogenic [71].

Bicyclic Antiestrogens

Scientists at the Upjohn Company, Kalamazoo, MI, focused much attention on the structure-activity relationships and properties of bicyclic (fixed ring)-based nonsteroidal antiestrogens [59–61]. Simple hydroxylated indenes [80, 81] that are superficially related to the structure of DES are potent estrogens. The structure-activity relationships of the indene nucleus have been investigated in the search for potent antifertility relationships [59] (Fig. 1.4). The 6-methoxy group is an advantage for activity but potent antifertility activity is determined by the substituted amine ethoxy side chain. Optimal activity is observed with the pyrrolidino side chain (IND 1, Fig. 1.4) and other substituted side chains (IND 2, 3, 4) have reduced activity. A morpholino side chain (IND 5) produces a compound with

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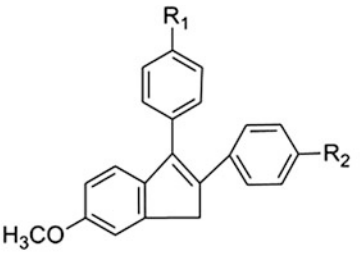

Compounds	R_1	R_2	
IND 1	OH_2CH_2CN	H	Potent Antifertility Action
IND 2	OH_2CH_2CN	H	
IND 3	OH_2CH_2CN	H	Reduced Antifertility Activity
IND 4	HOH_2CH_2CN (C_2H_5, C_2H_5)	H	
IND 5	OH_2CH_2CN	H	Low Antifertility Activity

Fig. 1.4 The relative antifertility activity of substituted indenes in the rat (Data adapted from Lednicer et al. [59])

253 approximately 1 % of the activity of IND 1 with the pyrrolidino side chain. In the
254 same study, Lednicer and coworkers [59] showed that the 6 phenols of IND 4 had
255 approximately 5 % of the potency of the methoxy compound. Hydroxylated

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derivative might be expected to have a shorter duration of action so that larger doses will be required to maintain adequate drug levels.

The 3,4-dihydronaphthalenes further exemplify the importance of the substituted side chain for optimal activity (Fig. 1.5). Nafoxidine (see Fig. 1.3 for comparison with other nonsteroidal antiestrogens) is the most potent compound of the series although the ether oxygen of the side chain can be replaced by carbon with very little loss of potency. However, decrease in the length of the side chain (NAF 1–3) (Fig. 1.5) reduces the antiestrogenic potency and in fact, removal of the side chain (NAF 6) results in the complete loss of the antagonist activity. The resulting compounds are estrogens [60, 61]. These observations led Lednicer et al. [60] to suggest that a basic group, at a given position in space, is required to obtain a molecule with estrogen antagonist activity. This point of view is further supported by the observation that dimethylation *ortho* to the aminoethoxy side chain in MER25 [82] and tamoxifen [83] reduces antiestrogen activity and receptor binding, respectively. The methyl substitutions reduce the number of positions in space that the side chain can adopt. A series of derivatives of tamoxifen with different polar side chains had been investigated [84]. The resulting biological activity related to structure is shown in Fig. 1.6. Trioxifene (available as the mesylate salt LY133314, Fig. 1.3) has been described [85] and phase I trials as a potential agent for breast cancer therapy were completed, but the drug was not developed. The unusual structural feature of trioxifene (Fig. 1.3) is the introduction of a ketone group linking the *p*-alkylaminoethoxyphenyl ring to the ethylenic bond. The structure therefore diverges from the usual triphenylethylene type. This, in the future, would turn out to be an important structural feature to create the antiestrogens with no estrogen-like actions in the uterus as raloxifene.

Centchroman (Fig. 1.3) has been studied in considerable detail in laboratory animals and women as it was investigated as a postcoital contraceptive agent [86, 87]. The structure-activity relationships of the chromans and the unsaturated chromenes have been given considerable attention. The structure with the greatest similarity to nafoxidine (a 3, 4-diphenylchromene) has very potent antifertility activity in rats. Substitution of hydrogen for two methyl groups at the 2 position gives a less active compound but reduction of the 3, 4 double bond restores potent antifertility activity (centchroman). It is important to note that two diastereoisomers are possible for the substituted chroman. Centchroman is the active *trans* isomer, whereas the *cis* isomer is virtually inactive [88, 89]. Like the 3, 4-dihydronaphthalenes, centchroman is antiestrogenic in the rat [90].

All the nonsteroidal antiestrogens have an alkylaminoethoxy side chain. As previously noted, moving the group further away from the double bond with the substitution of a ketone group (trioxifene) does not reduce antiestrogenic activity. Nevertheless, there seems to be a requirement for the nitrogen on the aminoethoxy side chain to be at a given position in space. A chain length of three atoms seems to be required to place the nitrogen group in the optimal position [60]. All of the studies in vivo with the structure-function relationships of antiestrogens as antifertility agents built up a strong conceptual model that the antiestrogens side chain was interacting actively with a select portion of the ER. Studies now evolved to

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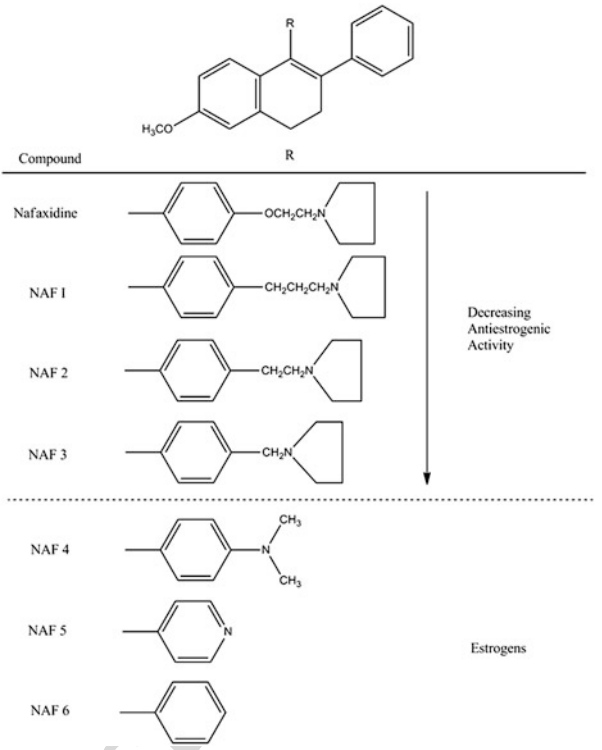

Fig. 1.5 The relative antiestrogenic activity of substituted 3,4-dihydronaphthalene in immature rats (Data adapted from Lednicer et al. [60, 61])

molecular mechanisms in the decades between 1970 and 2000 to predict efficacy of the ER ligand complex based on interrogation of ligand ER interactions.

The Molecular Modulation of the Estrogen Receptor by Nonsteroidal Antiestrogens

The description of the selective binding of [^{3}H]estradiol in the estrogen target tissues of the immature rat (uterus, vaginal) [2] and the subsequent isolation of the ER as an extractable protein from the rat uterus [91, 92] was not only an advance in molecular endocrinology but also an advance that would improve the therapeutics of breast cancer. The idea that by detecting the presence of the ER in a breast tumor would soon evolve from being a prediction test to decide the appropriateness of endocrine ablative surgery to become the target for antiestrogenic drugs was an important conceptual step [93]. Once it was found that the ER was extractable in the 1960s, it was possible to study and understand the binding of ligands to the ER and perhaps gain an insight into the mechanism of action of estrogens and antiestrogens. Early studies of the competitive binding of estrogens and antiestrogens with [^{3}H] estradiol for the ER in vitro [94, 95] were unable to distinguish between estrogens

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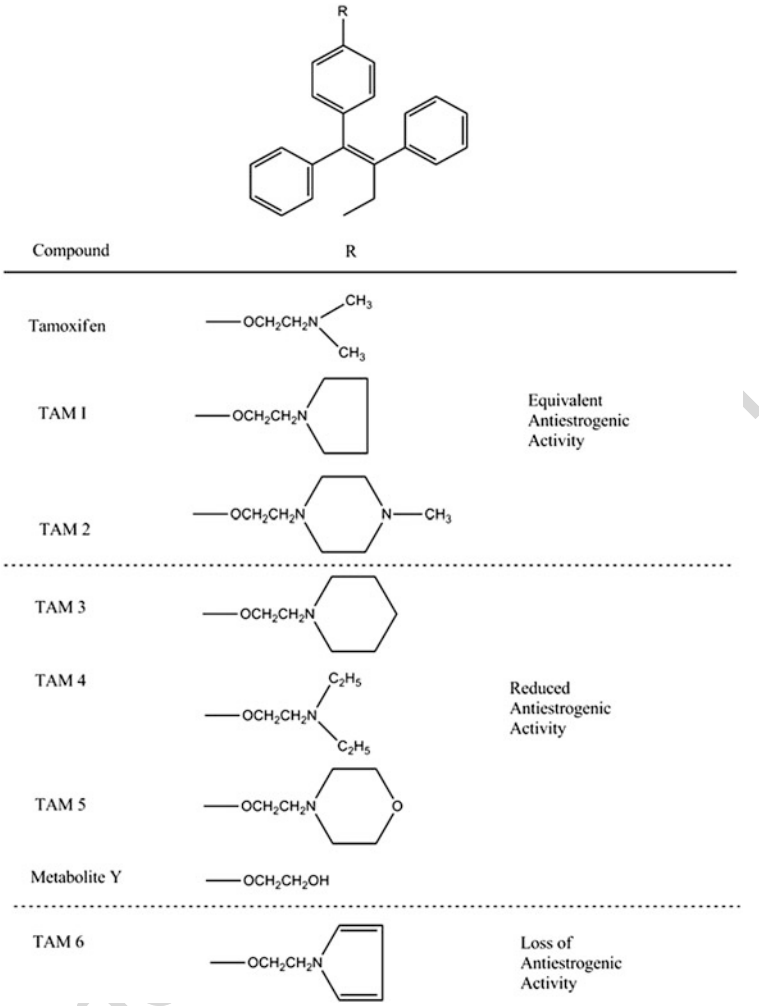

Fig 1.6 The effect of different side chains on the antiestrogenic activity of tamoxifen (Data adapted from Robertson et al. [84])

and antiestrogens biologically. All that could be concluded was that antiestrogens had low binding affinity for the ER and this, it was argued, was why such large doses were necessary to block estrogen action [94]. Also, it was concluded that the low affinity of antiestrogens for the ER was part of their mechanism of action: the ligand would not remain long enough bound to the receptor to activate estrogen action [94]. This proposal was all to change with the discovery of the pharmacological properties of 4-hydroxytamoxifen, a metabolite of tamoxifen then thought to be the principal metabolite of tamoxifen [96]. 4-Hydroxytamoxifen has a binding affinity for the ER equivalent to estradiols, so if it was possible to have high affinity

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antiestrogens, then low affinity was not the mechanism of antiestrogen action. The shape of the resulting complex was the key to efficacy and the subsequent modulation of signal transduction. 4-Hydroxytamoxifen was subsequently adopted as the standard laboratory antiestrogen in cell culture and 20 years later was used as an antiestrogenic ligand to be crystallized with the ligand-binding domain of the human ER [97].

In the 1970s, what was needed was a model cell system to study the structure-function relationships of ligands that bind to the ER. In this way, the intrinsic efficacy of the ligand ER complex could be deciphered, without concerns about pharmacokinetics and metabolism. The ER-positive breast cancer cell line MCF-7 had been described [98] but the fact that the cells apparently grew spontaneously in culture and would not respond to estradiol with growth but would when inoculated into athymic mice [99] led to considerable controversy in the field. Maybe estrogen was acting indirectly to promote breast cancer growth? Nevertheless, tamoxifen did block the spontaneous growth of MCF-7 cells and this blockade could be reversed with estradiol [100]. Interestingly enough, MCF-7 cells, or rather their ER, would be essential to create the first monoclonal antibodies to human ER [101, 102] and subsequently be the critical tool necessary to clone and sequence the human ER [103, 104].

The first cell system used to study the modulation of the ligand ER complex in vitro was primary cultures of the immature rat pituitary gland [105]. The target for the ER was the prolactin gene [22, 106]. The first publication validated the mechanism of actions of nonsteroidal antiestrogens at the ER to regulate estrogen-induced gene transcription as competitive inhibition of estradiol binding to the ER and that it was an advantage but not a requirement for an antiestrogen to be metabolically activated [106]. As with other drug receptor interactions, affinity and the intrinsic efficacy of the drug receptor complex are not interconnected for drug action. Numerous studies of structure-function relationships of triphenylethylenes described the structure-function relationships to modulate the ER complex between the extremes for estrogenic intrinsic efficacy and complete antiestrogen action [22, 107–111]. The structure-activity relationship [112] studies permitted the creation of a map of the hypothetical folding of the ER complex. However, it was the serendipitous advance in deciphering breast cancer cell replication in vitro that was to enhance the interpretation of all future laboratory studies.

In the mid-1980s, the Katzenellenbogen laboratory [113] made the critical discovery that ER-positive breast cancer cells had all been cultured in media containing high concentrations of a pH indicator, phenol red that contained a contaminant that was an estrogen (Fig. 1.7) [114, 115] (note: this is reminiscent of the anol-dianol controversy). Removal of the phenol red from media now permitted the structure-activity relationship studies of nonsteroidal antiestrogens to be extrapolated from prolactin gene modulation to the replication of breast cancer cell lines [116, 117].

However, the critical question to be addressed in molecular pharmacology was "what is the essential interaction of the antiestrogenic side chain with the ER that modulates estrogen-like and antiestrogen action?" A simple estrogen/antiestrogen

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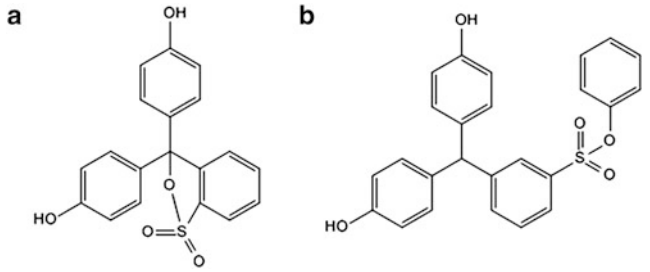

Fig. 1.7 pH indicator phenol red and contaminant bis(4-hydroxyphenyl)-[2-9phenoxy-sulfonul-phenyl]methane found in the growth medium that produced estrogenic effect of the MCF-7 cell line [114, 115]

model of the ER had been proposed as the "crocodile model" [118] with the jaws closed for estrogen action and the antiestrogen, a stick in the jaws to keep them open for antiestrogen action (Fig. 1.8). An antiestrogenic region (AER) that interacts with the appropriately positioned alkylaminoethoxy side chain on the ligand backbone had been proposed previously [22, 112, 118], but how to find it? Several advances were necessary before progress could occur. A model of acquired drug resistance to tamoxifen in athymic mice needed to be developed, the ER needed to be screened for mutations in drug resistant MCF-7 breast tumors, and ER needed to be stably transfected into ER-negative breast cancer cell and suitable gene modulated. All this was done to propose a hypothetical modulation of the antiestrogen ER complex prior to the crystallization of the ligand-binding domain with estradiol and raloxifene [119]. A biological clue was found in the human ER that would complement the structural knowledge of the ligand ER binding domain complex with functional information at a transforming growth factor-alpha (TGFα) gene target.

A mutation, asp 351 tyr, was noted in one MCF-7 tumor cell line with acquired resistance to tamoxifen [120]. The first transfection of the wild-type ER [121] into the ER-negative breast cancer cell line MDA-MB-231 eventually allowed any mutant ER to be transfected. The introduction of ERs with ligands could now be monitored at the estrogen-responsive binding domain with 4-hydroxytamoxifen and raloxifene [97, 119] indicating that while raloxifene's side chain shielded and possibly neutralized Asp351, the side chain of tamoxifen was shorter and barely interacted with Asp 351. To address the hypothesis that the side chain was preventing the interaction of Asp 351 with activating function 1 (AF-1) motif of the ER, ER complex was interrogated using mutations of Asp 351 and structural derivation of raloxifene [122–125] (Fig. 1.6). It was concluded that this amino acid was important to alter surface interactions with other co-regulators of hormone action.

The modulation of the ER complex through coactivator proteins went some way to explain SERM action, i.e., nonsteroidal antiestrogens switching on and switching off sites around a woman's body. But long before this concept was discovered and described in the mid-1980s [126], the literature was full of examples of the

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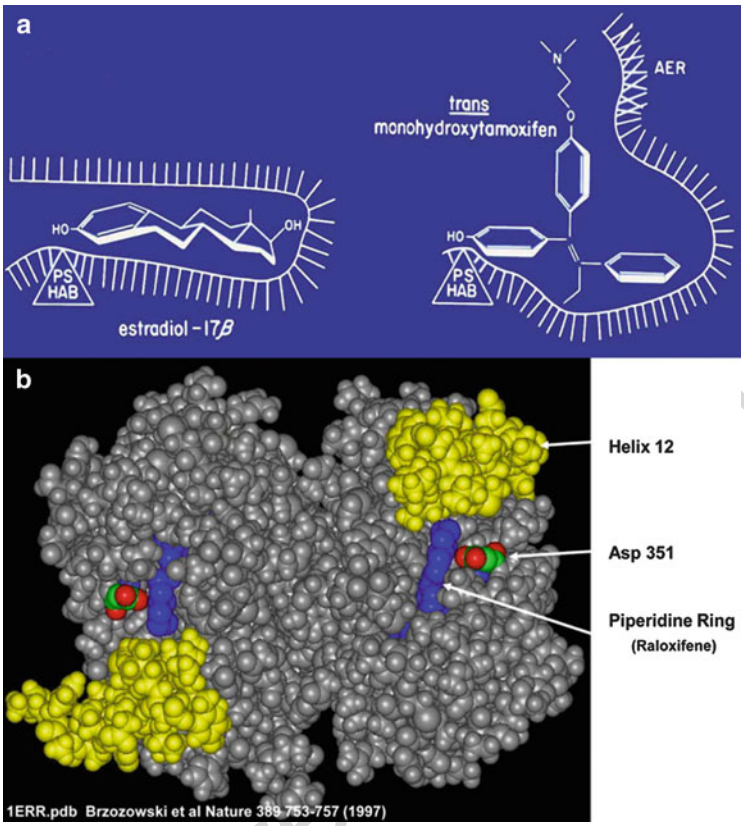

Fig. 1.8 The "crocodile" model of antiestrogenic action of 4-hydroxytamoxifen and its interaction with the antiestrogenic region of the ER [118], as well as the X-ray crystallography of the ligand-binding domain (LBD) of the ER interacting with the raloxifene piperidine ring via its Asp351 and thus producing an antagonistic conformation of the receptor and antiestrogenic biological effect (Front cover of [185])

species-specific pharmacology of nonsteroidal antiestrogens. We will illustrate this now but no adequate explanation has yet been offered or proven to explain the diverse pharmacology in different species.

Effect of Antiestrogens in Different Species

Lerner and coworkers [127] reported that the compound MER 25 antagonizes the actions of estradiol in rats and mice with no other demonstrable hormonal or antihormonal activity. In contrast, Emmens [128] found MER 25 to be only weakly active as an inhibitor of estradiol-stimulated vaginal cornification in the ovariectomized mouse. Nevertheless, the original claim of antiestrogenic activity has been

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adequately confirmed in a variety of interesting models. MER 25 inhibits diethylstilbestrol or estradiol-stimulated increases in the reticuloendothelial system [129] of the ovariectomized mouse. In the mature female rat, a large dose of MER 25 (20 mg) inhibits the estrogen-stimulated uterine ballooning observed at proestrus and doubling the dose also inhibits ovulation [130]. If MER 25 is administered after ovulation, there is inhibition of the estrogen-stimulated DNA, RNA, and protein synthesis that occurs during uterine decidualization [131]. The antiestrogenic action of MER 25 has also been reported at the level of the pituitary. Hypertrophy of the rat pituitary by continued estrogen administration is inhibited by the coadministration of MER 25 [132]. Similarly, estrogen-simulated prolactin release in the ovariectomized rat can be inhibited by large daily dose of MER 25 [133].

Although MER 25 is notable for its very low estrogenic activity in all species tested, some estrogenic responses in the uterus have been quantified. A single dose of MER 25 (5 mg) increases ovariectomized rat uterine glycogen, glucose, and percent water inhibition[134]. A striking short-lived increase in immature rat uterine glucose-6-phosphate dehydrogenase activity and a marked rise in uterine total lipid is observed after a single administration of MER 25 (10 mg) [135]. In ovariectomized mice, MER 25 has some estrogenic activity as evidenced by increases in uterine weight and a stimulation of the enzymes alkaline phosphatase and isocitrate dehydrogenase [76].

Overall, though, the pharmacology of MER 25 is established as an estrogen antagonist. Since the pharmacology of the related triphenylethylenes is so complex, this is presented in species-related groups.

Mouse

The antiestrogens based on triphenylethylene are generally considered to be estrogenic in the mouse. However, this statement is only true under precisely defined conditions. Tamoxifen (oral or SC) is typically estrogenic in the Allen-Doisy (vaginal smear) test using mature ovariectomized mice [75]. In comparative studies, tamoxifen [136, 137] and trioxifene [136] are estrogenic in the 3-day ovariectomized mouse uterine weight test. Similarly tamoxifen, ICI 47,699, enclomiphene, and zuclomiphene are fully uterotrophic in immature mice [138] and tamoxifen does not possess antiuterotrophic activity [139]. In contrast, nafoxidine [138] and trioxifene [136] are partially estrogenic with antiestrogenic properties in immature mice. It is of interest that trioxifene appears to be fully estrogenic in mature ovariectomized mice and antiestrogenic in immature mice, while tamoxifen is more estrogenic than trioxifene in both test systems [136]. Lee [140] pointed out that tamoxifen and nafoxidine are mitogenic in the ovariectomized mouse uterus and neither compound inhibits the mitogenic response to estrone. However, daily treatment of ovariectomized mice with tamoxifen for up to 14 days reduces estrone-stimulated uterine weight gain [141]. It is possible that the accumulation of tamoxifen may alter the pharmacology to produce an inhibitory effect.

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In this context, SC administration of a large dose of tamoxifen (or related *p*-methoxylated compounds) to ovariectomized mice produces a short period of estrogenic activity followed by a prolonged antiestrogenic and antifertility response [20, 142, 143]. The validity of the vaginal smear technique to assay prolonged antiestrogenic activity was initially questioned [144] although there is agreement about the reduced effectiveness of tamoxifen to produce a fully cornified vaginal epithelium [145].

Rat

The pharmacology of antiestrogens in the rat is dependent upon the target tissue or biochemical end point being studied. For this reason the effects of antiestrogens in different organs will be considered.

All of the nonsteroidal antiestrogens are able to stimulate a partial estrogenic response in the immature and ovariectomized rat uterus. Histological comparisons of estrogen and antiestrogen-stimulated uteri have demonstrated selective differences in both cell stimulation and mitotic activity. CI628 [146], tamoxifen, 4-hydroxytamoxifen [147], and nafoxidine [148] stimulate an enormous increase in the size of luminal epithelial cells. Estradiol increases the incorporation of [^{3}H] thymidine [146] and the mitotic activity [149] in luminal epithelial cells, whereas antiestrogens are much less active [146, 147]. In general antiestrogens produce hypertrophy rather than hyperplasia of luminal epithelial cells.

Much research with antiestrogens has focused on the estrogen control mechanisms of pituitary function. This, in part, is because of the early clinical applications of both clomiphene and tamoxifen as agents for the induction of ovulation in subfertile women [150, 151]. Estrogen-stimulated prolactin release in ovariectomized rats [152] is partially inhibited by nafoxidine [153] and tamoxifen [154]. Studies [155] have demonstrated that in the intact rat the cyclical release of prolactin at proestrus is inhibited by continuous tamoxifen therapy. This is consistent with the finding that tamoxifen, 4-hydroxytamoxifen, and trioxifene inhibit estrogen-stimulated prolactin synthesis by rat pituitary cells in culture [22]. Similarly, tamoxifen inhibits the growth and secretion of prolactin by the estrogen-induced pituitary tumor 7315a [156]. Furthermore, tamoxifen sensitizes the pituitary tumor cells to the inhibitory effects of bromocriptine on prolactin secretion in vitro [157].

Tamoxifen [158–160] and enclomiphene [158] (zuclomiphene is inactive) inhibit ovulation by blocking estrogen action at the level of the hypothalamus and pituitary. Gonadotropin release is inhibited in male and female rats by large doses of clomiphene (mixed isomers) [57] but it is possible that the estrogenic *cis* isomer is predominantly responsible for these effects. The ability of centchroman and clomiphene (mixed isomers) to alter serum FSH, LH, and prolactin in male and female rats has been compared [161]. Clomiphene lowers LH in male rats, slightly increases LH in female rates, but causes a large increase in prolactin in both species.

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Centchroman, with its rigid bicyclic structure, produces a similar effect to clomiphene on gonadotropin and prolactin levels in both sexes, although the compound is less estrogenic than clomiphene. Studies with the weakly estrogenic compound tamoxifen demonstrate that short-term (5 days) therapy of ovariectomized rats does not lower LH [162], whereas longer therapy (up to 4 weeks) results in a consistent decrease in LH levels [155]. Similarly, a large dose of tamoxifen is sufficiently estrogenic to decrease LH release in male rats [163].

Studies with rat pituitary cells in vitro demonstrates [164] that both estradiol and clomiphene (mixed isomers) sensitize the cells to the effects of LHRH (luteinizing hormone-releasing hormone). The antiestrogenic isomer enclomiphene is apparently only acting as an estrogen in this system. In contrast, Miller and Huang [165] observed that tamoxifen inhibits the estrogen sensitization of ovine pituitary cells to an LHRH analog. Furthermore, tamoxifen, CI628, and nafoxidine inhibit estrogen-stimulated LH release and reverse the inhibition of FSH release by estradiol in this system. To explain these contrasting results, it must be conceded that nothing is known about the pharmacology of antiestrogens in the sheep! Therefore, species differences may be responsible for differences in the action of the compounds. However, the fact that in vivo antiestrogens can cause increases or decreases in LH depending upon the physiological model used must point to the complex factors involved in the regulation of gonadotropin release.

Before considering other organ site effects of antiestrogens, one early observation with nonsteroidal estrogens in the pituitary is worthy of note. Continuous estradiol administration for several weeks can cause pituitary hypertrophy in the rat (F344), while administrating the estrogenic triphenylethylene TACE does not [37]. Of perhaps greater significance, TACE inhibits the hypertrophy of the pituitary produced by estradiol [166]. Current knowledge of the aberrant binding of triphenylethylene-based estrogen to the ER [167, 168] may actually be the reason for the different carcinogenic actions of differently shaped estrogens.

Several estrogen-modulated synthetic events in the liver have been considered as potential sites of antiestrogen action. For convenience, and because the effects of antiestrogens are similar, the rat and primate liver will be considered together. Rat and monkey liver have a well-defined ER system [169, 170], suggesting a mechanism for the effects of estrogen and antiestrogen. The continuous treatment of ovariectomized immature rats with tamoxifen or estradiol increases the synthesis of renin substrate [171]. Similarly, a comparison of ethinyl estradiol and nafoxidine has demonstrated that both are full agonists in stimulating plasma renin substrate in mature female rats [172]. During tamoxifen therapy, breast cancer patients have elevated circulating levels of sex hormone-binding globulin (SHBG) [173]. This observation is of interest because SHBG synthesis in the liver in under estrogen control. Overall, it seems that only estrogenic effects have been described for antiestrogens in the mammalian liver.

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Chick

Most studies with antiestrogens have focused on the effects in the oviduct and liver. The pharmacology of both tamoxifen [174] and 4-hydroxytamoxifen [175] in the oviduct is as a full antagonist. No estrogenic effects have been reported. In general, antiestrogens are full antagonists of estrogen action in the liver [176].

Conclusion

The purpose of our introductory chapter is to document the important evolutions of knowledge about nonsteroidal synthetic estrogens that really laid the foundation for all future work on nonsteroidal antiestrogens and then selective ER modulators (SERMs).

The intense interest in a study of nonsteroidal antiestrogens in the laboratory during the 1960s and 1970s as antifertility agents in different species or as laboratory tools to dissect estrogen action in its target tissues now slowly started to evolve from reproduction research to targeted cancer therapeutics. Tamoxifen would soon no longer be a laboratory tool and pharmacological curiosity, but an orphan breast cancer drug in search of an optimal strategy to best be deployed in the clinic. This now becomes the theme of our book.

Postscript. A series of chance meetings occurred at ICI Pharmaceuticals Division Alderley Park near my home in Cheshire. In 1967 I wanted to work in cancer research over my summer holiday (I had previously worked at the Yorkshire Cancer Research Campaign laboratory at Leeds University in the summer of 1966) so I went to ICI and I phoned up Dr. Steven Carter from the phonebook outside the laboratories in Alderley Park. He had just reported the unusual actions of cytochalasins in the journal Nature [177]. I asked him for a summer job and he asked me to set up an appointment. I said: "I'm outside ICI now," so he invited me in and the job was mine. Cytochalasins are a series of natural products but cytochalasin B caused polynuclear cells or at different concentration nuclear extrusion with a small cell membrane. This same natural product would later be used by Wayne Welshons to aid in discovering the actual location of the unoccupied ER [48, 178]. I had a great opportunity working in Steven Carter's laboratory on the electron microscopy of nuclear extrusions in mouse L cells. This was my introduction to ICI pharmaceuticals division. More importantly, I came to know Dora Richardson, the synthetic chemist who later would provide me with tamoxifen metabolites; Arthur (Walop) Walpole whose antifertility laboratory was opposite Steven Carter's; and Mike Barett, who was in charge of ICI's beta-blocker discovery program that was building on Jim Black's landmark discovery at ICI. Jim Black subsequently won the Nobel Prize in 1988 in Physiology and Medicine for "discoveries of important principles for drug treatment." Mike Barett's laboratory

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was next door to Steven Carter's. This again is where chance and opportunity take control, but you have to be ready to see the opportunity and be prepared to rise to the challenge. Mike Barett became the professor of Pharmacology at Leeds University. He apparently was impressed with my skill as a lecturer (I was a Ph.D. student at that time in the early 1970s) so he offered me a job as a lecturer in Pharmacology. I had no Ph.D. yet and no publications, but I was talent spotted!

I started my lifelong "love affair" with nonsteroidal estrogens and antiestrogens with the start of my Ph.D. thesis work entitled "A study of the oestrogenic and anti-oestrogenic activities of some substituted triphenylethylenes and triphenylethanes." I was supported by a Medical Research Council scholarship, which I only received by chance because I was originally on the waiting list. Someone declined their scholarship so there I was, a Ph.D. student in the Department of Pharmacology at the University of Leeds (1969–1972). I decided to study the ER with Dr. Edward Clark in the Department of Pharmacology at the University of Leeds. Dr. Jack Gorski had published an exciting series of reports showing that the ER could easily be extracted from the rat uterus and isolated by sucrose density gradient analysis. My project was going to be simple: I was to establish the new technique of sucrose density gradient analysis, isolate the receptor, and crystallize the protein with an estrogen and an antiestrogen. Through X-ray crystallography in the Astbury Department of Biophysics at the University of Leeds, we would establish the three-dimensional shape of the complexes to explain antiestrogenic action. The goal was to solve a fundamental question in pharmacology: What is the molecular mechanism of action for a drug? Progress was slow in establishing the receptor purification technique of sucrose gradient analysis, and I switched my thesis topic to study the structure-activity relationship of antiestrogens. As it turned out, this was a good, strategic decision, as it has taken the best efforts of the research community nearly 30 years to achieve success. The structure of the ER complex was solved by scientists at York University, England, in 1997. No one has yet succeeded in crystallizing the whole ER with an antiestrogen.

My study of failed contraceptives was less than inspiring as no one was recommending careers in a dead end. It was clear in the late 1960s that nonsteroidal antiestrogens would not be "morning-after pills." They were excellent in rats but did exactly the opposite in women. Also, as it turned out, the pharmaceutical industry chose to discontinue all their interest in these compounds because of too much toxicity or there was no money to be made. But chance meetings and my desire to be a part of developing a clinically useful drug for cancer would change that perspective.

The road to my Ph.D. was complicated in early 1972 as the university could not find an examiner. No one cared about failed contraceptives! Mike Barett solved the problem after Sir Charles Dodds declined with the words: "Sorry, I have not kept up with the literature during the past 20 years." He invited his former colleague Arthur Walpole to be my examiner, and after some grumbling by the university that it was inappropriate because "he was from industry," this set off a chain of events that would create tamoxifen as the gold standard for the treatment and prevention of

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breast cancer. This is the next chapter in our story as "Tamoxifen Goes Forward Alone."

During my Ph.D. studies, I learned all the names of the important players in estrogen action: Elwood Jensen, Jack Gorski, and Bill McGuire. Jensen and Gorski were world authorities on the ERs and my Ph.D. supervisor Ted Clark would often remark "look how many authors are on their papers; we cannot compete with these big groups." Bill McGuire was medically qualified and really drove the ER and progesterone receptor concept to predict the susceptibility of endocrine ablation into a clinical reality [179, 180]. Jensen and Gorski would eventually become my colleagues, coauthors, and then fellow members of the National Academy of Sciences. I remember well everyone congratulating Elwood Jensen at the pivotal meeting linking ER tumor level with response to ablative endocrine therapy in Bethesda, MD, in 1974 [181], when his election was announced. Elwood and I would receive the inaugural Dorothy P. Landon Award for translational research from the American Association for Cancer Research (AACR) in 2002, he for the ER target and me for the development of the "science of antiestrogens applied to cancer research" [182]. Bill McGuire and I would be close friends until his untimely death in 1992 [183, 184]. Each of these prominent scientists gave me help and support in the early years of my career with invitations to their laboratories to talk about my "orphan drug tamoxifen" or with letters of recommendation. Each was important for my career development as a young scientist in the 1970s.

In closing Chap. 1, I wish to state that my focused interest in the pharmacology of nonsteroidal antiestrogens started with Leonard Lerner's discovery of MER 25. I was thrilled to meet him at meetings in Mont-Tremblant, Canada, of "Recent Progress in Hormone Research" started by Gregory Pincus of the Worcester Foundation. Len and I talked endlessly about a "group of forgotten drugs," nonsteroidal antiestrogens. Two enthusiasts. I was more than thrilled to receive the Bruce Cain Award with Len from the AACR in 1989 [126]. At that time something that was nothing was being turned into something of medical significance.

References

1. Cook JW, Dodds EC, Hewett CL (1933) A synthetic oestrus-exciting compound. Nature 131:56
2. Jensen EV, Jacobson HI (1962) Basic guides to the mechanism of estrogen action. Recent Program Horm Res 18:387–414
3. Gorski J, Toft D, Shyamala G et al (1968) Hormone receptors: studies on the interaction of estrogen with the uterus. Recent Prog Horm Res 24:45–80
4. Jensen EV, DeSombre ER (1973) Estrogen-receptor interactions. Science 182:126–134
5. Allen E, Doisy EA (1923) An ovarian hormone. J Am Med Assoc 81:819–821
6. Kahnt LC, Doisy EA (1928) The vaginal smear method of assay of the ovarian hormone. Endocrinology 12:760–768
7. Emmens CW (1940/1941) Precursors of oestrogens. J Endocrinol 2:444–458
8. Emmens CW (1950) The intravaginal assay of naturally occurring oestrogens. J Endocrinol 6:302–307

Uncorrected Proof

Author's Proof

9. Emmens CW (1942) Oestrogens and pro-oestrogens related to stilbene and triphenylethylene. J Endocrinol 3:168–173
10. Emmens CW (1947) Halogen-substituted oestrogens related to triphenylethylene. J Endocrinol 5:170–173
11. Martin L, Claringbold PJ (1958) A highly sensitive assay for oestrogens. Nature 181:620–621
12. Martin L (1960) The use of 2-3-5-triphenyltetrazolium chloride in the biological assay of oestrogens. J Endocrinol 20:187–197
13. Bulbring E, Burn JH (1935) The estimation of oestrin and of male hormone in oily solution. J Physiol 85:320–333
14. Lauson HD, Heller OG, Golden JB, Sevringhaus EL (1939) The immature rat uterus in the assay of estrogenic substances and a comparison of estradiol, estrone, and estriol. Endocrinology 24:35–44
15. Evans JS, Varney RF, Koch FC (1941) The mouse uterine weight method for the assay of estrogens. Endocrinology 28:747–752
16. Astwood EB (1938) A six-hour assay for the quantitative determination of estrogen. Endocrinology 23:25–31
17. Rubin BL, Dorfman AS, Black L, Dorfman RI (1951) Bioassay of estrogens using the mouse uterine response. Endocrinology 49:429–439
18. Terenius L (1965) Uptake of radioactive oestradiol in some organs of immature mice. The capacity and structural specificity of uptake studied with several steroid hormones. Acta Endocrinol (Copenh) 50:584–596
19. Terenius L (1966) Effect of synthetic oestrogens and analogues on the uptake of oestradiol by the immature mouse uterus and vagina. Acta Pharmacol Toxicol (Copenh) 24:89–100
20. Jordan VC (1975) Prolonged antioestrogenic activity of ICI 46, 474 in the ovariectomized mouse. J Reprod Fertil 42:251–258
21. Jordan VC, Naylor KE (1979) The binding of [3H]-oestradiol-17 beta in the immature rat uterus during the sequential administration of non-steroidal anti-oestrogens. Br J Pharmacol 65:167–173
22. Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741–4745
23. Dodds EC, Lawson W (1936) Synthetic oestrogenic agents without the phenanthrene nucleus. Nature 137:996
24. Dodds EC, Lawson W (1937) A simple aromatic oestrogenic agent with an activity of the same order as that of oestrone. Nature 139:627
25. Zondek B, Bergmann E (1938) Phenol methyl ethers as oestrogenic agents. Biochem J 32:641–645
26. Dodds EC, Lawson W (1937) Oestrogenic activity of *p*-hydroxy propenyl benzene (Anol). Nature 139:1068
27. Campbell NR, Dodds EC, Lawson W (1938) Oestrogenic activity of anol: a highly active phenol isolated from the byproducts. Nature 142:1121
28. Campbell NR, Dodds EC, Lawson W (1938) Oestrogenic activity of dianol, a dimeride of *p*-propenyl-phenol. Nature 141:78
29. Dodds E (1938) Biological effects of the synthetic oestrogenic substance 4:4′-dihydroxy-alpha:beta-diethylstilbene. Lancet 232:1389–1391
30. Dodds EC, Goldberg L, Lawson W, Robinson R (1938) Oestrogenic activity of certain synthetic compounds. Nature 141:247–248
31. Dodds EC, Golberg L, Lawson W, Robinson R (1938) Oestrogenic activity of esters of diethylstilbestrol. Nature 142:211–212
32. Dodds EC, Golberg L, Lawson W, Robinson R (1938) Oestrogenic activity of alkylated stilbestrols. Nature 142:34
33. Robson JM, Schonberg A (1937) Oestrous reactions, including mating, produced by triphenylethylene. Nature 140:196

Uncorrected Proof

Author's Proof

34. Robson JM, Schonberg A, Fahim H (1938) Duration of action of natural and synthetic oestrogens. Nature 142:292–293
35. Robson JM, Schonberg A (1942) A new synthetic oestrogen with prolonged action when given orally. Nature 150:22–23
36. Robson JM, Ansari MY (1943) The fate of D.B.E. (alpha di-*p*-ethoxphenyl) (Beta-phenylbromoethylene) in the body. J Pharm Exptl Therap 79:340–345
37. Thompson CR, Werner HW (1951) Studies on estrogen tri-p-anisylchloroethylene. Proc Soc Exp Biol Med 77:494–497
38. Greenblatt RB, Brown NH (1952) The storage of estrogen in human fat after estrogen administration. Am J Obstet Gynecol 63:1361–1363
39. Thompson CR, Werner HW (1953) Fat storage of an estrogen in women following orally administered tri-p-anisylchloroethylene. Proc Soc Exp Biol Med 84:491–492
40. Haddow A, Watkinson JM, Paterson E, Koller PC (1944) Influence of synthetic oestrogens on advanced malignant disease. Br Med J 2:393–398
41. Kennedy BJ, Nathanson IT (1953) Effects of intensive sex steroid hormone therapy in advanced breast cancer. J Am Med Assoc 152:1135–1141
42. Dodds EC, Folley SJ, Glascock RF, Lawson W (1958) The excretion of microgram doses of hexoestrol by rabbits and rats. Biochem J 68:161–167
43. Glascock RF, Hoekstra WG (1959) Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep. Biochem J 72:673–682
44. Folca PJ, Glascock RF, Irvine WT (1961) Studies with tritium-labelled hexoestrol in advanced breast cancer. Comparison of tissue accumulation of hexoestrol with response to bilateral adrenalectomy and oophorectomy. Lancet 2:796–798
45. Tate AC, Jordan VC (1983) The estrogen receptor. In: Agarwal MK (ed) Textbook of Receptorology. Walter DeGruyter, Berlin, pp 381–463
46. Jordan VC, Tate AC, Lyman SD et al (1985) Rat uterine growth and induction of progesterone receptor without estrogen receptor translocation. Endocrinology 116:1845–1857
47. King WJ, Greene GL (1984) Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature 307:745–747
48. Welshons WV, Lieberman ME, Gorski J (1984) Nuclear localization of unoccupied oestrogen receptors. Nature 307:747–749
49. Welshons WV, Cormier EM, Wolf MF et al (1988) Estrogen receptor distribution in enucleated breast cancer cell lines. Endocrinology 122:2379–2386
50. Lerner LJ (1981) The first non-steroidal antioestrogen- MER 25. In: Sutherland RL, Jordan VC (eds) Non steroidal antioestrogens. Academic, Sydney, pp 1–16 AU5
51. Segal SJ, Nelson WO (1958) An orally active compound with anti-fertility effects in rats. Proc Soc Exp Biol Med 98:431–436 AU6
52. Chang MC (1959) Degeneration of ova in the rat and rabbit following oral administration of 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-anisylethanol. Endocrinology 65:339–342
53. Barnes LE, Meyer RK (1962) Effects of ethamoxytriphetol, MRL-37, and clomiphene on reproduction in rats. Fertil Steril 13:472–480
54. Kistner RW, Smith OW (1959) Observations on the use of a nonsteroidal estrogen antagonist: MER 25. Surg Forum 10:725–729
55. Kistner RW, Smith OW (1961) Observations on the use of nonsteroidal estrogen antagonist: MER 25. Fertil Steril 12:121–141
56. Smith OW, Kistner RW (1963) Action of MER 25 and of clomiphene on the human ovary. JAMA 184:878–886
57. Holtkamp DE, Greslin JG, Root CA, Lerner LJ (1960) Gonadotrophin inhibiting and anti-fecundity effects of chloramiphene. Proc Soc Exp Biol Med 105:197–201
58. Davidson OW, Wada K, Segal SJ (1965) Effects of clomiphene at different stages of pregnancy in the rat. Implications regarding possible action mechanisms. Fertil Steril 16:195–201

Uncorrected Proof

Author's Proof

59. Lednicer D, Babcock JC, Marlatt PE et al (1965) Mammalian antifertility agents. I. Derivatives of 2,3-diphenylindenes. J Med Chem 8:52–57
60. Lednicer D, Lyster SC, Duncan GW (1967) Mammalian antifertility agents. IV. Basic 3,4-dihydronaphthalenes and 1,2,3,4-tetrahydro-1-naphthols. J Med Chem 10:78–84
61. Lednicer D, Lyster SC, Aspergren BD, Duncan GW (1966) Mammalian antifertility agents. 3. 1-Aryl-2-phenyl-1,2,3,4-tetrahydro-1-naphthols, 1-aryl-2-phenyl-3,4-dihydronaphthalenes, and their derivatives. J Med Chem 9:172–176
62. Ray S, Grover PK, Kamboj VP et al (1976) Antifertility agents. 12. Structure-activity relationship of 3,4-diphenylchromenes and -chromans. J Med Chem 19:276–279
63. Greenwald GS (1965) Effects of a dihydronaphthalene derivative (U-11100a) on pregnancy in the rat. Fertil Steril 16:185–194
64. Harper MJ, Walpole AL (1967) Mode of action of I.C.I. 46,474 in preventing implantation in rats. J Endocrinol 37:83–92
65. Emmens CW (1970) Postcoital contraception. Br Med Bull 26:45–51
66. Emmens CW, Humphrey K, Martin L, Owen WH (1967) Antifertility properties of two non-oestrogenic steroids and MRL 37. Steroids 9:235–243
67. Palopoli FP, Feil VJ, Allen RE et al (1967) Substituted aminoalkoxytriarylhaloethylenes. J Med Chem 10:84–86
68. DiPietro DL, Sanders FJ, Goss DA (1969) Effect of *cis* and *trans* isomers of clomiphene citrate on uterine hexokinase activity. Endocrinology 84:1404–1408
69. Schulz KD, Haselmayer B, Holzel F (1971) The influence of clomid and its isomers on dimethylbenzanthracene-induced rat mammary tumors. In: Hubinot PO, Leroy F, Galand P (eds) Basic action of sex steroids on target organs. Karger, Basel
70. Self LW, Holtkamp DE, Kuhn WL (1967) Pituitary gonad related effects of isomers of clomiphene citrate. Fed Proc Fed Am Soc Exp Biol 26:534
71. Jordan VC, Haldemann B, Allen KE (1981) Geometric isomers of substituted triphenylethylenes and antiestrogen action. Endocrinology 108:1353–1361
72. Harper MJ, Walpole AL (1966) Contrasting endocrine activities of *cis* and *trans* isomers in a series of substituted triphenylethylenes. Nature 212:87
73. Bedford GR, Richardson DN (1966) Preparation and identification of *cis* and *trans* isomers of a substituted triarylethylene. Nature (London) 212:733
74. Kilbourn BT, Mais RHB, Owston PG (1968) Identification of isomers of a substituted triarylethylene: the crystal structure of 1-p-(2-dimethylaminoethoxyphenyl)-1,2-*cis*-diphenyl but-1-ene hydrobromide. Chem Commun 1:291
75. Harper MJ, Walpole AL (1967) A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 13:101–119
76. Collins DJ, Hobbs JJ, Emmens CW (1971) Antiestrogenic and antifertility compounds. 4. 1,1,2-triarylalkan-1-ols and 1,1,2-triarylalk-1-enes containing basic ether groups. J Med Chem 14:952–957
77. Callantine MR, Humphrey RR, Lee SL et al (1966) Action of an estrogen antagonist on reproductive mechanisms in the rat. Endocrinology 79:153–167
78. Jensen EV, Jacobson HI, Smith S et al (1972) The use of estrogen antagonists in hormone receptor studies. Gynecol Invest 3:108–123
79. Arbogast LY, DeSombre ER (1975) Estrogen-dependent in vitro stimulation of RNA synthesis in hormone-dependent mammary tumors of the rat. J Natl Cancer Inst 54:483–485
80. Salzer W (1946) Uber neue synthetische hochwirksame oestrogene. Hoppe-Seyler's Z. Physiol Chem 274:39–47
81. Silverman M, Bogert MT (1946) The synthesis of some indene and dihydronaphthalene derivatives related to stilbestrol. J Org Chem 11:34–49
82. Clark ER, Jordan VC (1976) Oestrogenic, anti-oestrogenic and fertility effects of some triphenylethanes and triphenylethylenes related to ethamoxytriphetol (MER 25). Br J Pharmacol 57:487–493

Uncorrected Proof

Author's Proof

83. Abbott AC, Clark ER, Jordan VC (1976) Inhibition of oestradiol binding to oestrogen receptor proteins by a methyl-substituted analogue of tamoxifen. J Endocrinol 69:445–446
84. Robertson DW, Katzenellenbogen JA, Hayes JR, Katzenellenbogen BS (1982) Antiestrogen basicity–activity relationships: a comparison of the estrogen receptor binding and antiuterotrophic potencies of several analogues of (Z)-1,2-diphenyl-1-[4-[2-(dimethylamino)ethoxy] phenyl]-1-butene (tamoxifen, Nolvadex) having altered basicity. J Med Chem 25:167–171
85. Jones CD, Suarez T, Massey EH et al (1979) Synthesis and antiestrogenic activity of [3,4-dihydro-2-(4-methoxyphenyl)-1-naphthalenyl][4-[2-(1-pyrrolidinyl)ethoxy]-phe nyl] methanone, methanesulfonic acid salt. J Med Chem 22:962–966
86. Kamboj VP, Setty BS, Chandra H et al (1977) Biological profile of centchroman–a new postcoital contraceptive. Indian J Exp Biol 15:1144–1150
87. Mukerjee SS, Sethi N, Srivastava GN et al (1977) Chronic toxicity studies of centchroman in rats and rhesus monkeys. Indian J Exp Biol 15:1162–1166
88. Dwani SAA, Saxena R, Setty BS, Gupta RC, Koll PL, Ray S, Anand N (1979) Secooestradiols and some non-steroidal estrogens: structural correlates of oestrogenic action. J Steroid Biochem 11:67
89. Saeed A, Durani N, Durani S et al (1984) Cis isomer of centchroman–a selective ligand for the microsomal antiestrogen binding site. Biochem Biophys Res Commun 125:346–352
90. Roy S, Datta JK (1976) Nature of estrogenic and anti-estrogenic actions of centchroman on rat uterus. Contraception 13:597–604
91. Toft D, Gorski J (1966) A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc Natl Acad Sci U S A 55:1574–1581
92. Toft D, Shyamala G, Gorski J (1967) A receptor molecule for estrogens: studies using a cell-free system. Proc Natl Acad Sci U S A 57:1740–1743
93. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44:30–38
94. Korenman SG (1970) Relation between estrogen inhibitory activity and binding to cytosol of rabbit and human uterus. Endocrinology 87:1119–1123
95. Skidmore J, Walpole AL, Woodburn J (1972) Effect of some triphenylethylenes on oestradiol binding in vitro to macromolecules from uterus and anterior pituitary. J Endocrinol 52:289–298
96. Jordan VC, Collins MM, Rowsby L, Prestwich G (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305–316
97. Shiau AK, Barstad D, Loria PM et al (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937
98. Soule HD, Vazguez J, Long A et al (1973) A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51:1409–1416
99. Shafie SM (1980) Estrogen and the growth of breast cancer: new evidence suggests indirect action. Science 209:701–702
100. Lippman ME, Bolan G (1975) Oestrogen-responsive human breast cancer in long term tissue culture. Nature 256:592–593
101. Greene GL, Fitch FW, Jensen EV (1980) Monoclonal antibodies to estrophilin: probes for the study of estrogen receptors. Proc Natl Acad Sci U S A 77:157–161
102. Greene GL, Nolan C, Engler JP, Jensen EV (1980) Monoclonal antibodies to human estrogen receptor. Proc Natl Acad Sci U S A 77:5115–5119
103. Greene GL, Gilna P, Waterfield M et al (1986) Sequence and expression of human estrogen receptor complementary DNA. Science 231:1150–1154
104. Green S, Walter P, Kumar V et al (1986) Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature 320:134–139
105. Lieberman ME, Maurer RA, Gorski J (1978) Estrogen control of prolactin synthesis in vitro. Proc Natl Acad Sci U S A 75:5946–5949

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Author's Proof

106. Lieberman ME, Jordan VC, Fritsch M et al (1983) Direct and reversible inhibition of estradiol-stimulated prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4734–4740
107. Jordan VC, Lieberman ME, Cormier E et al (1984) Structural requirements for the pharmacological activity of nonsteroidal antiestrogens in vitro. Mol Pharmacol 26:272–278
108. Jordan VC, Lieberman ME (1984) Estrogen-stimulated prolactin synthesis in vitro. Classification of agonist, partial agonist, and antagonist actions based on structure. Mol Pharmacol 26:279–285
109. Jordan VC, Koch R, Mittal S, Schneider MR (1986) Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. Br J Pharmacol 87:217–223
110. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48:812–815
111. Jordan VC, Koch R (1989) Regulation of prolactin synthesis in vitro by estrogenic and antiestrogenic derivatives of estradiol and estrone. Endocrinology 124:1717–1726
112. Jordan VC (1984) Biochemical pharmacology of antiestrogen action. Pharmacol Rev 36:245–276
113. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS (1986) Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A 83:2496–2500
114. Bindal RD, Carlson KE, Katzenellenbogen BS, Katzenellenbogen JA (1988) Lipophilic impurities, not phenolsulfonphthalein, account for the estrogenic activity in commercial preparations of phenol red. J Steroid Biochem 31:287–293
115. Bindal RD, Katzenellenbogen JA (1988) Bis(4-hydroxyphenyl)[2-(phenoxysulfonyl)phenyl] methane: isolation and structure elucidation of a novel estrogen from commercial preparations of phenol red (phenolsulfonphthalein). J Med Chem 31:1978–1983
116. Murphy CS, Langan-Fahey SM, McCague R, Jordan VC (1990) Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. Mol Pharmacol 38:737–743
117. Murphy CS, Parker CJ, McCague R, Jordan VC (1991) Structure-activity relationships of nonisomerizable derivatives of tamoxifen: importance of hydroxyl group and side chain positioning for biological activity. Mol Pharmacol 39:421–428
118. Jordan VC (1987) Laboratory models of breast cancer to aid the elucidation of antiestrogen action. J Lab Clin Med 109:267–277
119. Brzozowski AM, Pike AC, Dauter Z et al (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
120. Wolf DM, Jordan VC (1994) The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31:129–138
121. Jiang SY, Jordan VC (1992) Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. J Natl Cancer Inst 84:580–591
122. Schafer JI, Liu H, Tonetti DA, Jordan VC (1999) The interaction of raloxifene and the active metabolite of the antiestrogen EM-800 (SC 5705) with the human estrogen receptor. Cancer Res 59:4308–4313
123. MacGregor Schafer J, Liu H, Bentrem DJ et al (2000) Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine from aspartate at amino acid 351. Cancer Res 60:5097–5105
124. Liu H, Lee ES, Deb Los Reyes A et al (2001) Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor alpha complex. Cancer Res 61:3632–3639

Uncorrected Proof

Author's Proof

125. Liu H, Park WC, Bentrem DJ et al (2002) Structure-function relationships of the raloxifene-estrogen receptor-alpha complex for regulating transforming growth factor-alpha expression in breast cancer cells. J Biol Chem 277:9189–9198
126. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189
127. Lerner LJ, Holthaus FJ Jr, Thompson CR (1958) A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol. Endocrinology 63:295–318
128. Emmens CW, Cox RI, Martin L (1960) Oestrogen inhibition by steroids and other substances. J Endocrinol 20:198–209
129. Nicol T, Vernon-Roberts B, Quantock DC (1966) The effect of various anti-oestrogenic compounds on the reticulo-endothelial system and reproductive tract in the ovariectomized mouse. J Endocrinol 34:377–386
130. Shirley B, Wolinsky J, Schwartz NB (1968) Effects of a single injection of an estrogen antagonist on the estrous cycle of the rat. Endocrinology 82:959–968
131. Shelesnyak MC, Tic L (1963) Studies on the mechanism of decidualization. IV. Acta Endocrinol (Copenh) 42:465–472
132. Cutler A, Ober WB, Epstein JA, Kupperman HS (1961) The effect of 1-(p-2-diethylaminoethoxy-phenyl)-1-phenyl-2-panisyl ethanol upon rat pituitary and uterine responses to estrogen. Endocrinology 69:473–482
133. Jordan VC, Koerner S, Robison C (1975) Inhibition of oestrogen-stimulated prolactin release by anti-oestrogens. J Endocrinol 65:151–152
134. Wood JR, Wrenn TR, Bitman J (1968) Estrogenic and antiestrogenic effects of clomiphene, MER-25 and CN-55,945-27 on the rat uterus and vagina. Endocrinology 82:69–74
135. Harris DN, Lerner LJ, Hilf R (1968) The effects of progesterone and ethamoxytriphetol on estradiol-induced changes in the immature rat uterus. Trans N Y Acad Sci 30:774–782
136. Black LJ, Goode RL (1980) Uterine bioassay of tamoxifen, trioxifene and a new estrogen antagonist (LY117018) in rats and mice. Life Sci 26:1453–1458
137. Jordan VC, Rowsby L, Dix CJ, Prestwich G (1978) Dose-related effects of non-steroidal antioestrogens and oestrogens on the measurement of cytoplasmic oestrogen receptors in the rat and mouse uterus. J Endocrinol 78:71–81
138. Terenius L (1971) Structure-activity relationships of anti-oestrogens with regard to interaction with 17-beta-oestradiol in the mouse uterus and vagina. Acta Endocrinol (Copenh) 66:431–447
139. Terenius L (1970) Two modes of interaction between oestrogen and anti-oestrogen. Acta Endocrinol (Copenh) 64:47–58
140. Lee AE (1974) Effects of oestrogen antagonists on mitosis and (3H)oestradiol binding in the mouse uterus. J Endocrinol 60:167–174
141. Lee AE (1971) The limitations of using mouse uterine weight as an assay of oestrogen antagonism. Acta Endocrinol (Copenh) 67:345–352
142. Emmens CW (1971) Compounds exhibiting prolonged antioestrogenic and antifertility activity in mice and rats. J Reprod Fertil 26:175–182
143. Emmens CW, Carr WL (1973) Further studies of compounds exhibiting prolonged antioestrogenic and antifertility activity in the mouse. J Reprod Fertil 34:29–40
144. Martin L, Middleton E (1978) Prolonged oestrogenic and mitogenic activity of tamoxifen in the ovariectomized mouse. J Endocrinol 78:125–129
145. Martin L (1981) Effects of antiestrogens on cell proliferation in the rodent reproductive tract. In: Sutherland RL, Jordan VC (eds) Non-steroidal antiestrogens. Academic, Sydney, p 143
146. Kang YH, Anderson WA, DeSombre ER (1975) Modulation of uterine morphology and growth by estradiol-17beta and an estrogen antagonist. J Cell Biol 64:682–691
147. Jordan VC, Dix CJ (1979) Effect of oestradiol benzoate, tamoxifen and monohydroxy-tamoxifen on immature rat uterine progesterone receptor synthesis and endometrial cell division. J Steroid Biochem 11:285–291

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148. Clark JH, Hardin JW, Padykula HA, Cardasis CA (1978) Role of estrogen receptor binding and transcriptional activity in the stimulation of hyperestrogenism and nuclear bodies. Proc Natl Acad Sci U S A 75:2781–2784
149. Kaye AM, Sheratzky D, Lindner HR (1972) Kinetics of DNA synthesis in immature rat uterus: age dependence and estradiol stimulation. Biochim Biophys Acta 261:475–486
150. Greenblatt RB, Barfield WE, Jungck EC, Ray AW (1961) Induction of ovulation with MRL/41. Preliminary report. JAMA 178:101–104
151. Klopper A, Hall M (1971) New synthetic agent for the induction of ovulation: preliminary trials in women. Br Med J 1:152–154
152. Chen CL, Meites J (1960) Effects of estrogen and progesterone on serum and pituitary prolactin levels in ovariectomized rats. Endocrinology 66:96
153. Heuson JC, Waelbroeck C, Legros N et al (1971) Inhibition of DMBA-induced mammary carcinogenesis in the rat by 2-br- -ergocryptine (CB 154), an inhibitor of prolactin secretion, and by nafoxidine (U-11, 100 A), an estrogen antagonist. Gynecol Invest 2:130–137
154. Jordan VC, Koerner S (1976) Tamoxifen as an anti-tumour agent: role of oestradiol and prolactin. J Endocrinol 68:305–311
155. Jordan VC, Dix CJ, Allen KE (1981) Effects of antioestrogens in carcinogen-induced rat mammary cancer. In: Sutherland RL, Jordan VC (eds) Non-steroidal anti-oestrogens. Academic, Sydney, pp 261–280
156. de Quijada M, Timmermans HA, Lamberts SW (1980) Tamoxifen suppresses both the growth of prolactin-secreting pituitary tumours and normal prolactin synthesis in the rat. J Endocrinol 86:109–116
157. de Quijada M, Timmermans HA, Lamberts SW, MacLeod RM (1980) Tamoxifen enhances the sensitivity of dispersed prolactin-secreting pituitary tumor cells to dopamine and bromocriptine. Endocrinology 106:702–706
158. Labhsetwar AP (1970) Role of estrogens in ovulation: a study using the estrogen-antagonist, I.C.I. 46,474. Endocrinology 87:542–551
159. Bainbridge JG, Labhsetwar AP (1971) The role of oestrogens in spontaneous ovulation: location of site of action of positive feedback of oestrogen by intracranial implantation of the anti-oestrogen I.C.I. 46474. J Endocrinol 50:321–327
160. Billard R, McDonald PG (1973) Inhibition of ovulation in the rat by intrahypothalamic implants of an antioestrogen. J Endocrinol 56:585–590
161. Sheth AR, Vaidya RA, Arbatti NJ, Devi PK (1977) Effect of Centchroman on serum gonadotropins & prolactin in rats. Indian J Exp Biol 15:1191–1193
162. Dohler KDVSMA, Dohler U (1977) ICI46,474-lack of oestrogenic activity in the rat. ICRS Med Sci 5:185
163. Bartke A, Mason M, Dalterio S, Bex F (1978) Effects of tamoxifen on plasma concentrations of testosterone and gonadotrophins in the male rat. J Endocrinol 79:239–240
164. Hsueh AJ, Erickson GF, Yen SS (1978) Sensitisation of pituitary cells to luteinising hormone releasing hormone by clomiphene citrate in vitro. Nature 273:57–59
165. Miller WL, Huang ES (1981) Antiestrogens and ovine gonadotrophs: antagonism of estrogen-induced changes in gonadotropin secretions. Endocrinology 108:96–102
166. Segal SJ, Thompson CR (1956) Inhibition of estradiol-induced pituitary hypertrophy in rats. Proc Soc Exp Biol Med 91:623–625
167. Jordan VC, Schafer JM, Levenson AS et al (2001) Molecular classification of estrogens. Cancer Res 61:6619–6623
168. Bentrem D, Fox JE, Pearce ST et al (2003) Distinct molecular conformations of the estrogen receptor alpha complex exploited by environmental estrogens. Cancer Res 63:7490–7496
169. Aten RF, Weinberger MJ, Eisenfeld AJ (1980) Kinetics of in vivo nuclear translocation of the male and female rat liver estrogen receptors. Endocrinology 106:1127–1132
170. Aten RF, Dickson RB, Eisenfeld AJ (1979) Female and male green monkey liver estrogen receptor. Biochem Pharmacol 28:2445–2450

Uncorrected Proof

Author's Proof

171. Bichon M, Bayard F (1979) Dissociated effects of tamoxifen and oestradiol-17 beta on uterus and liver functions. J Steroid Biochem 10:105–107
172. Kneifel MA, Katzenellenbogen BS (1981) Comparative effects of estrogen and antiestrogen on plasma renin substrate levels and hepatic estrogen receptors in the rat. Endocrinology 108:545–552
173. Sakai F, Cheix F, Clavel M et al (1978) Increases in steroid binding globulins induced by tamoxifen in patients with carcinoma of the breast. J Endocrinol 76:219–226
174. Sutherland R, Mester J, Baulieu EE (1977) Tamoxifen is a potent "pure" anti-oestrogen in chick oviduct. Nature 267:434–435
175. Binart N, Catelli MG, Geynet C et al (1979) Monohydroxytamoxifen: an antioestrogen with high affinity for the chick oviduct oestrogen receptor. Biochem Biophys Res Commun 91:812–818
176. Lazier CB, Capony F, Williams DL (1981) Antioestrogen action in chick liver: effects on oestrogen receptors in oestrogen-induced proteins. In: Sutherland RL, Jordan VC (eds) Non-steroidal antioestrogens. Academic, Sydney, pp 215–230
177. Carter SB (1967) Effects of cytochalasins on mammalian cells. Nature 213:261–264
178. Welshons WV, Grady LH, Judy BM et al (1993) Subcellular compartmentalization of MCF-7 estrogen receptor synthesis and degradation. Mol Cell Endocrinol 94:183–194
179. Clark GM, McGuire WL, Hubay CA et al (1983) Progesterone receptors as a prognostic factor in stage II breast cancer. N Engl J Med 309:1343–1347
180. Horwitz KB, McGuire WL (1975) Predicting response to endocrine therapy in human breast cancer: a hypothesis. Science 189:726–727
181. McGuire WL, Carbone PP, Vollmer EP (1975) Estrogen receptors in human breast cancer. Raven, New York
182. Jensen EV, Jordan VC (2003) The estrogen receptor: a model for molecular medicine. Clin Cancer Res 9:1980–1989
183. Jordan VC (1995) Third annual William L. McGuire Memorial Lecture. "Studies on the estrogen receptor in breast cancer"–20 years as a target for the treatment and prevention of cancer. Breast Cancer Res Treat 36:267–285
184. Wolf DM, Jordan VC (1993) William L. McGuire Memorial Symposium. Drug resistance to tamoxifen during breast cancer therapy. Breast Cancer Res Treat 27:27–40
185. Jordan VC (2001) Selective estrogen receptor modulation: a personal perspective. Cancer Res 61:5683–5687

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Abstract	Tamoxifen (ICI 46,474), the *trans* isomer of a substituted triphenylethylene, was discovered in the fertility program at Imperial Chemical Industries, Pharmaceuticals Division, Cheshire, England. The plan was to use tamoxifen to regulate fertility, but this failed and interest refocused outside the company for applications to treat breast cancer. The initial application of the nonsteroidal antiestrogen was for the treatment of metastatic breast cancer in postmenopausal women and by the 1980s tamoxifen had replaced high-dose diethylstilbestrol therapy. Efficacy when compared with diethylstilbestrol was similar, but tamoxifen had fewer side effects. No other antiestrogens were developed by the pharmaceutical industry, as this was not considered a financially lucrative development strategy.	

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Chapter 2
Tamoxifen Goes Forward Alone

Abstract Tamoxifen (ICI 46,474), the *trans* isomer of a substituted triphenylethylene, was discovered in the fertility program at Imperial Chemical Industries, Pharmaceuticals Division, Cheshire, England. The plan was to use tamoxifen to regulate fertility, but this failed and interest refocused outside the company for applications to treat breast cancer. The initial application of the nonsteroidal antiestrogen was for the treatment of metastatic breast cancer in postmenopausal women and by the 1980s tamoxifen had replaced high-dose diethylstilbestrol therapy. Efficacy when compared with diethylstilbestrol was similar, but tamoxifen had fewer side effects. No other antiestrogens were developed by the pharmaceutical industry, as this was not considered a financially lucrative development strategy.

Introduction

History is lived forward but is written in retrospect. "We know the end before we consider the beginning and we can never wholly recapture what it was to know the beginning only" (C.V. Wedgewood, *William the Silent*). That is, unless one has lived through the evolving applications of tamoxifen.

Tamoxifen (ICI 46,474; Nolvadex), a nonsteroidal antiestrogen, started life as the endocrine treatment of choice for advanced breast cancer [1]. Adjuvant therapy with tamoxifen also proved to be effective [2] because a sustained survival advantage is noted for women with node-positive and node-negative disease. The Food and Drug Administration (FDA) approved the use of tamoxifen as an adjuvant therapy with chemotherapy (1986), as an adjuvant therapy alone (1988) in node-positive postmenopausal patients and pre- and postmenopausal node-negative patients with ER-positive disease (1990). Tamoxifen is used to treat breast cancer in men (1993). However, remarkably tamoxifen was also approved to reduce the risk of breast cancer in women at high risk (1998). Tamoxifen was also FDA approved for treatment of ductal carcinoma in situ (DCIS) (2000). No other cancer therapy is so widely approved and had so dramatic an impact on cancer care. Tamoxifen is,

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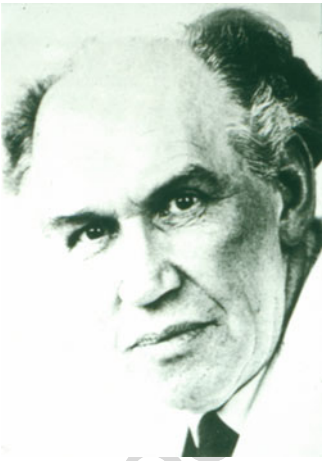

Fig. 2.1 Arthur Walpole who died suddenly on 2 July 1977. At the time of his death, he had retired as head of the Fertility Control Program at ICI's Pharmaceuticals Division at Alderley Park, near Macclesfield, Cheshire, but he had continued to work as a consultant on the joint research scheme between ICI and the Department of Pharmacology at the University of Leeds, England

however, one of those remarkable examples of a drug originally designed for one primary purpose that fails but is then steered by dedicated scientists toward a recognized secondary application where it becomes enormously successful.

The chief credit for the discovery of tamoxifen in 1962, and its subsequent application as an orphan drug treatment for metastatic breast cancer, must be given to Dr. Arthur L. Walpole (Fig. 2.1), then head of the fertility control program for Imperial Chemical Industries (ICI) Pharmaceuticals Division. Tamoxifen was identified as an effective postcoital contraceptive in rats [3–5] and there was a distinct possibility that antiestrogens could be developed as "morning-after" pills [6]. However, the basic pharmacology and physiology of ovulation and implantation are critically different in women and rats. When tamoxifen was tested in patients in preliminary clinical studies, it was found to induce ovulation rather than reduce fertility [7, 8] and so is marketed in some countries for the induction of ovulation in subfertile women [1].

The ovarian dependence of some breast cancers has long been recognized [9, 10] and the first antiestrogens [11, 12] were shown to be effective in their treatment, but the drugs then available were considered to be too toxic for chronic use [13–15] (Table 2.1). By the end of the 1960s, the direct role of estrogen in breast cancer growth was further substantiated with the description of ERs in breast tumors [18–20] and the subsequent clinical correlation with hormone dependency [21, 22]. However, clinical research with tamoxifen was not based on the ER but on proven antifertility activity as an antiestrogen in the rat. Walpole encouraged the clinical testing of the antiestrogen tamoxifen at the Christie Hospital and Holt Radium Institute in Manchester [16]. He had a long interest in cancer research [23] but also wanted to determine whether tamoxifen was an estrogen or an antiestrogen in humans because of the life between estrogens and breast cancer growth. A subsequent dose response was published by Dr. Harold Ward [17]. But in 1972, ICI Pharmaceutical Division

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Table 2.1 Comparison of the early chemical experience with antiestrogen as a treatment for metastatic breast cancer t1.1

Antiestrogen	Daily dose (mg)	Year	Response rate (%)	Toxicity	
Ethamoxytriphetol	500–4,500	1960	25	Acute psychotic episodes	t1.3
Clomiphene	100–300	1964–1974	34	Fear of cataracts	t1.4
Nafoxidine	180–240	1976	31	Cataracts, ichthyosis, photophobia	t1.5
Tamoxifen	20–40	1971–1973	31	Transient thrombocytopenia[a]	t1.6

[a]"The particular advantage of this drug is the low incidence of troublesome side effects" [16]. "Side effects were usually trivial" [17] t1.7

chose to abandon clinical development because there would be no financial gain for the limited applications in the treatment of metastatic breast cancer where only one in three patients respond for, on average, 2 years [24].

This chapter will trace the "resurrection" and development of tamoxifen for the treatment of advanced breast cancer in postmenopausal patients and consider the unusual set of circumstances that set the stage for the subsequent success of tamoxifen as a long-term adjuvant therapy in patients with node-positive and node-negative disease. In 1990, the fashion was to change again with a plan to test the worth of tamoxifen as a preventive in women at risk for breast cancer [25–27]. Much of the basic laboratory work in animal models was conducted in the period 1974–1992. This produced a strong rationale to move forward with clinical trials and the meticulous evaluations of pharmacology of tamoxifen (Fig. 2.2). This is our story.

ICI 46,474: The Early Years

In 1958, Lerner and coworkers described the first nonsteroidal antiestrogen MER 25. The drug was tested in clinical trials but proved to be toxic at the high doses required [28]. A successor compound, clomiphene (also known as chloramiphene or MRL41) (Fig. 2.2), now known to be a mixture of two geometric isomers with opposing biological activities, was a postcoital contraceptive in rats but was developed only clinically as a fertility drug [29] (see Chap. 1).

To understand the obstacles that had to be overcome before the successful clinical development of tamoxifen, it is necessary to recapture the mood of the times in the 1950s/1960s. Coronary heart disease was a primary target for drug development and was proving to be a lucrative market. However, one product—triparanol (MER29) (Fig. 2.2)—was to become a cause *célèbre* and a major issue in the relationship between product safety and regulatory authorities. Indeed, this case was taught to Craig Jordan as an undergraduate at Leeds University, in the Pharmacology Department (1965–1969), to illustrate how drug development can go very wrong.

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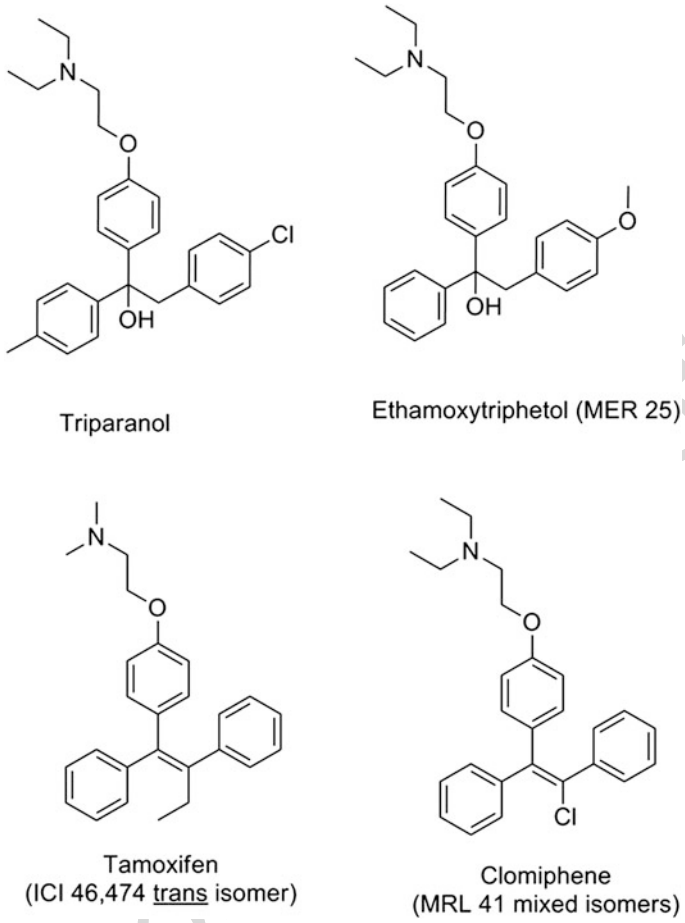

Fig. 2.2 Chemical structures of the first antiestrogens developed in the 1950s and 1960s, including tamoxifen

Triparanol was an orally active lipid-lowering agent developed by the Merrell Company during the 1960s [30]. Unfortunately, acute cataract formation was noted in young women treated with triparanol [31] and this ultimately led to the withdrawal of the medicine. The toxicity was linked to the accumulation of desmosterol as a consequence of the inhibition of cholesterol biosynthesis [32] (Fig. 2.3).

The punitive legal issues surrounding the withdrawal of triparanol forced the Merrell Company to avoid long-term treatments with any agents known to, or thought to, cause increases in the circulating levels of desmosterol. Triparanol [33], ethamoxytriphetol, and clomiphene [14] were all tested as treatments for breast cancer, but their potential to harm through cataract formation forced the Merrell Company to abandon work in the treatment of breast cancer. The administration of clomiphene for a few

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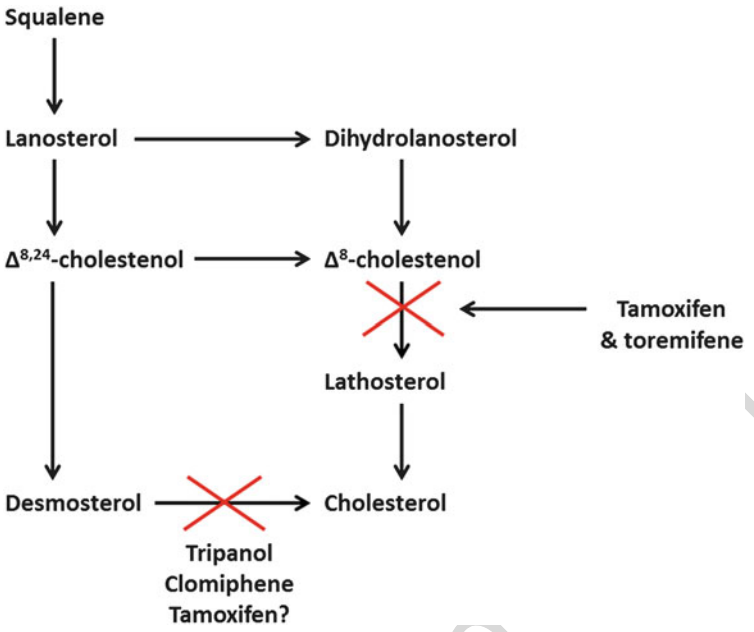

Fig. 2.3 The inhibition of cholesterol biosynthesis by triparanol, clomiphene, tamoxifen, and the chlorinated derivative of tamoxifen toremifene (see Chap. 3, Fig. 3.7)

days to induce ovulation was considered safe compared with the years of therapy necessary for breast cancer treatment.

Arthur Walpole, as head of the Fertility Control Program at ICI Pharmaceuticals Division Alderley Park, was already interested in the pharmacology of nonsteroidal estrogens and was asked to find a safer nonsteroidal antiestrogen in the early 1960s. Richardson was the synthetic organic chemist for the program (Fig. 2.4) and a young reproductive endocrinologist Michael J. K. Harper conducted the antifertility studies in the rat model (Fig. 2.4). The discovery of ICI 46,474 with reduced concerns about desmosterol accumulation was an advance.

From the time that tamoxifen was first available in clinical practice (1973) until the late 1980s, there were remarkably few concerns about the toxicity of tamoxifen, because the side effects from chemotherapy, by contrast, were so severe. Only with the extended use of tamoxifen as an adjuvant therapy in node-negative women, and the proposed use of tamoxifen as a chemopreventive, was there a return to an evaluation of the toxicity of tamoxifen, both by laboratory studies and by the analysis of randomized clinical trials. Despite the fact that tamoxifen was considered safe for long-term adjuvant therapy in women with breast cancer, analysis of the prevention trials organized and run by the National Surgical Adjuvant Bowel and Breast Project (NSABP) would demonstrate a small increase in cataracts and cataract operations for women without disease taking tamoxifen to reduce breast cancer incidence [34, 35].

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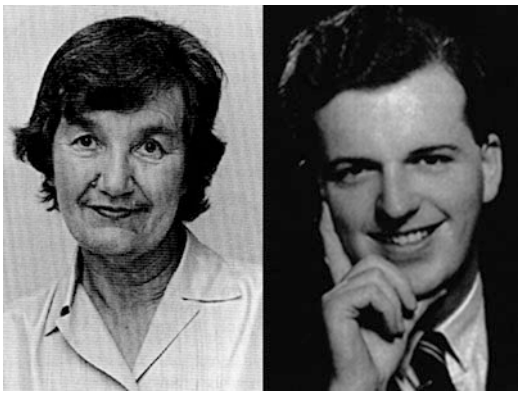

Fig. 2.4 (*Left*) Dora Richardson was a co-patent holder for ICI 46,474 and the organic chemist responsible for the synthesis of triphenylethylenes at ICI Pharmaceuticals. This photograph was taken on the occasion of her retirement in 1979. (*Right*) Mike Harper who discovered the opposing biological activities of the *cis* and *trans* isomers of substituted triphenylethylenes AU1

ICI 46,474 was first synthesized by Dr. Dora Richardson at ICI Ltd., Pharmaceuticals Division (Fig. 2.4), and was shown to be an antifertility agent in rodents [4, 5]. Dr. Michael Harper (Fig. 2.4) [3] made the discovery that the geometric isomers of substituted triphenylethylenes have opposing biological properties: the *cis* isomer ICI 47,699 is an estrogen, whereas the *trans* isomer ICI 46,474 has antiestrogenic activity. Thus the structure of the drug can program the cells for estrogenic or antiestrogenic properties [36–38]. Another observation made by Harper and Walpole was that ICI 46,474 exhibits species specificity; in short-term tests, the compound is an estrogen in the mouse and an antiestrogen in the rat [3, 4]. The triphenylethylene derivative blocks the binding of [^{3}H]estradiol to ERs derived from both rat and mouse target tissues [39–42], but no completely satisfactory subcellular mechanism for the species difference of ICI 46,474 has yet been established. In fact, the situation is probably more complex than may at first be appreciated. The long-term administration of tamoxifen to ovariectomized mice results in an initial estrogen-like effect in the vagina [40] and the uterus [43], but as treatment progresses both the uterus and vagina become refractory to the effects of exogenous estrogen, and ICI 46,474 becomes a complete antiestrogen in the vagina.

Preliminary clinical studies with ICI 46,474 to treat advanced breast cancer in postmenopausal women were conducted by Mary Cole and coworkers [16] at the Christie Hospital in Manchester. The confirmation that ICI 46,474 could be used successfully as palliative in advanced disease but produces few side effects [17, 44] acted as a catalyst to encourage the study of the mode of action of the drug in animal tumor models. Indeed the conversation between the laboratory and the clinic became the hallmark for the successful development of tamoxifen.

Animal studies were first started in 1973 at the Worcester Foundation for Experimental Biology Shrewsbury, Massachusetts [45–50]. The dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model, originally described a

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decade earlier by the Nobel Laureate Professor Charles Huggins [51], was used to study the efficacy and mode of action of ICI 46,474 under controlled laboratory conditions. The model was considered to be state of the art, because no other hormone-dependent models were then available for study. Rob Nicholson, then a graduate student at the Tenovus Institute for Cancer Research in Cardiff, Wales, also selected the DMBA-induced rat mammary carcinoma model for this study of the antitumor actions of ICI 46,474 and related compounds [52]. These parallel research ventures fully described the antitumor activity of the antiestrogen in vivo [41, 48–50, 53, 54] at a time when the efficacy of tamoxifen was being established widely in breast cancer clinical trials [55].

ICI 46,474 to Tamoxifen

In 1973, Nolvadex, the ICI brand of tamoxifen (as its citrate salt), was approved for the treatment of breast cancer by the Committee on the Safety of Medicines in the United Kingdom. Similar approval was given in the United States for the treatment of advanced disease in postmenopausal women by the Food and Drug Administration on 30 December 1977. Nolvadex is available in more than 110 countries as the first-line endocrine therapy for the treatment of breast cancer [1]. To mark this achievement, ICI Pharmaceutical Division was presented with the Queen's Award for Technological Achievement by the Lord Lieutenant of Cheshire, Viscount Leverhulme, on 6 July 1978. The remarkable success of tamoxifen encouraged a closer examination of its pharmacology with a view to further development and wider applications.

The metabolism of tamoxifen in animals and patients was first described by Fromson and coworkers [56, 57]. The major metabolic route to be described was hydroxylation to form 4-hydroxytamoxifen, which was subsequently shown to have high binding affinity for the estrogen receptor and to be a potent antiestrogen in its own right [58] with antitumor properties in the DMBA model [59]. Indeed it is an advantage for the tamoxifen to be metabolically activated to 4-hydroxytamoxifen [60], but this is not a prerequisite for antiestrogen action. The metabolite was subsequently shown to localize in target tissues after the administration of radioactive tamoxifen to rats [61]. Originally, 4-hydroxytamoxifen was believed to be the major metabolite in patients [57], but Hugh Adam [62] at ICI Pharmaceutical Division demonstrated that N-desmethyltamoxifen is the principal metabolite found in patients. There is usually a blood level ratio of 2:1 for N-desmethyltamoxifen that has twice the plasma half-life of tamoxifen (14 days vs. 7 days) [63]. The ubiquitous use of tamoxifen resulted in the publication of numerous methods to estimate tamoxifen and its metabolites in serum (reviewed in [64]). The metabolites that have been identified in patients are shown in Fig. 2.5. The minor metabolites, metabolite Y [65], metabolite Z [66], and 4-hydroxy-N-desmethyltamoxifen [67], all contribute to the antitumor actions of tamoxifen, because they are all antiestrogens which inhibit the binding of estradiol to the ER. The metabolism of tamoxifen will be considered in more detail in Chap. 3.

The next significant advance came with the availability of hormone-dependent human breast cancer cells to study antitumor mechanisms in the laboratory.

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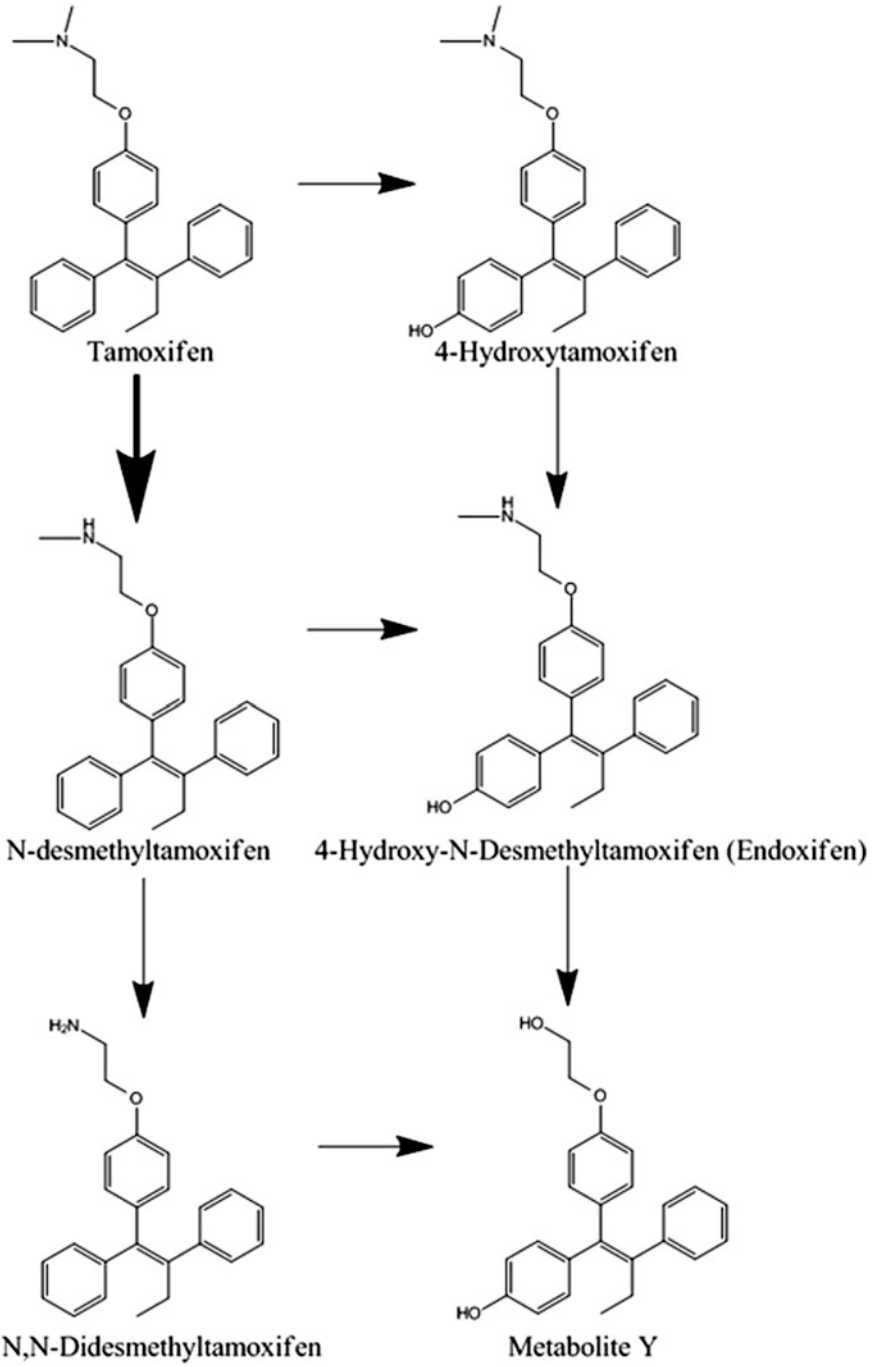

Fig. 2.5 The scheme of tamoxifen metabolism and the structures of its metabolites

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Marc Lippman [68] was the first to describe the ability of tamoxifen to inhibit the growth of MCF-7 ER-positive breast cancer cells [69] in culture and to demonstrate that the addition of estrogen could reverse the action of tamoxifen. Nearly a decade later, Kent Osborne [70] and Rob Sutherland [71] independently described the blockade by tamoxifen of breast cancer cells at the G_1 phase of the cell cycle.

Studies with the heterotransplantation of MCF-7 cells into athymic mice demonstrated that, unlike estradiol, tamoxifen does not support the growth of tumors [72]. Tamoxifen [73] and its metabolites [74] will block estrogen-stimulated tumor growth. However, very high circulatory levels (2,300 pg/ml) of estradiol in a low-tamoxifen environment (40 ng/ml) can partly reverse the inhibitory actions of tamoxifen for MCF-7 tumor growth [75]. Overall, these studies of the reversibility of tamoxifen action could have implications for its extended adjuvant use in premenopausal women.

These significant biological advances propelled tamoxifen forward to become the only nonsteroidal estrogen antagonist that would become the "gold standard" for the endocrine therapy of breast cancer for two decades. But none of this seemed possible in the 1970s when ICI Pharmaceutical Division was chauffeuring thousands of rats from Alderley Park to Leeds University. This investment in independent academic research would convert an orphan drug to be multibillion GBP blockbuster that saved millions of women's lives [76]. What is amazing is that the early work occurred without patent protection, but that changed.

Patenting Problems

Adequate patent protection is required to develop an innovation in a timely manner. In 1962, ICI Pharmaceuticals Division filed a broad patent in the United Kingdom (UK) (Application number GB19620034989 19620913). The application stated, "The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolaemic activity."

This was published in 1965 as UK Patent GB1013907, which described the innovation that different geometric isomers of substituted triphenylethylenes had either estrogenic or antiestrogenic properties [3]. Indeed, this observation was significant, because when scientists at Merrell subsequently described the biological activity of the separated isomers of their drug clomiphene, they inadvertently reversed the naming [77]. This was subsequently rectified [78].

Although tamoxifen was approved for the treatment of advanced breast cancer in postmenopausal women in 1977 in the United States (the year before ICI Pharmaceuticals Division received the Queen's Award for Technological Achievement in the UK), the patent situation was unclear. ICI Pharmaceuticals Division was repeatedly denied patent protection in the United States until the 1980s because of the perceived primacy of the earlier Merrell patents and because no advance (i.e., a safer, more

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specific drug) was recognized by the patent office in the United States. In other words, the clinical development advanced steadily for more than a decade in the United States without the assurance of exclusivity. This situation also illustrates how unlikely the usefulness of tamoxifen was considered to be by the medical advisors to the pharmaceutical industry in general. No other company chose to "steal" tamoxifen. Remarkably, when tamoxifen was hailed as the adjuvant endocrine treatment of choice for breast cancer by the National Cancer Institute in 1984 [79], the patent application, initially denied in 1984, was awarded through the court of appeals in 1985. This was granted with precedence to the patent dating back to 1965! So, at a time when worldwide patent protection was being lost, the patent protecting tamoxifen started a 17-year life in the United States. The unique and unusual legal situation did not go uncontested by generic companies, but AstraZeneca (as the ICI Pharmaceuticals Division is now called) rightly retained patent protection for their pioneering product, most notably, from Smalkin's decision in Baltimore, 1996 (Zeneca, Ltd. vs. Novopharm, Ltd.Civil Action No S95-163 United States District Court, D. Maryland, Northern Division, 14 March 1996).

Conclusion

The unprecedented advance of tamoxifen from the first unsure steps seems unbelievable but actually occurred. This situation was dependent on the correct prepared individuals being at the right place at the right time to advance a pioneering medicine that saves lives.

Postscript. In September 1972, at the time of the examination of my Ph.D. thesis by Dr. Arthur Walpole, I was unaware that the research director at ICI Pharmaceutical Division had ordered the termination of the clinical development of tamoxifen. This was a financial decision based on nonprofitability. My understanding is that all of the clinical research on tamoxifen (then ICI 46,474) had been reviewed in March 1972 at a symposium at Alderley Park [24].

The termination of tamoxifen's development toward registration and clinical use had resulted in Walpole requesting early retirement. Scientists at ICI Pharmaceutical Division did none of the laboratory work on tamoxifen as an antitumor agent; that was outsourced to me for a decade. But how did that happen?

I had already been recruited to the faculty as a lecturer in Pharmacology at Leeds, but first I was required to spend a couple of years in America to obtain my BTA (Been to America, a colloquial acronym as a prestigious research qualification). It had been arranged that I would go to the Worcester Foundation for Experimental Biology (the home of the oral contraceptive) to work with Mike Harper, who had left ICI Pharmaceutical Division some years earlier and now headed an Agency for International Development Program, to create a once a month contraceptive based on prostaglandins (the new research fashion!). I remember my conversation with Mike Harper on the telephone as I stood in the corridor on the phone in the old Medical School in Leeds. He asked three questions: "Could you start in September

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(1972)?" "Would $12,000 a year be acceptable?" and "Would you work on prostaglandins?" "Yes, yes, yes," I replied and headed off to the library to find out what prostaglandins were!

Walpole, my committee, and I met for my examination in the Department of Pharmacology at the Leeds University in early September 1972. This had become a matter of urgency as I had to complete the examination, drive to Southampton to board the QEII, and then travel from New York to Worcester, MA, to be a visiting scientist for 2 years at the Worcester Foundation for Experimental Biology.

When I arrived to the Worcester Foundation in September 1972—incidentally not knowing anything about prostaglandins—I discovered that Mike Harper had accepted a job with the World Health Organization in Geneva. My new boss Ed Klaiber said: "Next week give me a plan of research you propose to complete here in the next two years" and "You can do anything you like as long as some of it includes prostaglandins." Armed with a brand new Ph.D. in "failed contraceptives" (a topic not designed to equip me for a research career!), I immediately found myself as an independent investigator and planned my work on prostaglandins. However, my new circumstances would also allow me to explore my passion—to develop a drug to treat breast cancer.

A phone call to Walpole started the process of turning ICI 46,474 into tamoxifen, the gold standard for the endocrine treatment of breast cancer for the next 30 years. Walpole informed me the ICI Pharmaceuticals Division had just acquired Stuart Pharmaceuticals in Wilmington, Delaware, and they had created a new company ICI Americas. Lois Trench, the drug monitor for tamoxifen, would be the individual involved in the investment in my laboratory at the Worcester Foundation with an unrestricted research grant to determine how best to use tamoxifen in the clinic. But how to start? I was a pharmacologist with experience in "failed contraceptive" not a cancer research scientist. It seems that the way forward depends upon a clear plan, enthusiasm, and who you meet.

The National Cancer Act was passed in 1971 in the United States and the "war on cancer" began. The president of the Worcester Foundation Mahlon Hoagland realized that the research resources of the foundation in reproductive endocrinology could be steered toward endocrine-dependent cancers with the right advisor on the Scientific Advisory Board. Dr. Elwood Jensen, director of the Ben May Laboratory for Cancer Research at the University of Chicago, was a pioneer in the identification of the ER in estrogen target tissues in the rat and the application of this knowledge for the identification of estrogen-dependent breast tumors in women with metastatic breast cancer. The absence of ER in the tumor meant that there was no possibility of a response to endocrine ablation. Jensen spent a couple of days at the foundation in late 1972 and we spent time together going over my thesis work. I told him of my plans for tamoxifen and he generously agreed to have his staff (or rather Silvia Smith) teach me techniques of ER analysis and most importantly his colleague Dr. Gene DeSombre to teach me the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. My visit to Chicago to learn the techniques was a dream come true!

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Lois Trench arranged for me to receive a small collection of deep-frozen breast tumors so we started the program of translational research with the aid of Suzane Koerner, a superb technician. Lois insisted I became a consultant to ICI Americas to encourage clinicians in oncology groups to study tamoxifen in clinical trial. I lectured to the members of the Eastern Cooperative Oncology Group Breast Committee at their meetings in Miami and Jasper National Park in 1974. Too many adventures there to fit in the limited space here, I am afraid! Lois, then sponsored me to present the first study on tamoxifen as a preventive of mammary cancer in rats at the International Steroid Hormone Congress in Mexico City in September 1974 [45] (more adventures with my boss Ed Klaiber in Acapulco).

The idea of publishing my emerging data for the treatment and prevention of breast cancer did not occur immediately. Nobody in the scientific or clinical community really cared about the development of another (more expensive) endocrine therapy of limited effectiveness. However, that perspective was to change. Eliahu Caspi called me to his office one day in July 1974 and announced he had been charged with the responsibility of evaluating my CV and bibliography to explore the possibility of me staying at the foundation as a staff and not returning to Leeds University. He was rather frightening as an individual and stared at me across his desk. He reiterated that he had been told to interview me and evaluate my CV. He then said: "but you haven't got one as you have not published anything." After a stunned silence from me, I replied: "but I haven't discovered anything," to which he then gave me the best advice I had received about developing an academic career up to that point. "Tell them the story so far; each paper can be written within about 2 weeks and create a theme of interlocking research papers." I have followed his advice ever since.

I would like to recount an unanticipated honor that occurred by chance in 2002. At the commencement of the University of Massachusetts Medical School at the Mechanics Hall in Worcester in 2001, I was delivering my acceptance speech for an honorary Doctor of Science degree and told my Eliahu Caspi story about publication—emphasizing that if you don't publish, it never happened. A year later I was asked to deliver the inaugural Eliahu Caspi Memorial Lecture at the Worcester Foundation. It was then that I learned of the remarkable background of Dr. Caspi and had the pleasure of spending time with his accomplished family. As a young man in Poland, Caspi had survived a Russian prison camp, escaped to the emerging Israel, joined the Haganah (early Israeli Defense Forces), and then came to America to complete his Ph.D. at Clark University in Worcester. He then joined the Worcester Foundation having a distinguished career in glucocorticoid metabolism and synthesis until his death in May 2001.

Uncorrected Proof

References

1. Furr BJ, Jordan VC (1984) The pharmacology and clinical uses of tamoxifen. Pharmacol Ther 25:127–205
2. Group EBCTC (1992) Systematic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Lancet 339:1–15, and 71–85

Author's Proof

3. Harper MJ, Walpole AL (1966) Contrasting endocrine activities of *cis* and *trans* isomers in a series of substituted triphenylethylenes. Nature 212:87
4. Harper MJ, Walpole AL (1967) A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 13:101–119
5. Harper MJ, Walpole AL (1967) Mode of action of I.C.I. 46,474 in preventing implantation in rats. J Endocrinol 37:83–92
6. Emmens CW (1970) Postcoital contraception. Br Med Bull 26:45–51
7. Klopper A, Hall M (1971) New synthetic agent for the induction of ovulation: preliminary trials in women. Br Med J 1:152–154
8. Williamson JG, Ellis JD (1973) The induction of ovulation by tamoxifen. J Obstet Gynaecol Br Commonw 80:844–847
9. Beatson GT (1896) On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. Lancet 2:104–107
10. Boyd S (1900) On oophorectomy in cancer of the breast. Br Med J 2:1161–1167
11. Lerner LJ, Holthaus FJ Jr, Thompson CR (1958) A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol. Endocrinology 63:295–318
12. Holtkamp DE, Greslin JG, Root CA, Lerner LJ (1960) Gonadotrophin inhibiting and anti-fecundity effects of chloramiphene. Proc Soc Exp Biol Med 105:197–201
13. Kistner RW, Smith OW (1959) Observations on the use of nonsteroidal estrogen antagonist: MER 25. Surg Forum 10:725–729
14. Herbst AL, Griffiths CT, Kistner RW (1964) Clomiphene sitrate (Nsc-35770) in disseminated mammary carcinoma. Cancer Chemother Rep 43:39–41
15. Legha SS, Slavik M, Carter SK (1976) Nafoxidine–an antiestrogen for the treatment of breast cancer. Cancer 38:1535–1541
16. Cole MP, Jones CT, Todd ID (1971) A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer 25:270–275
17. Ward HW (1973) Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J 1:13–14
18. Sander S (1968) The in vitro uptake of oestradiol in biopsies from 25 breast cancer patients. Acta Pathol Microbiol Scand 74:301–302
19. Johansson H, Terenius L, Thoren L (1970) The binding of estradiol-17beta to human breast cancers and other tissues in vitro. Cancer Res 30:692–698
20. Korenman SG, Dukes BA (1970) Specific estrogen binding by the cytoplasm fof human breast carcinoma. J Clin Endocrinol Metab 30:639–645
21. Jensen EV, Block GE, Smith S et al (1971) Estrogen receptors and breast cancer response to adrenalectomy. Natl Cancer Inst Monogr 34:55–70
22. McGuire WLCP, Vollmer EP (1975) Estrogen receptors in human breast cancer. Raven, New York
23. Jordan VC (1988) The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. Breast Cancer Res Treat 11:197–209
24. Jordan VC (2006) Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 147(Suppl 1):S269–S276
25. Powles TJ, Hardy JR, Ashley SE et al (1989) A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. Br J Cancer 60:126–131
26. Powles TJ, Tillyer CR, Jones AL et al (1990) Prevention of breast cancer with tamoxifen–an update on the Royal Marsden Hospital pilot programme. Eur J Cancer 26:680–684
27. Fisher B (1992) The evolution of paradigms for the management of breast cancer: a personal perspective. Cancer Res 52:2371–2383
28. Lerner LJ (1981) The first nonsteroidal antioestrogen-MER 25. In: Sutherland RL, Jordan VC (eds) Non-steroidal antioestrogens: molecular pharmacology and antitumour activity. Academic, Sydney, pp 1–16
29. Clark JH, Markaverich BM (1982) The agonistic-antagonistic properties of clomiphene: a review. Pharmacol Ther 15:467–519

Uncorrected Proof

Author's Proof

30. Hollander W, Chobanian AV, Wilkins RW (1960) The effects of triparanol (MER-29) in subjects with and without coronary artery disease. JAMA 174:5–12
31. Laughlin RC, Carey TF (1962) Cataracts in patients treated with triparanol. JAMA 181:339–340
32. Avigan J, Steinberg D, Vroman HE et al (1960) Studies of cholesterol biosynthesis. I. The identification of desmosterol in serum and tissues of animals and man treated with MER-29. J Biol Chem 235:3123–3126
33. Kraft RO (1962) Triparanol in the treatment of disseminated mammary carcinoma. Cancer Chemother Rep 25:113–115
34. Fisher B, Costantino JP, Wickerham DL et al (2005) Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97:1652–1662
35. Fisher B, Costantino JP, Wickerham DL et al (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90:1371–1388
36. Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741–4745
37. Jordan VC, Koch R, Langan S, McCague R (1988) Ligand interaction at the estrogen receptor to program antiestrogen action: a study with nonsteroidal compounds in vitro. Endocrinology 122:1449–1454
38. Murphy CS, Langan-Fahey SM, McCague R, Jordan VC (1990) Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. Mol Pharmacol 38:737–743
39. Skidmore J, Walpole AL, Woodburn J (1972) Effect of some triphenylethylenes on oestradiol binding in vitro to macromolecules from uterus and anterior pituitary. J Endocrinol 52:289–298
40. Jordan VC (1975) Prolonged antioestrogenic activity of ICI 46, 474 in the ovariectomized mouse. J Reprod Fertil 42:251–258
41. Jordan VC, Jaspan T (1976) Tamoxifen as an anti-tumour agent: oestrogen binding as a predictive test for tumour response. J Endocrinol 68:453–460
42. Terenius L (1970) Two modes of interaction between oestrogen and anti-oestrogen. Acta Endocrinol (Copenh) 64:47–58
43. Jordan VC, Lababidi MK, Mirecki DM (1990) Anti-oestrogenic and anti-tumour properties of prolonged tamoxifen therapy in C3H/OUJ mice. Eur J Cancer 26:718–721
44. O'Halloran MJ, Maddock PG (1974) I.C.I. 46,474 in breast cancer. J Ir Med Assoc 67:38–39
45. Jordan VC (1974) Antitumor activity of the antiestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 5:354
46. Jordan VC (1974) The antiestrogen tamoxifen (ICI 46,474) as an antitumor agent. In: Proceedings of the Eastern cooperative oncology group, Miami
47. Jordan VC (1974) Tamoxifen: mechanism of antitumor activity in animals and man. In: Proceedings of the Eastern cooperative oncology group, Jasper
48. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
49. Jordan VC, Koerner S (1975) Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 11:205–206
50. Jordan VC, Koerner S (1976) Tamoxifen as an anti-tumour agent: role of oestradiol and prolactin. J Endocrinol 68:305–311
51. Huggins C, Grand LC, Brillantes FP (1961) Mammary cancer induced by a single feeding of polymucular hydrocarbons, and its suppression. Nature 189:204–207
52. Nicholson RI, Golder MP (1975) The effect of synthetic anti-oestrogens on the growth and biochemistry of rat mammary tumours. Eur J Cancer 11:571–579
53. Jordan VC, Dowse LJ (1976) Tamoxifen as an anti-tumour agent: effect on oestrogen binding. J Endocrinol 68:297–303

Uncorrected Proof

Author's Proof

54. Nicholson RI, Golder MP, Davies P, Griffiths K (1976) Effects of oestradiol – 17beta and tamoxifen on total and accessible cytoplasmic oestradiol – 17beta receptors in DMBA-induced rat mammary tumours. Eur J Cancer 12:711–717
55. Tamoxifen Workshop (1976) Proceedings of the annual spring meeting of the primary breast cancer therapy group, Key Biscayne, p 1409: 66
56. Fromson JM, Pearson S, Bramah S (1973) The metabolism of tamoxifen (I.C.I. 46,474). I. In laboratory animals. Xenobiotica 3:693–709
57. Fromson JM, Pearson S, Bramah S (1973) The metabolism of tamoxifen (I.C.I. 46,474). II. In female patients. Xenobiotica 3:711–714
58. Jordan VC, Collins MM, Rowsby L, Prestwich G (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305–316
59. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
60. Allen KE, Clark ER, Jordan VC (1980) Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 71:83–91
61. Borgna JL, Rochefort H (1981) Hydroxylated metabolites of tamoxifen are formed in vivo and bound to estrogen receptor in target tissues. J Biol Chem 256:859–868
62. Adam HK, Douglas EJ, Kemp JV (1979) The metabolism of tamoxifen in human. Biochem Pharmacol 28:145–147
63. Patterson JS, Settatree RS, Adam HK, Kemp JV (1980) Serum concentrations of tamoxifen and major metabolite during long-term nolvadex therapy, correlated with clinical response. Eur J Cancer 1:89–92
64. Jordan VC, Murphy CS (1990) Endocrine pharmacology of antiestrogens as antitumor agents. Endocr Rev 11:578–610
65. Jordan VC, Bain RR, Brown RR et al (1983) Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. Cancer Res 43:1446–1450
66. Kemp JV, Adam HK, Wakeling AE, Slater R (1983) Identification and biological activity of tamoxifen metabolites in human serum. Biochem Pharmacol 32:2045–2052
67. Lien EA, Solheim E, Lea OA et al (1989) Distribution of 4-hydroxy-N-desmethyltamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. Cancer Res 49:2175–2183
68. Lippman ME, Bolan G (1975) Oestrogen-responsive human breast cancer in long term tissue culture. Nature 256:592–593
69. Brooks SC, Locke ER, Soule HD (1973) Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J Biol Chem 248:6251–6253
70. Osborne CK, Boldt DH, Clark GM, Trent JM (1983) Effects of tamoxifen on human breast cancer cell cycle kinetics: accumulation of cells in early G1 phase. Cancer Res 43:3583–3585
71. Sutherland RL, Green MD, Hall RE et al (1983) Tamoxifen induces accumulation of MCF 7 human mammary carcinoma cells in the G0/G1 phase of the cell cycle. Eur J Cancer Clin Oncol 19:615–621
72. Shafie SM, Grantham FH (1981) Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. J Natl Cancer Inst 67:51–56
73. Osborne CK, Hobbs K, Clark GM (1985) Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res 45:584–590
74. Gottardis MM, Robinson SP, Jordan VC (1988) Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumoristatic action of tamoxifen. J Steroid Biochem 30:311–314
75. Iino Y, Wolf DM, Langan-Fahey SM et al (1991) Reversible control of oestradiol-stimulated growth of MCF-7 tumours by tamoxifen in the athymic mouse. Br J Cancer 64:1019–1024
76. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44: 30–38

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Author's Proof

77. Palopoli FP, Feil VJ, Allen RE et al (1967) Substituted aminoalkoxytriarylhaloethylenes. J Med Chem 10:84–86
78. Ernst S, Hite G, Cantrell JS et al (1976) Stereochemistry of geometric isomers of clomiphene: a correction of the literature and a reexamination of structure-activity relationships. J Pharm Sci 65:148–150
79. Consensus conference (1985) Adjuvant chemotherapy for breast cancer. JAMA 254:3461–3463

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Abstract	By the early 1970s, a number of metabolites of tamoxifen had been identified in animals and following administration to a few patients. The hydroxylated metabolite of tamoxifen, 4-hydroxytamoxifen, proved to be the most interesting. The discovery of its high binding affinity for the estrogen receptor made it a new laboratory tool for all future in vitro studies of antiestrogen action and also provided the clue for all future structure-function relationships studies of new antiestrogens. These compounds would subsequently be developed as selective estrogen receptor modulators (SERMs). Tamoxifen is a prodrug but it is the metabolite 4-hydroxy-N-desmethyltamoxifen or endoxifen that has attracted pharmacogenetic interest. Mutations of the CYP2D6 gene control endoxifen production and have been associated with drug efficacy in some clinical trials.	

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Chapter 3
Metabolites of Tamoxifen as the Basis of Drug Development

Abstract By the early 1970s, a number of metabolites of tamoxifen had been identified in animals and following administration to a few patients. The hydroxylated metabolite of tamoxifen, 4-hydroxytamoxifen, proved to be the most interesting. The discovery of its high binding affinity for the estrogen receptor made it a new laboratory tool for all future in vitro studies of antiestrogen action and also provided the clue for all future structure-function relationships studies of new antiestrogens. These compounds would subsequently be developed as selective estrogen receptor modulators (SERMs). Tamoxifen is a prodrug but it is the metabolite 4-hydroxy-N-desmethyltamoxifen or endoxifen that has attracted pharmacogenetic interest. Mutations of the CYP2D6 gene control endoxifen production and have been associated with drug efficacy in some clinical trials.

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Introduction

Tamoxifen is believed to be a prodrug and can be metabolically activated to 4-hydroxytamoxifen [1–4] or alternatively can be metabolically routed via N-desmethyltamoxifen to 4-hydroxy-N-desmethyltamoxifen [5, 6] (endoxifen) (Fig. 3.1). The hydroxylated metabolites of tamoxifen have a high binding affinity for the ER [1, 7]. The finding that the CYP2D6 subtype of cytochrome P450 activates tamoxifen to endoxifen [8] has implications for cancer therapeutics. It has been proposed that women with enzyme variants that cannot make endoxifen may not have as successful an outcome with tamoxifen therapy. Alternatively, women who have a wild-type enzyme may make high levels of the potent antiestrogen endoxifen and experience hot flashes. As a result, these women may take selective serotonin reuptake inhibitors (SSRIs) to ameliorate hot flashes but there are potential pharmacological consequences to this strategy. Some of the SSRIs are metabolically altered by the CYP2D6 enzyme [9]. It is therefore possible to envision a drug interaction whereby SSRIs block the metabolic activation of tamoxifen.

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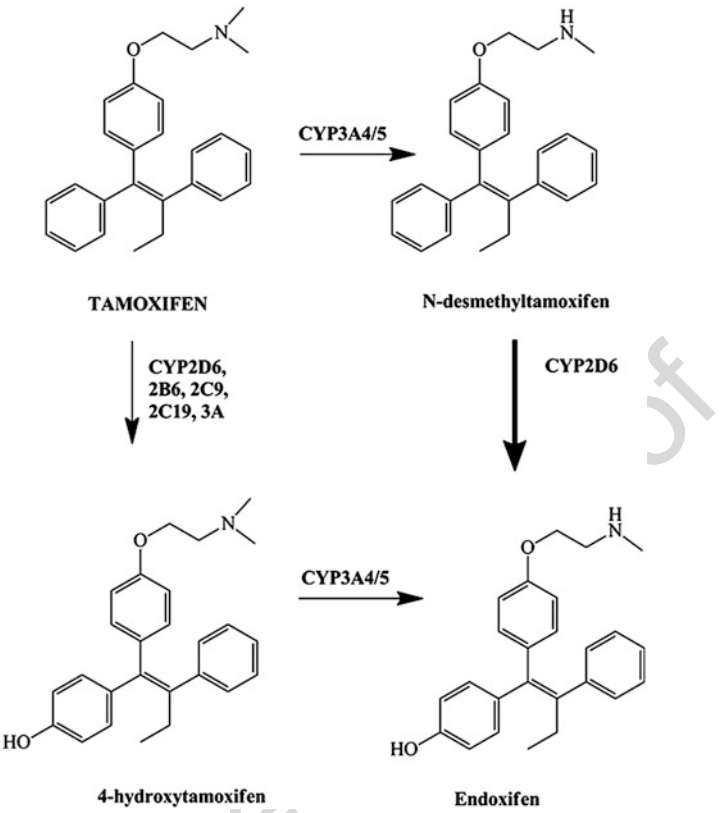

Fig. 3.1 The metabolic activation of tamoxifen to phenolic metabolites that have a high binding activity for the human ER. Both 4-hydroxytamoxifen and endoxifen are potent antiestrogens in vitro

This chapter will describe the scientific twists and turns that tamoxifen and its metabolites have taken over the past 30 years. The story is naturally dependent on the fashions in therapeutic research at the time. What seems obvious to us as a successful research strategy today, with millions of women taking tamoxifen, was not so 30 years ago at the beginning when the clinical community and pharmaceutical industry did not see "antihormones" as a priority at all for drug development [10]. In 1972, tamoxifen was declared an orphan drug with little prospect of successful clinical development [11].

Basic Mechanisms of Tamoxifen Metabolism

The original survey of the putative metabolites of tamoxifen was conducted in the laboratories of ICI Pharmaceuticals Division and published in 1973 [12]. A number of hydroxylated metabolites were noted (Fig. 3.2) following the administration of

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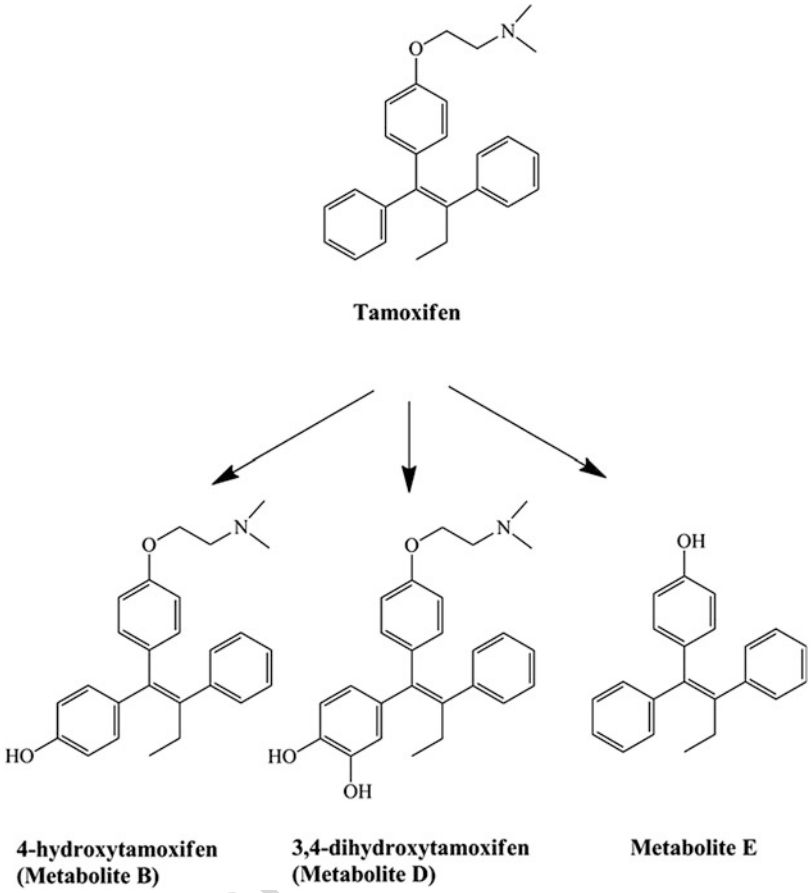

Fig. 3.2 The original hydroxylated metabolites of tamoxifen noted in animals by Fromson and coworkers [12]

^{14}C-labeled tamoxifen to various species (rat, mouse, monkey, and dog). The major route of excretion of radioactivity was in the feces. The rat and dog studies showed that up to 53 % of the radioactivity derived from tamoxifen was excreted via the bile and up to 69 % of this was reabsorbed via an enterohepatic recirculation until elimination eventually occurs [12]. The hydroxylated metabolites are excreted as glucuronides. However, no information about their biological activity was available until the finding that 4-hydroxytamoxifen had a binding affinity for the ER equivalent to 17β estradiol [1]. Similarly, 3, 4-dihydroxytamoxifen (Fig. 3.2) bound to the human ER but interestingly enough, 3, 4-dihydroxytamoxifen was not significantly estrogen-like in the rodent uterus despite being antiestrogenic [1, 4].

Additional studies on the metabolism of tamoxifen in four women [13] identified 4-hydroxytamoxifen as the primary metabolite using a thin layer chromatographic technique to identify ^{14}C-labeled metabolites. This assumption, coupled with the

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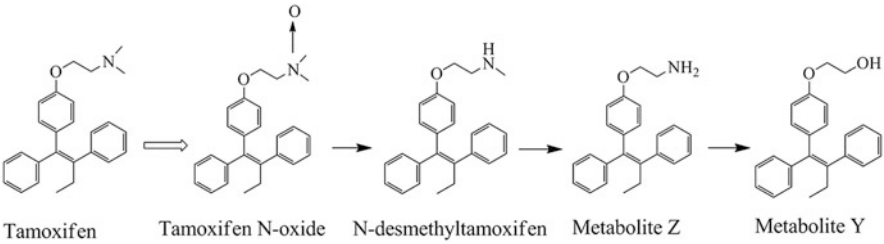

Fig. 3.3 The serial metabolic dimethylation and deamination of the antiestrogenic side chain of tamoxifen. Each of the metabolites is a weak antiestrogen with poor binding affinity for the ER

potent antiestrogenic actions of 4-hydroxytamoxifen [1] and the conclusion that it was an advantage, but not a requirement for tamoxifen to be metabolically activated [2, 14], seemed to confirm the idea that 4-hydroxytamoxifen was the active metabolite that bound in rat estrogen target tissues to block estrogen action [3]. However, the original analytical methods used to identify 4-hydroxytamoxifen as the major metabolite in humans were flawed [15] and subsequent studies identified N-desmethyltamoxifen (Fig. 3.3) as the major metabolite circulating in human serum [16]. The metabolite was found to be further demethylated to *N*-desdimethyltamoxifen (metabolite Z) [17] and then deaminated to metabolite Y, a glycol derivative of tamoxifen [18, 19]. The metabolites (Fig. 3.3) that are not hydroxylated at the 4 position of tamoxifen (equivalent to the three phenolic hydroxyl of estradiol) are all weak antiestrogens that would each contribute to the overall antitumor actions of tamoxifen at the ER based on their relative binding affinities for the ER and their actual concentrations locally.

At the end of the 1980s, the identification of another metabolite tamoxifen 4-hydroxy-N-desmethyltamoxifen in animals [20] and man [5, 6] was anticipated but viewed as obvious and uninteresting. The one exception that was of interest was metabolite E (Fig. 3.2 identified in the dog [12]. This phenolic metabolite without the dimethylaminoethyl side chain is a full estrogen [19, 21]. The dimethylaminoethoxy side chain of tamoxifen is necessary for antiestrogenic action [21].

It is not a simple task to study the actions of metabolites in vivo. Problems of pharmacokinetics, absorption, and subsequent metabolism all conspire to confuse the interpretation of data. Studies in vitro using cell systems of estrogen target tissues were defined and refined in the early 1980s to create an understanding of the actual structure-function relationships of tamoxifen metabolites. Systems were developed to study the regulation of the prolactin gene in primary cultures of immature rat pituitary gland cells [14, 22] or cell replication in ER-positive breast cancer cells [23–26]. Overall, these models were used to describe the importance of a phenolic hydroxyl to tether the triphenylethylenes appropriately in the ligand-binding domain of the ER and to establish the appropriate positioning of an "antiestrogenic" side chain in the "antiestrogen region" of the ER [22] to modulate gene activation and growth [14, 22, 27–30]. These structure-function studies that created hypothetical models of the ligand-ER complex were rapidly advanced with

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the first reports of the X-ray crystallography of the estrogen, 4-hydroxytamoxifen [31], or raloxifene ER ligand-binding domain [32] complexes. The ligand-receptor protein interaction was subsequently interrogated by examining the interaction of the specific amino acid asp 351 with the antiestrogenic side chain of the ligand [33]. A mutation was found as the dominant ER species in a tamoxifen-stimulated breast tumor grown in athymic mice [33, 34]. The structure-function relationships studies, that modulated estrogen action at a transforming growth factor-alpha gene target, demonstrated that the ligand shape would ultimately program the shape of the ER complex in a target tissue [35–39]. This concept is at the heart of metabolite pharmacology and is required to switch on and switch off target sites around the body. The other piece of the mechanism of the SERM puzzle that was eventually solved was the need for another player to partner with the ER complex. Coactivators [40] can enhance the estrogen-like effects of compounds at a target site [41]. However, in the early 1990s, the molecular and clinical use of this knowledge with the development and application of SERMs was in the future [42].

It is clear from this background about the early development of tamoxifen and the fact that tamoxifen was considered to be such a safe drug in comparison to other cytotoxic agents used in therapy during the 1970s and 1980s that there was little enthusiasm for in-depth studies of tamoxifen metabolism. However, this perspective was to change in the 1990s with the widespread use of tamoxifen as the gold standard for the treatment and prospect of clinical trials to evaluate the worth of tamoxifen for the prevention of breast cancer.

The urgent focus of translational research in the early1990s was to discover why tamoxifen was a complete carcinogen in rat liver [43, 44] and to determine whether there was a link between metabolism and the development of endometrial cancer noted in very small but significant numbers of postmenopausal women taking adjuvant tamoxifen [45, 46].

All interest in the metabolism of tamoxifen focused on the production of DNA adducts [47] that were responsible for rat liver carcinogenesis and, at the time, believed to be potentially responsible for carcinogenesis in humans [48]. Although many candidates were described [49–52], the metabolite found to be responsible for the initiation of rat liver carcinogenesis is α-hydroxytamoxifen [53–57] (Fig. 3.4). α-Hydroxytamoxifen has been resolved into R-(+) and S-(−)enantiomers. Metabolism by rat liver microsomes gave equal amounts of the two forms, but in hepatocytes the R form gave 8× the level of DNA adducts as the S form. As both had the same chemical reactivity toward DNA, Osborne et al. [58] suggested that the R form was a better sulfotransferase substrate. This enzyme is believed to catalyze DNA adduct formation. Subsequently, Osborne et al. [59] conducted studies with alpha-hydroxy-*N*-desmethyltamoxifen; the R-(+) gave 10× the level of adducts in rat hepatocytes as the S-(−).

There were reasonable concerns that the hepatocarcinogenicity of tamoxifen in rats would eventually translate to humans but fortunately this is now known to be untrue [60]. The demonstration of carcinogenesis in the rat liver appears to be related to poor DNA repair mechanisms in the inbred strains of rats. In contrast, it appears that the absence of liver carcinogenesis in women exposed to tamoxifen

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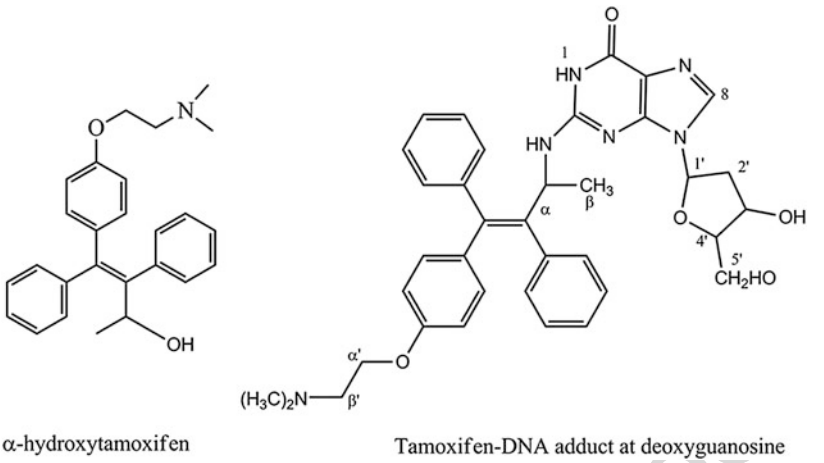

Fig. 3.4 The putative metabolite of tamoxifen, α- hydroxytamoxifen, that produces DNA adducts through covalent binding to deoxyguanosine in the rat liver

[61] is believed to result from the sophisticated mechanisms of DNA repair inherent in human cells. These concepts are described in more detail in Chap. 6.

The questions that next needed to be addressed were: Can improvements be made to the tamoxifen molecule? What happens to tamoxifen in patients?

Metabolic Mimicry

The demonstration [1, 2] that the class of compounds referred to as nonsteroidal antiestrogens were metabolically activated to compounds with high binding affinity for the ER created additional opportunities for the medicinal chemists within the pharmaceutical industry to develop new agents. An initial attempt was 3-hydroxytamoxifen (droloxifene) that was evaluated extensively in clinical trials. Those trials have been reviewed [62] but no advantages over tamoxifen were found. It is important to note that all studies used higher doses compared to tamoxifen. This emphasizes the principle that as droloxifene is a hydroxylated compound and is excreted more rapidly.

Drug discovery accelerated once the nonsteroidal antiestrogens [63] were recognized to be SERMs [64–66] and had applications not only for the treatment and prevention of breast cancer but also as potential agents to treat osteoporosis and coronary heart disease [67, 68]. The reader is referred to other recent review articles to obtain further details of new medicines under investigation [67, 68] but some current examples are worthy of note and will be mentioned briefly. Compounds of interest that have their structural origins from metabolites of nonsteroidal antiestrogens are summarized in Fig. 3.5.

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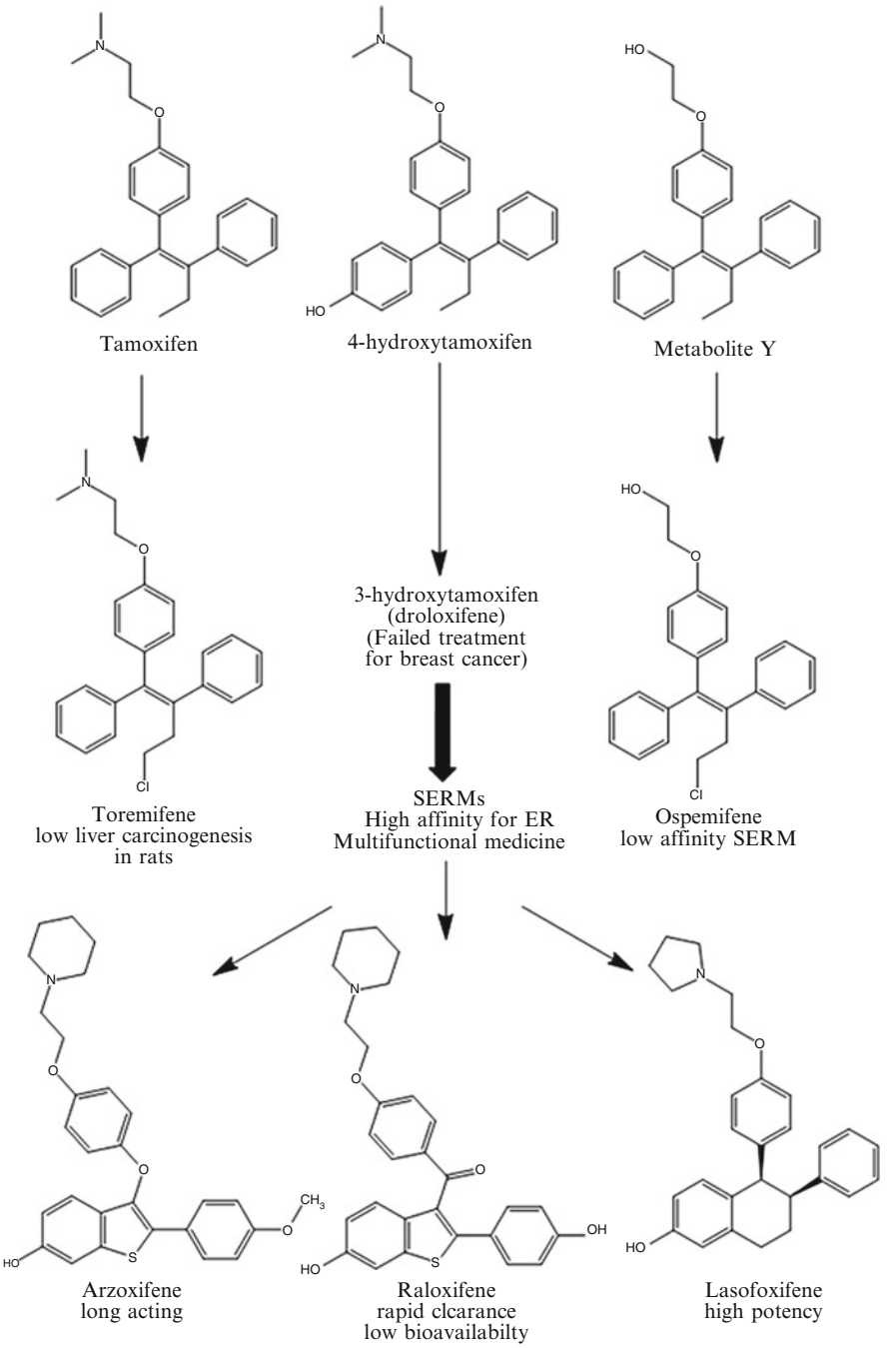

Fig. 3.5 The structures of new SERMs and their origins from other antiestrogens

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Raloxifene is an agent that originally was destined to be a drug to treat breast cancer but it failed in that application [69]. It appears that the pharmacokinetics and bioavailability of raloxifene are a challenge. Only about 2 % of administered raloxifene is bioavailable [70] but despite this, the drug is known to have a reasonable biological half-life of 27 h. The reason for this disparity is that raloxifene is a polyphenolic drug that can be glucuronidated and sulfated by bacteria in the gut so the drug cannot be absorbed [71, 72]. This phase II metabolism in turn controls enterohepatic recirculation and ultimately impairs the drug from reaching and interacting with receptors in the target. This concern has been addressed with the development of the long-acting raloxifene derivative arzoxifene that is known to be superior to raloxifene as a chemopreventive in rat mammary carcinogenesis [73]. One of the phenolic groups (Fig. 3.5) is methylated to provide protection from phase II metabolism.

Nevertheless, arzoxifene has not performed well as a treatment for breast cancer [74, 75]; higher doses are less effective than lower doses. These data imply that effective absorption is impaired by phase III metabolism. That being said, the results of trials evaluating the effects of arzoxifene as a drug to treat osteoporosis have been completed [76–78].

Unfortunately, the bioavailability of phenolic drugs is also dependent on phase II metabolism to inactive conjugates in the target tissue. 4-Hydroxytamoxifen [1] is only sulfated by three of seven sulfotransferase isoforms, whereas raloxifene is sulfated by all seven [79]. Maybe local phase II metabolism plays a role in neutralizing the antiestrogen action of raloxifene in the breast. Falany et al. [79] further report that SULT1E1, that sulfates raloxifene in the endometrium, is only expressed in the secretory phase. In contrast, 4- hydroxytamoxifen is sulfated at all stages of the uterine cycle.

Lasofoxifene is a diaryltetrahydronaphthalene derivative referred to as CP336156 [80] that has been reported to have high binding affinity for ER and have potent activity in preserving bone density in the rat [81, 82]. The structure of CP336156 is reminiscent of the putative antiestrogenic metabolic route for nafoxidine [83] (Chap. 1, Fig. 1.4) that failed to become a breast cancer drug because of unacceptable side effects [84]. CP336156 is the *l* enantiomer that has 20 times the binding affinity for the ER as the *d* enantiomer. Studies demonstrate that the *l* enantiomer had twice the bioavailability of the *d* enantiomer. The authors [80] ascribed the difference to enantioselective glucuronidation of the *d* isomer. An evaluation of CP336156 in the prevention and treatment of rat mammary tumors induced by *N*-nitroso-*N*-methylurea shows activity similar to that of tamoxifen [85].

Ospemifene or deaminohydroxytoremifene is related to metabolite Y formed by the deamination of tamoxifen [19]. Metabolite Y has a very low binding affinity for the ER [19, 86] and has weak antiestrogenic properties compared with tamoxifen. Ospemifene is a known metabolite of toremifene (4-chlorotoremifene) but unlike tamoxifen, there is little carcinogenic potential in animals [87]. It is possible that the large chlorine atom on the 4 position of toremifene and ospemifene reduces α-hydroxylation to the ultimate carcinogen related to α-hydroxytamoxifen (Fig. 3.5). Deaminohydroxytoremifene has very weak estrogenic and antiestrogenic

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properties in vivo [88] but demonstrates SERM activity in bone and lowers cholesterol. The compound is proposed to be used as a preventative for osteoporosis. Preliminary clinical data in healthy men and postmenopausal women demonstrate pharmacokinetics suitable for daily dosing between 25 and 200 mg [89]. Interestingly enough, unlike raloxifene, ospemifene has a strong estrogen-like action in the vagina but neither ospemifene nor raloxifene affects endometrial histology [90, 91].

Overall, the goal of developing a bone-specific agent is reasonable, but the key to commercial success will be the prospective demonstration of the prevention of breast and endometrial cancer as beneficial side effects. This remains a possibility based on prevention studies completed in the laboratory [92, 93].

Tamoxifen Metabolism Today

During the past decade, there has been considerable interest in the pharmacogenetics of tamoxifen-metabolizing enzymes in humans. The central hypothesis is that aberrant genes responsible for the metabolic activation of tamoxifen will influence therapeutics.

A comprehensive evaluation of the sequential biotransformation of tamoxifen has been completed by Desta and coworkers [8]. They used human liver microsomes and experiments with specifically expressed human cytochrome P450s to identify the prominent enzymes involved in phase I metabolism. Their results are summarized in Fig. 3.1 with the relevant CYP genes indicated for the metabolic transformations. The authors make a strong case that N-desmethyltamoxifen, the principal metabolite of tamoxifen that accumulates in the body, is converted to endoxifen by the enzyme variant CYP2D6. The CYP2D6 enzyme is also important to produce the potent primary metabolite 4-hydroxytamoxifen (this was first reported by David Kupfer at the Worcester Foundation 15 years ago! [94]), but the metabolite can also be formed by other enzymes: CYP2B6, CYP2C9, CY2C19, and CYP3A4.

The CYP2D6 phenotype is defined as the metabolic ratio (MR) by dividing the concentration of an unchanged probe drug, known to be metabolized by the CYP2D6 gene product, by the concentration of the relevant metabolite at a specific time. These measurements have resulted in the division of the CYP2D6 phenotype in four metabolic classes: poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM), and ultrarapid metabolizers (UM). Over 80 different single-nucleotide polymorphisms have been identified but there are inconsistencies in the precise definitions of ascribing a genotype to a phenotype [95, 96]. Bradford [96] and Raimundo and coworkers [97] have described the frequency of common alleles for CYP2D6. Pertinent to the current discussion of tamoxifen metabolism, the CYP2D6*4 allele [98] is estimated to have a frequency of12–23 % in Caucasians, 1.2–7 % in black Africans, and 0–2.8 % in Asians [95, 96]. A lower estimate of (<10 %) of the PM phenotype is presented by Bernard and coworkers [99].

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The molecular pharmacology of endoxifen has been reported [7, 100, 101]. Endoxifen and 4-hydroxytamoxifen were equally potent at inhibiting estrogen-stimulated growth of ER-positive breast cancer cells MCF-7, T47D, and BT474. Both metabolites are significantly superior in vitro to tamoxifen the parent drug. Additionally, the estrogen-responsive genes pS2 and progesterone receptor were both blocked to an equivalent degree by endoxifen and 4-hydroxytamoxifen [100, 101]. Lim and coworkers [101] have extended the comparison of endoxifen and 4-hydroxytamoxifen in MCF-7 cells by comparing and contrasting global gene regulation using the Affymetrix U133A Gene Chip Array. There were 4,062 total genes that were either up- or downregulated by estradiol, whereas, in the presence of estradiol, 4-hydroxytamoxifen or endoxifen affected 2,444 and 2,390 genes, respectively. Overall, the authors [101] demonstrated good correlation between RT-PCR and select genes from the microarray and concluded that the global effects of endoxifen and 4-hydroxytamoxifen were similar.

Stearns and coworkers [102]and Jin and coworkers [103] have confirmed and significantly extended Lien's original identification of endoxifen and observation [5, 6] that there are usually higher circulating levels of endoxifen than 4-hydroxytamoxifen in patients receiving adjuvant tamoxifen therapy. However, Flockhart's group [102] has advanced the pharmacogenomics and drug interactions surrounding tamoxifen therapy that should be a consideration in the antihormonal treatment of breast cancer.

The ubiquitous use of tamoxifen for the treatment of node-negative women [104] during the 1990s, the use of tamoxifen plus radiotherapy following lumpectomy for the treatment of ductal carcinoma in situ (DCIS) [105], as well as the option to use tamoxifen for chemoprevention in high-risk pre- and postmenopausal women [106] enhanced awareness of the menopausal side effects experienced by women when taking tamoxifen. Up to 45 % of women with hot flashes grade them as severe [106]; therefore, there have been efforts to improve quality of life. Treatments with the SSRIs are popular [102, 107, 108] (Fig. 3.6). The SSRIs are twice as effective as the "placebo" effect at reducing menopausal symptoms in randomized clinical trials [107–109], so there is naturally an increased usage of SSRIs with long-term tamoxifen treatment to maintain compliance. Unfortunately, the metabolism of tamoxifen to hydroxylated metabolites [94, 110, 111] and the metabolism of SSRIs [9, 112–115] both occur via the CYP2D6 gene product. Indeed Stearns and coworkers [102] showed that the paroxetine reduced the levels of endoxifen during adjuvant tamoxifen therapy and endoxifen levels decrease by 64 % in women with wild-type CYP2D6 enzyme. Patients were examined who were taking venlafaxine, sertraline, and paroxetine and compared with those women who were homozygotes for the CYP2D6*4/*4 inactive genotype. Patients with the wild-type gene who took the most potent inhibitor paroxetine had serum levels of endoxifen equivalent to the patients with the aberrant CYP2D6 gene. In fact, the clinical data were consistent with the inhibition constants for the inhibition of CYP2D6 by paroxetine (potent), fluoxetine, sertraline, citalopram (intermediate), and venlafaxine (weak) which are 0.05, 0.17, 1.5, 7, and 33 μmol/l, respectively.

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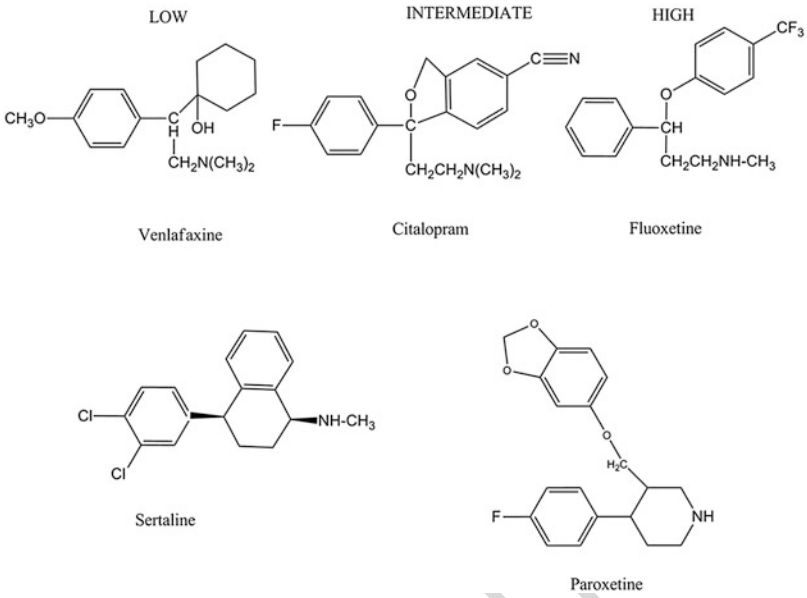

Fig. 3.6 The structures of selective serotonin reuptake inhibitors (SSRIs) that have low intermediate to high affinity for the CYP2D6 enzyme system. High affinity binders for CYP2D6 block the metabolic activation of tamoxifen to endoxifen (Fig. 3.1)

The CYP2D6 gene product that is fully functional (wild type) is classified as the CYP2D6*1. A large number of alleles are associated with no enzyme activity or reduced activity. Conversely, high metabolizers can have multiple copies of the CYP2D6 allele [116]. A recent study by Borges and coworkers [117] continues to expand our understanding of the detrimental effect of CYP2D6 variants plus concomitant administration of SSRIs on endoxifen levels. But, it is the clinical correlations with tumor responses and side effects that are of importance if pharmacogenomics is to be truly relevant in breast cancer therapy.

Clinical Correlations

The metabolic activation of tamoxifen to endoxifen by the CYP2D6 enzyme system still remains controversial to plan the treatment of patients with breast cancer. Since the discovery and description of the pharmacological properties of endoxifen, retrospective clinical trials were examined to determine the pharmacological relevance of endoxifen. The results of clinical trials, however, vary. Clinical investigation by Dieudonne and coworkers [118] have shown that patients with CYP2D6*4/*4 homozygous mutation, which reduces the levels of endoxifen in patients' plasma, are still responding to tamoxifen treatment and tamoxifen still has an effect

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on endometrial tissue and elevated the plasma levels of FSH and SHBG in those patients to the levels found in general tamoxifen-treated population. Study by Schroth and coworkers [119] have shown that there was an association of CYP2D6 genotype and clinical outcome for breast cancer patients, in particular the presence of two wild-type alleles correlated with better clinical outcomes and presence of mutant alleles with worse outcomes. Study by Kiyotani and coworkers [120] showed also that there is a significant correlation between the presence of risk alleles of CYP2D6, which are associated with lower plasma levels of endoxifen, and significantly lower recurrence-free survival in breast cancer patients that were taking tamoxifen as monotherapy. Study by Lammers and coworkers [121] also has demonstrated correlation between the overall survival of breast cancer patients that were taking tamoxifen 40 mg daily with poor metabolizer genotype, compared to patients with extensive metabolizer genotype. A study by Madlensky and coworkers [122] has shown that there is no association between breast cancer outcomes and the concentrations of 4-hydroxytamoxifen or endoxifen; however, they have demonstrated a threshold of endoxifen concentration, below which there is an increase in breast cancer recurrence rate and that about 80 % of patients are above that threshold. Interestingly, their threshold concentration of endoxifen is equivalent to concentrations found in patients with poor metabolizer genotype. In the study by Lash and coworkers [123], it was shown that there is virtually no correlation between recurrence rates of breast cancer in patients and the presence of one or two functional alleles. However, in 2012 results of studies from Arimidex, Tamoxifen, Alone or in Combination (ATAC) and Breast International Group (BIG) I-98 trails were published [124, 125]. The results concurrently showed no association between the recurrence rates and the genotypes of the postmenopausal patients taking tamoxifen alone or in combination with aromatase inhibitor. The results of the trials have sparked a controversy [126]. One thing that is certain is that endoxifen plasma levels do vary in patients taking tamoxifen depending on their metabolic genotype [127, 128]. It should be noted that in some of the trials the patients were postmenopausal or had previous chemotherapy. In 2012, we simulated the estrogen environment of postmenopausal women in vitro and test the antiestrogenic properties of tamoxifen and its metabolites in physiological concentrations on a panel of ER-positive human breast cancer cell lines (MCF-7, T47D, ZR-75-1, and BT474). The concentrations of estrogens (E1/E2) used to simulate postmenopausal women treated with tamoxifen were obtained from published studies [129, 130], as well as the concentrations of tamoxifen and its metabolites (N-desmethyltamoxifen, 4-hydroxytamoxifen, and endoxifen) based on the CYP2D6 genotype in postmenopausal breast cancer patients [128]. Our results demonstrate that irrespective of CYP2D6 genotype (extensive, intermediate, or poor metabolizers (EM, IM, and PM, respectively)), tamoxifen and its primary metabolites (N-desmethyltamoxifen and 4-hydroxytamoxifen) are able to inhibit completely the estrogen-stimulated growth of breast cancer cells in vitro. Additional endoxifen in any concentration corresponding to CYP2D6 genotype was not able to increase the antiestrogenic effect of tamoxifen and its primary metabolites. Moreover, we demonstrate that 4-hydroxytamoxifen is absolutely essential for

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inhibition of estrogen action. Based on our results, we can conclude that endoxifen is pharmacologically supportive but not essential for any genotype of CYP2D6 in a postmenopausal setting.

However, little is known on the role on the antiestrogenic impact of endoxifen in premenopausal women that are treated with tamoxifen. We have simulated premenopausal estrogen environment in vitro and used the same concentrations of tamoxifen and its metabolites found in different CYP2D6 genotypes. Our results show that tamoxifen and its primary metabolites are able to inhibit partially the estrogenic effect in the same panel of ER-positive human breast cancer cell lines; however, the addition of endoxifen, unlike in postmenopausal simulation, further inhibits the estrogens. Interestingly, the higher concentrations of endoxifen associated with EM and IM genotypes are inhibiting estrogens better, than at lower concentrations as found patients with PM genotype. It should be noted that addition of endoxifen at PM concentrations does not increase the antiestrogenic properties of tamoxifen and its primary metabolites in vitro. It was shown that the increase of tamoxifen dose in breast cancer patients increases the plasma levels of endoxifen [131–133]. In particular, it was shown by Irvin and coworkers [131] that the increase of tamoxifen dose to 40 mg daily administered by patients with IM and PM genotype increased the plasma levels of endoxifen. Using these levels of increased tamoxifen and its metabolites, we have simulated the average premenopausal estrogen setting in vitro and assessed the pharmacological impact of increased concentrations of endoxifen in IM and PM setting. Our results show that biologically there is no significant difference after treatments with tamoxifen primary metabolites and endoxifen at concentrations corresponding to 20 and 40 mg/daily. Interestingly, none of the tamoxifen treatments were able to fully inhibit the estrogen action in MCF-7 and T47D cells in the premenopausal setting; increasing the concentrations of endoxifen to levels higher than physiological was able to fully inhibit estrogen action. We conclude that endoxifen thus contributes to inhibition of estrogen action and growth of ER-positive breast cancer cells; however, endoxifen plays a supportive in a situation following chemotherapy in premenopausal patients.

Postscript. On my return from the United States to Leeds University in 1974, I was supported by the ICI Pharmaceutical Division Clinical Department and the Yorkshire Cancer Campaign. In 1975, I initially wished to study the hydroxylated metabolites of tamoxifen for two reasons: (1) would the metabolites be estrogens if low affinity was important for antiestrogenic activity or (2) would potent antiestrogen effects of the metabolites explain the potent antiestrogenic properties of tamoxifen in rats but the really weak antiestrogenic activity to block ER in vitro. Dora Richardson gave me their limited supply of the precious metabolites monohydroxytamoxifen (metabolite B) and dihydroxytamoxifen (metabolite D).

My students at Leeds University Clive Dix and Margaret Collins took the leading roles in discovering the pharmacological properties of 4-hydroxytamoxifen (the correct name of metabolite B). I recall telling Clive Dix to redo all ligand-binding experiments of 4-hydroxytamoxfien competition inhibiting the binding of

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[^{3}H]estradiol to rat uterine cytosolic ER. "Look Clive, there are no reports of a nonsteroidal antiestrogens binding to the ER with the same affinity as estradiol-learn to do your serial dilutions properly!" He was correct and it was an important discovery. When we discovered the potent antiestrogenic properties of 4-hydroxytamoxifen, I was informed by Sandy Todd at ICI Pharmaceutical Division that there were no patents for the metabolites. The scientists at ICI had clearly never believed the clinical development process would take off, as it did in the early 1970s with animal data to support clinical trials. A rule at ICI that all drug metabolites for a drug in active development and marketing had to be patented had been broken (remember the program was terminated in 1972). As a result, in 1976, I agreed to write up our paper, lodge it with ICI staff at ICI Pharmaceutical Division, and delay publishing until a patent was obtained for 4-hydroxytamoxifen. This occurred 1 year later. I also voluntarily agreed to not talk about our work in 1976 as it was important to get tamoxifen FDA approved in the United States. In 1976, I set off to Key Biscayne to the NSABP meeting to tell them all about tamoxifen [134].

What happened to 4-hydroxytamoxifen? It became the antiestrogen of choice for all laboratory studies in vitro for the next 30 years, but we also showed it was not the product to be developed instead of tamoxifen [135]. If tamoxifen was a prodrug, then 4-hydroxytamoxifen could be the active agent. The patent for 4-hydroxytamoxifen was sold to Besins International and a French physician Dr. Maurvais Jarvais, who advanced the proposal that breast cancer and breast pain could be resolved with a daily preparation rubbed on the breast [136]. Clinical trials have addressed this issue over the past 30 years.

I was subsequently awarded a Leeds University/ICI Pharmaceutical Division Joint Research scheme to evaluate the therapeutic potential of 6,7 alpha-substituted estradiol alkylated derivatives. We had discovered that substitution of the 6 and 7 positions of estradiol still retained significant binding affinity of the ligand for the receptor. The idea was to use the estradiol as the carrier molecule for an alkylating moiety to be delivered to the DNA precisely and kill ER-positive breast cancers. Alternatively, we could radiolabel the estradiol and subsequently discover the sites for estrogen-regulated genes. Neither of these ideas were successful. We published our findings [137] but did not follow up the 7-substituted estradiol with a $(CH_2)_{10}$ side chain. The further development of the idea was to result in the pure antiestrogen fulvestrant [138], but this was entirely the discovery of the Pharmaceutical Industry, with Alan Wakeling and his team.

We will find out what happened to the idea of estradiol with a long side chain at position 7 in Chap. 5.

References

1. Jordan VC, Collins MM, Rowsby L, Prestwich G (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305–316
2. Allen KE, Clark ER, Jordan VC (1980) Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 71:83–91

Uncorrected Proof

Author's Proof

3. Borgna JL, Rochefort H (1981) Hydroxylated metabolites of tamoxifen are formed in vivo and bound to estrogen receptor in target tissues. J Biol Chem 256:859–868
4. Jordan VC, Dix CJ, Naylor KE et al (1978) Nonsteroidal antiestrogens: their biological effects and potential mechanisms of action. J Toxicol Environ Health 4:363–390
5. Lien EA, Solheim E, Kvinnsland S, Ueland PM (1988) Identification of 4-hydroxy-N-desmethyltamoxifen as a metabolite of tamoxifen in human bile. Cancer Res 48:2304–2308
6. Lien EA, Solheim E, Lea OA et al (1989) Distribution of 4-hydroxy-N-desmethyltamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. Cancer Res 49:2175–2183
7. Johnson MD, Zuo H, Lee KH et al (2004) Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. Breast Cancer Res Treat 85:151–159
8. Desta Z, Ward BA, Soukhova NV, Flockhart DA (2004) Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: prominent roles for CYP3A and CYP2D6. J Pharmacol Exp Ther 310:1062–1075
9. Crewe HK, Lennard MS, Tucker GT et al (1992) The effect of selective serotonin re-uptake inhibitors on cytochrome P4502D6 (CYP2D6) activity in human liver microsomes. Br J Clin Pharmacol 34:262–265
10. Jordan VC, Brodie AM (2007) Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 72:7–25
11. Jordan VC (2003) Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2:205–213
12. Fromson JM, Pearson S, Bramah S (1973) The metabolism of tamoxifen (I.C.I. 46,474). I. In laboratory animals. Xenobiotica 3:693–709
13. Fromson JM, Pearson S, Bramah S (1973) The metabolism of tamoxifen (I.C.I. 46,474). II. In female patients. Xenobiotica 3:711–714
14. Lieberman ME, Jordan VC, Fritsch M et al (1983) Direct and reversible inhibition of estradiol-stimulated prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4734–4740
15. Adam HK, Gay MA, Moore RH (1980) Measurement of tamoxifen in serum by thin-layer densitometry. J Endocrinol 84:35–42
16. Adam HK, Douglas EJ, Kemp JV (1979) The metabolism of tamoxifen in humans. Biochem Pharmacol 27:145–147
17. Kemp JV, Adam HK, Wakeling AE, Slater R (1983) Identification and biological activity of tamoxifen metabolites in human serum. Biochem Pharmacol 32:2045–2052
18. Bain RR, Jordan VC (1983) Identification of a new metabolite of tamoxifen in patient serum during breast cancer therapy. Biochem Pharmacol 32:373–375
19. Jordan VC, Bain RR, Brown RR et al (1983) Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. Cancer Res 43:1446–1450
20. Robinson SP, Langan-Fahey SM, Jordan VC (1989) Implications of tamoxifen metabolism in the athymic mouse for the study of antitumor effects upon human breast cancer xenografts. Eur J Cancer Clin Oncol 25:1769–1776
21. Jordan VC, Gosden B (1982) Importance of the alkylaminoethoxy side-chain for the estrogenic and antiestrogenic actions of tamoxifen and trioxifene in the immature rat uterus. Mol Cell Endocrinol 27:291–306
22. Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741–4745
23. Katzenellenbogen JA, Carlson KE, Katzenellenbogen BS (1985) Facile geometric isomerization of phenolic non-steroidal estrogens and antiestrogens: limitations to the interpretation of experiments characterizing the activity of individual isomers. J Steroid Biochem 22:589–596
24. Katzenellenbogen BS, Norman MJ, Eckert RL et al (1984) Bioactivities, estrogen receptor interactions, and plasminogen activator-inducing activities of tamoxifen and hydroxytamoxifen isomers in MCF-7 human breast cancer cells. Cancer Res 44:112–119

Uncorrected Proof

Author's Proof

25. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS (1986) Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A 83:2496–2500
26. Murphy CS, Meisner LF, Wu SQ, Jordan VC (1989) Short- and long-term estrogen deprivation of T47D human breast cancer cells in culture. Eur J Cancer Clin Oncol 25:1777–1788
27. Jordan VC, Lieberman ME, Cormier E et al (1984) Structural requirements for the pharmacological activity of nonsteroidal antiestrogens in vitro. Mol Pharmacol 26:272–278
28. Jordan VC, Koch R, Mittal S, Schneider MR (1986) Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. Br J Pharmacol 87:217–223
29. Murphy CS, Langan-Fahey SM, McCague R, Jordan VC (1990) Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. Mol Pharmacol 38:737–743
30. Murphy CS, Parker CJ, McCague R, Jordan VC (1991) Structure-activity relationships of nonisomerizable derivatives of tamoxifen: importance of hydroxyl group and side chain positioning for biological activity. Mol Pharmacol 39:421–428
31. Shiau AK, Barstad D, Loria PM et al (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937
32. Brzozowski AM, Pike AC, Dauter Z et al (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
33. Wolf DM, Jordan VC (1994) The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31:129–138
34. Wolf DM, Jordan VC (1994) Characterization of tamoxifen stimulated MCF-7 tumor variants grown in athymic mice. Breast Cancer Res Treat 31:117–127
35. MacGregor Schafer J, Liu H, Bentrem DJ et al (2000) Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. Cancer Res 60:5097–5105
36. Levenson AS, Jordan VC (1998) The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. Cancer Res 58:1872–1875
37. Liu H, Lee ES, Deb Los Reyes A et al (2001) Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor alpha complex. Cancer Res 61:3632–3639
38. Levenson AS, Catherino WH, Jordan VC (1997) Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. J Steroid Biochem Mol Biol 60:261–268
39. Liu H, Park WC, Bentrem DJ et al (2002) Structure-function relationships of the raloxifene-estrogen receptor-alpha complex for regulating transforming growth factor-alpha expression in breast cancer cells. J Biol Chem 277:9189–9198
40. Onate SA, Tsai SY, Tsai MJ, O'Malley BW (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270:1354–1357
41. Jordan VC, O'Malley BW (2007) Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 25:5815–5824
42. Jordan VC, Gapstur S, Morrow M (2001) Selective estrogen receptor modulation and reduction in risk of breast cancer, osteoporosis, and coronary heart disease. J Natl Cancer Inst 93:1449–1457
43. Greaves P, Goonetilleke R, Nunn G et al (1993) Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. Cancer Res 53:3919–3924
44. Hard GC, Iatropoulos MJ, Jordan K et al (1993) Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. Cancer Res 53:4534–4541
45. Fornander T, Rutqvist LE, Cedermark B et al (1989) Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1:117–120

Uncorrected Proof

Author's Proof

46. Fisher B, Costantino JP, Redmond CK et al (1994) Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. J Natl Cancer Inst 86:527–537
47. Han XL, Liehr JG (1992) Induction of covalent DNA adducts in rodents by tamoxifen. Cancer Res 52:1360–1363
48. Rutqvist LE, Johansson H, Signomklao T et al (1995) Adjuvant tamoxifen therapy for early stage breast cancer and second primary malignancies. Stockholm Breast Cancer Study Group. J Natl Cancer Inst 87:645–651
49. Styles JA, Davies A, Lim CK et al (1994) Genotoxicity of tamoxifen, tamoxifen epoxide and toremifene in human lymphoblastoid cells containing human cytochrome P450s. Carcinogenesis 15:5–9
50. Lim CK, Yuan ZX, Lamb JH et al (1994) A comparative study of tamoxifen metabolism in female rat, mouse and human liver microsomes. Carcinogenesis 15:589–593
51. Moorthy B, Sriram P, Pathak DN et al (1996) Tamoxifen metabolic activation: comparison of DNA adducts formed by microsomal and chemical activation of tamoxifen and 4-hydroxytamoxifen with DNA adducts formed in vivo. Cancer Res 56:53–57
52. Pongracz K, Pathak DN, Nakamura T et al (1995) Activation of the tamoxifen derivative metabolite E to form DNA adducts: comparison with the adducts formed by microsomal activation of tamoxifen. Cancer Res 55:3012–3015
53. Potter GA, McCague R, Jarman M (1994) A mechanistic hypothesis for DNA adduct formation by tamoxifen following hepatic oxidative metabolism. Carcinogenesis 15:439–442
54. Phillips DH, Carmichael PL, Hewer A et al (1994) alpha-Hydroxytamoxifen, a metabolite of tamoxifen with exceptionally high DNA-binding activity in rat hepatocytes. Cancer Res 54:5518–5522
55. Phillips DH, Potter GA, Horton MN et al (1994) Reduced genotoxicity of [D5-ethyl]-tamoxifen implicates alpha-hydroxylation of the ethyl group as a major pathway of tamoxifen activation to a liver carcinogen. Carcinogenesis 15:1487–1492
56. Osborne MR, Hewer A, Hardcastle IR et al (1996) Identification of the major tamoxifen-deoxyguanosine adduct formed in the liver DNA of rats treated with tamoxifen. Cancer Res 56:66–71
57. Phillips DH, Carmichael PL, Hewer A et al (1996) Activation of tamoxifen and its metabolite alpha-hydroxytamoxifen to DNA-binding products: comparisons between human, rat and mouse hepatocytes. Carcinogenesis 17:89–94
58. Osborne MR, Hewer A, Phillips DH (2001) Resolution of alpha-hydroxytamoxifen; R-isomer forms more DNA adducts in rat liver cells. Chem Res Toxicol 14:888–893
59. Osborne MR, Hewer A, Phillips DH (2004) Stereoselective metabolic activation of alpha-hydroxy-N-desmethyltamoxifen: the R-isomer forms more DNA adducts in rat liver cells. Chem Res Toxicol 17:697–701
60. Phillips DH (2001) Understanding the genotoxicity of tamoxifen? Carcinogenesis 22:839–849
61. Jordan VC (1995) What if tamoxifen (ICI 46,474) had been found to produce rat liver tumors in 1973? A personal perspective. Ann Oncol 6:29–34
62. Jordan VC, Gradishar WJ (1997) Molecular mechanisms and future uses of antiestrogens. Mol Aspects Med 18:167–247
63. Jordan VC (1984) Biochemical pharmacology of antiestrogen action. Pharmacol Rev 36:245–276
64. Jordan VC (1988) Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 6:589–595
65. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189
66. Jordan VC (2001) Selective estrogen receptor modulation: a personal perspective. Cancer Res 61:5683–5687

Uncorrected Proof

Author's Proof

67. Jordan VC (2003) Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. J Med Chem 46:1081–1111
68. Ariazi EA, Ariazi JL, Cordera F, Jordan VC et al (2006) Estrogen receptors as therapeutic targets in breast cancer. Curr Top Med Chem 6:195–216
69. Buzdar AU, Marcus C, Holmes F et al (1988) Phase II evaluation of Ly156758 in metastatic breast cancer. Oncology 45:344–345
70. Snyder KR, Sparano N, Malinowski JM (2000) Raloxifene hydrochloride. Am J Health Syst Pharm 57:1669–1675, quiz 76–8
71. Kemp DC, Fan PW, Stevens JC (2002) Characterization of raloxifene glucuronidation in vitro: contribution of intestinal metabolism to presystemic clearance. Drug Metab Dispos 30:694–700
72. Jeong EJ, Liu Y, Lin H, Hu M (2005) Species- and disposition model-dependent metabolism of raloxifene in gut and liver: role of UGT1A10. Drug Metab Dispos 33:785–794
73. Suh N, Lamph WW, Glasebrook AL et al (2002) Prevention and treatment of experimental breast cancer with the combination of a new selective estrogen receptor modulator, arzoxifene, and a new rexinoid, LG 100268. Clin Cancer Res 8:3270–3275
74. Baselga J, Llombart-Cussac A, Bellet M et al (2003) Randomized, double-blind, multicenter trial comparing two doses of arzoxifene (LY353381) in hormone-sensitive advanced or metastatic breast cancer patients. Ann Oncol 14:1383–1390
75. Buzdar A, O'Shaughnessy JA, Booser DJ et al (2003) Phase II, randomized, double-blind study of two dose levels of arzoxifene in patients with locally advanced or metastatic breast cancer. J Clin Oncol 21:1007–1014
76. Bolognese M, Krege JH, Utian WH et al (2009) Effects of arzoxifene on bone mineral density and endometrium in postmenopausal women with normal or low bone mass. J Clin Endocrinol Metab 94:2284–2289
77. Downs RW Jr, Moffett AM, Ghosh A et al (2010) Effects of arzoxifene on bone, lipid markers, and safety parameters in postmenopausal women with low bone mass. Osteoporos Int 21:1215–1226
78. Kendler DL, Palacios S, Cox DA et al (2011) Arzoxifene versus raloxifene: effect on bone and safety parameters in postmenopausal women with osteoporosis. Osteoporos Int AU4
79. Falany JL, Pilloff DE, Leyh TS, Falany CN (2006) Sulfation of raloxifene and 4-hydroxytamoxifen by human cytosolic sulfotransferases. Drug Metab Dispos 34:361–368
80. Rosati RL, Da Silva JP, Cameron KO et al (1998) Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryltetrahydronaphthalene. J Med Chem 41:2928–2931
81. Ke HZ, Paralkar VM, Grasser WA et al (1998) Effects of CP-336,156, a new, nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. Endocrinology 139:2068–2076
82. Ke HZ, Qi H, Crawford DT et al (2000) Lasofoxifene (CP-336,156), a selective estrogen receptor modulator, prevents bone loss induced by aging and orchidectomy in the adult rat. Endocrinology 141:1338–1344
83. Tatee T, Carlson KE, Katzenellenbogen JA et al (1979) Antiestrogens and antiestrogen metabolites: preparation of tritium-labeled (+/−)-cis-3-[p-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phenoxyl]-1,2-propanediol (U-23469) and characterization and synthesis of a biologically important metabolite. J Med Chem 22:1509–1517
84. Legha SS, Slavik M, Carter SK (1976) Nafoxidine–an antiestrogen for the treatment of breast cancer. Cancer 38:1535–1541
85. Cohen LA, Pittman B, Wang CX et al (2001) LAS, a novel selective estrogen receptor modulator with chemopreventive and therapeutic activity in the N-nitroso-N-methylurea-induced rat mammary tumor model. Cancer Res 61:8683–8688
86. Robertson DW, Katzenellenbogen JA, Hayes JR, Katzenellenbogen BS (1982) Antiestrogen basicity–activity relationships: a comparison of the estrogen receptor binding and

Uncorrected Proof

Author's Proof

antiuterotrophic potencies of several analogues of (Z)-1,2-diphenyl-1-[4-[2-(dimethylamino) ethoxy]phenyl]-1-butene (tamoxifen, Nolvadex) having altered basicity. J Med Chem 25:167–171

87. Hellmann-Blumberg U, Taras TL, Wurz GT, DeGregorio MW et al (2000) Genotoxic effects of the novel mixed antiestrogen FC-1271a in comparison to tamoxifen and toremifene. Breast Cancer Res Treat 60:63–70
88. Qu Q, Zheng H, Dahllund J et al (2000) Selective estrogenic effects of a novel triphenylethylene compound, FC1271a, on bone, cholesterol level, and reproductive tissues in intact and ovariectomized rats. Endocrinology 141:809–820
89. DeGregorio MW, Wurz GT, Taras TL et al (2000) Pharmacokinetics of (deaminohydroxy) toremifene in humans: a new, selective estrogen-receptor modulator. Eur J Clin Pharmacol 56:469–475
90. Rutanen EM, Heikkinen J, Halonen K et al (2003) Effects of ospemifene, a novel SERM, on hormones, genital tract, climacteric symptoms, and quality of life in postmenopausal women: a double-blind, randomized trial. Menopause 10:433–439
91. Komi J, Lankinen KS, Harkonen P et al (2005) Effects of ospemifene and raloxifene on hormonal status, lipids, genital tract, and tolerability in postmenopausal women. Menopause 12:202–209
92. Namba R, Young LJ, Maglione JE et al (2005) Selective estrogen receptor modulators inhibit growth and progression of premalignant lesions in a mouse model of ductal carcinoma in situ. Breast Cancer Res 7:R881–R889
93. Wurz GT, Read KC, Marchisano-Karpman C et al (2005) Ospemifene inhibits the growth of dimethylbenzanthracene-induced mammary tumors in Sencar mice. J Steroid Biochem Mol Biol 97:230–240
94. Dehal SS, Kupfer D (1997) CYP2D6 catalyzes tamoxifen 4-hydroxylation in human liver. Cancer Res 57:3402–3406
95. Beverage JN, Sissung TM, Sion AM et al (2007) CYP2D6 polymorphisms and the impact on tamoxifen therapy. J Pharm Sci 96:2224–2231
96. Bradford LD (2002) CYP2D6 allele frequency in European Caucasians, Asians, Africans and their descendants. Pharmacogenomics 3:229–243
97. Raimundo S, Toscano C, Klein K et al (2004) A novel intronic mutation, 2988G>A, with high predictivity for impaired function of cytochrome P450 2D6 in white subjects. Clin Pharmacol Ther 76:128–138
98. Hanioka N, Kimura S, Meyer UA, Gonzalez FJ (1990) The human CYP2D locus associated with a common genetic defect in drug oxidation: a G1934—A base change in intron 3 of a mutant CYP2D6 allele results in an aberrant 3′ splice recognition site. Am J Hum Genet 47:994–1001
99. Bernard S, Neville KA, Nguyen AT, Flockhart DA (2006) Interethnic differences in genetic polymorphisms of CYP2D6 in the U.S. population: clinical implications. Oncologist 11:126–135
100. Lim YC, Desta Z, Flockhart DA, Skaar TC (2005) Endoxifen (4-hydroxy-N-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen. Cancer Chemother Pharmacol 55:471–478
101. Lim YC, Li L, Desta Z et al (2006) Endoxifen, a secondary metabolite of tamoxifen, and 4-OH-tamoxifen induce similar changes in global gene expression patterns in MCF-7 breast cancer cells. J Pharmacol Exp Ther 318:503–512
102. Stearns V, Johnson MD, Rae JM et al (2003) Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. J Natl Cancer Inst 95:1758–1764
103. Jin Y, Desta Z, Stearns V et al (2005) CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. J Natl Cancer Inst 97:30–39
104. Fisher B, Costantino J, Redmond C et al (1989) A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. N Engl J Med 320:479–484

Uncorrected Proof

Author's Proof

105. Fisher B, Dignam J, Wolmark N et al (1999) Tamoxifen in treatment of intraductal breast cancer: National Surgical Adjuvant Breast and Bowel Project B-24 randomised controlled trial. Lancet 353:1993–2000
106. Fisher B, Costantino JP, Wickerham DL et al (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90:1371–1388
107. Loprinzi CL, Kugler JW, Sloan JA et al (2000) Venlafaxine in management of hot flashes in survivors of breast cancer: a randomised controlled trial. Lancet 356:2059–2063
108. Loprinzi CL, Sloan JA, Perez EA et al (2002) Phase III evaluation of fluoxetine for treatment of hot flashes. J Clin Oncol 20:1578–1583
109. Stearns V, Beebe KL, Iyengar M, Dube E (2003) Paroxetine controlled release in the treatment of menopausal hot flashes: a randomized controlled trial. JAMA 289:2827–2834
110. Crewe HK, Ellis SW, Lennard MS, Tucker GT (1997) Variable contribution of cytochromes P450 2D6, 2C9 and 3A4 to the 4-hydroxylation of tamoxifen by human liver microsomes. Biochem Pharmacol 53:171–178
111. Coller JK, Krebsfaenger N, Klein K et al (2002) The influence of CYP2B6, CYP2C9 and CYP2D6 genotypes on the formation of the potent antioestrogen Z-4-hydroxy-tamoxifen in human liver. Br J Clin Pharmacol 54:157–167
112. Lessard E, Yessine MA, Hamelin BA et al (2001) Diphenhydramine alters the disposition of venlafaxine through inhibition of CYP2D6 activity in humans. J Clin Psychopharmacol 21:175–184
113. Albers LJ, Reist C, Vu RL et al (2000) Effect of venlafaxine on imipramine metabolism. Psychiatry Res 96:235–243
114. Yoon YR, Cha IJ, Shon JH et al (2000) Relationship of paroxetine disposition to metoprolol metabolic ratio and CYP2D6*10 genotype of Korean subjects. Clin Pharmacol Ther 67:567–576
115. Jeppesen U, Gram LF, Vistisen K et al (1996) Dose-dependent inhibition of CYP1A2, CYP2C19 and CYP2D6 by citalopram, fluoxetine, fluvoxamine and paroxetine. Eur J Clin Pharmacol 51:73–78
116. Andersson T, Flockhart DA, Goldstein DB et al (2005) Drug-metabolizing enzymes: evidence for clinical utility of pharmacogenomic tests. Clin Pharmacol Ther 78:559–581
117. Borges S, Desta Z, Li L et al (2006) Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. Clin Pharmacol Ther 80:61–74
118. Dieudonne AS, Lambrechts D, Claes B et al (2009) Prevalent breast cancer patients with a homozygous mutant status for CYP2D6*4: response and biomarkers in tamoxifen users. Breast Cancer Res Treat 118:531–538
119. Schroth W, Goetz MP, Hamann U et al (2009) Association between CYP2D6 polymorphisms and outcomes among women with early stage breast cancer treated with tamoxifen. JAMA 302:1429–1436
120. Kiyotani K, Mushiroda T, Imamura CK et al (2010) Significant effect of polymorphisms in CYP2D6 and ABCC2 on clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients. J Clin Oncol 28:1287–1293
121. Lammers LA, Mathijssen RH, van Gelder T et al (2010) The impact of CYP2D6-predicted phenotype on tamoxifen treatment outcome in patients with metastatic breast cancer. Br J Cancer 103:765–771
122. Madlensky L, Natarajan L, Tchu S et al (2011) Tamoxifen metabolite concentrations, CYP2D6 genotype, and breast cancer outcomes. Clin Pharmacol Ther 89:718–725
123. Lash TL, Cronin-Fenton D, Ahern TP et al (2011) CYP2D6 inhibition and breast cancer recurrence in a population-based study in Denmark. J Natl Cancer Inst 103:489–500
124. Regan MM, Leyland-Jones B, Bouzyk M et al (2012) CYP2D6 genotype and tamoxifen response in postmenopausal women with endocrine-responsive breast cancer: the breast international group 1–98 trial. J Natl Cancer Inst 104:441–451

Uncorrected Proof

Author's Proof

125. Rae JM, Drury S, Hayes DF et al (2012) CYP2D6 and UGT2B7 genotype and risk of recurrence in tamoxifen-treated breast cancer patients. J Natl Cancer Inst 104:452–460
126. Schroth W, Antoniadou L, Fritz P et al (2007) Breast cancer treatment outcome with adjuvant tamoxifen relative to patient CYP2D6 and CYP2C19 genotypes. J Clin Oncol 25:5187–5193
127. Lim JS, Chen XA, Singh O et al (2011) Impact of CYP2D6, CYP3A5, CYP2C9 and CYP2C19 polymorphisms on tamoxifen pharmacokinetics in Asian breast cancer patients. Br J Clin Pharmacol 71:737–750
128. Murdter TE, Schroth W, Bacchus-Gerybadze L et al (2011) Activity levels of tamoxifen metabolites at the estrogen receptor and the impact of genetic polymorphisms of phase I and II enzymes on their concentration levels in plasma. Clin Pharmacol Ther 89:708–717
129. Jordan VC, Fritz NF, Tormey DC (1987) Endocrine effects of adjuvant chemotherapy and long-term tamoxifen administration on node-positive patients with breast cancer. Cancer Res 47:624–630
130. Ravdin PM, Fritz NF, Tormey DC, Jordan VC (1988) Endocrine status of premenopausal node-positive breast cancer patients following adjuvant chemotherapy and long-term tamoxifen. Cancer Res 48:1026–1029
131. Irvin WJ Jr, Walko CM, Weck KE et al (2011) Genotype-guided tamoxifen dosing increases active metabolite exposure in women with reduced CYP2D6 metabolism: a multicenter study. J Clin Oncol 29:3232–3239
132. Barginear MF, Jaremko M, Peter I et al (2011) Increasing tamoxifen dose in breast cancer patients based on CYP2D6 genotypes and endoxifen levels: effect on active metabolite isomers and the antiestrogenic activity score. Clin Pharmacol Ther 90:605–611
133. Kiyotani K, Mushiroda T, Imamura CK et al (2012) Dose-adjustment study of tamoxifen based on CYP2D6 genotypes in Japanese breast cancer patients. Breast Cancer Res Treat 131:137–145
134. Jordan VC (1976) Antiestrogenic and antitumor properties of tamoxifen in laboratory animals. Cancer Treat Rep 60:1409–1419
135. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
136. Rouanet P, Linares-Cruz G, Dravet F et al (2005) Neoadjuvant percutaneous 4-hydroxytamoxifen decreases breast tumoral cell proliferation: a prospective controlled randomized study comparing three doses of 4-hydroxytamoxifen gel to oral tamoxifen. J Clin Oncol 23:2980–2987
137. Jordan VC, Fenuik L, Allen KE et al (1981) Structural derivatives of tamoxifen and oestradiol 3-methyl ether as potential alkylating antioestrogens. Eur J Cancer 17:193–200
138. Wakeling AE, Dukes M, Bowler J (1991) A potent specific pure antiestrogen with clinical potential. Cancer Res 51:3867–3873

Uncorrected Proof

Author's Proof

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Chapter No.: 3

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Chapter 4
Adjuvant Therapy: The Breakthrough

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Introduction

The initial success of adjuvant monotherapy with L-phenylalanine mustard [1] or combination chemotherapy [2] to delay the recurrence of node-positive breast cancer encouraged the investigation of other, perhaps less toxic, therapies. Most of the beneficial effects of adjuvant chemotherapy were noted in premenopausal women. In retrospect, this result was almost certainly a "chemical oophorectomy" produced by the cancer treatment. During the 1970s and 1980s, numerous reports [3, 4] described the changes in women's endocrinology as ovarian function is destroyed. Indeed, in the premenopausal women with breast cancer, combination cytotoxic chemotherapy can be considered to be endocrine therapy [5]. The low reported incidence of side effects noted with tamoxifen [6, 7] with modest efficacy naturally caused clinicians to consider adjuvant antiestrogen therapy. But the question to be addressed was "How long is long enough for adjuvant tamoxifen therapy?"

During the 1970s, at a time that tamoxifen was available in the United Kingdom for the treatment of metastatic breast cancer in postmenopausal women, and only being evaluated for that indication in the United States until approval by the FDA in

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December 1977. The laboratory studies in the 1970s would encourage the testing of long-term adjuvant treatment, but the change in conservative clinical philosophy about using a "palliative" treatment of low efficacy would take a decade [8].

Laboratory studies using the DMBA-induced rat mammary carcinoma model were first used to explore whether tamoxifen would be an effective adjuvant therapy and whether the drug produces a tumoristatic or tumoricidal effect in vivo. Studies with estrogen receptor (ER) in positive MCF-7 breast cancer cells in vitro had previously indicated that tamoxifen could be a tumoricidal drug [9], but the results from the DMBA studies in vivo (first reported at a breast cancer symposium at King's College, Cambridge, England, in September 1977) (Fig. 4.1) demonstrated that a short course of tamoxifen therapy (1 month) given 1 month after the carcinogenic insult only delayed the appearance of mammary tumors; continuous therapy (for 6 months) resulted in 90 % of the animals remaining tumor free (Fig. 4.2) [12, 13]. Indeed if tamoxifen therapy is stopped, tumors appear [14]. Thus, tamoxifen was shown to have a tumoristatic component to its mode of action, and the laboratory results indicated that long-term (up to 5 years) or indefinite therapy might be the best clinical strategy for adjuvant treatment. Subsequent laboratory studies using DMBA- or N-nitrosomethylurea (NMU)-induced rat mammary tumors [15–17] or human breast cancer cell lines inoculated into athymic mice [18–20] have all supported the initial observation. However, most attention has naturally focused on the clinical evaluation of adjuvant tamoxifen therapy.

Adjuvant Therapy with Tamoxifen

Several trials of tamoxifen monotherapy as an adjuvant to mastectomy were initiated toward the end of the 1970s. The majority of clinical trial organizations selected a conservative course of 1 year of adjuvant tamoxifen [21–27]. This decision was, however, based on a number of reasonable concerns. Patients with advanced disease usually respond to tamoxifen for 1 year, and it was expected that ER-negative disease would be encouraged to grow prematurely during adjuvant therapy. If this growth was to occur, then the physician would have already used a valuable palliative drug and would have only combination chemotherapy to slow the relentless growth of recurrent disease. A related argument involved the changing strategy for the application of adjuvant combination chemotherapy. Recurrent treatment cycles (2 years) of cytotoxic chemotherapy were found to be of no long-term benefit for the patient. An aggressive course of short-term treatment (6 months) with the most active cytotoxic drugs could have the best chance to kill tumor cells before the premature development of drug resistance. The same argument provided an intuitive reluctance to use long-term tamoxifen therapy because it would lead to premature drug resistance: longer might not be better.

Finally, there were sincere concerns about the side effects of adjuvant therapy and the ethical issues of treating patients who might never have recurrent disease.

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Fig. 4.1 Breast cancer symposium at King's College, Cambridge, England, in September 1977. The concept of extended adjuvant tamoxifen treatment was first proposed at this meeting. Clinical studies of 1-year adjuvant tamoxifen were in place; regrettably, a decade later, this approach was shown to produce little survival benefit for patients. In the insets, (*top*) V. Craig Jordan, who presented the new concept, and (*bottom left*) Dr. Helen Stewart, who was a participant at the conference. She would initiate a pilot trial in 1978 and, led by Sir Patrick Forest, would later guide the full randomized Scottish trial of the 5-year adjuvant tamoxifen treatment versus control in the 1980s. Both clinical trials were later proven to produce survival advantages for patients. The concept of longer tamoxifen treatment producing more survival benefits for patients was eventually established indirectly by the Oxford Overview Analysis in 1992 [10] and directly by the Swedish group led by Dr. Lars Rutqvist [11]

Although this argument primarily focused on chemotherapy and node-negative patients, it is fair to say that few women in the mid-1970s had received extended therapy with tamoxifen, so that long-term side effects were largely unknown. The majority of tamoxifen-treated patients had received only about 2 years of treatment for advanced disease before drug resistance occurred. Potential side effects of thrombosis, osteoporosis, and so on were only of secondary importance. The use of tamoxifen in the disease-free patient would change that perspective.

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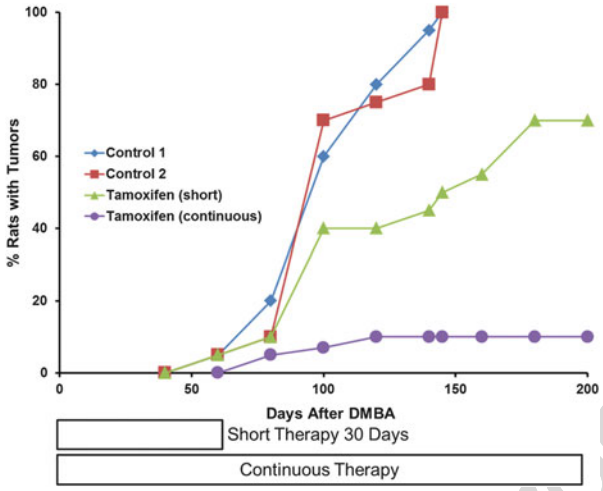

Fig. 4.2 The effectiveness of long-term tamoxifen treatment in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. The administration of 20-mg DMBA by gavage to 50-day-old female Sprague-Dawley rats in all animals developing mammary tumors 160 days later. The short-term (30 days) administration of different daily doses (12.5–800 μg) of tamoxifen between days 30 and 60 after DMBA results in a delay of tumor formation. However, not all animals are protected from the carcinogen. In contrast, the daily administration of a clinically relevant dose (50 μg daily = 0.25 mg/kg in rats or 20 mg daily to a 70 kg woman) of tamoxifen continuously, starting 30 days after DMBA, results in 90 % of animals remaining tumor free

In 1977, Dr. Douglass C. Tormey organized the first evaluation of long-term tamoxifen therapy in node-positive patients treated with combination chemotherapy plus tamoxifen [28, 29]. This pilot study was initiated to determine whether patients could tolerate 5 years of adjuvant tamoxifen therapy and whether metabolic tolerance would occur during long-term tamoxifen therapy. No unusual side effects of tamoxifen therapy were noted, and blood levels of tamoxifen and its metabolites N-desmethyltamoxifen and metabolite Y remained stable throughout the 5 years of treatment. Although this study was not a randomized trial, those patients who received long-term tamoxifen therapy continued to make excellent progress, and many patients took the drug for more than 14 years. We reported [30] that tamoxifen does not produce metabolic tolerance during 10 years of administration. Serum levels of tamoxifen and its metabolites are maintained.

The metabolic stability data and the DMBA-induced rat mammary carcinoma data [31] were used to support randomized Eastern Cooperative Oncology Group (ECOG) trials EST 4181 and 5181. An early analysis of EST 4181, which compares short-term tamoxifen with long-term tamoxifen (both with combination chemotherapy), demonstrated an increase in disease-free survival with long-term tamoxifen therapy [32]. In fact, the 5-year tamoxifen arm went through a second randomization either to stop the tamoxifen or to continue the antiestrogen indefinitely. The National Surgical Adjuvant Breast and Bowel Project (NSABP) clinical trial organization has conducted a registration study of 2 years of combination

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chemotherapy (L-PAM, 5-FU) plus tamoxifen with an additional year of tamoxifen alone [33] to build on the successes of the earlier trials that demonstrated the efficacy of tamoxifen in receptor-positive postmenopausal patients [34–36]. Overall, these investigators conclude that 3 years of tamoxifen confers a significant advantage for patients over 2 years of tamoxifen.

Although the 2-year adjuvant tamoxifen study that was conducted by the Nolvadex Adjuvant Trial Organization (NATO) was the first to demonstrate a survival advantage for women [37], subsequent clinical trials all evaluated a longer duration of tamoxifen therapy. A small, randomized clinical trial of 3 years of tamoxifen versus no treatment demonstrated a survival advantage for ER-positive patients who receive tamoxifen [38]. Similarly, the Scottish trial that evaluated 5 years of tamoxifen versus no treatment demonstrated a survival advantage for patients who take tamoxifen [39]. The Scottish trial is particularly interesting because it addresses the question of whether to administer tamoxifen early as an adjuvant or to save the drug until recurrence. This comparison was possible because most patients in the control arm received tamoxifen at recurrence. Early concerns that long-term adjuvant tamoxifen would result in premature drug resistance are unjustified, because the patients have a survival advantage on the adjuvant tamoxifen arm. Indeed, an analysis of non-cancer-related deaths in the Scottish trial demonstrated a significant decrease in fatal myocardial infarction for patients receiving adjuvant tamoxifen for 5 years [40]. A number of other studies also demonstrate a decrease in coronary heart disease with tamoxifen [41, 42] but there is no overall consensus on this point and the overview analysis of clinical trials does not support enhanced survival by reduced coronary heart disease in women taking tamoxifen.

Studies in Premenopausal Women

Tamoxifen was initially used in premenopausal women to treat menometrorrhagia [43] and to induce ovulation in infertile women [44, 45]. Subsequent evaluation of the endocrine effects of tamoxifen by Groom and Griffiths [46] revealed an increase in ovarian estrogen production.

Although concerns have been expressed about the potential for the reversal of tamoxifen's action in a high-estrogen environment, tamoxifen can effectively control the growth of advanced breast cancer in premenopausal patients [47–51], and small clinical trials have demonstrated that tamoxifen and oophorectomy [52, 53] have similar efficacy. Adjuvant monotherapy with tamoxifen has shown efficacy in node-positive premenopausal patients [54], but most experience has been derived from the study B_{14} of node-negative ER-positive premenopausal patients conducted by the NSABP [55]. Tamoxifen increases the disease-free survival and, perhaps most importantly, the antiestrogen is active in premenopausal women. The protocol used an initial treatment period of 5 years of adjuvant tamoxifen, continue tamoxifen for an additional 5-year period. No advantages were found for longer

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adjuvant therapy but there were more reported side effects [55]. However, this is a very small trial and the issue of extending tamoxifen therapy in the ATLAS (Adjuvant Tamoxifen: Longer Against Shorter) trial from 5 to 10 years is currently being addressed. The following questions have now been asked: (1) What are the advantages and disadvantages of 5 versus 10 years of adjuvant tamoxifen? (2) What are the improvements in mortality during and after 10 years of adjuvant tamoxifen? The initial results of the ATLAS trial with 12,984 women who have completed 5 years of adjuvant tamoxifen are randomized to stop or continue for a further 5 years. The report of 6,846 women with ER-positive disease is reported [56] and compared with the earlier analysis of no treatment versus 5 years of adjuvant tamoxifen [57]. These enormous data sets confirm that endometrial cancer is the only side effect of concern in postmenopausal women, but deaths from endometrial cancer do not offset the benefits of adjuvant tamoxifen with an enhanced 50 % decrease in mortality in the decade after 10 years of tamoxifen.

These data [56] will be compared with aTTom (adjuvant Tamoxifen Treatment—offer more?) in 2013 and regular follow-ups will occur with reporting over the next 2 years.

Tamoxifen is currently available to treat selected patients at each stage of breast cancer, but the overview analysis of randomized clinical trials has precisely described the worth of antiestrogen therapy. By way of an introduction, the overview analysis wonderfully demonstrated that "longer is better" for the effectiveness of different durations of adjuvant tamoxifen alone used to treat premenopausal women with ER-positive breast cancer. One year of adjuvant therapy is completely ineffective in improving either recurrence or survival (Fig. 4.3). Five years of tamoxifen produces 30 % decrease in mortality and a 50 % decrease in recurrence.

Overview of Clinical Trials

The first overview analysis of adjuvant therapy for breast cancer was conducted in 1984 by Richard Peto, Rory Collins, and Richard Gray leading the team for the Clinical Trials Unit of Oxford University. Analysis of clinical trials' results pertaining to tamoxifen demonstrated not only a decrease in recurrence-free survival for postmenopausal women receiving tamoxifen but also increase in overall survival. These data were refined, checked, and presented again at the National Cancer Institute Consensus Conference in Bethesda, Maryland, in 1985, where the panel concluded that adjuvant tamoxifen should be the standard of care for all postmenopausal women with ER-positive primary tumor and positive nodes [58].

As an aside, this was the year that ICI Pharmaceutical Division (Zeneca) was awarded the start of their "use patent" for tamoxifen as a treatment for breast cancer originally submitted and denied from 1965 onward (25 years!) (see Chap. 2). The patent would now extend into the twenty-first century creating the resources to advance chemoprevention and tamoxifen in the United States and the major clinical

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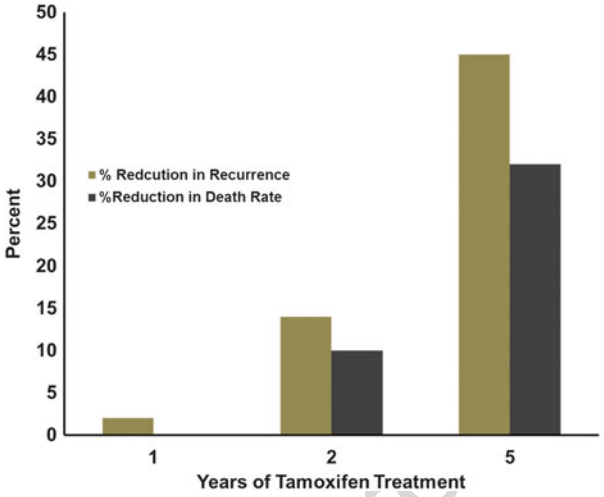

Fig. 4.3 The antitumorigenic action of tamoxifen in postmenopausal women. The results from the overview analysis have proven "the longer the better" concept for treatment with tamoxifen

trial of anastrozole, their aromatase inhibitor. Anastrozole versus tamoxifen and the combination (ATAC), then the single largest adjuvant endocrine clinical trial and became pivotal to lead progress with breast cancer therapy [59].

The overview of the clinical trials with tamoxifen was published in 1998 and 2005 [60, 61]. The 1998 and 2005 reports had three major therapeutic conclusions:

1. Tamoxifen was only effective as an adjuvant therapy in patients with an ER-positive breast tumor.
2. Longer was better than short adjuvant therapy in the treatment of ER-positive breast cancer. The power of this principle was best illustrated in premenopausal women receiving tamoxifen monotherapy: 1 year of adjuvant tamoxifen was completely ineffective at improving either recurrence rates or mortality but 5 years decreased recurrence by 50 % and mortality by 30 %. The scientific principles [8], published before any of the trials had started to recruit patients, were proven to have veracity.
3. The concern that the increased incidence of endometrial cancer during long-term adjuvant tamoxifen therapy might significantly reduce the value of tamoxifen as a cheap and effective life-saving medicine was calculated to be incorrect [60, 61].

We will now summarize the 2011 report of the relevance of breast cancer hormone receptors to the efficacy of adjuvant tamoxifen [57]. The meta-analysis of data was derived from 20 randomized clinical trials (n = 21,457) of adjuvant tamoxifen employing a 5-year treatment duration (80 % compliance). Again the continuing evaluation of adjuvant tamoxifen demonstrates the veracity of science in "the real world":

1. The ER positive disease (n = 10,645) tamoxifen reduced recurrence rates during the first 10 years but thereafter, there was no gain or loss out to 15 years.

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2. Marginal ER-positive disease (10–19 femtomoles/mg cytosol protein—from assays no longer used or quantitation employed) recurrence rates were substantial and significant.
3. Progesterone receptor was of no value to predict responsiveness to tamoxifen.
4. Breast cancer mortality was reduced by a third for the first 15 years.
5. All-cause mortality was substantially reduced despite small increases in thromboembolic and uterine cancer deaths (only women over 55 years of age) in women taking tamoxifen.

However, with the shift of the use of tamoxifen to aromatase inhibitors in postmenopausal patients, we felt it is appropriate to summarize the clinical trial to clarify the state of knowledge with the use of aromatase inhibitors versus tamoxifen.

Arrival of Aromatase Inhibitors as Adjuvant Therapy

The meta-analysis of the data from different trials (the Austrian Breast and Colorectal Cancer Study Group (ABCSG) XII trial, the Breast International Group (BIG) I-98/International Breast Cancer Study Group (IBCSG) 18–98 trial, and the ATAC trial) submitted to the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) was published in 2010 and described the comparison of the third-generation aromatase inhibitors (AI) against tamoxifen in breast cancer patients [62]. The patients were divided into two cohorts: cohort one comprised 9,856 patients that underwent treatment with AI immediately after surgery for 5 years and were compared to patients treated with tamoxifen; cohort two comprised 9,015 patients to assess the AI treatment with AI after 2–3 years of tamoxifen. The results of this analysis have shown that the administration of AI immediately after surgery for 5 years in the first cohort of patients has significantly reduced the recurrence of breast cancer by 23 % comparing to 5 years of tamoxifen. In the other cohort of patients, the efficacy of the switch to AI after 2–3 years of tamoxifen treatment was analyzed and it was shown that there was a 40 % reduction in risk of recurrence during the 3 following years after tamoxifen treatment. The authors of that study suggest that tamoxifen treatment after 3 years has sensitized the cancer cells to AI treatment; however, there is no experimental data supporting that. Also patients in both cohorts had follow-ups (5.8 years in cohort one and 3.9 years in cohort two) to assess the recurrence of the disease. The reduction of recurrence of breast cancer in both cohorts at 5 years after diagnosis was approximately 3 % and highly significant (2.9 %, SE = 0.7 % in cohort 1; and 3.1 %, SE = 0.6 % in cohort 2). The mortality rates in both cohorts were analogous at 5 years after diagnosis; however, there was a further decrease of mortality from breast cancers in the second cohort (AI after 2–3 years of tamoxifen). The authors concluded that AIs achieve "modest" improvements in breast cancer end points with significant reductions in recurrence in both cohorts of patients and specifically reduced mortality from breast cancer in

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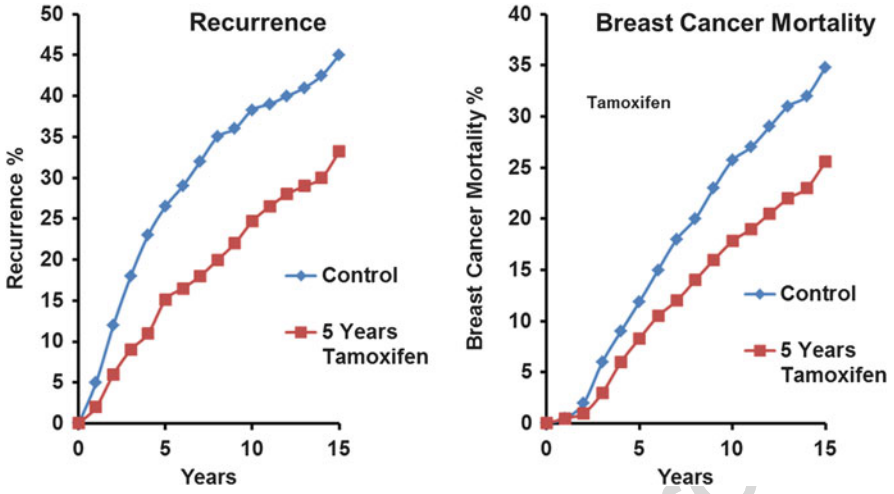

Fig. 4.4 The therapeutic action of tamoxifen after the treatment termination. The decrease of recurrence and mortality from breast cancer continues even 15 years after the treatment with tamoxifen stopped

the second cohort. However, it should be noted that AIs also have different side effects versus tamoxifen. AIs are associated with fewer endometrial cancers and thromboembolic events than tamoxifen but with increased incidents of arthralgia and bone fractures [63, 64].

Increasing Survivorship Following 5 Years of Adjuvant Tamoxifen

A significant mystery is why mortality continues to decrease following 5 years of adjuvant tamoxifen, i.e., after tamoxifen treatment has stopped [57, 61] (Fig. 4.4). Tamoxifen is a complete inhibitor of estrogen action at the tumor ER, so no drug would imply estrogen would bind to the unoccupied ER to cause tumor regrowth and increase mortality. But it does not!

However, a possible explanation occurred more by accident than design, through a study of acquired drug resistance to tamoxifen (Chap. 9). With the acceptance that long-term adjuvant tamoxifen was the appropriate strategy for the treatment of node-positive/node-negative breast cancer, in the late 1980s, it was imperative to develop a realistic model of acquired drug resistance to tamoxifen in the laboratory to determine mechanisms and diverse strategies for second-line therapy. The first transplantable model of acquired resistance was propagated in athymic mice. The ER-positive MCF-7 breast cancer cell line was used to develop the model [20]. AU5 Acquired resistance to tamoxifen developed within 2 years and once acquired either tamoxifen or physiologic estradiol utilizing the tumor ER to cause growth [65].

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However, the tumors could only be propagated in mice, and no successful transfer from tumor to tissue culture occurred. The model did not seem to replicate adjuvant therapy but rather metastatic breast cancer that fails tamoxifen treatments within 2 years. This seemed to be bad news but this became the good news as the unique tumor model could only be retained for study by routine propagation to tamoxifen-treated mice over years.

The finding that, following 5 years of retransplantation of tumors with acquired tamoxifen resistance into successive generations of tamoxifen-treated athymic mice, physiologic estrogen causes tumors to melt away was both mystifying and exciting. We will expand on this exciting new biology of estrogen-induced apoptosis in Chap. 9, but suffice to say it raised the possibility that acquired drug resistance to tamoxifen evolves and that the act of stopping tamoxifen after 5 years of adjuvant therapy causes the woman's own estrogen to seek out the appropriately reconfigured and sensitized breast cancer cells and triggers apoptosis. These data were first reported at the St. Gallen Breast Cancer Meeting with the hypothesis that the women's own estrogen caused the decrease in the patient mortality by killing appropriately sensitive microscopic foci of breast cancer cells [66].

In closing this chapter, it is important to stress that the hypothesis was not well received by the clinical community or the idea that physiologic estrogen administration might be of therapeutic significance. Despite the fact that no peer-reviewed funding was forthcoming, our research was sustained through philanthropy by the Lynn Sage Breast Cancer Foundation and the Robert H. Lurie Comprehensive Cancer Center at Northwestern University in Chicago, IL. Almost by chance, talented surgeons (Drs. Yao, Lee, England, and Bentrem) were looking for a project to exploit and this was it. They reproduced the Wolf data [66] over a 5-year period and it became clear that by year 5 of tamoxifen treatment, physiologic estrogen administration killed breast cancer cells with acquired resistance to tamoxifen. Estradiol killed the resistant cells but the remaining cells were again sensitive to antihormone therapy [67]. The process was cyclical (Fig. 4.5) and would eventually be tested in clinical trial and the molecular biology of estradiol-induced apoptosis clarified (Chap. 9). The concept was extended to the SERM raloxifene in an exceptionally long 10-year transplantation study of an MCF-7 study of acquired raloxifene resistance in athymic mice [68]. The original Wolf study and Balaburski study some 20 years apart are illustrated in Fig. 4.5.

Postscript. Perhaps the most important continuing support that ICI Pharmaceutical Division made to the development of tamoxifen (Nolvadex) was the hundreds of rats they chauffeured from Alderley Park nearby to Leeds University Medical School. Over the years (1974–1978), this strategy, instituted and paid for by Dr. Roy Cotton in the clinical department, was visionary. He was investing in a young enthusiastic pharmacologist who wanted to develop drugs to treat cancer. To a young faculty member in the Department of Pharmacology at Leeds University, armed with additional grants from the Yorkshire Cancer Research Campaign to purchase expensive ultracentrifuges (they were happy to invest in a BTA, Been to America), and ultimately an ICI/University of Leeds Joint Research Scheme

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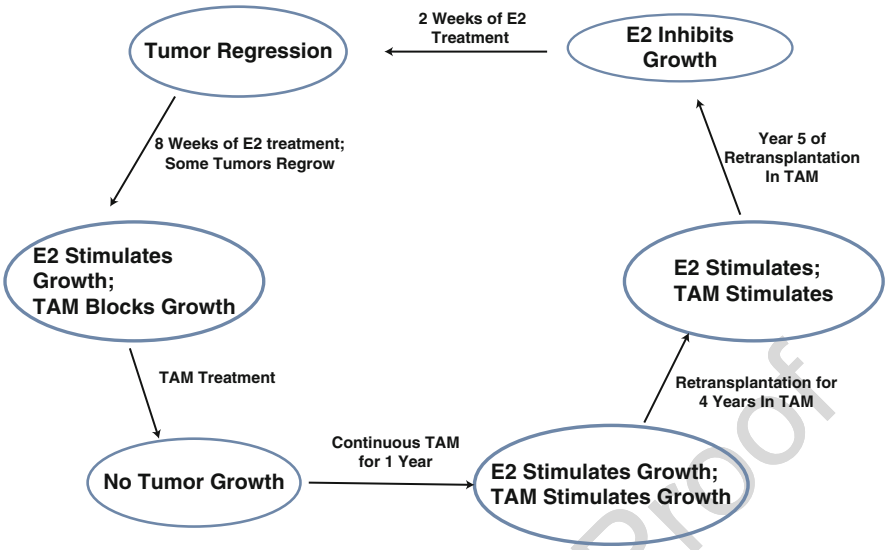

Fig. 4.5 The cyclical sensitivity and resistance of breast cancer cells to tamoxifen and estradiol. Estradiol is able to induce apoptosis in resistant cells; however, the remaining cells were again sensitive to tamoxifen treatment

co-headed by Walpole for ICI and I for Leeds to create 6,7-substituted alkylating estrogens, the unlimited animals were more valuable than gold. What did the investment yield? We did extensive studies on the mechanism of action of tamoxifen [69–75]; we were the first to discover the pharmacological properties of 4-hydroxytamoxifen [15, 17, 76–81], discovered the metabolic activation of tamoxifen, and most importantly created the strategy with animal models, to employ long-term adjuvant tamoxifen treatment for patients with ER-positive breast cancer [82–86].

We had two strategic goals with the studies of Karen Allen, an extremely talented technician who had trained in my group when she was an undergraduate in the Department of Pharmacology, and Clive Dix, an exceptional PhD student funded with an ICI Graduate Student Fellowship. Our first goal was to establish whether short-term high-dose tamoxifen administered to rats 30 days after the DMBA to induce mammary cancer for a short period of time (4 weeks which we considered to be equivalent to 1 year in a woman's life) would "cure the animals." It did not, but we realized that suppression of tumor development by tamoxifen was dose related, i.e., once the accumulated and slowly excreted tamoxifen was gone from the body, the tumors appeared. Clive demonstrated that continuous tamoxifen treatment was necessary to prevent tumorigenesis, almost completely, and was superior to oophorectomy [15]. Thus, long-term adjuvant therapy was going to be better to control the recurrence of ER-positive disease effectively after primary surgery.

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Our second goal was to determine whether 4-hydroxytamoxifen, a more potent antiestrogen than tamoxifen, was a more potent antitumor agent in the rat. It was not, although continuous therapy was effective at controlling tumor development [17]. We concluded that rapidly excreted hydroxylated antiestrogens were poor antitumor agents, a principle that was to recur with polyhydroxylated raloxifene [16] when used for the prevention of breast cancer [87] and proven over the next 30 years!

The opportunity to present our new concept for the adjuvant use of tamoxifen occurred in September 1977 at an ICI Pharmaceutical Division Breast Cancer Symposium at King's College, Cambridge, England. Michael Baum was the chair of my session and it was clear that plans were in place to increase the duration of adjuvant tamoxifen therapy from the standard 1 year to 2 years with the NATO trial (the acronym was based on the belief the Americans would read their subsequent papers and refer to them in their publications if they believed it was an American sponsored trial. The acronym actually stands for "Nolvadex Adjuvant Trial Organization") and the proposed 5 years for the Scottish trial. Each of the trialists considered their choice of trial design was arbitrary, but we already had the scientific basis in plan that would prove to be successful in their clinical trials.

The week following the King's College meeting I began a 3-month sabbatical at the University of Wisconsin Clinical Cancer Center, Madison, Wisconsin. There I proposed the "tamoxifen forever" clinical strategy as a forward thinking goal to accelerate tamoxifen's development and prevent disease recurrence. I should restate that tamoxifen at that time was not FDA approved in the United States even for the treatment of metastatic breast cancer. This would occur on 29 December 1977. Presentation of the strategy with compelling laboratory data to create potential survival advantages for patients with ER-positive breast cancer caught on with both the Eastern Cooperative Oncologic Group (ECOG) and the National Adjuvant Breast and Bowel Project (NSABP) as they advanced their adjuvant therapy trials from 2 to 5 years. This was a critical decision that saved hundreds of thousands of women's lives worldwide.

The good news for my career was that this 3-month sabbatical time in the Wisconsin Clinical Cancer Center in Madison resulted in a job offer because by this time I had lots of publications and Eliahu Caspi's lesson had been learned! (See Chap. 2.) After a year setting up the Ludwig Institute in Bern, Switzerland (1979–1980), and forging friendships that would last a career, I moved to Wisconsin to learn and recreate my Tamoxifen Team in America (Chap. 5).

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References

1. Fisher B, Carbone P, Economou SG et al (1975) 1-Phenylalanine mustard (L-PAM) in the management of primary breast cancer. A report of early findings. N Engl J Med 292:117–122
2. Bonadonna G, Brusamolino E, Valagussa P et al (1976) Combination chemotherapy as an adjuvant treatment in operable breast cancer. N Engl J Med 294:405–410

Author's Proof

3. Jordan VC, Fritz NF, Tormey DC (1987) Endocrine effects of adjuvant chemotherapy and long-term tamoxifen administration on node-positive patients with breast cancer. Cancer Res 47:624–630
4. Rose DP, Davis TE (1980) Effects of adjuvant chemohormonal therapy on the ovarian and adrenal function of breast cancer patients. Cancer Res 40:4043–4047
5. Jordan VC (1998) Chemotherapy is antihormonal therapy–how much proof do oncologists need? Eur J Cancer 34:606–608
6. Cole MP, Jones CT, Todd ID (1971) A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer 25:270–275
7. Ward HW (1973) Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J 1:13–14
8. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44:30–38
9. Lippman ME, Bolan G (1975) Oestrogen-responsive human breast cancer in long term tissue culture. Nature 256:592–593
10. Early Breast Cancer Trialists' Collaborative Group (1992) Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Lancet 339:1–15
11. Swedish Breast Cancer Cooperative Group (1996) Randomized trial of two versus five years of adjuvant tamoxifen for postmenopausal early stage breast cancer. J Natl Cancer Inst 88:1543–1549
12. Jordan VC (1978) Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. Rev Endocr Relat Cancer:49–55 AU6
13. Jordan VC, Dix CJ, Allen KE (1979) The effectiveness of long-term treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In: Salmon SE, Jones SE (eds) AU7
14. Robinson SP, Mauel DA, Jordan VC (1989) Antitumor actions of toremifene in the 7,12-dimethylbenzanthracene (DMBA)-induced rat mammary tumor model. Eur J Cancer Clin Oncol 24:1817–1821
15. Jordan VC, Allen KE, Dix CJ (1980) Pharmacology of tamoxifen in laboratory animals. Cancer Treat Rep 64:745–759
16. Gottardis MM, Jordan VC (1987) Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 47:4020–4024
17. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
18. Osborne CK, Hobbs K, Clark GM (1985) Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res 45:584–590
19. Osborne CK, Coronado EB, Robinson JP (1987) Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. Eur J Cancer Clin Oncol 23:1189–1196
20. Gottardis MM, Robinson SP, Jordan VC (1988) Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumoristatic action of tamoxifen. J Steroid Biochem 30:311–314
21. Ribeiro G, Palmer MK (1983) Adjuvant tamoxifen for operable carcinoma of the breast: report of clinical trial by the Christie Hospital and Holt Radium Institute. Br Med J (Clin Res Ed) 286:827–830
22. Rose C, Thorpe SM, Andersen KW et al (1985) Beneficial effect of adjuvant tamoxifen therapy in primary breast cancer patients with high oestrogen receptor values. Lancet 1:16–19
23. Ribeiro G, Swindell R (1985) The Christie Hospital tamoxifen (Nolvadex) adjuvant trial for operable breast carcinoma–7-yr results. Eur J Cancer Clin Oncol 21:897–900
24. Cummings FJ, Gray R, Davis TE et al (1985) Adjuvant tamoxifen treatment of elderly women with stage II breast cancer. A double-blind comparison with placebo. Ann Intern Med 103:324–329

Uncorrected Proof

Author's Proof

25. Baum MaomotNATO (1985) Controlled trial of tamoxifen as single adjuvant agent in management of early breast cancer. Lancet 1:836–840
26. Pritchard KI, Meakin JW, Boyd NF, Ambus K, DeBoer G, Paterson AHG, Sutherland DJA, Wilkinson RH, Bassett AA, Evans WK, Beale FA, Clark RM, Keane TJ (1984) A randomized trial of adjuvant tamoxifen in postmenopausal women with axillary node positive breast cancer. In: Jones SE, Salmon SE (eds) Adjuvant therapy of breast cancer. Grune and Stratton, New York, pp 339–348
27. Wallgren A, Baral E, Castensen J, Friberg S, Glas U, Hjalmar JL, Kargas M, Nordenskjold B, Skoog L, There N-O, Wilking N (1984) Should adjuvant tamoxifen be given for several years in breast cancer. In: Jones SE, Salmon SE (eds) Adjuvant therapy of breast cancer. Grune and Stratton, New York, pp 331–338
28. Tormey DC, Jordan VC (1984) Long-term tamoxifen adjuvant therapy in node-positive breast cancer: a metabolic and pilot clinical study. Breast Cancer Res Treat 4:297–302
29. Tormey DC, Rasmussen P, Jordan VC (1987) Long-term adjuvant tamoxifen study: clinical update. Breast Cancer Res Treat 9:157–158
30. Langan-Fahey SM, Tormey DC, Jordan VC (1990) Tamoxifen metabolites in patients on long-term adjuvant therapy for breast cancer. Eur J Cancer 26:883–888
31. Jordan VC (1983) Laboratory studies to develop general principles for the adjuvant treatment of breast cancer with antiestrogens: problems and potential for future clinical applications. Breast Cancer Res Treat 3(Suppl):S73–S86
32. Falkson HC, Gray R, Wolberg WH et al (1990) Adjuvant trial of 12 cycles of CMFPT followed by observation or continuous tamoxifen versus four cycles of CMFPT in postmenopausal women with breast cancer: an Eastern Cooperative Oncology Group phase III study. J Clin Oncol 8:599–607
33. Fisher B, Brown A, Wolmark N et al (1987) Prolonging tamoxifen therapy for primary breast cancer. Findings from the National Surgical Adjuvant Breast and Bowel Project clinical trial. Ann Intern Med 106:649–654
34. Fisher B, Redmond C, Brown A et al (1981) Treatment of primary breast cancer with chemotherapy and tamoxifen. N Engl J Med 305:1–6
35. Fisher B, Redmond C, Brown A et al (1983) Influence of tumor estrogen and progesterone receptor levels on the response to tamoxifen and chemotherapy in primary breast cancer. J Clin Oncol 1:227–241
36. Fisher B, Redmond C, Brown A et al (1986) Adjuvant chemotherapy with and without tamoxifen in the treatment of primary breast cancer: 5-year results from the National Surgical Adjuvant Breast and Bowel Project Trial. J Clin Oncol 4:459–471
37. Baum M, Brinkley DM, Dossett JA et al (1983) Controlled trial of tamoxifen as adjuvant agent in management of Early Breast Cancer. Interim analysis at four years by the Nolvadex Adjuvant Trial Orginisation. Lancet 321:257–261
38. Delozier T, Julien JP, Juret P et al (1986) Adjuvant tamoxifen in postmenopausal breast cancer: preliminary results of a randomized trial. Breast Cancer Res Treat 7:105–109
39. Scottish Cancer Trials Office (MRC) E (1987) Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee. Lancet 2:171–175
40. McDonald CC, Stewart HJ (1991) Fatal myocardial infarction in the Scottish adjuvant tamoxifen trial. The Scottish Breast Cancer Committee. BMJ 303:435–437
41. Rutqvist LE, Mattsson A (1993) Cardiac and thromboembolic morbidity among postmenopausal women with early-stage breast cancer in a randomized trial of adjuvant tamoxifen. The Stockholm Breast Cancer Study Group. J Natl Cancer Inst 85:1398–1406
42. Hackshaw A, Roughton M, Forsyth S et al (2011) Long-term benefits of 5 years of tamoxifen: 10-year follow-up of a large randomized trial in women at least 50 years of age with early breast cancer. J Clin Oncol 29:1657–1663
43. el Sheikha Z, Klopper A, Beck JS (1972) Treatment of menometrorrhagia with an anti-oestrogen. Clin Endocrinol (Oxf) 1:275–282

Uncorrected Proof

Author's Proof

44. Klopper A, Hall M (1971) New synthetic agent for the induction of ovulation: preliminary trials in women. Br Med J 1:152–154
45. Williamson JG, Ellis JD (1973) The induction of ovulation by tamoxifen. J Obstet Gynaecol Br Commonw 80:844–847
46. Groom GV, Griffiths K (1976) Effect of the anti-oestrogen tamoxifen on plasma levels of luteinizing hormone, follicle-stimulating hormone, prolactin, oestradiol and progesterone in normal pre-menopausal women. J Endocrinol 70:421–428
47. Manni A, Trujillo JE, Marshall JS et al (1979) Antihormone treatment of stage IV breast cancer. Cancer 43:444–450
48. Pritchard KI, Thomson DB, Myers RE et al (1980) Tamoxifen therapy in premenopausal patients with metastatic breast cancer. Cancer Treat Rep 64:787–796
49. Kalman AM, Thompson T, Vogel CL (1982) Response to oophorectomy after tamoxifen failure in a premenopausal patient. Cancer Treat Rep 66:1867–1868
50. Planting AS, Alexieva-Figusch J, Blonk-vdWijst J, van Putten WL (1985) Tamoxifen therapy in premenopausal women with metastatic breast cancer. Cancer Treat Rep 69:363–368
51. Sawka CA, Pritchard KI, Paterson AH et al (1986) Role and mechanism of action of tamoxifen in premenopausal women with metastatic breast carcinoma. Cancer Res 46:3152–3156
52. Ingle JN, Krook JE, Green SJ et al (1986) Randomized trial of bilateral oophorectomy versus tamoxifen in premenopausal women with metastatic breast cancer. J Clin Oncol 4:178–185
53. Buchanan RB, Blamey RW, Durrant KR et al (1986) A randomized comparison of tamoxifen with surgical oophorectomy in premenopausal patients with advanced breast cancer. J Clin Oncol 4:1326–1330
54. Party CABTW (1988) Cyclophosphamide and tamoxifen as adjuvant therapies in the management of breast cancer. Br J Cancer 57:604–607
55. Fisher B, Costantino J, Redmond C et al (1989) A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. N Engl J Med 320:479–484
56. Davies C, Hongchao P, Godwin J et al (2012) Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomized trial. Lancet
57. Davies C, Godwin J, Gray R et al (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378:771–784
58. Consensus Conference (1985) Adjuvant chemotherapy for breast cancer. JAMA 254:3461–3463
59. Howell A, Cuzick J, Baum M et al (2005) Results of the ATAC (arimidex, tamoxifen, alone or in combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 365:60–62
60. EBCTCG (1998) Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 351:1451–1467
61. EBCTCG (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 365:1687–1717
62. Dowsett M, Cuzick J, Ingle J et al (2010) Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 28:509–518
63. Lin NU, Winer EP (2008) Advances in adjuvant endocrine therapy for postmenopausal women. J Clin Oncol 26:798–805
64. Lonning PE, Geisler J (2008) Indications and limitations of third-generation aromatase inhibitors. Expert Opin Investig Drugs 17:723–739
65. Gottardis MM, Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 48:5183–5187
66. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127:23–33

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Author's Proof

67. Yao K, Lee ES, Bentrem DJ et al (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6:2028–2036
68. Balaburski GM, Dardes RC, Johnson M et al (2010) Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. Int J Oncol 37:387–398
69. Abbott AC, Clark ER, Jordan VC (1976) Inhibition of oestradiol binding to oestrogen receptor proteins by a methyl-substituted analogue of tamoxifen. J Endocrinol 69:445–446
70. Jordan VC (1976) Antiestrogenic and antitumor properties of tamoxifen in laboratory animals. Cancer Treat Rep 60:1409–1419
71. Jordan VC, Prestwich G (1977) Binding of [3H]tamoxifen in rat uterine cytosols: a comparison of swinging bucket and vertical tube rotor sucrose density gradient analysis. Mol Cell Endocrinol 8:179–188
72. Jordan VC, Dix CJ, Rowsby L, Prestwich G (1977) Studies on the mechanism of action of the nonsteroidal antioestrogen tamoxifen (I.C.I. 46,474) in the rat. Mol Cell Endocrinol 7:177–192
73. Jordan VC, Naylor KE (1979) The binding of [3H]-oestradiol-17 beta in the immature rat uterus during the sequential administration of non-steroidal anti-oestrogens. Br J Pharmacol 65:167–173
74. Jordan VC, Prestwich G (1978) Effect of non-steroidal anti-oestrogens on the concentration of rat uterine progesterone receptors. J Endocrinol 76:363–364
75. Jordan VC, Rowsby L, Dix CJ, Prestwich G (1978) Dose-related effects of non-steroidal antioestrogens and oestrogens on the measurement of cytoplasmic oestrogen receptors in the rat and mouse uterus. J Endocrinol 78:71–81
76. Jordan VC, Collins MM, Rowsby L, Prestwich G (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305–316
77. Jordan VC, Dix CJ, Naylor KE et al (1978) Nonsteroidal antiestrogens: their biological effects and potential mechanisms of action. J Toxicol Environ Health 4:363–390
78. Jordan VC, Dix CJ (1979) Effect of oestradiol benzoate, tamoxifen and monohydroxy-tamoxifen on immature rat uterine progesterone receptor synthesis and endometrial cell division. J Steroid Biochem 11:285–291
79. Dix CJ, Jordan VC (1980) Subcellular effects of monohydroxytamoxifen in the rat uterus: steroid receptors and mitosis. J Endocrinol 85:393–404
80. Allen KE, Clark ER, Jordan VC (1980) Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 71:83–91
81. Dix CJ, Jordan VC (1980) Modulation of rat uterine steroid hormone receptors by estrogen and antiestrogen. Endocrinology 107:2011–2020
82. Jordan VC, Dowse LJ (1976) Tamoxifen as an anti-tumour agent: effect on oestrogen binding. J Endocrinol 68:297–303
83. Jordan VC, Jaspan T (1976) Tamoxifen as an anti-tumour agent: oestrogen binding as a predictive test for tumour response. J Endocrinol 68:453–460
84. Jordan VC, Jaspan T (1976) Oestrogen binding as a predictive test for DMBA-induced tumour response to tamoxifen therapy. In: Hellman K, Conors TA (eds) Chemotherapy. Plenum, New York, pp 89–94 AU8
85. Jordan VC (1978) Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. Rev Endocr Relat Cancer:49–55 (October supplement)
86. Jordan VC, Dix CJ, Allen KE (1979) The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In: Salmon SE, Jones SE (eds) Adjuvant therapy of cancer II. Grune and Strattin, New York, pp 19–26
87. Vogel VG, Costantino JP, Wickerham DL et al (2010) Update of the National Surgical Adjuvant Breast and Bowel Project study of tamoxifen and raloxifene (STAR) P-2 trial: preventing breast cancer. Cancer Prev Res (Phila) 3:696–706

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AU4	Please check sentence starting "The ER positive disease...." for sense.	
AU5	Please check latter part of sentence starting "Acquired resistance to..." for completeness.	
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Abstract	The idea that tamoxifen could potentially be employed to prevent breast cancer in populations of women with high risk naturally mandated an extensive laboratory and clinical investigation of potential toxicological concerns. It was reasoned that if estrogen was necessary to maintain bone density and protect women from coronary heart disease, then an “antiestrogen” might prevent breast cancer but increase the risks of osteoporosis and coronary heart disease. Laboratory results and translation to clinical trial proved the reverse was true, and the new drug group, selective estrogen receptor modulators (SERMs), was discovered. Tamoxifen (and raloxifene) paradoxically prevented bone loss in ovariectomized rats (estrogen-like) but prevented rat mammary carcinogenesis (antiestrogen-like). The same was true in patients with tamoxifen (and raloxifene) maintaining bone density but preventing breast cancer. Additionally, circulating cholesterol decreased (an estrogen-like effect) in patients. However, an estrogen-like effect of tamoxifen that became a concern was the discovery that in the laboratory, tamoxifen prevented breast cancer growth but enhanced the growth of endometrial cancer.	

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Chapter 5
The Wisconsin Story in the 1980s: Discovery of Target Site-Specific Estrogen Action

Abstract The idea that tamoxifen could potentially be employed to prevent breast cancer in populations of women with high risk naturally mandated an extensive laboratory and clinical investigation of potential toxicological concerns. It was reasoned that if estrogen was necessary to maintain bone density and protect women from coronary heart disease, then an "antiestrogen" might prevent breast cancer but increase the risks of osteoporosis and coronary heart disease. Laboratory results and translation to clinical trial proved the reverse was true, and the new drug group, selective estrogen receptor modulators (SERMs), was discovered. Tamoxifen (and raloxifene) paradoxically prevented bone loss in ovariectomized rats (estrogen-like) but prevented rat mammary carcinogenesis (antiestrogen-like). The same was true in patients with tamoxifen (and raloxifene) maintaining bone density but preventing breast cancer. Additionally, circulating cholesterol decreased (an estrogen-like effect) in patients. However, an estrogen-like effect of tamoxifen that became a concern was the discovery that in the laboratory, tamoxifen prevented breast cancer growth but enhanced the growth of endometrial cancer.

Introduction

In the early 1980s, Professor Trevor Powles, the head of the Breast Cancer Unit at the Royal Marsden hospital, took the bold step to initiate a pilot clinical trial of tamoxifen to treat healthy women with a high risk of breast cancer. The goals were to determine whether healthy women without disease would take tamoxifen for years, monitor side effects, and use the experience gained as a vanguard for a large placebo-controlled chemotherapeutics study of tamoxifen. The scientific rationale was based on two dominant facts: (1) In the laboratory, tamoxifen was known to prevent the initiation and promotion of mammary cancer by estrogen in the DMBA-induced rat mammary carcinoma model [1, 2]. (2) Tamoxifen, used as an adjuvant

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therapy, was noted in a letter to the Lancet [3] to reduce the incidence of contralateral breast cancer.

In other words, tamoxifen inhibited rat mammary carcinogens in the standard laboratory model used in breast cancer research at the time, and tamoxifen actually inhibited the incidence of primary breast cancer.

At this time in the 1980s, tamoxifen was classified as a nonsteroidal antiestrogen [4] and clinical trials with long-term adjuvant therapy were reporting a good safety profile for the drug administered between 2 years and potentially indefinite therapy [5–7]. However, the idea of treating healthy women with an "antiestrogen" raised some important issues that had to be addressed. If estrogen was important to maintain bone density and, at the time, there was the conviction that estrogen protected women from coronary heart disease, then the administration of an "antiestrogen" might well prevent half a dozen breast cancers per year in a 1,000 high-risk women, but the antiestrogenic interaction would expose the majority of women to crushing osteoporosis and an increased risk of dying from coronary heart disease. The target site pharmacology needed to be investigated in the laboratory, and steps had to be taken to translate the findings to clinical practice.

Two approaches were addressed that were ultimately to change clinical perceptions about "nonsteroidal antiestrogens" and, more importantly, to change the application of these drugs in medicine. We will describe the developing set of laboratory studies that would result in a new understanding of the pharmacology of tamoxifen and raloxifene (then called keoxifene) and then describe the clinical studies that occurred simultaneously that opened the door to the descriptions of a new drug group—the selective estrogen receptive modulators or SERMs. This program was unique to the Wisconsin Comprehensive Cancer Center, so we will describe the associated information from others that confirmed or supported our research strategy during the 1980s.

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Laboratory Studies on the Target Site-Specific Pharmacology of "Nonsteroidal Antiestrogens"

The early studies in the literature concerning ICI 46,474 (later tamoxifen) described its antifertility and antiestrogenic properties in the immature rat uterus and in ovariectomized rat Allen-Doisy tests [8, 9]. Paradoxically, tamoxifen was estrogenic in the mouse uterus [10–12]. Tamoxifen was also known to lower circulating cholesterol in the rat with no significant increase in circulating desmosterol [8]. In contrast, LY156758 (keoxifene to become raloxifene) and LY117018 were both antiestrogens in the rat and mouse uterus and blocked estrogen and tamoxifen induced increase in uterine weight [13–17]. There was initially no information about circulating cholesterol in animals, as all interest was then focused upon the use of keoxifene as a treatment for breast cancer, an indication for which it was eventually to fail, and work was discontinued at Eli Lilly in the late 1980s.

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There was interest in comparing and contrasting the actions of tamoxifen and keoxifene on the rodent uterus, rat bone density, and carcinogen-induced rat mammary cancers and human tumors (breast and endometrial) grown in athymic mice. The differential effects of tamoxifen in the athymic mouse uterus transplanted with a growing estrogen-stimulated ER-positive MCF-7 tumor was particularly interesting. Administration of estradiol caused an increase in uterine weight and the growth of the MCF-7 tumor. However, tamoxifen caused an increase in mouse uterine weight but did not cause MCF-7 tumor growth. In fact, tamoxifen blocked estrogen-stimulated growth. We analyzed the tamoxifen metabolites in both estrogen target organs and found they were comparable, so we concluded "that the drug can selectively stimulate or inhibit events in the target tissues of different species without metabolic intervention" [18]. It was realized, however, that the target site specificity had clinical relevance to the application of tamoxifen as a long-term adjuvant therapy and as a potential chemopreventive.

Dr. Satyaswaroop at Penn State Medical School in Hershey, Pennsylvania, had dedicated considerable efforts to establish human endometrial cancer that grew in athymic mice [19]. He also noted that tamoxifen would increase the growth of human endometrial cancers [20] but had not stated that these data could be translated to clinical practice. In a pioneering experiment that hereafter changed clinical practice, human endometrial cancer and an MCF-7 tumor were transplanted into athymic mice and treated with both physiologic estrogen and tamoxifen. The goal was to establish whether tamoxifen would stop the estrogen-stimulated growth of both human tumors in the same mouse. The results (Fig. 5.1) demonstrated that tamoxifen inhibited estrogen-stimulated tumor growth but enhanced the growth of the human endometrial tumor. It was concluded that "these findings suggest that the disparate pharmacology of TAM is a tissue-specific phenomenon" [21] and suggested that "Until the influence of TAM and other antiestrogens on endometrial cancers has been fully investigated, vigilance by physicians treating patients with these agents is needed to establish the clinical relevance (if any) of these observations." In other words, it was possible that tamoxifen could prevent the growth of breast cancer but enhance the growth of endometrial cancer. The clinical community was quick to replicate the same target tissue concept in patients treated with long-term adjuvant tamoxifen therapy [22] with tamoxifen decreasing contralateral breast cancer but increasing the incidence of endometrial cancer in postmenopausal women. It was clear that tamoxifen was enhancing the growth of some target tissues but blocking the growth of others, so tamoxifen may not be appropriate in postmenopausal women at high risk of breast cancer.

A new dimension was necessary. Chemoprevention was to be a reality with antiestrogens and that new dimension would be keoxifene (raloxifene). Raloxifene was compared with tamoxifen in rats to prevent mammary carcinogenesis [23] and endometrial cancer [24].

There was a concern about "nonsteroidal antiestrogens" inhibiting bone regeneration and causing osteoporosis during long-term adjuvant tamoxifen treatment or during the use of tamoxifen as a chemopreventive, so there was a focus on

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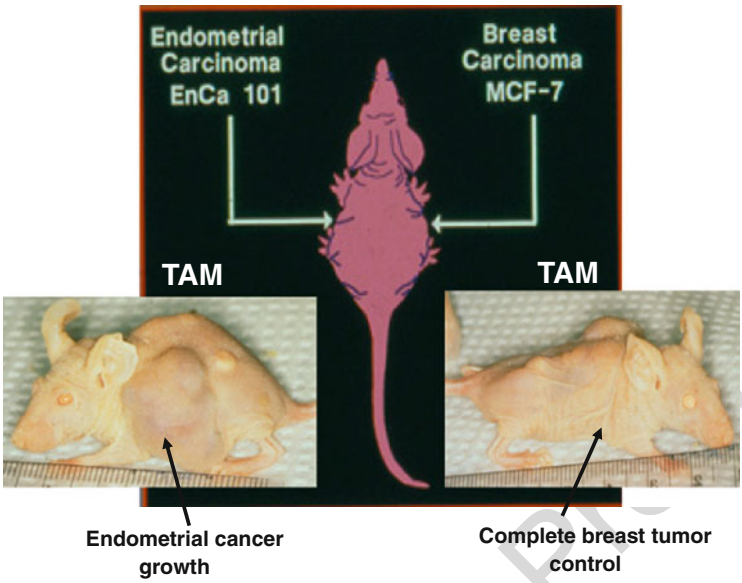

Fig. 5.1 The pioneering bitransplantation study by Gottardis with an ER-positive breast tumor (MCF-7) implanted in one axilla and an ER-positive endometrial tumor (EnCa 101) in the other axilla. Tamoxifen blocks estrogen-stimulated growth of the breast tumor, but tamoxifen encourages the growth of the endometrial tumor

measurements of rat bone following ovariectomy and antiestrogen treatment. Earlier, Beall and coworkers [25] had reported that clomiphene (a mixture of estrogenic and antiestrogenic geometric isomers) maintained bone density in ovariectomized retired breeder rats. However, since the administered drug was an impure mixture and not an antiestrogenic drug specifically, there was no proof that the estrogenic isomer had not caused an increase in bone density.

In contrast, the same model was used in the rat to determine the effect of the pure antiestrogenic isomer tamoxifen, and the results were compared with raloxifene, an antiestrogen with less estrogen-like actions than tamoxifen in the rat uterus and a fixed ring structures. Both antiestrogens maintained bone density, and in fact a combination of antiestrogens and estrogen was additive [26]. A study of tamoxifen and raloxifene to prevent rat mammary carcinogens demonstrated efficacy for both antiestrogens, but tamoxifen was shown to be superior and raloxifenes' effectiveness was found to be not long lasting [23]. More than 20 years later, these data were to be relevant in the STAR trial (Chap. 8) with tamoxifen having long-term and lasting actions to prevent breast tumor incidence, but raloxifene was not able to sustain the antitumor effect after treatment was stopped [27].

Finally, raloxifene was less effective at stimulating the growth of human endometrial cancer in laboratory models [24] and less effective at stimulating the growth of rodent uterine in vivo [15]. Taken together, these data generated in the same laboratory over a period of 2–3 years described the target site-specific actions of

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nonsteroidal antiestrogens to switch on and switch off estrogen target sites around the body. Those data lead to the proposal first stated at the First International Chemoprevention Conference in New York [28].

> . . .an extensive clinical investigation of available antioestrogens. Could analogs be developed to treat osteoporosis or even retard the development of atherosclerosis? Should the agent also retain anti-breast tumour actions then it might be expected to act as a chemosuppressive on all developing breast cancers.
>
> . . . . . .a bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century.

This vision became a reality and it led to the further clinical evaluation of tamoxifen in bone and then raloxifene as a selective estrogen/antiestrogen in target sites around a human's body. Tamoxifen was the drug of choice to study because it was approved clinically. The agent of choice by the clinical community to study chemoprevention in high-risk women was tamoxifen and raloxifene (aka keoxifene) that was unavailable for clinical testing. AU3

The Wisconsin Tamoxifen Study

A preliminary study of bone mineral density in women treated with adjuvant tamoxifen showed no detrimental effects at 2 years, i.e., the antiestrogenic actions of tamoxifen did not decrease bone density [29]. These data encouraged the establishment of a double-blind placebo-controlled trial of node-negative postmenopausal (no menses for 12 months) breast cancer patients with a diagnosis up to 10 years previously.

It is important to emphasize that in the late 1980s, adjuvant tamoxifen treatment was not the standard of care for the node-negative patient. Women were randomized to either tamoxifen or placebo for 2 years (Fig. 5.2) with evaluations for bone density, symptoms, and cardiovascular risk factors at baseline, 3, 6, 12, 18, and 24 months later.

The main results were reported in a series of publications in the early 1990s [30–32]. The changes in cardiovascular risk factors during tamoxifen treatment were encouraging for long-term safety of adjuvant tamoxifen and as a potential chemopreventive agent in high-risk women. Total cholesterol decreased by 12 % during the 2-year period and this remained statistically significant ($P < 0.001$). The main effect was driven by a specific decrease of 20 % in low-density lipoprotein (LDL) cholesterol ($P < 0.0001$) with stable high-density lipoprotein (HDL) cholesterol. Fibrinogen rapidly decreased by 20 % at 6 months ($P < 0.0003$) and a 7 % decrease in platelets with a significant decrease in antithrombin III was observed in tamoxifen-treated women.

The bone parameters were highly significant and established the idea that tamoxifen could maintain or build bone translated from the laboratory [26, 33–35] to clinical practice. The placebo group had a decrease in radius of

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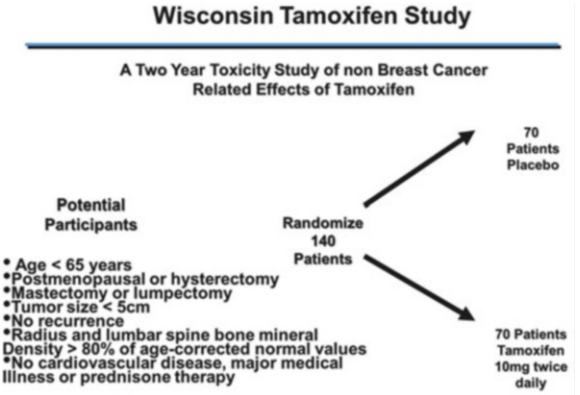

Fig. 5.2 The design of the Wisconsin Tamoxifen Study recruited 140 node-negative breast cancer patients to be randomized to either tamoxifen (20 mg/daily) or placebo. Bone mineral density was measured by dual-photon absorptiometry at regular intervals, and bloods were drawn to determine circulating lipids and clotting factors

1–1.292 % per year ($P < 0.0001$) and spine of −0.9967 % per year ($P < 0.0008$). Tamoxifen-treated women lost bone in the radius from baseline of −0.878 % per year ($P < 0.0002$) and lumbar spine a gain of 0.611 % per year ($P < 0.04$). A comparison of both lumbar spine linear rates was highly significant by 2 years ($P < 0.0001$). Symptoms were consistent with prior reports with only a modest rise in hot flashes compared with placebo. Gynecological symptoms increased modestly when vaginal discharge, vaginal dryness, bleeding, and genital pruritus were identified. Interestingly, there were fewer headaches.

In general, these data from the Wisconsin Tamoxifen Study were confirmed by other publications around this time [36–40].

Translational Research

The results with tamoxifen and raloxifene in the ovariectomized rat in the mid-1980s were subsequently confirmed by others, first for tamoxifen [33–35] and then eventually raloxifene [41–43]. The clinical research on tamoxifen was set to demonstrate that circulating cholesterol was reduced and postmenopausal bone density was maintained in contrast to placebo-treated controls. The links between tamoxifen and endometrial cancer (Chap. 6) and rat liver carcinogenesis were naturally of concern for the testing of tamoxifen as a chemopreventive (Chap. 6), but a new strategy was in place in the refereed literature when Leonard Lerner and I were awarded the Bruce F. Cain Award by the American Association for Cancer Research for laboratory research that resulted in a successful strategy to treat cancer [44]. Simply stated, the roadmap for pharmaceutical industry to follow was as follows:

> Is this the end of the possible applications for antioestrogens? Certainly not. We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered

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about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be post-menopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer.

Numerous companies followed the roadmap but not before keoxifene was renamed raloxifene and became the first SERM to treat and prevent osteoporosis and prevent breast cancer in postmenopausal women.

Postscript. During the 1980s, the nascent Breast Cancer Program led by Dr. Douglass Tormey, former head of the Breast Cancer Program at the National Cancer Institute, was building rapidly to create a multidisciplinary group able to conduct important translational research with the potential to "export" ideas to the Eastern Cooperative Oncology Group. Tormey had been the chief of the Breast Committee throughout the 1970s and was instrumental in recruiting me to Wisconsin. Lois Trench was also key, as the drug monitor for tamoxifen, and was the one to "get me started" at the Worcester Foundation for Experimental Biology (1972–1974). I was ICI America's first scientific consultant to use my laboratory results to facilitate clinical trials in America. I arrived in Madison, Wisconsin, in 1980 following a period establishing a breast cancer center for the Ludwig Institute for Cancer Research in Bern, Switzerland (1979–1980). My brief in Madison was to establish a major center for tamoxifen research and act as a link between basic science at the University of Wisconsin and clinical trials. Remember, tamoxifen was only approved to treat metastatic breast cancer by the FDA at the end of 1977, but we had plans! To achieve this, all my students had Dr. Jack Gorski on their Ph. D. thesis committee, and I recruited (with Jack's encouragement) numerous of his trainees to my laboratory at the Comprehensive Cancer Center. The late Mara Lieberman, Wade Welshons, and Mike Fritsch were all outstanding.

Another important scientist of note at the cancer center was Dr. David Rose who introduced me to a range of new antiestrogens (LY117018, trioxifene, LY156758) from Eli Lilly. David left Madison in the early 1980s, and it was decided that I should assume the responsibility for his staff, his laboratory space, and the ER clinical laboratory that served the hospitals in Southern Wisconsin. This was a frightening turn of events, so I called my mentor Bill McGuire in San Antonio to explain that I did not feel prepared for the task. He replied that I was looking at this incorrectly—"it's an opportunity" and so it was. In 1988, I was appointed as the director of the Breast Cancer Research and Treatment Program for the cancer center.

Wisconsin created the optimal environment to advance exciting translational science and create new careers. There we created an outstanding Tamoxifen Team in the Department of Human Oncology for 14 years; everyone was excellent, played their part, and contributed important skills and publications that changed medicine. It was a superb cancer center where young ambitions could be realized in a nurturing environment of an outstanding community focused on science. But from

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the many in my Tamoxifen Team, several must be mentioned because they either changed medical practice, created new knowledge in tamoxifen pharmacology that would change the way we perceived mechanisms, or created new models that would be critical for future advances.

Anna Riegel (née Tate) demonstrated outstanding skills as an undergraduate student at Leeds University Department of Pharmacology where I was her tutor, and she received a first-class honors degree in pharmacology, a distinction in her master's degree in steroid endocrinology, and was awarded a Fulbright Hays Scholarship to study for a Ph.D. with me at the McArdle Laboratory for Cancer Research at the University of Wisconsin-Madison. She published a pivotal paper in cancer research with myself, Elwood Jensen, and Geoffrey Greene, on the shape of the estrogen and 4-hydroxytamoxifen ER complex conceived through as study of antibodies to the human ER [45]. This model complemented studies I was conducting with Jack Gorski [46] that presaged (rather accurately) the subsequent crystallization of the ligand-binding domain of the ER with estrogens and antiestrogens some 15 years later [47, 48]. Anna was also an important part of our team that contributed to the debate in the early 1980s about the localization of the ER within the cells of estrogen target tissues. The two-step hypothesis stated that estrogen diffuses into the cell, binds with high affinity to the cytoplasmic ER, and is translocated to the nucleus where it is transformed (activated) to initiate estrogen-specific gene transcription (protein synthesis and growth) [49]. However, McGuire's group in San Antonio and others had suggested that unoccupied ER was actually in the nucleus [50]. Two pieces of evidence swayed scientific opinion to create a new model of estrogen action: monoclonal antibodies demonstrated nuclear ER in breast cancer cells in an estrogen-free environment [51] and the Gorski group used cytochalasin B with GH3 rat pituitary cells to create nucleoplasts and cytoplasts to show ER only on the nucleoplasts [52]. It was strange to recall I had worked as a summer student with Steven Carter, at ICI Pharmaceuticals Division in the summer of 1967, who discovered the cell enucleation property of the natural product cytochalasin B [53].

In 1983, we reported to the Endocrine Society in San Antonio that tamoxifen analogs that could not be metabolically activated to 4-hydroxytamoxifen switched on growth of the immature rat uterus and induced progesterone synthesis but apparently without translocating the ER complex from the cytoplasm to the nucleus. The person responsible for these studies was the late Barbara Gosden whom Anna recommended for a job in my laboratory for 2 years. Anna and Barbara were students of the master's course in steroid endocrinology at Leeds in 1979 when Barbara completed her studies in vivo. She was concerned that she had the wrong answer—but she had made a discovery. This was exploited and confirmed using triphenylethylene estrogens that only weakly bound to the ER in the rat uterus. The uterus grew and progesterone receptors were made, but the ER "appeared" to remain in the cytosolic fraction (or cytoplasmic) and not in the nuclear fraction. We got the same result as the metabolically resistant tamoxifen analogs and proposed using this example of tamoxifen structural pharmacology to

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suggest that it was the technique of uterine cell disruption that caused the abnormal result inconsistent with the 2-step model—but nobody cared [54]!

Marco M. Gottardis was a superb experimentalist with animal models. I inherited him from David Rose in 1983, and he accepted my invitation to become a Ph.D. student on the Human Oncology Ph.D. Training Program in 1984. The publications from his Ph.D. changed medicine. Marco demonstrated the chemopreventive actions of tamoxifen and raloxifene in carcinogen-induced rat mammary carcinoma model [23]. He concluded that raloxifene in the long term would not be superior to tamoxifen. The update of the STAR trial (Chap. 8) was to prove his data correct some 20 years later [27]. In the mid-1980s, at a time when long-term adjuvant tamoxifen treatment was being tested, there was no knowledge about acquired drug resistance to tamoxifen. Marco established the first laboratory model of acquired tamoxifen resistance in athymic mice [55]. Tamoxifen resistance is unique in the transplantable model as it takes the form of tamoxifen-stimulated growth. He showed that estrogen withdrawal (a decade later this was equivalent to aromatase inhibitor treatment) and used the first pure antiestrogen [56] to demonstrate that these strategies were appropriate second-line therapies to be used in clinic.

Perhaps of greatest significance clinically was the superb experimental model of bitransplantation of a human endometrial and breast tumor in athymic mice. The tumors were both ER positive, but tamoxifen only blocked estrogen-stimulated growth of the breast tumor but enhanced the growth of the endometrial tumor. The clinical significance was clear. Women taking long-term tamoxifen needed to be checked for endometrial cancer growth. I presented a pivotal lecture in Italy during a celebration of the 900th anniversary of the University of Bologna, and this was noted by clinicians in the audience. Dr. Hardell from Sweden immediately reported about our laboratory finding in a letter to the Lancet and described several anecdotal cases he had observed of endometrial cancer in tamoxifen-treated patients. I replied [57] that we needed a placebo-controlled clinical evaluation to settle the matter once and for all. Fornander and colleagues [22] showed that 5 years of adjuvant tamoxifen would increase the detection of endometrial cancer by fivefold in postmenopausal women compared to placebo-treated women. The standard of care changed for women treated with tamoxifen with the introduction of routine gynecological examinations. This saved lives and is an excellent example of the potential for improvements in women's health with rapid clinical translation. The process from conceiving the laboratory study to publicizing and publishing the results in Cancer Research, followed by correspondence to the Lancet and the fast clinical publications in the Lancet, was 2–3 years.

Shun Yen Jiang came to my laboratory on a 4-year scholarship from Taiwan to learn molecular biology. However, she gave my Tamoxifen Team far more with a succession of firsts. She created two estrogen deprivation-resistant breast cancer cell lines from MCF-7 cells. These are MCF7:5C [58] that was to be so critical for our understanding of estrogen-induced apoptosis. These cells were waiting for Joan Lewis to "discover" in the deep freeze a decade later (Chap. 9). Shun Yen also created the MCF7:2A cells, the only breast cancer cells with a high molecular

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weight ER protein completely characterized by John Pink and found to be 6 and 7 exon repeats in the ligand-binding domain [59, 60]. John also documented the two different systems regulating ER synthesis in breast cancer [61] and with Cathy Murphy the first ER-positive to ER-negative transition in breast cancer cell lines during estrogen deprivation [62, 63]. Shun Yen Jiang subsequently reversed the process by creating the first stable transfectant of the ER gene into an ER-negative breast cancer cell line MDA-MB-231 [64]. This advance in cell biology was passed on to Bill Catherino who created a stable transfectant of a natural mutant ER asp351tyr (BC2) [65] from a tamoxifen-stimulated tumor developed by Doug Wolf [66] who discovered the mutant ER in one particular tumor cell line [66].

All my students start with multiple projects in the expectation that one would bear fruit. With Doug, all bore fruit but this was not clear at the time. But this is what good (and reliable) results in the laboratory really are. I gave Doug another couple of projects to address and to discover the mechanism of tamoxifen-stimulated growth. One hypothesis at the time in the early 1990s was that metabolic activation of tamoxifen to the 4-hydroxytamoxifen metabolite would also produce an estrogenic *cis* isomer to cause growth (Cathy Murphy demonstrated that this was not true as not all isomers were antiestrogenic [67]). Doug used a fixed ring tamoxifen analog that could not isomerize to prove that it was the actual drug not an isomer that caused growth [68]. All of this closely interconnected research passed from generations of students to the next as the optimal model for progress. Progress and knowledge to aid patients was achieved in the nested environment at UW-Madison. A big breakthrough for us at the UW-Madison was yet to come. In the early 1990s, growth factor pathways were the answer to cancer. I set Doug Wolf the problem of characterizing estrogen and tamoxifen-stimulated tumor growth through their growth factor pathways in Marco's model of tamoxifen resistance. However, when Doug addressed the question, all the physiologic estrogen-stimulated tumors derived from acquired tamoxifen-resistant tumors disappeared. He was embarrassed and very apologetic that he had repeated the experiment several times—tamoxifen-stimulated tumors grew just as Marco described 5 years earlier, but estrogen caused tumors to melt away. He believed he had failed to deliver the expected result from Marco's work, but he had made a discovery—estrogen-induced apoptosis [69]. This was confirmed at a new institution, the Robert Lurie Comprehensive Cancer Center at North Western University [70], and ultimately changed medicine through first providing us with data to be funded by the Department of Defense to study mechanisms that would be used to develop treatment for antihormone-resistant breast cancer [71] and the results of the WHI estrogen-alone study where there is a significant decrease in breast cancers and mortality [72].

But it did not end there with innovation of discovery by students. Mei Wei Jeng was a student from Taiwan, who had obtained a master's degree in Iowa. She made several important advances in cellular pharmacology. Using Shun Yen Jiang's stable transfectants of wild-type ER in MDA-MB-231 cells (S-10s, all my students named their own cell lines!), Mei Wei Jeng addressed what seemed the obvious hypothesis that the cause of estrogen action to stop growth of the S-10 cells was

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because it blocked TGF-α (a growth-stimulating hormone) production but increased TGF-β (a growth inhibitor) production. This was not true [73], it was the other way around, but new knowledge gave the Tamoxifen Team standard estrogen target gene TGF-α for all our subsequent work. Meey-Huey Jeng was also very keen to discover the role of the progestogens in the modulation of TGF-β. Instead, she discovered that 19-nortestosterone derivatives of the oral contraceptives were estrogens on MCF-7 cell growth [74], as was the antiprogentin RU486 at high doses [75].

So why did we ever do a bone study? Dr. Urban Lindgren, from the Karolinska Institute in Stockholm, was doing a sabbatical at the UW-Madison. He approached me to consider creating a rapidly developing osteoporosis model in ovariectomized rats. Nothing was really known about the effect of individual nonsteroidal antiestrogens on rat bone, so it seemed fairly simple as an experiment: antiestrogens would create bone loss in the ovariectomized rat. I obviously selected tamoxifen as there was really nothing known clinically about the action on bone, and it might aid the move to clinical testing of tamoxifen as a preventive for breast cancer. After Eli Lilly abandoned their anticancer program to create a rival to tamoxifen with keoxifene for breast cancer treatment, I was left with a large quantity of the nonsteroidal antiestrogen in the laboratory. I selected keoxifene as a competitor to tamoxifen. The reason was because keoxifene was less estrogenic in the uterus than tamoxifen [15]; this would probably make bone loss much worse. Lindgren taught Eric Phelps, an exchange student at UW-Madison, how to do the ash density study and then to our surprise another discovery! Tamoxifen was estrogen-like in bone as was keoxifene, and the combination with estradiol benzoate was additive [26]. These data were repeatedly rejected in "Bone" journals, so I wrote our results up for the refereed journal Breast Cancer Research and Treatment as I guessed correctly that the medical community would be interested in our findings. The results with tamoxifen were confirmed by others, and the Wisconsin Tamoxifen Study was propelled forward with other clinicians committed to the idea that tamoxifen would built bone [40]. Keoxifene became raloxifene, and funnily enough, the target site specificity of a combination of estrogen and a nonsteroidal antiestrogen being clinically valuable has now evolved into bazedoxifene and conjugated equine estrogen being used to control menopausal symptoms but with uterine and breast safety (Chap. 10)! A lot was initiated in Wisconsin that would change medical science with selective estrogen receptor modulators (SERMs).

All of this decade of discovery at the Wisconsin Comprehensive Cancer Center would provide a foundation for the subsequent interrogation of the modulation of the ER by selective ER modulator by Jennifer MacGregor-Schafer [76, 77] and Hong Liu [78, 79] at the Robert H. Laurie Cancer Center, Northwestern University, Chicago. The Wisconsin scientists would pass the baton of estrogen-induced apoptosis to the Northwestern Medical Scientists Kathy Yao, Dave Bentrem [70, 80], Clodio Osipo [81], Hong Liu [82], and Joan Lewis [83] (Chap. 9). It has always been a Tamoxifen Team effort from generation to generation.

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References

1. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
2. Allen KE, Clark ER, Jordan VC (1980) Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 71:83–91
3. Cuzick J, Baum M (1985) Tamoxifen and contralateral breast cancer. Lancet 2:282
4. Jordan VC (1984) Biochemical pharmacology of antiestrogen action. Pharmacol Rev 36:245–276
5. (1983) Controlled trial of tamoxifen as adjuvant agent in management of early breast cancer. Interim analysis at four years by Nolvadex Adjuvant Trial Organisation. Lancet 1:257–261
6. Tormey DC, Jordan VC (1984) Long-term tamoxifen adjuvant therapy in node-positive breast cancer: a metabolic and pilot clinical study. Breast Cancer Res Treat 4:297–302
7. Scottish Cancer Trials Office (MRC) E (1987) Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee. Lancet 2:171–175
8. Harper MJ, Walpole AL (1967) A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 13:101–119
9. Harper MJ, Walpole AL (1967) Mode of action of I.C.I. 46,474 in preventing implantation in rats. J Endocrinol 37:83–92
10. Harper MJ, Walpole AL (1966) Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. Nature 212:87
11. Terenius L (1970) Two modes of interaction between oestrogen and anti-oestrogen. Acta Endocrinol (Copenh) 64:47–58
12. Terenius L (1971) Structure-activity relationships of anti-oestrogens with regard to interaction with 17-beta-oestradiol in the mouse uterus and vagina. Acta Endocrinol (Copenh) 66:431–447
13. Black LJ, Goode RL (1980) Uterine bioassay of tamoxifen, trioxifene and a new estrogen antagonist (LY117018) in rats and mice. Life Sci 26:1453–1458
14. Black LJ, Goode RL (1981) Evidence for biological action of the antiestrogens LY117018 and tamoxifen by different mechanisms. Endocrinology 109:987–989
15. Black LJ, Jones CD, Falcone JF (1983) Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 32:1031–1036
16. Jordan VC, Gosden B (1983) Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 113:463–468
17. Jordan VC, Gosden B (1983) Differential antiestrogen action in the immature rat uterus: a comparison of hydroxylated antiestrogens with high affinity for the estrogen receptor. J Steroid Biochem 19:1249–1258
18. Jordan VC, Robinson SP (1987) Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc 46:1870–1874
19. Satyaswaroop PG, Zaino RJ, Mortel R (1983) Human endometrial adenocarcinoma transplanted into nude mice: growth regulation by estradiol. Science 219:58–60
20. Satyaswaroop PG, Zaino RJ, Mortel R (1984) Estrogen-like effects of tamoxifen on human endometrial carcinoma transplanted into nude mice. Cancer Res 44:4006–4010
21. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48:812–815
22. Fornander T, Rutqvist LE, Cedermark B et al (1989) Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1:117–120
23. Gottardis MM, Jordan VC (1987) Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 47:4020–4024

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Author's Proof

24. Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC (1990) Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 50:3189–3192
25. Beall PT, Misra LK, Young RL et al (1984) Clomiphene protects against osteoporosis in the mature ovariectomized rat. Calcif Tissue Int 36:123–125
26. Jordan VC, Phelps E, Lindgren JU (1987) Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10:31–35
27. Vogel VG, Costantino JP, Wickerham DL et al (2010) Update of the National Surgical Adjuvant Breast and Bowel Project study of tamoxifen and raloxifene (STAR) P-2 trial: preventing breast cancer. Cancer Prev Res (Phila) 3:696–706
28. Jordan VC (1988) Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 6:589–595
29. Love RR, Mazess RB, Tormey DC et al (1988) Bone mineral density in women with breast cancer treated with adjuvant tamoxifen for at least two years. Breast Cancer Res Treat 12:297–302
30. Love RR, Newcomb PA, Wiebe DA et al (1990) Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 82:1327–1332
31. Love RR, Wiebe DA, Newcomb PA et al (1991) Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann Intern Med 115:860–864
32. Love RR, Mazess RB, Barden HS et al (1992) Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 326:852–856
33. Turner RT, Wakley GK, Hannon KS, Bell NH (1987) Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. J Bone Miner Res 2:449–456
34. Turner RT, Wakley GK, Hannon KS, Bell NH (1988) Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. Endocrinology 122:1146–1150
35. Turner RT, Evans GL, Wakley GK (1993) Mechanism of action of estrogen on cancellous bone balance in tibiae of ovariectomized growing rats: inhibition of indices of formation and resorption. J Bone Miner Res 8:359–366
36. Jones AL, Powles TJ, Treleaven JG et al (1992) Haemostatic changes and thromboembolic risk during tamoxifen therapy in normal women. Br J Cancer 66:744–747
37. Kedar RP, Bourne TH, Powles TJ et al (1994) Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial. Lancet 343:1318–1321
38. Powles TJ, Jones AL, Ashley SE et al (1994) The Royal Marsden Hospital pilot tamoxifen chemoprevention trial. Breast Cancer Res Treat 31:73–82
39. Powles TJ, Hickish T, Kanis JA et al (1996) Effect of tamoxifen on bone mineral density measured by dual-energy x-ray absorptiometry in healthy premenopausal and postmenopausal women. J Clin Oncol 14:78–84
40. Turken S, Siris E, Seldin D et al (1989) Effects of tamoxifen on spinal bone density in women with breast cancer. J Natl Cancer Inst 81:1086–1088
41. Black LJ, Sato M, Rowley ER et al (1994) Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 93:63–69
42. Evans G, Bryant HU, Magee D et al (1994) The effects of raloxifene on tibia histomorphometry in ovariectomized rats. Endocrinology 134:2283–2288
43. Evans GL, Bryant HU, Magee DE, Turner RT (1996) Raloxifene inhibits bone turnover and prevents further cancellous bone loss in adult ovariectomized rats with established osteopenia. Endocrinology 137:4139–4144
44. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189

Uncorrected Proof

Author's Proof

45. Tate AC, Greene GL, DeSombre ER et al (1984) Differences between estrogen- and antiestrogen-estrogen receptor complexes from human breast tumors identified with an antibody raised against the estrogen receptor. Cancer Res 44:1012–1018
46. Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741–4745
47. Brzozowski AM, Pike AC, Dauter Z et al (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
48. Shiau AK, Barstad D, Loria PM et al (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937
49. Jensen EV, DeSombre ER (1973) Estrogen-receptor interaction. Science 182:126–134
50. Zava DT, McGuire WL (1977) Estrogen receptor. Unoccupied sites in nuclei of a breast tumor cell line. J Biol Chem 252:3703–3708
51. King WJ, Greene GL (1984) Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature 307:745–747
52. Welshons WV, Lieberman ME, Gorski J (1984) Nuclear localization of unoccupied oestrogen receptors. Nature 307:747–749
53. Carter SB (1967) Effects of cytochalasins on mammalian cells. Nature 213:261–264
54. Jordan VC, Tate AC, Lyman SD et al (1985) Rat uterine growth and induction of progesterone receptor without estrogen receptor translocation. Endocrinology 116:1845–1857
55. Gottardis MM, Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 48:5183–5187
56. Gottardis MM, Jiang SY, Jeng MH, Jordan VC (1989) Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res 49:4090–4093
57. Jordan VC (1989) Tamoxifen and endometrial cancer. Lancet 1:733–734
58. Jiang SY, Wolf DM, Yingling JM et al (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 90:77–86
59. Pink JJ, Jiang SY, Fritsch M, Jordan VC (1995) An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. Cancer Res 55:2583–2590
60. Pink JJ, Wu SQ, Wolf DM et al (1996) A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. Nucleic Acids Res 24:962–969
61. Pink JJ, Jordan VC (1996) Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. Cancer Res 56:2321–2330
62. Murphy CS, Meisner LF, Wu SQ, Jordan VC (1989) Short- and long-term estrogen deprivation of T47D human breast cancer cells in culture. Eur J Cancer Clin Oncol 25:1777–1788
63. Pink JJ, Bilimoria MM, Assikis J, Jordan VC (1996) Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. Br J Cancer 74:1227–1236
64. Jiang SY, Jordan VC (1992) Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. J Natl Cancer Inst 84:580–591
65. Catherino WH, Wolf DM, Jordan VC (1995) A naturally occurring estrogen receptor mutation results in increased estrogenicity of a tamoxifen analog. Mol Endocrinol 9:1053–1063
66. Wolf DM, Jordan VC (1994) The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31:129–138
67. Murphy CS, Langan-Fahey SM, McCague R, Jordan VC (1990) Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. Mol Pharmacol 38:737–743

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68. Wolf DM, Langan-Fahey SM, Parker CJ et al (1993) Investigation of the mechanism of tamoxifen-stimulated breast tumor growth with nonisomerizable analogues of tamoxifen and metabolites. J Natl Cancer Inst 85:806–812
69. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127:23–33
70. Yao K, Lee ES, Bentrem DJ et al (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6:2028–2036
71. Ellis MJ, Gao F, Dehdashti F et al (2009) Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 302:774–780
72. Anderson GL, Chlebowski RT, Aragaki AK et al (2012) Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. Lancet Oncol 13:476–486
73. Jeng MH, Jiang SY, Jordan VC (1994) Paradoxical regulation of estrogen-dependent growth factor gene expression in estrogen receptor (ER)-negative human breast cancer cells stably expressing ER. Cancer Lett 82:123–128
74. Jeng MH, Parker CJ, Jordan VC (1992) Estrogenic potential of progestins in oral contraceptives to stimulate human breast cancer cell proliferation. Cancer Res 52:6539–6546
75. Jeng MH, Langan-Fahey SM, Jordan VC (1993) Estrogenic actions of RU486 in hormone-responsive MCF-7 human breast cancer cells. Endocrinology 132:2622–2630
76. MacGregor Schafer J, Liu H, Bentrem DJ et al (2000) Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. Cancer Res 60:5097–5105
77. Schafer JI, Liu H, Tonetti DA, Jordan VC (1999) The interaction of raloxifene and the active metabolite of the antiestrogen EM-800 (SC 5705) with the human estrogen receptor. Cancer Res 59:4308–4313
78. Liu H, Lee ES, Deb Los Reyes A et al (2001) Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor alpha complex. Cancer Res 61:3632–3639
79. Liu H, Park WC, Bentrem DJ et al (2002) Structure-function relationships of the raloxifene-estrogen receptor-alpha complex for regulating transforming growth factor-alpha expression in breast cancer cells. J Biol Chem 277:9189–9198
80. Bentrem D, Fox JE, Pearce ST et al (2003) Distinct molecular conformations of the estrogen receptor alpha complex exploited by environmental estrogens. Cancer Res 63:7490–7496
81. Osipo C, Gajdos C, Liu H et al (2003) Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 95:1597–1608
82. Liu H, Lee ES, Gajdos C et al (2003) Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 95:1586–1597
83. Lewis JS, Meeke K, Osipo C et al (2005) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97:1746–1759

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Abstract	The laboratory study to show that tamoxifen was likely to increase the risk of endometrial cancer in women was initially rapidly confirmed by examination of an adjuvant clinical trials database. However, there was a concern raised that tamoxifen was producing high-grade endometrial cancer, but this claim turned out to be unsubstantiated. In general, the NSABP (P-1) study showed low-grade good prognosis disease with no deaths from endometrial cancer. In contrast, the laboratory finding in the early 1990s that select strains of rats were vulnerable to hepatocarcinoma following lifetime exposure to high daily doses of tamoxifen was of concern and caused labeling changes for tamoxifen. The concerns that there would be significant increases in fatal hepatocellular carcinomas were unfounded on examination of clinical trials data and subsequently ongoing monitoring of epidemiology databases.	

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Chapter 6
Carcinogenesis and Tamoxifen

Abstract The laboratory study to show that tamoxifen was likely to increase the risk of endometrial cancer in women was initially rapidly confirmed by examination of an adjuvant clinical trials database. However, there was a concern raised that tamoxifen was producing high-grade endometrial cancer, but this claim turned out to be unsubstantiated. In general, the NSABP (P-1) study showed low-grade good prognosis disease with no deaths from endometrial cancer. In contrast, the laboratory finding in the early 1990s that select strains of rats were vulnerable to hepatocarcinoma following lifetime exposure to high daily doses of tamoxifen was of concern and caused labeling changes for tamoxifen. The concerns that there would be significant increases in fatal hepatocellular carcinomas were unfounded on examination of clinical trials data and subsequently ongoing monitoring of epidemiology databases.

Introduction

The toxicological requirements to develop a drug as a breast cancer therapy contrast dramatically from the requirements necessary for approval for a drug to be used in well women. Metastatic breast cancer is fatal, so a small therapeutic index between toxicity and clinical benefit is appropriate. In contrast, drugs must be rigorously tested and demonstrate no toxicological issues in tests of mutagenesis and carcinogenesis in preclinical models prior to FDA approval for use in humans without disease.

Tamoxifen was launched as a treatment for metastatic breast cancer in postmenopausal women in the United Kingdom in 1973, and similar approvals occurred in the United States in December 1977. Toxicology was based on short-term tests in two species, and clinical data showed efficiency with a remarkable lack of side effects [1, 2]. However, the successful use of tamoxifen as an adjuvant therapy in node-positive breast cancer and the expanded use of tamoxifen as an adjuvant therapy in node-negative breast cancer, where the majority of patients are cured

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by early surgery and radiation, enhanced enthusiasm to use tamoxifen to prevent breast cancer in high-risk populations of well women. The laboratory data supported the development of prospective clinical trials [3, 4], and it was already known that tamoxifen, used as an adjuvant therapy, reduced the incidence of contralateral breast cancer by 50 % [5]. The idea that tamoxifen would be used in well women therefore mandated a renewed evaluation of the toxicology of tamoxifen despite the fact that the drug had been successfully used ubiquitously in breast cancer therapy for 20 years.

A surprise was in store. Firstly was the finding that tamoxifen was target site specific and enhanced endometrial cancer growth but, at the same time, prevented estrogen-stimulated breast tumor growth [6] (Fig. 5.1, Chap. 5). Secondly was the findings of long-term carcinogenesis studies in the rat; tamoxifen was a liver carcinogen.

Tamoxifen and the Endometrial Carcinoma

The association between tamoxifen and endometrial carcinoma in humans is based upon clinical observations during the period 1988–1994. There is believed to be an increased incidence of endometrial carcinoma associated with breast cancer; therefore, physicians need to take extra precautions for the routine care of their patients. Tamoxifen is known to have estrogen-like properties in the uterus of some patients [7–9], so treatment would be expected to encourage the growth of preexisting disease, a principle which was first illustrated in the laboratory (Fig. 5.1, Chap. 5). When a breast tumor and endometrial carcinoma are co-transplanted into athymic mice, tamoxifen will block the estrogen-stimulated growth of the breast tumor while stimulating the endometrial carcinoma to grow [6, 10]. This is a demonstration of tamoxifen's target site specificity.

When evaluating reports of tamoxifen-induced endometrial carcinoma, it is important to appreciate that the incidence of occult endometrial tumors found in autopsy specimens is approximately five times the reported incidence in the general population [11]. The estrogen-like properties of tamoxifen can cause uterine hyperemia and proliferation, facilitating the growth of occult disease and leading to symptoms such as spotting and bleeding. Deaths from endometrial carcinoma occurred during tamoxifen therapy for breast cancer, initially raising the possibility that an aggressive form of the disease could be caused by tamoxifen. However, it should be remembered that only one-third of metastatic endometrial cancer is hormonally responsive, so tamoxifen would not be expected to control the majority of advanced endometrial cancer.

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Deaths from Endometrial Carcinoma

Magriples and coworkers [12] completed a computer search of the Yale New Haven Hospital tumor registry for the decade 1980–1990 and identified 53 patients with a history of breast cancer who subsequently developed endometrial cancer. Fifteen of these patients received tamoxifen and 38 did not. A total of 3,457 women were initially identified with breast cancer, but the proportion receiving tamoxifen was not stated. Interestingly enough, all of the tamoxifen-treated patients received 40-mg tamoxifen daily rather than the standard 20 mg daily. Five patients died of endometrial carcinoma during tamoxifen therapy, and the tumors from tamoxifen-treated patients were in general (67 %) poorly differentiated endometrial carcinomata (Table 6.1). The authors concluded "it appears that women receiving tamoxifen as treatment for breast cancer who subsequently develop uterine cancer are at risk for high-grade endometrial cancers that have a poor prognosis." Examination of the duration of tamoxifen therapy received by women before detection and subsequent death from endometrial carcinoma shows that three patients received tamoxifen for 12 months or less.

Deaths in women taking tamoxifen for relatively short time periods were also reported in the Stockholm study [13] (Table 6.2) and the NSABP study B14 [14] (Table 6.3). In the Stockholm study, 931 patients were randomized to receive either 2 or 5 years of tamoxifen 40 mg daily. Seventeen patients have been diagnosed with endometrial carcinoma; however, examination of patient records shows that each of the women received tamoxifen for less than 2 years, and the reported tumors were grades 1 and 2. One of the major conclusions of the study was that the probability of developing endometrial carcinoma was increased with duration of tamoxifen therapy [15]. However, examination of the 17 cases of endometrial carcinoma detected in the nearly 1,000 patients shows that 13 of the women who developed endometrial carcinoma received less than 2 years of tamoxifen treatment [13].

In the NSABP study [14], 1,419 patients were randomized to receive 20-mg tamoxifen daily for 5 years, and 1,220 patients were recruited and registered to receive at least 5 years of tamoxifen. Twenty-three women developed endometrial carcinoma with an average time of evaluation of 8 years and 5 years for randomized and registered patients, respectively. Six patients in the tamoxifen-treated arms died after a diagnosis of endometrial carcinoma (Table 6.3). Three of the six women took tamoxifen for less than 2 years, and one woman never took tamoxifen, although she was included in the analysis based on intention to treat. Overall, eight of the total of 23 women taking tamoxifen received the drug for less than 2 years.

Based on an analysis of current clinical trials data available in the 1990s, it was possible to address the question [12] of whether an aggressive high-grade disease develops during tamoxifen therapy.

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t1.1 **Table 6.1** Clinical and pathological features of tamoxifen-treated breast cancer patients who died of endometrial carcinoma in the Yale Haven Cancer Survey [12]

t1.2	Patient	Age	Months on tamoxifen	Endometrial histology	FIGO stage
t1.3	1	71	120	Adenosquamous FG3	NS
t1.4	2	85	96	Endometrial	IIIC
t1.5	3	60	12	Endometrioid FG3	NS
t1.6	4	71	12	MMT	IVB
t1.7	5	87	3	Papillary serous	NS

t1.8 *NS* not stated, *MMT* mixed Mullerian tumor

t2.1 **Table 6.2** Clinical and pathological features of tamoxifen-treated patients who died of endometrial carcinoma in the Stockholm trial [13]

t2.2	Age	Months on tamoxifen	Patient	Endometrial histology	FIGO stage
t2.3	68	24	1	NS grade I	I
t2.4	69	13	2	NS grade II	I
t2.5	70	11	3	NS grade II	IV

t3.1 **Table 6.3** Characteristics and pathological feature of tamoxifen-treated breast cancer patients who died of endometrial carcinoma (EC) in the NSABP B14 trial [14]

t3.2	Patient	Age	Months on tamoxifen	Off tamoxifen to diagnosis (months)	Histology	FIGO stage	Cause of death
t3.3	1	68	65	0	Papillary	IVG1	PE
t3.4	2	54	42	23	Carcinosarcoma	11BG3	EC
t3.5	3	58	22	73	Papillary	1BG3	EC
t3.6	4	68	5	0	Endometrioid	1A	CV disease
t3.7	5	63	9	0	Endometrioid	1BG2	EC
t3.8	6	66	0	0	Endometrioid	1BG1	EC

t3.9 *CV* cardiovascular, *PE* pulmonary embolus

106 Tamoxifen and the Stage of Endometrial Carcinoma

107 The discovery that high doses of tamoxifen will cause adduct formation in rat liver
108 DNA [16] occurred at the same time that Magriples and coworkers [12] reported
109 tamoxifen was associated with high-grade endometrial carcinoma. This naturally
110 lead to the possibility that tamoxifen may be causing progression of preexisting
111 disease. However, randomized clinical trial [14] and an epidemiology study [17]
112 did not support this proposition, although, in each case, the authors state that the
113 numbers are too low to draw any definite conclusions. Fisher and coworkers [14]
114 compared the stages of endometrial carcinoma and tumor grades found in their
115 study and in the Yale Tumor Registry Study and the Swedish Trail. An epidemiol-
116 ogy study from the Netherlands Cancer Institute is included for comparison [17]
117 (Table 6.4). It is difficult to make absolute comparisons of these data, but several
118 points can be made. The studies all found that the majority of tumors reported were
119 stage 1 endometrial carcinoma. The percentage of low-grade tumors was variable

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with 78 %, 33 %, 53 %, and 52 % for the NSABP, Yale, Swedish, and Netherlands studies, respectively. Additionally, for comparison purposes, a Gynecologic Oncology Group Study [18] of 222 patients found the distribution of cases to be 82 % low-grade cases (FIGO I and 2) and 18 % high-grade cases (FIGO 3). Overall, the Yale group stood alone having the largest proportion of high-grade tumors, with 67 %. However, the fact that the events were so low, and patients with already advanced endometrial carcinoma were being given tamoxifen to treat breast cancer, made this fact not unexpected. Based on this analysis of available data, there was insufficient evidence to support the statement that "women receiving tamoxifen as treatment for breast cancer who subsequently develop uterine cancer are at high risk for high-grade endometrial cancers that have a poor prognosis [12]." Nevertheless, the fact that there was an increase in the incidence of endometrial cancer was a major clue with the use of tamoxifen for both treatment and prevention in the 1990s. How bad was the fear of tamoxifen for some patients? In the mid-1990s, one patient said to me: "Thank God! I have been diagnosed with ER-negative breast cancer and I don't have to take tamoxifen."

Incidence of Endometrial Cancer with Tamoxifen

It is now possible to give a precise rate for the incidence of endometrial carcinoma in tamoxifen-treated patients. The results from the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) [19] have shown that tamoxifen increases the incidence of endometrial cancer and was strongly correlated with age. The risk of incidence or death from endometrial cancer was very little in the younger age group (<45, or 45–54 years) with only one death and 11 incidents of endometrial cancer in the <45 years group with ER-positive breast cancer and seven deaths and 71 incident cases of endometrial cancer in the 55–69 years group with ER-positive breast cancer (incidence 3.8 % in the tamoxifen group vs. 1.1 % in the control group; absolute increase 2.6 %, 95 % CI) [19].

Most importantly, the new knowledge about the small but significant increase in endometrial cancer in postmenopausal patients treated with long-term adjuvant tamoxifen therapy acted as a forewarning for the NCI NSABP P-1 prevention trial to remain vigilant for signs of spotting and bleeding on protocol. Accurate results for the detection of endometrial cancer in pre- and postmenopausal women at risk for breast cancer are documented in Table 6.4. There is a significant increase in endometrial cancer in postmenopausal population, but not in premenopausal women at risk for breast cancer [20, 21]. It is important to note that no patient died from endometrial cancer in the NCI/NSABP P-1 study, probably because of the meticulous surveillance practices during the study.

Finally, another clinical trial Study of Tamoxifen and Raloxifene (STAR), also known as NSABP P-2 trial, concluded, as well, that tamoxifen treatment increases the incidence of invasive uterine cancer in comparison to women with high risk of breast cancer treated with raloxifene [22]. Increase by 45 % (RR, 0.55; 95 % CI, AU1

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t4.1 **Table 6.4** Comparison of the uterine cancers in tamoxifen-treated and control patients [12–14, 17]

t4.2		NSABP		Yale tumor registry			Swedish trial				Netherlands cancer institute			
t4.3		Tamoxifen n = 25		Tamoxifen n = 15		No tamoxifen n = 38	Tamoxifen n = 17		No tamoxifen n = 5		Tamoxifen n = 23		No tamoxifen n = 75	
t4.4		Events	%	Events	%	Events %	Events	%	Events	%	Events	%	Events	%
t4.5	Stage													
t4.6	I	21	88	7	78	23 88	14	82	4	100	17	85	62	87
t4.7	II–IV	3	12	2	22	3 12	3	18	0	0	3	15	9	13
t4.8	Total no. staged	24		9		26	17		4		20		71	
t4.9	Histological grade													
t4.10	Low (good)	18	78	5	33	26 74	8	53	4	100	12	52[a]	24	32[a]
t4.11	High (poor)	5	22	10	67	9 26	7	47	0	0	11	48	51	68
t4.12	Total no. graded	23		15		35	15		4		23		75	

t4.13 [a]Calculated from a statement made by the authors in the discussion of the paper [17]. No breakdown of histological grade was presented in the results, although the morphological classification for users and nonusers of tamoxifen was in the same proportions. The proportion of well-differentiated tumors in the no tamoxifen group of this study is very low in comparison to all the studies and the survey in [18]

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0.36–0.83) for invasive uterine cancer, and 80 % higher incidence of endometrial cancer in tamoxifen-treated group than in raloxifene treated (RR, 0.19; 95 %CI, 0.12–0.29).

Tamoxifen and Rat Liver Carcinogenesis

It is now clear that if I had pursued the idea of giving high doses of tamoxifen to prevent rat mammary carcinogenesis [4], then rat liver carcinogenesis would have been discovered in 1973 [23], and there would have been no tamoxifen, and hundreds of thousands of women would now be dead of breast cancer. There would probably be no aromatase inhibitors or SERMs. The pharmacological industry would not have advanced a known carcinogen for long-term therapy (adjuvant therapy) or chemoprevention, so the "gold standard" would not have existed for others to beat.

High daily doses of tamoxifen will produce hepatocellular carcinoma in the rat (Table 6.5) if administered for up to half the animal's lifetime. This is particularly true at a 45.2 mg/kg dose, when tumors are formed within 6 months in 29 % of the animals [24]. There is general agreement that high daily doses of tamoxifen result in the premature death of rats. In the study by Greaves and coworkers [25], 50 % of control female rats were alive and well at about 104 weeks (2 years), but treatment with 35 mg/kg tamoxifen daily produced 50 % deaths by 42 weeks. Interestingly, the low dose of 5 mg/kg/day increases the survival of male and female rats at 2 years (males, 30 % deaths in treated vs. 70 % deaths in controls; females, 25 % deaths in treated vs. 50 % deaths in controls). The authors note [25] that their low tamoxifen dose (5 mg/kg/day) completely inhibited the incidence of adenomas in the pituitary gland and adenocarcinomas of the mammary gland in female rats and almost completely inhibited adenomas of the pituitary gland and parathyroid gland in male rats.

The published studies indicate that there is a threshold level for liver carcinogenicity, which is approximately 3 mg/kg/day [24]. However, the study by Dragan and coworkers [27], using a different rat strain and experimental design, observed no hepatocellular carcinomata after 15 months of treatment. The design of the study divided carcinogenesis into initiation and promotion. Carcinogenesis was initiated with diethylnitrosamine (DEN 10 mg/kg oral) in partially hepatectomized Fischer F344 rats, and promotion to carcinogenesis was completed with tamoxifen in the feed at 250 ppm. Blood levels of tamoxifen were 230 $\pm$ 30 ng/ml (i.e., in the range of clinical experience [27]). It can be estimated that a 200-g rat consumes 10 g of food containing 2.5 mg tamoxifen per day, so a rat received a daily dose of 12.5 mg/kg, which is within the 10–30 mg/kg/day dosing regimens of other studies [24]. No hepatocellular carcinomata were observed if DEN, the initiator, was omitted, but tumors were seen if DEN was given with tamoxifen, leading the authors to conclude that tamoxifen is a promoter of hepatocellular carcinoma in the Fischer rat. However, all the other studies, mainly using Sprague-Dawley strains of rats and bolus administration of drug by lavage, suggest that tamoxifen is a complete carcinogen at high doses.

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t5.1 **Table 6.5** The occurrence of hepatocellular carcinoma in various rat strains during long-term tamoxifen treatment

t5.2 Strain of rat	Daily dose (mg/kg)	*n*	Duration (months)	Hepatocellular carcinoma %	(*n*)	Reference
t5.5 1. Sprague-Dawley (Crl:CD(BR))	2.8	57	15	0	(22)	[24]
t5.6	11.3	57	15	45	(11)	
t5.7	45.2	57	12	75	(4)	
t5.8 2. Wistar (Alpk: ApfSD)	5	52	24	16	(51)	[25]
t5.9	20	52	24	64	(51)	
t5.10	35	52	24	64	(51)	
t5.11 3. Sprague-Dawley (Crl:CD(BR))	11.3	84	12	44	(36)	[26]
t5.12	22.6	75	12	100	(24)	
t5.13 4. Fischer F344	12.5[a]	20	15	0	(8)	[27]

AU2

204 Tamoxifen and DNA Adduct Formation

205 Carcinogenesis requires genotoxicity, so it is important to correlate the formation of
206 DNA adducts with the formation of tumors in a particular organ for a sensitive
207 species. Mani and Kupfer [28] first showed that in human and rat liver microsome
208 systems in vitro [^{14}C], tamoxifen was metabolized by an NADPH-dependent
209 cytochrome P450-mediated activation system to intermediate(s) which covalently
210 bound to microsomal proteins. Han and Liehr [16] subsequently showed that the
211 administration intraperitoneally (i.p.) of tamoxifen (20 mg/kg/day) to Sprague-
212 Dawley rats resulted in two DNA adducts after only 1 day and up to six adducts
213 after 6 consecutive days of treatment. A similar result was observed by Hard and
214 associates [26] using 48 mg/kg/day tamoxifen for 7 days in Sprague-Dawley rats.
215 It is clear that large doses of tamoxifen can produce DNA adducts, but White and
216 coworkers [29] have investigated the dose adduct relationship in rats. Seven days of
217 dosing with between 5 and 45 mg tamoxifen/kg/day produced an almost linear
218 dose-dependent increase in DNA adducts in the Fischer 344 rat. At doses of less
219 than 5 mg/kg/day, tamoxifen did not alter the chromatograph from ^{32}P post-labeled
220 DNA from treated rats. It would appear, therefore, that there is a threshold for the
221 appearance of adducts with tamoxifen and the induction of liver tumors. The
222 metabolite α-hydroxytamoxifen was subsequently found to be responsible for
223 DNA adducts in rats (see Chap. 3).
224 White and colleagues [29] also examined whether adduct formation occurs in the
225 mouse, which does not produce liver tumors in response to tamoxifen. There is
226 DNA adduct formation in both C57B1/6 and DBA/2 mice; however, this is approx-
227 imately 30 % of that observed with a similar dosing schedule in the Fischer rats
228 [29], raising questions about the correlation between adduct formation and clini-
229 cally evident tumors.

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In humans, DNA adducts were not observed in the livers of tamoxifen-treated women; however, only limited samples were screened [30]. A study in vitro [31] demonstrated the ability to form DNA adducts with human and rat liver microsomes using 100-μM tamoxifen. Although the levels of DNA adducts are low and in the range of the studies in vivo with mice, the human liver was two to three times more effective at producing DNA adducts than the rat. The Sprague-Dawley rat livers used in the studies in vitro [31] are from a strain that is extremely sensitive to the carcinogenic actions of tamoxifen in vivo. Adduct formation in vitro can be dramatically altered by adding different cofactors [31], and the level of DNA adduct formation that is required for carcinogenesis may be dose related, as in the rat in vivo [29]. The level of adducts, 1–3 $\times$ 10^8 nucleotides, observed in the study of rat liver microsomes in vitro [31] is not in the carcinogenic range in vivo [29], although caution must be used when comparing in vivo and in vitro studies.

Overall, these data demonstrated that DNA adducts could be formed in vitro and in vivo, but the level of adduct formation seems critical for carcinogenesis. Adduct formation using human microsomes is very low, but this can be enhanced into the mouse range using cumene hydroperoxide as a cofactor [31]. However, mice do not produce liver tumors after long-term treatment. Thus, the most important issues in the 1990s were the species differences, the correlation between liver carcinogenesis and DNA adduct formation, the effect of the rate of repair of DNA in different species, and the relative doses used to demonstrate the carcinogenic effects of tamoxifen. However, the epidemiology of human liver cancer did not support patient risk evaluations in women taking tamoxifen. No correlation has been noted to this day, but in the 1990s, the concern was justified with the move to prevention and the possibility that the liver carcinogenesis could occur decades after taking the drug.

Doses of Tamoxifen in Animals and Man

A key argument made regarding rat liver carcinogenesis studies was that since the serum concentrations of tamoxifen obtained in the rat (Table 6.6) were within the range of serum concentrations achieved during the treatment of breast cancer, then the results are clinically relevant. It is generally believed that toxicology testing should be conducted to mimic human pharmacokinetics. However, the rat and mouse clear tamoxifen from the body at a much faster rate than the human so that higher doses must be administered to maintain the blood level in the human range used for treatment. Examination of the relative dosage regimens in different species and the resulting serum levels of tamoxifen illustrate the point. Serum levels of tamoxifen during the treatment of breast cancer with 10 mg twice daily (approximately 285 μg/kg daily for a 70-kg postmenopausal woman) are usually between 100 and 200 ng/ml [32]. In contrast, the administration of 50- or 100-μg tamoxifen

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daily to ovariectomized mature mice (approximately 2.5 mg/kg for a 20-g mouse) or immature rats (approximately 3 mg/kg for a 35-g rat) for 7–10 days results in pharmacological effects but produces serum levels of tamoxifen often below the level of detection by high performance liquid chromatography [33]. Only by giving high doses of tamoxifen (200 mg/kg) to animals can one adequately study circulating levels of drug [33]. We studied the circulating levels of tamoxifen in patients receiving high daily doses of tamoxifen. Increasing the daily dose to the limits of toxicity (10 mg/kg) [35] in humans reaches the dose range (5–35 mg/kg) used to treat rats in the liver carcinogenesis studies (Table 6.6). However, the blood levels are tenfold higher in the human. Comparable serum levels in the rat and human during tamoxifen treatment can only be produced by treating rats with high doses of tamoxifen. The schedules that are used to demonstrate liver carcinogenesis in the rat (5–40 mg/kg) are 20 times greater than the standard treatment regimen in women (20 mg daily or 285 μg/kg).

Testing at Comparable Therapeutic Levels

Tamoxifen, at a daily dose of 50 μg (250 μg/kg), inhibits the growth and development of dimethylbenzanthracene-induced rat mammary tumors [36]. This is equivalent to the therapeutic dose used to treat metastatic breast cancer and as an adjuvant therapy in node-positive and node-negative disease. The duration of therapy for the treatment of breast cancer can be indefinite in some clinical trials [37, 38], but most treatment plans use 5 years of adjuvant tamoxifen at a dose of 20 mg daily. With the life expectancy of most women being 80 years of age, this translates into about 6 % of a woman's lifetime, and most women are treated during their postmenopausal years. In contrast, studies of rat liver carcinogenesis employ a test system that starts at 6 weeks of age (just post-puberty) and treats daily with approximately 20 times the human dose for the rest of the animals' life. At a dose of 11.3 mg/kg, approximately half the rats develop liver tumors within a year [26] (Table 6.7).

It is important to state that the general need for carcinogenic testing is to establish whether an agent is carcinogenic per se not just at the level of therapeutic value. To achieve this, animals are tested with a high dose, with lower doses approaching the therapeutic range. A positive result in the animal test does not mean that human therapeutic levels will be carcinogenic but provides a warning of such a possibility. A treatment regimen of tamoxifen, 0.25 mg/kg daily, for 2–3 months during the second year of the rats' life would be an equivalent bioassay. This approach would give a realistic view of the toxicological risks observed in patients. Since the doses to be used are far below the level that causes adduct formation [29] and repair mechanisms occur after the cessation of therapy, there is little probability that animals will develop liver tumors, thus duplicating clinical experience.

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Table 6.6 Circulating serum levels obtained with different dosage regimens in the rat, mouse, and human (70-kg postmenopausal women) t6.1

Species	Dosage per day (mg/kg)	Duration	Tamoxifen concentration	Reference	
Human	0.28	>2 years	148	[32]	t6.3
Rat	3	7 days	<1	[33]	t6.4
Rat	200	7 days	1,000	[33]	t6.5
Mouse	2.5	7 days	<10	[33]	t6.6
Mouse	200	10 days	300	[33]	t6.7
Human	4.9	1 year	1,300	[33, 34]	t6.8
Human	Approx. 10	11 days	1,855	[35]	t6.9

Table 6.7 The levels of circulating tamoxifen achieved with the dosing regimens used in rats during carcinogenesis experiments t7.1

Rats	Dosage regimen (mg/kg)	Tamoxifen concentration (ng/ml)	Liver tumors	Reference	
1. Mature Wistar	5	166	Yes	[24]	t7.3
	20	644			t7.4
	35	636			t7.5
2. Mature Sprague-Dawley	11.3	138 ± 41	Yes	[25]	t7.6
	22.6	172 ± 103			t7.7
3. Mature Fischer	12.5[a]	230 ± 30	Yes	[27]	t7.8

[a]Based on estimate of daily food intake of 10 g per day of 250-mg tamoxifen/kg feed t7.9

Toxicological testing of new drugs in development to reduce the risks to patients is crucial, but tamoxifen has received extensive clinical testing over the past 40 years without producing major toxicities. Although it is argued that a decade is required for iatrogenic carcinogenesis in patients [39], there is currently little or no information to demonstrate that tamoxifen is a significant liver carcinogen in the human, as has been demonstrated for the rat [24]. The divergence of effects in rats and women is because of differences in the dose, duration and timing of tamoxifen treatment, differential metabolism, rapid repair responses in humans, and the susceptibility of some inbred strains of rat to hepatocellular carcinogens.

Conclusion

Overall, the effective translational research on the link between tamoxifen and the growth of endometrial cancer with the important step of taking our laboratory finding [6] to the clinical community [15] resulted in lives saved and put in place new gynecologic procedures that remain to this day. It was specifically stated: "Until the influence of TAM and other antiestrogens on endometrial cancers has been fully investigated, vigilance by physicians treating patients with these agents is needed to establish the clinical relevance (if any) of these observations." However, the other toxicological issue, rat liver carcinogenesis was not to evaporate so

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easily and is a lasting example of those observations in laboratory that do not necessarily translate to the clinic. However, Zeneca (originally ICI pharmaceuticals division) formally required a "black box" designation to comply with the toxicological findings. A lesson learned, but not unlike the fact the tamoxifen was a superb antifertility agent in the laboratory but did exactly the opposite in clinical practice!

Postscript. The results of the pioneering experiment by my Ph.D. student Marco Gottardis [6] on the target site specificity in breast cancer, and endometrial cancer was used by us to appeal to the clinical community to monitor their adjuvant clinical trials. This story is told in the Postscript to Chap. 5. Marco and I traveled to ICI pharmaceutical division in 1987, and he presented his work at Alderley Park for their staff. The staff at ICI took immediate action and contacted the Stockholm adjuvant clinical trial group to look at their database with different durations of tamoxifen [15, 40]. The results of their data collection process replicated our laboratory study, fewer contralateral breast cancers and more endometrial cancers with tamoxifen. It is interesting to observe that an examination of their paper published in 1989 shows that the axis for the duration of patient monitoring of their adjuvant tamoxifen trail for endometrial cancer extends for 10.5 years. In other words, they already had the data by the year 1987 when we first talked about our animal studies of human disease in 1987. The NSABP followed up with their evaluation of tamoxifen and endometrial cancer in 1991 [41]. All of these translational research successes were essential to prepare the clinical trials community for monitoring the proposed chemoprevention trials in women without breast cancer for endometrial cancer.

Wisconsin with its two cancer centers: the Wisconsin Comprehensive Cancer Center and McArdle Laboratory for Cancer Research had significant researchers in carcinogenesis. Henry Pitot, a former director of the McArdle Laboratory, was a world authority on hepatocarcinogenesis. Here was a superb opportunity to join forces on a "hot topic" in toxicology—rat carcinogenesis with nonsteroidal antiestrogens. Henry had a talented, keen, and enthusiastic postdoc. . .who successfully wrote up our proposal, and Henry generously resulted that I was the principal investigator as this was a "topical tamoxifen issue," and I was better positioned to be successful. He was correct and numerous publications subsequently followed. This is what cancer centers are all about—collaboration to aid and understanding of topics that will affect the well-being of patients, in this case the concern was about liver cancer with tamoxifen use. It also provided me with the opportunity to participate in the debate about the safety of tamoxifen at the national level and especially at hearings in the state of California. All through the development of tamoxifen, I had a philosophy of looking at "the good, the bad, and the ugly" of tamoxifen. Patient safety and patient mortality was always the goal.

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References

1. Cole MP, Jones CT, Todd ID (1971) A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer 25:270–275
2. EBCTCG (1988) Effects of adjuvant tamoxifen and of cytotoxic therapy on mortality in early breast cancer. An overview of 61 randomized trials among 28,896 women. N Engl J Med 319:1681–1692
3. Jordan VC (1974) Antitumour activity of the antiestrogen ICI 46,474 (Tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 5:354
4. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
5. Cuzick J, Baum M (1985) Tamoxifen and contralateral breast cancer. Lancet 2:282
6. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48:812–815
7. Cross SS, Ismail SM (1990) Endometrial hyperplasia in an oophorectomized woman receiving tamoxifen therapy. Case report. Br J Obstet Gynaecol 97:190–192
8. Gal D, Kopel S, Bashevkin M et al (1991) Oncogenic potential of tamoxifen on endometria of postmenopausal women with breast cancer–preliminary report. Gynecol Oncol 42:120–123
9. De Muylder X, Neven P, De Somer M et al (1991) Endometrial lesions in patients undergoing tamoxifen therapy. Int J Gynaecol Obstet 36:127–130
10. Satyaswaroop PG, Zaino RJ, Mortel R (1984) Estrogen-like effects of tamoxifen on human endometrial carcinoma transplanted into nude mice. Cancer Res 44:4006–4010
11. Horwitz RI, Feinstein AR (1986) Estrogens and endometrial cancer. Responses to arguments and current status of an epidemiologic controversy. Am J Med 81:503–507
12. Magriples U, Naftolin F, Schwartz PE, Carcangiu ML (1993) High-grade endometrial carcinoma in tamoxifen-treated breast cancer patients. J Clin Oncol 11:485–490
13. Fornander T, Hellstrom AC, Moberger B (1993) Descriptive clinicopathologic study of 17 patients with endometrial cancer during or after adjuvant tamoxifen in early breast cancer. J Natl Cancer Inst 85:1850–1855
14. Fisher B, Costantino JP, Redmond CK et al (1994) Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. J Natl Cancer Inst 86:527–537
15. Fornander T, Rutqvist LE, Cedermark B et al (1989) Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1:117–120
16. Han XL, Liehr JG (1992) Induction of covalent DNA adducts in rodents by tamoxifen. Cancer Res 52:1360–1363
17. van Leeuwen FE, Benraadt J, Coebergh JW et al (1994) Risk of endometrial cancer after tamoxifen treatment of breast cancer. Lancet 343:448–452
18. Boronow RC, Morrow CP, Creasman WT et al (1984) Surgical staging in endometrial cancer: clinical-pathologic findings of a prospective study. Obstet Gynecol 63:825–832
19. Davies C, Godwin J, Gray R et al (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378:771–784
20. Fisher B, Costantino JP, Wickerham DL et al (2005) Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97:1652–1662
21. Fisher B, Costantino JP, Wickerham DL et al (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90:1371–1388

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22. Runowicz CD, Costantino JP, Wickerham DL et al (2011) Gynecologic conditions in participants in the NSABP breast cancer prevention study of tamoxifen and raloxifene (STAR). Am J Obstet Gynecol 205:535 e1–535 e5
23. Jordan VC (1995) What if tamoxifen (ICI 46,474) had been found to produce rat liver tumors in 1973? A personal perspective. Ann Oncol 6:29–34
24. Williams GM, Iatropoulos MJ, Djordjevic MV, Kaltenberg OP (1993) The triphenylethylene drug tamoxifen is a strong liver carcinogen in the rat. Carcinogenesis 14:315–317
25. Greaves P, Goonetilleke R, Nunn G et al (1993) Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. Cancer Res 53:3919–3924
26. Hard GC, Iatropoulos MJ, Jordan K et al (1993) Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. Cancer Res 53:4534–4541
27. Dragan YP, Fahey S, Street K et al (1994) Studies of tamoxifen as a promoter of hepatocarcinogenesis in female Fischer F344 rats. Breast Cancer Res Treat 31:11–25
28. Mani C, Kupfer D (1991) Cytochrome P-450-mediated activation and irreversible binding of the antiestrogen tamoxifen to proteins in rat and human liver: possible involvement of flavin-containing monooxygenases in tamoxifen activation. Cancer Res 51:6052–6058
29. White IN, de Matteis F, Davies A et al (1992) Genotoxic potential of tamoxifen and analogues in female Fischer F344/n rats, DBA/2 and C57BL/6 mice and in human MCL-5 cells. Carcinogenesis 13:2197–2203
30. Martin EA, Rich KJ, White IN et al (1995) 32P-postlabelled DNA adducts in liver obtained from women treated with tamoxifen. Carcinogenesis 16:1651–1654
31. Pathak DN, Bodell WJ (1994) DNA adduct formation by tamoxifen with rat and human liver microsomal activation systems. Carcinogenesis 15:529–532
32. Langan-Fahey SM, Tormey DC, Jordan VC (1990) Tamoxifen metabolites in patients on long-term adjuvant therapy for breast cancer. Eur J Cancer 26:883–888
33. Robinson SP, Langan-Fahey SM, Johnson DA, Jordan VC (1991) Metabolites, pharmacodynamics, and pharmacokinetics of tamoxifen in rats and mice compared to the breast cancer patient. Drug Metab Dispos 19:36–43
34. Jordan VC, Bain RR, Brown RR et al (1983) Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. Cancer Res 43:1446–1450
35. Trump DL, Smith DC, Ellis PG et al (1992) High-dose oral tamoxifen, a potential multidrug-resistance-reversal agent: phase I trial in combination with vinblastine. J Natl Cancer Inst 84:1811–1816
36. Jordan VC (1983) Laboratory studies to develop general principles for the adjuvant treatment of breast cancer with antiestrogens: problems and potential for future clinical applications. Breast Cancer Res Treat 3(Suppl):S73–S86
37. Falkson HC, Gray R, Wolberg WH et al (1990) Adjuvant trial of 12 cycles of CMFPT followed by observation or continuous tamoxifen versus four cycles of CMFPT in postmenopausal women with breast cancer: an Eastern Cooperative Oncology Group phase III study. J Clin Oncol 8:599–607
38. Tormey DC, Gray R, Abeloff MD et al (1992) Adjuvant therapy with a doxorubicin regimen and long-term tamoxifen in premenopausal breast cancer patients: an Eastern Cooperative Oncology Group trial. J Clin Oncol 10:1848–1856
39. Jordan VC, Lababidi MK, Mirecki DM (1990) Anti-oestrogenic and anti-tumour properties of prolonged tamoxifen therapy in C3H/OUJ mice. Eur J Cancer 26:718–721
40. Fornander T, Rutqvist LE (1989) Adjuvant tamoxifen and second cancers. Lancet 1:616
41. Fisher B, Redmond C (1991) New perspective on cancer of the contralateral breast: a marker for assessing tamoxifen as a preventive agent. J Natl Cancer Inst 83:1278–1280

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Abstract	Tamoxifen was first shown to prevent the initiation and promotion of rat mammary carcinogenesis in the 1970s. During the 1990s, numerous trials were initiated to test the worth of tamoxifen to decrease the incidence of breast cancer in otherwise healthy women. The Royal Marsden study was first with a vanguard study in the 1980s followed by the National Surgical Breast and Bowel Project (NSABP) P-1 trial, the Italian Study of women not at risk, and the International Breast Cancer Study Group (IBIS). Multiple subsequent analyses all showed some efficacy to reduce breast cancer incidence, but the NSABP study was the strongest powered clinical trial uniformly demonstrating a 50 % decrease in incidence for both pre- and postmenopausal women at risk. As predicted, endometrial cancer was the most troublesome side effect, but only in postmenopausal women taking tamoxifen.	

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Chapter 7
Chemoprevention: Cinderella Waiting for the Ball

Abstract Tamoxifen was first shown to prevent the initiation and promotion of rat mammary carcinogenesis in the 1970s. During the 1990s, numerous trials were initiated to test the worth of tamoxifen to decrease the incidence of breast cancer in otherwise healthy women. The Royal Marsden study was first with a vanguard study in the 1980s followed by the National Surgical Breast and Bowel Project (NSABP) P-1 trial, the Italian Study of women not at risk, and the International Breast Cancer Study Group (IBIS). Multiple subsequent analyses all showed some efficacy to reduce breast cancer incidence, but the NSABP study was the strongest powered clinical trial uniformly demonstrating a 50 % decrease in incidence for both pre- and postmenopausal women at risk. As predicted, endometrial cancer was the most troublesome side effect, but only in postmenopausal women taking tamoxifen.

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Introduction

The idea of the prevention of breast cancer is not new, but significant practical progress has been made, through translational research, to make the idea feasible in some women. It is now possible to reduce the incidence of breast cancer through the inhibition of estrogen action.

Professor Antoine Lacassagne [1] stated a vision for the prevention of breast cancer at the annual meeting of the American Association of Cancer Research in Boston in 1936.

> If one accepts the consideration of adenocarcinoma of the breast as the consequence of a special hereditary sensibility to the proliferative actions of estrone, one is led to imagine a therapeutic preventative for subjects predisposed by their heredity to this cancer. It would consist – perhaps in the very near future when the knowledge and use of hormones will be better understood – in the suitable use of a hormone antagonistic or excretory, to prevent the stagnation of estrone in the ducts of the breast.

But no agent that was "antagonistic to prevent the stagnation of oestrone in the breast" was available to the clinician for clinical trial until tamoxifen [2, 3]. AU1

P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
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Tamoxifen became the "antiestrogen" of choice because of the following (a) There was a large body of basic biological evidence that this was a valid hypothesis to test (b) Tamoxifen was noted to reduce the incidence of contralateral breast cancer when used as an adjuvant therapy to treat micrometastases from the original primary tumor. (c) There was a huge and expanding clinical experience with tamoxifen as a long-term treatment for node-positive and node-negative breast cancer. The later point was important as the majority of patients with estrogen receptor (ER)-positive node-negative breast cancers are cured by surgery (plus radiation) alone, so 5 years of adjuvant tamoxifen was essentially already being used in the majority of these cured "well women" [4, 5].

In this chapter, the changing fashions in endocrine chemoprevention will be described. The change in fashion occurred because of significant advances in our AU2 understanding of the pharmacology of the drug group called the "nonsteroidal antiestrogens" [6] that underwent a metamorphosis in the mid-1980s [7] to become the new drug group called the selective ER modulators (SERMs) [8, 9]. See Chap. 5.

The Link Between Estrogen and Breast Cancer

The topic has recently been reviewed [10] in the refereed research literature so only essential facts will be considered here. The link between estrogen action for breast cancer growth of the original tumor, ER, and 5 years of adjuvant tamoxifen therapy to block tumor growth is compelling and proven in randomized clinical trials [11]. The findings can be simply summarized: breast tumors that are ER negative do not respond to tamoxifen treatment, tamoxifen dramatically reduces recurrence and mortality during 5 years of treatment for patients with ER-positive breast cancer, and this is maintained for at least 15 years following completion of therapy (see Chap. 4). Tamoxifen reduces the incidence of contralateral breast cancer by 50 % and this is sustained, but tamoxifen also increases the incidence of endometrial cancer in postmenopausal women (and mortality). The negative actions of adjuvant tamoxifen, such as deaths from endometrial cancer or thromboembolic disease, do not affect the overall benefit of treatment [11], but do impact on the use of tamoxifen for chemoprevention. Profound target site-specific actions of tamoxifen on the uterus in the recent overview [11] recapitulate and confirm the translational research with tamoxifen completed in the 1980s [12, 13] with the recognition of a small but significant increase in the incidence of endometrial cancer in postmenopausal women treated with tamoxifen. This finding eventually resulted in the paradigm shift away from tamoxifen to new opportunities, but this advances our story too quickly. In the 1980s, tamoxifen was the only medicine available for testing therapeutic and chemopreventive strategies with SERMs in the 1990s. The clinical community advanced with a responsibility to weigh risks and benefits in clinical trials to ensure the safety and long-term health of women at risk for breast cancer.

The treatment trials database and translational research were essential to address the hypothesis that tamoxifen, a nonsteroidal antiestrogen, could effectively block

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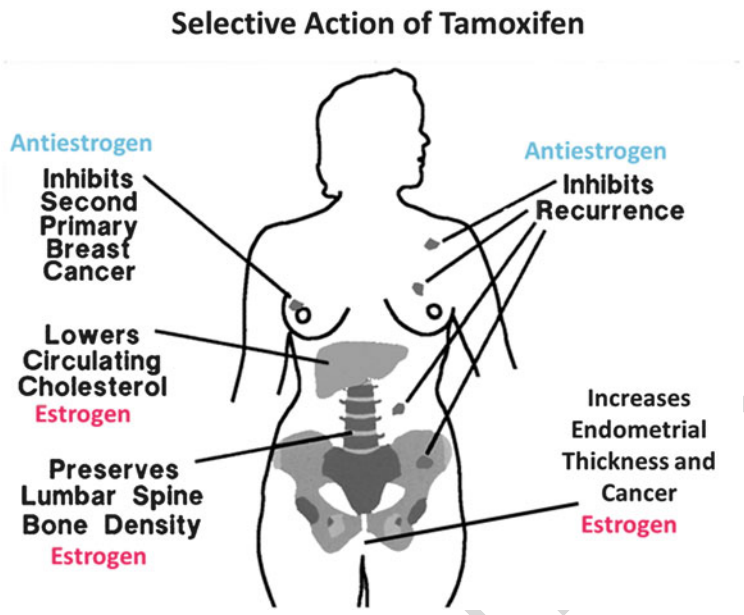

Fig. 7.1 Selective action of tamoxifen in target tissues. Tamoxifen is a SERM and has antiestrogenic action in the breast, but estrogenic properties in the bone, endometrium

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the genesis and growth of ER-positive breast cancer but would be ineffective against the growth of ER-negative disease. Nevertheless in the 1980s, estrogen was also considered to be an essential component of women's health by maintaining bone density and preventing coronary heart disease. Thus, if tamoxifen, an antiestrogen, prevented the development and growth of ER-positive breast cancer in half a dozen high-risk women per year per thousand [14], hundreds of other women in the selected population might subsequently develop osteoporosis and coronary heart disease. The intervention with tamoxifen would be detrimental to public health. The good news was tamoxifen was not an antiestrogen everywhere; it was the lead compound of the drug group that selectively modulated ER target tissues around the body (Fig. 7.1). The original work (described in Chap. 5) to investigate the target site pharmacology of tamoxifen in the laboratory was to provide a database with which to predict clinical outcomes and safety for future chemoprevention trials. This discovery ultimately facilitated the development of a new strategy for the utilization of new SERMs as chemopreventives in breast cancer.

Prevention of Mammary Cancer in Rodents

The expanding literature on the prevention of rodent mammary cancer was used to support the clinical use of tamoxifen to prevent breast cancer. As mentioned earlier, Lacassagne predicted that a therapeutic intervention could be developed that would

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"prevent or antagonize the congestion of estrone in the breast." Unfortunately, no therapeutic agent was available and all his predictions were based upon the known effect of early oophorectomy on the development of mammary cancer in high-incidence strains of mice [1]. Clearly, the indiscriminate oophorectomy of young women would be an inappropriate intervention. The animal studies with tamoxifen were undertaken for two reasons: first, to establish the efficacy of tamoxifen in well-described models of carcinogenesis and, second, to discover whether tamoxifen would always be an inhibitor or whether the drug would ever exacerbate tumorigenesis. Two animal model systems were used extensively: the carcinogen-induced rat mammary carcinoma model and mouse mammary tumor virus (MMTV)-infected strains of mice.

The mammary carcinogens 7,12-dimethylbenz[α]anthracene (DMBA) [15] and N-nitrosomethylurea (NMU) [16] induce tumors in young female rats. The timing of the carcinogenic insult is very important, because as the animals age they become resistant to the mammary carcinogens. Tumorigenesis does not occur in oophorectomized animals, and the sooner oophorectomy is performed after the carcinogenic insult, the more effective it is in preventing the development of tumors [17].

The administration of tamoxifen to carcinogen-treated rats prevents the initiation of carcinogenesis, and animals remain tumor-free [18, 19]. The short-term administration of tamoxifen at different times after the carcinogenic insult is effective in reducing the number of tumors that develop [20, 21], although most animals develop at least one tumor after therapy is stopped.

Continuous tamoxifen therapy that is started at 1 month after the administration of carcinogens completely inhibits the appearance of mammary tumors [22, 23]. Under these circumstances, tamoxifen is preventing promotion and suppressing the appearance of occult disease. In fact, if treatment is stopped prematurely (i.e., a 3–4-month duration of therapy), the microfoci of transformed cells grow into palpable tumors. Because the timing of initiation in human breast cancer is unknown, and unlike the laboratory model not all women will develop tumors, tamoxifen will be given to target populations to suppress, and there is expectation that this will reverse the promotional effects of estrogen during carcinogenesis. Lacassagne performed his pioneering mammary tumor experiments linking estrogen with carcinogens in the high-risk mouse [24], so this was another model to use.

Until 1989, there was a paucity of information about the efficacy of tamoxifen to inhibit mouse mammary tumorigenesis. This was true in part because tamoxifen is estrogenic in short-term tests in oophorectomized [25] and immature mice [26]. However, the finding that long-term tamoxifen therapy renders the oophorectomized mouse vagina [27] and athymic mouse uterus [12] refractory to estrogenic stimuli prompted a reconsideration of the value of tamoxifen as a preventive in mouse mammary tumor models.

High-incidence strains of mice that develop mammary tumors are infected with MMTV, which is transferred to the offspring in the mothers' milk [28]. Tumorigenesis appears to be ovarian dependent, because the highest incidence of tumors appears in females, and tumorigenesis can be delayed or prevented depending upon the age at

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oophorectomy [29]. Steroid hormones activate the pro-viral MMTV [30], which in turn can initiate an increase in growth factors from the viral integration site Int. 2 [31]. Promotion of the initiated cells with steroid hormones and prolactin then completes tumorigenesis.

Long-term tamoxifen therapy, after an early cycle of pregnancy and weaning to facilitate early tumorigenesis, is equivalent to an ovariectomy performed at 4 months in reducing tumorigenesis to 50 % at 14 months of age. However, tamoxifen is superior to oophorectomy, even after therapy is stopped, because ovariectomized animals continue to develop tumors, whereas animals previously treated with tamoxifen do not develop any more tumors [32].

We followed up on initial observations with an investigation of tumorigenesis in virgin mice. In this study design, mice develop mammary tumors during their second year of life. Again, long-term tamoxifen therapy started at 3 months of age is superior to oophorectomy at 3 months. Fifty percent of the oophorectomized animals develop tumors by the third year of life, whereas 90 % of tamoxifen-treated mice remain tumor-free [33]. These studies are illustrated in Fig. 7.2.

Overall, the results of the studies in the mouse model are particularly interesting because they changed our view of the interspecies pharmacology of tamoxifen. Long-term treatment with tamoxifen results in an initial classification of tamoxifen as an estrogen, but within a few weeks the pharmacology changes and tamoxifen becomes an antiestrogen. An understanding of this process was seen to have important implications for the long-term use of tamoxifen as an adjuvant therapy and a preventive.

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Tamoxifen: The First SERM for the Prevention of Breast Cancer in High-Risk Populations

Forty years ago, tamoxifen was shown to prevent the induction [18] and promotion [20] of carcinogen-induced mammary cancer in rats. Similarly, tamoxifen was also shown to prevent the development of mammary cancer induced by ionizing radiation in rats [34]. These laboratory observations, coupled with the emerging preliminary clinical observation that adjuvant tamoxifen could prevent contralateral breast cancer in women [35], provided a rationale for Dr. Trevor Powles, who, in 1986, established the vanguard study at the Royal Marsden Hospital in England to test whether tamoxifen could prevent breast cancer in high-risk women [36].

During the 1990s, much progress was achieved to answer the question: "Does tamoxifen have worth in the prevention of breast cancer in select high-risk women?" The results of four international trials that address this question—the Royal Marsden study, the NSABP/NCI study, the Italian study, and the IBIS trial—have been reported. These data will be presented in detail as well as their subsequent updates in the past decade. A summary of trial characteristics and findings are presented in Table 7.1.

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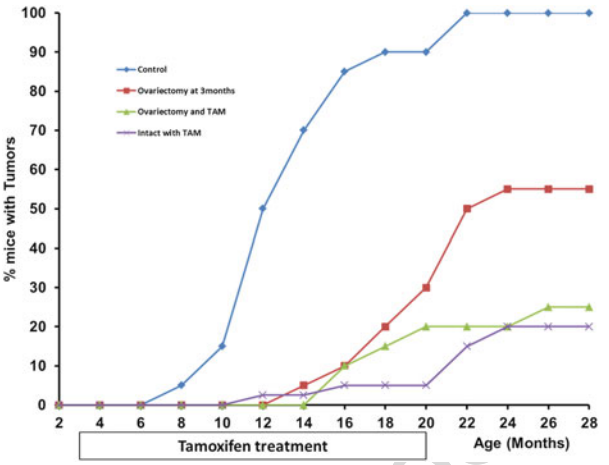

Fig. 7.2 The ability of long-term tamoxifen treatment or ovariectomy on the development of the mammary tumors in virgin C3H/OUJ mice. Long-term tamoxifen therapy is more effective as a chemopreventive than ovariectomy

t1.1 **Table 7.1** Comparison of the characteristics of various breast cancer treatment trials

t1.2 Characteristic	NSABP	Royal Marsden	Italian	IBIS
t1.3 Sample size	13,388	2,471	5,408	7,152
t1.4 Women years of follow-up	46,858	12,355	5,408	29,800
t1.5 Participants <50	40 %	62 %	36 %	52 %
t1.6 Breast cancer incidence per 1,000				
t1.7 Placebo	6.7	5.5	2.3	6.7
t1.8 Tamoxifen	3.4	4.7	2.1	4.7

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176 Royal Marsden Study

177 Powles and coworkers recruited 2,484 women aged 30–70 to a placebo-controlled
178 trial using 20 mg of tamoxifen daily for up to 8 years. Women were eligible if their
179 risk of breast cancer was increased due to family history. Each participant had at
180 least one first-degree relative with breast cancer under age 50; or a first-degree
181 relative affected at any age, plus an additional affected first- or second-degree
182 relative; or a first-degree relative with bilateral breast cancer. Women with a history
183 of benign breast biopsy and an affected first-degree relative of any age were also
184 eligible. Women with a history of venous thrombosis, any previous malignancy, or
185 an estimated life expectancy of fewer than 10 years were excluded [37, 38]. A total
186 of 2,494 women consented to participate in the study, and 23 were excluded from
187 final analysis due to the presence of preexisting ductal carcinoma in situ (DCIS) or
188 invasive breast carcinoma [38]. The trial was undertaken to evaluate the problems
189 of accrual, acute symptomatic toxicity, compliance, and safety as a basis for
190 subsequent large national, multicenter trials designed to test whether tamoxifen
191 can prevent breast cancer. However, the trial has also been analyzed for breast
192 cancer incidence [38].

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Acute symptomatic toxicity was low for participants on tamoxifen or placebo, and compliance remained correspondingly high: 77 % of women on tamoxifen and 82 % of women on placebo remained on medication at 5 years, as predicted. There was a significant increase in hot flashes (34 % vs. 20 %), mostly in premenopausal women ($P < 0.005$); vaginal discharge (16 % vs. 4 %; $P < 0.005$); and menstrual irregularities (14 % vs. 9 %; $P < 0.005$), respectively. At the most recent follow-up, 320 women had discontinued tamoxifen and 176 had discontinued placebo prior to the study's completion ($P < 0.005$).

Until their report in 1994 [37], the Marsden group observed no thromboembolic episodes; a detailed analysis of other coagulation parameters in a sequential subset of women also found no significant changes in protein S, protein C, or cross-linked fibrinogen degradation products. At 70 months, no significant difference in the incidence of deep vein thrombosis or pulmonary embolism was observed between groups. A significant fall in total plasma cholesterol occurred within 3 months and was sustained over 5 years of treatment [39–41]. The decrease affected low-density lipoprotein, with no change in apolipoproteins A and B or high-density lipoprotein cholesterol.

In contrast, tamoxifen exerted antiestrogenic or estrogenic effects on bone density, depending on menopausal status. In premenopausal women, early findings demonstrated a small but significant ($P < 0.05$) loss of bone in both the lumbar spine and hip at 3 years [41]. In contrast, postmenopausal women had increased bone mineral density in the spine ($P < 0.005$) and hip ($P < 0.001$) compared to nontreated women.

Finally, the Marsden group made an extensive study of gynecological complications associated with tamoxifen treatment in healthy women. Since ovarian and uterine assessment by transvaginal ultrasound became available sometime after the trial's start, many subjects did not have a baseline evaluation. Ovarian screening demonstrated a significantly increased risk ($P < 0.005$) of detecting benign ovarian cysts in premenopausal women who had received tamoxifen for more than 3 months compared to controls. There were no changes in ovarian appearance in postmenopausal women [37]. A careful examination of the uterus with transvaginal ultrasonography using color Doppler imaging in women taking tamoxifen showed that the organ was usually larger; moreover, women with histological abnormalities had significantly thicker endometria [42]. Of particular interest in this regard was the observation that 20 mg of tamoxifen daily exerted a time-dependent proliferation of the endometrium in premenopausal and early postmenopausal women. This effect appeared to be mediated by the stromal component, since no cases of cancer or even epithelial hyperplasia were observed among the tamoxifen-treated group in the Italian study with 33 women [43].

Although the vanguard study has provided invaluable information about the biological effects of tamoxifen in healthy women, the trial was not designed to answer the question of whether tamoxifen prevents breast cancer. In spite of this, an analysis of breast cancer incidence was reported at a median follow-up of 70 months, when 42 % of the participants had completed therapy or withdrawn [38]. During the study, 336 women on tamoxifen and 305 on placebo received hormone-replacement

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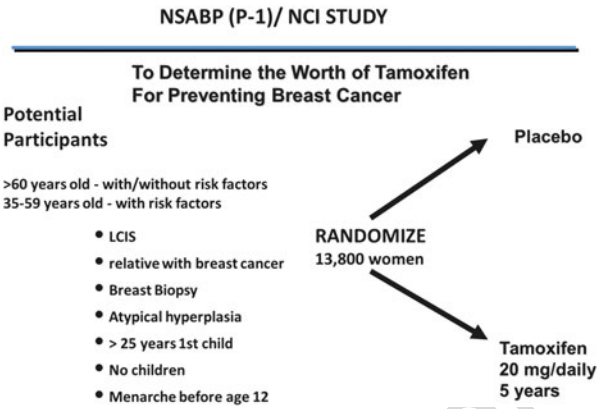

Fig. 7.3 The study design for the NSABP/NCI P-1 trial. On the *left* are the risk factors, according to the Gail model of breast cancer risk assessment based on which the participants of the study were selected

therapy. No difference in the incidence of breast cancer was observed between the groups. There were 34 carcinomas in the tamoxifen group and 36 in the placebo group-a relative risk of 1.06. Of the 70 cancers, only 8 were ductal carcinoma in situ. An analysis of the subset of women on hormone-replacement therapy did not demonstrate an interaction with tamoxifen treatment.

NSABP/NCI P-1 Study

This study opened in the United States and Canada in May of 1992 with an accrual goal of 16,000 women to be recruited at 100 North American sites. It closed after accruing 13,338 in 1997 due to the high-risk status of the participants. The study design is illustrated in Fig. 7.3. Those eligible for entry included any woman over the age of 60 or women between the ages of 35 and 59 whose 5-year risk of developing breast cancer, as predicted by the Gail model [14], was equal to that of a 60-year-old woman. Additionally, any woman over age 35 with a diagnosis of lobular carcinoma in situ (LCIS) treated by biopsy alone was eligible for entry to the study. In the absence of LCIS, the risk factors necessary to enter the study varied with age, such that a 35-year-old woman must have had a relative risk (RR) of 5.07, whereas the required RR for a 45-year-old woman was 1.79. Routine endometrial biopsies to evaluate the incidence of endometrial carcinoma in both arms of the study were also performed.

The breast cancer risk of women enrolled in the study was extremely high, with no age group having an RR of less than 4—including the over-60s group. Recruitment was also balanced, with about one-third younger than 50 years, one-third between 50 and 60 years, and one-third older than 60 years. Secondary end points of the study included the effect of tamoxifen on the incidence of fractures and cardiovascular deaths. Most importantly, the study planned to provide the first information about the role of genetic markers in the etiology of breast cancer. It was hoped to establish whether tamoxifen has a role to play in the treatment of

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women who are found to carry somatic mutations in the BRCA-1 gene. This did not occur as the number of patients with BRCA-1/2 mutations was not significant in the population [44].

The first results of the NSABP study were reported in September 1998, after a mean follow-up of 47.7 months [45]. There were a total of 368 invasive and noninvasive breast cancers in the participants: 124 in the tamoxifen group and 224 in the placebo group. A 49 % reduction in the risk of invasive breast cancer was seen in the tamoxifen group, and a 50 % reduction in the risk of noninvasive breast cancer was observed. A subset analysis of women at risk due to a diagnosis of LCIS demonstrated a 56 % reduction in this group. The most dramatic reduction was seen in women at risk due to atypical hyperplasia, where risk was reduced by 86 %.

The benefits of tamoxifen were observed in all age groups, with a relative risk of breast cancer ranging from 0.45 in women aged 60 and older to 0.49 for those in the 50- through 59-year-old age group, and 0.56 for women aged 49 and younger. A benefit for tamoxifen was also observed for women with all levels of breast cancer risk within the study, indicating that the benefits of tamoxifen are not confined to a particular lower risk or higher risk subset. Benefits were observed in women at risk on the basis of family history and those whose risk was due to other factors.

As expected, the effect of tamoxifen occurred on the incidence of ER-positive tumors, which were reduced by 69 % per year. The rate of ER-negative tumors in the tamoxifen group (1.46 per 1,000 women) did not significantly differ from the placebo group (1.20 per 1,000 women). Tamoxifen reduced the rate of invasive cancers of all sizes, but the greatest difference between the groups was the incidence of tumors 2.0 cm or less. Tamoxifen also reduced the incidence of both node-positive and node-negative breast cancer. The beneficial effects of tamoxifen were observed for each year of follow-up in the study. After year 1, the risk was reduced by 33 % and, in year 5, by 69 %.

Tamoxifen also reduced the incidence of osteoporotic fractures of the hip, spine, and radius by 19 %. However, the difference approached, but did not reach, statistical significance. This reduction was greatest in women aged 50 and older at study entry. No difference in the risk of myocardial infarction, angina, coronary artery bypass grafting, or angioplasty was noted between groups.

The study confirmed the association between tamoxifen and endometrial carcinoma (Figs. 7.4 and 7.5). The relative risk of endometrial cancer in the tamoxifen group was 2.5. The increased risk was seen in women aged 50 and older, whose relative risk was 4.01 (Fig. 7.5). There was no significance in the incidence of endometrial carcinoma in tamoxifen- or placebo-treated premenopausal women. All endometrial cancers in the tamoxifen group were grade 1 and none of the women on tamoxifen died of endometrial cancer. There was 1 endometrial cancer death in the placebo group. Although there is no doubt that tamoxifen increases the risk of endometrial cancer, it is important to recognize that this increase translates to an incidence of 2.3 women per 1,000 per year who develop endometrial carcinoma. More women in the tamoxifen group developed deep vein thrombosis (DVT) than in the placebo group (Fig. 7.6). Again, this excess risk was confined to women

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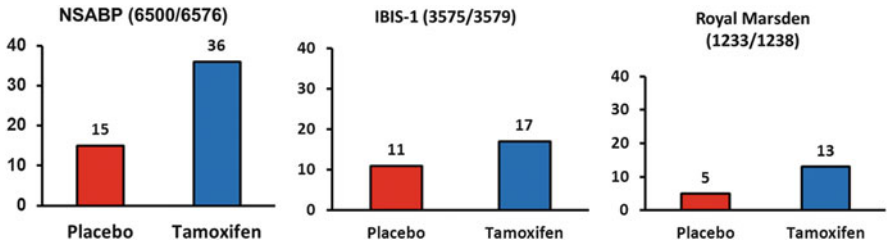

Fig. 7.4 The correlation between the increase in endometrial carcinoma incidence and tamoxifen treatment. In all three clinical trials show an increase in the incidence of endometrial carcinomas in tamoxifen-treated cohorts

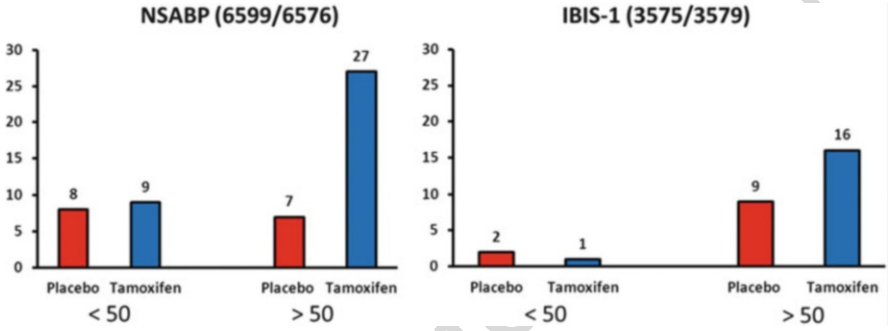

Fig. 7.5 The correlation between increase of endometrial carcinoma incidence and the age of the patient. The results of all three clinical trials showed that the increase in endometrial carcinoma significant incidence occurred in postmenopausal patients (>50 years of age) treated with tamoxifen in comparison to premenopausal patients (<50 years of age)

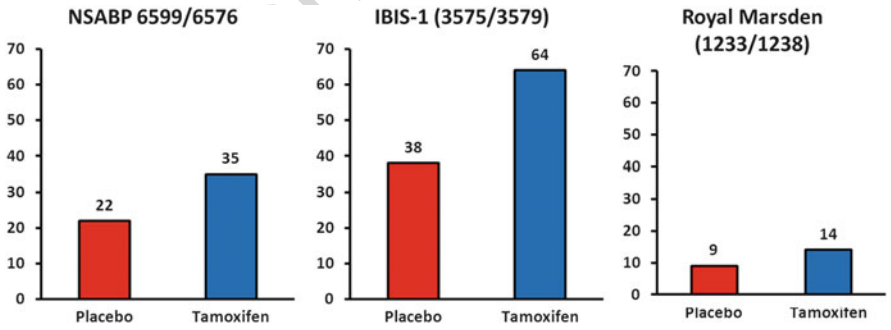

Fig. 7.6 Incidence of deep vein thrombosis (*DVT*) is significantly increased in tamoxifen-treated patients

aged 50 and older. The relative risk of DVT in the older age group was 1.71. (95 % CI 0.85–3.58). An increase in pulmonary emboli was also seen in the older women taking tamoxifen, with a relative risk of approximately 3 (Fig. 7.7). Three deaths

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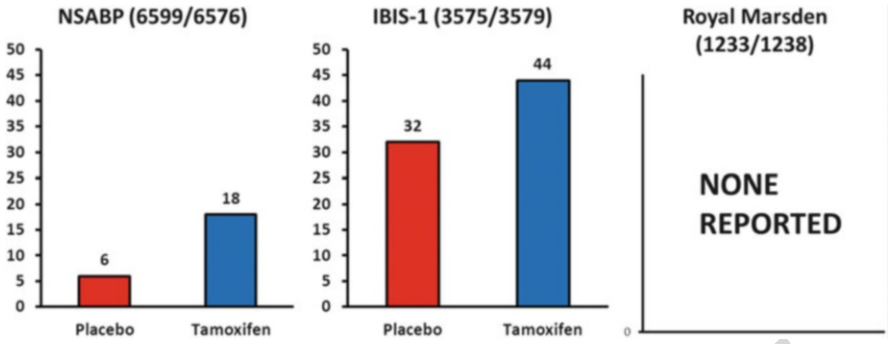

Fig. 7.7 Incidence of pulmonary embolism is increased in tamoxifen-treated patients. Observed in NSABP/P-1 and IBIS trials, but none were reported in the Royal Marsden trials

from pulmonary emboli occurred in the tamoxifen arm, but all were in women with significant comorbidities. An increased incidence of stroke (RR 1.75) was also seen in the tamoxifen group, but this did not reach statistical significance.

An assessment of the incidence of cataract formation was made using patient self-report. A small increase in cataracts was noted in the tamoxifen group—a rate of 24.8 women per 1,000 compared to 21.7 in the placebo group. There was also an increased risk of cataract surgery in the women on tamoxifen. These differences were marginally, statistically significant, and observed in the older patients in the study. This finding emphasizes the ocular safety of tamoxifen first predicted by Harper and Walpole in the 1960s [25], but as will be seen in Chap. 8, raloxifene does not have this effect. These findings emphasize the need to assess the patient's overall health status before making a decision to use tamoxifen for breast cancer risk reduction.

An assessment of quality of life showed no difference in depression scores between groups [46]. Hot flashes were noted in 81 % of the women on tamoxifen compared to 69 % of the placebo group, and the tamoxifen-associated hot flashes appeared to be of greater severity than those in the placebo group. Moderately bothersome or severe vaginal discharge was reported by 29 % of the women in tamoxifen group and 13 % in the placebo group [47]. No differences in the occurrence of irregular menses, nausea, fluid retention, skin changes, or weight gain or loss were reported.

Italian Study

The third tamoxifen prevention study, performed in Italy, began in October 1992 and randomized 5,408 women aged 35–70 to 20 mg of tamoxifen daily for 5 years [48]. Women were required to have had a hysterectomy for a nonneoplastic condition to obviate concerns about an increased risk of endometrial carcinoma.

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There was no requirement that participants be at risk for breast cancer development, and in fact, those who underwent premenopausal oophorectomy with hysterectomy actually had a slightly reduced risk of breast cancer development. Women with endometriosis, cardiac disease, and deep venous thrombosis were excluded from the study. Although 5,408 women were randomized into this study, 1,422 withdrew and only 149 completed 5 years of treatment.

The incidence of breast cancer did not differ between groups, with 19 cases in the tamoxifen group and 22 in the placebo group. Tumor characteristics, including size, grade, lymph node status, and receptor status, also did not differ between groups.

The incidence of thrombophlebitis was increased in the tamoxifen group. A total of 64 events were reported, 38 in the tamoxifen group and 18 in the placebo group ($P = 0.0053$). However, 42 of these were superficial phlebitis.

No differences in the incidence of cerebrovascular ischemic events were observed [48].

In 2003, a brief communication was published on the Italian Study that also compared the effectiveness of tamoxifen in cohorts of women who were using hormone-replacement therapy (HRT) or not. The results showed no significant difference between women taking tamoxifen or placebo in women who never used HRT and were in low-risk group ($P = 0.44$), and among women in the same cohort but in the high-risk group, there was a nonsignificant difference in favor of tamoxifen ($P = 0.099$). In the cohort of women that have used HRT during the trial and were in the low-risk group, there was also no statistically significant difference in women taking tamoxifen or placebo ($P = 0.31$); however, in the high-risk group there was a significant difference in favor of tamoxifen ($P = 0.009$).

The International Breast Cancer Intervention Study (IBIS-I)

The IBIS-I trial was a double-blind placebo-controlled randomized trial of tamoxifen [49]. Women at high risk (7,152) of breast cancer, between ages of 35 and 7 years were randomized into two groups. Women were randomized either into the placebo group (3,574) and women treated with 20 mg daily tamoxifen group (3,578). A total of 13 patients were excluded from the study, and the remaining were followed up for 5 years. The primary outcome measure was the incidence of breast cancer. After a median 50-month follow-up, 69 breast cancer cases were reported in the 5,378 women group treated with tamoxifen, and 101 cases in the 3,566 women placebo group, thus indicating a 32 % reduction ($P = 0.013$). Endometrial cancer was increased not significantly (11 vs. 2, $P = 0.2$) (Fig. 7.4), and thromboembolic events were significantly increased in the tamoxifen-treated group (43 vs. 17, $P = 0.001$) (Fig. 7.7). Based on these results, the authors concluded that preventive administration of tamoxifen is contradicted in women at high risk of thromboembolic disease. Tamoxifen should be stopped as an antithrombotic

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measure after surgeries or immobilization. However, tamoxifen does reduce the incidence of breast cancer by about a third, and non-breast-cancer causes of death are not increased by tamoxifen [49].

Follow-Up of Chemoprevention Studies with Tamoxifen

The main result from all the studies is that once tamoxifen is stopped, the antitumor effects sustained, systemic symptomology disappears, but the major side effect of an increase in endometrial cancer continues to accumulate in postmenopausal women [50–52]. Again it is tempting to speculate that the nascent breast cancer have been altered to survive in an environment of continuous tamoxifen, acquired resistance evolves, and then a woman's own estrogen causes apoptosis and tumoricidal actions in the "prepared" breast cancer cells after tamoxifen is stopped. This concept is discussed in detail in Chap. 9.

Tamoxifen again became a pioneering medicine but this time as the first drug to be FDA approved to reduce the risk of developing cancer, specifically ER-positive breast cancer. However, the translational research on endometrial cancer risk with tamoxifen [12, 53] demanded a safer solution to chemoprevention with SERMs. A strategy was already in place (Chap. 5) to move forward the first SERM to prevent osteoporosis and prevent breast cancer at the same time without the risk of endometrial cancer being increased. Keoxifene, the failed breast cancer drug, became raloxifene.

Two Approaches to the Chemoprevention of Breast Cancer

The successful clinical completion of the chemoprevention studies in women at high risk of developing breast cancer during the late 1990s resulted in FDA approval of tamoxifen for risk reduction in pre- and postmenopausal women in 1998. Despite reservations about tamoxifen and its toxicology (Chap. 6) for chemoprevention, the drug remains a cheap and lifesaving drug for the treatment of breast cancer worldwide. The data of endometrial cancer, deep vein thrombosis, and pulmonary embolism appear mainly in postmenopausal women [50]. However, the drug has both efficacy and an excellent safety profile in premenopausal women.

A recent review of the literature [54] concluded that "the risk of endometrial cancer, deep vein thrombosis and pulmonary embolism is low in women <50 years who take tamoxifen for breast cancer prevention. The risk decreases from the active to follow-up phase of treatment. Education and counseling are the cornerstones of breast cancer chemoprevention."

Nevertheless, despite the safety issues being low in premenopausal women, no other country has approved tamoxifen for chemoprevention in women with a high risk of developing breast cancer.

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Chemoprevention of breast cancer did, however, expanded dramatically throughout the 1990s based upon the laboratory work conducted with the discovery of selective estrogenic and antiestrogenic actions of estrogen target sites around the body. This work at the Wisconsin Clinical Cancer Center (Chap. 5) would subsequently be known in the literature as selective ER modulation. The strategic view described earlier (Chap. 5) was further refined to create a roadmap for drug development by the pharmaceutical industry. Simply stated, the proposal was to develop multifunctional medicines to aim at reducing the morbidity and mortality of a major disease affecting millions of women after menopause but, at the same time, reducing the risk of breast cancer. In 1990, this proposal was published in Cancer Research, the flagship journal of the American Association for Cancer Research [7]. This was the B.F. Cain Memorial Lecture for laboratory advances in cancer research that were having therapeutic impact in clinical applications and refined the original SERM concept (Chap. 5).

> We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines, and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids; it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high-risk group to prevent breast cancer.

Raloxifene: Abandoned and Resurrected

Raloxifene, originally called keoxifene, was first reported by scientists at Eli Lilly, Indianapolis, to be an antiestrogen with a high affinity for the estrogen receptor (ER) [55]. Much like its earlier analog, LY117018, raloxifene has only mild estrogen-like properties in the uterus [56].In fact, at very high doses, LY117018 can even block the antiuterotropic effects of a variety of steroidal and nonsteroidal compounds in the rat [57]. The drug has antitumor effects in the rat, but is less potent than tamoxifen [23, 58]. Although the original direction for raloxifene's clinical development was breast cancer therapy, Eli Lilly chose to abandon this approach toward the end of the 1980s. However, the discovery that raloxifene might prevent osteoporosis, [59] prevent breast cancer, [23] and, at the same time, have minor estrogen-like effects in the uterus laid the foundation for the subsequent confirmation of bone data in animals [56]. These discoveries also led to the completion of clinical trials that demonstrated maintenance of bone density in postmenopausal women at risk for osteoporosis [60].

As part of a safety profile for any estrogen-like drug for the prevention of osteoporosis, raloxifene had to be evaluated for breast safety. To this end, Eli Lilly organized an independent oncology advisory committee to adjudicate all breast cancers diagnosed in the randomized, placebo-controlled trials for the

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Table 7.2 The Raloxifene Oncology Advisory Committee formed by Eli Lilly t2.1

The Raloxifene Oncology Advisory Committee[a] t2.2
Alberto Costa, M.D.—European Institute for Oncology, Milan (Breast Surgeon, Co-PI Italian Tamoxifen Prevention Trial) t2.3
V. Craig Jordan, Ph.D., D.Sc.—Northwestern University Medical School, Chicago (Committee Chairperson) t2.4
Marc E. Lippman, M.D.—Georgetown University Medical School, Washington DC (Director, Lombardi Comprehensive Cancer Center) t2.5
Monica Morrow, M.D.—Northwestern University Medical School, Chicago (Breast Surgeon, Director, Lynn Sage Breast Cancer Program) t2.6
Larry Norton, M.D.—Memorial Sloan-Kettering Cancer Center, New York (Head, Division of Oncology) t2.7
Trevor J. Powels, FRCP, Ph.D.—Royal Marsden Hospital, London (Medical Oncologist, PI Royal Marsden, Tamoxifen Prevention Study) t2.8

[a]Responsible for the evaluation and adjudication of breast cancer cases in the 10,533 patients participating in randomized, placebo-controlled trials to prevent osteoporosis t2.9

prevention of osteoporosis. The committee (Table 7.2) was assembled to provide expertise in diagnosis, breast cancer prevention, and breast medical oncology. Committee members met every 6 months to review pathology, mammograms, and patient records to determine whether disease was preexisting at the time of entry to the trial and whether the cancer was invasive or noninvasive. All patients who developed breast cancer in all trials were adjudicated blind, and the results were then collated and analyzed by Biostatistician Steven Eckert of Eli Lilly.

The pivotal registration trial to establish the efficacy and value of raloxifene for the treatment and prevention of osteoporosis was called Multiple Outcomes of Raloxifene Evaluation (MORE) [61]. The MORE trial was a randomized double-blind trial that recruited 7,705 postmenopausal women (mean age 66.5 years) with osteoporosis defined as prior vertebral fractures or femoral neck or a spine T score 2.5SD or more below that of non-osteoporotic women. Participants were randomized to placebo or two raloxifene treatment groups: 60 or 120 mg daily.

Based on the positive results from the MORE trial, raloxifene is currently FDA approved for the prevention of osteoporosis. Raloxifene, 60 mg daily, produces a 1–2 % increase in postmenopausal bone density—an increase equivalent to that noted with tamoxifen. Raloxifene also reduces fractures by about 30–40 %. In addition, raloxifene is also approved to prevent osteoporosis in Europe and in more than a dozen other countries.

As part of the evaluation of osteoporosis in the MORE trial, there were several preplanned additional outcomes measures: histologically confirmed breast cancer, transvaginal ultrasonography to evaluate uterine effects of raloxifene in 1,781 randomly chosen participants, and an assessment of DVT and pulmonary embolism by chart review.

The MORE trial, analyzed at 3 years of follow-up, documented 27 cases of breast cancer in the control (2,576 women) but only a total of 13 cases in these

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treated with raloxifene (5,129 women). In other words, 126 women would need to be treated to prevent osteoporosis to prevent one case of breast cancer: the original hypothesis and roadmap [7, 62] was valid [63]!

Most importantly, the decrease in the risk of breast cancer was confined to ER-positive disease; there was a 90 % decrease in ER-positive breast cancer but no change in ER-negative breast cancer. Unlike previous experience with tamoxifen in postmenopausal women, there was no increase in the risk of endometrial cancer during raloxifene treatment. However, there was a threefold increase in venous thrombotic disease equivalent to that reported for both tamoxifen and estrogen in postmenopausal women. It is recommended that raloxifene, tamoxifen, or estrogen replacement is not taken by women with a history of thromboembolic disorders. The analysis of the MORE trail for breast cancer incidences at 3 years was confirmed with a 4 years reanalysis [64], demonstrating a 72 % decrease in the incidence of invasive breast cancer compared to placebo. The decision was made to revise and extend the MORE trial with Continuing Outcomes Relevant to Evista (CORE) trial.

During the 8 years of the MORE/CORE trials, the incidence of invasive breast cancer and ER-positive breast cancer was reduced by 66 % and 76 % respectively with no increase in the risk of endometrial cancer ($P < 0$), no endometrial hyperplasia ($P > 0.99$), and no vaginal bleeding ($P = 0.087$).

However, the fact that raloxifene was proven to reduce the risk of breast cancer but not increase the risk of endometrial cancer mandated that tamoxifen (the FDA-approved standard of care) and raloxifene must be tested head to head in postmenopausal women at high risk for the prevention of breast cancer.

The scene was now set for the NCI/NSAP P-2 study to go forward in high-risk postmenopausal women that would put tamoxifen versus raloxifene with a primary end point: the prevention of breast cancer. No placebo arm was recruited as it was considered unethical not to use tamoxifen, the approved drug of choice known to reduce the risk of breast cancer by 50 %.

However, wisely, the MORE trial was simultaneously extended out to 8 years of raloxifene treatment for women at risk for osteoporosis. All women who volunteered to continue on raloxifene (60 mg daily) had previously taken either 60 or 120 mg raloxifene. A total of 3,510 women were in the raloxifene are compared to 1,703 women in placebo arm [65]. During the CORE trial invasive breast cancer was decreased by 59 % and ER-positive breast cancer by 66 % compared to placebo. Overall, for the continued MORE/CORE trial, invasive breast cancer was reduced by 66 % and ER-positive breast cancer by 76 %.

Although the study of long-term raloxifene in the MORE/CORE trail was necessary because the treatment and prevention of osteoporosis requires continuous treatment (no drug benefit), the data was to be important once the results of the STAR trail were evaluated (Chap. 8).

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Conclusion

In the 20 years between the 1990s and 2010, not one but two agents were shown to reduce the incidence of invasive breast cancer in postmenopausal women at high risk to develop the disease. Raloxifene was approved for the prevention of osteoporosis in high-risk women with a dramatic reduction in the incidence of breast cancer as a beneficial side effect. The side effect of endometrial cancer with tamoxifen was solved. Overall, a triumph for translational research, the creation of a roadmap to follow and a new drug group called the SERMs.

Postscript. The first study that I ever completed and presented at the International Steroid Hormone Congress in Mexico City in 1974 was on the prevention of rat mammary carcinogenesis with tamoxifen. Arthur Walpole and I had previously discussed the results and we both appreciated the significance of the data for women's health. But the idea and these data were 20 years too soon! Tamoxifen was not even FDA approved for the treatment of breast cancer until December 1977, and this was for metastatic breast cancer. There was a long way to go before the NCI would fund Dr. Fisher's NSABP trial and it would start in 1992. Over the years our Tamoxifen Teams provided most of the translational information about safety (endometrial cancer), strategies with long-term therapy and bone safety. The story of "who did what" in the laboratory at Wisconsin to "set the scene" for the exploitation of SERMs has been told in the Postscript to Chap. 5.

Uncorrected Proof

References

1. Lacassagne A (1936) Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer 27:217–225
2. Jordan VC (2003) Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2:205–213
3. Jordan VC (2006) Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 147(Suppl 1):S269–S276
4. Scottish Cancer Trials Office (MRC), Edinburgh (1987) Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee. Lancet 2:171–175 AU4
5. Fisher B, Costantino J, Redmond C et al (1989) A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. N Engl J Med 320:479–484
6. Jordan VC (1984) Biochemical pharmacology of antiestrogen action. Pharmacol Rev 36:245–276
7. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189
8. Jordan VC (2006) The science of selective estrogen receptor modulators: concept to clinical practice. Clin Cancer Res 12:5010–5013
9. Jordan VC (2007) Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nat Rev Cancer 7:46–53

Author's Proof

10. Jordan VC (2009) A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 69:1243–1254
11. EBCTG (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378:771–784
12. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48:812–815
13. Fornander T, Rutqvist LE, Cedermark B et al (1989) Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1:117–120
14. Gail MH, Brinton LA, Byar DP et al (1989) Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. J Natl Cancer Inst 81:1879–1886
15. Huggins C, Grand LC, Brillantes FP (1961) Mammary cancer induced by a single feeding of polymucular hydrocarbons, and its suppression. Nature 189:204–207
16. Gullino PM, Pettigrew HM, Grantham FH (1975) N-nitrosomethylurea as mammary gland carcinogen in rats. J Natl Cancer Inst 54:401–414
17. Dao TL (1962) The role of ovarian hormones in initiating the induction of mammary cancer in rats by polynuclear hydrocarbons. Cancer Res 22:973–981
18. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
19. Turcot-Lemay L, Kelly PA (1980) Characterization of estradiol, progesterone, and prolactin receptors in nitrosomethylurea-induced mammary tumors and effect of antiestrogen treatment on the development and growth of these tumors. Cancer Res 40:3232–3240
20. Jordan VC, Allen KE, Dix CJ (1980) Pharmacology of tamoxifen in laboratory animals. Cancer Treat Rep 64:745–759
21. Wilson AJ, Tehrani F, Baum M (1982) Adjuvant tamoxifen therapy for early breast cancer: an experimental study with reference to oestrogen and progesterone receptors. Br J Surg 69:121–125
22. Jordan VC (1983) Laboratory studies to develop general principles for the adjuvant treatment of breast cancer with antiestrogens: problems and potential for future clinical applications. Breast Cancer Res Treat 3(Suppl):S73–S86
23. Gottardis MM, Jordan VC (1987) Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 47:4020–4024
24. Lacassagne A (1955) Endocrine factors concerned in the genesis of experimental mammary carcinoma. J Endocrinol 13:ix–xviii
25. Harper MJ, Walpole AL (1967) A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 13:101–119
26. Terenius L (1971) Structure-activity relationships of anti-oestrogens with regard to interaction with 17-beta-oestradiol in the mouse uterus and vagina. Acta Endocrinol (Copenh) 66:431–447
27. Jordan VC (1975) Prolonged antioestrogenic activity of ICI 46, 474 in the ovariectomized mouse. J Reprod Fertil 42:251–258
28. Bittner JJ (1939) Relation of nursing to the extra-chromosomal theory of breast cancer in mice. Am J Cancer 35:90–97
29. Lathrop AE, Loeb L (1916) Further investigations on the origin of tumors in mice. III. On the part played by internal secretion in the spontaneous development of tumors. J Cancer Res 1:1–19
30. Hynes NE, Groner B, Michalides R (1984) Mouse mammary tumor virus: transcriptional control and involvement in tumorigenesis. Adv Cancer Res 41:155–184
31. Peters G, Brookes S, Placzek M et al (1989) A putative int domain for mouse mammary tumor virus on mouse chromosome 7 is a 5′ extension of int-2. J Virol 63:1448–1450

Uncorrected Proof

Author's Proof

32. Jordan VC, Lababidi MK, Mirecki DM (1990) Anti-oestrogenic and anti-tumour properties of prolonged tamoxifen therapy in C3H/OUJ mice. Eur J Cancer 26:718–721
33. Jordan VC, Lababidi MK, Langan-Fahey S (1991) Suppression of mouse mammary tumorigenesis by long-term tamoxifen therapy. J Natl Cancer Inst 83:492–496
34. Welsch CW, Goodrich-Smith M, Brown CK, Miglorie N, Clifton KH (1981) Effect of an estrogen antagonist (tamoxifen) on the initiation and progression of gamma-irradiation-induced mammary tumors in female Sprague–Dawley rats. Eur J Cancer Clin Oncol 17:1255–1258
35. Cuzick J, Baum M (1985) Tamoxifen and contralateral breast cancer. Lancet 2:282
36. Powles TJ, Hardy JR, Ashley SE et al (1989) A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. Br J Cancer 60:126–131
37. Powles TJ, Jones AL, Ashley SE et al (1994) The Royal Marsden Hospital pilot tamoxifen chemoprevention trial. Breast Cancer Res Treat 31:73–82
38. Powles T, Eeles R, Ashley S et al (1998) Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomised chemoprevention trial. Lancet 352:98–101
39. Jones AL, Powles TJ, Treleaven JG et al (1992) Haemostatic changes and thromboembolic risk during tamoxifen therapy in normal women. Br J Cancer 66:744–747
40. Powles TJ, Tillyer CR, Jones AL et al (1990) Prevention of breast cancer with tamoxifen—an update on the Royal Marsden Hospital pilot programme. Eur J Cancer 26:680–684
41. Powles TJ, Hickish T, Kanis JA, Tidy A, Ashley S (1996) Effect of tamoxifen on bone mineral density measured by dual-energy x-ray absorptiometry in healthy premenopausal and post-menopausal women. J Clin Oncol 14:78–84
42. Kedar RP, Bourne TH, Powles TJ et al (1994) Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial. Lancet 343:1318–1321
43. Decensi A, Fontana V, Bruno S et al (1996) Effect of tamoxifen on endometrial proliferation. J Clin Oncol 14:434–440
44. King MC, Wieand S, Hale K et al (2001) Tamoxifen and breast cancer incidence among women with inherited mutations in BRCA1 and BRCA2: National Surgical Adjuvant Breast and Bowel Project (NSABP-P1) Breast Cancer Prevention Trial. JAMA 286:2251–2256
45. Fisher B, Costantino JP, Wickerham DL et al (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90:1371–1388
46. Ganz PA (2001) Impact of tamoxifen adjuvant therapy on symptoms, functioning, and quality of life. J Natl Cancer Inst Monogr 30:130–134
47. Day R, Ganz PA, Costantino JP et al (1999) Health-related quality of life and tamoxifen in breast cancer prevention: a report from the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Clin Oncol 17:2659–2669
48. Veronesi U, Maisonneuve P, Costa A et al (1998) Prevention of breast cancer with tamoxifen: preliminary findings from the Italian randomised trial among hysterectomised women. Italian Tamoxifen Prevention Study. Lancet 352:93–97
49. Cuzick J, Forbes J, Edwards R et al (2002) First results from the International Breast Cancer Intervention Study (IBIS-I): a randomised prevention trial. Lancet 360:817–824
50. Fisher B, Costantino JP, Wickerham DL et al (2005) Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97:1652–1662
51. Cuzick J, Forbes JF, Howell A (2006) Re: Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 98:643, author reply 643–644
52. Powles TJ (1999) Re: Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 91:730
53. Jordan VC, Morrow M (1994) Should clinicians be concerned about the carcinogenic potential of tamoxifen? Eur J Cancer 30A:1714–1721

Uncorrected Proof

Author's Proof

54. Iqbal J, Ginsburg OM, Wijeratne TD et al (2012) Endometrial cancer and venous thromboembolism in women under age 50 who take tamoxifen for prevention of breast cancer: a systematic review. Cancer Treat Rev 38:318–328
55. Black LJ, Jones CD, Falcone JF (1983) Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 32:1031–1036
56. Black LJ, Sato M, Rowley ER et al (1994) Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 93:63–69
57. Jordan VC, Gosden B (1983) Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 113:463–468
58. Clemens JA, Bennett DR, Black LJ, Jones CD (1983) Effects of a new antiestrogen, keoxifene (LY156758), on growth of carcinogen-induced mammary tumors and on LH and prolactin levels. Life Sci 32:2869–2875
59. Jordan VC, Phelps E, Lindgren JU (1987) Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10:31–35
60. Delmas PD, Bjarnason NH, Mitlak BH et al (1997) Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N Engl J Med 337:1641–1647
61. Ettinger B, Black DM, Mitlak BH et al (1999) Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 282:637–645
62. Jordan VC (1988) Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 6:589–595
63. Cummings SR, Eckert S, Krueger KA et al (1999) The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA 281:2189–2197
64. Cauley JA, Norton L, Lippman ME et al (2001) Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial. Multiple outcomes of raloxifene evaluation. Breast Cancer Res Treat 65:125–134
65. Martino S, Cauley JA, Barrett-Connor E et al (2004) Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 96:1751–1761

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Abstract	The toxicological concern with the potential of tamoxifen to increase the incidence of endometrial cancer or hepatocellular carcinoma mandated a new approach to chemoprevention. The SERM raloxifene does not have the toxicological concern of tamoxifen and is approved for the treatment and prevention of osteoporosis but at the same time reduces breast cancer incidence. The Study of Tamoxifen and Raloxifene (STAR) demonstrated that the two SERMs were equivalent in reducing breast cancer incidence but raloxifene had a better safety profile. However, tamoxifen can reduce breast cancer incidence during therapy for 5 years, and this is maintained for at least a decade after treatment. In contrast, raloxifene must be given continuously.	

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Chapter 8
Tamoxifen and Raloxifene Head to Head: The STAR Trial

Abstract The toxicological concern with the potential of tamoxifen to increase the incidence of endometrial cancer or hepatocellular carcinoma mandated a new approach to chemoprevention. The SERM raloxifene does not have the toxicological concern of tamoxifen and is approved for the treatment and prevention of osteoporosis but at the same time reduces breast cancer incidence. The Study of Tamoxifen and Raloxifene (STAR) demonstrated that the two SERMs were equivalent in reducing breast cancer incidence but raloxifene had a better safety profile. However, tamoxifen can reduce breast cancer incidence during therapy for 5 years, and this is maintained for at least a decade after treatment. In contrast, raloxifene must be given continuously.

The STAR trial recruitment and evaluation was unprecedented in the history of clinical cancer trials (Fig. 8.1). The STAR trial was a phase III, double-blind trial that screened 184,480 postmenopausal women (mean age 58.5 years) for a full year with breast cancer risk over 1.65 %, and 19,747 were subsequently randomized to receive either tamoxifen (20 mg daily) or raloxifene (60 mg daily) for 5 years (Fig. 8.2). The primary aim of the trial was to assess the occurrence of invasive breast cancer in postmenopausal high-risk women with raloxifene and compare the preventive efficacy with, by then an established drug, tamoxifen. The secondary aim was to establish the efficacy of raloxifene treatment, such as cardiovascular, bone density, and general toxicities. Three groups of women were eligible: postmenopausal women over 60, irrespective of their risk of breast cancer; postmenopausal women who were diagnosed previously with lobular carcinoma in situ (LCIS); and postmenopausal women between the ages of 35 and 59, who have a high risk of developing breast cancer based on the presence of a combination of risk factors. The risk factors were assessed by using a modified Gail model that was used in the NSABP/P-1 trial. The main risk factors included age; number of first-degree relatives who have been diagnosed with breast cancer; whether the woman has had any children and the age of the first delivery; history of biopsies, especially if the results have shown atypical hyperplasia; and the age of the woman's first menstrual period.

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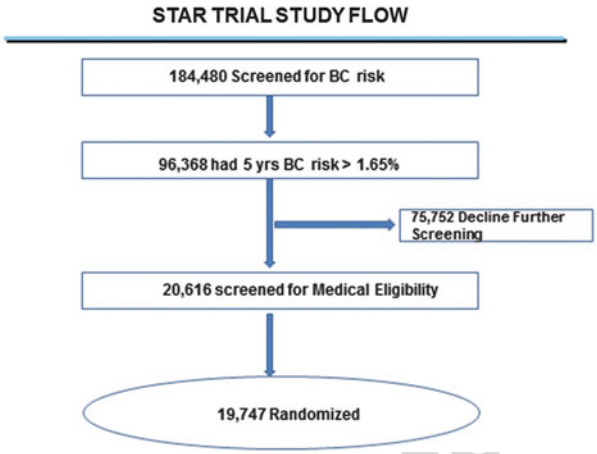

Fig. 8.1 STAR trial recruitment scheme. A total of 19,747 postmenopausal women were selected based on their eligibility to participate in the study

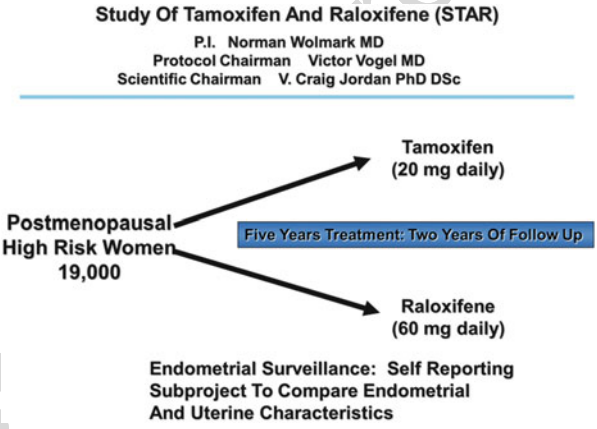

Fig. 8.2 STAR trial randomization scheme. A total of 19,747 selected women were randomized to be treated with either 20 mg of tamoxifen daily or 60 mg of raloxifene daily

A preplanned analysis was triggered when a total of 327 incidents of invasive breast cancers occurred. The trial was conducted beginning 1 July 1999 and was assessed at a cutoff date of 31 December 2005. The data reported initially were 6 years and 5 months after the STAR trial initiated recruitment [1].

There were a total of 168 invasive breast cancers in the raloxifene-treated group and 163 invasive breast cancers in the tamoxifen-treated group (Fig. 8.3). A control arm was not considered to be appropriate as tamoxifen was the FDA-approved medicine and the standard of care, but an estimate of invasive breast cancer in a hypothetical control arm based on the level of risk in an equivalent number of women not treated with a SERM was estimated at 312 (Fig. 8.3). Thus, both tamoxifen and raloxifene are producing about a 50 % decrease in breast cancer incidence. There were however fewer noninvasive breast cancer (57 cases) in the tamoxifen-treated group compared with the raloxifene-treated group (80 cases), but this was barely statistically significant ($P = 0.052$) (Fig. 8.4). However, a later

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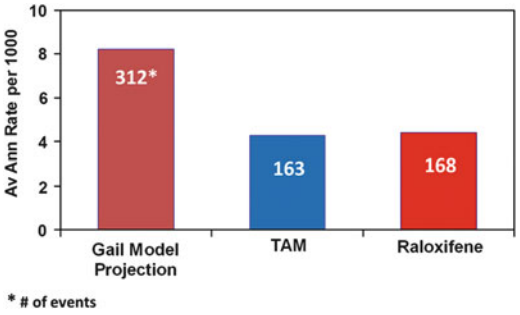

Fig. 8.3 The results of invasive breast cancer reduction in STAR trial. Raloxifene virtually was equivalent to tamoxifen in reducing the incidence of invasive breast cancer by 50 %, as compared to the projected untreated control. It was considered unethical to use untreated control as an approved breast cancer treatment with tamoxifen already was available at the time

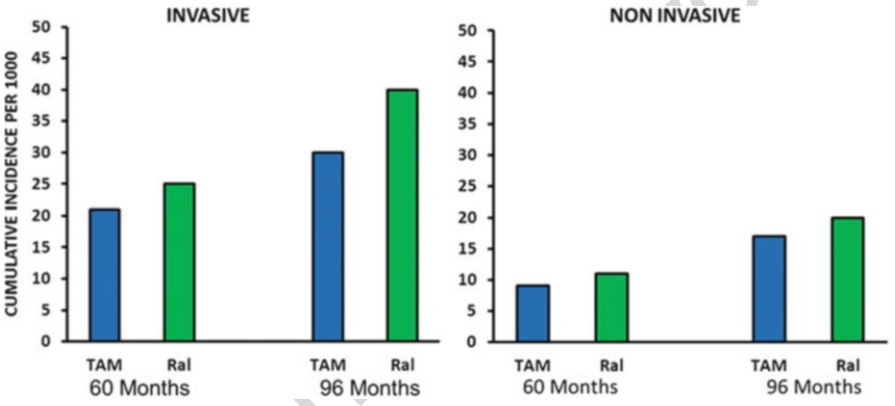

Fig. 8.4 Cumulative incidence of invasive breast cancer and noninvasive breast cancer in women treated with tamoxifen and raloxifene and followed up at 60 and 96 months post randomization

statistical study was initiated to assess the actual benefit/risk for breast cancer prevention for postmenopausal women [2]. The data were pooled from the Women's Health Initiative, STAR trial, and End Results Program and were used to develop a benefit/risk assessment index, which could be used for assessing the chemoprevention benefits with either raloxifene or tamoxifen. The results of the statistical analysis demonstrated that benefit/risk index was dependent on age, race, and history of hysterectomy. Postmenopausal women with no hysterectomy treated with raloxifene generally have better index than those treated with tamoxifen and so do premenopausal women with prior hysterectomy.

In contrast, there were fewer endometrial cancer (23 cases) in patients treated with raloxifene then those treated with tamoxifen (36 cases), though this does not reveal statistical significance ($P < 0.07$) (Fig. 8.5). However, this is deceptive as tamoxifen has a fundamentally different effect on the uterus than raloxifene.

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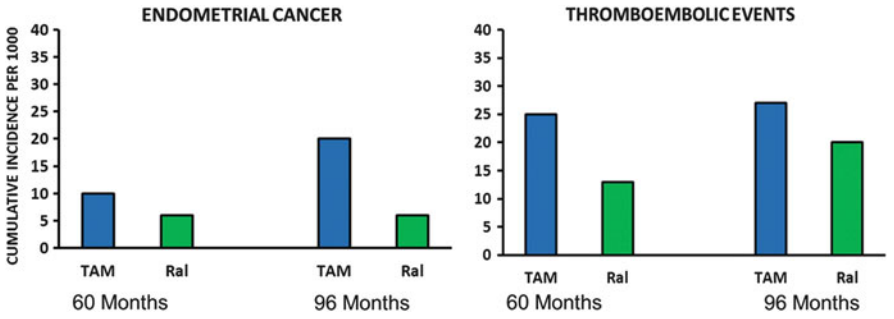

Fig. 8.5 Cumulative incidence of uterine cancers and thromboembolic events in women treated with either tamoxifen or raloxifene and followed up at 60 and 96 months post randomization

t1.1 **Table 8.1** The rates of developed cataracts and cataract surgeries during STAR trial. A total of 8,341 women were treated with tamoxifen and 8,336 were treated with raloxifene

t1.2		Events		Rate per 1,000	
t1.3	Cataracts and cataract surgery	Tamoxifen	Raloxifene	Tamoxifen	Raloxifene
t1.4	Developed cataracts	739	603	14.58	11.69
t1.5	Cataracts followed by surgery	575	462	11.18	8.85

Women elected to have 244 hysterectomies on tamoxifen can be compared to 111 hysterectomies in patients taking raloxifene. Similarly, there were fewer thrombotic events ($P = 0.01$) (Fig. 8.5), cataracts ($P = 0.002$), and cataract surgeries ($P = 0.03$) in women being treated with raloxifene (Table 8.1).

Therefore, overall, tamoxifen and raloxifene are equivalent during the treatment phase, for reducing the risk of breast cancer in high-risk postmenopausal women, but raloxifene appears to have a better safety profile than tamoxifen during treatment. However, this is where the pharmacology becomes interesting.

A subsequent analysis of the STAR trial at 90 months after initiating recruitment was reported [3]. Interestingly, the efficacy of raloxifene and tamoxifen did not remain equivalent in the post treatment phase of the study. The 5-year "pulse" of tamoxifen treatment seemed to have changed the breast cancer tissue or changed the tumor environment so that even 5 years after the therapy cessation, the occurrence of contralateral breast cancer was still prevented [4]. Similar results were shown in animals, where the number of breast tumors in tamoxifen-treated rats never reached the same levels as in control animals [5]. The efficacy of raloxifene to reduce the incidence of invasive breast cancer decreases so that within 2–3 years after treatment, raloxifene only retain 76 % of the ability of tamoxifen to prevent the occurrence of invasive breast cancer in post treatment period. However, based on the Martino study [6], raloxifene should be consulted as a continuous therapy and should not be stopped at 5 years.

Concerning safety with raloxifene, there was now a significant decrease in the incidence of endometrial cancers ($P = 0.003$), thrombotic events ($P = 0.007$),

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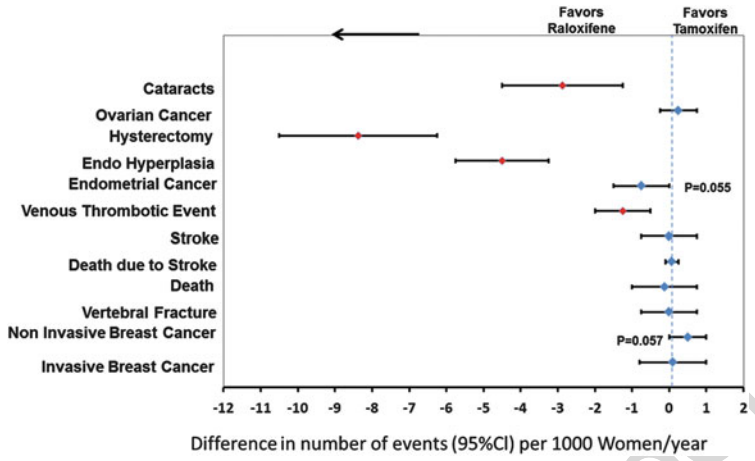

Fig. 8.6 Efficacy and important outcomes in favor of raloxifene and tamoxifen in the STAR trial

(Fig. 8.6) cataracts, and cataract operations (RR = 0.80; 95 % CI, 0.72–0.89 and RR = 0.79; 95 % CI, 0.70–0.90, respectively) (Fig. 8.6). As a summary of the efficacy and important outcomes of the STAR trial, Fig. 8.6 addresses outcomes in favor of either raloxifene or tamoxifen.

Raloxifene was approved by the FDA for the reductions of incidence of breast cancer in high-risk postmenopausal women and breast cancer in postmenopausal women with osteoporosis on 24 July 2007.

Postscript. During the past 40 years, the idea of preventing breast cancer in women at high risk for developing the disease has advanced from just that, an idea [7] to become a practical reality with not one but two FDA-approved medicines. Forty years ago, there was no tamoxifen, only ICI 46,474, a failed "morning-after pill" that was reinvented as an antiestrogen throughout the 1970s, and a strategy was established to enable progress to move forward in the clinic for both chemoprevention and long-term adjuvant therapy with an antihormonal agent [8]. Indeed, at that time, the word "chemoprevention" was not even in the English language. It was still for Michael Sporn to invent the idea of using chemicals to prevent cancer [9, 10] and establish the word chemoprevention. Raloxifene the failed "breast cancer drug" was conceptually reinvented as the first SERM to treat osteoporosis and prevent breast cancer at the University of Wisconsin Comprehensive Cancer Center.

At the start of the STAR trial, Dr. Norman Wolmark, principal investigator of the NCI grant and of the NSABP, appointed me as the scientific chair for the clinical trial. His goal was to recruit a qualified scientist to address unanticipated issues of importance to our patients, should they arise. My expertise was translational research on both SERMs. Fortunately, and remarkably, no cases arose during the tenure of the STAR trial (in contrast to the NCI/NSABP P-1 trail).

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The question was asked to me more than once: "How will you feel if raloxifene proves to be superior to tamoxifen in the STAR trial?" "Delighted" would be my reply as the scientific foundations for the applications of both SERMs (and the concept of the new SERM drug group—Chap. 5) had both emerged from my laboratory. Firstly with the reinvention of tamoxifen, a failed contraceptive to a potential chemopreventive at the Worcester Foundations and as a long-term adjuvant therapy at the University of Leeds in the early 1970s [11, 12]. Raloxifene was a failed breast cancer drug originally called keoxifene that was abandoned in the late 1980s by the pharmaceutical industry but reinvented in my laboratory at the Wisconsin Clinical Cancer Center as a potential candidate medicine with the goal: "prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer" [13]. Our laboratory data was subsequently confirmed [14], and clinical testing for raloxifene to treat and prevent osteoporosis advanced from about 1992 through clinical testing by the pharmaceutical industry. AU1

The evaluation of the use of two SERMs with different characteristics in the STAR trial taught important lessons in translational research. In the laboratory during the 1980s, it was clear that the structurally related polyhydroxylated compounds LY117,018 and LY156,758 were short acting and rapidly excreted drugs compared to tamoxifen [15–17]. Much higher daily doses of rapidly excreted antiestrogens were necessary for effective antitumor action [5, 17], and the antiestrogenic effects of the polyhydroxylated compounds disappeared rapidly once the drug is stopped. In contrast, tamoxifen accumulates. Thus, large daily doses of raloxifene are necessary to achieve the same efficacy as tamoxifen, and this is true with raloxifene (tamoxifen is used at a standard dose of 20 mg daily; the MORE trial used 60 vs. 120 mg daily of raloxifene). Additionally, the relative clearance rate of raloxifene and tamoxifen would have implications for a correlation between compliance and the actions of the SERMs to be chemopreventive agents in breast cancer. Tamoxifen accumulates but the drug can still be detected in the circulations 6 weeks after the last dose. Raloxifene is cleared rapidly within a few days of the last dose. Thus, missing a few tamoxifen tablets is of little consequence to the efficacy of the drug, but regularly missing raloxifene doses exposes the patient to estrogen-induced proliferations of nascent breast tumors. There is also another important aspect of tamoxifen's pharmacology that is only recently being understood. Long-term adjuvant tamoxifen therapy [18, 19] and chemoprevention [20–22] retain antitumor actions long after tamoxifen is stopped. This was noted in the NSABP P-1 trail [21] and in the NSABP/STAR P-2 trial [3]. In contrast, raloxifene was unable to maintain long-term antitumor effects in the STAR trial [3], confirming the earlier laboratory data [5, 17]. It was this finding that prompted the conclusion of recommendations from the NSABP: "It is unlikely that the optimal durations of raloxifene for chemoprevention will be evaluated in a breast cancer prevention setting; however, the use of raloxifene in treating and preventing osteoporosis is approved for an indefinite period time. Therefore, continuing raloxifene therapy beyond 5 years might be an approach that would preserve its full chemopreventive activity" [3].

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References 154

1. Vogel VG, Costantino JP, Wickerham DL et al (2006) Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 295:2727–2741
2. Freedman AN, Yu B, Gail MH et al (2011) Benefit/risk assessment for breast cancer chemoprevention with raloxifene or tamoxifen for women age 50 years or older. J Clin Oncol 29:2327–2333
3. Vogel VG, Costantino JP, Wickerham DL et al (2010) Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: preventing breast cancer. Cancer Prev Res (Phila) 3:696–706
4. Early Breast Cancer Trialists' Collaborative Group (1998) Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 351:1451–1467
5. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
6. Martino S, Cauley JA, Barrett-Connor E et al (2004) Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 96:1751–1761
7. Lacassagne A (1936) Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer 27:217–225
8. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44:30–38
9. Sporn MB, Dunlop NM, Newton DL, Smith JM (1976) Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). Fed Proc 35:1332–1338
10. Sporn MB (1976) Approaches to prevention of epithelial cancer during the preneoplastic period. Cancer Res 36:2699–2702
11. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
12. Jordan VC (1974) Antitumour activity of the antiestrogen ICI 46,474 (Tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 5:354
13. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189
14. Black LJ, Sato M, Rowley ER et al (1994) Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 93:63–69
15. Jordan VC, Gosden B (1983) Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 113:463–468
16. Jordan VC, Gosden B (1983) Differential antiestrogen action in the immature rat uterus: a comparison of hydroxylated antiestrogens with high affinity for the estrogen receptor. J Steroid Biochem 19:1249–1258
17. Gottardis MM, Jordan VC (1987) Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 47:4020–4024
18. EBCTCG (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 365:1687–1717
19. Davies C, Godwin J, Gray R et al (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378:771–784
20. Cuzick J, Forbes JF, Sestak I et al (2007) Long-term results of tamoxifen prophylaxis for breast cancer – 96-month follow-up of the randomized IBIS-I trial. J Natl Cancer Inst 99:272–282

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21. Fisher B, Costantino JP, Wickerham DL et al (2005) Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97:1652–1662
22. Powles TJ, Ashley S, Tidy A et al (2007) Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. J Natl Cancer Inst 99:283–290

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Abstract	The clinical acceptance and validation of the therapeutic strategy of long-term adjuvant tamoxifen treatment mandated an examination of acquired drug resistance under laboratory conditions. The first model in vivo of acquired resistance of ER-positive breast cancer cells transplanted into immune deficient mice demonstrated tamoxifen-stimulated tumor growth after about 2 years of continuous treatment. When tamoxifen was stopped, tumors also grew with physiologic estradiol. The model showed that no estrogen (similar to the use of aromatase inhibitors) or a pure antiestrogen to destroy ER (fulvestrant) presaged this therapeutic approach in clinical trials a decade later. However, the long-term retransplantation of breast tumors with acquired tamoxifen resistance for at least 5 years demonstrated a vulnerability of these tumors. Tamoxifen-stimulated tumor growth but physiologic estrogen now caused tumor regression and apoptosis. The new biology of estrogen-induced apoptosis now is used to explain the decrease in mortality after adjuvant tamoxifen is stopped in patients and also the value of conjugated equine estrogens to reduce breast cancer incidence in women treated in their 60s.	

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Chapter 9
Acquired Resistance to Tamoxifen: Back to the Beginning

Abstract The clinical acceptance and validation of the therapeutic strategy of long-term adjuvant tamoxifen treatment mandated an examination of acquired drug resistance under laboratory conditions. The first model in vivo of acquired resistance of ER-positive breast cancer cells transplanted into immune deficient mice demonstrated tamoxifen-stimulated tumor growth after about 2 years of continuous treatment. When tamoxifen was stopped, tumors also grew with physiologic estradiol. The model showed that no estrogen (similar to the use of aromatase inhibitors) or a pure antiestrogen to destroy ER (fulvestrant) presaged this therapeutic approach in clinical trials a decade later. However, the long-term retransplantation of breast tumors with acquired tamoxifen resistance for at least 5 years demonstrated a vulnerability of these tumors. Tamoxifen-stimulated tumor growth but physiologic estrogen now caused tumor regression and apoptosis. The new biology of estrogen-induced apoptosis now is used to explain the decrease in mortality after adjuvant tamoxifen is stopped in patients and also the value of conjugated equine estrogens to reduce breast cancer incidence in women treated in their 60s.

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Introduction

The idea that the determination of the estrogen receptor (ER) content in the breast tumor of a patient with metastatic breast cancer would predict response to ablative endocrine surgery (oophorectomy, adrenalectomy, hypophysectomy) became a clinical reality and requirement for each breast cancer patient in the mid-1970s [1]. This was established on a National Cancer Institute sponsored meeting in Bethesda, Maryland, at the Holiday Inn in 1974. The rationale was that if the ER was not present in the tumor, then the patient should not have ablative surgery. This would be 30 % of all patients and there would be no response. There would be a response of about 60 % in patients with an ER-positive tumor. Tamoxifen, however, was not considered in these deliberations as it was not yet in clinical trial in America.

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Tamoxifen was Food and Drug Administration (FDA) approved and available to treat metastatic breast cancer in December 1977. By then, the ER had evolved into the target for tamoxifen action [2] and has subsequently become a drug to be used as a potential long-term adjuvant therapy, and there was laboratory data to indicate there was potential for tamoxifen as the first chemopreventive for breast cancer.

The fact that all metastatic breast tumors with ER did not respond to tamoxifen treatment, and those tumors that do respond, do so for about 1–2 years [3], created a classification of intrinsic resistance where treatment fails to control tumor growth at the 2-month evaluation point and acquired resistance where the tumor eventually escapes from estrogen blockade of the ER by tamoxifen and grows autonomously.

The prevalent theory in the 1970s for acquired resistance to endocrine therapy was that tumors were heterogeneous and those cells containing ER were controlled and died out, and the ER-negative tumor cells overgrew and become dominant. Thus, a tumor would evolve from ER positive to become ER negative. However, this was inconsistent with clinical experience by the medical oncology community. Select AU1 breast cancer could respond to, the then standard of care, high-dose diethylstilbestrol (DES) fail therapy evidenced by tumor regrowth, have a withdrawal response by stopping (DES) treatment, once the tumor regrew the clinician would try high-dose androgen or progestin therapy. This whole process of alternating endocrine therapies is called the endocrine treatment cascade and is used successfully to this day in selected patients before using cytotoxic combination chemotherapy. The practice of medicine therefore was not consistent with the theory that resistance occurred with a trend to ER-negative cell populations: the theory must therefore be incorrect. The solution to the problem was to come from studies utilizing athymic mice to grow human breast cancer cell lines and to study acquired resistance to tamoxifen.

The MCF-7 Breast Cancer Cell Line

The important cell line created by Soule and coworkers [4]at the Michigan Cancer Foundation (MCF) was from a pleural effusion from a nun, Sister Catherine Frances, initially treated with high-dose DES (tamoxifen was not available at that time). The cell line is ER positive [5] and became the "work horse" in the laboratory for the study of hormone-dependent breast cancer. Tamoxifen blocked spontaneous growth of MCF-7 cells in culture [6] and estradiol reversed the tamoxifen blockade. Estrogen did not however enhance growth in culture but it did in athymic mice [7] leading to the idea that a second factor in vivo was required. Within the decade of the 1980s, the Katzenellenbogen laboratory would discover that the ubiquitous phenol red indicator used in culture media contained a potent estrogenic contaminant [8, 9] (Fig. 1.7, Chap. 1).

Nevertheless, it was probably fortunate that the ER-positive MCF-7 cells were always grown in an estrogen-containing environment to maintain their hormone-responsive characteristics. Without estrogen in the media, the estrogen-responsive cells die [10].

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The value of the MCF-7 cell line to breast cancer research has been reviewed previously [11]. Throughout the 1980s and 1990s, numerous reports of tamoxifen resistance or resistance to raloxifene-like molecules or pure antiestrogens were published using MCF-7 cells in vitro or with other ER-positive cells [12–23]. We will, however, focus here on the importance of the transplantation of MCF-7 cells into athymic mice to create a breakthrough in deciphering acquired drug resistance. Shafie and Granthan [24] first showed that MCF-7 tumors grow in athymic mice with estradiol treatment, but not with tamoxifen treatment. Osborne [25] demonstrated that tamoxifen would control estrogen-stimulated MCF-7 growth in athymic mice but eventually the MCF-7 derived tumors would grow despite tamoxifen treatment [26]. Osborne concluded that part of the action of tamoxifen was tumoristatic; therefore, long-term treatment was necessary[26] . This was consistent with original data generated in carcinogen-induced rat mammary cancer models [27]. However, the finding that MCF-7 tumors with acquired resistance to tamoxifen grew *because* of estrogen or tamoxifen treatment not despite the tamoxifen was a discovery [28]. The tumors were transplantable and had adapted a mechanism for the tamoxifen-ER complex to cause growth somewhat similar to estrogen. Additionally, the resistance (tamoxifen-stimulated growth) is not athymic mouse specific, i.e., some strange metabolic difference or difference in NK cells. Tamoxifen-stimulated MCF-7 tumor growth occurs in either athymic rats or beige (NK deficient) mice [29]. Unfortunately (or as it turned out fortunately!), cell lines of tamoxifen-resistant tumors could not be treated and retain the tamoxifen-resistant growth phenotype. However, to explain the estrogen-like action of tamoxifen, an interesting hypothesis emerged in the early 1990s to explain tamoxifen-stimulated growth via the generation of estrogenic isomers of tamoxifen metabolites. This will be described briefly, as an interesting clinical approach was used to address the proposed molecular mechanism of acquired tamoxifen resistance. It is an excellent example of how basic structure-function relationship can resolve important clinical questions in the laboratory.

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Tamoxifen Metabolism Hypothesis

Tamoxifen is metabolized to numerous hydroxylated compounds, some with estrogen-like actions and others with antiestrogenic actions (Chap. 3). The metabolism hypothesis with subsequent geometric isomerization to putative estrogens was based on the known estrogenic and antiestrogenic properties of the cis (ICI 47,699) and trans (ICI 46,474) isomers of tamoxifen [30–33]. It was noted that there is less tamoxifen in tumors with acquired resistance [31] and an increase in (E) 4-hydroxytamoxifen as a putative estrogen. Similar findings were made in patients failing tamoxifen therapy, i.e., lower levels of tamoxifen in the tumor and the ratio of E to Z isomers of 4-hydroxytamoxifen were higher [32]. Additionally [33], metabolite E, a weak estrogen, was identified in patients with tamoxifen refractory tumors. To evaluate the hypothesis a series of non-isomerizable fixed ring

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derivatives and isomers of 4-hydroxytamoxifen and metabolite E were synthesized. The E isomer of 4-hydroxytamoxifen is actually a very weak antiestrogen (not a full estrogen) [34, 35]. The Z isomer of metabolite E is only a very weak estrogen (Fig. 3.2, Chap. 3).

To address the tamoxifen metabolism hypothesis, further in vivo, a fixed ring version of tamoxifen was synthesized so that any metabolite that we produced could not isomerize to potent estrogens. Fixed ring tamoxifen is equally able to stimulate the acquired tamoxifen treatment tumors as the parent drug [36]. Thus, other resistance mechanisms, based on growth factor driven tumor growth have now become most useful for developing future therapeutic strategies either as second time treatments or if given with tamoxifen initially may prevent resistance occurring in the first place.

Growth Factor-Driven Acquired and Intrinsic Resistance

There is compelling evidence that HER 2/neu can subvert hormone-responsive growth completely in ER-positive cells. Stable transfection of MCF-7 cells with the HER 2/neu gene results in tumor growth in athymic mice not regulated by tamoxifen [37].

Over the past 20 years the Osborne Group in Texas have refined the growth factor driven MCF-7 model in vivo and defined precisely the ways of blocking either the receptors or their tyrosine kinases singly or together to create long-term responses or "cures" in vivo while applying either tamoxifen or estrogen deprivations equivalent to aromatase inhibitors treatment strategies.

In recent years antibodies targeting HER2, or tyrosine kinase inhibitors that target the HER family (1–4) have become available for clinical evaluation. Among anti-HER2 monoclonal antibodies tested, trastuzumab is now a well-known and approved drug that is established though clinical trials as an important component of the first-line treatment of patients with HER2 amplified metastatic breast cancer [38–40]. When trastuzumab is administered in a preoperative setting, this strategy increases the pathological complete response rate [38]. In a large phase III trial investigating adjuvant therapy in HER2 positive early breast cancer, the addition of trastuzumab to chemotherapy increases both the disease-free and overall survival [41]. Aside from trastuzumab there is also pertuzumab, which is a monoclonal antibody against HER2 that blocks dimerization with HER1 and HER3 [42]. Although pertuzumab has therapeutic activity in HER2 positive breast cancer patients, combination therapy with trastuzumab has proven to be more effective [43]. Ertumaxomab is another monoclonal antibody against HER2 that has demonstrated strong immunological responses in HER2 positive breast cancer patients in phase I clinical trial [44].

Aside from monoclonal anti-HER2 antibodies, the use of tyrosine kinase inhibitors to target HER2 is proving to be effective. In particular, lapatinib, a dual tyrosine kinase inhibitor of both HER1 and HER2 and of Akt and mitogen-activated AU2

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protein kinase (MAPK), has been demonstrated to inhibit cell growth and induce apoptosis in several breast cancer cell lines [45]. Results from phase I and II clinical trials have shown that lapatinib has therapeutic value in a number of tumors, in particular in breast cancer patients [46–48]. In a xenograft mouse model, lapatinib is able to prevent tamoxifen resistance [49], showing the role of increased growth factor signaling pathways in resistance [50] and the potential benefit of targeting the increased growth factor signaling to reverse tamoxifen resistance in the clinic. A systemic review of the databases from clinical trials (including phase III) demonstrates that combination therapy of HER2-positive HR-positive metastatic breast cancers in postmenopausal women with lapatinib and anastrozole is superior to lapatinib monotherapy and superior to tamoxifen treatment [51].

Thus, it is now clear that exogenous inhibitors of the HER-signaling network and other mitogenic pathways can abrogate or improve the response rate of breast cancer with acquired resistance [52].

An Evolving Model of Acquired Resistance to SERMs and Aromatase Inhibitors

The transplantable model of acquired tamoxifen resistance in ER-positive breast cancer cell lines develops within about a year [28, 53]. This is the same time that resistance to tamoxifen treatment occurs in ER-positive metastatic breast cancer. Thus, the model recapitulates acquired resistance to tamoxifen in metastatic breast cancer, and therapeutic studies in the mice mimic the second-line responses of tumors in aromatase inhibitors or fulvestrant after tamoxifen failure in clinical trial [54, 55]. The laboratory-derived tumor of acquired resistance to tamoxifen does not grow without physiologic estrogen action. This is induced by the fact that no estrogen treatment maybe like aromatase inhibitor treatment or a pure antiestrogen ICI 164,384 (the lead compound that resulted in fulvestrant [56, 57]) blocks tumor growth [58].

At this time, in the mid-1980s, it was clear that long-term adjuvant tamoxifen therapy for years or indefinitely [59–61] was showing promise for enhanced survival, and there was no early recurrence of micrometastatic disease as a result of the development of early acquired resistance. Something was conceptually wrong with the link between the endocrine treatment of metastatic breast cancer with its greater bulk and the responsiveness of micrometastatic disease that is undetectable during adjuvant therapy.

One plausible explanation was proposed in the early 1990s based on the transplantable model of acquired resistance to tamoxifen maintained through serial transplantations into further generations of tamoxifen-treated year after year athymic mice. After about 5 years of retransplantations, the tamoxifen-stimulated MCF-7 tumors changed their survival characteristics and responsiveness to estrogen. Physiologic estrogen was no longer a survival signal causing tumor growth in

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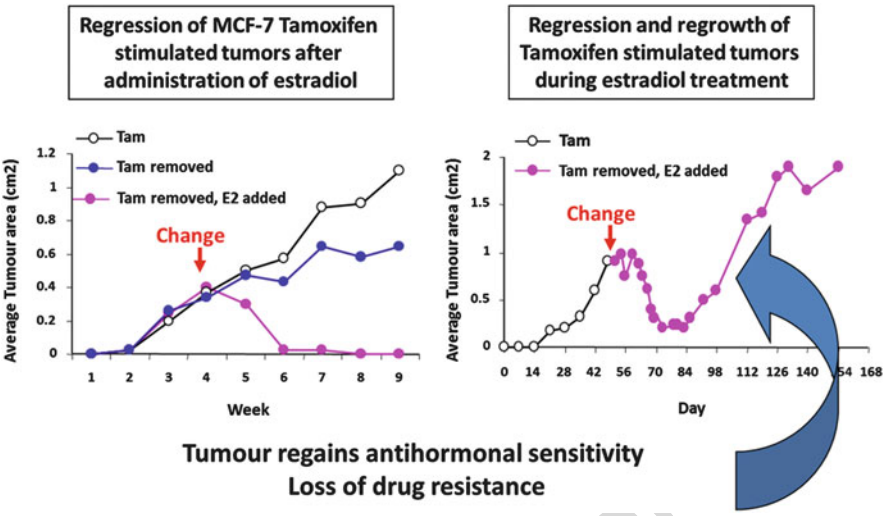

Fig. 9.1 Cyclic changes in sensitivity and resistance of breast tumors grown in vivo after treatments with tamoxifen and estradiol

the animals [28] but rather an inhibitor of tumor growth causing small tamoxifen-stimulated tumors to just melt away [62] (Fig. 9.1). It was suggested that following long-term adjuvant tamoxifen therapy, it was actually the act of stopping tamoxifen that reinforced and enhanced patient survival [63]. A woman's own estrogen was now killing the prepared and sensitized micrometastasis. Further study expanded the hypothesis to become a cyclical event with physiologic estrogen causing the destruction of a novel form of acquired antiestrogen resistance, but then the tumor was again responsive to antihormonal therapies such as aromatase inhibitors or indeed tamoxifen treatment (Fig. 9.1). It was suggested that physiologic estrogen could be used as a salvage therapy [64]. However, this was extremely controversial. The suggestion that administering estradiol to breast cancer patients after failing repeated antihormone therapies for breast cancer was unacceptable to IRBs in the 1990s and especially so to women's advocate groups. Nevertheless, with the therapeutic drift from tamoxifen to the aromatase inhibitors during the first decade of the twenty-first century, acquired resistance to estrogen deprivation became an important scientific issue.

In the 1970s and 1980s, Richard Santen [65, 66] had systematically and rigorously examined the clinical endocrine pharmacology of aminoglutethimide as an inhibitor of estrogen production, but the drug was not specific from the aromatase enzyme and glucocorticoids had to be coadministered. Angela Brodie had pioneered the practical applications of developing specific drugs to destroy the aromatase enzyme first in the 1970s at the Worcester Foundation and subsequently at the University of Maryland. Her discovery of the properties of 4-hydroxyandrostenedione went from the laboratory to clinical trial with approval in Europe [67–70]. The new aromatase inhibitors started to become the AU4

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antihormonal standard of care as long-term adjuvant therapy attention turned to acquired drug resistance. Santen's group reported that long-term estrogen-deprived (LTED) cells from the MCF-7 line would respond to estrogen in vitro initially claimed to have "acquired hypersensitivity" to minute amounts of estrogen in the environment to accomplish an apparent "estrogen-independent growth response" [71, 72]. Santen would subsequently show that LTED cells would respond to estrogen with apoptosis [73]. This was an explanation of Haddow's original chemical therapy for breast cancer, i.e., high-dose estrogen to treat postmenopausal patients with metastatic breast cancer [74, 75]. However, the new twist was that the antihormone therapies has now sensitized breast cancer cells to low doses of estrogen therapy, perhaps in the physiologic range. This concept could be used in the clinic as a salvage therapy and to explain the paradoxal new data with the estrogen replacement (CEE) in the Women's Health Initiative (WHI) of hysterectomized women [76]. There were fewer breast cancers!

Back to the Beginning

Paul Ehrlich created the first chemical therapy (chemotherapy) when he discovered Salvarsan for the treatment of syphilis in the later part of the nineteenth century. In the early years of the twentieth century, he turned his attentions to treating cancer and chose to develop animal models to facilitate drug testing. He had created this successful translational research process with his work on syphilis, so why not build on success? The year before his death in 1915, Ehrlich conceded defeat stating, "I have wasted 15 years of my life on experimental cancer research."

Sir Alexander Haddow accepted the challenge in the 1940s when he found that carcinogenic polycyclic hydrocarbons cause tumor regression in animal models. Clearly, it was not going to be possible to use these same hydrocarbons in patients, but he reasoned that the new synthetic estrogens diethylstilbestrol and the triphenylethylenes had multiple phenyl rings, so he tested them. Tumor regressions occurred in the animals, so high-dose estrogen therapy was tested in patients and produced therapeutic effect in about 30 % of metastatic breast cancer in postmenopausal women over 60 [74]. High-dose estrogen therapy remained the palliative treatment of choice until tamoxifen become the standard of care, and because it was safer and therefore more versatile, its applications extended to long-term adjuvant therapy and chemoprevention during the 1980s [3, 77].

Returning to high-dose estrogen therapy pre-tamoxifen, Haddow was the inaugural Karnofsky Memorial Lecturer at ASCO [78, 79]. In his lecture, Haddow expressed his concern about progress in cancer therapeutics. He did not believe there would ever be a cancer-specific target as Ehrlich had proposed; cancer was self, Haddow reasoned, unlike the story of antibiotics that could be tested in the laboratory to determine the correct antibiotic for the appropriate treatment of the actual disease. The crude cancer therapies were nonspecific and tried on the patient as the only way to determine whether the tumor was sensitive or not. He stated:

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t1.1 **Table 9.1** Objective response rates in postmenopausal women with metastatic breast cancer undergoing high dose estrogen therapy. The patients were divided based on years after menopause (Basil Stoll. Breast Cancer Management Early and Late. William Herman Medical Books Ltd., London pp. 133–146)

t1.2	Age since menopause	Patient #'s	% Regression
t1.3	Postmenopausal 0–5 years	63	9 %
t1.4	Postmenopausal > 5 years	344	35 %

260 the need exists for some method of prior screening to indicate the optimal choice (of
261 chemotherapy) in particular cases . . . efforts thus far have been disappointing.

262 He also stated:

263 . . . the extraordinary extent of tumour regression observed in perhaps 1 % of post-
264 menopausal cases (with oestrogen) has always been regarded as of major theoretical
265 importance, and it is a matter for some disappointment that so much of the underlying
266 mechanisms continues to elude us . . . [79]

267 The one bright glimmer of hope reason was the fact that high-dose DES was
268 extremely effective in some breast cancers. Haddow, it should be noted, also used
269 his preliminary data [74] to conduct a multicentric clinical trial through the Royal
270 Society of Medicine. He had a discovery:

271 When the various reports were assembled at the end of that time, it was fascinating to
272 discover that rather general impression, not sufficiently strong from the relatively small
273 numbers in any single group, became reinforced to the point of certainty; namely, the
274 beneficial responses were three times more frequent in women over the age of 60 years than
275 in those under that age; that oestrogens may, on the contrary, accelerate the course of
276 mammary cancer in younger women, and that their therapeutic use should be restricted to
277 cases 5 years beyond the menopause. Here was an early and satisfying example of the
278 advantages which may accrue from cooperative clinical trial.

279 This observation in clinical practice was supported by Dr. Basil Stoll whose
280 personal experience with high-dose DES for the treatment of metastatic breast
281 cancer in postmenopausal women replicated Haddow's observations (Table 9.1).
282 Thus, estrogen deprivation is the key to success for estrogen therapy, both for the
283 clinical use of high-dose therapy and for the interpretation of the CEE trial alone in
284 the WHI [76]. The women in the trial were an average 68 years of age! But can we
285 now seek a mechanism for the chain of events that causes the estrogen-ER complex
286 to trigger apoptosis?

287 Mechanisms of Estrogen-Induced Apoptosis

288 Studies of the molecular mechanisms of estradiol-induced apoptosis have occurred
289 only during the last decade. The study by Santen's group showed that estrogen
290 increases Fas ligand in LTED MCF-7 [73] cells but, by contrast, estradiol increases
291 Fas receptor in apoptotic long-term tamoxifen-resistant (phase II) MCF-7 tumors [80],

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both pointing to an extrinsic mechanism through "death receptors." However, these early studies were not time dependent but only snapshots of the apoptosis process at random times.

During the early 1990s, a couple of important estrogen-deprived cell lines were cloned from a cell population of MCF-7:WS8s following long-term (>1 year) estrogen deprivation in phenol-red-free media containing triple charcoal stripped serum. The two cell lines MCF-7:5C [81] and MCF-7:2A [82] were created in the anticipation of eventually being able to elucidate resistance to aromatase inhibitors, but they were placed in liquid nitrogen and stored for that day.

Lewis and coworkers [83] focused efforts in vitro on the MCF-7:5C cell line to describe the development of early apoptotic responses to estradiol. Rapid apoptotic events occurred at the intrinsic mitochondrial level with release of cytochrome C and a rise in proapoptotic gene products (BAX, BIM, and NOXA). Apoptosis was completely blocked by both fulvestrant (that destroys the cellular ER) and 4-hydroxytamoxifen, though the latter SERM did not affect the cell cycle in MCF-7:5C cells (i.e., these cells are resistant to SERMs). Flow cytometry was used to confirm the development of estrogen-induced apoptosis with increased annexin V and DAPI staining was used to confirm apoptosis by microscopy.

The MCF-7:2A cells only slowly go through apoptosis during the second week of estradiol treatment but this can be accelerated by using buthionine sulfoximine (BSO) to prevent glutathione synthesis [84]. The reduction of mechanisms to protect cells from reactive oxygen species is clearly an important protective measure to ensure survival of aromatase resistant cells.

The unique cell lines that are so sensitive either to estradiol-induced growth MCF-7:WS8 or rapidly apoptotic MCF-7:5C cells and slowly apoptotic MCF-7:2A cells have formed the foundations for an extensive study of the mechanistic studies of basal gene levels of activations between estrogen-responsive and estrogen-independent cell growth and the timed gene responses of all those cell lines over a 96-h period and the rate of gene activation of the MCF-7:2A cells over the second week of estrogen exposure [85].

Eric Ariazi at the Fox Chase Cancer Center working with Heather Cunliffe at Translational Genomics in Arizona created a superb Agilent gene array database for a "movie" of pathway analysis in the life and death of breast cancer cells. Essentially the study [85] creates a sequenced cooperative enrichment analysis of inflammatory responses, ER signaling, inflammation, and folding protein responsiveness in the endoplasmic reticulum during the timed move to full apoptosis. Ping Fan has described AP-1 synthesis and activation to initiate apoptosis through the accumulation of reactive oxygen species (ROS), all of which can be blocked by 4-hydroxytamoxifen or paradoxically a cSrc inhibitor [86]. But with the cascade of caspases created by estrogen action in MCF-7:5C cells and its modulations by arachidonic acid [85], the question must be asked: "What is it about the ER that triggers apoptosis in the correctly conditioned estrogen-deprived cells?" To address the question and find an answer, one must first examine the relationship between the ligand, the ER and the actual shape of the ER complex. It is this interrogation that exposed the mechanism of the "Haddow paradox" [79].

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A New Classification of Estrogens

The crystallization of the human ER ligand-binding domain with estradiol, raloxifene [87] diethylstilbestrol and 4-hydroxytamoxifen [88] precisely revealed the nature of the structural changes in the ER complex to create a mechanism of estrogen action that neatly dovetailed with the structure-activity relationships first described for modulation with the prolactin gene by the ER complex [35, 89–92] and the studies of the modulation of the transforming growth factor-alpha (TGF-α) gene by mutant ER-α in the 1990s [93, 94] and the 2000s [95–98]. Simply summarized, these studies defined the interaction of antiestrogenic side chains, correctly positioned to interact, neutralize, or shield the exposed amino acid 351 once the activation function-2 (AF-2) binding site for coactivators on helix 12 has been pushed open like the jaws of a crocodile. Pharmacologically, the angular triphenylethylenes that form the backbone of the SERMs only become antiestrogenic at appropriate target sites like the breast or uterus with a correctly positioned side chain.

But it was Geoffrey Greene [88] who used the phrase "the bulky antiestrogenic side chain" that created our next conceptual advance, as the antiestrogenic side chain was a finger like alkylaminoethoxy side chain, a trivial amount of molecular "bulk." However, Greene was including the nonplanar phenyl ring! We hypothesized that the planar and angular nonsteroidal estrogens would fit the ER ligand-binding domain differently. All estrogens were not equal. A precise biological assay of two different cell lines derived using the ER-negative MDA-MB-231 cell line either stably transfected with wild-type ER or the asp 351gly mutant. Planar (class I) estrogens such as DES and estradiol and nonplanar (class II) triphenylethylene estrogens were compared and contrasted to switch on or off the TGF-α gene. The results were a simple yes/no answer. A planar estrogen (class I) would easily fit in the binding pocket of the LBD to activate AF 2 coactivator binding formed from a closed helix 12 sealing the ligand inside. Both cell lines would activate TGF-α. In contrast, the estrogen-like activity of 4-hydroxytamoxifen with a short antiestrogenic side chain results from the negatively charged aspartate 351 communicating with AF-1 to cause estrogen action (weak as it is) and activation of the TGF-α gene. In the cells with the asp351gly mutation, there would be no activation of TGF-α [99]. A triphenylethylene estrogen had some estrogen action with wild-type ER and an exposed asp 351, but with the asp 351 gly mutant with no charge, there was none [99]. It was proof that the shape of the ER complex with a triphenylethylene had a pushed back helix 12. Simply stated, crocodile jaws closed for a class I estrogen, jaws open for a class II estrogen. In the paper, it was stated that the authors had no idea what this would mean in biology [99] but there was a claim that it could be important. We showed the effect was reproducible by classifying the estrogen-like contaminant of the nonsteroidal didesmethyl methoxychlor (DDM) as a class 2 estrogen [100].

However, the fact that 4-hydroxytamoxifen completely blocked the action of estradiol to cause apoptosis in MCF-7:5C cells opened the door to prove that shape

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mattered for the estrogen-ER complex to trigger apoptosis. Did the "jaws of the crocodile" need to be closed to trigger apoptosis?

The first clue that the hypothesis was going to prove to be correct and control apoptosis was the triphenylethylene estrogen-ER complexes were shown not to be down regulated in MCF-7 cells like estradiol, but to accumulate like 4-hydroxytamoxifen. The ER complex for these nonplanar estrogens was like an antiestrogen! The shape of the ER with different types of estrogen did, in fact, control an important biological process—estrogen-induced apoptosis! Further studies exhaustively demonstrated that the triphenylethylenes stimulated the growth of MCF-7 cells just like estradiol, but with less potency, and confirmed and massively expanded earlier studies that triphenylethylene estrogens did block apoptosis. Triphenylethylene complex with ER did not bind the coactivator SRC-3 as avidly at the promoter regions of estrogen-responsive genes [101], and these data beautifully confirmed the observation in complimentary studies that SRC-3 was important for estrogen-induced apoptosis [102]. By studying SRC3-interacting proteins, one could decipher the early events in estrogen-induced apoptosis in vitro [102] and in vivo (...). AU6

However, during this conversation with nature to decipher the mechanism of estrogen-induced apoptosis, very important one fact was inconsistent. If estrogenic triphenylethylenes block estrogen-induced apoptosis in a cell like MCF-7:5C in the laboratory, then why did Haddow observe his best responses with estrogen-induced tumor regress with estrogenic triphenylethylenes used for the treatment of metastatic breast cancer in late postmenopausal women [74]? A clinical reality with tumor regression with estrogen trumps a laboratory study every time! This inconsistency was solved with that the triphenylethylenes kill the cells in culture in 2 weeks. The time course is extended with class II angular estrogens so the triggering process is only occurring slowly. In the patient the long-term retention and storage of triphenylethylenes in a woman's body fat provides a continuous high estrogen environment to produce optimal antitumor actions. A conversation with nature does work!

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Final Thoughts on Four Decades of Discovery to Advance the Value of the ER Target in Breast Cancer

We begin and end our story with the actions of synthetic estrogens to kill breast cancer cells that have been prepared for sacrifice through estrogen deprivations. The best current example of the value of this knowledge in women's health are the results of the Women's Health Initiative with conjugated equine estrogens alone in hysterectomized women to reduce breast cancer incidence and mortality for women in their mid-60s [76]. The 40 years starting with the development of tamoxifen from a failed contraceptive to being the gold standard that saved the lives of millions of women through the prudent application of the laboratory principle of long-term adjuvant therapy [2] resulted in the mandatory laboratory study of acquired drug

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resistance to long-term tamoxifen therapy. Acquired resistance would surely occur, but no one could have predicted the development of tamoxifen-stimulated breast cancer growth or the evolution of acquired resistance to expose a fatal vulnerability in breast cancer so that physiologic estrogen triggered apoptosis. Each discovery was in the hands of young scientists as generations of Tamoxifen Teams that turned ideas into lives saved. Progress occurred through their outstanding skill in the laboratory and the philosophy that if Nature gives us the "wrong answer" to our question, Nature does not lie. The answer is the true answer to the question that must be considered as the solution to the problem to be solved.

Postscript. During his Ph.D. training, Doug Wolf discovered multiple valuable clues to understand SERMs, drug resistance, and estrogen-induced apoptosis, but at the time all of this was speculative with no real basis in scientific fact. The two discoveries that Doug contributed were both serendipity. In fact, all advances are serendipity in basic science, but it is the recognition of the new knowledge that becomes the key to discovery. One spots the clue and expands on the observation because it is a "conversation with Nature." The unimaginative scientist throws the clue away as it does not fit the model of what is correct or incorrect in their mind at the time.

In a search for mechanisms to explain acquired tamoxifen resistance, Doug was focused in two directions in his Ph.D. thesis. The two main questions were as follows: "Is acquired drug resistance to tamoxifen because a mutation of the estrogen receptor occurs to change the pharmacology of tamoxifen from an antiestrogen to an estrogen?" and secondly "What growth factor receptors and receptor signal transduction pathways are responsible for estradiol-stimulated growth of tumors with acquired tamoxifen resistance and does tamoxifen use the same pathways as estradiol?"

To address the issue of a mutation of the ER enhancing the estrogen-like effects of tamoxifen, Doug created a number of tamoxifen-stimulated tumor lines and screened them for ER mutations [62]. All tumor lines had wild-type ER except one with a large proportion of an ER with an asp 351 tyr mutation [62, 103]. We had a no idea at the time what this was going to mean for understanding the mechanics of SERM action but it was destined to be profound. Bill Catherino, an M.D., Ph.D. student in my laboratory at Wisconsin, subsequently created the BC-2 stably transfected cell line in MDA-MB-231 cells using a cDNA for the mutant receptor [104]. Anna Levenson, a postdoctoral fellow and then a research assistant professor at Northwestern used a transforming growth factor (TGF)-α target (discovered by Mei Huey Jeng) [105] to compare and contrast the estrogenic and antiestrogenic action of tamoxifen and raloxifene. The Asp351Tyr ER turned out to be the first and, to date, the only natural mutation of the human ER to change the pharmacology of a nonsteroidal antiestrogen from a complete antiestrogen to an estrogen [93, 106]. We were mystified why a mutation buried in the ligand-binding domain (LBD) of the ER could influence the pharmacology of raloxifene, but the reason became clear with the subsequent publications of the crystal structure of the raloxifene-ER LBD [87]. However, if one examines the X-ray crystallography in the papers it is almost impossible to interpret in "the real world" of protein-protein

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interactions. That is for the outside! The fact that we realized that Asp 351 was a surface amino acid on the ER complex was the key to finding the "antiestrogen region" was had predicted in paper published 15 years earlier [90, 107]. But the discovery was by chance and this chance created opportunities for a productive scientific collaboration. I had been invited to Signal Pharmaceuticals in California to discuss a new SERM, but as I was waiting for my taxi to take me to my hotel, I started to wander the corridors and struck up a conversation with a young man Jim Zapf who was "playing" on his computer. "What do you do?" says I. Jim replied, "I do docking of ligands with the ER ligand binding domain." "OK," I said. "How good is your program? Can you show me the outside of the ER complex dimer—this is what other proteins see?" "No problem," Jim replies. "Let me ask you this. Color in where helix 12 is with the estradiol or raloxifene ER complex?" In a second or two I exclaim, "It really is the crocodile model of estrogen and antiestrogen action." We had proposed this 15 years earlier [90]. "OK so where is aspartate 351 in the estradiol ER complex?" I inquired. Jim replies, "It's here under helix 12 on the surface of the complex but it does not play a role." As we switch to the raloxifene-ER complex, the significance of aspartate 351 was clear through its interaction with the "antiestrogenic side chain" of raloxifene. The pyrolidine ring shields and neutralized the aspartate producing a complete antiestrogen, but tamoxifen has a side chain that is a few Å shorter and cannot do the job completely and is promiscuous with estrogen-like actions. This chance meeting resulted in collaboration and a half a dozen publications of ER modulation. We subsequently interrogated the ligand asp 351 interactions (Chap. 5, Postscript) and this was reviewed by Levenson [108].

One of the Doug's other tasks was to utilize the Marco Gottardis athymic mouse model of acquired resistance to tamoxifen [28] to discover the growth factor pathways, responsible for estradiol or tamoxifen-stimulated tumor growth. At this time, in the early 1990s, growth factor signaling was the fashion [109] and primarily spearheaded by Dr. Marc E. Lippman who had just become the director of the Lombardi Cancer Center in Washington, DC. He had moved, with all his staff, from the National Cancer Institute in Bethesda where he was the head of the Breast program. Doug's project was simple. Grow up some of the tamoxifen-stimulated tumors with tamoxifen in athymic mice and then switch to either tamoxifen or physiologic estrogen released from subcutaneous capsules. Then, harvest growing tumors and measure all known growth factors and their receptors to answer the question: "Is estrogen or tamoxifen induced-growth stimulated by the same or different growth factors?" The tumors did not grow with physiologic estradiol; they disappeared—they just melted away in a few weeks! I suggested that the long-term tamoxifen exposure had somehow accelerated a natural sensitivity to estrogen-induced tumor cell death. It was the explanations of Haddow's landmark observation in patients 50 years before [74, 78]! These data at Wisconsin [63] were presented at the St. Gallen Breast Cancer Conference in 1992 and were replicated at Northwestern by a superb team of resident surgeons Kathy Yao, Eun-Sook Lee, Dave Bertram, Gale England, a medical oncology fellow Ruth O'Regan, and my Ph.D. student Jennifer MacGregor Shafer [64]. Their data showed that Doug's work

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was reproducible and the phenomenon occurred over a 5-year period (i.e., in the last 2 years of transplantation in tamoxifen-treated mice). Gale England showed this beautifully in her notebook and Dave Bentram stepped in to perform biotransplant tumor experiments requested by the referees for Kathy Yao's paper [64]. They thought the animals had changed, not the tumor. Dave showed that it was the tumor, not the animals. These data opened a new door of discovery for the next decade with the exploitation of the principle of successfully treating patients with acquired antihormone therapy with low doses of estradiol [110], the study of mechanisms [73, 80, 83, 85, 111] that answered Haddow's statement of dismay in his 1971 Karnofsky lecture:

> ... the extraordinary extent of tumour regression observed in perhaps 1 % of post-menopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us ...

I was thrilled to be selected as the 38th winner of the Karnofsky Memorial Lecture and selected as my title, "The paradoxical actions of estrogen in breast cancer: survival or death?" As Haddow and I were (are) British and my Tamoxifen Team have, through serendipity, now discovered the molecular mechanism of estrogen-induced apoptosis, this seemed to me to be the appropriate tribute to his pioneering advance in chemical therapy.

Our subsequent work also provided the basis for the explanation of the antitumor effects of physiologic estrogen when used as estrogen replacement therapy [76]. A valuable conversation with nature that could have been so easily abandoned in 1993 with the "wrong answer" from Doug's experiment that could not reproduce estrogen response in Marco's Model. But another twist was necessary to advance our Tamoxifen Team tale.

It is worth emphasizing the significant role that Dr. Joan Lewis-Wambi played in this story, our knowledge of estrogen-induced apoptosis and the cell model she breathed life into—by chance. Dr. Shun-Yuan Jiang created both the MCF-7:5C [81] cells and MCF-7:2A cells [82]. Both cell lines were cloned out of populations that were estrogen deprived for almost a year. The majority of cells died but some survived and grew under estrogen-free conditions. The MCF-7:5C [81] are ER positive and PgR negative, and we reported they did not respond to estrogen or antiestrogens. Joan Lewis almost 10 years later was given the task of studying these cells at a time that it was clear that the aromatase inhibitors would be an essential treatment option for breast cancer patients in the medical oncologists' armamentarium. What did she do? She charged the serum conditions to grow our MCF-7:5C cells and did not follow the essential tradition of repeating exactly what Shun-Yuan had done in her paper [81]. Amazingly, the MCF-7:5C cells grew spontaneously, but apoptosis occurred rapidly with physiologic estrogen in vitro and in vivo. We had never had a cell line that responded to estrogen as a cidal stimulus—and now we did [112]. She created a pivotal paper on the intrinsic mechanism of apoptosis [83] and followed this up with a super description of the delayed apoptosis (estrogen took a week longer to cause cell death in the MCF-7:2A cells) observed

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in the 2A cells that could be advanced to immediate cell death with estrogen if glutathione synthesis was blocked [84]. I should document that her husband Dr. Chris Wambi was conducting research on the redox role of glutathione using buthionine sulfoximine (BSO) to stop glutathione synthesis, and it was this husband and wife team that found our mechanism of cell survival that could be neutralized so that estrogen now caused rapid cell death. This is a good husband/wife synergy in science.

Without these cell models, our expanding experience and publications with acquired tamoxifen and raloxifene resistance in vivo, we could not have successfully competed for our Department of Defense Center of Excellence Grant. These studies and models passed on one to another over decades by my trainees significantly advanced women health and helped families stay together longer through the increased survival of women either with breast cancer or at risk of breast cancer.

References

1. McGuire WL, Carbone PP, Vollmer EP, United States. National Cancer Institute. Breast Cancer Treatment Committee (1975) Estrogen receptors in human breast cancer. Raven Press, New York, xiv, 284 pp
2. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44:30–38
3. Ingle JN, Ahmann DL, Green SJ et al (1981) Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. N Engl J Med 304:16–21
4. Soule HD, Vazguez J, Long A et al (1973) A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51:1409–1416
5. Brooks SC, Locke ER, Soule HD (1973) Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J Biol Chem 248:6251–6253
6. Lippman ME, Bolan G (1975) Oestrogen-responsive human breast cancer in long term tissue culture. Nature 256:592–593
7. Shafie SM (1980) Estrogen and the growth of breast cancer: new evidence suggests indirect action. Science 209:701–702
8. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS (1986) Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A 83:2496–2500
9. Bindal RD, Katzenellenbogen JA (1988) Bis(4-hydroxyphenyl)[2-(phenoxysulfonyl)phenyl] methane: isolation and structure elucidation of a novel estrogen from commercial preparations of phenol red (phenolsulfonphthalein). J Med Chem 31:1978–1983
10. Welshons WV, Jordan VC (1987) Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. Eur J Cancer Clin Oncol 23:1935–1939
11. Levenson AS, Jordan VC (1997) MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res 57:3071–3078
12. Nawata H, Bronzert D, Lippman ME (1981) Isolation and characterization of a tamoxifen-resistant cell line derived from MCF-7 human breast cancer cells. J Biol Chem 256:5016–5021
13. Miller MA, Lippman ME, Katzenellenbogen BS (1984) Antiestrogen binding in antiestrogen growth-resistant estrogen-responsive clonal variants of MCF-7 human breast cancer cells. Cancer Res 44:5038–5045

Uncorrected Proof

Author's Proof

14. Bronzert DA, Greene GL, Lippman ME (1985) Selection and characterization of a breast cancer cell line resistant to the antiestrogen LY 117018. Endocrinology 117:1409–1417
15. van den Berg HW, Lynch M, Martin J et al (1989) Characterisation of a tamoxifen-resistant variant of the ZR-75-1 human breast cancer cell line (ZR-75-9a1) and ability of the resistant phenotype. Br J Cancer 59:522–526
16. Wiseman LR, Johnson MD, Wakeling AE et al (1993) Type I IGF receptor and acquired tamoxifen resistance in oestrogen-responsive human breast cancer cells. Eur J Cancer 29A:2256–2264
17. Brunner N, Frandsen TL, Holst-Hansen C et al (1993) MCF7/LCC2: a 4-hydroxytamoxifen resistant human breast cancer variant that retains sensitivity to the steroidal antiestrogen ICI 182,780. Cancer Res 53:3229–3232
18. Lykkesfeldt AE, Madsen MW, Briand P (1994) Altered expression of estrogen-regulated genes in a tamoxifen-resistant and ICI 164,384 and ICI 182,780 sensitive human breast cancer cell line, MCF-7/TAMR-1. Cancer Res 54:1587–1595
19. Borras M, Jin L, Bouhoute A et al (1994) Evaluation of estrogen receptor, antiestrogen binding sites and calmodulin for antiestrogen resistance of two clones derived from the MCF-7 breast cancer cell line. Biochem Pharmacol 48:2015–2024
20. Brunner N, Boysen B, Jirus S et al (1997) MCF7/LCC9: an antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen. Cancer Res 57:3486–3493
21. Larsen SS, Madsen MW, Jensen BL, Lykkesfeldt AE (1997) Resistance of human breast-cancer cells to the pure steroidal anti-estrogen ICI 182,780 is not associated with a general loss of estrogen-receptor expression or lack of estrogen responsiveness. Int J Cancer 72:1129–1136
22. Parisot JP, Leeding KS, Hu XF et al (1999) Induction of insulin-like growth factor binding protein expression by ICI 182,780 in a tamoxifen-resistant human breast cancer cell line. Breast Cancer Res Treat 55:231–242
23. Chan CM, Lykkesfeldt AE, Parker MG, Dowsett M (1999) Expression of nuclear receptor interacting proteins TIF-1, SUG-1, receptor interacting protein 140, and corepressor SMRT in tamoxifen-resistant breast cancer. Clin Cancer Res 5:3460–3467
24. Shafie SM, Grantham FH (1981) Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. J Natl Cancer Inst 67:51–56
25. Osborne CK, Hobbs K, Clark GM (1985) Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res 45:584–590
26. Osborne CK, Coronado EB, Robinson JP (1987) Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. Eur J Cancer Clin Oncol 23:1189–1196
27. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
28. Gottardis MM, Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 48:5183–5187
29. Gottardis MM, Wagner RJ, Borden EC, Jordan VC (1989) Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro. Cancer Res 49:4765–4769
30. Harper MJ, Walpole AL (1966) Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. Nature 212:87
31. Osborne CK, Coronado E, Allred DC et al (1991) Acquired tamoxifen resistance: correlation with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxytamoxifen. J Natl Cancer Inst 83:1477–1482

Uncorrected Proof

Author's Proof

32. Osborne CK, Wiebe VJ, McGuire WL et al (1992) Tamoxifen and the isomers of 4-hydroxytamoxifen in tamoxifen-resistant tumors from breast cancer patients. J Clin Oncol 10:304–310
33. Wiebe VJ, Osborne CK, McGuire WL, DeGregorio MW (1992) Identification of estrogenic tamoxifen metabolite(s) in tamoxifen-resistant human breast tumors. J Clin Oncol 10:990–994
34. Murphy CS, Langan-Fahey SM, McCague R, Jordan VC (1990) Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. Mol Pharmacol 38:737–743
35. Jordan VC, Koch R, Langan S, McCague R (1988) Ligand interaction at the estrogen receptor to program antiestrogen action: a study with nonsteroidal compounds in vitro. Endocrinology 122:1449–1454
36. Wolf DM, Langan-Fahey SM, Parker CJ et al (1993) Investigation of the mechanism of tamoxifen-stimulated breast tumor growth with nonisomerizable analogues of tamoxifen and metabolites. J Natl Cancer Inst 85:806–812
37. Benz CC, Scott GK, Sarup JC et al (1992) Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res Treat 24:85–95
38. Slamon DJ, Leyland-Jones B, Shak S et al (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344:783–792
39. Marty M, Cognetti F, Maraninchi D et al (2005) Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. J Clin Oncol 23:4265–4274
40. Gasparini G, Gion M, Mariani L et al (2007) Randomized Phase II Trial of weekly paclitaxel alone versus trastuzumab plus weekly paclitaxel as first-line therapy of patients with Her-2 positive advanced breast cancer. Breast Cancer Res Treat 101:355–365
41. Robert N, Leyland-Jones B, Asmar L et al (2006) Randomized phase III study of trastuzumab, paclitaxel, and carboplatin compared with trastuzumab and paclitaxel in women with HER-2-overexpressing metastatic breast cancer. J Clin Oncol 24:2786–2792
42. Bernard-Marty C, Lebrun F, Awada A, Piccart MJ (2006) Monoclonal antibody-based targeted therapy in breast cancer: current status and future directions. Drugs 66:1577–1591
43. Cortes J, Fumoleau P, Bianchi GV et al (2012) Pertuzumab monotherapy after trastuzumab-based treatment and subsequent reintroduction of trastuzumab: activity and tolerability in patients with advanced human epidermal growth factor receptor 2-positive breast cancer. J Clin Oncol 30:1594–1600
44. Kiewe P, Hasmuller S, Kahlert S et al (2006) Phase I trial of the trifunctional anti-HER2 x anti-CD3 antibody ertumaxomab in metastatic breast cancer. Clin Cancer Res 12:3085–3091
45. Konecny GE, Pegram MD, Venkatesan N et al (2006) Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. Cancer Res 66:1630–1639
46. Gomez HL, Doval DC, Chavez MA et al (2008) Efficacy and safety of lapatinib as first-line therapy for ErbB2-amplified locally advanced or metastatic breast cancer. J Clin Oncol 26:2999–3005
47. Nelson MH, Dolder CR (2006) Lapatinib: a novel dual tyrosine kinase inhibitor with activity in solid tumors. Ann Pharmacother 40:261–269
48. Cameron D, Casey M, Press M et al (2008) A phase III randomized comparison of lapatinib plus capecitabine versus capecitabine alone in women with advanced breast cancer that has progressed on trastuzumab: updated efficacy and biomarker analyses. Breast Cancer Res Treat 112:533–543
49. Xia W, Bacus S, Hegde P et al (2006) A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. Proc Natl Acad Sci U S A 103:7795–7800

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Author's Proof

50. Shou J, Massarweh S, Osborne CK et al (2004) Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst 96:926–935
51. Riemsma R, Forbes CA, Amonkar MM et al (2012) Systematic review of lapatinib in combination with letrozole compared with other first-line treatments for hormone receptor positive(HR+) and HER2+ advanced or metastatic breast cancer (MBC). Curr Med Res Opin 28(8):1263–1279
52. Maximov PY, Lewis-Wambi JS, Jordan VC (2009) The paradox of oestradiol-induced breast cancer cell growth and apoptosis. Curr Signal Transduct Ther 4:88–102
53. Schafer JM, Lee ES, O'Regan RM et al (2000) Rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice. Clin Cancer Res 6:4373–4380
54. Osborne CK, Pippen J, Jones SE et al (2002) Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. J Clin Oncol 20:3386–3395
55. Howell A, Robertson JF, Quaresma Albano J et al (2002) Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. J Clin Oncol 20:3396–3403
56. Wakeling AE, Bowler J (1987) Steroidal pure antioestrogens. J Endocrinol 112:R7–R10
57. Wakeling AE, Dukes M, Bowler J (1991) A potent specific pure antiestrogen with clinical potential. Cancer Res 51:3867–3873
58. Gottardis MM, Jiang SY, Jeng MH, Jordan VC (1989) Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res 49:4090–4093
59. Tormey DC, Jordan VC (1984) Long-term tamoxifen adjuvant therapy in node-positive breast cancer: a metabolic and pilot clinical study. Breast Cancer Res Treat 4:297–302
60. Scottish Cancer Trials Office (MRC), Edinburgh (1987) Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee. Lancet 2:171–175
61. Fisher B, Brown A, Wolmark N et al (1987) Prolonging tamoxifen therapy for primary breast cancer. Findings from the National Surgical Adjuvant Breast and Bowel Project clinical trial. Ann Intern Med 106:649–654
62. Wolf DM, Jordan VC (1994) Characterization of tamoxifen stimulated MCF-7 tumor variants grown in athymic mice. Breast Cancer Res Treat 31:117–127
63. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127:23–33
64. Yao K, Lee ES, Bentrem DJ et al (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6:2028–2036
65. Santen RJ, Samojlik E, Wells SA (1980) Resistance of the ovary to blockade of aromatization with aminoglutethimide. J Clin Endocrinol Metab 51:473–477
66. Santen RJ, Worgul TJ, Samojlik E et al (1981) A randomized trial comparing surgical adrenalectomy with aminoglutethimide plus hydrocortisone in women with advanced breast cancer. N Engl J Med 305:545–551
67. Brodie AM, Schwarzel WC, Brodie HJ (1976) Studies on the mechanism of estrogen biosynthesis in the rat ovary–I. J Steroid Biochem 7:787–793
68. Brodie AM, Schwarzel WC, Shaikh AA, Brodie HJ (1977) The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. Endocrinology 100:1684–1695
69. Brodie AM, Garrett WM, Hendrickson JR et al (1981) Inactivation of aromatase in vitro by 4-hydroxy-4-androstene-3,17-dione and 4-acetoxy-4-androstene-3,17-dione and sustained effects in vivo. Steroids 38:693–702
70. Coombes RC, Goss P, Dowsett M et al (1984) 4-hydroxyandrostenedione in treatment of postmenopausal patients with advanced breast cancer. Lancet 2:1237–1239

Uncorrected Proof

Author's Proof

71. Masamura S, Santner SJ, Heitjan DF, Santen RJ (1995) Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. J Clin Endocrinol Metab 80:2918–2925
72. Shim WS, Conaway M, Masamura S et al (2000) Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. Endocrinology 141:396–405
73. Song RX, Mor G, Naftolin F et al (2001) Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 93:1714–1723
74. Haddow A, Watkinson JM, Paterson E, Koller PC (1944) Influence of synthetic oestrogens on advanced malignant disease. Br Med J 2:393–398
75. Kennedy BJ, Nathanson IT (1953) Effects of intensive sex steroid hormone therapy in advanced breast cancer. J Am Med Assoc 152:1135–1141
76. Anderson GL, Chlebowski RT, Aragaki AK et al (2012) Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. Lancet Oncol 13:476–486
77. Jordan VC (2003) Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2:205–213
78. Haddow A (1971) Cancer research: the great debate. N Engl J Med 285:24–28
79. Haddow A, David A (1970) Karnofsky memorial lecture. Thoughts on chemical therapy. Cancer 26:737–754
80. Osipo C, Gajdos C, Liu H et al (2003) Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 95:1597–1608
81. Jiang SY, Wolf DM, Yingling JM et al (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 90:77–86
82. Pink JJ, Jiang SY, Fritsch M, Jordan VC (1995) An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. Cancer Res 55:2583–2590
83. Lewis JS, Meeke K, Osipo C et al (2005) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97:1746–1759
84. Lewis-Wambi JS, Kim HR, Wambi C et al (2008) Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. Breast Cancer Res 10:R104
85. Ariazi EA, Cunliffe HE, Lewis-Wambi JS et al (2011) Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci U S A 108:18879–18886
86. Fan P, McDaniel RE, Kim HR et al (2012) Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines. Eur J Cancer 48(18):3488–3498
87. Brzozowski AM, Pike AC, Dauter Z et al (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
88. Shiau AK, Barstad D, Loria PM et al (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937
89. Jordan VC, Koch R, Mittal S, Schneider MR (1986) Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. Br J Pharmacol 87:217–223
90. Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741–4745
91. Lieberman ME, Jordan VC, Fritsch M et al (1983) Direct and reversible inhibition of estradiol-stimulated prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4734–4740
92. Jordan VC, Lieberman ME (1984) Estrogen-stimulated prolactin synthesis in vitro. Classification of agonist, partial agonist, and antagonist actions based on structure. Mol Pharmacol 26:279–285

Uncorrected Proof

Author's Proof

93. Levenson AS, Catherino WH, Jordan VC (1997) Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. J Steroid Biochem Mol Biol 60:261–268
94. Levenson AS, Tonetti DA, Jordan VC (1998) The oestrogen-like effect of 4-hydroxytamoxifen on induction of transforming growth factor alpha mRNA in MDA-MB-231 breast cancer cells stably expressing the oestrogen receptor. Br J Cancer 77:1812–1819
95. Bentrem D, Dardes R, Liu H et al (2001) Molecular mechanism of action at estrogen receptor alpha of a new clinically relevant antiestrogen (GW7604) related to tamoxifen. Endocrinology 142:838–846
96. MacGregor Schafer J, Liu H, Bentrem DJ et al (2000) Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. Cancer Res 60:5097–5105
97. Liu H, Park WC, Bentrem DJ et al (2002) Structure-function relationships of the raloxifene-estrogen receptor-alpha complex for regulating transforming growth factor-alpha expression in breast cancer cells. J Biol Chem 277:9189–9198
98. Liu H, Lee ES, Deb Los Reyes A et al (2001) Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor alpha complex. Cancer Res 61:3632–3639
99. Jordan VC, Schafer JM, Levenson AS et al (2001) Molecular classification of estrogens. Cancer Res 61:6619–6623
100. Bentrem D, Fox JE, Pearce ST et al (2003) Distinct molecular conformations of the estrogen receptor alpha complex exploited by environmental estrogens. Cancer Res 63:7490–7496
101. Maximov P, Sengupta S, Lewis-Wambi JS et al (2011) The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: A hypothesis. Horm Mol Biol Clin Investig 5:27–34
102. Hu ZZ, Kagan BL, Ariazi EA et al (2011) Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. PLoS One 6:e20410
103. Wolf DM, Jordan VC (1994) The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31:129–138
104. Catherino WH, Wolf DM, Jordan VC (1995) A naturally occurring estrogen receptor mutation results in increased estrogenicity of a tamoxifen analog. Mol Endocrinol 9:1053–1063
105. Jeng MH, Jiang SY, Jordan VC (1994) Paradoxical regulation of estrogen-dependent growth factor gene expression in estrogen receptor (ER)-negative human breast cancer cells stably expressing ER. Cancer Lett 82:123–128
106. Levenson AS, Jordan VC (1998) The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. Cancer Res 58:1872–1875
107. Jordan VC (1984) Biochemical pharmacology of antiestrogen action. Pharmacol Rev 36:245–276
108. Levenson AS, MacGregor Schafer JI, Bentrem DJ et al (2001) Control of the estrogen-like actions of the tamoxifen-estrogen receptor complex by the surface amino acid at position 351. J Steroid Biochem Mol Biol 76:61–70
109. Dickson RB, Lippman ME (1995) Growth factors in breast cancer. Endocr Rev 16:559–589
110. Ellis MJ, Gao F, Dehdashti F et al (2009) Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 302:774–780
111. Liu H, Lee ES, Gajdos C et al (2003) Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 95:1586–1597
112. Lewis JS, Osipo C, Meeke K, Jordan VC (2005) Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 94:131–141

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Chapter No.: 9

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AU2	Please check if edit to sentence starting "In particular, lapatinib...." is okay.	
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Abstract	Tamoxifen, the first targeted therapy to treat breast cancer, has dramatically changed medicine. Study of the pharmacology of tamoxifen created a successful adjuvant treatment strategy to save lives, created the first chemopreventive to prevent any cancer in humans, and was the pioneering selective estrogen receptor modulator (SERM) that resulted in the new drug group, the SERMs. New agents such as lasofoxifene and bazedoxifene show a promise in the range of beneficial effects they demonstrate in clinical trial to treat multiple diseases in women. Additionally, new agents and approaches with conjugated equine estrogen are being explored to prevent hot flashes, thereby enhancing the likelihood that compliance with SERMs improves.	

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Chapter 10
The Legacy of Tamoxifen

Abstract Tamoxifen, the first targeted therapy to treat breast cancer, has dramatically changed medicine. Study of the pharmacology of tamoxifen created a successful adjuvant treatment strategy to save lives, created the first chemopreventive to prevent any cancer in humans, and was the pioneering selective estrogen receptor modulator (SERM) that resulted in the new drug group, the SERMs. New agents such as lasofoxifene and bazedoxifene show a promise in the range of beneficial effects they demonstrate in clinical trial to treat multiple diseases in women. Additionally, new agents and approaches with conjugated equine estrogen are being explored to prevent hot flashes, thereby enhancing the likelihood that compliance with SERMs improves.

Introduction

During the 1970s and 1980s, the pharmaceutical industry worked diligently to study the structure-activity relationships of nonsteroidal antiestrogens to find a competitor for tamoxifen. The list includes droloxifene (3-hydroxytamoxifen), trioxifene, LY117,018, toremifene, and idoxifene [1]. Clinical trials were, in the main, unable to show any significant advantages over tamoxifen. The bench mark to predict success was less uterotrophic activity and LY117,018, which as a result evolved to become raloxifene via LY156,758. Toremifene was registered for the treatment of metastatic breast cancer but is not appropriate for adjuvant therapy in the United States. There has been interest in the use of toremifene for the treatment of prostate cancer [2, 3]. Tamoxifen, uniquely, remained the sole agent of choice as an adjuvant therapy for about 20 years.

ICI Pharmaceutical Division chose another direction to solve the "estrogenic tickle" of tamoxifen with a plan for the development of fulvestrant as an injectable pure antiestrogen.

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P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
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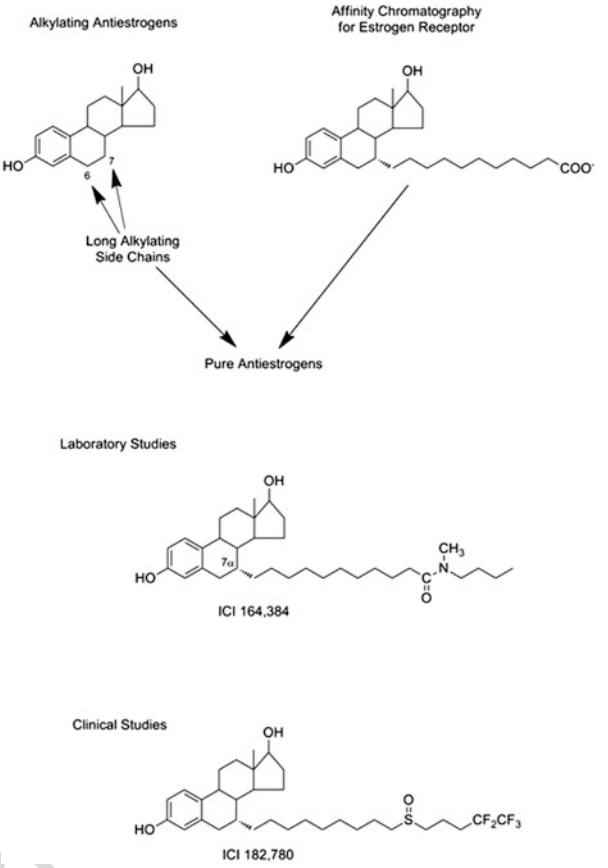

Fig. 10.1 The progress of two unrelated ideas coming together to create a new drug group: the pure antiestrogens. Estradiol derivatives substituted at 6 and 7 positions were created to deliver an alkylating agent via the ER to DNA. In contrast, estradiol was attached to long hydrocarbon chains on the Sephadex column to purify the ER. Both aspects of estradiol chemistry came together to create the pure antiestrogens at ICI Pharmaceuticals Division in the early 1980s

Pure Antiestrogens

The possibility that a pure antiestrogen could be developed with high binding affinity for the ER combines the observation that MER 25, the first antiestrogen, has virtually no estrogenic properties in any animal species [4], with the knowledge that binding affinity and biological activity are separate functions of the same molecule [5]. The antiestrogens ICI164,384 and ICI182,780 are derivatives of estradiol with an optimal binding affinity for the ER, but these structural analogs are unique because they do not have any estrogenic properties and they have a novel subcellular mechanism of action [6] (Fig. 10.1). The serendipitous discovery of pure antiestrogens occurred through two essentially unsuccessful research endeavors that converged thus providing the optimal intellectual environment for new drug discovery. Derivatives of estradiol or estrone substituted in the 6 and 7 positions were being evaluated as potential alkylating antiestrogens in the late 1970s through an ICI-Leeds University joint research scheme [7]. Independently, scientists in France were attempting to purify the ER using estradiol linked at the 7 position through a ten-membered carbon side chain to

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Sephadex columns [8]. Dr. Alan Wakeling brought both of these independent ideas together to discover the structure-function relationships of a new class of compounds that have no estrogenic properties in any test system [9–11]. The pure antiestrogen ICI 164,384 has been used extensively in laboratory studies [6], but the more potent ICI182,780 [11] is currently approved for the treatment of breast cancer in postmenopausal women metastatic breast cancer.

The compound is used as a 250-mg injectable 1-month sustained release preparation with therapeutic equivalence to anastrozole following failure of tamoxifen therapy [12, 13]. However, the endocrine option of fulvestrant has never achieved "first-line" status and as such never been evaluated as an adjuvant therapy. Nevertheless, 2-week strategies deserve mention.

The idea of combining an aromatase inhibitor with fulvestrant versus an aromatase inhibitor alone has merit from laboratory studies but has produced one result which was an improvement for the combination versus the aromatase inhibitor alone. By contrast, a second trial using the same treatment [14] protocol showed no difference for the combination versus the aromatase inhibitor alone [15]. It seems that the trial that showed no improvement for the combination [15] had a higher population of patients who had been exposed to tamoxifen treatment previously. Another issue is dosage. The pharmacokinetics of fulvestrant from the 250-mg depot injection is poor with low circulating levels [16]. To address this critically important issue, the CONFIRM trial has compared 250 versus 500 mg monthly injections [17]. The higher dose provides a superior response so this should now be considered to be the dosage of choice.

Angela Brodie's dedicated and pioneering work [18–20] was essential as proof of principle that a selective aromatase inhibitor could be discovered with clinical efficacy. The problem with her discovery, 4-hydroxyandrostenedione, was that it was an injectable rather than a more convenient oral preparation. However, the fact that the failed "morning-after pill" ICI46,474 was transformed successfully into the "gold standard" tamoxifen for the adjuvant treatment of breast cancer provided a new target (the aromatase enzyme) to improve antihormonal therapy in breast cancer. With profits expanding from sales of tamoxifen in the United States after 1990, the key issue for the successful drug development of an aromatase inhibitor would be satisfied: profits. The patent from tamoxifen would be running out in America by 2000, and aromatase inhibitors would be substituted, but only for the postmenopausal patients. Three orally active third-generation aromatase inhibitors were subsequently successfully developed for adjuvant therapy: anastrozole, letrozole, and exemestane. Each was demonstrated to have a small but consistent improvement over 5 years of tamoxifen alone whether given instead of tamoxifen in postmenopausal patients, after 5 years of tamoxifen, or switching after a couple of years of tamoxifen [22–29]. There has even been a successful trial of exemestane as a prevention in postmenopausal high-risk women [30]. However, it is hard to see how this approach would be superior to a sophisticated third-generation SERM functioning as a multifunctional medicine in women's health.

The advantages of aromatase inhibitors for postmenopausal patients are clear in large population trials and for healthcare systems. Patents for aromatase inhibitors are now running out or have run out and cheap generics are becoming available.

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The aromatase inhibitors were initially priced extremely high compared to tamoxifen to compensate for each only securing about 1/3 of the original tamoxifen market. A disease-free survival advantage is noted for adding an aromatase inhibitor to the treatment plan compared to tamoxifen alone [31] and concerns about endometrial cancer and blood clots are diminished. Current clinical studies to improve endocrine response rates seek to exploit emerging knowledge about the molecular mechanisms of antihormone resistance to aromatase inhibitors [32]. Combinations of letrozole and lapatinib, an inhibitor of the HER2 pathway, show some advantages over letrozole alone in ER-positive and HER-positive metastatic breast cancer [33]. A similar improvement in responsiveness to aromatase inhibitors is noted with a combination with the mTor inhibitor everolimus [34–36]. None of this would have come about but for 20 years of endocrine therapy using tamoxifen as the pioneer. AU1

SERM Successes

A failed "morning-after pill," ICI46,474, becomes tamoxifen and a failed "breast cancer drug," LY156,758, becomes raloxifene to give us the science of selective estrogen receptor modulators (SERMs). There two "wrongs" gave women's health a path that was the "right" research track. As a result the lives of millions of women were improved worldwide. The women who survived through tamoxifen treatment provided strength and support for their families, and the drug continues to fulfill that role in society. Grandmothers now see their grandchildren grow up and mothers see their children married to have families of their own. Women who use raloxifene to prevent osteoporosis have fewer breast cancers, perhaps 20,000 fewer breast cancers if the half a million women taking the drug continue to do so for a decade. Less morbidity occurs with the treatment of cancer and possibly less deaths from breast cancer in the long run. AU2

What is perhaps unique is that without tamoxifen there would be no raloxifene as there had to be a leader to beat. What is unusual is that the pharmacological basis for the development of two orphan drugs from two separate drug companies in separate continents should spring from the same laboratory. The Tamoxifen Team laboratory chose to move, after being talent spotted from Leeds University, to Switzerland and then Wisconsin. These were the opportunities presented and seized upon to be in the right place at exactly the right time, trained and ready to exploit the stream of scientific discoveries that charged medicine twice by the 1990s.

SERMs did not end with raloxifene and the principle created successes and failures over the years. We will close with the pharmacological success of SERMs despite the unsuccessful struggle in this harsh economic climate to create a viable economic model for new compounds. But that initially was the stages of both tamoxifen and raloxifene; the key to success was first to market with tamoxifen earning billions over the past 40 years after being abandoned as being financially unviable and raloxifene earning billions too after being totally abandoned for clinical development for half a decade!

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The market for the prevention of osteoporosis is much bigger than breast cancer so considerable effort has gone into the development of SERMs for this indication. We will not consider arzoxifene as it has been tested unsuccessfully as a breast cancer therapy and its effectiveness in osteoporosis is proven, and there are consistent decreases in breast cancer incidence. Development is terminated because of toxicity. We will consider ospemifene and lasofoxifene as agents modeled on earlier antiestrogens and bazedoxifene as a SERM with an interesting twist—combination with conjugated equine estrogen.

Lasofoxifene (CP-336156, Fablyn)

Lasofoxifene is interesting as its structure has its origins to the early days of the 1960s when Lednicer and coworkers [37–39] were seeking the optimal postcoital contraceptive (Fig. 10.2). Nafoxidine was the result that then evolved into a potential breast cancer drug that failed [40]. The search for SERMs defined and refined the possible structural components necessary for the new target—osteoporosis. The discovery and preliminary preclinical pharmacology of CP-33156 were first reported in 1998 and since then there has been a steady stream of important publications about this interesting compound. David Thompson's group has contributed most of the new knowledge describing the actions of CP-33156 in the rat with a particular focus on bone, circulating cholesterol, and the uterus [41–44]. The crystallography of lasofoxifene with the ligand-binding domain (LBD) of the ER is resolved [45]. The conformations of the complex is consistent with prior structure of 4-hydroxytamoxifen [46] and raloxifene [47] which adopt the antagonist conformation with helix 12 pushed back and unable to seal the lasofoxifene into the LBD.

An extremely interesting aspect of the pharmacology of lasofoxifene is the enhanced bioavailability of the levorotatory (l-) enantiomer being more potent in terms of ER binding affinity as well as enhanced bioavailability compound of the dextrorotatory (d-) enantiomer [48]. The potency in vivo is enhanced because the (l) isomer lasofoxifene is a poor substrate for glucuronidation.

A whole range of clinical trials with lasofoxifene have been completed for the prevention of osteoporosis [49, 50] with beneficial effects of significantly reducing strokes, coronary heart disease, and breast cancer [51] without increasing endometrial cancer. These are all the properties originally proposed for the potential of SERMs [52].

Finally one interesting aspect of lasofoxifene is the enhanced improvement in vaginal atrophy observed with treatment and increased vaginal lubrication [53]. However, despite the fact that lasofoxifene is approved for the treatment and prevention of osteoporosis in the European Union at doses 1/100 of those used for raloxifene, the SERM still is unable to control hot flashes. This is a serious barrier to compliance and quality of life. However, the road to development of the SERM bazedoxifene has produced an interesting solution.

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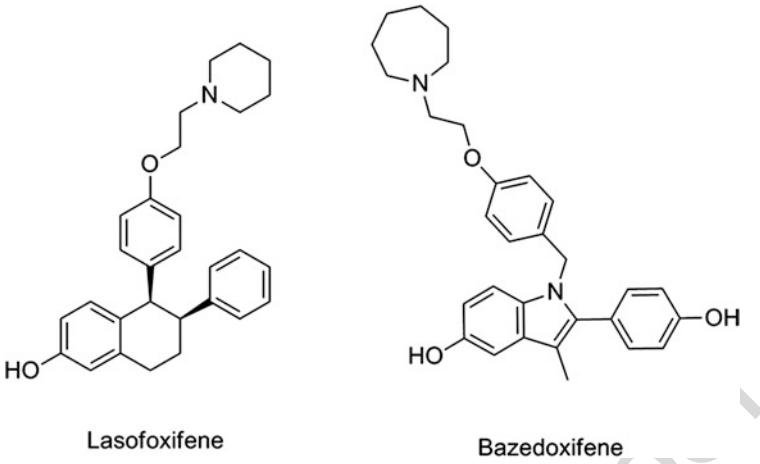

Fig. 10.2 Chemical structures of new SERMs lasofoxifene and bazedoxifene

Bazedoxifene (TSE-424, WAY-140424)

Bazedoxifene is an indole derivative, almost obviously developed from the earlier compound zindoxifene (Fig. 10.2) by attaching an alkylaminoethoxy phenyl side chain in the appropriate "antiestrogen" position of the molecule. The original metabolites of zindoxifene were actually estrogenic in laboratory tests [54] and zindoxifene was without activity for the treatment of breast cancer [55]. Initial laboratory studies with bazedoxifene showed activity as an antiestrogen in MCF-7 breast cancer cells but also was effective in causing cell death [56] in aromatase-resistant breast cancer cells derived from the MCF-7 cell line [57]. Bazedoxifene is a typical SERM which maintains bone density in the ovariectomized rat [58] and the cynomolgus monkey over an 18-month treatment period [59]. Clinical studies demonstrate the value of bazedoxifene from the treatment and potential of osteoporosis. But it is the pairing of bazedoxifene with conjugated equine estrogen (CEE) that enhances effects of lowering lipids and improving bone density while reducing vasomotor effects [60]. In fact, a comparison with lasofoxifene and raloxifene suggests a unique gene profiling for bazedoxifene and CEE on breast cancer cells [61].

Ospemifene (FC-1271a)

This triphenylethylene is a metabolite of toremifene with a unique glycol side chain. This transformation by deamination of the side chain of a nonsteroidal antiestrogen was first noted with tamoxifen when metabolite Y was first discovered [62, 63]. The same transformation occurs with toremifene. Ospemifene is a typical SERM in the rat [64]. Lowering cholesterol, building bone, and blocking estrogen stimulated uterine weight. A range of studies have demonstrated a lack of

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genotoxicity [65] significant antitumor actions in mice [66] and ability to block the growth of premalignant lesions in a mouse model of DCIS [67]. Indeed the pharmacological effects of ospemifene have been documented in rhesus macaque monkeys [68] as well as humans.

Refining the SERM Concept Further

The fact that there are two ERs, ERα and ERβ [69], naturally has caused a search for ER-specific subtype drugs. Most of our knowledge of the role of each ER subtype has come from a study of knockout mice for one or the other ER [70]. Pharmacologically the main difference between the ER seems to be that AF-1 region [71]. The ligand-binding pockets of ERα and ERβ are very similar with two amino acids Leu and Met in ERα replaced by Met and Leu in ERβ [72].

Despite the difficulties that need to be advanced for subtype-specific agents in very similar proteins, the quest for new medicines has been a priority; changing the antiestrogenic dimethylaminoethoxy side chain to an acrylic side chain creates ERα-specific activity in stimulating endometrial cancer cells [73]. The ERβ-specific agonist SERBA-1 caused involution of the mouse prostate with no effects on ventral prostrate or testicular weight [74]. The Wyeth ERβ-specific agonist ERβ-041 has a dramatic effect in preclinical models of adjuvant-induced arthritis [75]. Most importantly numerous pharmaceutical companies are addressing the issue of controlling hot flashes for a more acceptable SERM. Both Eli Lilly and Johnson & Johnson [76, 77] have compounds shown to control changes in skin temperature in the morphine-dependent rat models.

However, the SERM principle has now been applied to all members of the nuclear receptor superfamily to create selective nuclear receptor modulation to treat diseases with greater specificity not previously believed to be possible. There are now selective androgen receptor modulators (SARMs) [78], selective progesterone receptor modulators (SPRMs) [79], selective glucocorticoid receptor modulators (SGRMs) [80], selective mineralocorticoid receptor modulators (SMRMs) [81], selective thyroid receptor modulators (STRMs) [82], and selective peroxisome proliferator-activated receptor modulators (SPPARMs) [83]. The idea of switching on and off target sites around the body to improve human health and survival is very appealing as we increase longevity.

However, as we bring our story to a close, it is perhaps ironic to reflect that all this progress in a new pharmacology of receptor action was made possible by a potent postcoital contraceptive in the rat, originally designed to prevent life. Much good came from that failed contraceptive tamoxifen that has dramatically enhanced life expectancy, prevented breast cancers, created the SERMs, and dramatically enhanced the prospects of a longer healthier life.

Postscript. Breast cancer is the most prevalent cancer of women and death rates are only secondary to lung cancer. However, lung cancer has a known cause, smoking; the targeting of women by the advertising industry in the 1980s to encourage

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smoking as a positive lifestyle advance has had consequences with a rising mortality. Breast cancer has no such cause and effect solution to prevent the disease. Yet despite the huge problem of "where to start" in treatment and prevention of breast cancer, the last four decades of research has heralded a new era in personalized medicine for cancer in general, in large part because of the breakthroughs in breast cancer treatment.

The understanding of the links between hormones and breast cancer was to mature for over a century [84] but was, as with all breakthroughs, dependent on the fashions in research. Change occurred in 1971 with passing of the National Cancer Act. This important political step was to articulate a plan to sponsor research and translate the profound breakthroughs that would result into improved patient care. This would be achieved through a nationwide system of clinical cancer centers where laboratory scientists and clinical scientists would interact daily to decrease the mortality from cancer. I have had the privilege of either directing breast cancer programs (University of Wisconsin Comprehensive Cancer Center at Madison, Wisconsin, with Monica Morrow, M.D., perhaps the most accomplished breast cancer surgeon in the world; Robert H. Luire Comprehensive Cancer Center, Northwestern University, Chicago) or as the vice president of Medical Science (Fox Chase Cancer Center, Philadelphia) or as the scientific director (Georgetown University Lombardi Comprehensive Cancer Center, Washington, DC). But it was the experience of the first cancer center I experienced at the University of Wisconsin (Madison) that was critical for my development as a cancer scientist. The opportunity to be recruited was the reason I went to America. This was a wonderful place to learn and develop my ideas. I had the pleasure of working with Director Paul Carbone, Lasker Prize winner (for the development of MOPP and the treatment of Hodgkin's disease) and also the head of the Eastern Cooperative Group. I was talent spotted because of what I could achieve if given the chance to develop tamoxifen to its full potential. This clearly was a success and the wonderful environment of talented scientific colleagues and first-rate graduate students gave medicine SERMs. But it is my interaction with Harold Rusch the inaugural and then former director of the clinical cancer center that I cherish the most. Harold had his office next to mine and we talked every day. He taught me valuable lessons in scientific leadership and the requirement to advance the career development of one's staff. To this day I answer my phone with "How can I help you?" His book is a "must read." Something attempted, something done." He was also the first director of the McArdle Laboratory and built it to be a world-class center of excellence in cancer research. Through the tragedy of his daughter's death from breast cancer, he became one of this nation's strongest advocates for clinical cancer centers to take ideas to the clinic to save lives. There had to be a path to clinical trials and patient care, and he was strategically situated on the President's Cancer Panel to advocate change. He became the first director of the Wisconsin Clinical Cancer Center and then recruited Paul Carbone to continue the task. I was honored when Harold told me that on his death he would like me to speak at his memorial service. He had been diagnosed with prostate cancer and had but a short time to live. To me it was important to obtain a letter of gratitude for all Dr. Rusch had achieved for cancer

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research in the United States. I went to the president of the United States. This letter was received just in time at a ceremony at Dr. Rusch's home with the letter read and presented by Donna Shala, then chancellor of the University of Wisconsin-Madison. At Harold's memorial service, I read that same letter as my mark of respect for a great yet humble man, who thought of his staff and colleagues always before himself.

With regard to hormones and cancer, the "epicenter" for positive change I believe was the Worcester Foundation for Experimental Biology in Shrewsbury, Massachusetts. This was the home of the oral contraceptive and the founding Director Gregory Pincus [85] created a world-renowned research institution with a principal theme of reproduction research. It was at the foundation that Pincus turned the dream of oral contraception into a practical reality. His drive and commitment accelerated clinical testing with a progestin which was the culmination of a decade of laboratory investigations. But luck takes control, as often as not. I partially like the story of the first trials with a synthetic progestin that were found to contain an impurity. The progestin was purified and less effective as a contraceptive. The impurity was an estrogen so the combined oral contraceptives "so to speak" conceived. But by the late 1960s, fashions in research were changing and cancer research was to move center stage. With the passing of the National Cancer Act in 1971 came opportunity for funding. For one of us (VCJ), who was a visiting scientist at the foundation (1972–1974), from the University of Leeds, England, this was an important time and valuable to learn and exchange ideas. But the opportunities from the environment of the foundation catalyzed the conversion of ICI46,474 to tamoxifen (with a *big* push from Lois Trench).

The philosophy of the foundation was to advance new ideas and concepts. The first systemic studies with tamoxifen as a breast cancer drug were started [86] but remarkably, in a laboratory not more than 100 yards away from mine, Angela and Harvey Brodie were taking the first steps to create 4-hydroxyandronestedione [18] as the first specific aromatase inhibitor successfully tested in patients [21]. Angela's tenacity and vision was critical for the future development of new aromatase inhibitors. The subsequent pharmaceutical development of tamoxifen as the first long-term adjuvant endocrine therapy targeted to the ER and chemopreventive made the improvements with aromatase inhibitors certain. Tamoxifen and the aromatase inhibitors all continue to reduce mortality. These are the therapeutic cornerstones of the modern era of targeted treatments for breast cancer. All the successes in hormones and breast cancer started at the foundation to be a practical approach to the treatment and prevention.

Furthermore, it is remarkable to note that the scientists at the Worcester Foundation had already changed the world with the oral contraceptive and M. C. Chang had conducted seminal studies on in vitro fertilization with the discovery of sperm capacitation within the uterus. This immediately was used first in animals; I liked the stories I heard that Chang had taken sperm and egg from a mink and a stoat that normally would never mate to create a *stink*! The animal work was necessary to set the stage for the birth of Louise Brown at 11:47 p.m., 25 July 1978 (coincidentally my birthday). By 8 June 1980, health authorities in Virginia announced the first US-built

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clinic using the Edwards-Steptoe's method. Society has much to be grateful for from the research on hormones initiated in the confines of a couple of acres of land in Massachusetts and the vision of Gregory Pincus. There are four major advances in women's health: the oral contraceptive, in vitro fertilization, a clinical plan for tamoxifen, and the first specific aromatase inhibitor!

References

1. Gradishar WJ, Jordan VC (1997) Clinical potential of new antiestrogens. J Clin Oncol 15:840–852
2. Stein S, Zoltick B, Peacock T et al (2001) Phase II trial of toremifene in androgen-independent prostate cancer: a Penn cancer clinical trials group trial. Am J Clin Oncol 24:283–285
3. Raghow S, Hooshdaran MZ, Katiyar S, Steiner MS (2002) Toremifene prevents prostate cancer in the transgenic adenocarcinoma of mouse prostate model. Cancer Res 62:1370–1376
4. Lerner LJ, Holthaus FJ Jr, Thompson CR (1958) A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol. Endocrinology 63:295–318
5. Jordan VC, Collins MM, Rowsby L, Prestwich G (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305–316
6. Wakeling AE (1994) A new approach to breast cancer therapy-total estrogen ablation with pure antiestrogens. In: Jordan VC (ed) Long-term tamoxifen treatment for breast cancer. University of Wisconsin Press, Madison, pp 219–234
7. Jordan VC, Fenuik L, Allen KE et al (1981) Structural derivatives of tamoxifen and oestradiol 3-methyl ether as potential alkylating antioestrogens. Eur J Cancer 17:193–200
8. Bucourt R, Vignau M, Torelli V (1978) New biospecific adsorbents for the purification of estradiol receptor. J Biol Chem 253:8221–8228
9. Wakeling AE, Bowler J (1987) Steroidal pure antioestrogens. J Endocrinol 112:R7–R10
10. Bowler J, Lilley TJ, Pittam JD, Wakeling AE (1989) Novel steroidal pure antiestrogens. Steroids 54:71–99
11. Wakeling AE, Dukes M, Bowler J (1991) A potent specific pure antiestrogen with clinical potential. Cancer Res 51:3867–3873
12. Osborne CK, Pippen J, Jones SE et al (2002) Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. J Clin Oncol 20:3386–3395
13. Howell A, Robertson JF, Quaresma Albano J et al (2002) Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. J Clin Oncol 20:3396–3403
14. Mehta RS, Barlow WE, Albain KS et al (2012) Combination anastrozole and fulvestrant in metastatic breast cancer. N Engl J Med 367:435–444
15. Bergh J, Jonsson PE, Lidbrink EK et al (2012) FACT: an open-label randomized phase III study of fulvestrant and anastrozole in combination compared with anastrozole alone as first-line therapy for patients with receptor-positive postmenopausal breast cancer. J Clin Oncol 30:1919–1925
16. Howell A, DeFriend DJ, Robertson JF et al (1996) Pharmacokinetics, pharmacological and anti-tumour effects of the specific anti-oestrogen ICI 182780 in women with advanced breast cancer. Br J Cancer 74:300–308
17. Di Leo A, Jerusalem G, Petruzelka L et al (2010) Results of the CONFIRM phase III trial comparing fulvestrant 250 mg with fulvestrant 500 mg in postmenopausal women with estrogen receptor-positive advanced breast cancer. J Clin Oncol 28:4594–4600
18. Brodie AM, Schwarzel WC, Shaikh AA, Brodie HJ (1977) The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. Endocrinology 100:1684–1695

Uncorrected Proof

Author's Proof

19. Brodie AM, Longcope C (1980) Inhibition of peripheral aromatization by aromatase inhibitors, 4-hydroxy- and 4-acetoxy-androstene-3,17-dione. Endocrinology 106:19–21
20. Brodie AM, Marsh D, Brodie HJ (1979) Aromatase inhibitors – IV. Regression of hormone-dependent, mammary tumors in the rat with 4-acetoxy-4-androstene-3,17-dione. J Steroid Biochem 10:423–429
21. Coombes RC, Goss P, Dowsett M et al (1984) 4-hydroxyandrostenedione in treatment of postmenopausal patients with advanced breast cancer. Lancet 2:1237–1239
22. Baum M, Budzar AU, Cuzick J et al (2002) Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. Lancet 359:2131–2139
23. Howell A, Cuzick J, Baum M et al (2005) Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 365:60–62
24. Thurlimann B, Keshaviah A, Coates AS et al (2005) A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. N Engl J Med 353:2747–2757
25. Coates AS, Keshaviah A, Thurlimann B et al (2007) Five years of letrozole compared with tamoxifen as initial adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer: update of study BIG 1-98. J Clin Oncol 25:486–492
26. Coombes RC, Hall E, Gibson LJ et al (2004) A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. N Engl J Med 350:1081–1092
27. Boccardo F, Rubagotti A, Puntoni M et al (2005) Switching to anastrozole versus continued tamoxifen treatment of early breast cancer: preliminary results of the Italian Tamoxifen Anastrozole Trial. J Clin Oncol 23:5138–5147
28. Goss PE, Ingle JN, Martino S et al (2003) A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. N Engl J Med 349:1793–1802
29. Goss PE, Ingle JN, Martino S et al (2005) Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17. J Natl Cancer Inst 97:1262–1271
30. Goss PE, Ingle JN, Ales-Martinez JE et al (2011) Exemestane for breast-cancer prevention in postmenopausal women. N Engl J Med 364:2381–2391
31. Dowsett M, Cuzick J, Ingle J et al (2010) Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 28:509–518
32. Roop RP, Ma CX (2012) Endocrine resistance in breast cancer: molecular pathways and rational development of targeted therapies. Future Oncol 8:273–292
33. Riemsma R, Forbes CA, Amonkar MM et al (2012) Systematic review of lapatinib in combination with letrozole compared with other first-line treatments for hormone receptor positive(HR+) and HER2+ advanced or metastatic breast cancer(MBC). Curr Med Res Opin 28:1263–1279
34. Bachelot T, Bourgier C, Cropet C et al (2012) Randomized phase II trial of everolimus in combination with tamoxifen in patients with hormone receptor-positive, human epidermal growth factor receptor 2-negative metastatic breast sancer with prior exposure to aromatase inhibitors: A GINECO study. J Clin Oncol 30:2718–2724
35. Baselga J, Campone M, Piccart M et al (2012) Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med 366:520–529
36. Baselga J, Semiglazov V, van Dam P et al (2009) Phase II randomized study of neoadjuvant everolimus plus letrozole compared with placebo plus letrozole in patients with estrogen receptor-positive breast cancer. J Clin Oncol 27:2630–2637
37. Lednicer D, Babcock JC, Marlatt PE et al (1965) Mammalian antifertility agents. I. Derivatives of 2,3-diphenylindenes. J Med Chem 8:52–57

Uncorrected Proof

Author's Proof

38. Lednicer D, Lyster SC, Aspergren BD, Duncan GW (1966) Mammalian antifertility agents. 3. 1-aryl-2-phenyl-1,2,3,4-tetrahydro-1-naphthols, 1-aryl-2-phenyl-3,4-dihydronaphthalenes, and their derivatives. J Med Chem 9:172–176
39. Lednicer D, Lyster SC, Duncan GW (1967) Mammalian antifertility agents. IV. Basic 3,4-dihydronaphthalenes and 1,2,3,4-tetrahydro-1-naphthols. J Med Chem 10:78–84
40. Legha SS, Slavik M, Carter SK (1976) Nafoxidine – an antiestrogen for the treatment of breast cancer. Cancer 38:1535–1541
41. Ke HZ, Paralkar VM, Grasser WA et al (1998) Effects of CP-336,156, a new, nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. Endocrinology 139:2068–2076
42. Ke HZ, Qi H, Crawford DT et al (2000) Lasofoxifene (CP-336,156), a selective estrogen receptor modulator, prevents bone loss induced by aging and orchidectomy in the adult rat. Endocrinology 141:1338–1344
43. Ke HZ, Qi H, Chidsey-Frink KL et al (2001) Lasofoxifene (CP-336,156) protects against the age-related changes in bone mass, bone strength, and total serum cholesterol in intact aged male rats. J Bone Miner Res 16:765–773
44. Ke HZ, Foley GL, Simmons HA et al (2004) Long-term treatment of lasofoxifene preserves bone mass and bone strength and does not adversely affect the uterus in ovariectomized rats. Endocrinology 145:1996–2005
45. Vajdos FF, Hoth LR, Geoghegan KF et al (2007) The 2.0 A crystal structure of the ERalpha ligand-binding domain complexed with lasofoxifene. Protein Sci 16:897–905
46. Shiau AK, Barstad D, Loria PM et al (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937
47. Brzozowski AM, Pike AC, Dauter Z et al (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
48. Rosati RL, Da Silva Jardine P, Cameron KO et al (1998) Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryltetrahydronaphthalene. J Med Chem 41:2928–2931
49. Cummings SR, Ensrud K, Delmas PD et al (2010) Lasofoxifene in postmenopausal women with osteoporosis. N Engl J Med 362:686–696
50. Moffett A, Ettinger M, Bolognese M et al (2004) Lasofoxifene, a next generation SERM, is effective in preventing loss of BMD and reducing LDL-C in postmenopausal women. J Bone Miner Res 19:S96
51. LaCroix AZ, Powles T, Osborne CK et al (2010) Breast cancer incidence in the randomized PEARL trial of lasofoxifene in postmenopausal osteoporotic women. J Natl Cancer Inst 102: 1706–1715
52. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189
53. Gennari L (2009) Lasofoxifene, a new selective estrogen receptor modulator for the treatment of osteoporosis and vaginal atrophy. Expert Opin Pharmacother 10:2209–2220
54. Robinson SP, Jordan VC (1987) Reversal of the antitumor effects of tamoxifen by progesterone in the 7,12-dimethylbenzanthracene-induced rat mammary carcinoma model. Cancer Res 47: 5386–5390
55. Jabara AG, Toyne PH, Harcourt AG (1973) Effects of time and duration of progesterone administration on mammary tumours induced by 7,12-dimethylbenz(a)anthracene in Sprague–Dawley rats. Br J Cancer 27:63–71
56. Chan O, Inouye K, Akirav E et al (2005) Insulin alone increases hypothalamo-pituitary-adrenal activity, and diabetes lowers peak stress responses. Endocrinology 146:1382–1390
57. Lewis-Wambi JS, Kim H, Curpan R et al (2011) The selective estrogen receptor modulator bazedoxifene inhibits hormone-independent breast cancer cell growth and down-regulates estrogen receptor alpha and cyclin D1. Mol Pharmacol 80:610–620

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58. Kanasaki H, Bedecarrats GY, Kam KY et al (2005) Gonadotropin-releasing hormone pulse frequency-dependent activation of extracellular signal-regulated kinase pathways in perifused LbetaT2 cells. Endocrinology 146:5503–5513
59. Smith S, Minck D, Jolette J et al (2005) Bazedoxifene prevents ovariectomy-induced bone loss in the Cynomolgus monkey. J Bone Miner Res 20:S174
60. Kharode Y, Bodine PV, Miller CP et al (2008) The pairing of a selective estrogen receptor modulator, bazedoxifene, with conjugated estrogens as a new paradigm for the treatment of menopausal symptoms and osteoporosis prevention. Endocrinology 149:6084–6091
61. Chang KC, Wang Y, Bodine PV et al (2010) Gene expression profiling studies of three SERMs and their conjugated estrogen combinations in human breast cancer cells: insights into the unique antagonistic effects of bazedoxifene on conjugated estrogens. J Steroid Biochem Mol Biol 118:117–124
62. Bain RR, Jordan VC (1983) Identification of a new metabolite of tamoxifen in patient serum during breast cancer therapy. Biochem Pharmacol 32:373–375
63. Jordan VC, Bain RR, Brown RR et al (1983) Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. Cancer Res 43:1446–1450
64. Qu Q, Zheng H, Dahllund J et al (2000) Selective estrogenic effects of a novel triphenylethylene compound, FC1271a, on bone, cholesterol level, and reproductive tissues in intact and ovariectomized rats. Endocrinology 141:809–820
65. Hellmann-Blumberg U, Taras TL, Wurz GT, DeGregorio MW (2000) Genotoxic effects of the novel mixed antiestrogen FC-1271a in comparison to tamoxifen and toremifene. Breast Cancer Res Treat 60:63–70
66. Wurz GT, Read KC, Marchisano-Karpman C et al (2005) Ospemifene inhibits the growth of dimethylbenzanthracene-induced mammary tumors in Sencar mice. J Steroid Biochem Mol Biol 97:230–240
67. Namba R, Young LJ, Maglione JE et al (2005) Selective estrogen receptor modulators inhibit growth and progression of premalignant lesions in a mouse model of ductal carcinoma in situ. Breast Cancer Res 7:R881–R889
68. Wurz GT, Hellmann-Blumberg U, DeGregorio MW (2008) Pharmacologic effects of ospemifene in rhesus macaques: a pilot study. Basic Clin Pharmacol Toxicol 102:552–558
69. Nilsson S, Gustafsson JA (2011) Estrogen receptors: therapies targeted to receptor subtypes. Clin Pharmacol Ther 89:44–55
70. Nilsson S, Makela S, Treuter E et al (2001) Mechanisms of estrogen action. Physiol Rev 81: 1535–1565
71. McInerney EM, Weis KE, Sun J et al (1998) Transcription activation by the human estrogen receptor subtype beta (ER beta) studied with ER beta and ER alpha receptor chimeras. Endocrinology 139:4513–4522
72. Pike AC, Brzozowski AM, Hubbard RE et al (1999) Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. EMBO J 18:4608–4618
73. Hoekstra WJ, Patel HS, Liang X et al (2005) Discovery of novel quinoline-based estrogen receptor ligands using peptide interaction profiling. J Med Chem 48:2243–2247
74. Norman BH, Dodge JA, Richardson TI et al (2006) Benzopyrans are selective estrogen receptor beta agonists with novel activity in models of benign prostatic hyperplasia. J Med Chem 49:6155–6157
75. Follettie MT, Pinard M, Keith JC Jr et al (2006) Organ messenger ribonucleic acid and plasma proteome changes in the adjuvant-induced arthritis model: responses to disease induction and therapy with the estrogen receptor-beta selective agonist ERB-041. Endocrinology 147: 714–723
76. Jain N, Xu J, Kanojia RM et al (2009) Identification and structure-activity relationships of chromene-derived selective estrogen receptor modulators for treatment of postmenopausal symptoms. J Med Chem 52:7544–7569

Uncorrected Proof

Author's Proof

77. Wallace OB, Lauwers KS, Dodge JA et al (2006) A selective estrogen receptor modulator for the treatment of hot flushes. J Med Chem 49:843–846
78. Mohler ML, Bohl CE, Jones A et al (2009) Nonsteroidal selective androgen receptor modulators (SARMs): dissociating the anabolic and androgenic activities of the androgen receptor for therapeutic benefit. J Med Chem 52:3597–3617
79. Chabbert-Buffet N, Meduri G, Bouchard P, Spitz IM (2005) Selective progesterone receptor modulators and progesterone antagonists: mechanisms of action and clinical applications. Hum Reprod Update 11:293–307
80. Hudson AR, Roach SL, Higuchi RI (2008) Recent developments in the discovery of selective glucocorticoid receptor modulators (SGRMs). Curr Top Med Chem 8:750–765
81. Baxter JD, Funder JW, Apriletti JW, Webb P (2004) Towards selectively modulating mineralocorticoid receptor function: lessons from other systems. Mol Cell Endocrinol 217:151–165
82. Yoshihara HA, Scanlan TS (2003) Selective thyroid hormone receptor modulators. Curr Top Med Chem 3:1601–1616
83. Berger JP, Petro AE, Macnaul KL et al (2003) Distinct properties and advantages of a novel peroxisome proliferator-activated protein [gamma] selective modulator. Mol Endocrinol 17: 662–676
84. Jordan VC (2009) A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 69:1243–1254
85. Speroff L (2009) A good man: Gregory Goodwin Pincus: the man, his story, the birth control pill. Arnica Pub., Portland, xxi, 359 pp
86. Jordan VC (2006) Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 147(Suppl 1):S269–S276

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Author Queries

Chapter No.: 10

Query Refs.	Details Required	Author's response
AU1	Please check sentence starting "None of this. . ." for sense.	
AU2	Please check if edit to sentence starting "Women who use. . ." is okay.	
AU3	Please provide appropriate opening quotes for sentence starting ". . . something done."	
AU4	Please check if edit to sentence starting "I partially like the...." is okay.	
AU5	Usage of all caps for emphasis has been changed to italics. Please check if okay.	
AU6	Please check if edit to sentence starting "This immediately was. . ." is okay.	

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Appendix A: Four Decades of Discovery in Breast Cancer Research and Treatment: An Interview with V. Craig Jordan

Marc Poirot

The past is never dead. It is not even the past.—William Faulkner

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Abstract

V. Craig Jordan is a pioneer in the molecular pharmacology and therapeutics of breast cancer. As a teenager, he wanted to develop drugs to treat cancer, but at the time in the 1960s, this was unfashionable. Nevertheless, he saw an opportunity and, through his mentors, trained himself to reinvent a failed "morning-after pill" to become tamoxifen, the gold standard for the treatment and prevention of breast cancer. It is estimated that at least a million women worldwide are alive today because of the clinical application of Jordan's laboratory research. Throughout his career, he has always looked at "the good, the bad, and the ugly" of tamoxifen. He was the first to raise concerns about the possibility of tamoxifen increasing endometrial cancer. He described selective estrogen receptor modulation (SERM), and he was the first to describe both the bone protective effects and the breast chemopreventive effects of raloxifene. Raloxifene did not increase endometrial cancer and is now used to prevent breast cancer and osteoporosis. The scientific strategy he introduced of using long-term therapy for treatment and prevention caused him to study acquired drug resistance to SERMs. He made the paradoxical discovery that physiological estrogen can be used to treat and to prevent breast cancer once exhaustive antihormone resistance develops. His philosophy for his four decades of discovery has been to use the conversation between the laboratory and the clinic to improve women's health.

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Abbreviations. AACR, American Association for Cancer Research; ASCO, American Society of Clinical Oncology; CEE, Conjugated equine estrogen, DES, Diethylstilbestrol; DMBA, Dimethylbenzanthracene; EBCTCG, Early Breast Cancer Trialists' Collaborative Group; ECOG, Eastern Cooperative Oncology Group; FDA, Food and Drug Administration; ICI, Imperial Chemical Industries; SERM, Selective estrogen receptor modulator; STAR, Study of Tamoxifen and Raloxifene; TGF-α, Transforming growth factor-alpha; WFEB, Worcester Foundation for Experimental Biology; WHI, Women's Health Initiative.

Tamoxifen, originally classified as a nonsteroidal antiestrogen but now known as the first selective estrogen receptor modulator (SERM), is a pioneering medicine that for more than 20 years was the gold standard for the adjuvant treatment of breast cancer in pre- and postmenopausal patients with ER-positive tumors [1]. Millions of women continue to live longer and healthier lives because of tamoxifen treatment. Tamoxifen is also a pioneering medicine, as it is the first drug to be approved in the United States of America by the Food and Drug Administration (FDA) for the reduction of the incidence of breast cancer in high-risk pre- and postmenopausal women [2].

Craig Jordan grew up with a passion for chemistry, but was specifically intrigued by the prospect of using organic chemistry to design drugs to treat cancer. At the age of 13, his mother allowed him to convert his bedroom into a chemistry laboratory, where he often got into difficulties during his experiments, either setting the curtains on fire as a rather overreactive experiment was being thrown out of the window or destroying the lawn outside. However, he did convince his mother that by using the chemistry of fertilizers, he could regrow the lawn again, but when he did, it came out an interesting shade of blue! Craig had a passion for teaching, and the chemistry and biology teachers at his school, Moseley Hall Grammar School in Cheadle, Cheshire, England, allowed him to have a laboratory to teach biochemistry. It was these same teachers who convinced his parents that he should apply to university. By contrast, Craig was more content with the idea of becoming an organic chemistry technician at the research laboratories of Imperial Chemical Industries (ICI) near where he lived.

Craig was given an opportunity for interview at only one university (Leeds University, West Yorkshire, England), but he succeeded in convincing the two faculty interviewers, Dr. Ronnie Kaye and Dr. Edward Clark, that he should have a chance in the Pharmacology Department. Years later, Craig found out that the reason he was given an interview was that they had been intrigued at the Headmaster's letter, which stated the candidate was "an unusual young man" and then repeated the statement in capitals. On July 18, 2001, Craig received the first honorary Doctor of Medicine degree from the University of Leeds for humanitarian research that has changed healthcare. The citation, presented by the Chancellor Lord Melvyn Bragg, starts: "Craig Jordan is one of the most distinguished medical scientists of the last one hundred years." He was delighted to be able to invite

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Drs. Clark and Kaye to the luncheon and the ceremony (Fig. A.1). These were the two individuals who talent-spotted Craig; Dr. Kaye was his tutor for his 4 years as an undergraduate, and Dr. Clark persuaded him to become a graduate student armed with the last available Medical Research Council studentship in the United Kingdom for the year 1969 (Fig. A.2). Someone had declined their studentship, thus allowing Craig to do a Ph.D.! Dr. Clark's project, which Craig found so attractive, was the prospect of extracting the ER from the rodent uterus, purifying it and then crystallizing the ER protein with an estrogen and a nonsteroidal antiestrogen. The x-ray crystallography would be completed at the Astbury Department of Biophysics at the University of Leeds, and all the work was estimated to take the 3 years of the scholarship. At that time, the nonsteroidal antiestrogens had failed to fulfill their promise in the pharmaceutical industry as "morning-after pills"; they were perfect in rats, but in women they did exactly the opposite and enhanced fertility by inducing ovulation.

The project in crystallizing the ER did not go as planned, so he rapidly changed his topic with a new title: "A study of the oestrogenic and anti-oestrogenic activities of some substituted triphenylethylenes and triphenylethanes" (Fig. A.3). This was a good strategic research choice, as no one has yet succeeded in crystallizing the whole ER with either an estrogen or antiestrogen. But further difficulties were to arise in Craig's journey to a career in cancer research.

As a Ph.D. student, Craig was talent spotted for an immediate tenure track faculty position because of his skill as a lecturer. He had no publications and his Ph.D. topic was going nowhere. No one was recommending careers in failed contraceptives! During the interview with the University Committee charged with making the appointment, he was told that he would have to go to America to get his BTA (been to America) before he could start the job. First, however, he had to get a Ph.D., and to do that, it had to be examined. However, the university could find no one in the country qualified for the task. Sir Charles Dodds, the discoverer of the synthetic estrogen, diethylstilbestrol (DES), declined with regrets as he had not kept up with the literature for the past 20 years! But here is where luck and chance take control. He was in the right place at the right time and, by meeting the right people, changed medicine.

Dr. Arthur Walpole was head of the Fertility Control Program at ICI's Pharmaceuticals Division and a personal friend of the chairman of Craig's Pharmacology Department. The university reluctantly accepted Dr. Walpole (despite the fact that he was from industry!) to be Craig's examiner, and he was also able to organize a 2-year visit to the Worcester Foundation for Experimental Biology (WFEB) in Shrewsbury, Massachusetts, to study with Dr. Michael Harper on new methods of contraception. Harper and Walpole had completed all the early work on ICI 46,474 as a contraceptive at ICI Pharmaceuticals in the early 1960s. Craig vividly remembers the transatlantic telephone call with Dr. Harper: "Can you come in September?" "Will $12,000 a year be enough?" "Will you work on prostaglandins?" "Yes, yes, yes," he replied and went off to the library to find out what prostaglandins were! But when he got to the WFEB in September 1972, he was told that Dr. Harper had gone to Geneva to be head of Contraception Research

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Fig. A.1 Photograph before the ceremony for the degree of Doctor of Medicine *honoris causa* at Leeds University on 18 July 2001. Dr. Edward R. Clark, my Ph.D. supervisor (1969–1972) (*left*), and Dr. Ronnie Kaye, head of my degree course (1965–1969) (*center*), formally from the Department of Pharmacology, University of Leeds, England. I am on the *right side* with my signature glass of Burgundy

Fig. A.2 I always love dressing up. The University of Leeds is my *alma mater*, and I have attended four ceremonies there: (**a**) Bachelor of Science, First Class Honours, 1969; (**b**) Doctor of Philosophy, 1973; (**c**) Doctor of Science, earned by examination. A select committee evaluated my refereed publications to establish a contribution to science, 1985; (**d**) Honorary Doctor of Medicine for Humanitarian Research, 2001

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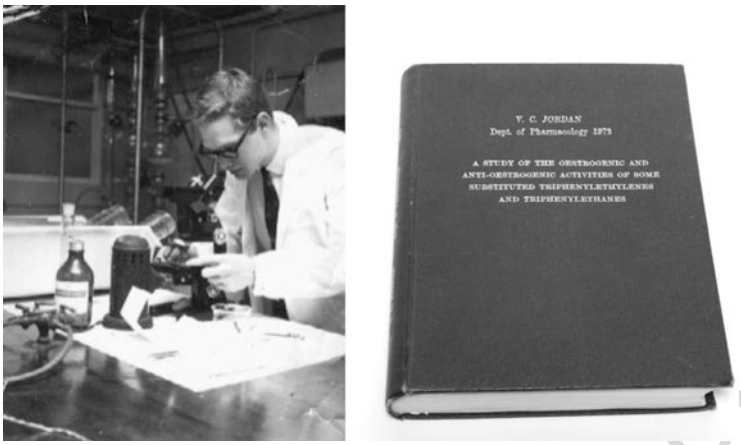

Fig. A.3 My first publicity photograph during the time that I was a Ph.D. student at the Department of Pharmacology, University of Leeds, England, 1969–1972. It was necessary as I had been selected as the Medical Research Council's student representative to the Nobel Prize Winner's meeting in Lindau, Germany, in 1972. I am examining cells from mouse vaginal smears; big science. Also shown is my Ph.D. that nobody wanted to examine

at the World Health Organization. Craig was told to sit down, write up what he would do for the next 2 years, and organize his own laboratory. He was now an independent investigator.

A phone call to Dr. Walpole explained his dilemma at the WFEB, but he felt that there was an opportunity for the failed morning-after pill, ICI 46,474, to be used for the treatment of breast cancer. This call was rewarded by Dr. Walpole arranging for funding and contacts with Ms. Lois Trench at ICI America for Craig to conduct the translational research on the drug that would become tamoxifen. As an independent investigator, the research funding from ICI was an unrestricted research grant, but as Craig was not a cancer research scientist and he was at WFEB, the home of the oral contraceptive, what was the first step to be? Again, it is who you meet. After the National Cancer Act in 1971, the WFEB director had made the decision to bring a cancer research specialist onto the Board of Scientific Advisors to help with future funding opportunities in hormones and cancer research. Dr. Elwood Jensen was the director of the Ben May Laboratory for Cancer Research in Chicago, Illinois, and was credited with the translational research where he described the ER in immature rat estrogen target tissues and then used this knowledge to propose a test for the hormone dependency of metastatic breast cancers. Simply stated, if the ER is absent in the tumor, the patient was unlikely to respond to endocrine ablation (oophorectomy, adrenalectomy, or hypophysectomy), but if the tumor was ER positive, there was a high probability that the tumor would respond to estrogen withdrawal. It was a practical test to avoid morbidity from unnecessary operations that require hospitalization.

Craig spent the day with Dr. Elwood Jensen in November 1972 and told him what he wanted to do with ICI 46,474. Craig subsequently traveled to the Ben May

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Laboratory for Cancer Research to be taught techniques of ER analysis and to learn all about the dimethylbenzanthracene (DMBA) rat mammary carcinoma model and then to Dr. Bill McGuire's laboratory in San Antonio, Texas, to learn complementary analytical methods for the ER. Armed with these techniques and resources from ICI throughout the 1970s (his first decade of discovery), he created the laboratory principles of targeting the tumor ER and advocating the use of long-term adjuvant tamoxifen therapy as the appropriate clinical strategy to save lives (Fig. A.4) [3, 4]. This proposition by Craig was not at all popular, as throughout the 1970s and 1980s in the United Kingdom, it was strongly believed there was no correlation between tamoxifen use and the presence of the ER in breast tumors. Additionally, nobody was interested in a new antihormone therapy, as combination cytotoxic chemotherapy was king. It was going to cure cancer. However, Craig persevered and had the courage of his convictions that his laboratory research would save lives. As it turned out, tamoxifen has probably saved more lives than any other cancer therapeutic drug.

Craig also learned an important lesson at the WFEB around the time he was to leave and return to Leeds. A senior scientist at the WFEB, Dr. Eliahu Caspi, invited Craig to his office for an interview to explore the possibility of Craig staying at the WFEB. Craig recalls this was a very frightening experience, for Dr. Caspi had a no-nonsense personality, judged people, and said what he thought. He stated that he had been asked to evaluate my CV, as everybody was of the opinion that I would be a useful asset at the WFEB. He stared at Craig across the desk and said, "You don't have a CV, as you have no publications." After the initial shock, Craig responded, "But I haven't discovered anything yet." The advice Craig received was some of the best advice he had received thus far in his career. He was told "to tell them the story so far and link together several related publications to create a theme." Craig has done this ever since, creating the theme of tamoxifen. In 1998, with the release of the successful chemoprevention trial with tamoxifen, Craig was referred to as the "Father of Tamoxifen" by the *Chicago Tribune,* a title that has stuck to this day.

Although many people published using tamoxifen in their studies as a laboratory tool or used it in the 1960s in reproduction research, Craig's focus from the outset was clear; the goal was to develop a medicine for the treatment and prevention of breast cancer (he conducted the first chemopreventive study in the laboratory in 1974 [7], 3 years before the drug was approved by the FDA for the treatment of metastatic breast cancer in postmenopausal women). Craig stresses that but for the unrestricted support from ICI, meeting the right people and his uncompromising determination (many referred to this at the time as poor career judgment), tamoxifen would probably not have happened. Scientists at ICI did not conduct any studies with the drug as an antitumor agent. Indeed, in late 1972, all of the data with ICI 46,474 was reviewed and the research director terminated clinical trials and stopped the development project. The Marketing Department had decided that a treatment for metastatic breast cancer was not going to generate sufficient revenue.

Arthur Walpole was toward the end of his career and chose to take early retirement, but only agreed to remain an employee if funds could be given to a young man he had met, Craig Jordan, who (as he did) wanted to turn ICI 46,474 into

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Fig. A.4 The I.C.I. Pharmaceuticals at King's College, Cambridge, Meeting in the summer of 1977. The goal of the meeting was physician education about research being done with tamoxifen. This was the first time I presented in public my ideas about targeting the tumor ER and using long-term treatment with tamoxifen as the best strategy to be applied to adjuvant therapy [5]. *Reviews on Endocrine-related Cancer* (49–55). However, the major presentation that made everything change clinically was in Arizona in 1979 [6]. In the above picture, Michael Baum (*right*), was the Chair of the session at King's College and stated that they had plans to use 2 years of tamoxifen as an adjuvant therapy (*on a hunch*). Helen Stewart (*left*) was considering starting a pilot trial in Scotland using 5 years of adjuvant tamoxifen for the treatment of patients. For the placebo arm, patients would be treated with tamoxifen at first recurrence. If toxicity was acceptable, they would

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a drug to treat breast cancer. Walpole and Craig subsequently worked together on an ICI/University joint research scheme when Craig returned as lecturer in the Department of Pharmacology at the University of Leeds in September 1974. Earlier in his career, Dr. Walpole was an accomplished cancer research scientist, but had not been allowed to work in this area by ICI because fertility control was considered to be potentially more lucrative [8]. Dr. Walpole died suddenly on July 2, 1977, before he could witness the success of Craig's laboratory strategy for the treatment and prevention of breast cancer.

The clinical development of tamoxifen was very progressive and validated all your assumptions. Could you tell us how you were involved in the clinical evaluation and how you convinced the company to invest in what may have been very challenging trials?

I think it's fair to say that this was not the real story, but the real story is unbelievable. I have always considered my research as being a conversation between the laboratory and the clinic, and I had the privilege of first introducing tamoxifen to clinical trials' organizations in America. My objective was to provide a scientific rationale for the clinical studies in treatment and prevention. My research and qualifications were required to obtain approval for tamoxifen as a medicine in both Japan and Germany, and I was delighted to be the only person invited from outside of ICI Pharmaceuticals to attend a celebration in 1977, of the Queen's Award for Technological Achievement for tamoxifen. The surprising part about the tamoxifen story is that although patents for the drug were obtained by ICI Pharmaceuticals around the world, in the mid-1960s, these same patents were denied in the United States of America. Thus, all of the work I was completing on the antitumor actions of tamoxifen in the United States was done without patent protection for ICI. Looked at another way, it was clear that all the other pharmaceutical companies had no interest in the clinical development of tamoxifen, because either the drug was not going to work very well or not generate enough revenue. But it was my clinical strategy of long-term adjuvant therapy that saved lives and made revenues [9]. Clinical testing went ahead and when the patents expired in the rest of the world, ICI was awarded the patent for the use of tamoxifen in the treatment of breast cancer in 1985, but backdated to the original patent application in 1965. Now, extended adjuvant therapy was the practical solution for effective treatment. Thus, for the next 20 years, ICI was able to generate

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Fig. A.4 (continued) move forward to test the idea of early long-term treatment or late treatment at first recurrence. Both trials showed survival advantages for long-term adjuvant tamoxifen. The week after the King's College Meeting, I was at the University of Wisconsin at their Comprehensive Cancer Center to convince clinicians of the Eastern Cooperative Oncology Group (ECOG) that longer was going to be better. At the time, tamoxifen was not on the market in America but I was talent spotted by Paul Carbone, the Head of ECOG and the director of the Comprehensive Cancer Center, to be recruited to the University of Wisconsin, Department of Human Oncology. Eventually, I would be the director of their Breast Cancer Research and Treatment Program

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enormous revenues in the United States, as tamoxifen was the standard of care for long-term adjuvant tamoxifen therapy and the only game in town. This money catalyzed the advent of ICI marketing antiandrogens for prostate cancer and the aromatase inhibitors for breast cancer.

Watching your scientific activity since the beginning, you always seem fascinated by the development of small molecules since their conception up to their development. Is that what gives you much fun in your work?

I absolutely love experiments involving the structure-function relationships of the antiestrogens. My basic scientific research has been to create models of gene modulation or replication to determine the structure of the ER antiestrogen complex that subsequently could be interrogated. This passion resulted in a whole series of publications focused on the modulation of the prolactin gene [10–12] which then went through a metamorphosis to study the modulation of the SERM ER complex and the way that the ligand can interact with specific amino acids, thereby switching on or switching off the complex at target genes [13]. We actually found the only natural mutation of the human ER in a laboratory model of tamoxifen-stimulated tumor growth. We engineered the mutant ER into ER-negative breast cancer cells and found it would make the antiestrogen, raloxifene, an estrogen at the transforming growth factor-alpha (TGF-α) target gene. For me, this was important as one amino acid in the ER could change the pharmacology of raloxifene. In other words, this provided a fascinating insight into the relationship of the antiestrogenic side chain and a specific amino acid at the surface of the ER protein [14–17].

Do you think that a drug may have a commercial future in the chemoprevention of cancer?

As you know, we have made enormous progress with advancing the failed breast cancer drug, raloxifene, and millions of women are now benefiting from its use for the treatment of osteoporosis, but with a reduction in breast cancer incidence at the same time. This is the practical reality of our early translational research completed at the University of Wisconsin in the second decade of discovery (1980s). The "Tamoxifen Team" discovered selective estrogen receptor modulation and tamoxifen and raloxifene were both now classified as SERMs [18]. But the realization that tamoxifen could not possibly have widespread use because it increases the risk (though this is very small) of endometrial cancer in postmenopausal women [19], naturally guided us to our new SERM strategy in the late 1980s. We discovered that SERMs maintain bone density [20] and therefore could potentially prevent osteoporosis with the beneficial antiestrogenic side effect of preventing breast cancer [21]. We had solid translational research, as we had found that tamoxifen built bone both in the laboratory [20] and in clinical trial [22]. Raloxifene has a better safety profile and does not increase the risk of endometrial cancer [23], but it does not reduce the risk of coronary heart disease. I think the new SERM, lasofoxifene [24], is very good, as it prevents osteoporosis, breast cancer, coronary heart disease, and strokes, but without an increase of endometrial cancer. The problem is how to

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advance in a crowded market with low budgets for marketing. Lasofoxifene is approved but not marketed in the European Union.

No molecule targeting estrogen receptor has, to date, proved to be more efficient than tamoxifen in patients despite the development of a number of promising compounds. How do you explain that? Was it a choice of the pharmaceutical industry because of the cost of the development of such compound?

The issue with tamoxifen is unique. It was clearly lucky that tamoxifen had an acceptable toxicology profile for the treatment of cancer. It came onto the market at a time when the standard of care was combination cytotoxic chemotherapy, so tamoxifen looked good to patients. Tamoxifen was not supposed to succeed but advanced from strength to strength for 20 years. However, things change very rapidly in the arena of patient preference. In the early 1990s, when tamoxifen was being considered for testing as a chemopreventive and the specter of endometrial cancer translated from the laboratory [19] to clinical practice, this was clearly not good news for well women. Worse still, tamoxifen was found to produce DNA adducts in rat liver and initiate rat liver hepatocarcinogenesis [25]. Although liver tumors did not translate to clinical practice, this did not lessen concern, as the drug ended up with a black box label as a human carcinogen. Timing is everything with discovery and competitors could never catch up with clinical testing, despite the fact they may have been safer. We will never know.

To demonstrate that natural or synthetic molecules can prevent the occurrence of cancer is long and expensive. This raises the question of the life of the patents but also the natural molecules, which may not be patentable. Do you think there may be solutions to these problems?

I think it's currently impossible to find a solution to this dilemma. Clearly, the pharmaceutical industry will never advance with 20 year studies because the patents will run out. But here is a controversial point: the success of healthcare has now created the situation of increased longevity, so that drugs that enhance survival through prevention can only make matters worse. What is society to do? How does society find the resources to support an aging population?

You have developed recently a very provocative approach using estrogens for the treatment of breast cancers. This can be considered as a paradoxical use of estrogens? Could you explain us a little bit about that.

The third and fourth decades have been a wonderful surprise in our journey of discovery. We posed the question (based upon the clinical acceptance of long-term antihormonal therapy [9] as the most appropriate adjuvant treatment for breast cancer), what would be the mechanism and the timeframe for acquired antihormone resistance? Our first model clearly showed something unique as far as drug resistance is concerned—SERM-stimulated growth, something that is not seen with any other drug in cancer therapy [26]. This form of resistance occurred within a year or two and was consistent with the development of acquired resistance to tamoxifen in

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metastatic breast cancer. However, here was the dilemma: this model did not replicate the outstanding success observed with 5 years of adjuvant tamoxifen treatment [27]. In fact, 5 years of treatment continues to enhance decreases in mortality for more than a decade once tamoxifen is stopped. By a series of lucky accidents, one of my students (Doug Wolf) discovered that physiologic estrogen could cause dramatic tumor regression after 5 years of tamoxifen treatment, i.e., serial transplantation of tamoxifen-resistant tumors into generations of tamoxifen-treated mice [28]. This discovery reminded me of the words of Sir Alexander Haddow, FRS in 1970 during the Inaugural Karnofsky Lecture at the American Society of Clinical Oncology (ASCO): "... the extraordinary extent of tumour regression observed in perhaps 1 % of post-menopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us ..." [29]. It is now clear that aggressive estrogen deprivation with aromatase inhibitors or SERMs can rapidly reconfigure breast cancer cells through an evolution of drug resistance, which exposes a vulnerability that could not be anticipated—physiological estrogen-induced apoptosis [30, 31]. When Haddow did his original work using high-dose DES for the treatment of metastatic breast cancer in women during their late 60s and 70s, the best therapeutic results occurred the further away the patient was from the menopause. Antihormone therapy accelerates all of that in breast cancer, so physiologic estrogen can initiate the same triggering mechanism. Indeed, this is possibly the same mechanism that is occurring in the Women's Health Initiative (WHI) by conjugated equine estrogen (CEE) alone actually produces a decrease in the incidence of breast cancer in hysterectomized postmenopausal women [32]. What is particularly interesting about these data is the 6 years of monitoring after CEE is stopped, there is a continued reduction in the incidence of breast cancer, i.e., the estrogen has destroyed the nascent breast cancer cells in the ducts [33]. Our current laboratory work is focused entirely on deciphering the molecular mechanism of estrogen-induced apoptosis [34]. In this way, we may find the vulnerability triggered by the ER estrogen complex for cellular destruction; that vulnerable site in the cancer cell may be the next target for a new class of selective anticancer agents applicable to sites other than breast cancer.

Your contributions to medicine have received a lot of recognition but how does one become the "Diana, Princess of Wales Professor of Cancer Research"?

Life is all about chance meetings. In the mid-1990s, I was invited to organize a Breast Cancer Symposium in Chicago, and Diana was my keynote speaker (Fig. A.5). She came on a 3-day visit to Northwestern University and the Robert H. Lurie Comprehensive Cancer Center. Naturally, it was a very special time and when she left to return to London, we agreed to correspond and I sent her copies of my books on tamoxifen. There was even talk of a return trip for either her or Prince William or Prince Harry to open one of our new research buildings. Regrettably, everything changed with her untimely death in a tragic car accident in Paris on August 31, 1997. An anonymous donation was subsequently made to the Robert

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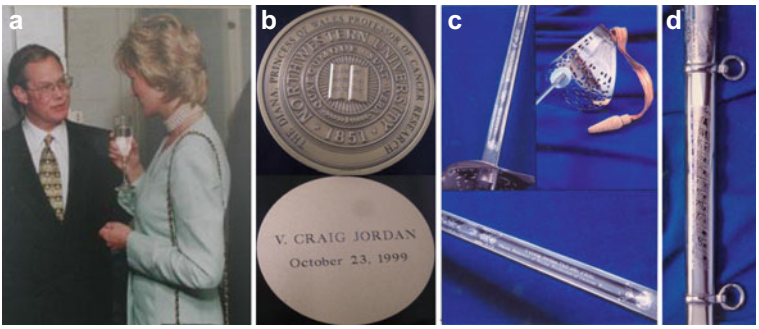

Fig. A.5 The Diana, Princess of Wales Chair of Cancer Research. In June 1996, Diana, the Princess of Wales visited Chicago for 3 days and we first met (**a**) at the evening reception at the home of the President of Northwestern University, Henry Bienen. The Chair was anonymously endowed at the Robert H. Lurie Comprehensive Cancer Center after Diana's untimely death on 31 August 1997. I was inaugurated on 23 October 1999, being presented with a unique Professorial medal (**b**) with copies being sent to her sons Prince William and Harry and also kept by my daughters, Helen and Alexandra. My students presented me with an engraved sword (**c**) to commemorate the event and their names, and the dates of the award of their Ph.D. degrees are engraved on the scabbard (**d**)

H. Lurie Comprehensive Cancer Center, and with letters from Lady Sarah McCorquodale (her sister) and the Earl Spencer (her brother), it was agreed that I would hold a professorship at Northwestern University in her name. Essentially, it was my British citizenship, a British medicine (tamoxifen), and our meeting and correspondence that was important to the family. On October 23, 1999, the Professorship was conferred on me by Henry Bienen, the president of Northwestern University, and over a 2-day period, there was a symposium in my honor by my former Ph.D. students, and during the celebration dinner, attended by representatives from the British Embassy, Barry Furr (the Chief Scientist from ICI), family, friends, and colleagues, my students presented me with an engraved sword (Fig. A.5) with each of the dates of their Ph.D. engraved on the scabbard as battle honors—very moving!

You have contributed more than 600 research and review papers to the literature with more than 23,000 citations and an h-index of 80. If you had to select ten of your research papers and three reviews, which would they be and why?

- Jordan V. C. (1976). *Eur J Cancer* 12: 419–424. Literally my first cancer research paper with tamoxifen that was rejected in 1974, but with kind and generous comments from one of the reviewers. I persevered and eventually this was one of the papers from my work used to justify the chemoprevention trials.
- Jordan V. C. and Allen K. E. (1980). *Eur J Cancer* 16: 239–251. The paper makes three points: this is the first refereed article that longer treatment is going to be better than shorter treatment; our discovery of 4-hydroxytamoxifen's

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pharmacology as a potent antiestrogen with a binding affinity for ER equivalent to estradiols [35] naturally made us think that this would be a more powerful anticancer agent—not true, cleared too quickly—and finally, we stated that antiestrogen treatment followed by estrogen deprivation would be a good strategy for people—true.

- Gottardis M. M., et al. (1988). *Cancer Res* 48: 812–815. This was the paper that warned the clinical community that tamoxifen could potentially increase the incidence of endometrial cancer in patients—true.
- Gottardis M. M. and Jordan V. C. (1988). *Cancer Res* 48: 5183–5187. This was the first report that acquired drug resistance with tamoxifen was unique and stimulated by SERMs—true.
- Love R. R., et al. (1992). *New Engl J Med* 326: 852–856. This was the randomized clinical trial based on our laboratory evidence and subsequently those of others that tamoxifen would maintain bone density in people. This paper opened the door to raloxifene.
- Levenson A. S. and Jordan V. C. (1998). *Cancer Res* 58: 1872–1875. A clean demonstration that a mutant ER found in a tamoxifen-stimulated tumor by a previous Ph.D. student (Doug Wolf) could change an antiestrogen to an estrogen. This could be done by a natural process.
- Cummings S. R., et al. (1999). *JAMA* 281: 2189–2197. Proof of principle that the concept we first articulated back in the late 1980s that you could develop a SERM to prevent osteoporosis and prevent breast cancer at the same time—true.
- Yao K., et al. (2000). *Clin Cancer Res* 6: 2028–2036. The first refereed publication to demonstrate that drug resistance to tamoxifen evolves and exposes a vulnerability to permit physiologic estrogen to cause tumor regression. Subsequently translated to the clinic—true.
- Vogel V. G., et al. (2006). The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA*. 295: 2727–2741. Two discarded drugs from the pharmaceutical industry that were reinvented in the same pharmacology laboratory to become the pioneering chemopreventive agents and FDA-approved—true.
- Vogel V. G., et al. (2010). *Cancer Prev Res* 3: 696–706. A follow-up of the trial several years after stopping SERM treatment, confirmed the predictions of one of my Ph.D. students (Marco Gottardis) in 1987 that tamoxifen would be the better chemopreventive in the long term.

I've always viewed an invitation to write a review article from a journal as a wonderful opportunity to project your personality, express your views, and, most importantly, reach out to young scientists and graduate students as theirs is the future. Here are my three choices:

- Jordan V. C. (1984). *Pharm Rev* 36: 245–276. This was my first major review when I first came to America. No one had really treated the topic as an issue in pharmacology, as all of the previous reviews in the 1960s and 1970s were about the control of fertility. I wanted a summary of the mechanisms of action of antiestrogens. It was all of our knowledge up to that point (423 citations).

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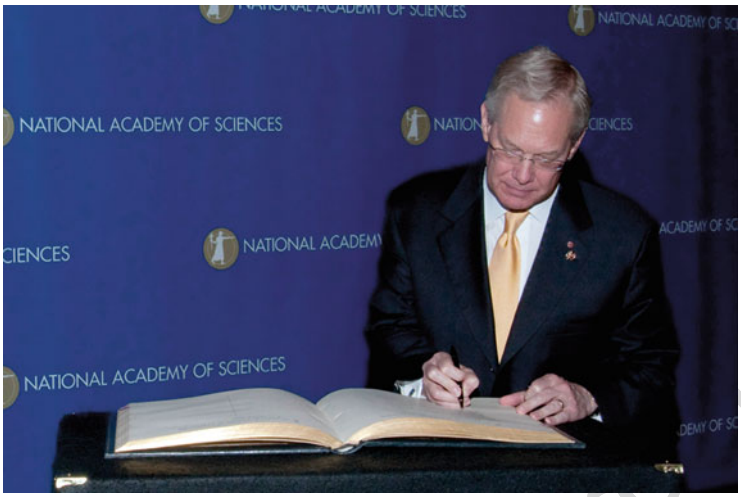

Fig. A.6 Signing the "Great Book" of Members of the National Academy of the Sciences USA during the Induction Ceremony on April 24, 2010

- Jordan V. C. (2006). *Br J Pharmacol* 147: S269–S276. I was thrilled to be asked by the British Pharmacological Society to write the story of my research in a Special Issue of our Journal. I got wonderful feedback from students.
- Jordan V. C. (2009). *Cancer Res*. 69: 1243–1254. I was proud to be asked by the American Association for Cancer Research (AACR) to contribute a review of progress in hormone dependent tumors as a part of a series to celebrate the 100th anniversary of AACR.

I see that you received the David A. Karnofsky Award in 2008 from ASCO, but it is stated in the regulations for the Award that it is given in "recognition of innovative clinical research and developments that have changed the way oncologists think about the general practice of oncology." You are a laboratory scientist and not a clinician; didn't this surprise you?

When I received the telephone call from the chair of the Awards Committee, Gabriel Hortobagyi, I was absolutely dumbfounded, because naturally, I knew I was not a clinician! All previous recipients were clinicians. This is ASCO's highest award, and I was being asked to join the legends of clinical practice. For the first 15 min of my conversation with Gabriel, I examined with him every reason why I should not be their recipient. After 15 min, he became exasperated and said, "Is this a 'Yes, I accept'?" I accepted the honor. Apparently, I learned that the reason the committee selected my work was because as a laboratory scientist and a pharmacologist, I had always been present at clinical breast cancer meetings over the decades, putting forward my point of view in cancer treatment with SERMs. For me, the promise of life was the most important goal. But safety was essential. The involvement I had every day with the clinical evaluation of tamoxifen [22], AU1

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Fig. A.7 Honorary Fellowship of the Royal Society of Medicine awarded by Professor Ilora Finlay, Baroness Finlay of Llandaff, president of the Royal Society of Medicine (2008). This honor is awarded to individuals of international standing who have eminently distinguished themselves in the service of medicine and the fields which influence it. The Society permits, at most, 100 people into this elite group at any one time. In 2008, there were only 89 Honorary Fellows worldwide. In 2009, I received the Jephcott Medal from the Royal Society of Medicine, and in 2010, I was elected as the president of the Royal Society of Medicine Foundation in North America

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followed by leadership positions for the evaluation of raloxifene [23], and then as the scientific chair of the Study of Tamoxifen and Raloxifene (STAR) [36, 37] allowed me to deploy the knowledge generated by my "Tamoxifen Team" over decades to save lives and advance women's health [38]. Please remember that when I started this improbable and unlikely journey at the beginning of the 1970s, cancer therapeutics with a targeted agent, chemoprevention, and the drug group, SERMs (or even tamoxifen for that matter!) did not exist. Cancer research was not recommended as a career for the pharmacologist and the pharmacologist would not knowingly venture into women's health. All of the revenues in the pharmaceutical industry were derived from heart drugs and drugs that affected the central nervous system (e.g., tranquilizers) (Fig. A.6).

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When I was starting the research for my Ph.D. at Leeds University, Sir Alexander Haddow, FRS in the Inaugural Karnofsky Lecture [29], was dismayed at the prospect for cancer therapeutics. Unlike the success noted with antibiotics for the treatment of different infectious diseases, there were no laboratory tests to establish whether chemotherapy would be effective or not. The physician just had to give it to the patient and see if it worked! Haddow was also not convinced that a cancer-specific drug could be developed because cancer was self. In Haddow's Karnofsky Lecture publication, there was one glimmer of hope: Haddow had used the first chemical therapy to treat any cancer, i.e., high-dose estrogen to treat metastatic breast cancer in women in their late 60s and 70s. He observed that some of the responses just melted the tumors away. But he was dismayed that the mechanisms had remained elusive. I am pleased to say that we have now solved the question surrounding the mechanism of estrogen-induced apoptosis [34] (Fig. A.7).

It is fair to say that the work that has evolved and developed on the treatment and prevention of breast cancer over the past four decades has changed our outlook and replaced pessimism with hope. The first decade of discovery was essential to move forward in the field [9]. It has not only been possible to create change in medical practice, but the laboratory principles all translated to patient care to save or at least extend lives. That is what pharmacology is.

In closing, I must end where we began. I have thanked Drs. Kaye and Clark (Fig. A.1) many times for the opportunity they gave me with a place at Leeds University. The reply I received was usually "we were only doing our job." Good words to remember and live by.

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References

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1. Jordan VC (2003) Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2:205–213
2. Jordan VC (2007) Chemoprevention of breast cancer with selective oestrogen receptor modulators. Nat Rev Cancer 7:46–53
3. Jordan VC, Koerner S (1975) Tamoxifen (ICI46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 11:205–206
4. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the nonsteroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
5. Jordan VC (1978) Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. Rev Endocr Relat Cancer Suppl:49–55
6. Jordan VC, Dix CJ, Allen KE (1979) The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In: Salmon SE, Jones SE (eds) Adjuvant therapy of cancer II. Grune & Stratton, Philadelphia, pp 19–26
7. Jordan VC (1976) Effect of tamoxifen (ICI46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
8. Jordan VC (1988) The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. Breast Cancer Res Treat 11:197–209

Author's Proof

9. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44:30–38
10. Lieberman ME, Jordan VC, Fritsch M, Santos MA, Gorski J (1983) Direct and reversible inhibition of estradiol-stimulated prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4734–4740
11. Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741–4745
12. Jordan VC, Lieberman ME (1984) Estrogen-stimulated prolactin synthesis in vitro classification of agonists, partial agonist and antagonist actions based on structure. Mol Pharm 26:279–285
13. Wolf DM, Jordan VC (1994) The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31:129–138
14. Levenson AS, Jordan VC (1998) The key to the antiestrogenic mechanism of raloxifene is Amino Acid 351 (Asp) in the estrogen receptor. Cancer Res 58:1872–1875
15. Macgregor-Schafer JI, Liu H, Bentrem D, Zapf J, Jordan VC (2000) Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex by substituting glycine for aspartate at amino acid 351. Cancer Res 60:5097–5105
16. Liu H, Lee ES, De Los Reyes A, Zapf JW, Jordan VC (2001) Silencing and reactivation of the selective estrogen receptor modulator (SERM)-ER alpha complex. Cancer Res 61:3632–3639
17. Liu H, Park W, Bentrem DJ, McKian KP, De Los Reyes A, Macgregor-Schafer J, Zapf JW, Jordan VC (2002) Structure-function relationships of the raloxifene-estrogen receptor alpha complex for regulating transforming growth factor-alpha expression in breast cancer cells. J Biol Chem 277:9189–9198
18. Jordan VC (2001) Selective estrogen receptor modulation: a personal perspective. (Perspectives in Cancer Research). Cancer Res 61:5683–5687
19. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48:812–815
20. Jordan VC, Phelps E, Lindgren JU (1987) Effects of antiestrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10:31–35
21. Gottardis MM, Jordan VC (1987) The antitumor actions of keoxifene (raloxifene) and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 47:4020–4024
22. Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, Carbone PP, Demets DL (1992) Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Eng J Med 326:852–856
23. Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, Norton L, Nickelsen T, Bjarnason NH, Morrow M, Lippman ME, Black D, Glusman JE, Costa A, Jordan VC (1999) The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. JAMA 281:2189–2197
24. Cummings SR, Ensrud K, Delmas PD, Reid DM, Goldstein S, Sriram U, Lee A, Thompson J, Armstrong RA, Thompson DD, Powles T, Zanchetta J, Kendler D, Neven P, Eastell R (2010) Lasofoxifene in postmenopausal women with osteoporosis. N Engl J Med 362:686–696
25. Jordan VC (1995) What if tamoxifen (ICI46,474) had been found to produce liver tumors in rats in 1973? Ann Oncol 6:29–43
26. Gottardis MM, Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 48:5183–5187

Uncorrected Proof

Author's Proof

27. Early Breast Cancer Trialists' Collaborative Group (EBCTCG) (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378:771–784
28. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127:23–33
29. Haddow A, David A (1970) Karnofsky memorial lecture. Thoughts on chemical therapy. Cancer 26:737–754
30. Yao K, Lee ES, Bentrem DJ, England G, Schafer JIM, O'Regan R, Jordan VC (2000) Antitumor action of physiologic estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6:2028–2036
31. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li Y, Bell E, Chandel NS, Jordan VC (2005) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97:1746–1759
32. la Croix AZ, Chlebowski RT, Manson JE, Aragaki AK, Johnson KC, Martin L, Margolis KL, Stefanick ML, Brzyski R, Curb JD, Howard BV, Lewis CE, Wactawski-Wende J (2011) Health outcomes after stopping conjugated equines estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. JAMA 205:1305–1314
33. Jordan VC, Ford LS (2011) Paradoxical clinical effect of estrogen on breast cancer risk: A "new" biology of estrogen-induced apoptosis. Cancer Prev Res 4:633–637
34. Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, Tapia C, Kim HR, Yerrum S, Sharma CGN, Nicolas E, Balagurunathan Y, Ross EA, Jordan VC (2011) Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer via stress responses as identified by global gene expression across time. Proc Natl Acad Sci U S A 108 (47):18879–18886 AU4
35. Jordan VC, Collins MM, Rowsby L, Prestwich G (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305–316
36. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER, Wade JL, Robidoux A, Margolese RG, James J, Lippman SM, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N (2006) The Study of Tamoxifen and Raloxifene (STAR): report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. JAMA 295:2727–2741
37. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER, Wade JL, Robidoux A, Margolese RG, James J, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N (2010) Update of the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: preventing breast cancer. Cancer Prev Res 3:696–706
38. Jordan VC (2008) The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer–survival or death? J Clin Oncol 26:3073–3082
39. Jordan VC (1984) Biochemical pharmacology of antiestrogen action. Pharm Rev 36:245–276
40. Jordan VC (2006) 75th Anniversary Edition British Journal of Pharmacology Special Issue. Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 147:S269–S276
41. Jordan VC (2009) A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 69:1243–1254

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Appendix B: Selected Awards That Recognize the Contribution of Tamoxifen and Raloxifene to Medicine

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2012	ASPET Goodman and Gilman Award	586
2011	St. Gallen International Breast Cancer Award	587
2009	Elected to National Academy of Sciences (USA)	588
2009	Fellow of the Academy of Medical Sciences (UK)	589
2008	Honorary Fellowship of the Royal Society of Medicine	590
2008	American Society of Clinical Oncology 38th David A. Karnofsky Award	591
2007	Gregory G. Pincus Award and Medal. Worcester Foundation for Biomedical Research	592
2006	American Society Award for Chemoprevention, American Society of Clinical Oncology	593
2005–2009	Endowed Chair: Alfred G Knudson Chair in Basic Science, Fox Chase Comprehensive Cancer Center	594
2005	Honorary Doctor of Science Degree. University of Bradford, England	595
2003	The Charles F. Kettering Prize of the General Motors Cancer Research Foundation.	596
2002	Officer of the Most Excellent Order of the British Empire (OBE)	597
2002	American Cancer Society Medal of Honor (Basic Research Award).	598
2002	Inaugural Dorothy P. Landon American Association for Cancer Research (AACR) Prize in Translational Research.	599
2001	Bristol Myers Squibb Award and Medal for Distinguished Achievement in Cancer Research	600
2001	Honorary Doctor of Medicine Degree. University of Leeds, England	601
2001	Honorary Doctor of Science Degree. University of Massachusetts	602
2000	Strang Award. Cornell Medical School	603
2000	Honorary Fellowship Award and Medal. Faculty of Medicine of University Colllege, Dublin	604
1999–2004	Endowed Chair: Diana, Princess of Wales Professor of Cancer Research, Robert H. Lurie Cancer Center, Northwestern University Feinberg School of Medicine	605
1994	William L. McGuire Memorial Award. San Antonio Breast Cancer Symposium	606
1993	Cameron Prize Award. University of Edinburgh	607
1993	American Society for Pharmacology and Experimental Therapeutics Award for Experimental Therapeutics. Contributions to research on human disease	608
1993	Gaddum Memorial Award for research contributions to pharmacology, British Pharmacological Society	609

(continued)

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1992	Inaugural Brinker International Breast Cancer Award for Basic Science. Susan G. Komen Foundation
1989	Eighth Bruce F. Cain Memorial Award American Association for Cancer Research

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Review

Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer

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ABSTRACT

This article describes the origins and evolution of "antiestrogenic" medicines for the treatment and prevention of breast cancer. Developing drugs that target the estrogen receptor (ER) either directly (tamoxifen) or indirectly (aromatase inhibitors) has improved the prognosis of breast cancer and significantly advanced healthcare. The development of the principles for treatment and the success of the concept, in practice, has become a model for molecular medicine and presaged the current testing of numerous targeted therapies for all forms of cancer. The translational research with tamoxifen to target the ER with the appropriate duration (5 years) of adjuvant therapy has contributed to the falling national death rates from breast cancer. Additionally, exploration of the endocrine pharmacology of tamoxifen and related nonsteroidal antiestrogen (e.g. keoxifene now known as raloxifene) resulted in the laboratory recognition of selective ER modulation and the translation of the concept to use raloxifene for the prevention of osteoporosis and breast cancer.

However, the extensive evaluation of tamoxifen treatment revealed small but significant side effects such as endometrial cancer, blood clots and the development of acquired resistance. The solution was to develop drugs that targeted the aromatase enzyme specifically to prevent the conversion of androstenedione to estrone and subsequently estradiol. The successful translational research with the suicide inhibitor 4-hydroxyandrostenedione (known as formestane) pioneered the development of a range of oral aromatase inhibitors that are either suicide inhibitors (exemestane) or competitive inhibitors (letrozole and anastrozole) of the aromatase enzyme. Treatment with aromatase inhibitors is proving effective and is associated with reduction in the incidence of endometrial cancer and blood clots when compared with tamoxifen and there is also limited cross resistance so treatment can be sequential. Current clinical trials are addressing the value of aromatase inhibitors as chemopreventive agents for postmenopausal women.

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The enthusiasm with which the clinical community has embraced the use of antiestrogenic therapy to treat breast cancer is based upon the proven record of success that first the nonsteroidal antiestrogen tamoxifen and then the aromatase inhibitor have demonstrated in clinical trial. The reasons for the enthusiasm are obvious. Antihormonal therapy, particularly aromatase inhibition to create a "no estrogen state" in the postmenopausal breast cancer patient is effective, saves women's lives, is contributing successfully to reducing the national mortality from breast cancer, is relatively cheap and has fewer side effects and easy administration (oral) than any other anticancer strategy. However, the successful application of a therapeutic strategy to block the known growth stimulation property of estrogen in breast cancer was not greeted with such enthusiasm 40 years ago.

Estrogen is essential for life. Without the critical role of estrogenic steroids, reproduction would not be possible. Based on emerging knowledge from laboratory studies, the value of modulating the steroid environment during the menstrual cycle was advanced to clinical testing during the 1950s as a means of oral contraception. The results of these studies were to change society forever.

The Worcester Foundation for Experimental Biology is the place where Gregory Pincus established the scientific principles necessary to propose clinical testing of the oral contraceptive and M.C. Chang subsequently established the first protocols to perform *in vitro* fertilization. Simply stated, the Worcester Foundation was, at that time, the world center for steroid endocrinology and reproductive biology. Over the years, hundreds of scientists have trained at the Foundation and subsequently spread their knowledge throughout the world [1]. However, fashions in research change and new opportunities emerge.

In 1971, President Nixon made a national commitment to seek a cure for cancer by signing the National Cancer Act. Mahlon Hoagland, the President of the Worcester Foundation, responded to the initiative by appointing Professor Elwood V. Jensen, Director of the Ben May Cancer Research Laboratory at the University of Chicago, to be a member of the Foundation's Scientific Advisory Board. Jensen had discovered the estrogen receptor (ER) as the putative mechanism of estrogen action in its target tissues [2]. The known link between estrogen and breast cancer suggested that "antiestrogenic strategies" might have potential as therapeutic agents [3]. Jensen applied knowledge of ER action to breast cancer treatment by devising the ER assay to identify breast cancers that would respond to endocrine ablation [4] but not all breast cancers responded. Hoagland's plan was to encourage the exploitation of the rich resources in endocrinology at the Foundation to be used for cancer research. The scene was set for independent investigators to work in cancer endocrinology but it is fair to say no one in academic medical oncology was interested in development of new antihormone therapies. Combination cytotoxic chemotherapy was king. Industry and clinical trial groups were respectively convinced that (1) developing anticancer drugs was a very risky business and (2) the right combination of cytotoxic agents applied at the right time would cure cancer. The principle was working in childhood leukemia, why not breast cancer?

The authors first met at the Worcester Foundation during the closing months of 1972. By coincidence, we were both English and grew up in the same county of Cheshire. One of us (VCJ) had conducted a PhD (1968–1972) on the structure activity relationships of a group of failed contraceptives, the nonsteroidal antiestrogens, the other (AMHB) had worked on hormones and breast cancer at the Christie Hospital in Manchester where the first preliminary study of ICI 46,474 was subsequently completed [5]. This was before ICI 46,474 was renamed tamoxifen (Fig. 1).

We have started this review with an account of our individual experiences that led to the development of tamoxifen and the aromatase inhibitors. Our perspective is followed by a

description of the therapeutic target, the estrogen signal transduction system and we close with current clinical advances in antihormonal therapy.

General Motors Prize Awards Ceremony—Washington, DC, 2005

The Charles F. Kettering Prize from the General Motors Cancer Research Foundation is awarded annually for the most outstanding recent contributions to the diagnosis or treatment of cancer. V. Craig Jordan (VCJ) and Angela H. Brodie (AMHB) are recognized on separate occasions for their pioneering studies that defined the scientific principles used clinically for the targeted treatment and prevention of breast cancer. Their body of work using the selective estrogen receptor modulators, tamoxifen and raloxifene (VCJ) and the first suicide inhibitor of the aromatase enzyme 4-hydroxyandrstendione (AMHB) has been individually recognized by several of the worlds leading prizes including the Brinker International Award from the Susan G. Komen Breast Cancer Foundation (VCJ 1993, AMHB 2000), the Dorothy P. Landon/American Association for Cancer Research Prize for Translational Research (VCJ 2002, AMHB 2006) and the Charles F. Kettering Prize from the General Motors Cancer Research Foundation (VCJ 2003, AMHB 2005) (see photograph). Jordan and Brodie have been members and attended the Endocrine Society since 1981 and 1962, respectively.

1. V. Craig Jordan: ICI 46,474 to tamoxifen

In 1967 Arthur Walpole and Mike Harper at the Imperial Chemical Industries (ICI) Pharmaceutical Division in Alderley Park, Cheshire reported the antiestrogenic and antifertility properties of a substituted triphenylethylene ICI 46,464 [6,7]. The Alderley Park team had been tasked during the 1960s to discover compounds to modulate fertility. Although Walpole also had an interest in anticancer chemotherapy, [8] as head of the fertility control program, he did not conduct any laboratory investigations of ICI 46,474 as an anticancer agent. He did, however, ensure that ICI Pharmaceuticals Division patented the compound with the statement, "The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolaemic activity." Nevertheless, there was no patent for ICI 46,474 in the United States in 1972.

Harper had moved to the Worcester Foundation in the late 1960s and was investigating the potential of prostaglandins to be used as a once a month contraceptive. Although it was clear that prostaglandins were too toxic for systemic use, it is perhaps relevant to point out that a prostaglandin is currently used with mifipristone (RU486) as an abortifacient.

In 1972 I had completed my PhD on the "Structure Activity Relations of Substituted Triphenylethylenes and Triphenylethanes" but the University of Leeds was having difficulty securing an appropriate external examiner for my thesis. Nobody cared about the topic and it was only after considerable negotiation that Arthur Walpole (from industry!) was permitted to undertake the task. This experience started a collaboration that only ended with his untimely death in 1977.

In that same year, I took a 2 year leave of absence from the Department of Pharmacology at the University of Leeds originally to work with Mike Harper on the contraceptive properties of prostaglandins but when I arrived, he had left to work in the World Health Organization in Geneva and I was told—do anything you like, as long as some of it involves prostaglandins! My passion was the application of chemistry to medicine and I had always wanted to develop targeted anticancer drugs. Two events occurred in 1972–1973 that permitted me to pursue my passion. A meeting between Jensen and me at the Worcester Foundation in November 1972 would solve the problem of how to conduct a systematic laboratory examination of the antitumor actions of ICI 46,474. Jensen offered to teach me the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model [9] and techniques to measure estrogen receptors (ERs) in animal and human tumors. These techniques were essential to reinvent ICI 46,474 (tamoxifen) as an antitumor agent targeted to the ER. A phone call to Arthur Walpole in the United Kingdom secured funding to support the work and introduced me to Lois Trench, the newly appointed drug monitor for ICI 46,474 at the recently acquired Stuart Pharmaceuticals in Wilmington, Delaware. The company quickly evolved into ICI Americas and now 30 years later is known as AstraZeneca. Lois Trench provided human breast cancers for me to establish that tamoxifen blocked, the binding of estradiol to the ER [10], and I was also asked to introduce ICI 46,474 first to the Eastern Cooperative Oncology Group (ECOG), [11] and subsequently to the National Surgical and Bowel Project (NSABP) [12]. The NSABP particularly would propel ICI 46,474 from obscurity in the 1960/1970s to center stage

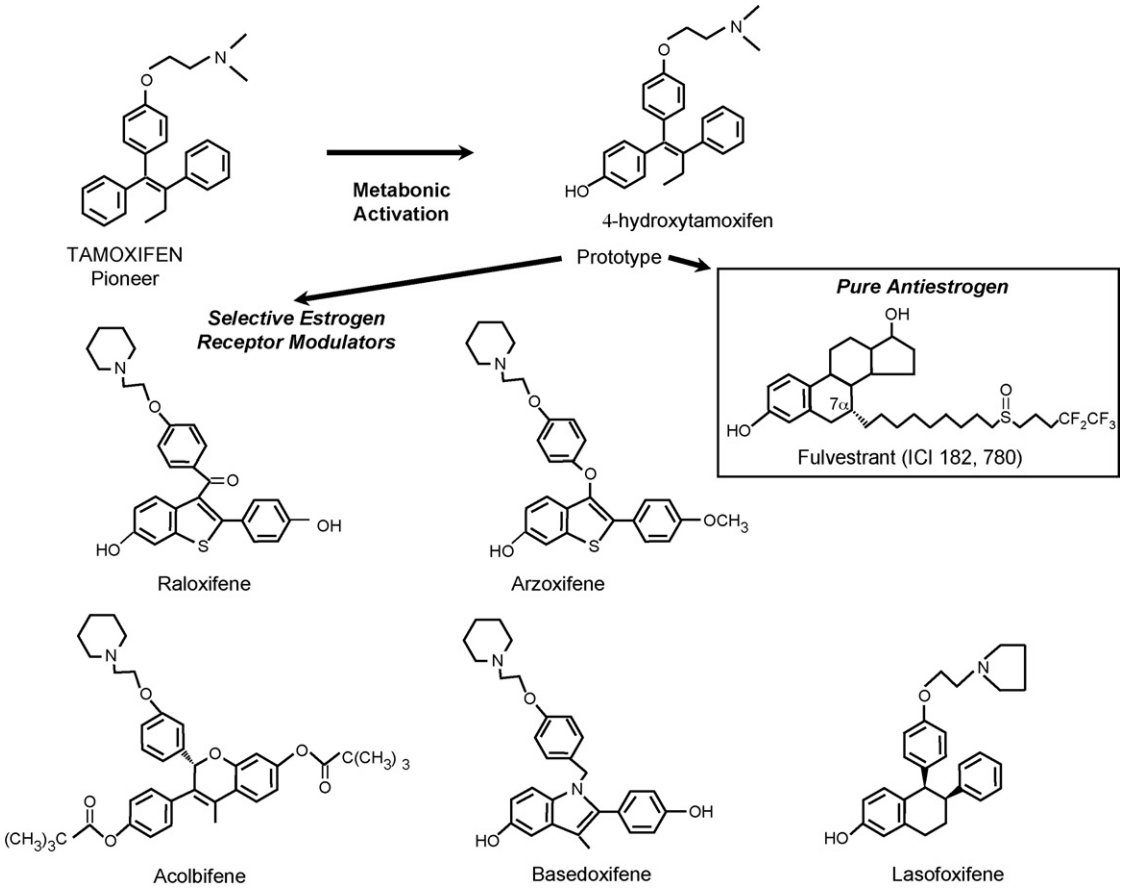

Fig. 1 – The activation of the pro drug tamoxifen to 4-hydroxytamoxifen, which has a high binding affinity for ER [103]. This knowledge resulted in the development of numerous new agents for use as selective estrogen ER modulations (SERMs) for the prevention of breast cancer and osteoporosis or the pure antiestrogen fulvestrant used as a treatment for ER positive advanced breast cancer following the failure of either tamoxifen treatment or an aromatase inhibitor.

in the treatment and prevention of breast cancer during the 1980/1990s.

1.1. *Translational research with tamoxifen*

A scientific strategy for the appropriate clinical application of tamoxifen was developed in the laboratory during the 1970s to target the drug to the tumors that were the most likely to respond [13,14]. Tamoxifen blocked the binding of estradiol to human breast and rat mammary tumor ERs and prevented the induction and growth of ER positive carcinogen-induced rat mammary carcinomas [10,15–17] These early studies raised the question of whether tamoxifen could prevent the majority of breast cancers, i.e.: ER positive breast cancer. However, the finding that long term tamoxifen treatment in animals with early mammary cancer, i.e., a low tumor burden [18–20] could create a tumor-free state suggested longer was going to be better than shorter durations of adjuvant therapy. The laboratory observations and pilot clinical studies [21,22] were to prove remarkably effective as an approach to treat women with early node positive and node negative ER positive breast cancer. However, the original clinical strategy in the 1970s for the evaluation of tamoxifen was to use 1 year of adjuvant treatment after surgery. The reason for this was that tamoxifen was only effective for the treatment of advanced breast cancer for about a year and there was a sincere concern that longer adjuvant treatment durations would result in premature drug resistance. This approach was to change.

An enormous advance in medicine is the introduction of meta-analysis or Overview analysis of small randomized clinical trials that individually show little or no benefits for agents under investigation but together provide a statistically secure result. The Overview analysis of breast cancer clinical trials was first conducted at Heathrow airport in 1984 [23]. The results when they were published in full in 1988 demonstrated a significant advantage for postmenopausal patients receiving tamoxifen [24]. Most importantly, a Consensus Conference held in Bethesda, MD recommended that tamoxifen should be used as an adjuvant therapy for postmenopausal ER positive, node positive patients with breast cancer [25]. The year 1985 was a good time for ICI Pharmaceuticals Division (now AstraZeneca) to be awarded a use patent for tamoxifen from the US Court of Appeals. The award of a patent for tamoxifen in 1985 started a 17 year exclusivity use patent in the US just at the time when the patent for tamoxifen had expired worldwide and just at the time that tamoxifen was poised to change healthcare. Thus, the accumulative 40-year patent for tamoxifen was to be the financial engine that facilitated

the development of a whole range of cancer therapies including aromatase inhibitors from AstraZeneca and subsequently other companies.

2. Angela M.H. Brodie—aromatase inhibitors: developing 4-hydroxyandrostenedione

I had received my PhD degree from Manchester University and was awarded an NIH Postdoctoral Training Fellowship, which brought me to the Worcester Foundation in 1962. The exciting atmosphere of cutting edge research enticed me to remain there after my fellowship. By the early 1970s, I had married a fellow scientist, Harry Brodie, and joined his lab working on the biochemistry of aromatase, the key enzyme in the biosynthesis of estrogens. Harry, an organic chemist, had begun developing inhibitors of aromatase as potential contraceptive agents and reported the first of these compounds in 1973 [26].

With my background in breast cancer at the Christie Hospital in the UK and the death of the previous lab director from the disease still on my mind, I was very interested in the possibility that aromatase inhibitors might be of value in the treatment of breast cancer. Clinical trials with ICI 46,474 (tamoxifen) had begun about this time. Although the antiestrogen was effective, it clearly did not yet have the impact on the disease that eventually brought it to the important position it has today. However, at that time, tamoxifen and other antiestrogens were known to be partial estrogen agonists as well as antagonists [6], raising concerns that they may not be optimally effective against breast cancer and may have adverse estrogenic effects. We reasoned that by using a different approach, compounds that blocked the production of estrogen without having significant estrogenic activity themselves might be identified. Results showed this to be the case. For the same reason, the possibility existed that aromatase inhibitors might also be more effective in treating breast cancer than antiestrogens. A number of laboratory studies were carried out which demonstrated the efficacy of the most potent aromatase inhibitor, 4-hydroxyandrostenedione (4-OHA) [27]. Some time later, we found that this compound acts not only by rapid competitive inhibition but also by inactivation of the enzyme. This effect is long lasting or irreversible (Fig. 2); see further below on steroidal inhibitors [28]. With some help from Craig, who had become proficient in developing mammary tumors with DMBA in rats, we showed that 4-OHA was effective in suppressing ovarian estrogen levels and causing regression of rodent mammary tumors [27]. In contrast to tamoxifen, 4-OHA was not estrogenic on other tissues such as the rat uterus. 4-OHA also inhibited peripheral (non-ovarian) estrogen synthesis in non-human primates in studies carried out in collaboration with Chris Longcope at the Worcester Foundation [29].

2.1. *Efforts to bring 4-OHA into the clinic*

Although these studies demonstrated that 4-OHA is highly effective, clinical studies were difficult to initiate, despite encouragement from the Decision Network Group at NCI who set aside funds to carry out toxicity studies. By 1978, Harry had left research, and I had moved to the University of Maryland in 1979. I was hopeful that the Cancer Center would be interested in bringing 4-OHA into the clinic. Tamoxifen had by then been shown to be effective and there was a lack of enthusiasm to investigate other approaches, not only at the University of Maryland but also in pharmaceutical companies. One approach, however, that did help the cause of aromatase inhibitors was the use of aminoglutethimide (AG) (Fig. 3) in treating breast cancer patients. Aminoglutethimide was a drug developed for treating epilepsy. However, it was found to cause adrenal suppression by inhibiting multiple cytochrome P-450 enzymes [30]. As adrenalectomy had been shown to be effective in treating breast cancer by Charles Huggins, Richard Santen and colleagues in the late 1970s began using aminoglutethimide as a medical approach to suppressing adrenal steroids in breast cancer patients [31–33]. Because aminoglutethimide inhibited a number of steroidogenic P-450 enzymes including CYP11, patients were given cortisol replacement. Santen was able to show that the main beneficial effect of aminoglutethimide then was inhibition of estrogen synthesis. However, aminoglutethimide had a number of significant side effects. Thus, it was my good fortune that some oncologists experienced with using aminoglutethimide were receptive to testing our selective aromatase inhibitor, 4-OHA. In the fall of 1981, I was invited to a conference in Rome to give a presentation about my research. Afterwards, an oncologist from London, Charles Coombes expressed interest in testing 4-OHA in breast cancer patients. Soon after my return to Maryland, a letter arrived from the Royal Marsden Hospital in London suggesting collaboration to bring 4-OHA, the first selective aromatase inhibitor into the clinic. In my laboratory at the University of Maryland we were able to produce a kilogram of 4-OHA by combining several batches of material. The toxicology was carried out through the Cancer Research Campaign in the UK. Paul Goss joined Charles Coombes as a PhD student and cared for the first patients that were treated with 4-OHA. Mitch Dowsett was also an important part of the team and measured estrogen and drug levels in the patients. Significant responses were seen in these first series of patients many of whom had relapsed from tamoxifen treatment. With these exciting results, Charles Coombes and I traveled to Horesham to Ciba-Geigy with the proposition that they take on 4-OHA

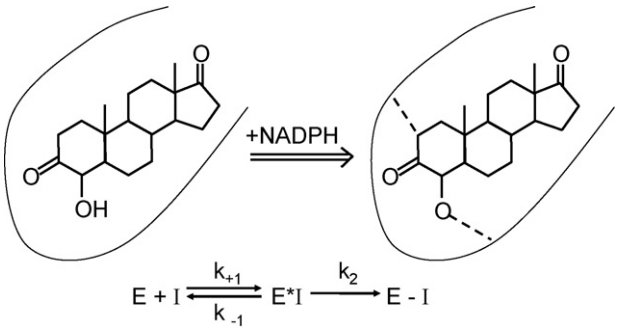

Fig. 2 – Aromatase inactivators: several steroidal inhibitors have been demonstrated to bind irreversibly or very tightly to the active site of aromatase e.g., 4-hydroxyandrostenedione (4-OHA).

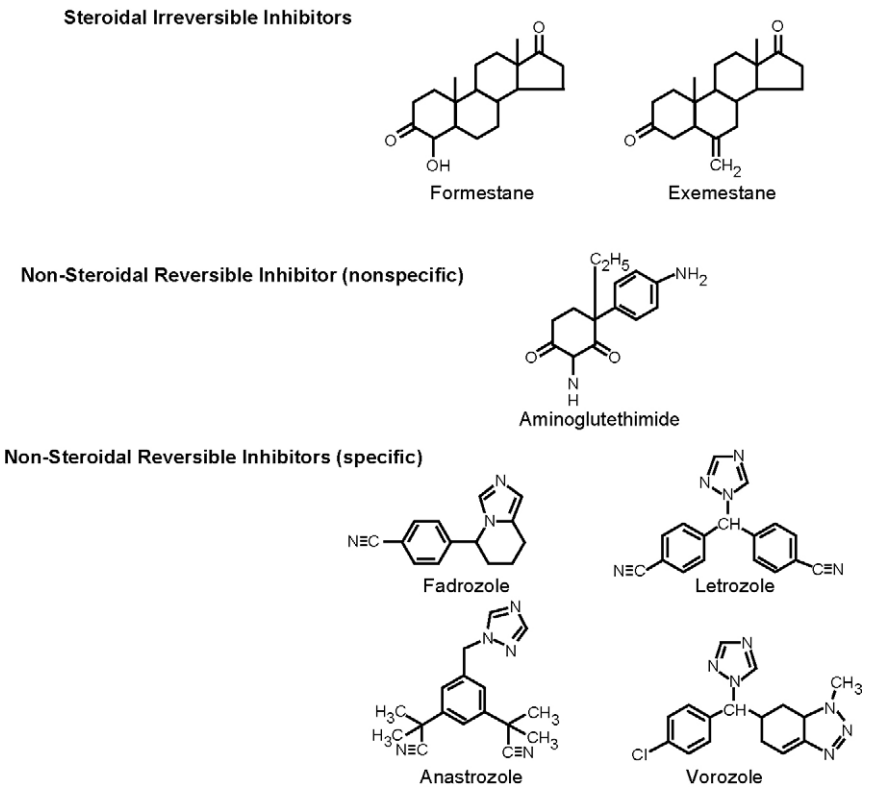

Fig. 3 – The structures of various aromatase inhibitors tested clinically for the treatment of breast cancer. The compounds are classified based on their mode of action and specificity for the aromatase enzyme.

and expand the clinical trials. Ciba-Geigy produced AG and it was quickly appreciated by the late Stuart Hughes that selective aromatase inhibitors such as 4-OHA would have distinct advantages. Clinical trials proceeded and Formestane (4-OHA) was the first selective aromatase inhibitor to become available and was the first new treatment of breast cancer in 10 years at that time.

As 4-OHA was of benefit in patients who had relapsed on tamoxifen, interest gradually grew in the possible benefits of using additional "hormonal" agents that are well tolerated. Before long, a number of pharmaceutical companies began producing aromatase inhibitors. Several of these were highly effective in inhibiting estrogen synthesis and some were more potent than 4-OHA. Although several US companies had produced excellent inhibitors, these did not come to clinical trials, largely due to internal company decisions. The field eventually thinned to three companies who had developed highly potent inhibitors.

One of these, exemestane, was a steroidal compound similar to formestane and developed by Farmitalia. The company had a history of making steroid and androgenic compounds mostly for anabolic activity. Exemestane (Fig. 3) has proved to be potent and effective in patients and is now approved by the FDA for breast cancer treatment. The two other FDA approved aromatase inhibitors came from pharmaceutical companies who investigated existing drug types for example, antifungal agents that inhibited cytochrome P-450 enzymes. The challenge was to modify such agents to be selective for aromatase. The result of these endeavors initially included vorazole (Fig. 3) also an inhibitor in this class. It was later discontinued despite good efficacy in breast cancer patients. However, the third generation agents letrozole and anastrozole (Fig. 3) were shown to be highly selective, yet reversible inhibitors of the aromatase enzyme.

2.2. *The estrogen ER signal transduction pathway as a model for molecular targeting*

Because of the importance of estrogen as a stimulus to the development and progression of breast cancer, estrogen synthesis (via aromatase) and action (via ER) continue to be exceptional targets for the treatment and chemoprevention of breast cancer [34]. Thirty years ago the idea of targeting and blocking estrogen action to treat breast cancer with tamoxifen or the idea of blocking the estrogen synthetase (aromatase) enzyme appeared to be simple and straightforward concepts. Today, these simple approaches have become multifaceted with many layers of complexity that are being explored to enhance tissue selectively, address intrinsic resistance and block the development of acquired antihormonal resistance.

2.3. *Mechanism of estrogen synthesis*

Aromatase mediates the conversion of the steroidal C-19 androgens to C-18 estrogens, which is the critical step in the biosynthesis of estrogens. This enzyme, therefore, has important functions in female development and reproduction. In the human, aromatase is expressed primarily by the

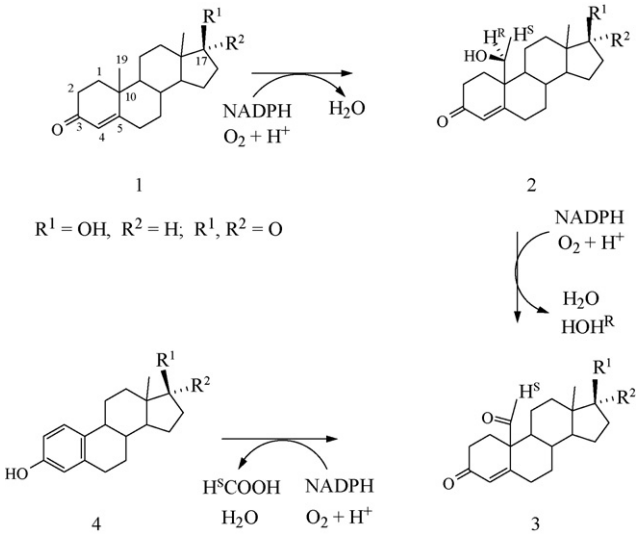

Fig. 4 – Aromatase mediates conversion of androgens to estrogens. Three hydroxylation steps are postulated.

ovary in premenopausal women [35]. However, central aromatization is necessary for the manifestations of many sex behavioral, neuroendocrine and developmental responses of several species [36,37]. In addition, aromatase is expressed in a number of other tissues throughout the body. The most important sites of non-gonadal estrogen synthesis are muscle and adipose tissue [38], where production increases with age in both sexes. Peripheral aromatization is the main source of estrogens in postmenopausal women [39] with significant production equivalent to premenopausal levels occurring in the breast [40].

Aromatization of androgens to estrogens occurs via a series of reactions (Fig. 4). An understanding of the mechanisms involved is important to the development of effective aromatase inhibitors. The aromatase complex consists of a cytochrome P450 hemoprotein and a flavoprotein, NADPH-cytochrome P450 reductase. The latter is common to most cell types and functions to donate electrons to the cytochrome P450. The P450 aromatase (P450 arom) binds the C-19 androgen substrates, androstenedione and testosterone and catalyzes their conversion to estrone and estradiol. This reaction is thought to involve three steps, each utilizing 1 M equiv. of NADPH and oxygen [41]. The first step is hydroxylation at the C-l9 of the androgen substrate. This appears to be a characteristic cytochrome P450 hydroxylation [42,43]. Based on site-directed mutagenesis studies of the enzyme [44–46], it is suggested that hydrogen bonding of the l9-hydroxylation intermediate to an acidic side-chain residue Glu-302 is of critical importance in the aromatization process [46]. Hydrogen bonding of the 3-ketone may also occur at a polar active site (His-128 residue). This anchors the intermediate and assures stereospecific removal of the C-19 pro-R hydrogen by a heme iron-oxo species during the second hydroxylation step. Because of the high electrophilicity of the aldehyde, the usual ferric peroxide breakdown may be circumvented and the normal hydroxylation cycle altered. A number of theories have been postulated to explain the mechanisms involved in the last step. The C10–C19 bond is cleaved resulting in aromatization of the steroid A-ring and release of formic acid. Recently, Hackett et al. [46] showed that the 1β-hydrogen atom removal by an iron-oxo intermediate from the substrate in the presence of the 2,3-enol meets little resistance (5.3–7.8 kcal/mol), whereas in the keto tautomer, this same process encounters barriers of 17.0–27.1 kcal/mol. Although the residues involved in the enolization of C-3 toward C-2 have not yet been identified, they would be essential for the final catalytic step.

Aromatization is a unique reaction in steroid biosynthesis and may therefore be inhibited by selective compounds that do not interfere with other P450 enzymes. Since aromatization is the last step in the biosynthetic sequence of steroid production, blockade of aromatization should not affect production of other steroids. For these reasons, aromatase is a particularly suitable target for inhibition (Table 1).

2.4. *Steroidal aromatase inhibitors*

The first selective aromatase inhibitors were reported in 1973 and were a number of C-19 steroids [26]. These compounds were substrate analogs and exhibited properties typical of competitive inhibitors. They included l,4,6-androstatriene-3,17-dione [47] 4-hydroxyandrostenedione (4-OHA) [48] and 4-acetoxyandrostenedione [49]. Interestingly, some of these inhibitors were later found to cause inactivation of the enzyme [28] and appear to be functioning as mechanism-based inhibitors. While not intrinsically reactive, inhibitors of this type are thought to compete rapidly with the natural substrate and subsequently interact with the active site of the enzyme (Fig. 2). They bind either very tightly or irreversibly to the enzyme, thus causing its inactivation [50]. Because they bind to the active site, these inhibitors should be quite specific and should also have lasting effects *in vivo* as a result of

Table 1 – Randomized Phase III trials of aromastase inhibitors vs. tamoxifen as first-line therapy in metastatic breast Cancer

Efficacy results, A/I tamoxifen	ORR (%)	Clinical benefit (%)	TTP (months)
Anastrozole, N = 1021 (pooling)	29/27	57/52	8.5/7.0
Letrozole, N = 907 (1 trial)	30/20[a]	49/38[a]	9.4/6.0[a]
Exemestane, N = 382 (randomized Phase II/III trial)	44/29[a]	72/66	10.9/6.7[a]

Data from: Bonneterre et al. [86], Mouridsen et al. [87], Paridaens et al. [89] and Pritchard [90].

[a] Statistically significant; ORR = overall response; TTP = time to progression.

inactivating the enzyme. Thus, the continued presence of the drug to maintain inhibition is not necessary and the chance of toxic side effects, therefore, will be low.

Dr. Chen and co-workers [51] have expressed a structurally stable and functionally active human aromatase in *E. coli*. Using this purified preparation, molecular features of the interaction of androstenedione (substrate) and exemestane (steroidal inhibitor) with aromatase have been studied by UV/vis spectral analysis. In addition, proteomic studies combined with MOLDJ-TOF MS revealed a 3-D overall folding of human aromatase, similar to that of the recently published 3-D theoretical computer model [52]. Proteomic results suggest that aromatase forms a symmetric dimer in solution through the interaction of the F helix and the F-G loop. The B and C helices and the B–C loop of aromatase appear to undergo major conformational changes when the enzyme binds to substrate or steroidal inhibitors. Reaction intermediate analysis suggested that residues E134, D309, T310, 5478, and 1-1480 are involved in enzyme catalysis. From inhibitory profile analysis and time-dependent inhibitory studies, residues E302, D309, and 5478 are thought to participate in the mechanism of the suicide inhibition of aromatase with exemestane.

A number of steroidal aromatase inhibitors in addition to 4-OHA and exemestane have been shown to cause inactivation. Brueggemeier et al [53,54] studied a number of 7α-substituted androstenedione derivatives, several of which cause inactivation of aromatase. The 1-methylandrosta-l,4-diene-3,17-dione (SH 489) [55] was shown to cause inactivation *in vitro*. Metcalf et al. [56] reported l0-(2-propynyl)estr-4-ene-3, 17-dione (MDL 18962) as the most potent aromatase inhibitor in their series. Two compounds with demonstrated biological activity are 6-methylen-androsta-1,4-diene-3,I7-dione (Exemestane, FCE 24304) and 4-aminoandrosta-l,4,6-triene-3,l7-dione (FCE 24928), [57] also cause inactivation of aromatase.

In rats, a single oral dose of 25 mg exemestane was found to cause a long-lasting reduction in plasma and urinary estrogen levels. Maximal suppression of circulating estrogens occurred 2–3 days after dosing and persisted for 4–5 days [58]. The lengthy duration of estrogen suppression is thought to be related to the irreversible nature of the drug-enzyme interaction rather than pharmacokinetic properties of the compound. Exemestane causes a marked decrease in serum and urine estrogen levels, and has no effect on other endocrine factors [59–61].

3. Non-steroidal aromatase inhibitors

Non-steroidal aromatase inhibitors contain a heteroatom (e.g., N, S, O) possessing a free electron pair for coordination with the heme iron (Fe^{3+}) and a substituent for interaction with other regions of the enzyme (Fig. 5). This type of binding is reflected in Soret band changes (usually bathochromic with respect to Type I inhibitors). Compounds that carry a nitrogen heteroatom have been the most studied and their binding with cytochrome P-450 enzymes give rise to a Type II difference spectrum with Soret maximum at 421–430 nM and minimum at 390–410 nM [62,63]. Intrinsically, nonsteroidal inhibitors are likely to be less enzyme specific than steroidal substrate analogs and can inhibit other cytochrome P450-mediated

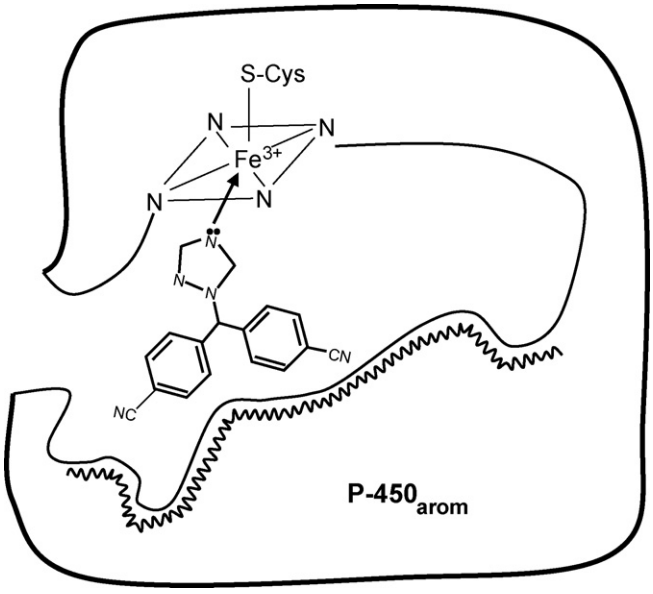

Fig. 5 – Interaction of non-steroidal inhibitors with aromatase.

hydroxylations as was the case for aminoglutethimide [30,64]. The newer non-steroidal inhibitors, anastrozole and letrozole, are triazole derivatives are potent, reversible inhibitors with high specificity. As they do not interact significantly with other P450 enzymes, they have few side effects in patients and have low toxicity.

3.1. *Selective modulation of aromatase*

The value of aromatase inhibitors as chemopreventives for postmenopausal women would be enhanced by tissue targeting. Remarkably, the regulation of aromatase appears to be different in different tissues. Several tissue-specific promoter regions have been identified upstream from the CYP19 gene [65–67]. Promoter PI.1 is the major promoter used in placental tissues and is the farthest upstream. Promoter II is utilized in the ovary. PII contains a cAMP response element and is predominant in breast cancer tissue as a result of a switch in promoters. Thus, aromatase can be stimulated by prostaglandin PGE_2 by increasing cAMP levels. Promoters PI.3, PI.4, PI.6, and PI.7 are the promoters used in other extraglandular sites. Promoter PI.3 is also present in adipose tissues such as normal breast tissue, and is increased in breast cancer tissue. Promoter PI.4 is the main promoter used in normal adipose tissue and responds to glucocorticoids and cytokines (e.g., IL-1β, IL-6 and TNFα). Because of this tissue specific regulation, there is interest in the possibility of identifying aromatase inhibitors that are selective for breast cancer by acting via promoter regulation [68]. Safi et al., [68] reports that orphan nuclear receptor liver receptor homolog-1 (LRH-1) is a specific transcriptional activator of aromatase in human breast preadipocytes and proposes LRH-1 as a target for selective inhibition of aromatase in the breast. Recently, Bruggemeier and colleagues found that a novel series of sulfonilide analogs could suppress aromatase activity and transcription indepen-

dent of Cox-2 [69]. This separation of activities may provide a basis for developing tissue specific aromatase inhibitors.

3.2. *The estrogen receptor signal transduction pathway*

A model of the current thinking about estrogen action is illustrated in Fig. 6. There are two ligand activated ER signal transduction molecules (ER alpha and ER beta) located at sites through the body. The assumption that is made is that specific receptors can modify the activation or suppression of genes in a particular target site thereby creating cellular homeostasis. The developmental and growth of breast cancer appears to be mediated through a balance of ER alpha and ER beta molecules. There is evidence to suggest that in an environment with an excess of ER beta this will create a growth inhibiting state and modifying the action of tamoxifen by enhancing antiestrogenic properties of the complex. In contrast, when ER alpha dominates the equation, as in breast cancer, estrogen can enhance breast cancer cell survival and tumor growth. Simply stated, the development of drugs to block estrogen regulated tumor growth (tamoxifen, raloxifene [SERMS]) or aromatase inhibitors (4-hydroxyandrostenedione) was predicted to prevent tumor growth, but the molecular biology of the transduction system has now provided a wealth of new information about drug resistance (see later section) and receptor modulation that is currently being applied to develop new medicines. It is clear from the investigation of the "nonsteroidal antiestrogens" tamoxifen and raloxifene that the receptor is modulated to enhance or suppress gene activation in different target tissues [70]. One proposed mechanism [71] is the balance of corepressor or coactivator proteins that can bind to the SERM ER protein complex to switch off (corepressor) or switch on (coactivator) the transcriptional potential of the complex. Alternatively in an estrogen-deprived environment, the unliganded ER would initially remain inactivated bound to a corepressor molecule.

The natural turnover of ER is accomplished by ubiquitinilation and destruction through the proteosome system. Fulvestrant, a pure antiestrogen is a derivative of the natural hormone estradiol with a long hydrophobic side chain substituted at the 7α position on the B ring of the steroid nucleus [72]. The steroid binds to the ligand binding domain of the ER in an inverted configuration with the hydrophobic side chain disrupting the ER shape by binding with the grove that can be occupied by Helix 12 of the ER [73] As a result of the unusual shape of the ER complex, it is rapidly ubiquitinilated and destroyed by the proteosome [74]. Pure antiestrogens cause a down regulation of ER so no genes can be activated. In contrast, a promiscuous SERM-like tamoxifen binds to ER

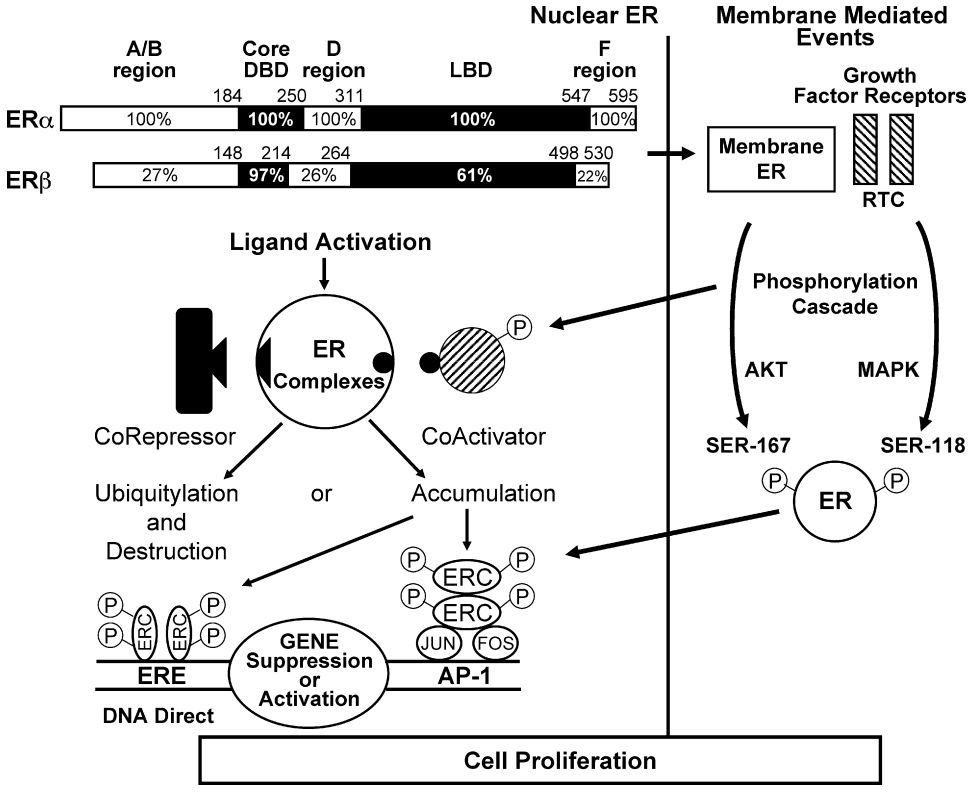

Fig. 6 – A diagramatics representation of the estrogen signal transduction pathway. The ligand estrogen receptor (either alpha or beta) (ER) complex is modulated in its actions by coactivators (CoA) or the unoccupied receptor is neutralized by corepressors (CoR). Overall the pathway can be further modified by phosphorylation of the ER or modulator molecules via growth factor receptor signaling pathways at the cell membrane (and membrane bound ER). These phosphorylation pathways are enhanced in antihormone resistance to allow the ER to promote unregulated gene activation through genomic and tethered mechanisms. This aids survival by preventing cancer cell apoptosis. Previously published in [14] and reprinted and adapted with permission.

and the complexes can accumulate thereby activating multiple genes via genomic and tethered mechanisms with the promoter region of target genes (Fig. 6).

The simple signal transduction pathway of estrogen action is also modified in cancer to cause either intrinsic or acquired resistance to antihormonal agents. The ER can be phosphorylated in cells with high levels of growth factor (HER2, EGFR, IGFR) signaling and redeploying from the nucleus to interact with protein kinases at the cell membrane. The ER then becomes part of the phosphorylation signal cascade that auto activates the ER and coactivators. It is essential to appreciate that a current understanding of the integration of the cross talk between ER and growth factor signal transduction pathways is important in antihormonal drug resistance and can be addressed logically in the treatment of breast cancer. This knowledge now provides a window of opportunity to apply new treatment approaches to control tumor growth. Unfortunately, the ER system eventually becomes redundant and the tumor is unresponsive to further antihormonal therapies. This survey of therapeutic target for the treatment of breast cancer can now be melded into a description of the advance in breast cancer treatment and prevention that, 30 years ago, would have seemed unlikely.

3.3. *Adjuvant therapy with tamoxifen*

Based on the successive analysis of accumulative randomized worldwide clinical trials, it is possible to summarize the main conclusions for tamoxifen therapy. At the time 20 years ago, when the Overview analysis first occurred, tamoxifen was the only universally used antihormonal agent. With no other competition, tamoxifen became the "gold standard" and established the principles of tumor targeting and identified the appropriate treatment strategy to aid survivorship [24,75–77].

- Five years of adjuvant tamoxifen enhances disease free survival. There is a 50% decrease in recurrences observed in ER positive patients 15 years after diagnosis.
- Five years of adjuvant tamoxifen enhances survival with a decrease in mortality 15 years after diagnosis.
- Adjuvant tamoxifen does not provide an increase in disease free or overall survival in ER negative breast cancer.
- Five years of adjuvant tamoxifen alone is effective in premenopausal women with ER.
- The benefits of tamoxifen in lives saved from breast cancer, far outweighs concerns about an increased incidence of endometrial cancer in postmenopausal women.
- Tamoxifen does not increase the incidence of second cancers other than endometrial cancer.
- No non-cancer related overall survival advantage is noted with tamoxifen when given as adjuvant therapy.

It is known that cytotoxic chemotherapy causes ovarian ablation by destroying the supply of follicles. However in young women the follicles are too numerous for complete destruction and menses continue after chemotherapy. Although tamoxifen is known to increase ovarian steroidogenesis in women with intact ovaries, [78,79] the antiestrogen is effective and approved for the treatment of premenopausal patients. That being the case, the question arises "should women without ovarian failure following chemotherapy receive antihormonal therapy?" The evidence suggests that this is a reasonable course of action and tamoxifen profoundly increases the control of recurrences in young premenopausal women following chemotherapy [80].

3.4. *The road to adjuvant treatment with aromatase inhibitors*

Two important observations stimulated further clinical development of aromatase inhibitors. The first was the meta-analysis discussed above showing the benefits of tamoxifen treatment [24] in ER+ breast cancer patients. The second was that it was later determined that after 5 years of adjuvant tamoxifen, no further benefit was observed [81]. This opened the door for the introduction of other treatment approaches. Interest was already turning towards aromatase inhibitors to fill the void.

The first clinical studies with formestane (4-OHA) were in patients who had relapsed from all available treatment. This included tamoxifen and second-line treatment with megace (medroxyprogesterone acetate) and aminoglutethimide. Although patients were often heavily pre-treated, response to 4-OHA was equal to or better than that of other agents. In addition, the compound was well tolerated and had fewer side effects than either Megace or aminoglutethimide. Eventually, 4-OHA was tested against tamoxifen as first-line treatment and was found to have equivalent efficacy [82]. The compound, 4-OHA was approved in most countries for treating patients with advanced breast cancer. Although 4-OHA was more effective in inhibiting peripheral aromatase and better tolerated than aminoglutethimide, subsequent aromatase inhibitors were able to block estrogen synthesis almost completely. Nevertheless, 4-OHA led the way for more potent aromatase inhibitors.

By the early 1990s, clinical trials had begun with aromatase inhibitors produced by several pharmaceutical companies. Novartis had developed oral non-steroidal aromatase inhibitors, fadrozole and letrozole, in addition to having 4-OHA. Letrozole proved especially potent. Oral doses as low as 0.1 and 0.25 mg in patients with metastatic breast cancer caused marked suppression of plasma estradiol, estrone and estrone sulfate levels and were observed within 24 h of the first administration [83]. These results suggest that this compound is a very powerful and selective aromatase inhibitor *in vivo*.

All three FDA approved compounds inhibited peripheral aromatization between 97 and 99%. Two randomized, double-blind studies demonstrated that anastrozole (1 mg daily) was slightly more effective than tamoxifen (20 mg daily) as first-line therapy in postmenopausal women with advanced breast cancer. Among those with ER+ tumors [84–86], the benefit was significant in terms of partial and complete responses including stable disease as well as time to progression. In a multicenter, randomized, double-blind study in advanced breast cancer, letrozole proved to be significantly better than tamoxifen in response rate, clinical benefit, time to progression, and time to treatment failure [87]. Exemestane was also significantly more effective than tamoxifen as first-line therapy in postmenopausal women with advanced breast cancer [88].

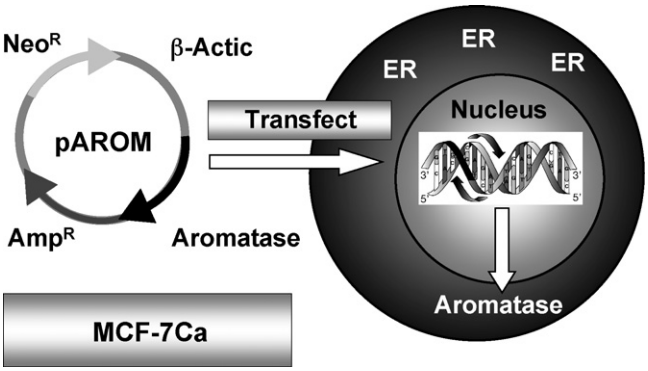

Fig. 7 – A human breast cancer cell line stably transfected with aromatase is sensitive to aromatase inhibitors and antiestrogens.

Once it was evident that aromatase inhibitors were more effective than tamoxifen, the focus of clinical trials soon moved to the use of the agents in the adjuvant setting for the treatment of early breast cancer [91,92]. The trials studied the effectiveness of aromatase inhibitors following tamoxifen, of aromatase inhibitors alone, and/or of the combination of aromatase inhibitors and tamoxifen in adjuvant therapy. These trials had their foundations in preclinical studies. We had developed a xenograft model that simulated the postmenopausal breast cancer patient. In this model, tumors are grown in ovariectomized, immunodeficient mice from MCF-7 human breast cancer cells stably transfected with the aromatase gene (MCF-7Ca) (Fig. 7). The possibility that blockade of estrogen action and estrogen synthesis may be synergistic was explored by treating mice with the aromatase inhibitor letrozole and the antiestrogen tamoxifen alone and in combination. However, the results in the model indicated that letrozole alone was better than tamoxifen or combined treatments [93]. This result was analogous to results later reported for the ATAC trial [94]. In addition, when tamoxifen treatment was no longer effective, tumor growth was significantly reduced in mice switched to letrozole treatment. Similar conclusions were reached in the MA-17 trial with letrozole [95] and the IES trial with exemestane following tamoxifen in early breast cancer [96]. Based on data from these and other multiple, large randomized trials, it was recommended by the American Society of Clinical Oncology (ASCO) technology assessment panel [97] that optimal adjuvant hormonal therapy for a postmenopausal woman with receptor-positive breast cancer include an aromatase inhibitor as initial therapy or after treatment with tamoxifen.

The aromatase inhibitors are all well tolerated. Patients experienced less gynecologic symptoms such as endometrial cancer, vaginal bleeding, and vaginal discharges. There were fewer cerebrovascular and venous thromboembolic events in patients receiving aromatase inhibitors than in those on tamoxifen. However, a low incidence of bone toxicity and musculoskeletal effects are associated with aromatase inhibitors. The latter includes small but significant increases in arthritis, arthralgia, and/or myalgia with aromatase inhibitors compared to tamoxifen. Fractures were increased with all three aromatase inhibitors compared to tamoxifen or placebo. The ATAC trial reported a fracture incidence of 7.1% in the anastrozole arm and 4.4% in the tamoxifen arm [94,98]. The fracture rate of letrozole-treated patients in the MA-17 trial was 3.6% versus 2.9% in placebo, following 5 years of tamoxifen treatment [95]. Similar small increases in osteoporosis and/or fractures (7.41%) were associated with the steroidal aromatase inhibitor, exemestane compared to tamoxifen (5.7%) in the IES trial [96].

It is suggested that patients are evaluated for baseline bone mineral density and receive bisphosphonate therapy if indicated [99]. No significant changes in serum cholesterol, HDL cholesterol, LDL Cholesterol, triglycerides or Lp(a) occur in non-hyperlipidemic postmenopausal women treated for 3 years following 5 years of adjuvant tamoxifen [100].

Other studies are on-going to compare the three aromatase inhibitors and/or combination therapies in early stage breast cancer or in the chemoprevention setting. A randomized, Phase III, double-blind trial (BIG 1-98) of the Breast International Group is comparing several adjuvant endocrine therapies in postmenopausal women with ER+ breast cancer. Letrozole versus tamoxifen treatment was compared in the first analysis of the monotherapy arms of the BIG1-98 study. After a median follow-up of 25.8 months, adjuvant treatment with letrozole was found to reduce the risk of recurrences significantly compared with tamoxifen [101]. The MA-27 study is a Phase III adjuvant trial in postmenopausal women with primary breast cancer comparing exemestane to anastrozole, with or without celecoxib, a COX-2 inhibitor. Overall, the aromatase inhibitors are proving to be superior agents to tamoxifen in the treatment of postmenopausal women with all stages of breast cancer. However, the pharmacology of tamoxifen and other non-steroidal antiestrogens was to provide new therapeutic opportunities for improving women's health.

3.5. *Selective estrogen receptor modulation and chemoprevention*

Twenty years ago, tamoxifen was classified as a nonsteroidal antiestrogen [102]. Of interest, however, was the observation that tamoxifen was metabolically activated to 4-hydroxytamoxifen, a nonsteroidal antiestrogen with a high binding affinity for the ER [103,104] (Fig. 1). This observation would provide a scientific foundation for future structure activity studies and subsequent drug development in this area. Now a whole range of new compounds with high affinity for the ER are available as therapeutic agents (Fig. 1).

In pharmacological terms tamoxifen was described as a partial agonist (estrogen-like) in target tissues such as the immature rat uterus but it was antiestrogenic because it blocked the full action of estradiol alone. In 1986, it was plausible that if estrogen was necessary to fend off osteoporosis and coronary heart disease the long-term administration of an antiestrogen to node negative women could eventually have a deleterious effect on bone density and produce a potential increase in the incidence of coronary heart disease for the majority of women. The potential side effects would be even worse for women only at high risk to develop breast cancer. Only a small minority of women would have a reduced risk of

breast cancer, but all women would be exposed to potential "antiestrogenic" toxicities. However, the classification of nonsteroidal antiestrogens was to change just after 1986. Today the concept is known as selective ER modulation (SERM).

In 1986, virtually nothing was known about the actions of nonsteroidal antiestrogens on bone density. A single report from NASA scientists showed that clomiphene, an impure isomeric mixture of a nonsteroidal estrogen and antiestrogen used for the induction of ovulation, would preserve bone density in ovariectomized rats [105]. Clearly there were efforts to prevent osteoporosis during space flight but the choice of experimental compound was flawed and frankly, a little bizarre. Since nonsteroidal antiestrogen such as tamoxifen reduce libido in men maybe that was the rationale!

However, the interpretation of the NASA results was not that simple. If clomiphene is an impure mixture of estrogenic and antiestrogenic isomers, which isomer is affecting bone? The consistent laboratory finding that tamoxifen the pure *trans* antiestrogenic isomer of a triphenylethylene maintained bone density in ovariectomized rats [106–108] seemed to translate to postmenopausal women [109], but would prospective clinical studies really show benefit? The Wisconsin Tamoxifen Study was started in 1986 to explore the potential toxicity of tamoxifen on bone density. The study demonstrated, in a double blind placebo controlled clinical trial, that tamoxifen could preserve bone in the postmenopausal woman [110]. Bone building would clearly be an advantage for chemoprevention studies, thereby enhancing the possibility that the worth of tamoxifen to prevent breast cancer could be tested safely. In the same studies, tamoxifen lowered low density lipoprotein [111,112] and, by inference, would appear not to increase the risk of coronary heart disease. These results were good. The bad was the laboratory discovery that although tamoxifen prevented the estrogen-stimulated growth of human breast cancers, the drug stimulated the growth of human endometrial cancers grown in the same athymic mouse [113]. This again was selective ER modulation. Stimulate one target site to produce growth and block the growth of another target site.

There was a very quick response from the clinical community to the warnings [113] that long-term tamoxifen treatment could be associated with an increase in the incidence of endometrial cancer [114–116] but not all reported studies [117,118] found increases in endometrial cancer associated with tamoxifen treatment. These studies were either too small or data was just not collected. There was also a question of whether the high dose of tamoxifen (40 mg. daily) used by Fornander and coworkers [116] was responsible for their findings but the report by Fisher [119] neutralized the argument because NSABP studies all use 20 mg. tamoxifen daily. Endometrial cancer again became an issue during recruitment to the pioneering tamoxifen chemoprevention study by Fisher and the NSABP when it was suggested that extremely dangerous endometrial cancer could be caused by tamoxifen treatment [120]. Nevertheless, results from the prospective chemoprevention study with tamoxifen actually showed that only postmenopausal women developed an excess of early stage mainly grade one endometrial cancers. There were no fatalities from endometrial cancer associated with tamoxifen in the study. [121,122].

The recognition that the so called "nonsteroidal antiestrogens" had estrogenic and antiestrogenic actions at different sites in the ovariectomized female rat and that these data translated to women to prevent bone loss and breast cancer created a new dimension in drug development. The fact that tamoxifen and the failed breast cancer drug keoxifene (LY156,758) [123] both prevented the development of carcinogen-induced rat mammary carcinomas [124] and maintained bone density in ovariectomized rates [106] indicated that this was a class effect. The significance of these observations for public health and chemoprevention of breast cancer was immediately recognized. At the first International Chemoprevention Meeting hosted by Dr. Ezra Greenspan, a group of scientists and clinicians were invited to New York in 1987 to share their vision of the possibilities and potential of chemoprevention [125]. The future of drug development was clear.

"The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused upon the identification of a population of high-risk women to test "chemopreventive" agents. But are resources being used less than optimally? An alternative would be to seize upon the developing clues provided by an extensive clinical investigation of available antiestrogens. Could analogs be developed to treat osteoporosis or even retard the development of atherosclerosis? If this proved to be true then a majority of women in general could be treated for these conditions as soon as menopause occurred. Should the agent also retain anti-breast tumor actions then it might be expected to act as a chemosuppressive. A bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century" [125]. This blueprint to improve healthcare was subsequently restated at the annual meeting of the American Association of Cancer Research in San Francisco, 1989 [126].

Compounds of the keoxifene class (LY117018 and LY156758) were obvious candidates for study despite the fact that the program to develop the drugs to treat breast cancer had been abandoned by Eli Lilly in 1988. The compounds were known to be less uterotrophic than tamoxifen in rodents [127] but they were short acting [128], which could explain their poor antitumor properties when compared with tamoxifen. Interestingly enough, keoxifene was already known to partially inhibit the growth of tamoxifen-stimulated human endometrial tumors under laboratory conditions [129].

Keoxifene, an estrogen that had failed to be developed as a drug to treat breast cancer [123] was reinvented in the early 1990s as raloxifene, a SERM. A use patent for the treatment and prevention of osteoporosis was filed by Eli Lilly in 1992. Raloxifene has now been available for the treatment and prevention of osteoporosis in postmenopausal women since 1999 based on the prospective clinical trials demonstrating an approximately 40% decrease in spinal fractures [130] with the advantage over hormone replacement therapy of causing a 70% decrease in the incidence of breast cancer [131,132]. The anticipated result in reducing the risk of breast cancer as a beneficial side effect of treating osteoporosis propelled raloxifene into clinical trial versus tamoxifen for the prevention of breast cancer as the primary endpoint. The results

from the study of tamoxifen and raloxifene (STAR)[1] in high risk postmenopausal women show that tamoxifen and raloxifene are equivalent in reducing the incidence of breast cancer but there is a decrease in the incidence of endometrial cancer, pulmonary emboli, deep vein thrombosis and endometrial hyperplasia noted with raloxifene. Raloxifene and tamoxifen, as would be expected for two SERMs, both have equivalent activity in preventing fractures [133].

Raloxifene also causes decreases in circulating low density lipoprotein cholesterol [134] and for this reason was evaluated as a preventive for coronary heart disease in the study named raloxifene use for the heart (RUTH) (see footnote 1). Although raloxifene prevents an increase in breast cancer incidence in the RUTH trial, there is no benefit in protecting against coronary heart disease and myocardial infarction [135]. Clearly, further studies with different agents will be needed to rethink the SERM strategy for a multifunctional drug that can prevent cancer (breast/uterus), osteoporosis and coronary heart disease.

Nevertheless, results with raloxifene are part fulfillment of the predicted promise [125,126] of the SERMs as medicines to prevent cancer and osteoporosis. As a result, there are now numerous new SERMs (e.g. lasofoxifene, basedoxifene, arzoxifene, etc.) being evaluated [136,137] (Fig. 1). Additionally, the concept is being applied throughout the steroid receptor super family so the impact on medicine, in the years to come with selective androgens, glucocorticoids or progestins will be considerable.

4. Aromatase inhibitors as chemopreventive agents

Aromatase inhibitors have potential for chemoprevention in women with increased risk of developing breast cancer for many of the same reasons as tamoxifen. Thus, reducing the number of proliferative events by inhibiting the stimulatory effects of estrogen will reduce the number of mutations that would otherwise occur. Evidence to support the value of aromatase inhibitors in the prevention setting comes from the adjuvant clinical trials that compare and contrast tamoxifen with an aromatase inhibitor. All studies show that an aromatase inhibitor is more effective than tamoxifen at preventing the development of contralateral breast cancer [95,101,138]. Based on the recent findings from the STAR trial [133], there is active interest in comparing an aromatase inhibitor with raloxifene. The National Surgical Adjuvant Breast and Bowel Project will compare raloxifene with letrozole in their next clinical trial in postmenopausal women at high risk for breast cancer. The success of such chemoprevention trials will depend on not only reduction in tumor incidence but also the long-term tolerability of the agents. The International Breast Cancer Intervention Group is currently comparing anastrozole versus placebo in a prevention study, and the accompanying DCIS study is comparing tamoxifen versus anastrozole in women with locally excised ductal carcinoma *in situ* (DCIS) [139]. A three-arm prevention study organized by the National Cancer Institute of Canada, will compare placebo versus exemestane versus exemestane and celecoxib [139]. Although increased risk of stroke and endometrial hyperplasia have not been associated with aromatase inhibitors, other potential side effects such as increased osteoporosis will also be important criteria in considering aromatase inhibitors for preventing breast cancer. Bisphosphonates are currently used by many postmenopausal women to prevent osteoporosis and could be used with aromatase inhibitors if bone loss is a concern.

The possibility that aromatase inhibitors can prevent breast cancer by an additional mechanism has been proposed by several investigators [140]. This hypothesis suggests that estrogens are metabolized to catechol estrogens, 2-hydroxy-estradiol, and 4-hydroxy-estradiol. Evidence exists that 4-hydroxy-estradiol but not 2-hydroxy-estradiol is potentially carcinogenic by forming depurinating estrogen-DNA adducts. Therefore, preventing the formation of estrogens and of their subsequent metabolism to catechol estrogen 3,4-quinones, may provide better protection from breast cancer than targeting the estrogen receptor. Recent studies by Russo et al., [141] demonstrated that exposure of the MCF-10F ER negative cell line to four 24 h alternate periods of 70 nM estradiol induced anchorage-independent growth, loss of ductulogenesis in collagen, invasiveness in Matrigel, and loss of 9p11-13. Only invasive cells that exhibited a 4p15.3-16 deletion were tumorigenic in nude mice. Tumors were poorly differentiated ER-α and PR-negative adenocarcinomas and expressed keratins, EMA, and E-cadherin. The complete transformation of the ER negative MCF-10F cells *in vitro* that resulted in tumor formation *in vivo* supports the concept that estrogen may act as an initiator of breast cancer in women.

4.1. *Drug resistance to antihormones*

Overall, the long-term, or perhaps indefinite use of antihormonal therapy will result in the development of antihormone resistance. To investigate the mechanisms of resistance to aromatase inhibitors, one approach is to use the xenograft model with tumors of human breast cancer MCF-7Ca cells stably transfected with aromatase [142,143] (Fig. 7). Mice were treated with letrozole until tumors eventually began to grow. The expression of signaling proteins was determined in tumors during the course of letrozole treatment compared to tumors of control mice. Tumors initially upregulated the ER while responding to treatment, but subsequently receptor levels decreased in tumors unresponsive to letrozole. Nevertheless, p-ER (Ser167) was increased suggesting that ligand independent activation of ER may enhance proliferation in tumors treated with letrozole. Tyrosine kinase receptor protein HER-2 and adapter proteins (p-Shc and Grb-2) as well as the signaling proteins in the MAPK cascade (p-Raf, p-Mekl/2, and p-MAPK), but not in the Pl3/Akt pathway, were all increased in tumors no longer responsive to letrozole, suggesting the possibility that ER may be phosphorylated by MAPKinase in the absence of ligand. To investigate whether sensitivity to

[1] The STAR trial compared and contrasted the efficacy of tamoxifen and raloxifene to reduce the incidence of breast cancer in postmenopausal women at high risk for breast cancer. Side effect profiles were a secondary endpoint. The RUTH trial is a placebo-controlled study to determine whether raloxifene would reduce the risk of coronary heart disease in high risk women.

letrozole could be regained, cells were isolated from the letrozole resistant tumors (LTLT) and treated with inhibitors of the MAPKinase pathway (P098059 and U0126). These compounds reduced MAPK activity and increased ER expression. EGFR/HER-2 inhibitors, gefitinib and AEE78S although not effective in the parental MCF-7Ca cells, also restored the sensitivity of LTLT cells to letrozole. Since there appears to be cross-talk between the ER and the tyrosine kinase receptor, the hypothesis was tested that degrading the ER with fulvestrant may prevent development of resistance. In xenografts, beginning treatment with letrozole *plus* fulvestrant to down regulate the ER prevented increases in HER-2, activation of MAPK, and was highly effective in inhibiting tumor growth throughout 29 weeks of treatment. These results suggest that disrupting the ER or blocking growth factor-mediated transcription may delay development of resistance to aromatase inhibitors and maintain growth inhibition of ER+ breast cancer by hormonal agents.

Numerous studies have been published on the estrogen deprivation of ER positive breast cancer cells *in vitro*. Two types of cellular response can occur based on the organization of the ER system [144]. In MCF-7 cells, ER is upregulated during conditions of estrogen deprivation whereas T47D cells undergo ER down regulation. As a result MCF-7 cells eventually grow spontaneously in the absence of estrogen [144–146], remain unresponsive to estrogen, but retain ER. In contrast T47D cells loose ER and become hormone independent [147].

There have been extensive laboratory investigations of the development of drug resistance to SERM (tamoxifen and raloxifene) treatment [148]. These studies have established some general principles that not only have applications in the treatment of breast cancer but also provide some unanticipated insights into the potential failure of second or third line treatment with the pure antiestrogen fulvestrant. Additionally, long-term antihormone action has also exposed a vulnerability of the breast cancer cells. Although estrogen is considered to be a survival signal for breast cancer cells, physiologic estrogen can cause rapid apoptosis of estrogen deprived breast cancer cells [149,150], tamoxifen resistant breast cancer cells [151] and raloxifene resistant breast cancer cells [152]. Most interestingly, combinations of physiologic estrogen and fulvestrant can cause a reversal of the apoptotic action of estrogen and robust tumor growth. [153]. Clearly, these observations can have potential implications for the treatment of advanced breast cancer once multiple antihormonal therapies have failed. There are current proposals to use physiological estrogen to reduce tumor burden in sensitive tumors that have responded and failed two consecutive antihormonal therapies. The clinical basis for estrogen responsiveness has already been demonstrated in this patient group [154] but the use of a 12 week course of low dose estrogen followed by the reintroduction of aromatase inhibition plus fulvestrant would not only incorporate the current laboratory results for the design of a clinical trial but also improve patient disease control prior to chemotherapy [155].

4.2. *Perspective*

We have described our personal experiences over the past 35 years with the investigation and development of antihormones for the treatment and prevention of breast cancer. We have been fortunate to have both been at the right places at the right times to be able to contribute to the advance in therapeutics. However, it should be clear from our narrative that we have also been unfashionable and proposed solutions to treatment at the wrong times. Nevertheless fashions in cancer research often take a decade to change. In our case, the philosophy that cytotoxic combination chemotherapy would cure breast cancer was tested by the clinical community and failed. Unfortunately, the clinical trials community can only test what is provided by the pharmaceutical industry and the idea of killing cancer cells seemed "a good idea at the time". Fortunately, when alternatives to chemotherapy were sought and changes in fashion were occurring, new agents and treatment strategies were in place to fill a potential treatment void. We independently target the same signal transduction pathway but at different points to provide an alternative approach to cytotoxic chemotherapy. Today, the concept of drug targeting has come of age as every new agent being developed by the pharmaceutical industry is designed to target the tumor specifically thereby potentially reducing toxicity for the patient. It remains for a future generation to integrate the new menu of targeted medicines into the treatment plan and to ensure that the appropriate affordable drug is made available to the appropriate woman.

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REFERENCES

[1] Hoagland H. The road to yesterday. Worcester MA; 1974.
[2] Jensen EV, Jacobson HI. Basic guides to the mechanism of estrogen action. Recent Prog Hormone Res 1962;18:387–414.
[3] Kennedy BJ. Hormone therapy for advanced breast cancer. Cancer 1965;18:1551–7.
[4] Jensen EV, Block GE, Smith S, Kyser K, DeSombre ER. Estrogen receptors and breast cancer response to adrenalectomy. Natl Cancer Inst Monogr 1971;34:55–70.
[5] Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. Br J Cancer 1971;25:270–5.
[6] Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 1967;13:101–19.

[7] Harper MJ, Walpole AL. Mode of action of I.C.I. 46,474 in preventing implantation in rats. J Endocrinol 1967;37:83–92.
[8] Jordan VC. The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. Breast Cancer Res Treat 1988;11:197–209.
[9] Huggins C, Grand LC, Brillantes FP. Mammary cancer induced by a single feeding of polynuclear hydrocarbons and their suppression. Nature 1961;189:204.
[10] Jordan VC, Koerner S, Tamoxifen. (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 1975;11:205–6.
[11] Jordan VC. The antioestrogen tamoxifen (ICI 46,474) as an antitumour agent. In: Proceedings Eastern Cooperative Oncology Group Meeting. 1974.
[12] Jordan VC. Antiestrogenic and antitumor properties of tamoxifen in laboratory animals. Cancer Treat Rep 1976;60:1409–19.
[13] Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discovery 2003;2:205–13.
[14] Jordan VC, Tamoxifen. (ICI 46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 2006;147:S269–76.
[15] Jordan VC, Dowse LJ. Tamoxifen as an anti-tumour agent: effect on oestrogen binding. J Endocrinol 1976;68: 297–303.
[16] Jordan VC, Jaspan T. Tamoxifen as an antitumour agent: oestrogen binding as a predictive test for tumour response. J Endocrinol 1976;68:453–60.
[17] Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. Eur J Cancer 1976;12:419–24.
[18] Jordan VC. Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. Rev Endocr Relat Cancer 1978:49–55. October Supplement.
[19] Jordan VC, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. Adj Ther Cancer 1979;2:19–26.
[20] Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 1980;16:239–51.
[21] Tormey DC, Jordan VC. Long-term tamoxifen adjuvant therapy in node-positive breast cancer: a metabolic and pilot clinical study. Breast Cancer Res Treat 1984;4:297–302.
[22] Tormey DC, Rasmussen P, Jordan VC. Long-term adjuvant tamoxifen study: clinical update. Breast Cancer Res Treat 1987;9:157–8.
[23] Anonymous. Review of mortality results in randomized trials in early breast cancer. Lancet 1984;ii:1205.
[24] EBCTCG. Effects of adjuvant tamoxifen and of cytotoxic therapy on mortality in early breast cancer. An overview of 61 randomized trials among 28,896 women. N Engl J Med 1988; 319:1681–92.
[25] Consensus Conference. Adjuvant chemotherapy for breast cancer. J Am Med Assoc 1985;254:3461–3.
[26] Schwarzel WC, Kruggel WG, Brodie HJ. Studies on the mechanism of estrogen biosynthesis. 8. The development of inhibitors of the enzyme system in human placenta. Endocrinology 1973;92:866–80.
[27] Brodie AMH, Schwarzel WC, Shaikh AA, Brodie HJ. The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen dependent processes in reproduction and breast cancer. Endocrinology 1977;100:1684–95.
[28] Brodie AM, Garrett WM, Hendrickson JR, Tsai-Morris CH, Marcotte PA, Robinson CH. Inactivation of aromatase in vitro by 4-hydroxy-4-androstene-3,17-dione and 4-acetoxy-4-androstene-3, 17-dione and sustained effects in vivo. Steroids 1981;38:693–702.
[29] Brodie AM, Longcope C. Inhibition of peripheral aromatization by aromatase inhibitors, 4-hydroxy- and 4-acetoxy-androstene-3,17-dione. Endocrinology 1980;106:19–21.
[30] Samojlik E, Santen RJ, Wells SA. Adrenal suppression with aminoglutethimide. II. Differential effects of aminoglutethimide on plasma androstenedione and estrogen levels. J Clin Endocrinol Metab 1977;45:480–7.
[31] Lipton A, Santen RJ. Medical adrenalectomy using aminoglutethimide and dexamethasone in advanced breast cancer. Cancer 1974;33:503–12.
[32] Santen RJ, Worgul TJ, Samojlik E, Interrante A, Boucher AE, Lipton A, et al. A randomized trial comparing surgical adrenalectomy with aminoglutethimide plus hydrocortisone in women with advanced breast cancer. N Engl J Med 1981;305:545–51.
[33] Santen RJ, Wells SA. The use of aminoglutethimide in the treatment of patients with metastatic carcinoma of the breast. Cancer 1980;46:1066–74.
[34] Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. Clin Cancer Res 2003;9:1980–9.
[35] Inkster SE, Brodie AM. Expression of aromatase cytochrome P-450 in premenopausal and postmenopausal human ovaries: an immunocytochemical study. J Clin Endocrinol Metab 1991;73:717–26.
[36] MacClusky NJ, Naftolin F. Sexual differentiation of the central nervous system. Science 1981;211:1294–303.
[37] Balthazart, J, Steroid metabolism and the activation of social behavior. In: Balthazart J, editor. Advances in comparative and environmental physiology, vol. 3. Germany, Berlin: Springer; 1989. p. 105–9.
[38] Longcope C, Pratt JH, Schneider SH, Fineberg SE. Aromatization of androgens by muscle and adipose tissue in vivo. J Clin Endocrinol Metab 1978;46:146–52.
[39] Hemsell DL, Grodin JM, Brenner PF, Siiteri PK, MacDonald PC. Plasma precursors of estrogen. II. Correlation of the extent of conversion of plasma androstenedione to estrone with age. J Clin Endocrinol Metab 1974;38:476–9.
[40] van Landeghem AA, Poortman J, Nabuurs M, Thijssen JH. Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue. Cancer Res 1985;45:2900–6.
[41] Blankenstein MA, Szymczak J, Daroszewski J, Milewicz A, Thijssen JH. Estrogens in plasma and fatty tissue from breast cancer patients and women undergoing surgery for non-oncological reasons. Gynecol Endocrinol 1992;6:13–7.
[42] Covey DF, Hood WF, Beusen DD, Carrell HL. Hydroperoxides as inactivators of aromatase: 10 beta-hydroperoxy-4-estrene-3,17-dione, crystal structure and inactivation characteristics. Biochemistry 1984;23:5398–406.
[43] Hahn EF, Fishman J. Immunological probe of estrogen biosynthesis. Evidence for the 2 beta-hydroxylative pathway in aromatization of androgens. J Biol Chem 1984;259:1689–94.
[44] Cole PA, Bean JM, Robinson CH. Conversion of a 3-desoxysteroid to 3-desoxyestrogen by human placental aromatase. Proc Natl Acad Sci USA 1990;87:2999–3003.
[45] Kao YC, Korzekwa KR, Laughton CA, Chen S. Evaluation of the mechanism of aromatase cytochrome P450. A site-directed mutagenesis study. Eur J Biochem 2001;268:243–51.
[46] Hackett JC, Brueggemeier RW, Hadad CM. The final catalytic step of cytochrome p450 aromatase: a density functional theory study. J Am Chem Soc 2005;127:5224–37.

[47] Brodie A, Garrett W, Hendrickson JM, Marsh DA, Brodie HJ. The effect of 1,4,6-androstatriene-3,17-dione (ATD) on DMBA-induced mammary tumors in the rat and its mechanism of action in vivo. Biochem Pharmacol 1982;31:2017–23.
[48] Brodie AMH, Schwarzel WC, Brodie HJ. Studies on the mechanism of estrogen biosynthesis in the rat ovary-1. J Steroid Biochem 1976;7:787–93.
[49] Brodie AMH, Marsh DA, Brodie HJ. Aromatase inhibitors IV. Regression of estrogen-dependent mammary tumors in the rat with 4-acetoxy-4-androstene-3,17-dione. J Steroid Biochem 1979;10:423–9.
[50] Sjoerdsma A. Suicide inhibitors as potential drugs. Clin Pharm Ther 1981;30:3–22.
[51] Hong Y, Yu B, Sherman M, Yuan YC, Zhou D, Chen S. Molecular basis for the aromatization reaction and exemestane-mediated irreversible inhibition of human aromatase. Mol Endocrinol 2006; in press.
[52] Favia AD, Cavalli A, Masetti M, Carotti A, Recanatini M. Three-dimensional model of the human aromatase enzyme and density functional parameterization of the iron-containing protoporphyrin IX for a molecular dynamics study of heme-cysteinato cytochromes. Proteins 2006;62:1074–87.
[53] Brueggemeier R, Li P-K, Snider CE, Darby MV, Katlic NE. 7α-Substituted androstenediones as effective in vitro and in vivo inhibitors of aromatase. Steroids 1987;50:163–78.
[54] Brueggemeier R, Li P-K, Chen HH, Moh PP, Katlic NE. Biochemical pharmacology of new 7-substituted androstenediones as inhibitors of aromatase. J Steroid Biochem Mol Biol 1990;37:379–85.
[55] Henderson D, Norbirath G, Kerb U. 1-Methyl-1,4-androstadiene-3, 17-dione (SH 489):Characterization of an irreversible inhibitor of estrogen biosynthesis. J Steroid Biochem Mol Biol 1986;24:303–6.
[56] Metcalf BW, Wright CL, Burkhart JP, Johnston JO. Substrate-induced inactivation of aromatase by allenic and acetylenic steroids. J Am Chem Soc 1981;103:3221–2.
[57] Zaccheo T, Giudici D, Ornati G, Panzeri A, DiSalle E. Comparison of the effects of the irreversible aromatase inhibitor exemestane with atamestane and MDL-18962 in rats with DMBA-induced mammary tumors. Eur J Cancer 1991;27:1145–50.
[58] Evans T, DiSalle E, Ornati G, et al. Phase I and endocrine study of exemestane (FCE 24304), a new aromatase inhibitor, in postmenopausal women. Cancer Res 1992;52:5933–9.
[59] Bajetta EZN, Noberasco C, et al. The minimal effective exemestane dose for endocrine activity in advanced breast cancer. Eur J Cancer 1997;33:587–91.
[60] Geisler JKN, Anker G, et al. In vivo inhibition of aromatization by exemestane, a novel irreversible aromatase inhibitor, in postmenopausal breast cancer patients. Clin Cancer Res 1998;4:29–093.
[61] Lonning P, Bajetta E, Murray R, et al. Activity of exemestane in metastatic breast cancer after failure of nonsteroidal aromatase inhibitors: a phase II trial. J Clin Oncol 2000;18:2234–44.
[62] Jefcoate CR. Measurement of substrate and inhibitor binding to microsomal cytochrome P-450 by optical-difference spectroscopy. Meth Enzymol 1978;52:258–79.
[63] Dawson JH, Andersson LA, Sono M. Spectroscopic investigations of ferric cytochrome P-450-CAM ligand complexes. Identification of the ligand trans to cysteinate in the native enzyme. J Biol Chem 1982;257:3606–17.
[64] Cash R, Brough AJ, Cohen MNP, Satoh PS. Aminoglutethimide (Elipten-CIBA) as an inhibitor of adrenal steroidogenesis. Mechanism of action and therapeutic trial. J Clin Endocrinol Metab 1967;27: 1239–48.
[65] Simpson E, Mahendroo MS, Means GD, et al. Tissue-specific promoters regulate aromatase cytochrome P450 expression. J Steroid Biochem Mol Biol 1993;44:321–30.
[66] Zhao Y, Agarwal VR, Mendelson CR, et al. Transcriptional regulation of CYP19 gene (aromatase) expression in adipose stromal cells in primary culture. J Steroid Biochem Mol Biol 1997;61:203–10.
[67] Simpson E, Clyne C, Rubin G, et al. Aromatase—a brief overview. Annu Rev Physiol 2002;64:93–127.
[68] Safi R, Kovacic A, Gaillard S, Murata Y, Simpson ER, McDonnell DP, et al. Coactivation of liver receptor homologue-1 by peroxisome proliferator-activated receptor gamma coactivator-1alpha on aromatase promoter II and its inhibition by activated retinoid X receptor suggest a novel target for breast-specific antiestrogen therapy. Cancer Res 2005;65:11762–70.
[69] Su B, Diaz-Cr ES, Landini S, Brueggemeier RW. Novel sulfonanilide analogues suppress aromatase expression and activity in breast cancer cells independent of COX-2 inhibition. J Med Chem 2006;49:1413–9.
[70] Jordan VC. Selective estrogen receptor modulation: a personal perspective. Cancer Res 2001;61:5683–7.
[71] Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev 2004;25:45–71.
[72] Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. Cancer Res 1991;51:3867–73.
[73] Pike AC, Brzozowski AM, Walton J, Hubbard RE, Thorsell A, Li Y, et al. Structural insights into the mode of action of a pure antiestrogen. Structure 2001;9:145–53.
[74] Wijayaratne AL, McDonnell DP. The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. J Biol Chem 2001;276:35684–92.
[75] EBCTCG. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Lancet 1992;339:1–15.
[76] EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 1998;354:1451–67.
[77] EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687–717.
[78] Ravdin PM, Fritz NF, Tormey DC, Jordan VC. Endocrine status of premenopausal node-positive breast cancer patients following adjuvant chemotherapy and long-term tamoxifen. Cancer Res 1988;48:1026–9.
[79] Jordan VC, Fritz NF, Langan-Fahey S, Thompson M, Tormey DC. Alteration of endocrine parameters in premenopausal women with breast cancer during long-term adjuvant therapy with tamoxifen as the single agent. J Natl Cancer Inst 1991;83:1488–91.
[80] Colleoni M, Gelber S, Goldhirsch A, Aebi S, Castiglione-Gertsch M, Price KN, et al. Tamoxifen after adjuvant chemotherapy for premenopausal women with lymph node-positive breast cancer: International Breast Cancer Study Group Trial 13-93. J Clin Oncol 2006;24:1332–41.
[81] Fisher B, Dignam J, Bryant J, Wolmark N. Five versus more than five years of tamoxifen for lymph node-negative breast cancer: updated findings from the National Surgical Adjuvant Breast and Bowel Project B-14 randomized trial. J Natl Cancer Inst 2001;93:684–90.

[82] Perez-Carrion R, Alberola V, Calabresi F, Michel R-T, Santos R, Fehling M, et al. Randomized trial of the selective aromatase inhibitor formestane (Lentaron) versus tamoxifen as first-line hormonal therapy in postmenopausal patients with advanced breast cancer. Breast 1993;3:198–9.
[83] Demers LM, Lipton A, Harvey HA, Kambic KB, Grossberg H, Brady C, et al. The efficacy of CGS 20267 in suppressing estrogen biosynthesis in patients with advanced stage breast cancer. J Steroid Biochem Mol Biol 1993;44:687–91.
[84] Bonneterre J, Thurlimann B, Robertson JF, Krzakowski M, Mauriac L, Koralewski P, et al. Anastrozole versus tamoxifen as first-line therapy for advanced breast cancer in 668 postmenopausal women: results of the Tamoxifen or Arimidex Randomized Group Efficacy and Tolerability study. J Clin Oncol 2000;18:3748–57.
[85] Nabholtz JM, Buzdar A, Pollak M, Harwin W, Burton G, Mangalik A, et al. Anastrozole is superior to tamoxifen as first-line therapy for advanced breast cancer in postmenopausal women: results of a North American multicenter randomized trial. Arimidex Study Group. J Clin Oncol 2000;18:3758–67.
[86] Bonneterre J, Buzdar A, Nabholtz JM, Robertson JF, Thurlimann B, von Euler M, et al. Anastrozole is superior to tamoxifen as first-line therapy in hormone receptor positive advanced breast carcinoma. Cancer 2001;92:2247–58.
[87] Mouridsen H, Gershanovich M, Sun Y, Perez-Carrion R, Boni C, Monnier A, et al. Superior efficacy of letrozole versus tamoxifen as first-line therapy for postmenopausal women with advanced breast cancer: results of a phase III study of the International Letrozole Breast Cancer Group. J Clin Oncol 2001;19:2596–606.
[88] Paridaens R, Dirix L, Lohrisch C, Beex L, Nooij M, Cameron D, et al. Mature results of a randomized phase II multicenter study of exemestane versus tamoxifen as first-line hormone therapy for postmenopausal women with metastatic breast cancer. Ann Oncol 2003;14:1391–8.
[89] Paridaens R, Therasse P, Dirix L. First-line hormonal treatment (HT) for metastatic breast cancer (MBC) with exemestane (E) or tamoxifen (T) in postmenopausal patients (pts)—a randomized phase III trial of the EORTC Group. J Clin Oncol 2004:22.
[90] Pritchard KI. Endocrine therapy of advanced disease: analysis and implications of the existing data. Clin Cancer Res 2003;9:460S–7S.
[91] Brodie A, Lu Q, Long B. Aromatase and its inhibitors. J Steroid Biochem Mol Biol 1999;69:205–10.
[92] Santen RJ, Harvey HA. Use of aromatase inhibitors in breast carcinoma. Endocr Relat Cancer 1999;6:75–92.
[93] Long BJ, Jelovac D, Handratta V, Thiantanawat A, MacPherson N, Ragaz J, et al. Therapeutic strategies using the aromatase inhibitor letrozole and tamoxifen in a breast cancer model. JNCI 2004;96:456–65.
[94] Baum M, Budzar AU, Cuzick J, Forbes J, Houghton JH, Klijn JG, et al. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomized trial. Lancet 2002;359:2131–9.
[95] Goss PE, Ingle JN, Marino S, Robert NJ, Muss HB, Piccart MJ, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. N Engl J Med 2003;349:1–10.
[96] Coombes RC, Hall E, Gibson LJ, Paridaens R, Jassem J, Delozier T, et al. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. N Engl J Med 2004;350:1081–92.
[97] Winer EP, Hudis C, Burstein HJ, Wolff AC, Pritchard KI, Ingle JN, et al. American Society of Clinical Oncology technology assessment on the use of aromatase inhibitors as adjuvant therapy for postmenopausal women with hormone receptor-positive breast cancer: status report 2004. J Clin Oncol 2005;23:619–29.
[98] Baum M, Buzdar A, Cuzick J, Forbes J, Houghton J, Howell A, et al. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early-stage breast cancer: results of the ATAC (Arimidex, Tamoxifen Alone or in Combination) trial efficacy and safety update analyses. Cancer 2003;98:1802–10.
[99] Ingle J. Aromatase inhibition and antiestrogen therapy in early breast cancer treatmen and chemoprevention. Oncology (Huntingt) 2001;15:28–34.
[100] Wasan KM, Goss PE, Pritchard PH, Shepherd L, Palmer MJ, Liu S, et al. The influence of letrozole on serum lipid concentrations in postmenopausal women with primary breast cancer who have completed 5 years of adjuvant tamoxifen (NCIC CTG MA.17L). Ann Oncol 2005;16: 707–15.
[101] Thurlimann B, Keshaviah A, Coates AS, Mouridsen H, Mauriac L, Forbes JF, et al. BIG (Breast International Group) and Group, I-C. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. N Engl J Med 2005;353:2747–57.
[102] Jordan VC. Biochemical pharmacology of antiestrogen action. Pharmacol Rev 1984;36:245–76.
[103] Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 1977;75:305–16.
[104] Allen KE, Clark ER, Jordan VC. Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 1980;71:83–91.
[105] Beall PT, Misra KL, Young RL, Spiut HJ, Evan HJ, LeBlanc A. Clomiphene protects against osteoporosis in the mature ovariectomized rat. Calcif Tissue Int 1985;36:123–5.
[106] Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987;10:31–5.
[107] Turner RT, Wakley GK, Hannon KS, Bell NH. Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. J Bone Miner Res 1987;2:449–56.
[108] Turner RT, Wakley GK, Hannon KS, Bell NH. Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. Endocrinology 1988;122:1146–50.
[109] Turken S, Siris E, Seldin D, Flaster E, Hyman G, Lindsay R. Effects of tamoxifen on spinal bone density in women with breast cancer. J Natl Cancer Inst 1989;81:1086–8.
[110] Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, et al. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 1992;326:852–6.
[111] Love RR, Newcomb PA, Wiebe DA, Surawicz TS, Jordan VC, Carbone PP, et al. Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 1990;82:1327–32.
[112] Love RR, Wiebe DA, Newcomb PA, Cameron L, Leventhal H, Jordan VC, et al. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann Intern Med 1991;115:860–4.
[113] Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 1988;48:812–5.

[114] Hardell L. Tamoxifen as risk factor for carcinoma of corpus uterus. Lancet 1988;ii:563.
[115] Jordan VC. Tamoxifen and endometrial cancer [letter]. Lancet 1988;2:1019.
[116] Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1989;1:117–20.
[117] Neven P, DeMylder X, VanBelle Y, Vanderick G, DeMylder E. Tamoxifen and the uterus and endometrium. Lancet 1989:375.
[118] Stewart HJ, Knight GM. Tamoxifen and the uterus and endometrium. Lancet 1989;i:375–6.
[119] Fisher B, Costantino JP, Redmond CK, Fisher ER, Wickerham DL, Cronin WM. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14 [see comments]. J Natl Cancer Inst 1994;86:527–37.
[120] Magriples U, Naftolin F, Schwartz PE, Carcangui ML. High grade endometrial carcinoma in tamoxifen treated breast cancer patients. J Clin Oncol 1993;11:485–90.
[121] Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998;90:1371–88.
[122] Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 2005;97:1652–62.
[123] Buzdar A, Marcus C, Holmes F, Hug V, Hortobagyi G. Phase II evaluation of Ly156758 in metastatic breast cancer. Oncology 1988;45:344–5.
[124] Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the *N*-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987;47:4020–4.
[125] Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 1988;6:589–95.
[126] Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. Cancer Res 1990;50:4177–89.
[127] Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 1983;32:1031–6.
[128] Jordan VC, Gosden B. Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 1983;113:463–8.
[129] Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 1990;50:3189–92.
[130] Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators [see comments] [published erratum appears in JAMA 1999 Dec 8;282(22):2124]. JAMA 1999;282:637–45.
[131] Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. multiple outcomes of raloxifene evaluation. JAMA 1999;281:2189–97.
[132] Cauley JA, Norton L, Lippman ME, Eckert S, Krueger KA, Purdie DW, et al. Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial. Multiple outcomes of raloxifene evaluation. Breast Cancer Res Treat 2001;65:125–34.
[133] Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. The study of tamoxifen and raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. JAMA 2006;295:2727–41.
[134] Walsh BW, Kuller LH, Wild RA, Paul S, Farmer M, Lawrence JB, et al. Effects of raloxifene on serum lipids and coagulation factors in healthy postmenopausal women. JAMA 1998;279:1445–51.
[135] Barrett-Connor E, Mosca L, Collins P, Geiger MJ, Grady D, Kornitzer M, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 2006;355:125–37.
[136] Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. J Med Chem 2003;46:1081–111.
[137] Ariazi EA, Ariazi JL, Cordera F, Jordan VC. Estrogen receptors as therapeutic targets in breast cancer. Curr Top Med Chem 2006;6:195–216.
[138] Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, Forbes JF, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 2005;365:60–2.
[139] Goss PE. Breast cancer prevention—clinical trials strategies involving aromatase inhibitors. J Steroid Biochem Mol Biol 2003;86:487–93.
[140] Cavalieri E, Chakravarti D, Guttenplan J, Hart E, Ingle J, Jankowiak R, et al. Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. Biochim Biophys Acta 2006;1766:63–78.
[141] Russo J, Fernandez SV, Russo PA, Fernbaugh R, Sheriff FS, Lareef HM, et al. 17-Beta-estradiol induces transformation and tumorigenesis in human breast epithelial cells. FASEB J 2006;20:1622–34.
[142] Jelovac D, Sabnis G, Long BJ, Goloubeva OG, Brodie AMH. Activation of MAPK in xenografts and cells during prolonged treatment with aromatase inhibitor letrozole. Cancer Res 2005;65:5380–9.
[143] Jelovac D, Macedo L, Goloubeva OG, Handratta V, Brodie AMH. Additive antitumor effect of aromatase inhibitor letrozole and antiestrogen fulvestrant in a postmenopausal breast cancer model. Cancer Res 2005;65:5439–44.
[144] Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. Cancer Res 1996;56:2321–30.
[145] Welshons WV, Jordan VC. Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. Eur J Cancer 1987;232:1935–9.
[146] Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocr 1992;90: 77–80.
[147] Pink JJ, Bilimoria MM, Assikis J, Jordan VC. Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. Br J Cancer 1996;74:1227–36.
[148] Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. Cancer Cell 2004;5:207–13.
[149] Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen induced apoptosis in breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 2005;94:131–41.

[150] Lewis JS, Meeke K, Osipo C, Bell E, Kidawi N, Chandel NS, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. JNCI 2005;97:1746–59.
[151] Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000;6:2028–36.
[152] Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, et al. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 2003;95:1586–97.
[153] Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant on estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 2003;95:1597–607.
[154] Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jorgensen LM, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat 2001;67:111–6.
[155] Jordan VC, Lewis JS, Osipo C, Cheng D. The apoptotic action of estrogen following exhaustive antihormonal therapy: a new clinical treatment strategy. Breast 2005;14:624–30.

Successful Translation Research with Selective Oestrogen Receptor Modulators to Treat and Prevent Breast Cancer

Erfolgreiche translationale Forschung über selektive Östrogenrezeptor-Modulatoren in der Behandlung und Prävention des Mammakarzinoms

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Schlüsselwörter
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- Prävention
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- SERM
- DICS

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Zusammenfassung

Vor 30 Jahren spielte die Antiöstrogentherapie mit Tamoxifen nur eine zweitrangige Rolle in der Mammakarzinomtherapie. Die Hoffnungen, die Heilung des metastasierten Mammakarzinoms erreichen zu können, fokusierten sich auf die Entwicklung neuer Zytostatika und der Hochdosischemotherapie mit Knochenmarktransplantation. Ähnliche Strategien wurden in der adjuvanten Behandlung des Mammakarzinoms verfolgt. Vereinfacht gesagt, das Ziel war es, die Krebszellen mit unspezifischen zytotoxischen Substanzen zu töten, während der Patient durch supportive Maßnahmen am Leben gehalten wurde. Aber die medizinische Forschung verläuft nicht nur gradlinig und alternative Ansätze, die die Kontrolle des Tumorwachstums mit minimalen Nebenwirkungen erlauben, wurden entwickelt – die sogenannte targeted therapy. Die Elemente dieser neuen Strategien waren seit 20 Jahren gestützt durch die Ergebnisse der Grundlagenforschung etabliert. Der Ansatz der Langzeitantihormontherapie zur Kontrolle des Zellwachstums über „targeting" des Tumor-Östrogenrezeptors hat die Behandlung des Mammakarzinoms revolutioniert. Der Erfolg dieser Strategie wird an der Abnahme der Mortalität des Mammakarzinoms abgelesen. Die translationale Forschung über den Tumor-Östrogenrezeptor mit einer ganzen Bandbreite neuer antiöstrogener Substanzen (Aromatasehemmer, reine Antiöstrogene) lässt neue Therapiemodelle der „targeted therapy" prophezeien. Wenn man sich die Pharmakologie der „Antiestrogene" genauer anschaut, wird man erstaunt sein. Die nicht steroidalen „Antiöstrogene" sind selektive Östrogenmodulatoren (SERMs). Mit anderen Worten, die Substanzen wirken an der Mamma als Antiöstrogene, als Östrogene im Knochen und vermindern den Cholesterinspiegel. Dieses Wissen ermöglicht einen praktischen Ansatz in der Chemoprävention des Mammakar-

Abstract

Thirty years ago, antiestrogen therapy with tamoxifen played only a secondary role in breast cancer care. All hopes to cure metastatic breast cancer were still pinned on either the discovery of new cytotoxic drugs or a dose dense combination of available cytotoxic drugs with bone marrow transplantation. A similar strategy with combination chemotherapy was employed as an adjuvant for primary breast cancer. Simply stated, the goal was to kill the cancer with nonspecific cytotoxic drugs while keeping the patient alive with supportive care. However, medical research does not travel in straight lines and alternative approaches emerged to solve the problem of controlling tumour growth with minimal side effects. The approach was targeted therapy. All the elements of the new strategy were in place twenty years ago supported by scientific principles derived from laboratory research. The approach of using long-term antihormone therapy to control early stage breast cancer growth would revolutionise cancer care by targeting the tumour oestrogen receptor (OER). The success of the strategy would be evidenced by lives saved and contribute to a decrease in the national mortality figures for breast cancer. More importantly, translational research that targeted the tumour OER with a range of new antioestrogenic drugs (aromatase inhibitors, pure antioestrogens) would presage the current fashion of blocking survival pathways for the tumour by developing novel targeted treatments. But a surprise was in store when the pharmacology of "antioestrogens" was studied in detail. The nonsteroidal "antioestrogens" are selective oestrogen modulators (SERMs). In other words, the compounds are antioestrogens in the breast, oestrogens in the bone and lower circulating cholesterol. This knowledge would establish a practical approach to breast cancer chemopre-

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Bibliography
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zinoms bei Frauen mit hohem Risiko (Tamoxifen) und niedrigem Risiko (Raloxifene).

vention for both high risk (tamoxifen) and low risk (raloxifene) women.

Widmung

Das Manuskript wurde Herrn Professor Dr. Dr. h.c. Manfred Kaufmann, Direktor der Universitäts-Frauenklinik Frankfurt, zu seinem 60. Geburtstag gewidmet.

Introduction

I am delighted to be invited to celebrate the 60th birthdays of Professor Manfred Kaufmann and his wife, Brigitte. Our careers are intertwined, so I wish to offer my personal observations on the specific changes that have occurred in the approach to the endocrine treatment of breast cancer over this period. These changes are considerable. As a new PhD graduate in pharmacology who had studied "antioestrogens" as an academic exercise between 1969 – 72, I was told I could now do anything I wanted. I chose to contribute to the reinvention of an orphan drug, ICI 46474 into tamoxifen.

When I was a teenager, my passion was chemistry. However, I wanted to use chemistry to treat cancer because I was inspired by Paul Ehrlich whose work succeeded in treating disease selectively. For this reason, I chose to study pharmacology at Leeds University and take the unfashionable step, in the late 1960's, of exploring the possibility of using targeted drugs to treat cancer selectively. Unfortunately, the obstacles in place to prevent progress were enormous.

Each generation creates and defends its own fashion in cancer research and this concept is well illustrated by the early resistance to change that occurred in the approach to the treatment of breast cancer. Virtually no one was interested in "another endocrine therapy" as combination cytotoxic chemotherapy was predicted to cure cancer.

In contrast, it is now clear that the approach to health care has changed in the past 30 years not once but twice as a result of advances in endocrine therapy. Tamoxifen is the first targeted treatment for breast cancer based on the successful translational work focused on the oestrogen receptor (OER) that has helped extend the lives of millions of women [1]. Perhaps equally important in this advance was the fact that tamoxifen has a beneficial therapeutic ratio that facilitated its use as a long-term adjuvant therapy. The application of the laboratory strategy of long-term antihormonal therapy targeted to the OER [2,3] saved lives which in turn has contributed significantly to the national reductions in mortality [4,5].

Secondly, the knowledge gained with tamoxifen propelled the drug forward for testing as the first chemopreventive for any cancer, created a new drug group, the selective oestrogen receptor modulators (SERMs) and resurrected keoxifene, a failed breast cancer drug [6] to be the first SERM for the treatment

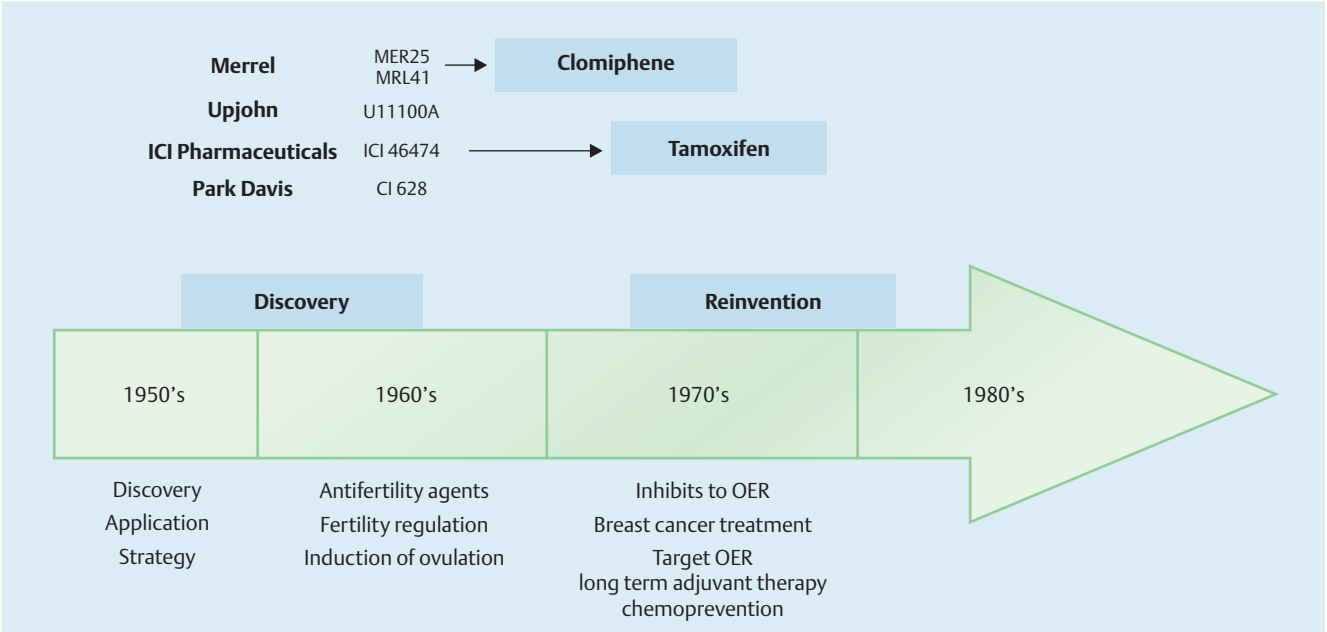

Fig. 1 The reinvention of nonsteroidal antioestrogens as pioneering agents for the effective treatment of breast cancer. Nonsteroidal antioestrogens were discovered by scientists in the pharmaceutical industry during the late 1950's and early 1960's. Initial optimism that they would be effective morning after pills were found to be invalid when clinical trials demonstrated that the compounds induced ovulation in subfertile women. Clomiphene and tamoxifen were both marketed for the induction of ovulation in subfertile women. During the 1960's, interest in using nonsteroidal antioestrogens in the clinic declined. During the 1970's, the nonsteroidal antioestrogen ICI 46474 was reinvented to be a targeted therapy for the long-term treatment of breast cancer [2]. The target was the oestrogen receptor and the principles established in the laboratory in the 1970's were to provide the basis for successful long-term adjuvant therapy targeted to patients with oestrogen receptor (OER) positive breast cancers and to explore the possibility of chemoprevention in high risk populations.

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and prevention of osteoporosis but with the ability to reduce the risk of breast and endometrial cancer [7].

The story of the discovery of tamoxifen as a "morning after pill" in the 1960's to then being reinvented as a targeted breast cancer therapy in the 1970's, has been recounted recently [2,3]. Nevertheless, the tale is important to retell as it illustrates how the changing fashions in research can influence progress. The development of the oral contraceptive by Pincus and colleagues at the Worcester Foundation during the 1950's changed society forever. Despite enthusiasm to create new ways to manipulate reproduction, the fashion of research in reproductive biology declined steadily throughout the 1960's with a decreased investment in the development of new contraceptive methods. Political and legal fashions were changing. But, in its place, the "War on Cancer" was declared in the United States in 1971.

The tale of tamoxifen also illustrates the length of time that must be taken to evaluate successful treatment strategies to affect changes in healthcare (**Fig. 1**). Nevertheless, momentum to change the approach for the treatment of breast cancer, and indeed of any cancer accelerated with tamoxifen. The drug became a ubiquitous tool to test targeting in breast cancer. More importantly, tamoxifen set the stage for the current optimism that important advances in cancer research are within our grasp and the sincere belief that clever people will solve problems and develop practical ways to kill cancer cells selectively with minimal side effects for the patient.

However, the question that surfaces is "why did the process of concept to changes in healthcare take such a long time for a novel targeted therapy with few side effects?"

The Lost Decade – Tamoxifen on Life Support

In April 1972, all the preliminary clinical data on ICI 46474 was reviewed by scientists at ICI Pharmaceuticals Division at Alderley Park but there was reluctance to pursue the development of the drug as a short term palliative treatment for breast cancer. Several factors were considered in the decision not to develop ICI 46474. The compound had no patent protection in the United States; this would only be granted in 1985! Most importantly at the time, there was estimated to be no significant market for a palliative drug that would only be effective for about a year for one out of three metastatic breast cancer patients. In the early 1970's, the total incidence of metastatic breast cancer in the United Kingdom was only a few thousand patients per year. Worldwide figures were obviously larger but the drug was to be priced 10 times more expensive than the standard endocrine treatment (diethylstilbestrol). The turnover of the drug was estimated to be £ 500 000 per annum, at most, with only £ 50 000 profit. It would always be an orphan drug and clinical development was stalled. With the wisdom of hindsight, there was also no infrastructure at Alderley Park to support a breast cancer programme. ICI Pharmaceuticals Division was not a "cancer company" and there was also no pipeline of compounds to replace ICI 46474 should subsequent studies (as they did 20 years later with rat liver carcinogensis) reveal unacceptable toxicities. Development could not be taken seriously. In the clinical community, it was generally accepted that another endocrine therapy would add almost nothing to the medical armamentarium of breast cancer therapies. Overall, there was little initial enthusiasm for the use of a new antihormonal therapy that benefited a minority of patients for a short period.

A.L. Walpole, Head of the Fertility Control Programme at ICI Pharmaceuticals Division discovered the antifertility properties of the molecule ICI 46474 in the early 1960's. The compound was an effective "morning after pill" in rats [8] but induced ovulation in women [9]. The project did not achieve its goal. Nevertheless, the observations that ICI 46474 had equivalent antitumour activity but reduced side effects compared to standard endocrine therapies used to treat advanced breast cancer [10,11] convinced Walpole that the drug should be marketed at least as an option for treatment. The drug was available for experimentation but looking for applications. Although no studies were conducted by scientists at ICI Pharmaceuticals Division, Walpole ensured that my laboratory would be supported to find those applications. Regrettably, Walpole died suddenly in 1977 and never saw the benefits that tamoxifen was to bring [2,12].

A scientific strategy for the appropriate clinical application of tamoxifen was developed in the laboratory to target the drug to the tumours that were the most likely to respond. Tamoxifen blocked the binding of estradiol to human breast and rat mammary tumour ER's and prevented the induction and growth of ER positive carcinogen-induced rat mammary carcinomas [2,3]. These early studies raised the question of whether tamoxifen could prevent the majority of breast cancers i.e.: ER positive breast cancer. However, the finding that long-term tamoxifen treatment in animals with early mammary cancer i.e., a low tumour burden [2] could create a tumour-free state suggested longer was going to be better than shorter durations of adjuvant therapy. On a personal note, I am extremely proud of the fact that the regulatory authorities in Germany required information on my work prior to granting permission for the application of tamoxifen to treat breast cancer.

The laboratory observations were to prove remarkably effective as an approach to treat women with early node positive and node negative ER positive breast cancer. However, the original clinical strategy in the 1970's for the evaluation of tamoxifen was to use one year of adjuvant treatment after surgery. The reason for this was that tamoxifen was only effective for the treatment of advanced breast cancer for about a year and there was a sincere concern that longer adjuvant treatment durations would result in premature drug resistance. This approach was to change.

With these observations as background, all of the pieces of the puzzle were about to come together in 1986 (20 years ago) to create a significant advance that would change healthcare twice.

An Overview of Adjuvant Clinical Trials

An enormous advance in medicine is the introduction of meta-analysis or Overview analysis of small randomised clinical trials that individually show little or no benefits for agents under investigation but together provide a valid result. The Overview analysis of breast cancer clinical trials was first conducted at Heathrow airport in 1984 [13]. The results when they were published in full in 1988 demonstrated a significant advantage for postmenopausal patients receiving tamoxifen [14].

Based on the successive analysis of accumulative randomized worldwide clinical trials, it is possible to summarise the main conclusions for tamoxifen. At the time 20 years ago, when the Overview analysis first occurred, tamoxifen was the only universally used antihormonal agent. With no other competition, tamoxifen became the "gold standard" and established the princi-

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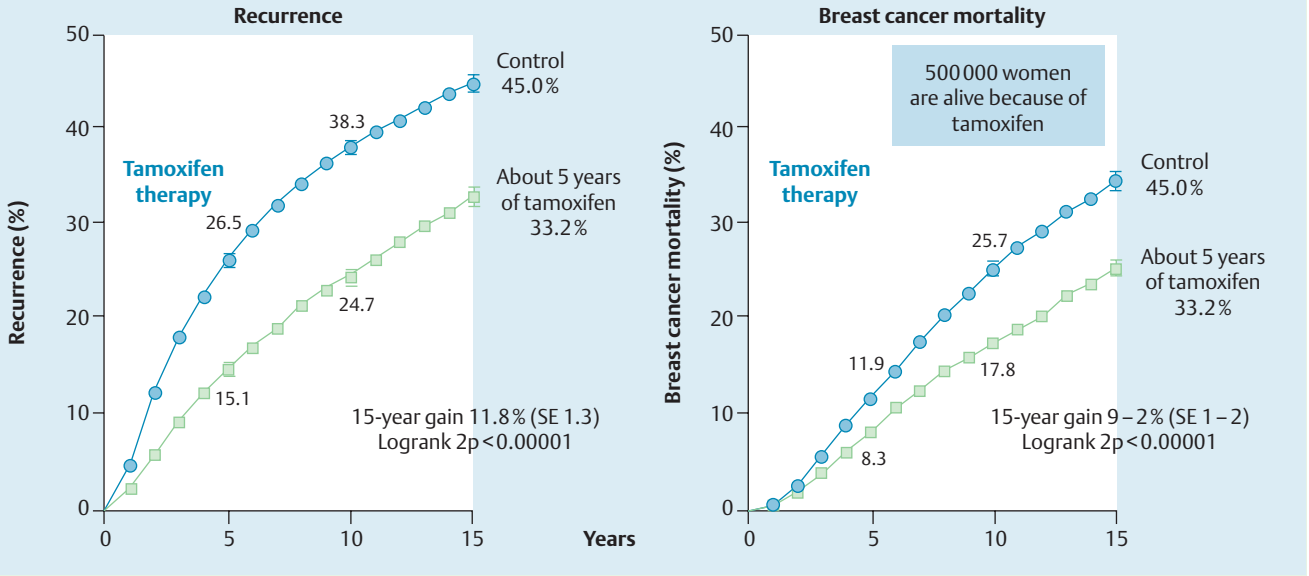

Fig. 2 The effectiveness of 5 years adjuvant tamoxifen therapy in OER positive and node negative breast cancer patients. These data on the percentage recurrence and breast cancer mortality are derived with permission from Early Breast Cancer Clinical Trialists Organization [5].

ples of tumour targeting and identified the appropriate treatment strategy to aid survivorship [4,5,14,15].

- Five years of adjuvant tamoxifen enhances disease free survival. There is a 50% decrease in recurrences observed in ER positive patients fifteen years after diagnosis (**Fig. 2**).
- Five years of adjuvant tamoxifen enhances survival with a decrease in mortality fifteen years after diagnosis (**Fig. 2**).
- Adjuvant tamoxifen does not provide an increase in disease free or overall survival in ER negative breast cancer.
- Five years of adjuvant tamoxifen alone is effective in premenopausal women with ER positive breast cancer.
- The benefits of tamoxifen in lives saved from breast cancer far outweighs concerns about an increased incidence of endometrial cancer in postmenopausal women.
- Tamoxifen does not increase the incidence of second cancers other than endometrial cancer.
- No non-cancer related overall survival advantage is noted with tamoxifen when given as adjuvant therapy.

Overall, the clinical conclusions obtained by the Overview process based on worldwide experience were all consistent with the parallel laboratory studies that guided clinical care. This might have been the end of the tale but for the continuing examination of tamoxifen and related drugs in the laboratory.

The Changing Pharmacology of Antioestrogens

Twenty years ago, tamoxifen was classified as a non-steroidal antioestrogen [16]. In pharmacological terms tamoxifen was described as a partial agonist (oestrogen-like) in target tissues such as the immature rat uterus but it was antioestrogenic because it blocked the full action of estradiol alone. In 1986, it was plausible that if oestrogen was necessary to fend off osteoporosis and coronary heart disease the long-term administration of an antioestrogen to node negative women could eventually have a deleterious effect on bone density and produce a potential increase in the incidence of coronary heart disease for the majority of women. The potential side effects would be even worse for women only at high risk to develop breast cancer. Only a small minority of women would have a reduced risk of breast cancer, but all women would be exposed to potential "antioestrogenic" toxicities. However, the classification of nonsteroidal antioestrogens was to change just after 1986. Today the concept is known as selective oestrogen receptor modulation.

In 1986, virtually nothing was known about the actions of nonsteroidal antioestrogens on bone density. A single report showed that clomiphene, a drug used for the induction of ovulation, would preserve bone density in ovariectomized rats [17]. However, the interpretation of these data was not that simple. Clomiphene is an impure mixture of oestrogenic and antioestrogenic isomers. Which isomer was affecting bone? The consistent laboratory finding that tamoxifen the pure *trans antioestrogenic isomer* of a triphenylethylene maintained bone density in ovariectomized rats [18–20] seemed to translate to postmenopausal women [21], but would prospective clinical studies really show benefit? The Wisconsin Tamoxifen Study was started in 1986 to explore the potential toxicity of tamoxifen on bone density. The study demonstrated in a double blind placebo controlled clinical trial that tamoxifen could preserve bone in the postmenopausal woman [22]. Bone building would clearly be an advantage for chemoprevention studies, thereby enhancing the possibility that the worth of tamoxifen to prevent breast cancer could be tested safely. In the same studies, tamoxifen lowered low density lipoprotein [23,24] and, by inference, would appear not to increase the risk of coronary heart disease. These results were good. The bad was the laboratory discovery that although tamoxifen prevented the oestrogen-stimulated growth of human breast cancers, the drug stimulated the growth of human endometrial cancers grown in the same athymic mouse [25]. This again was selective oestrogen receptor modulation. Stimulate one target site to produce growth but at the same time block another target site.

There was a very quick response from the clinical community to the warnings [25] that long-term tamoxifen treatment could be

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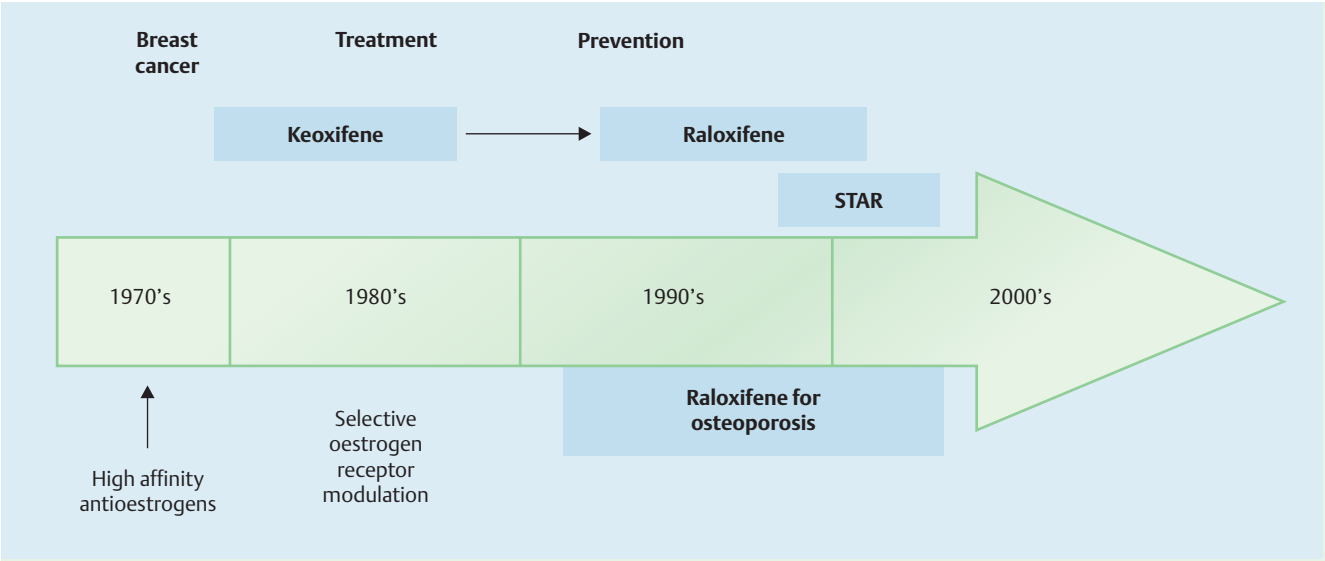

Fig. 3 Development of the concepts of selective oestrogen receptor modulations and the reinvention of keoxifene, a failed breast cancer drug to raloxifene, a successful agent for the prevention of osteoporosis and breast cancer. During the 1970's, a metabolite of tamoxifen called 4 hydroxy-tamoxifen was found to have a high binding affinity to the oestrogen receptor [48]. This discovery led to the synthesis of keoxifene (LY156758) [34]. The drug was initially used as a treatment for breast cancer but was unsuccessful and Eli Lilly discontinued investigation of this application. In the laboratory, the nonsteroidal antioestrogens were found to actually be selective oestrogen receptor modulators (SERMs). The compounds maintained bone density and oestrogenic action but prevented the development of mammary cancers and antioestrogenic action. This knowledge [32] was used to advance the evaluation of SERMs for the prevention of osteoporosis but with the prevention of breast cancer as a beneficial side effect. Keoxifene was reinvented as raloxifene and has now been successfully shown to prevent both osteoporosis in high risk women. Also, the recent study of tamoxifen and raloxifene (STAR trial) [39] has demonstrated that tamoxifen and raloxifene are equivalent in preventing the development of invasive breast cancer in high risk postmenopausal women.

associated with an increase in the incidence of endometrial cancer [26–28]. The advance in patient care was that women taking tamoxifen to treat breast cancer were also forewarned about gynecological complications.

By the 1990's, it was clear that the revelations about tamoxifen were not going to be helpful in bringing a proven agent that reduces the risk of breast cancer in pre and postmenopausal women by 50% [29, 30] to a broad constituency of high risk women. However, a new chemopreventive strategy was already in place by the end of the 1980's.

Selective OER Modulators

The recognition that the so called "nonsteroidal antioestrogens" had oestrogenic and antioestrogenic actions at different sites in the ovariectomized female rat and that these data translated to women to prevent osteoporosis and breast cancer created a new dimension in drug development. The fact that tamoxifen and the failed breast cancer drug keoxifene (LY156,758) [6] both prevented the development of carcinogen-induced rat mammary carcinomas [31] and maintained bone density in ovariectomized rates [18] indicated that this was a class effect. The significance of these observations for public health and chemoprevention of breast cancer was immediately recognized. The future of SERM drug development was clear.

"The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused upon the identification of a population of high-risk women to test 'chemopreventive' agents. But are resources being used less than optimally? An alternative would be to seize upon the developing clues provided by an extensive clinical investigation of available antioestrogens. Could analogues be developed to treat osteoporosis or even retard the development of atherosclerosis? If this proved to be true then a majority of women in general could be treated for these conditions as soon as menopause occurred. Should the agent also retain anti-breast tumour actions then it might be expected to act as a chemosuppressive. A bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century" [32].

This blueprint to improve healthcare was subsequently restated at the annual meeting of the American Association of Cancer Research in San Francisco, 1989 [33].

Compounds of the keoxifene class (LY117018 and LY156758) were obvious candidates for study despite the fact that the programme to develop the drugs to treat breast cancer had been abandoned by Eli Lilly in 1988. The compounds were known to be less uterotrophic than tamoxifen in rodents [34] but they were short acting [35] which could explain their poor antitumour properties when compared with tamoxifen. Interestingly enough, keoxifene was already known to partially inhibit the growth of tamoxifen-stimulated human endometrial tumours under laboratory conditions [36].

Keoxifene, the failed breast cancer drug was reinvented in the early 1990's as raloxifene, a SERM (**Fig. 3**). A use patent for the treatment and prevention of osteoporosis was filed by Eli Lilly in 1992.

Raloxifene has now been available for the treatment and prevention of osteoporosis in postmenopausal women since 1999 based on the prospective clinical trials demonstrating an approximately 40% decrease in spinal fractures [37] with the ad-

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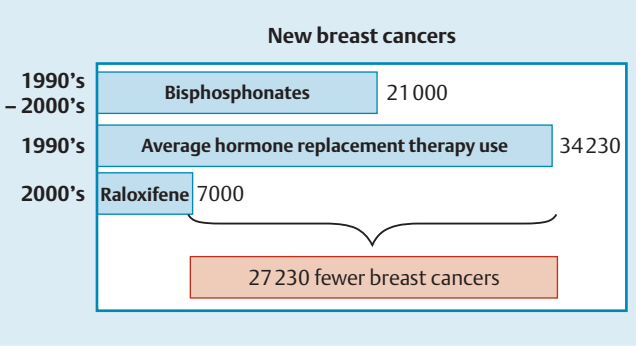

Fig. 4 Progress in the chemoprevention of breast cancer over the past 20 years. About half a million women are currently taking raloxifene for the prevention of osteoporosis but with the beneficial side effect of preventing breast cancer [39]. During the 1990's, a woman at risk for osteoporosis could have taken either bisphosphonate or legitimately used hormone replacement therapy to maintain bone density. If half a million women were taking bisphosphonates for a decade with risk factors, based upon the osteoporosis study conducted by Martino and colleagues [41], then those patients would have accumulated 21 000 new breast cancers. The bisphosphonates have no effect on breast cancer incidence. In contrast, based on the elevated risk factors observed in the Women's Health Initiative, [49] or the Million Women's Study [50], the average elevation in breast cancer incidence for the half million women taking hormone replacement therapy for 10 years would be 34 230 new breast cancers. In contrast, if half a million women give up hormone replacement therapy and take raloxifene for 10 years, there will only be an incidence of 7000 breast cancers. In other words, 27 230 fewer breast cancers would be observed in this population over a decade. (Taken with permission from the European Journal of Cancer [42]).

vantage over hormone replacement therapy of causing a 70% decrease in the incidence of breast cancer [7,38]. The anticipated result in reducing the risk of breast cancer as a beneficial side effect of treating osteoporosis propelled raloxifene into clinical trial vs. tamoxifen for the prevention of breast cancer as the primary endpoint. The study of tamoxifen and raloxifene (STAR) in high risk postmenopausal women now shows equivalent effects of tamoxifen and raloxifene to prevent breast cancer but raloxifene is safer [39].

A STAR is Born

The NSABP recruitment organisation randomised a total of 19 747 postmenopausal women with an increase 5 year risk of breast cancer (mean 4.03%) to receive either tamoxifen (20 mg daily) or raloxifene (60 mg daily) for 5 years to be followed by a 2 year observation period. The results were clear cut. Tamoxifen and raloxifene are equivalent as agents to reduce the risk of invasive breast cancer and no statistical significance was noted with the incidence of non invasive breast cancers (ductal carcinoma in situ plus lobular carcinoma in situ). Thus, based on the placebo controlled trial referred to as P-1 [30] where tamoxifen produced a 50% decrease in invasive breast cancer, raloxifene can be stated to do the same. In fact, the placebo controlled trial, Raloxifene use for the Heart (RUTH), demonstrated just that. The study [40] was designed to evaluate the value of raloxifene to reduce death from coronary heart disease. However, the trial showed no advantage for raloxifene. Nevertheless, raloxifene did reduce breast cancer incidence by 50% and, most importantly, there was no elevation in endometrial cancer.

In the STAR trial, the side effect profile benefited raloxifene. There were fewer endometrial cancers, fewer hysterectomies, fewer cataracts and fewer cataract operations. Additionally, there was fewer thromboemobolic events with raloxifene. Thus, raloxifene has advanced chemoprevention as a suitable, safer alternative to tamoxifen for high risk postmenopausal women.

The clinical advance with raloxifene is, however, also an advance in public health. The fact that raloxifene can reduce the risk of breast cancer in postmenopausal women being treated long term to prevent fractures [41] is important and validates the initial evidenced based hypothesis that this would become the reality [32,33]. It now has become possible to calculate the impact of raloxifene on public health for a population of half a million women taking the drug for a decade [42]. It is estimated that with prescribing practices changing from hormone replacement therapy to raloxifene for the prevention of osteoporosis, that more than 27 000 women with *not* have a diagnosis of breast cancer (**Fig. 4**).

Progress and Lessons Learned

Patents produce progress and the tales of tamoxifen and raloxifene are prime examples of this principle. Both drugs failed in their primary application and were successfully reinvented which, in turn, produced wide clinical usage. Tamoxifen did not break into the headlines one day but rather sneaked up on the cancer community and was established as the "standard of care" for endocrine treatment by the early 1980's. So much so that the World Health Organization declared tamoxifen an essential drug for the treatment of breast cancer. It is cheap, remarkably nontoxic, easily administered and saves lives. Progress is measured by the hundreds of thousands of women who are alive today who would have died if they had been diagnosed with breast cancer in the 1970's. The principle of long-term antihormonal therapy targeted to the OER has dramatically improved survivorship in breast cancer.

But tamoxifen might not have happened. There was no programme or pipeline to replace tamoxifen at the beginning. With no progress with tamoxifen in the 1970's, how long would it have been before aromatase inhibitors were developed with no lead adjuvant agent to pioneer targeted antihormonal therapy and develop the market? Lives would have been lost [43] and it might have been another decade before another approach of receptor targeting was shown to save lives. Reinvention became the path to progress. The reinvention of keoxifene to become raloxifene reinforces the lesson that "observations in one field of science become major discoveries in another" [33]. The SERM field is following closely on the heels of raloxifene with many new medicines [44].

Overall, it is clear that there are no short-term solutions to therapeutic changes in healthcare. Drug targeting, clinical trials and advances in chemoprevention require decades of dedicated effort. However, the lessons learned from endocrine therapy demonstrate the principle that there are also consequences to the patient when new treatments are introduced and constant re-examination of clinical results and persistent challenge to dogma are required. Laboratory research is currently defining the evolution of long-term antihormonal therapy [45] with the remarkable discovery that minute concentrations of oestrogen can kill

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breast cancers following years of antihormone treatment [46, 47] Learning to use our new knowledge of oestrogen action in clinical trials may be an unanticipated bonus of antihormone therapy.

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References

1 *Jensen EV, Jordan VC.* The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. Clin Cancer Res 2003; 9: 1980 – 1989
2 *Jordan VC.* Tamoxifen: a most unlikely pioneering medicine. Nature Reviews Drug Discovery 2003; 2: 205 – 213
3 *Jordan VC.* Tamoxifen (ICI 46474) as a targeted therapy to treat and prevent breast cancer. Brit J Pharmacol 2006; 147: S269 – S276
4 *Early Breast Cancer Trialists' Collaborative Group (EBCTCG).* Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 1998; 354: 1451 – 1467
5 *Early Breast Cancer Trialists' Collaborative Group (EBCTCG).* Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005; 365: 1687 – 1717
6 *Buzdar A, Marcus C, Holmes F, Hug V, Hortobagyi G.* Phase II evaluation of Ly156758 in metastatic breast cancer. Oncology 1988; 45: 344 – 345
7 *Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, Norton L, Nickelsen T, Bjarnason NH, Morrow M, Lippman ME, Black D, Glusman JE, Costa A, Jordan VC.* The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA 1999; 281: 2189 – 2197
8 *Harper MJ, Walpole AL.* Mode of action of I.C.I. 46474 in preventing implantation in rats. J Endocrinol 1967; 37: 83 – 92
9 *Williamson JG, Ellis JP.* The induction of ovulation by tamoxifen. J Obstet Gynaec Br Commonw 1973; 80: 844 – 847
10 *Cole MP, Jones CT, Todd ID.* A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. Br J Cancer 1971; 25: 270 – 275
11 *Ward HW.* Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J 1973; 1: 13 – 14
12 *Jordan VC.* The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. Breast Cancer Res Treat 1988; 11: 197 – 209
13 *Anonymous.* Review of mortality results in randomized trials in early breast cancer. Lancet 1984; ii: 1205
14 *EBCTCG.* Effects of adjuvant tamoxifen and of cytotoxic therapy on mortality in early breast cancer. An overview of 61 randomized trials among 28,896 women. N Engl J Med 1988; 319: 1681 – 1692
15 *EBCTCG.* Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Lancet 1992; 339: 1 – 15
16 *Jordan VC.* Biochemical pharmacology of antiestrogen action. Pharmacol Rev 1984; 36: 245 – 276
17 *Beall PT, Misra KL, Young RL, Spiut HJ, Evan HJ, LeBlanc A.* Clomiphene protects against osteoporosis in the mature ovariectomized rat. Calcif Tissue Int 1985; 36: 123 – 125
18 *Jordan VC, Phelps E, Lindgren JU.* Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987; 10: 31 – 35
19 *Turner RT, Wakley GK, Hannon KS, Bell NH.* Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. J Bone Miner Res 1987; 2: 449 – 456
20 *Turner RT, Wakley GK, Hannon KS, Bell NH.* Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. Endocrinology 1988; 122: 1146 – 1150
21 *Turken S, Siris E, Seldin D, Flaster E, Hyman G, Lindsay R.* Effects of tamoxifen on spinal bone density in women with breast cancer. J Natl Cancer Inst 1989; 81: 1086 – 1088
22 *Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, Carbone PP, DeMets DL.* Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 1992; 326: 852 – 856
23 *Love RR, Newcomb PA, Wiebe DA, Surawicz TS, Jordan VC, Carbone PP, DeMets DL.* Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 1990; 82: 1327 – 1332
24 *Love RR, Wiebe DA, Newcomb PA, Cameron L, Leventhal H, Jordan VC, Feyzi J, DeMets DL.* Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann Intern Med 1991; 115: 860 – 864
25 *Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC.* Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 1988; 48: 812 – 815
26 *Hardell L.* Tamoxifen as risk factor for carcinoma of corpus uterus [letter]. Lancet 1988; 2(8610): 563
27 *Jordan VC.* Tamoxifen and endometrial cancer [letter]. Lancet 1988; 2(8618): 1019
28 *Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, Skoog L, Somell A, Theve T, Wilking N, Hjalmar M-L.* Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1989; 1: 117 – 120
29 *Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, Atkins JN, Margolese RG, Runowicz CD, James JM, Ford LG, Wolmark N.* Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 2005; 97: 1652 – 1662
30 *Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N.* Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998; 90: 1371 – 1388
31 *Gottardis MM, Jordan VC.* Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987; 47: 4020 – 4024
32 *Jordan VC.* Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 1988; 6: 589 – 595
33 *Lerner LJ, Jordan VC.* The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. Cancer Res 1990; 50: 4177 – 4189
34 *Black LJ, Jones CD, Falcone JF.* Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 1983; 32: 1031 – 1036
35 *Jordan VC, Gosden B.* Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 1983; 113: 463 – 468
36 *Gottardis MM, Jiang SY, Jeng MH, Jordan VC.* Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res 1989; 49: 4090 – 4093
37 *Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, Christiansen C, Delmas PD, Zanchetta JR, Stakkestad J, Gluer CC, Krueger K, Cohen FJ, Eckert S, Ensrud KE, Avioli LV, Lips P, Cummings SR.* Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators [see comments] [published erratum appears in JAMA 1999; 282: 2124]. JAMA 1999; 282: 637 – 645
38 *Cauley JA, Norton L, Lippman ME, Eckert S, Krueger KA, Purdie DW, Farrerons J, Karasik A, Mellstrom D, Ng KW, Stepan JJ, Powles TJ, Morrow M, Costa A, Silfen SL, Walls EL, Schmitt H, Muchmore DB, Jordan VC, Ste-Marie LG.* Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial.

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Multiple outcomes of raloxifene evaluation. Breast Cancer Res Treat 2001; 65: 125 – 134
39 *Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon (jr) ER, Wade JL, Robidoux A, Margolese RG, James J, Lippman SM, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N.* The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial JAMA 2006; 295: 2727 – 2741
40 *Barrett-Connor E, Mosca L, Collins P, Geiger MJ, Grady D, Kornitzer M, McNabb MA, Wenger NK.* Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 2006; 355: 125 – 137
41 *Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, Secrest RJ, Cummings SR.* For the CORE Investigators Continuing Outcomes Relevant to Evista: Breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 2004; 96: 1751 – 1761
42 *Jordan VC.* Optimising endocrine approaches for the chemoprevention of breast cancer. Beyond the Study of Tamoxifen and Raloxifene (STAR) Trial. European Journal of Cancer 2006; 42: 2909 – 2913
43 *Jordan VC.* What if tamoxifen (ICI 46474) had been found to produce rat liver tumors in 1973? A personal perspective. Ann Oncol 1995; 6: 29 – 34
44 *Jordan VC.* Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. J Med Chem 2003; 46: 1081 – 1111
45 *Jordan VC.* Selective estrogen receptor modulation: concept and consequences in cancer. Cancer Cell 2004; 5: 207 – 213
46 *Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, Jordan VC.* Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000; 6: 2028 – 2036
47 *Lewis JS, Meeke K, Osipo C, Bell E, Kidawi N, Chandel NS, Jordan VC.* Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. JNCI 2005; 97: 1746 – 1759
48 *Jordan VC, Collins MM, Rowsby L, Prestwich G.* A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 1977; 75: 305 – 316
49 *Writing Group for the Women's Health Initiative Investigators: Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J.* Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women. JAMA 2002; 288: 321 – 332
50 *Million Women Study Collaborators.* Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet 2003; 362: 419 – 427

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SPECIAL ARTICLE

The 38th David A. Karnofsky Lecture: The Paradoxical Actions of Estrogen in Breast Cancer—Survival or Death?

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ABSTRACT

During the first David A. Karnofsky Award lecture entitled "Thoughts on Chemical Therapy" in 1970, Sir Alexander Haddow commented about the dramatic regressions observed with estrogen in some breast cancers in postmenopausal women, but regrettably the mechanism was unknown. He was concerned that a cancer-specific target would remain elusive, without tests to predict response to therapy. At that time, I was conducting research for my PhD on an obscure group of estrogen derivatives called nonsteroidal antiestrogens. Antiestrogens had failed to fulfill their promise as postcoital contraceptives and were unlikely to be developed further by the pharmaceutical industry. In 1972, that perspective started to change and ICI 46,474 was subsequently reinvented as the first targeted therapy for breast cancer. The scientific strategy of targeting the estrogen receptor (ER) in the tumor, treating patients with long-term adjuvant therapy, examining active metabolites, and considering chemoprevention all translated through clinical trials to clinical practice during the next 35 years. Hundreds of thousands of women now have enhanced survivorship after their diagnosis of ER-positive breast cancer. However, it was the recognition of selective ER modulation (SERM) that created a new dimension in therapeutics. Nonsteroidal antiestrogens selectively turn on or turn off estrogen target tissues throughout the body. Patient care was immediately affected by the recognition in the laboratory that tamoxifen would potentially increase the growth of endometrial cancer during long-term adjuvant therapy. At that time, a failed breast cancer drug, keoxifene, was found to maintain bone density of rats (estrogenic action) while simultaneously preventing mammary carcinogenesis (antiestrogenic action). Perhaps a SERM used to prevent osteoporosis could simultaneously prevent breast cancer? Keoxifene was renamed raloxifene and became the first SERM for the treatment and prevention of osteoporosis as well as the prevention of breast cancer, but without an increase in endometrial cancer. There the story might have ended had the study of antihormone resistance not revealed a vulnerability of cancer cells that could be exploited in the clinic. The evolution of antihormone resistance over years of therapy reconfigures the survival mechanism of the breast cancer cell, so estrogen no longer is a survival signal but a death signal. Remarkably, remaining tumor tissue is again responsive to continuing antihormone therapy. This new discovery is currently being evaluated in clinical trials but it also solves the mystery mechanism of chemical therapy with estrogen noted by Haddow in the first Karnofsky lecture.

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INTRODUCTION

By looking back, we can see the way forward. In 1970, Sir Alexander Haddow, FRS presented the first David A. Karnofsky Memorial Lecture entitled "Thoughts on Chemical Therapy."[1] Paul Ehrlich, MD, was the individual who revolutionized therapeutics when he first created a "chemotherapy" (chemical therapy) through rational synthesis, followed by predictive testing in laboratory models, and then clinical trials to demonstrate the cure of syphilis with Salvarsan.[2] He next turned to the treatment of cancer, but after more than a decade, he declared the year before he died in 1915: "I have wasted fifteen years of my life in experimental cancer research."[3] In his Karnofsky lecture, Haddow echoed Ehrlich's sentiment with the statements "the fact that the cancer cell is but a modification of the normal somatic cell holds out little prospect of a chemotherapia specifica in Ehrlich's sense" and "the need exists for some method of prior screening to indicate the optimal choice (of chemotherapy) in particular cases. . . . efforts thus far have been disappointing."[1] Haddow did, nevertheless, mention his results with the first chemical therapy for the treatment of any cancer—high-dose estrogen therapy. Haddow's work in 1944[4] showed that 25% of patients with advanced breast cancer treated with high doses of estrogen had clear responses. In 1944, the steroid estradiol was not available for therapeutics. Instead,

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synthetic estrogens called triphenylethylenes (made by Imperial Chemical Industries [ICI], now AstraZeneca) were used because they were cheap, effective, and long acting. Haddow noted "the extraordinary extent of tumor regression observed in perhaps 1% of postmenopausal cases has always been regarded as of *major theoretical importance* and it is a matter of *some disappointment that so much of the underlying mechanisms continue to elude us.*"[1] It should be stressed that Haddow's studies were a paradox, as a link between ovarian estrogen and breast cancer growth had already been established.[5-7] What was the mysterious anticancer mechanism of high doses of synthetic estrogens?

On the other side of the Atlantic in England, armed with a Medical Research Council Scholarship, I was struggling with a PhD thesis (1969 to 1972) entitled "Structure activity relationships of some substituted triphenylethylenes" at the University of Leeds. These estrogenic compounds had evolved into contraceptives or morning after pills, but had failed because they did the exact opposite in women—they induced ovulation.[8] No one was recommending a career studying triphenylethylenes in 1972; in fact, only after repeated failures did the Leeds University Medical School secure an examiner for my thesis. He was Arthur Walpole, PhD, who many years before had been interested in cancer therapy[9] but, in 1972, was Head of the Fertility Control program at ICI. He had discovered a triphenylethylene derivative, ICI 46,474, a contraceptive in rats which failed in that indication in women. ICI 46,474 was a drug looking for an application. as an antiestrogen,[10] so it could possibly be useful as palliative therapy for advanced breast cancer. However, no laboratory studies then supported this indication.

From the age of 16, I was completely enthralled with organic chemistry, but I wanted to apply chemical therapy to treat cancer. This was a very unfashionable career choice in the 1970s (Table 1) and there were no career opportunities for me at that time. Only a 2-year appointment at the Worcester Foundation for Experimental Biology in Massachusetts to work with Mike Harper (the other patent holder of ICI 46, 474) would change everything. Harper had left the Foundation when I arrived in September 1972, and I was told that I could do anything I wanted for 2 years. I chose to call Arthur Walpole about converting ICI 46,474 into a breast cancer drug but targeted to estrogen receptor (ER)–positive disease in patients.[11] What I did not know at the time was that the administration at ICI had terminated the clinical development program but Walpole had threatened to resign unless the orphan project went forward.[11,12] My call, and our friendship, secured funding to conduct the first systematic laboratory study of the potential applications of ICI 46,474 as a targeted anticancer agent.[12] No studies in this area other than antifertility studies were conducted by ICI staff. The subsequent continuing investment by ICI Pharmaceuticals Division in my laboratory at the University of Leeds (Pharmacology Department, 1974 to 1979) would shape the clinical application of tamoxifen as a long-term adjuvant therapy[13,14] targeted to the ER[15] and as the first agent approved to reduce the incidence of any cancer in high risk pre- and postmenopausal women.[16-19]

Table 1. Clinical Situation in 1972 for the Treatment of Breast Cancer

Treatment	Fact
Cytotoxic chemotherapy	An appropriate strategy to kill cancer cells
	Kills all rapidly replicating cells with no targeting to cancer
Estrogen receptor	Not yet a target for antiestrogenic drugs or even an established predictive test for endocrine ablation for breast cancer treatment
	Antiestrogens are failed contraceptives
Chemotherapy	The way to cure cancer

TRANSITION TO TAMOXIFEN

A number of laboratory principles were defined in the 1970s during the evaluation of tamoxifen's antitumor pharmacology. These principles would ultimately have implications for the successful application of tamoxifen as an adjuvant therapy and as a chemopreventive agent in women at high risk for breast cancer. At that time, the principles as a whole were not embraced by the clinical community primarily because nearly all hopes were pinned on combination cytotoxic chemotherapy to cure both metastatic breast cancer and node-positive breast cancer.[20] A palliative "hormone" (as tamoxifen was then classified) was unlikely to provide benefit. The key to success was the application of the antiestrogen to patients with a potentially responsive tumor (ER positive), with micrometastatic disease (stage I/II) but for the appropriate duration of adjuvant treatment.

In the 1960s, there was sufficient evidence to conclude that some breast cancers grew in response to estrogenic hormones.[21] The discovery of the ER[22] and the development of the ER assay[21] to predict which patients would not respond to endocrine ablative surgery became an important practical advance. The idea was simple. Patients whose tumors had no ERs would not respond to estrogen withdrawal because estrogen was not required for tumor growth. An unnecessary ablative operation (oophorectomy, adrenalectomy, or hypophysectomy) would be avoided.[23] At that time, the clinical application of nonsteroidal antiestrogen (triphenylethylene derivatives) as breast cancer therapies were disappointing with numerous toxic adverse effects,[11] except for ICI 46,474.[24,25]

Lois Trench was the first drug monitor for ICI 46,474 in the United States, and in general, she played a pivotal role in the development of tamoxifen. Specifically, she arranged for ER-positive breast tumors to be dispatched to my laboratory at the Worcester Foundation. I also went to Elwood Jensen's laboratory at the Ben May Laboratory for Cancer Research (University of Chicago) to learn sucrose density gradient analysis to measure ERs in breast tumors and to learn how to create hormone-dependent tumors in rats by the oral administration of the mammary carcinogen dimethylbenzanthracene (DMBA).[26] Armed with these techniques, I returned to the Worcester Foundation and, with resources from ICI Americas, my laboratory demonstrated that tamoxifen blocked estrogen binding to the human tumor ER[15] and that two sustained release injections of tamoxifen would almost completely prevent rat mammary carcinogenesis.[16,17] Lois Trench arranged for me to introduce tamoxifen first to the Eastern Cooperative Oncology Group in 1974,[27,28] and I was subsequently asked to introduce the pharmacology of tamoxifen to the National Surgical Adjuvant Breast and Bowel Project in 1976.[29] This started an association with both organizations that developed the idea of long-term adjuvant tamoxifen therapy[30-32] and more recently, breast cancer risk reduction with the selective ER modulators (SERMs) tamoxifen and raloxifene.[33]

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CLINICAL OUTCOMES WITH TAMOXIFEN

The idea that tamoxifen should be applied as a long-term adjuvant therapy for patients with ER-positive primary breast cancer was first publicly presented in the United Kingdom at Cambridge University in September, 1977[34] and subsequently at the second Adjuvant Therapy of Cancer Meeting in Tucson, AZ, in 1979.[35] The specific conclusion, based on the DMBA model system, was that long-term tamoxifen was the most effective suppressant of occult mammary tumor growth and short-term therapy was unlikely to be effective in clinical trial. At that time, in the mid-1970s, there were sincere concerns that only short-term therapy with tamoxifen should be tested because the drug was effective only in 30% of unselected patients and the average duration of the response was only about 1 year. Longer therapy was "guaranteed" to encourage the rapid development of drug resistance in the occult micrometastases. Michael Baum, who led the NATO group, (Nolvadex Adjuvant Trial Organization, but called NATO to enhance the likelihood that US clinicians would read the papers in the erroneous belief that it was a US clinical trials organization) was the first to report that 2 years of tamoxifen enhanced survival of unselected patients with breast cancer.[36] However, it was the report from the Scottish Trials Office[37] (by coincidence, on my birthday, July 25, 1987) that definitively showed a remarkable survival advantage for unselected women who received 5 years of adjuvant tamoxifen compared with a control group who only received tamoxifen on disease recurrence. Longer was better than shorter therapy, as none of the 1-year adjuvant trials showed a survival benefit; only the overview analysis of randomized clinical trials showed a clear pattern of success for the laboratory concept, especially in premenopausal women with ER-positive breast cancer.[14,38]

Interest in developing a strategy to address the chemoprevention of breast cancer grew and evolved during the early years of the 1980s.[39] However, based on the laboratory data with the DMBA-induced rat mammary carcinoma model[16,17] and the subsequent finding that tamoxifen inhibited the development of contralateral primary breast cancer,[40] Trevor Powles, at the Royal Marsden Hospital in England, initiated the first pilot study in high-risk women[41] to ascertain volunteer compliance and to eventually address issues of cardiovascular and gynecological safety and the effects of tamoxifen on bone density.[42-44] In contrast, studies conducted at the Wisconsin Comprehensive Cancer Center followed the translational research path from the laboratory to the clinic (see SERM: Laboratory Observations to Clinical Practice). Overall, the published safety data (with the exception of tamoxifen-induced rat liver cancer[45-48]) translated from the laboratory[10,49-51] to patients[41,48,52-54] and provided an appropriate basis to advance chemoprevention trials. Although the Fisher et al study[18,19] was definitive and the most comprehensive, several smaller studies supported the general conclusions that tamoxifen reduced the risk of breast cancer, not only during treatment[55] but for perhaps a decade thereafter when drug-related adverse effects are minimal.[19,56,57]

What has been learned through the experience of adjuvant tamoxifen treatment is that compliance is essential to receive the full benefit of long-term therapy, and that longer therapy is better than shorter therapy.[14,38] Early studies demonstrated that metabolic tolerance to long-term adjuvant tamoxifen treatment does not occur even after a decade of treatment.[30,58] In other words, tamoxifen does not get metabolized to estrogen-like metabolites or become rapidly excreted. However, there are wide interpatient variations in circulating levels of both tamoxifen and metabolites, which this has been a mystery until recently. Hot flashes, or other menopausal symptoms, are the main reason for stopping therapy prematurely, but as it turns out, menopausal symptoms are associated with a good prognosis and with an improved control of disease recurrence.[59,60]

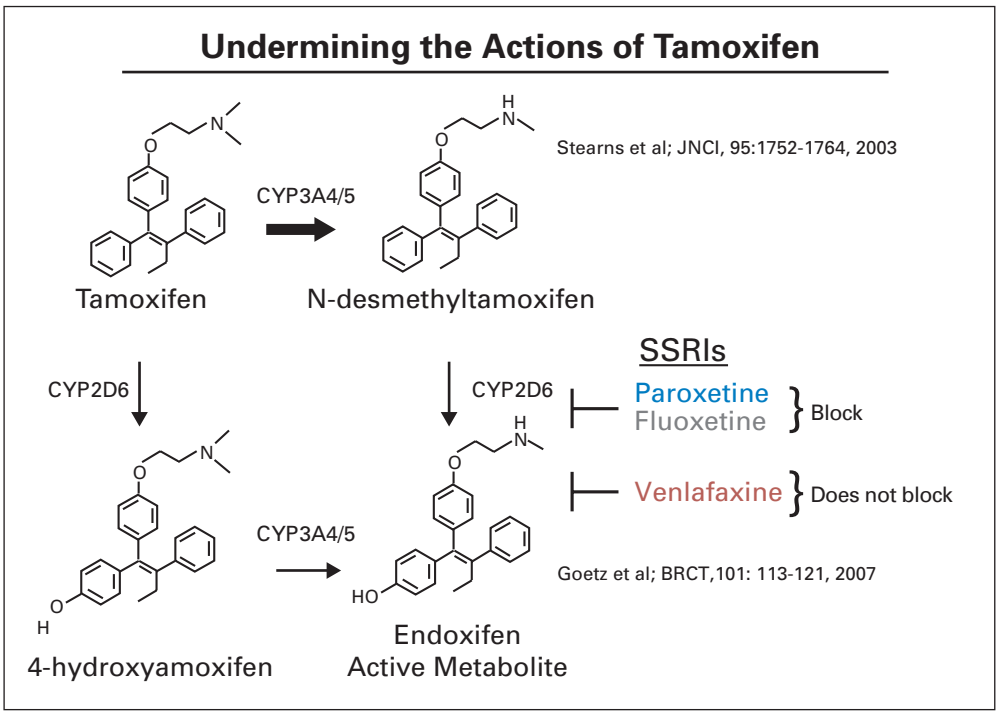

Fig 1. The metabolism of tamoxifen and the potential of selective serotonin reuptake inhibitors (SSRIs) to block metabolism of tamoxifen to endoxifen. Venlafaxine has a low affinity for the *CYP2D6* gene product so this is the agent of choice to block hot flashes.

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The metabolites of tamoxifen are antiestrogenic (Fig 1) and the conversation of tamoxifen to 4-hydroxytamoxifen is an advantage—but not a requirement—for antiestrogenic activity.[61,62] 4-hydroxytamoxifen continues to be an important laboratory tool for the laboratory study of antiestrogen action[63,64] and has been used to study the crystal structure of the ER with estrogens and antiestrogens.[65] However, a related metabolite endoxifen or 4-hydroxy-N-desmethyl tamoxifen[66] is the major antiestrogenic metabolite of tamoxifen in patients and is produced by the enzyme CYP2D6 (Fig 1).[67] Variants of the enzyme can either increase or decrease tamoxifen metabolism in patients producing more or less endoxifen. It is believed that elevated endoxifen can cause hot flashes which may suggest that the application of a selective serotonin reuptake inhibitor (SSRI) to alleviate these symptoms would be a reasonable course of action to maintain patient compliance. However, certain SSRIs, such as fluoxetine and paroxetine, block CYP2D6 and are contraindicated for patients taking adjuvant tamoxifen (Fig 1).[68-70] Venlafaxine is the SSRI of choice because it has a low affinity for CYP2D6. The general principle is to ensure appropriately high levels of endoxifen are produced to provide the best chance for therapeutic success with tamoxifen (Fig 1).

SERM: LABORATORY OBSERVATIONS TO CLINICAL PRACTICE

The received wisdom in the 1980s was that estrogen could prevent both osteoporosis and coronary heart disease (the latter was subsequently proven to be incorrect in the Women's Health Initiative nearly two decades later).[71] The proposed clinical evaluation of tamoxifen, a so-called antiestrogen, as a chemopreventive in healthy pre- and postmenopausal women, raised the concern that an antiestrogen would prevent the development of breast cancer, but increase the risk of crushing osteoporosis and death from coronary heart disease. In my laboratory at the Wisconsin Comprehensive Cancer Center (Madison, WI), we initiated a program to evaluate the pharmacology of tamoxifen so we could predict the extent of toxic adverse effects in subsequent clinical trials. At that time, we were positioning the overall program at Wisconsin to conduct a chemoprevention study.

We discovered that tamoxifen exhibited target site-specific actions as an estrogen in the mouse uterus[72] and human endometrial cancer,[50] as an antiestrogen in rat mammary carcinogenesis[13,17,73] and in human breast cancer cells,[72] but was an estrogen-like drug able to preserve bone density in ovariectomized rats.[49] Our findings that the target-specific action of tamoxifen-induced endometrial cancer growth[50] had immediate clinical consequences that were to improve health care.[74,75] The public discussions that followed caused clinical trials organizations to evaluate their emerging data. An elevated incidence of endometrial cancer in postmenopausal patients was noted in those women who received tamoxifen.[51,76] Initially, the description of this adverse effect caused unprecedented concern that there would be a high incidence of poor-grade endometrial cancer,[77] but the results of Fisher et al's chemoprevention study[18] clearly demonstrated that there was no elevation in endometrial cancer in premenopausal women, but a four- to five-fold increase in endometrial cancer with good grade (early detection) in postmenopausal women. The involvement of gynecologists in the treatment plan for breast cancer provided the necessary safeguards for patients. Overall, it is now established that the benefits of long-term adjuvant tamoxifen treatment far outweigh the risks of endometrial cancer,[14,38] but it was clear even in 1989 that an alternative approach to chemoprevention was necessary.[78,79] The idea was simple: "We have obtained valuable clinical information about this *group* of drugs that can be applied in other disease states. Research does not travel straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high-risk group to prevent breast cancer."[79]

This strategic prediction was not made in isolation. We had already completed laboratory studies with a chemical cousin of tamoxifen, called keoxifene, to show it prevented rat mammary carcinogenesis[73] and almost completely blocked tamoxifen-stimulated endometrial cancer growth[80] but prevented bone loss in ovariectomized rats.[49,81] However, at that time in 1990, nobody cared.

KEOXIFENE RESURRECTED AS RALOXIFENE

The compound known as LY156758 or keoxifene[82] started life as an antiestrogen and all initial efforts in testing were focused on an application as a breast cancer drug. It was to be a competitor for tamoxifen. However, keoxifene failed in that application[83] because the drug group has poor bioavailability[84] and crossresistance with tamoxifen.[85] As with tamoxifen, keoxifene was a drug looking for an application. Scientists at Eli Lilly eventually confirmed[86] the earlier results that keoxifene preserved bone density[49] and like tamoxifen[10] also lowered circulating cholesterol (tamoxifen already had a patent as a hypocholesteremic agent[11]).

The trial Multiple Outcomes of Raloxifene Evaluation (MORE) addressed the hypothesis that raloxifene could reduce the incidence of fractures in high-risk osteoporotic postmenopausal women. The results showed raloxifene did reduce spinal fractures by approximately 50% during the 3-year treatment period.[87] Raloxifene was the first SERM approved to treat and prevent women at risk for osteoporosis. The second preplanned evaluation was breast and endometrial safety. I was the chair of the Oncology Advisory Committee established to monitor breast cancer incidence. We found a significant 70% decrease after 3 years of raloxifene[88] in the incidence of breast cancers and after 4 years[89] of raloxifene treatment for osteoporosis. A subsequent evaluation of a placebo-controlled trial called Raloxifene Use for the Heart (RUTH), designed to evaluate the cardio protective actions of the SERM,[90] also noted a significant decrease in invasive breast cancer incidence and more importantly, both MORE[88] and RUTH[90] showed no elevation in endometrial cancers. However, the RUTH trial showed no improvement or benefit for patients at risk for dying from cardiac disease if they took raloxifene.[90]

As a public health intervention, the original proposal[78,79] that a SERM used to prevent osteoporosis in women at risk for osteoporosis could simultaneously reduce the incidence of breast cancer appears to be valid. With the current shift in the prescribing of hormone replacement therapy in the wake of the Women's Health Initiative[71] in the

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United States and the Million Women's Study[91] in the United Kingdom, a decrease in the incidence of ER-positive breast cancer has been noted by Ravdin.[92] With the availability of raloxifene as long-term therapy to treat and prevent osteoporosis, it is clear that there will potentially be a reduction in breast cancer incidence in the general population. This anticipated decrease in breast cancer incidence with long-term raloxifene use is evidenced by the data published by Martino et al.[93] These data were recently used to estimate decreases in breast cancer incidence in large populations of women not identified as at risk for breast cancer.[94]

The good safety and efficacy profile for raloxifene made it the agent of choice to compare head-to-head against tamoxifen in the Study of Tamoxifen and Raloxifene (STAR) to reduce breast cancer incidence in postmenopausal women deemed at high risk. Norman Wolmark invited me to be the scientific chair on the STAR trial advisory board just in case there were any toxicological or pharmacologic surprises. None occurred. Overall, the results[33] were another important step forward in chemoprevention; tamoxifen and raloxifene reduced the incidence of breast cancer equally, but the safety profile of raloxifene is superior. Based on the clinical trials,[19,33,55,93,95] it is now possible to summarize progress in chemoprevention (Table 2[19,33,39,88,96]), because agents can now be applied selectively to patient populations. However, each agent has been reinvented and then transitioned from the laboratory through clinical trials to an advance in health care, a process that extended over 30 years. It is perhaps important to state that the prudent use of tamoxifen or raloxifene to reduce the risk of breast cancer in the appropriate groups of high risk women is an important advance in therapeutics. Regrettably, there is reluctance to use these approved agents within the high-risk population, but often this is because of misinformation about the risks as physicians are now in a position to pick the right agent for the right patient.

DRUG RESISTANCE TO SERMS

The acceptance of the concept of long-term antihormone therapy to target, treat, and prevent breast cancer[20] raised the specter of drug resistance to SERMs. Twenty years ago, my team took a long-term view by creating a whole range of breast and endometrial cancer models resistant to tamoxifen and raloxifene.[97-101] Our goal was to anticipate the clinical development of drug resistance and to understand mechanisms so that second-line therapies could be deployed rationally. The models were developed naturally by first establishing estrogen stimulated tumor growth in athymic mice followed by long-term SERM treatment to identify SERM-resistant tumors. All our models were retransplanted into subsequent generations of mice so that the impact of long-term SERM therapy could be evaluated in hormone-responsive breast and endometrial cancer. What is unique about SERM resistance is that both breast and endometrial tumors grow in response to either SERMs or estrogen. No estrogen (mimicking aromatase inhibitor treatment) or the use of a pure antiestrogen (ICI 164,384[102] or fulvestrant[103,104]) prevent SERM resistant tumor growth. This is why aromatase inhibitors or fulvestrant are effective second-line therapies after tamoxifen failure.[105,106]

However, the early models of SERM resistance did not reflect the majority of clinical experience. The natural laboratory models developed during a year of therapy[97,107] and therefore reflected drug resistance in patients with metastatic breast cancer who are only treated successfully for 1 year. In other laboratories, ER-positive models were developed that were engineered by stable transfection of the HER2/*neu* gene.[108,109] These tumors are resistant to tamoxifen but reflect a small subset of clinical disease, including ER/HER2/*neu*–positive breast cancer. We took the strategic decision to determine what would occur if breast tumors were retransplanted into successive generations of tamoxifen stimulated mice for 5 years or more (ie, to replicate the actual clinical conditions employed during long-term adjuvant therapy). Remarkably, drug resistance evolves (Fig 2[99,110]) and the survival signaling pathway in tamoxifen resistant tumors becomes reorganized so that instead of estrogen being a survival signal, physiologic estrogen now inhibits tumor growth. This discovery[99,111] provided an invaluable insight into the evolution of drug resistance to SERMs and prompted the reclassification of the process through phase I (SERM/estrogen stimulated growth) and phase II (SERM stimulated growth estrogen inhibited growth). This new knowledge now provides an opportunity to treat patients with low-dose estrogen after exhaustive antihormone therapy.

NEW BIOLOGY OF ESTROGEN ACTION: CLINICAL TRANSLATION

The apoptotic action of physiological estrogen to cause dramatic tumor regression of long-term tamoxifen-resistant ER-positive breast cancers grown in athymic mice[99,111] was subsequently extended to long-term raloxifene resistance[112] and ER-positive breast cancer cells maintained in an estrogen-deprived environment for prolonged periods.[110,113-116] Most importantly, the apoptotic results observed with estrogen-deprived cells were noted both in vitro and in vivo by inoculation into athymic mice.[110]

Mechanistic studies, using our unique laboratory models, demonstrate that the antihormone resistant cells have reconfigured the ER signal transduction pathway so despite the fact that the ER still regulates the appropriate estrogen-regulated genes (including pS_2 and *myc*)[117] there is a profound effect of estrogen to activate the fas (death)

Table 2. Practice of Prevention 2008[19,33,39,88,94,96]

Drug	Group/Reason	Advantage
Tamoxifen	High-risk postmenopausal women	No increase in blood clots or endometrial cancer
Raloxifene	High-risk premenopausal women	No increase in endometrial cancer
	Treatment and prevention of osteoporosis	Reduction in the risk of breast cancer and no increase in endometrial cancer

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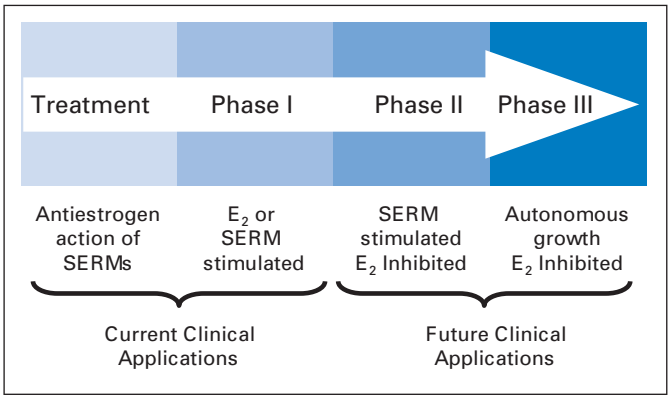

Fig 2. Evolution of drug resistance to selective estrogen receptor modulations (SERMs). Acquired resistance occurs during long-term treatment with a SERM and is evidenced by SERM-stimulated breast tumor growth. Tumors also continue to exploit estrogen for growth when the SERM is stopped, so a dual signal transduction process develops. The aromatase inhibitors prevent tumor growth in SERM-resistant disease and fulvestrant that destroys the estrogen receptor (ER) is also effective. This phase of drug resistance is referred to as phase I resistance. Continued exposure to a SERM results in continued SERM-stimulated growth, but eventually autonomous growth occurs that is unresponsive to fulvestrant or aromatase inhibitors. The event that distinguishes phase I from phase II acquired resistance is a remarkable switching mechanism that now causes apoptosis, rather than growth, with physiologic levels of estrogen. These distinct phases of laboratory-drug resistance[99,110] have their clinical parallels and this new knowledge is being integrated into the treatment plan.

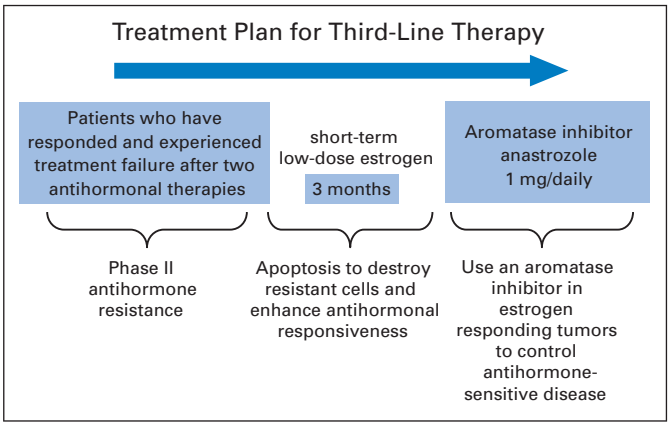

Fig 3. Clinical protocol to investigate the efficacy of estradiol induced apoptosis in long-term endocrine refractory breast cancer. An anticipated treatment plan for third-line endocrine therapy. Patients must have responded and experience treatment failure with two successive antihormone therapies to be eligible for a course of low-dose estradiol therapy for 3 months. The anticipated response rate is 30%[121] and responding patients will be treated with anastrozole until relapse. Validation of the treatment plan will establish a platform to enhance response rates with apoptotic estrogen by integrating known inhibitors of tumor survival pathways into the 3-month debulking "estrogen purge". The overall goal is to increase response rates and maintain patients for longer on antihormone strategies before chemotherapy is required.

receptor system[115,118] or to alternatively have a direct effect on mitochondrial function via the bcl2 system.[111,119] Thus, an understanding of the paradoxical actions of estrogen has emerged that depend on the state of estrogen deprivation of the breast cancer cell. In an estrogen rich environment, the estradiol-ER complex is a survival system promoting tumor growth. In contrast, in an estrogen-deprived environment (treatment with tamoxifen or an aromatase inhibitor) estrogen action is replaced by internal survival signaling based on the selection of cells with enhanced growth factor receptors. The growth factor receptors[120] initiate cascades that phosphorylate either unoccupied ER or ER liganded by SERMs. This model would also explain the earlier observations why high-dose estrogen therapy was only effective as a treatment for breast cancer in women many years after the menopause.[1] Natural estrogen deprivation had occurred. The process is accelerated and enhanced, however, in patients treated long-term with SERMs or aromatase inhibitors so that only low doses of estrogen are necessary to cause experimental tumors to regress. The question now becomes, can this new laboratory knowledge be translated to patient care?

Several clinical trial groups are currently addressing this issue. In our own case, we are recruiting patients with metastatic breast cancer who have succeeded and experienced treatment failure with at least two successive endocrine therapies (Fig 3) and we are determining the efficacy of a 12-week purge of high-dose estradiol (30 mg daily) therapy. The goal is to confirm and extend the previously study published by Lonning and colleagues[121] and then to determine the minimum dose of estradiol necessary to induce the anticipated 30% response rate.[121] Based on our previous laboratory studies,[99] we propose to retreat responding patients with anastrozole to determine efficacy.

Overall, our clinical program is part of a multi-institutional Center of Excellence grant BCO50277 entitled "A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis" that will map the survival and death pathways of our models and integrate clinical material to determine the validity of the laboratory-derived molecular mechanisms and, ultimately, to address the issue of why the majority of tumors do not respond to estrogen alone. Knowledge of the new apoptotic biology of estrogen could be enhanced in the future in much the same way as the modest responses initially observed were enhanced to benefit patients with tamoxifen and raloxifene. The philosophy is to deploy the right treatment at the right time and for the right patient.

PROGRESS IN TREATING DISEASE?

In closing, it is perhaps pertinent to re-examine Haddow's comments delivered during the first David A. Karnofsky lecture in 1970. He saw little evidence that specific chemical therapies could be developed and there was really no predictive test to identify tumors that could respond to a chemical therapy. The idea of a targeted drug was to be advanced soon thereafter during the 1970s[20] when the ER assay evolved from being a predictive test for endocrine ablation to become the target for a failed contraceptive to be reinvented as tamoxifen and to be used for long durations in the treatment and prevention of breast cancer.[11] However, translational research does not travel in straight lines: one needs luck so the unanticipated can be integrated into the treatment plan and perhaps, if one is lucky, new innovations in therapy can be developed.

SERM was unanticipated and much luck led to progress in treatment. Issues over the increased risk of endometrial cancer caused by tamoxifen treatment coupled with the recognition that the drug group called the nonsteroidal antiestrogens[122] could enhance bone density in animals[49,123] and man[54] opened the door for the development of raloxifene[81] as the first SERM for the treatment and prevention of osteoporosis as well as the reduction of risk for breast cancer,[33,88] but

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with no increase in endometrial cancer risk. Chemoprevention has now extended from an idea[16,17,124] to a clinical reality (Table 2).

The enormous impact that tamoxifen has had on the treatment of breast cancer for 25 years (1978 to 2003) naturally encouraged efforts to improve treatment responses and reduce the adverse effects noted with tamoxifen.[125] This goal has been achieved with the introduction of a range of aromatase inhibitors for the treatment of breast cancer in postmenopausal women.[125,126] The principles of treatment remain the same: targeting the ER and then employing long-term therapy now for perhaps up to 15 years to exploit the trend observed in MA-17 (tamoxifen followed by an aromatase inhibitor).[127] Tamoxifen surprisingly did not go away, but remains the treatment of choice for premenopausal women with breast cancer, the appropriate agent for risk reduction in premenopausal women, a major drug of interest for the study of pharmacogenomics, and the major life-saving antihormone in countries throughout the world that do not have the sophisticated and wealthy health care system we have in the United States. Furthermore, the laboratory principle from the 1970s that "longer is better" for adjuvant therapy[13,128] continues to be evaluated in the Adjuvant Tamoxifen Long Against Short (ATLAS) trial that compares 10 years of tamoxifen with 5 years of tamoxifen. If 10 years of tamoxifen treatment is superior to 5 years, then the public health impact will be profound as this cheap and easily accessible drug can continue to provide benefit in lives saved. The current approaches and advances in the antihormone therapy of breast cancer are summarized in Figure 4.

Finally, the paradox of estrogen action in dictating the survival or death of breast cancer cells has become transparent, closing a circle of knowledge left hanging in the wake of Haddow's Karnofsky presentation in 1970.[1] The dramatic results he observed with high-dose estrogen therapy in a small fraction of women[1] was a powerful testament to the potential of chemical therapy. Unfortunately, there was no knowledge about the mechanisms to further exploit the concept. Fashions in therapy began to move toward blocking estrogen action and shifted from the more toxic high doses of estrogen to the less toxic but equally efficacious tamoxifen.[129] Now we find ourselves returning to the beginning of "chemical therapy" because unusual and unanticipated laboratory observations were placed on the web of knowledge. This knowledge has remained dormant until it could now be called to the center of the web when the fashion in research again changes. The discovery of apoptosis as a natural process to destroy aberrant cells[130] would probably have never be linked in the same sentence with "hormone" therapy. However, it is now clear that antihormone drug resistance can reprogram some hormone responsive cancer cells to be supersensitive to the apoptotic actions of physiological estrogen.[99,111] These tantalizing laboratory observations now provide another opportunity for chemical therapy to aid patients. The knowledge is already finding its way into clinical trials, so that in the future it may be possible that the antihormone resistant disease from select patients can be destroyed by an "estrogen purge" and then patients could again be maintained for a longer period on an antihormone therapy.

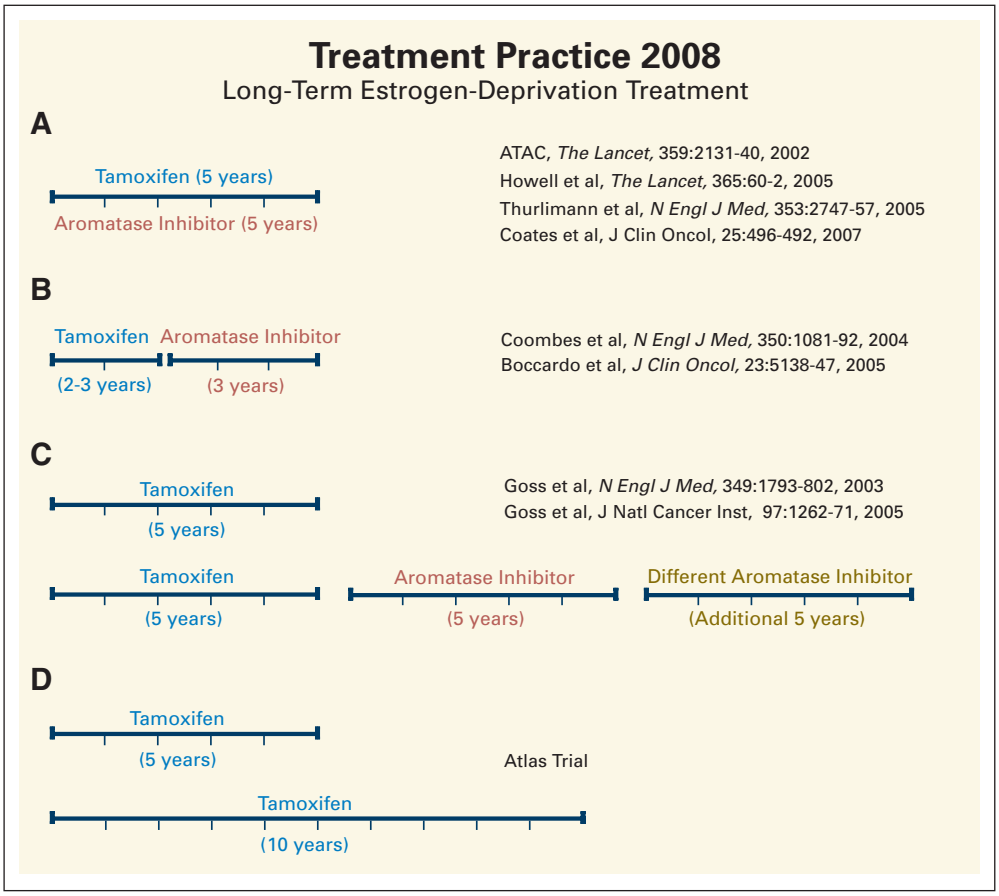

Fig 4. Adjuvant antihormone strategies for the treatment of estrogen receptor–positive breast cancer.[126,127] ATLAS, Adjuvant Tamoxifen Long Against Short.

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We have perhaps researched the zenith of our abilities to manipulate the ER with our current armamentarium. So, is this then the end of our story? Certainly not. There is much still to be accomplished. The SERM concept has now been extended to include all members of the steroid receptor superfamily[20,131] so that in the future diseases may be selectively treated that until now had been thought to be untreatable. New specific medicines are now being developed to achieve this goal.[131,132] But, where could the estrogen-induced apoptosis story take us? It may be that the modest results observed in select sensitive patients with ER-positive metastatic breast cancer could be amplified by the prudent use of selective survival inhibitors. If the cancer cell is prevented from surviving, then perhaps the mild estrogen apoptotic trigger will kill more tumor cells. Indeed, if we can work out how the ER complex naturally seeks out its intracellular trigger, then perhaps that trigger could be the next target for chemical therapy for a range of cancers beyond breast cancer.

AUTHOR'S DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

REFERENCES

1. Haddow A, David A: Karnofsky memorial lecture: Thoughts on chemical therapy. Cancer 26: 737-754, 1970

2. Baumler E: Paul Ehrlich, Scientist for Life. New York, New York, Holmes & Meier, 1984

3. Schrek R: Fashions in cancer research, in The Year Book of Pathology and Clinical Pathology. Chicago, IL, The Year Book Publishers, 1959, pp. 26-39

4. Haddow A, Watkinson JM, Paterson E: Influence of synthetic oestrogens upon advanced malignant disease. BMJ 2:393-398, 1944

5. Beatson GT: On the treatment of inoperable cases of carcinoma of the mamma: Suggestions for a new method of treatment with illustrative cases. Lancet 2:104-107, 1896

6. Boyd S: On oophorectomy in cancer of the breast. BMJ ii:1161-1167, 1900

7. Lacassagne A: Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer 27:217-225, 1936

8. Klopper A, Hall M: New synthetic agent for the induction of ovulation: Preliminary trial in women. BMJ 1:152-154, 1971

9. Jordan VC: The development of tamoxifen for breast cancer therapy: A tribute to the late Arthur L. Walpole. Breast Cancer Res Treat 11:197-209, 1988

10. Harper MJ, Walpole AL: A new derivative of triphenylethylene: Effect on implantation and mode of action in rats. J Reprod Fertil 13:101-119, 1967

11. Jordan VC: Tamoxifen: A most unlikely pioneering medicine. Nature Reviews Drug Discovery 2:205-213, 2003

12. Jordan VC: Tamoxifen (ICI 46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 147:S269–S276, 2006

13. Jordan VC, Allen KE: Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239-251, 1980

14. EBCTCG: Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. Lancet 365:1687-1717, 2005

15. Jordan VC, Koerner S: Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 11:205-206, 1975

16. Jordan VC: Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 5:354, 1974

17. Jordan VC: Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA- induced rat mammary carcinoma. Eur J Cancer 12:419-424, 1976

18. Fisher B, Costantino JP, Wickerham DL, et al: Tamoxifen for prevention of breast cancer: Report of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 90:1371-1388, 1998

19. Fisher B, Costantino JP, Wickerham DL, et al: Tamoxifen for the prevention of breast cancer: Current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97:1652-1662, 2005

20. Jordan VC: Tamoxifen: Catalyst for the change to targeted therapy. Eur J Cancer 44:30-38, 2008

21. Jensen EV, Jordan VC: The estrogen receptor: A model for molecular medicine: The Dorothy P: Landon AACR Prize for Translational Research. Clin Cancer Res 9:1980-1989, 2003

22. Jensen EV, Jacobson HI: Basic guides to the mechanism of estrogen action. Recent Progr Hormone Res 18:387-414, 1962

23. McGuire WL, Carbone PP, Sears ME, et al: Estrogen receptors in human breast cancer: An overview, in McGuire WL, Carbone PP, Volmer EP (eds): Estrogen Receptor in Human Breast Cancer. New York, New York, Raven Press, 1975, pp. 1-7

24. Cole MP, Jones CT, Todd ID: A new anti-oestrogenic agent in late breast cancer: An early clinical appraisal of ICI 46474. Br J Cancer 25:270-275, 1971

25. Ward HW: Anti-oestrogen therapy for breast cancer: A trial of tamoxifen at two dose levels. BMJ 1:13-14, 1973

26. Huggins C, Grand, LC, Brillantes, FP.: Mammary cancer induced by a single feeding of polynuclear hydrocarbons and their suppression. Nature 189:204-207, 1961

27. Jordan VC: The antioestrogen tamoxifen (ICI 46,474) as an antitumour agent. Proceedings Eastern Cooperative Oncology Group Meeting, Miami, FL, February 11-12, 1974

28. Jordan VC: Tamoxifen mechanism of antitumour activity in animals and man. Proceedings Eastern Cooperative Oncology Group Meeting, Jasper, Alberta, Canada, June 22-25, 1974

29. Jordan VC: Antiestrogenic and antitumor properties of tamoxifen in laboratory animals. Cancer Treat Rep 60:1409-1419, 1976

30. Tormey D, Jordan VC: Long-term tamoxifen adjuvant therapy in node-positive breast cancer: A metabolic and pilot clinical study. Breast Cancer Res Treat 4:297-302, 1984

31. Fisher B, Brown A, Wolmark N, et al: Prolonging tamoxifen therapy for primary breast cancer: Findings from the National Surgical Adjuvant Breast and Bowel Project clinical trial. Ann Intern Med 106:649-654, 1987

32. Falkson HC, Gray R, Wolberg WH, et al: Adjuvant trial of 12 cycles of CMFPT followed by observation or continuous tamoxifen versus four cycles of CMFPT in postmenopausal women with breast cancer: An Eastern Cooperative Oncology Group phase III study. J Clin Oncol 4:559-607, 1990

33. Vogel VG, Costantino JP, Wickerham DL, et al: The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 trial. JAMA 295:2727-2741, 2006

34. Jordan VC: Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. Reviews on Endocrine-Related Cancer 49-55, 1978

35. Jordan VC, Dix CJ, Allen KE: The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. Adjuvant Therapy of Cancer 2:19-26, 1979

36. Baum M, Brinkley DM, Dossett JA, et al: Improved survival among patients treated with adjuvant tamoxifen after mastectomy for early breast cancer. Lancet 2:450, 1983

37. Edinburgh SCTO: Adjuvant tamoxifen in the management of operable breast cancer: The Scottish Trial: Report from the Breast Cancer Trials Committee. Lancet 2:171-175, 1987

38. EBCTCG: Tamoxifen for early breast cancer: An overview of the randomised trials. Lancet 354: 1451-1467, 1998

39. Jordan VC: Tamoxifen or raloxifene for breast cancer chemoprevention: A tale of two choices–point. Cancer Epidemiol Biomarkers Prev 16: 2207-2209, 2007

40. Cuzick J, Baum M: Tamoxifen and contralateral breast cancer. Lancet 2:282, 1985

41. Powles TJ, Hardy JR, Ashley SE, et al: A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. Br J Cancer 60:126-131, 1989

42. Powles TJ, Jones AL, Ashley SE, et al: The Royal Marsden Hospital pilot tamoxifen chemoprevention trial. Breast Cancer Res Treat 31:73-82, 1994

43. Powles TJ, Hickish T, Kanis JA, et al: Effect of tamoxifen on bone mineral density measured by dual-energy x- ray absorptiometry in healthy premenopausal and postmenopausal women. J Clin Oncol 14:78-84, 1996

44. Kedar RP, Bourne TH, Powles TJ, et al: Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial. Lancet 343:1318-1321, 1994

45. Han XL, Liehr JG: Induction of covalent DNA adducts in rodents by tamoxifen. Cancer Res 52: 1360-1363, 1992

46. Greaves P, Goonetilleke R, Nunn G, et al: Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. Cancer Res 53:3919-3924, 1993

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47. Jordan VC, Morrow M: Should clinicians be concerned about the carcinogenic potential of tamoxifen? Eur J Cancer 30A:1714-1721, 1994

48. Jordan VC: Tamoxifen and tumorigenicity: A predictable concern. J Natl Cancer Inst 87:623-626, 1995

49. Jordan VC, Phelps E, Lindgren JU: Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10:31-35, 1987

50. Gottardis MM, Robinson SP, Satyaswaroop PG, et al: Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48:812-815, 1988

51. Fornander T, Rutqvist LE, Cedermark B, et al: Adjuvant tamoxifen in early breast cancer: Occurrence of new primary cancers. Lancet 1:117-120, 1989

52. Love RR, Newcomb PA, Wiebe DA, et al: Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 82:1327-1332, 1990

53. Love RR, Wiebe DA, Newcomb PA, et al: Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann Intern Med 115:860-864, 1991

54. Love RR, Mazess RB, Barden HS, et al: Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 326:852-856, 1992

55. Cuzick J, Powles T, Veronesi U, et al: Overview of the main outcomes in breast-cancer prevention trials. Lancet 361:296-300, 2003

56. Powles TJ, Ashley S, Tidy A, et al: Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. J Natl Cancer Inst 99:283-290, 2007

57. Cuzick J, Forbes JF, Sestak I, et al: Long-term results of tamoxifen prophylaxis for breast cancer–96-month follow-up of the randomized IBIS-I trial. J Natl Cancer Inst 99:272-282, 2007

58. Langan-Fahey SM, Tormey DC, Jordan VC: Tamoxifen metabolites in patients on long-term adjuvant therapy for breast cancer. Eur J Cancer 26: 883-888, 1990

59. Goetz MP, Rae JM, Suman VJ, et al: Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. J Clin Oncol 23:9312-9318, 2005

60. Mortimer JE, Flatt SW, Parker BA, et al: Tamoxifen, hot flashes and recurrence in breast cancer. Breast Cancer Res Treat 108:421-426, 2007

61. Jordan VC, Collins MM, Rowsby L, et al: A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305-316, 1977

62. Allen KE, Clark ER, Jordan VC: Evidence for the metabolic activation of non-steroidal antioestrogens: A study of structure-activity relationships. Br J Pharmacol 71:83-91, 1980

63. Lieberman ME, Gorski J, Jordan VC: An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741-4745, 1983

64. Tate AC, Greene GL, DeSombre ER, et al: Differences between estrogen- and antiestrogen-estrogen receptor complexes from human breast tumors identified with an antibody raised against the estrogen receptor. Cancer Res 44:1012-1018, 1984

65. Shiau AK, Barstad D, Loria PM, et al: The structural basis of estrogen receptor/co-activator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927-937, 1998

66. Lien EA, Solheim E, Kvinnsland S, et al: Identification of 4-hydroxy-N-desmethyltamoxifen as a metabolite of tamoxifen in human bile. Cancer Res 48:2304-2308, 1988

67. Desta Z, Ward BA, Soukhova NV, et al: Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: Prominent roles for CYP3A and CYP2D6. J Pharmacol Exp Ther 310:1062-1075, 2004

68. Stearns V, Johnson MD, Rae JM, et al: Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. J Natl Cancer Inst 95:1758-1764, 2003

69. Jin Y, Desta Z, Stearns V, et al: CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. JNCI 97:30-39, 2005

70. Borges S, Desta Z, Li L, et al: Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: Implication for optimization of breast cancer treatment. Clin Pharmacol Ther 80:61-74, 2006

71. Rossouw JE, Anderson GL, Prentice RL, et al: Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results from the Women's Health Initiative randomized controlled trial. JAMA 288:321-333, 2002

72. Jordan VC, Robinson SP: Species-specific pharmacology of antiestrogens: Role of metabolism. Fed Proc 46:1870-1874, 1987

73. Gottardis MM, Jordan VC: Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 47:4020-4024, 1987

74. Hardell L: Tamoxifen as risk factor for carcinoma of corpus uterus. Lancet ii:563, 1988

75. Jordan VC: Tamoxifen and endometrial cancer. Lancet 2:1019, 1988

76. Fisher B, Costantino JP, Redmond CK, et al: Endometrial cancer in tamoxifen-treated breast cancer patients: Findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. J Natl Cancer Inst 86:527-537, 1994

77. Magriples U, Naftolin F, Schwartz PE, et al: High grade endometrial carcinoma in tamoxifen treated breast cancer patients. J Clin Oncol 11:485-490, 1993

78. Jordan VC: Chemosuppression of breast cancer with tamoxifen: Laboratory evidence and future clinical investigations. Cancer Invest 6:589-595, 1988

79. Lerner LJ, Jordan VC: The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award lecture. Cancer Res 50:4177-4189, 1990

80. Gottardis MM, Ricchio ME, Satyaswaroop PG, et al: Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 50:3189-3192, 1990

81. Lewis JS, Jordan VC: Case histories: Raloxifene, in Taylor J, Triggle D (eds): Comprehensive Medicinal Chemistry II (ed 8). Oxford, United Kingdom, Elsevier Limited, 2007, pp. 103-121

82. Black LJ, Jones CD, Falcone JF: Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 32:1031-1036, 1983

83. Buzdar A, Marcus C, Holmes F, et al: Phase II evaluation of Ly156758 in metastatic breast cancer. Oncology 45:344-345, 1988

84. Jordan V, Gosden B: Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 113:463-468, 1983

85. O'Regan RM, Gajdos C, Dardes RC, et al: Effects of raloxifene after tamoxifen on breast and endometrial tumor growth in athymic mice. J Natl Cancer Inst 94:274-283, 2002

86. Black LJ, Sato M, Rowley ER, et al: Raloxifene (LY139481 HCl) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 93:63-69, 1994

87. Ettinger B, Black DM, Mitlak BH, et al: Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: Results from a 3-year randomized clinical trial Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 282:637-645, 1999

88. Cummings SR, Eckert S, Krueger KA, et al: The effect of raloxifene on risk of breast cancer in postmenopausal women: Results from the MORE randomized trial. JAMA 281:2189-2197, 1999

89. Cauley JA, Norton L, Lippman ME, et al: Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trialBreast Cancer Res Treat 65:125-134, 2001

90. Barrett-Connor E, Mosca L, Collins P, et al: Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 355:125-137, 2006

91. Million Women Study Collaborators: Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet 362:419-427, 2003

92. Ravdin PM, Cronin KA, Howlader N, et al: The decrease in breast-cancer incidence in 2003 in the United States. N Engl J Med 356:1670-1674, 2007

93. Martino S, Cauley JA, Barrett-Connor E, et al: For the CORE Investigators Continuing Outcomes Relevant to Evista: Breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 96:1751-1761, 2004

94. Jordan VC: Optimising endocrine approaches for the chemoprevention of breast cancer: Beyond the Study of Tamoxifen and Raloxifene (STAR) trial. Eur J Cancer 42:2909-2913, 2006

95. Cummings SR, Norton L, Eckert S, et al: Raloxifene reduces the risk of breast cancer and may decrease the risk of endometrial cancer in postmenopausal women: Two-year finding from the Multiple Outcomes of Raloxifene Evaluation (MORE) trial. Proc Am Soc Clin Oncol 2a:1998 (abstr 3)

96. Jordan VC: Chemoprevention of breast cancer with selective oestrogen receptor modulators. Nat Rev Cancer 7:46-53, 2007

97. Gottardis MM, Jordan VC: Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 48:5183-8187, 1988

98. Wolf DM, Langan-Fahey SM, Parker CJ, et al: Investigation of the mechanism of tamoxifen-stimulated breast tumor growth with nonisomerizable analogues of tamoxifen and metabolites. J Natl Cancer Inst 85:806-812, 1993

99. Yao K, Lee ES, Bentrem DJ, et al: Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6:2028-2036, 2000

100. Schafer JM, Lee ES, O'Regan RM, et al: Rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice. Clin Cancer Res 6:4373-4380, 2000

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101. Osipo C, Meeke K, Liu H, et al: Trastuzumab therapy for tamoxifen-stimulated endometrial cancer. Cancer Res 65:8504-8513, 2005

102. Gottardis MM, Jiang SY, Jeng MH, et al: Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res 49:4090-4093, 1989

103. Osborne CK, Coronado-Heinsohn EB, Hilsenbeck SG, et al: Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. J Natl Cancer Inst 87:746-750, 1995

104. Lee ES, MacGregor-Schafer JI, Yao K, et al: Cross resistance of triphenylethylene-type antiestrogen but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6:4893-4899, 2000

105. Osborne CK, Pippen J, Jones SE, et al: Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: Results of a North American trial. J Clin Oncol 20:3386-3395, 2002

106. Howell A, Robertson JFR, Quaresma Albano J, et al: Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. J Clin Oncol 20:3396-3403, 2002

107. Osborne CK, Coronado EB, Robinson JP: Human breast cancer in the athymic nude mouse: Cytostatic effects of long-term antiestrogen therapy. Eur J Cancer Clin Oncol 23:1189-1196, 1987

108. Benz CC, Scott GK, Sarup JC, et al: Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res Treat 24:85-95, 1992

109. Osborne CK, Bardou V, Hopp TA, et al: Role of the estrogen receptor coactivator AIB1 (SRC3) and HER2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst 95:353-361, 2003

110. Lewis JS, Meeke K, Osipo C, et al: Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. JNCI 97:1746-1759, 2005

111. Wolf DM, Jordan VC: A Laboratory Model to Explain the Survival Advantage Observed in Patients Taking Adjuvent Tamoxifen Therapy: Recent Results in Cancer. Res Heidelberg, Springer-Verlag, 1993, pp 23-33

112. Liu H, Lee ES, Gajdos C, et al: Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 95:1586-1597, 2003

113. Pink JJ, Jiang SY, Fritsch M, et al: An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. Cancer Res 55:2583-2590, 1995

114. Jiang SY, Wolf DM, Yingling JM, et al: An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol and Cell Endo 90:77-80, 1992

115. Song RX, Mor G, Naftolin F, et al: Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 93:1714-1723, 2001

116. Lewis JS, Osipo C, Meeke K, et al: Estradiol induced apoptosis in a breast cancer cell line resistant to estrogen deprivation. J Steroid Biochem 3:131-141, 2005

117. Osipo C, Meeke K, Cheng D, et al: Role for HER2/neu and HER3 in fulvestrant-resistant breast cancer. Int J Oncol 30:509-520, 2007

118. Osipo C, Gajdos C, Liu H, et al: Paradoxical action of fulvestrant on estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 95:1597-1607, 2003

119. Song RX, Zhang Z, Mor G, et al: Down-regulation of Bcl-2 enhances estrogen apoptotic action in long-term estradiol-depleted ER+ breast cancer cells. Apoptosis 10:667-668, 2005

120. Ali S, Coombes RC: Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer 2:101-112, 2002

121. Lonning PE, Taylor PD, Anker G, et al: High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat 67:111-116, 2001

122. Jordan VC: Biochemical pharmacology of antiestrogen action. Pharmacol Rev 36:245-276, 1984

123. Turner RT, Wakley GK, Hannon KS, et al: Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. J Bone Miner Res 2:449-456, 1987

124. Sporn MB: Approaches to prevention of epithelial cancer during the preneoplastic period. Cancer Res 36:2699-2702, 1976

125. Jordan VC, Brodie AM: Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 72:7-25, 2007

126. Goss PE, Strasser K: Aromatase inhibitors in the treatment and prevention of breast cancer. J Clin Oncol 19:881-894, 2001

127. Goss PE, Ingle JN, Martino S, et al: Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: Updated findings from NCIC CTG MA. 17. J Natl Cancer Inst 97:1262-1271, 2005

128. Jordan VC, Allen KE, Dix CJ: Pharmacology of tamoxifen in laboratory animals. Cancer Treat Rep 64:745-759, 1980

129. Ingle JN, Ahmann DL, Green SJ, et al: Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. N Engl J Med 304:16-21, 1981

130. Youle RJ, Strasser A: The BCL-2 protein family: Opposing activities that mediate cell death. Nature 9:47-59, 2008

131. Jordan VC, O'Malley BW: Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 25:5815-5824, 2007

132. Ariazi EA, Ariazi JL, Cordera F, et al: Estrogen receptors as therapeutic targets in breast cancer. Current Topics in Medicinal Chemistry 6:195-216, 2006

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A Century of Deciphering the Control Mechanisms of Sex Steroid Action in Breast and Prostate Cancer: The Origins of Targeted Therapy and Chemoprevention

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Abstract

The origins of the story to decipher the mechanisms that control the growth of sex hormone–dependent cancers started more than 100 years ago. Clinical observations of the apparently random responsiveness of breast cancer to endocrine ablation (hormonal withdrawal) provoked scientific inquiries in the laboratory that resulted in the development of effective strategies for targeting therapy to the estrogen receptor (ER; or androgen receptor in the case of prostate cancer), the development of antihormonal treatments that dramatically enhanced patient survival, and the first successful testing of agents to reduce the risk of developing any cancer. Most importantly, elucidating the receptor-mediated mechanisms of sex steroid–dependent growth and the clinical success of antihormones has had broad implication in medicinal chemistry with the synthesis of new selective hormone receptor modulators for numerous clinical applications. Indeed, the successful translational research on the ER was the catalyst for the current strategy for developing targeted therapies to the tumor and the start of "individualized medicine." During the past 50 years, ideas about the value of antihormones translated effectively from the laboratory to improve clinical care, improve national survival rates, and significantly reduced the burden of cancer. [Cancer Res 2009;69(4):1243–54]

Beginnings at the Dawn of the 20th Century

Schinzinger (1) is credited with suggesting that oophorectomy could be used to treat breast cancer; however, this suggestion did not seem to have been adopted. In contrast, the report by Beaston (2) that oophorectomy could initiate a regression of metastatic breast cancer in two premenopausal women was a landmark achievement. Although it is often stated that Beaston's work was empirical clinical research, the rationale to conduct an oophorectomy was, in fact, an example of early translational research. Beaston was aware of the essential role of removing the ovary in maximizing milk production in cows. He reasoned there was potentially some factor that traveled in the blood supply to the breast as there was no known connection through the nerves. Interestingly enough, he also conducted laboratory experiments in rabbits before his clinical experiment, so the work was bench-to-bedside (2). By 1900, Stanley Boyd (3) had assembled the results of all the available clinical cases of oophorectomy to treat breast cancer in Great Britain in perhaps the first "clinical trial." Boyd concluded that only one-third of metastatic breast tumors responded to oophorectomy. This clinical result and overall response rate has remained the same to this day.

Unfortunately, responses were of limited duration and enthusiasm waned that this approach was the answer to cancer treatment. The approach of endocrine ablation was only relevant to breast cancer (and subsequently prostate cancer; ref. 4), thus, the approach was only effective in a small subset of all cancer types. At the dawn of the 20th Century, there was no understanding of the endocrine system or hormones. Nevertheless, laboratory studies started to decipher the biological control mechanisms responsible for the clinical observations.

Links between Sex Steroids and Cancer

The trend in breast cancer research in the early years of the 20th century was to use inbred strains of mice to study the growth and incidence of spontaneous mammary cancer. Lathrop and Loeb (5) found that before age 3 months was the optimal time for oophorectomy to prevent the development of mammary cancer, but obviously, this knowledge could not be translated to the clinical setting; who would one treat? The mechanism was also unknown until Allen and Doisy (6), using an ovariectomized mouse vaginal cornification assay, showed that a principle, that they called estrogen (identified as estrone, the principal steroid), was present in ovarian follicular fluid. Their major advance set the scene for the subsequent breakthroughs in molecular endocrinology and therapeutics in the latter half of the 20th century (Fig. 1).

The idea that breast cancer might be a preventable disease was extended by Professor Antoine Lacassagne (7, 8) who first showed that estrogen could induce mammary tumors in mice. Lacassagne (9) hypothesized, "*If one accepts the consideration of adenocarcinoma of the breast as the consequence of a special hereditary sensibility to the proliferative action of oestrone, one is led to imagine a therapeutic preventive for subjects predisposed by their heredity to this cancer. It would consist—perhaps in the very near future when the knowledge and use of hormones will be better understood—in the suitable use of a hormone, antagonistic or excretory, to prevent the stagnation of oestrone in the ducts of the breasts.*" However, when Lacassagne stated his vision at the annual meeting of the American Association for Cancer Research in Boston in 1936, there were no lead compounds that antagonized estrogen action, but the Allen Doisy mouse assay could be used to study structure activity relationships to find synthetic estrogens. Within a decade, a landmark discovery was to occur in "chemical therapy" that was to expand the treatment of metastatic breast cancer to include postmenopausal women who are, in fact, the majority who develop metastatic disease.

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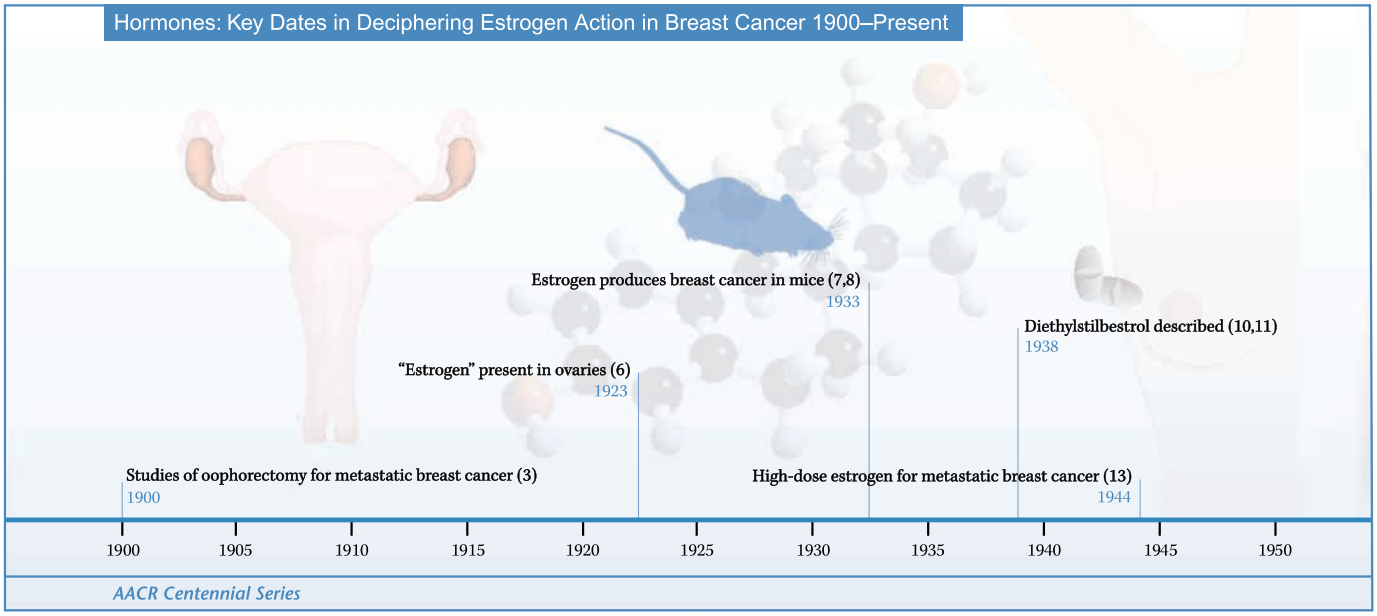

Figure 1. Timeline of the major landmarks in estrogen action and its application for the treatment and prevention of breast cancer.

During the 1930s, there were significant advances in the knowledge of the precise structural requirements for estrogen action in its target tissue, the vagina. Synthetic compounds based on stilbene (10, 11) and triphenylethylene (12) were screened using the Allen Doisy ovariectomized mouse vaginal cornification assay to define compounds with optimal structures and duration of estrogen action. Sir Alexander Haddow found that carcinogenic polycyclic hydrocarbons would cause tumor regression in animals. However, these could not be used to treat humans. The nonsteroidal triphenylethylene-based estrogens had similar structures to polycyclic hydrocarbons and also caused tumor regression in animals. With this clue, Sir Alexander Haddow (13) used the first chemical therapy to treat patients. His results published in 1944 showed that high-dose estrogen therapy was effective in causing tumor regressions in postmenopausal patients with breast cancer and men with prostate cancer. There was, however, no understanding of a mechanism. Indeed he stated in 1970: *"In spite of the extremely limited practicability of such measure [high dose estrogen], the extraordinary extent of tumor regression observed in perhaps 1% of postmenopausal cases has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us"* (14). These experimental data were also an apparent paradox as endocrine ablation to remove estrogens and their precursors was the dogma of the time (15).

In the past 50 years, the progress in deciphering the control mechanisms of estrogen action in breast cancer (and androgen action in prostate cancer), has accelerated with advances in technology and an understanding of cell biology. But progress in research does not travel in straight lines, yet chance observations can create a major breakthrough. This has happened repeatedly in the story of the treatment and prevention of breast cancer.

Conceptual Progress through Scientific Serendipity

It is perhaps relevant to illustrate a few astute observations by scientists that accelerated progress immensely in deciphering the complexities of hormone action and the control of breast cancer growth.

Sir Charles Dodds (11) is credited with the synthesis of the potent synthetic estrogen diethylstilbestrol (Fig. 2) that was subsequently used for the treatment of both prostate cancer and breast cancer, and regrettably was also applied to prevent recurrent abortions (16), which caused an increase in clear cell carcinoma of the vagina in the children (17). During the race to describe the minimal molecular structure that would trigger vaginal cornification in the ovariectomized mouse vagina, controversy erupted in the 1930s over the reproducibility of results concerning the compound anethole. The authors were minimalistic in reporting the synthetic methodology, so replication proved impossible to create the correct biology. Rather the product was correct, but the method used by the *original* authors was not reported accurately and caused dimerization of anethole to an impurity dianethole an estrogen. This active impurity was structurally similar to parallel research endeavors that concluded with the synthesis of the potent estrogen diethylstilbestrol. Thus, the purity of chemicals for testing was critical for successful science.

A similar story was also immensely important in allowing scientists to understand the direct actions of estrogen on the breast cancer cell *in vitro*. The MCF-7 estrogen receptor (ER)-positive breast cancer cell line (18) has been the work horse for the study of estrogen-stimulated growth. However, early examination of MCF-7 cells in the 1970s could not uniformly show estrogen-stimulated growth. Antiestrogens inhibited the apparently constitutive growth of MCF-7 cells, but estradiol did reverse the inhibitory actions of

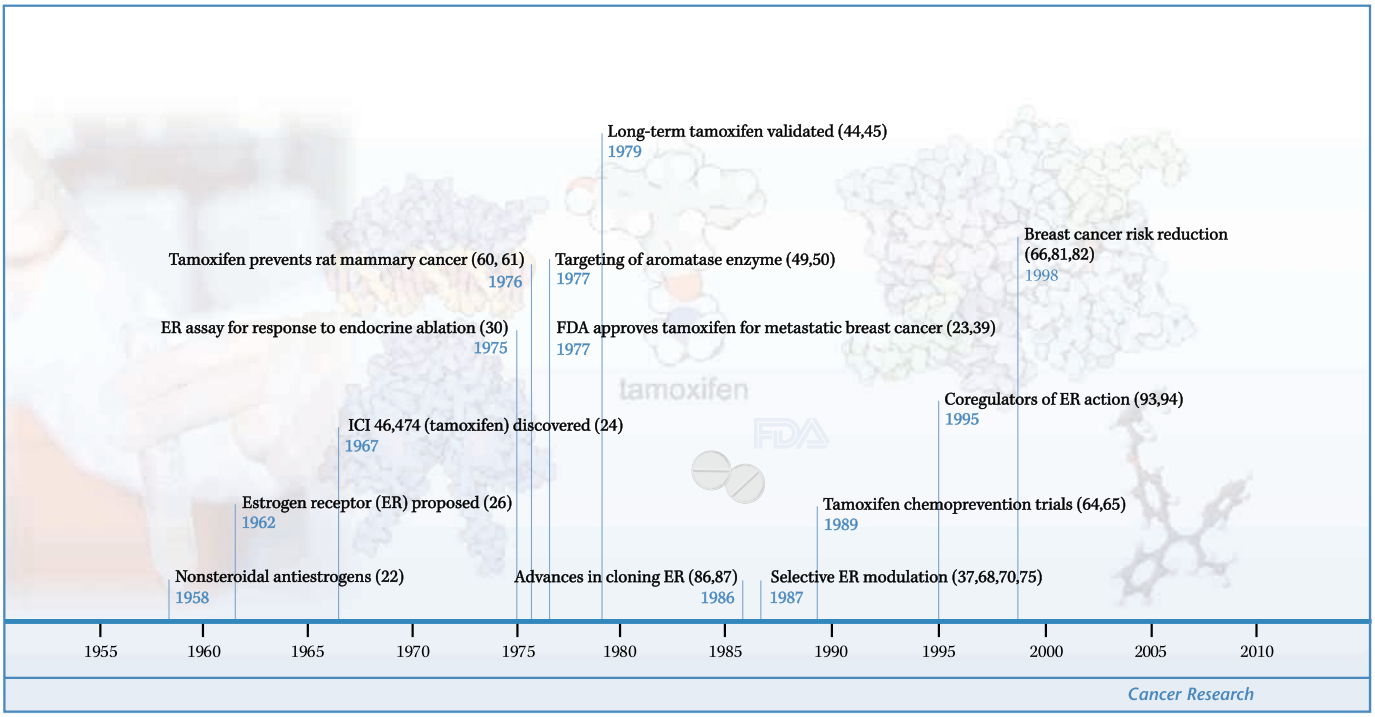

Figure 1. *Continued.*

antiestrogens on growth (19). The mystery deepened when studies *in vitro* could not show estrogen-stimulated growth but MCF-7 cells inoculated into athymic mice would grow into tumors only with estrogen treatment. There was clearly a second factor required for estrogen-stimulated tumor growth *in vivo*! (20).

The astute observations of John and Benita Katzenellenbogen solved the mystery of why estrogen did not stimulate MCF-7 breast cancer cell growth *in vitro*. It seems that all cells had been grown for more than a decade in standard medium containing large concentrations of a pH indicator called phenol red. The Katzenellenbogens realized that the structure of phenol red was similar to nonsteroidal estrogens and removal of the indicator from cell culture media caused cell growth rate to decrease and only then would exogenous estrogen cause growth (21). In other words, the cells were already growing maximally in phenol red containing medium. Subsequent studies revealed that the culprit was, in fact,

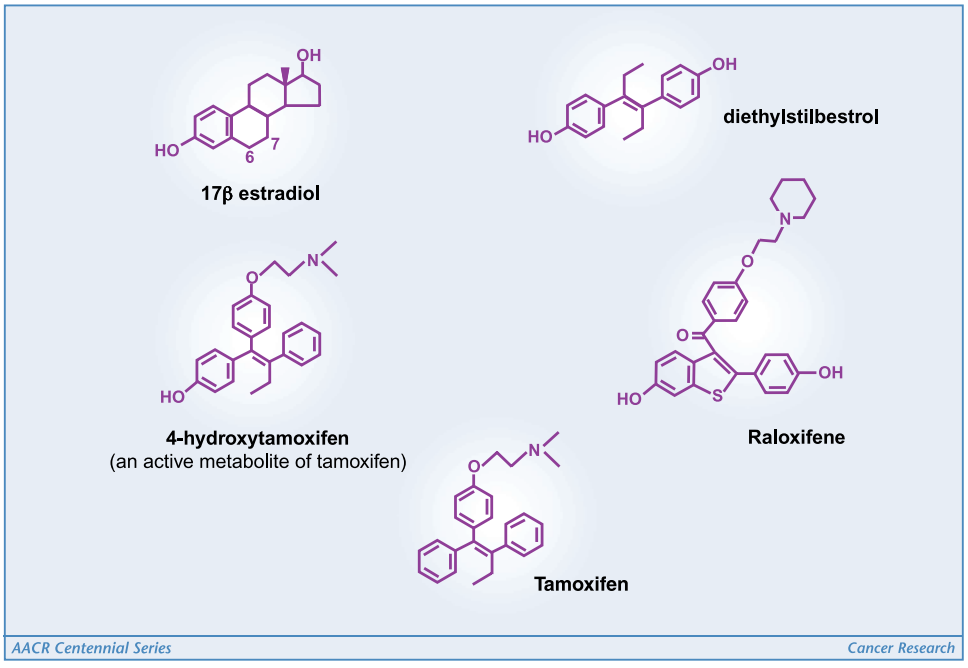

Figure 2. The structures of estrogens, antiestrogens, and SERMs mentioned in the text. The position 6 and 7 on the estradiol molecule indicate where tritium atoms were inserted to first describe estrogen binding to target tissue (26). The metabolite 4-hydroxytamoxifen (121) is an active metabolite of tamoxifen that has been the standard laboratory antiestrogen and crystallized with the ligand binding domain of the ER (95).

a partially dimerized chemical contaminant of phenol red. This critical technical advance permitted all of the subsequent understanding of the molecular biology of direct estrogen action.

Leonard Lerner (22) was a young research endocrinologist employed by Merrell Dowe to study nonsteroidal estrogen pharmacology. He noticed that the structure of one of the compounds being tested for the control of coronary artery disease was a triphenylethanol similar to the estrogenic triphenylethylenes and he asked to test this chemical as an estrogen. To his surprise, the compound, subsequently renamed MER25 or ethamoxytriphetol, was antiestrogenic in all species tested and had no estrogen-like actions in any animal tests. Lerner (22) had discovered the first nonsteroidal antiestrogen. Although the compound was too toxic and not potent enough for clinical use, Lerner went on to be involved in the discovery of the first triphenylethylene antiestrogen called chloramiphene (MRL41) later to be known as clomiphene (23). Originally, the nonsteroidal antiestrogens were predicted, based on animal studies, to be potent postcoital contraceptives, which in the early 1960s had a huge potential market as "morning after pills." However, clomiphene did exactly the opposite; it induced ovulation in women (23). Enthusiasm waned and there was general disinterest in this area of research until ICI 46,474, another nonsteroidal antiestrogen discovered in the fertility program of ICI Pharmaceutical Ltd (now AstraZeneca; ref. 24) was reinvented as the first targeted therapy for breast cancer and the first chemopreventive for any cancer (25).

A Target for Treatment and Prevention

The early theory for estrogen action in its target tissues, e.g., uterus, vagina, etc., was that there was chemical transformation between estrone and the less abundant 17β estradiol (Fig. 2) to control the redox potential of the tissue environment. In the late 1950s, Jensen (Fig. 3) and Jacobsen (26) chose another approach at the Ben May Laboratories of the University of Chicago. They synthesized (6, 7) [^{3}H] estradiol (Fig. 2) with very high-specific activity. After its injection into the immature female rats, the unchanged steroid bound to and was retained by the estrogen target tissues: the uterus, vagina, and pituitary gland. In contrast, [^{3}H] estradiol bound to, but was not retained, by nontarget tissues, e.g., muscle, lung, heart. There was clearly a receptor mechanism at play that could be blocked (27) by the coadministration of the first nonsteroidal antiestrogen MER-25 (22).

The mystery of why only about one third of advanced breast cancers responded to either endocrine ablation (3) or high-dose estrogen therapy (15) was solved by the application of basic endocrinology to the practical issue of excluding women with metastatic breast cancer who would not significantly benefit from

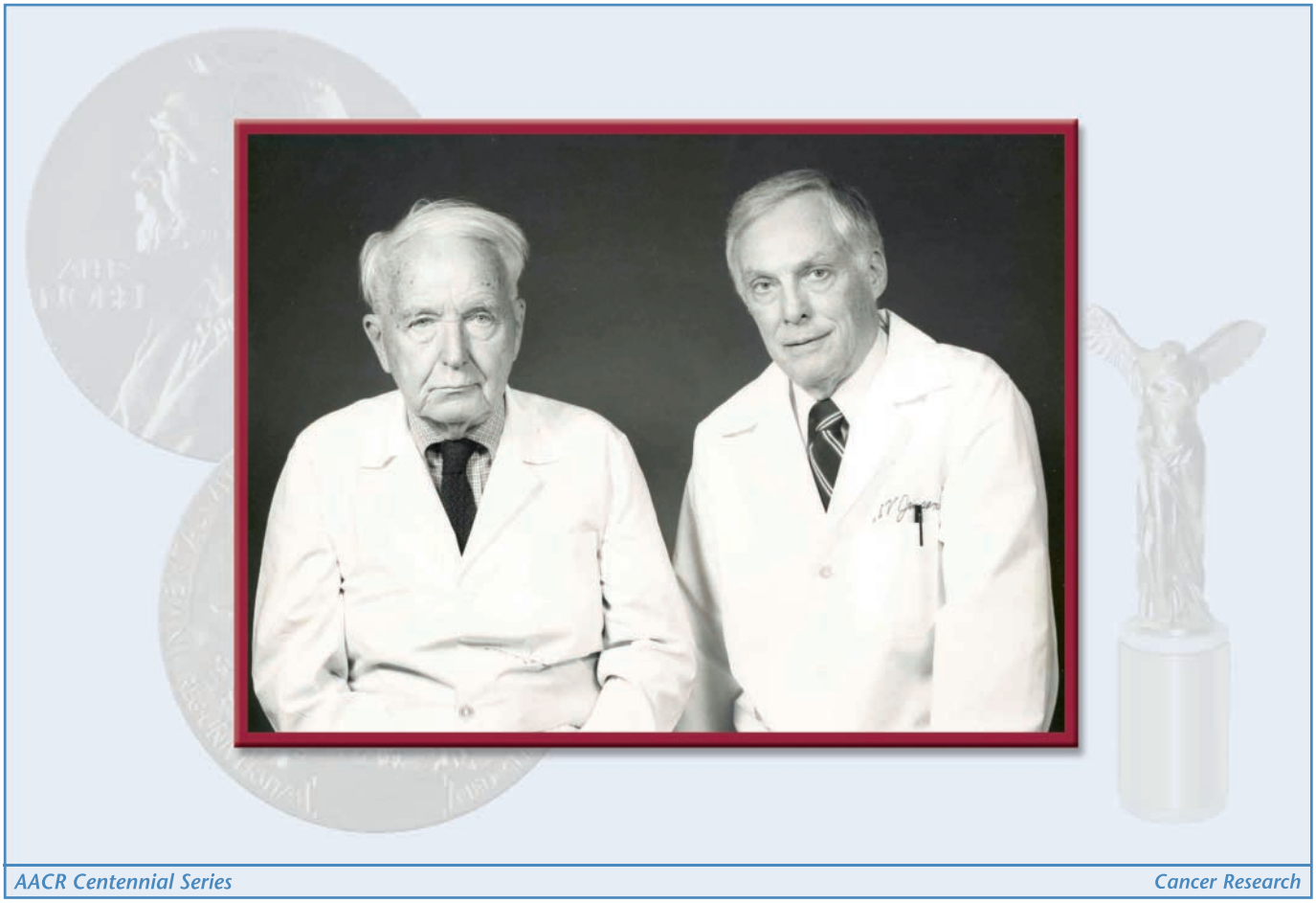

Figure 3. Professor Charles Huggins (*left*) and Elwood Jensen were to receive the Nobel Prize for Physiology and Medicine (1966) and the Lasker Award (2004) for their work on androgen action in cancer and the role of ER in physiology and cancer, respectively.

unnecessary endocrine ablative surgery (oophorectomy, adrenalectomy, or hypophysectomy). The ER was found to be an extractable protein from the rat uterus that would bind [^{3}H] estradiol in the extraction cytosol (28, 29). During the late 1960s, numerous methods were described to identify and quantitative ER levels in tumor biopsies (30) and these data were subsequently correlated with clinical outcomes in metastatic breast cancer (30). Breast tumors without the ER were unlikely to respond to endocrine ablation and therefore should not be treated with this modality. The ER assay was introduced as the standard of care in the mid-1970s to predict endocrine responsiveness to endocrine ablation. It should be stressed that tamoxifen was not available in medical practice until the Food and Drug Administration (FDA) approved this "hormone therapy" in December 1977 for the treatment of metastatic breast cancer in postmenopausal women (23). Indeed, research with the value of the ER assay to predict responsiveness to antiestrogens was unconvincing (23) and the value of adding another hormone therapy to the treatment armamentarium was uncertain. In the 1970s, all hopes in medical oncology were focused on discovering the correct combination of high dose cytotoxic therapies to cure breast cancer much in the same way as both childhood leukemias and Hodgkin's Disease had been cured. This was not to be but translational research took another route; using the ER as a drug target instead of as a predictive test for endocrine ablation (31).

An Unlikely Therapeutic Solution

Professor Paul Ehrlich (1854–1915) established a model for the development of chemical therapies (chemotherapy) to treat infectious disease. A range of chemical therapies would be synthesized to study structure function relationships in appropriate laboratory models that replicated human disease (32). A clinical study would then be performed on the most promising candidate. Ehrlich's pioneering work to develop Salvarsan for the successful treatment of syphilis is a landmark achievement (32). He was, however, unsuccessful in applying the same principles to cancer chemotherapy. Indeed, even as recently as 1970, Sir Alexander Haddow (14) stated that there was unlikely to be a "chemotherapia specifica" like Paul Ehrlich envisioned because cancer was so similar to the tissue of origin. There was also no target or effective tests or models to predict efficacy in cancer treatment before administration to the patient. The key to the successful development of tamoxifen, a failed contraceptive (23), was the application of Ehrlich's principles of developing an effective treatment strategy by using disease specific laboratory models and the use of the tumor ER as a target for drug action (25).

Available laboratory models for the study of the antitumor actions of antiestrogenic drugs were strains of mice with a high incidence of spontaneous mammary tumors (5) or the carcinogen-induced rat mammary carcinoma (33). The mouse models had fallen out of fashion with the discovery of the "Bittner milk factor," a virus that transmits mammary carcinogenesis to subsequent generations through the mother's milk (34). The research community also began to realize that breast cancer was not a viral disease. Nevertheless, the knowledge of mouse mammary carcinogenesis proved to be pivotal for developing precise and targeted promoters to initiate mammary cancer with oncogenes using transgenic mice (35). Another problem with tumor testing of tamoxifen in mice was the unusual observation that tamoxifen, or ICI 46,474 as it was then known, was an estrogen in the mouse (24, 36). This pharmacologic peculiarity became important later with the recognition of selective ER modulation (37). Most importantly, work did not advance quickly in the 1960s and early 1970s, as there was no enthusiasm about introducing a new "hormonal therapy" into clinical practice (25). All early compounds had failed to advance past early clinical studies and only tamoxifen was marketed (23) for the induction of ovulation or the general treatment of late-stage breast cancer in postmenopausal women (38–40).

In the late 1960s, the 7,12-dimethybenz(a)anthracene–induced (DMBA) rat mammary carcinoma model (33) was extremely fashionable for research on the endocrinology of rat mammary carcinogenesis (41, 42). However, the parallels with breast cancer are few, as the tumors do not metastasize and are regulated primarily by prolactin secreted by the pituitary gland in direct response to estrogen action (43). Be that as it may, there was no alternative. Therefore, the DMBA rat mammary carcinoma model was adapted to determine the appropriate strategy for the use of antihormonal therapy as an adjuvant. At that time in the mid-1970s, the early adjuvant trials with tamoxifen did not target patients with ER-positive breast cancer and used only short-term (1 year) tamoxifen treatment to avoid premature drug resistance. This duration of tamoxifen that was selected as the antiestrogen only controlled the growth of metastatic breast cancer for about a year (39). The value of short- and long-term (1- or 6-month treatment equivalent to 1 or 6 years of adjuvant treatment in patients) antihormone administration was determined starting treatment 1 month after DMBA administration to 60-day-old Sprague-Dawley rats. Long-term therapy was remarkably effective at controlling the appearance of mammary tumors and was far superior to short term treatment (44, 45). The concepts of targeting the ER and using long-term adjuvant therapy effectively translated through clinical trials to improve national survival rates for breast cancer (46, 47).

Targeting Treatment for Breast Cancer

The early clinical work of Santen (48) established the practical feasibility of using aminogluthemide, an agent that blocks both adrenal steroidogenesis and the CYP19 aromatase enzyme to stop conversion of testosterone and androstenedione to estradiol and estrone, respectively. Unfortunately, aminoglutethimide must be given with a natural glucocorticoid; therefore, long-term therapy is not a practical possibility. Brodie and coworkers (49, 50) advanced knowledge of the specific targeting of the CYP19 aromatase enzyme with the identification and subsequent development of 4 hydroxyandrostenedione (51) as the first practical suicide inhibitor of the aromatase enzyme (Fig. 4). Incidentally, the pivotal work with both tamoxifen and 4-hydroxyandrostenedione (Figs. 2 and 4) was initiated at the Worcester Foundation for Experimental Biology in Massachusetts in the early 1970s (52). Brodie's contribution eventually became the catalyst to create a whole range of agents (e.g., anastrozole; Fig. 3) targeted to the aromatase enzyme for the treatment of breast cancer in postmenopausal women (53). The clinical application of aromatase inhibitors has reduced the side effects noted with tamoxifen in postmenopausal women such as blood clots and endometrial cancer and there has been a small but significant improvement in disease control for the postmenopausal patient when results are compared with tamoxifen (54, 55).

However, recent research into the pharmacogenetics of tamoxifen has suggested that CYP2D6 enzyme product is important for

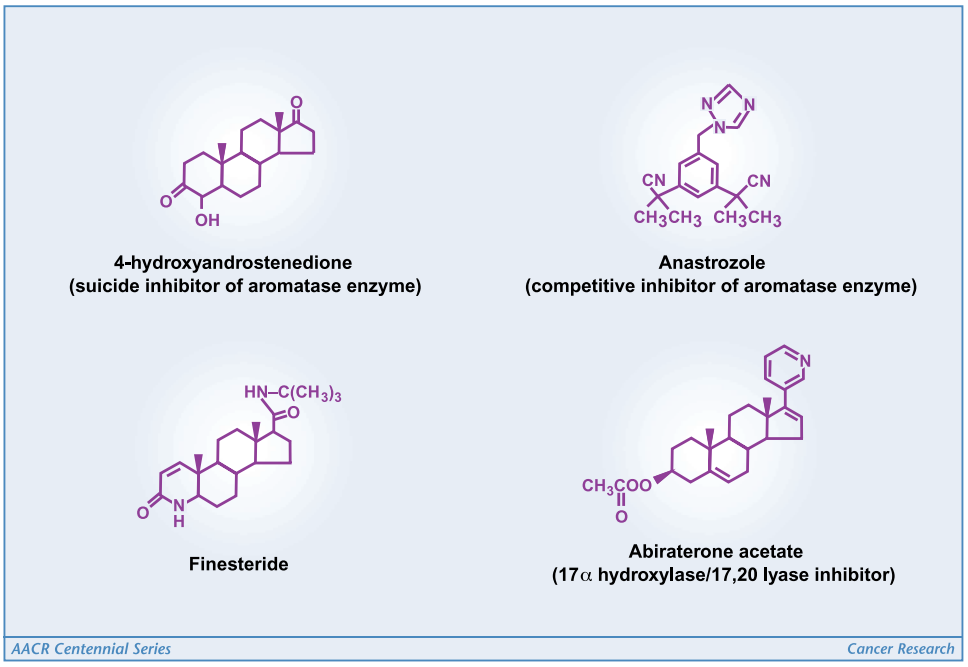

Figure 4. Structures of inhibitors of estrogen and androgen biosynthesis.

metabolism to the active antiestrogen endoxifene (4-hydroxy-N-desmethyltamoxifen; ref. 56), and the use of certain selective serotonin reuptake inhibitors to reduce hot flashes seems to be contraindicated because of drug interaction at the CYP2D6 enzyme (57, 58). Current research is also exploring the hypothesis that a mutated and ineffective CYP2D6 gene product undermines the therapeutic activity of tamoxifen (57, 58). It may be that patients could eventually be selected for optimal effective tamoxifen treatment in cases of ER-positive breast cancer. This would be worthwhile for the chemoprevention of breast cancer. Clearly, the identification of patients for optimal long-term use of tamoxifen should exclude those high-risk women with a mutant CYP2D6 gene who choose to use chemoprevention, as tamoxifen treatment may possibly be suboptimal.

Chemoprevention of Breast Cancer

In the middle of the 1970s, Sporn (59) advanced the concept of the chemoprevention of cancer and strongly advocated this approach as the optimal and clearly most rational way to reduce the burden of cancer. Practical chemoprevention articulated by Lacassagne (9) has its foundations with the finding that tamoxifen prevents DMBA-induced rat mammary carcinogenesis (60, 61). These laboratory findings (45, 60, 61) and the subsequent clinical finding that adjuvant tamoxifen treatment reduces the incidence of contralateral breast cancer (62) prompted Powles (63, 64) to initiate the first exploratory trial to test the worth of tamoxifen to reduce the incidence of breast cancer in high risk women. Although numbers were small, the Powles study did ultimately show the ability of tamoxifen to reduce breast cancer incidence many years after the treatment had stopped (65). In contrast, the large study by Fisher (66, 67) definitively showed the efficacy of tamoxifen to reduce the incidence of ER-positive breast cancer initially and continues to do so after therapy stops in both premenopausal and postmenopausal women at high risk. Tamoxifen became the first medicine approved by the FDA for risk reduction of any cancer. However, concerns based on laboratory findings (68), about the potential of tamoxifen to increase the risk of endometrial cancer in postmenopausal women and the carcinogenic potential of tamoxifen as a hepatocarcinogen (69), demanded that there had to be a better way to reduce the risk of breast cancer as a public health initiative.

The recognition of selective estrogen receptor modulator (SERM) action by nonsteroidal antiestrogens that stimulate some estrogen target tissues but block estrogen-stimulated tumor growth in others, (70) introduced a new dimension into therapeutics and advanced chemoprevention. Raloxifene has its origins as a nonsteroidal antiestrogen for the treatment of breast cancer (71, 72) as LY156758 or keoxifene. The drug failed in that indication (73) and further development was abandoned (74). The discovery that both tamoxifen and keoxifene would maintain bone density in ovariectomized rats (75), block rat mammary carcinogenesis (76), but that keoxifene was less estrogen-like than tamoxifen in the rodent uterus (71) and was less effective in stimulating the growth of endometrial cancer, (77) suggested a new therapeutic strategy (78). Simply stated (79): *"We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids, so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high-risk group to prevent breast cancer."*

Several years later, keoxifene was renamed raloxifene (Fig. 2) and was shown to maintain bone density in osteoporotic or osteopenic women (80), and simultaneously reduce the incidence of invasive

breast cancer without causing an increase in the incidence of endometrial cancer (81). Raloxifene went on to be tested against tamoxifen in the Study of Tamoxifen and Raloxifene trial (82) and was FDA approved both for the treatment and prevention of osteoporosis in postmenopausal women and for the reduction of invasive breast cancer incidence in postmenopausal women at elevated risk. The clinical advances with SERMs-modulating estrogen target tissues has provided exceptional opportunities to treat and prevent multiple diseases. However, for the future it is the study of the molecular events of estrogen action that holds the promise of further breakthroughs in patient care.

Molecular Mechanisms of Estrogen and SERM Action

It is not possible to provide a comprehensive review of the explosion of interest in receptor-mediated molecular mechanisms of action of estrogen, so the reader is referred to significant reviews to appreciate the evolution of the topic (83, 84). What will be presented is an evolving guide to current thinking. There are two ERs called α and β (Figs. 5 and 6). The receptor ERα is the traditional ER (26, 28), but it should be stressed that the development of monoclonal antibodies to ER (85) was the essential step for ERα cloning (86, 87) that provided the clues to discover ERβ (88). The receptor proteins encode on different chromosomes and have homology as members of the steroid receptor superfamily, but there are distinct patterns of distribution and distinct and subtle differences in structure and ligand binding affinity. An additional dimension that may be significant for tissue modulation is the ratio of ERα and ERβ at a target site. A high ERα/ERβ ratio correlates well with very high levels of cellular proliferation, whereas the predominance of functional ERβ over ERα correlates with low levels of proliferation (89, 90). The ratio of ERs in normal and neoplasic breast tissue may be an important factor for the long-term success of chemoprevention with SERMs. There is, as a result, much interest in synthesizing ER subtype specific ligands.

There are functional differences between ERα and ERβ that can be traced to the differences in the Activating Function 1 (AF-1) domain located in the amino terminus of the ER (Fig. 6). The amino acid homology of AF-1 is poorly conserved (only 20%). In contrast, AF-2 region located at the C terminus of the ligand binding domain, differs only by one amino acid: D545 in ERα and N496 in ERβ. Because the AF-1 and AF-2 regions are critical for the interaction with other coregulatory proteins and gene transcription, the structural differences between AF-1 provides a clue about the potential functional differences between ER α and β. Studies using chimeras of ER α and β by switching the AF-1 regions show that this region contributes to the cell and promoter specific differences in transcriptional activity. In general, SERMs can partially activate engineered genes regulated by an estrogen response element through ERα but not ERβ (91, 92). In contrast, 4-hydroxytamoxifen and raloxifene can stimulate activating protein-1–regulated reporter genes with both ERα and ERβ in a cell-dependent fashion.

The simple model for estrogen action, with either ERα or ERβ controlling estrogen-regulated events, has now evolved into a fascinating mix of protein partners that have the potential to modulate gene transcription (Fig. 5). It is more than a decade since the first steroid receptor coactivator was first described (93). Now dozens of coactivator molecules are known, and also corepressor molecules exist to prevent the gene transcription by unliganded receptors (94).

It is reasonable to ask how does the ligand program the receptor complex to interact with other proteins? X-ray crystallography of the ligand binding domains of the ER liganded with either estrogens or antiestrogens show the potential of ligands to promote coactivator binding or prevent coactivator binding based on the shape of the estrogen or anti-ER complex (95, 96). Evidence has accumulated that the broad spectrum of ligands that bind to

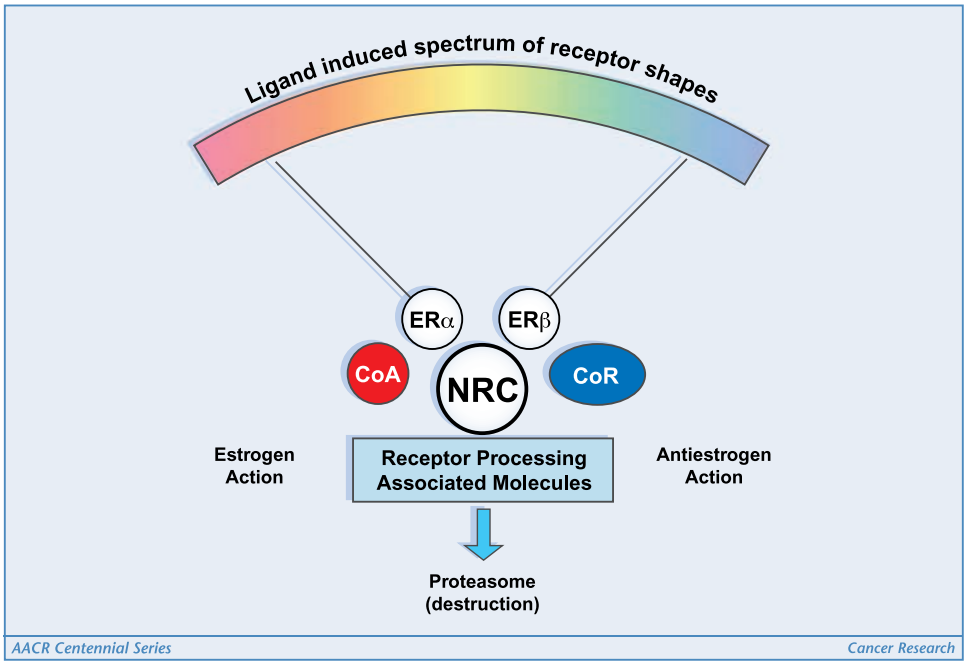

Figure 5. Molecular mechanisms of estrogens, antiestrogens, and SERMs in estrogen target tissues. The nuclear receptor complex (*NRC*) that results from ligand binding to either ERα or ERβ can interact with either coactivators (*CoA*) or corepressors (*CoR*) to initiate estrogenic or antiestrogenic responses, respectively. The activation of transcription at a promoter site of an estrogen responsive gene occurs through the binding of the complex that is cyclically destroyed through the proteosome and then the reassembling of a new complex.

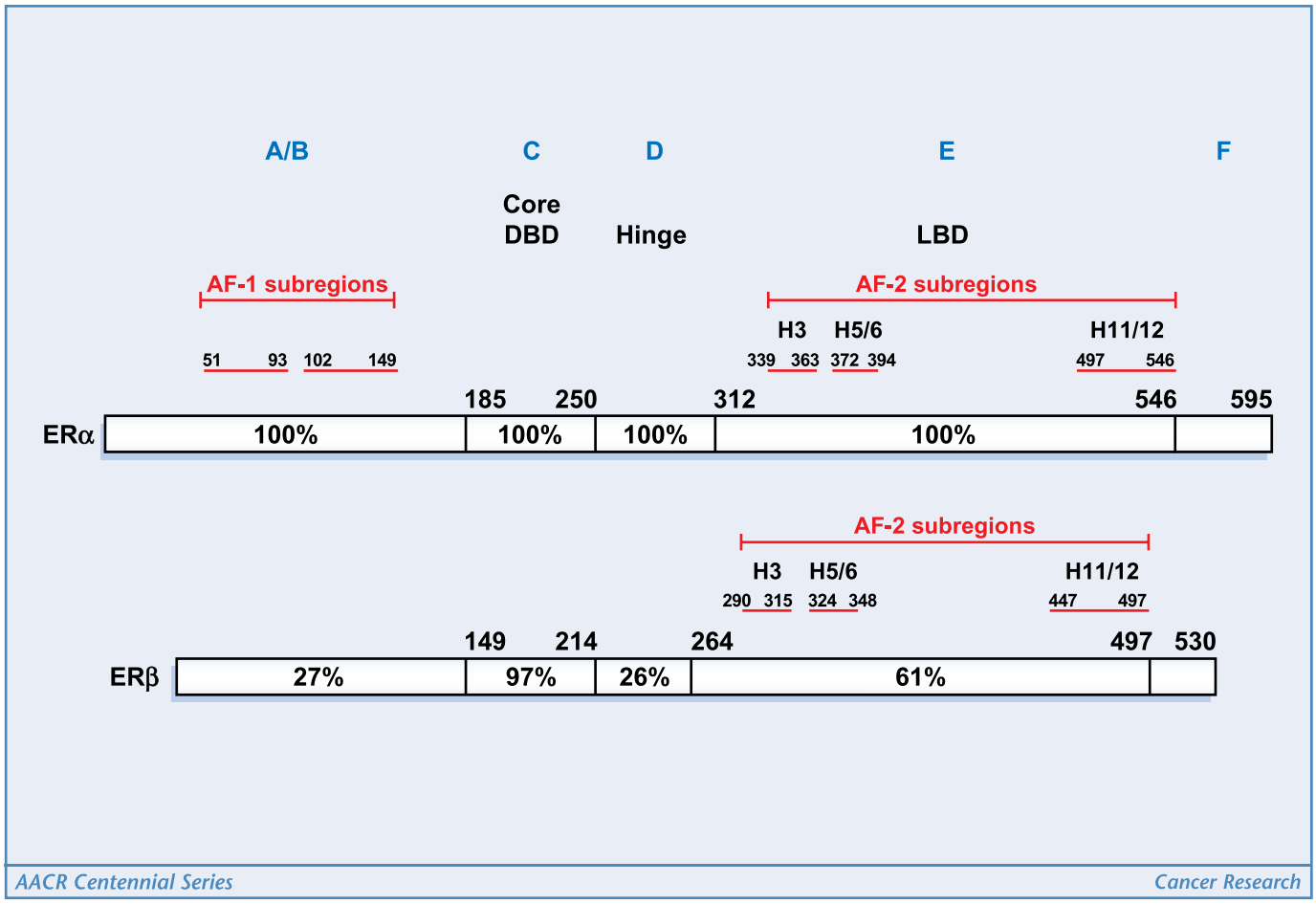

Figure 6. A comparison of the percent homology of the domains of ERs α and β abbreviations: DNA binding domain (*DBD*), ligand binding domain (*LBD*), activating functions (*AF*).

the ER can create a broad range of ER complexes that are either fully estrogenic or antiestrogenic at a particular target site (97). Thus, a mechanistic model of estrogen action and antiestrogen action (Fig. 5) has emerged based on the shape of the ligand that programs the complex to adopt a particular shape that ultimately interacts with coactivators or corepressors in target cells to determine the estrogenic or antiestrogenic response, respectively.

Not surprisingly, the coactivator model of steroid hormone action has now become enhanced into multiple layers of complexity thereby amplifying the molecular mechanisms of modulation (98).The ER complex with its core coactivator (e.g., SRC3) positions itself in the promoter region of an ER responsive gene and attracts associated molecules that engages RNApolII to start transcription. However, the complex of associated molecules also acetylates or deacetylates histones on DNA, thereby regulating the exposure of DNA to modulate transcription. Additionally, associated molecules are recruited to the receptor complex that are members of a family of enzymes that ubiquitinylate proteins in the complex for destruction. Estrogen action is therefore a dynamic process of complex assembly and destruction at the target gene (99).

The complicated modulation of estrogen action at individual target sites is challenging to comprehend but provides opportunities to develop new targeted treatments for sex steroids.

Current Insights into Sex Steroid Modulation

The accumulated knowledge about modulating the ER complex through coregulators interacting at AF-2 and AF-1 create new opportunities for novel drug discovery. The target site modulation of the ER with SERMs has been expanded to the androgen receptor (AR) with selective AR modulators (SARM; refs. 100, 101). existing nonsteroidal SARMs are being used to define tissue specific gene expression that will lead to clinically useful selective anabolic therapies without stimulating the prostate (102).

Studies of the molecular pharmacology of selective nuclear receptor modulators are focused on the relationship between the external shape of the ligand receptor complex and coregulator binding at AF-2 (103, 104). Combinatorial phage display can identify external regions of the receptor complex to map SARM action or create peptide antagonists that will block coactivator binding with potential as new therapies for prostate cancer. Indeed, this approach is now being extended to orphan nuclear receptors that do not need a small ligand for gene regulation (105). Progress with defining cofactors to study the biology of estrogen-related receptor α (ERR-α) is an important advance with significance for new targeted therapeutic agents. The recent description of the role of ERR-α in angiogenesis of ER-negative tumors (106) is a potential practical application of this work.

Posttranslational modifications of sex steroid receptors at AF-1 through phosphorylation cascades have their origins from the cell surface growth factor receptors (107, 108) This knowledge has a potential application to understand the molecular biology of antihormone resistance. However, our evolving knowledge of antihormonal drug resistance has important therapeutic consequences.

Drug Resistance to SERMs

The acceptance of the concept of long-term antihormonal therapy to target, treat, and prevent breast cancer (25) raised the specter of drug resistance to SERMs and SARMs. However, the early models of SERM resistance did not reflect the majority of clinical experience. The natural laboratory models of antihormone resistance caused stimulation of tumor growth during a year of therapy (109), and therefore, reflected drug resistance in patients with metastatic breast cancer who are only treated successfully for a year. The early laboratory models of drug resistance did not replicate clinical experience with adjuvant therapy for 5 years. Remarkably, drug resistance evolves (Fig. 7) and the survival signaling pathways in tamoxifen-resistant tumors becomes reorganized so that instead of estrogen being a survival signal, physiologic estrogen now inhibits tumor growth (110). This discovery provides an invaluable insight into the evolution of drug resistance to SERMS and prompted the reclassification of the process through phase I (SERM/estrogen stimulated) to phase II (SERM-stimulated/estrogen-inhibited growth; ref. 111).

This model would also explain the earlier observations (13) why high-dose estrogen therapy was only effective as a treatment for breast cancer in women many years after the menopause. Natural estrogen deprivation had occurred. The process is accelerated and enhanced, however, in patients treated long term with SERMs or aromatase inhibitors so that only low doses of estrogen are necessary to cause experimental tumors to regress. The new knowledge of the apoptotic action of estrogen (or androgen—see next section) could potentially lead to the discovery of a precise apoptotic trigger initiated naturally by steroid hormone receptors (111). Discovery of this apoptotic trigger might result in an application that targets critical survival signals with new drugs.

Parallel Path of the Prostate

Charles Huggins (Fig. 3; ref. 112) resurrected the use of endocrine ablation for the treatment hormone-dependent breast cancers. His focus, however, was the regulation of the growth of the prostate gland and the application of that knowledge for the treatment of prostate cancer (4). He received the Nobel Prize for Physiology and Medicine in 1966. The process for deciphering the molecular mechanisms of androgen action in its target tissues and prostate cancer has tended to lag behind the pathfinder estrogen. Nevertheless, the basic model for the regulation of nuclear hormone receptor action is consistent but the details of androgen action are distinctly different than estrogen action, which in turn created novel therapeutic opportunities to stop the biosynthesis of each active steroidal agent. The similarities and differences in the molecular actions of estrogen and androgen action are illustrated in Fig. 8. The two significant differences (yet similarities) in the biosynthetic pathways between estrogens and androgens are as follows: (*a*) the aromatization of the A ring of testosterone to create the high-affinity ER binding ligand 17β estradiol in women. This bioactivation led to the development of aromatase inhibitors to block estrogen synthesis (50); and (*b*) the reduction of testosterone to the high-affinity AR binding ligand dihydroxytestosterone in men. This knowledge led to the development of the 5α reductase inhibitor finesteride (Fig. 4) that was tested successfully for risk reduction for prostate cancer in men (113). Unfortunately, as yet, finesteride has failed to advance for use as a chemopreventive for prostate cancer because of overstated concerns about the accelerated development of potentially more aggressive prostate cancers in those men who did not have tumorigenesis prevented. In contrast, aromatase

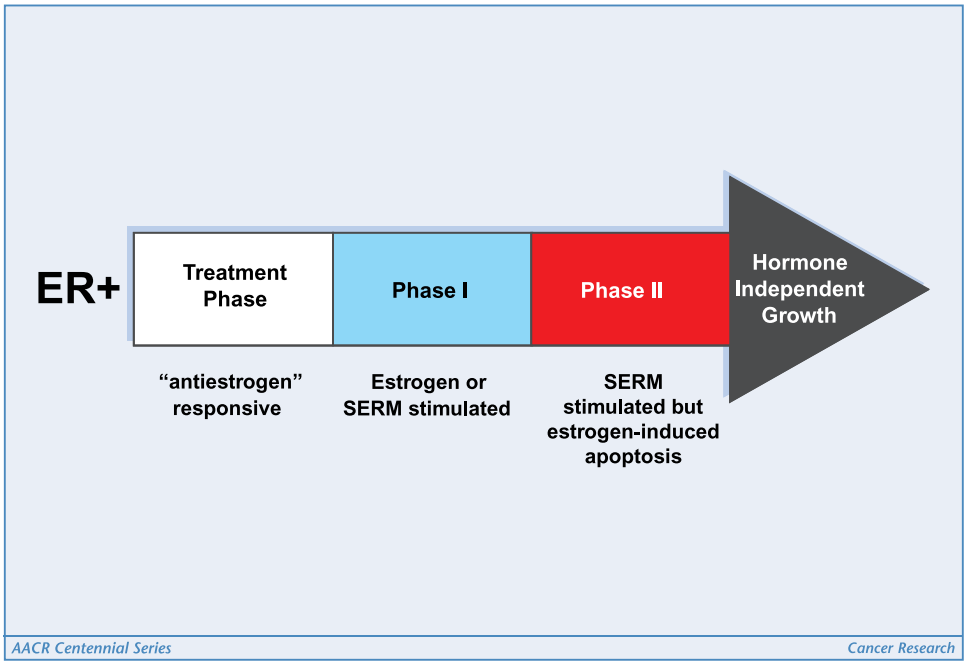

Figure 7. The evolution of resistance to selective ER modulators (SERMS: tamoxifen or raloxifene) long-term therapy.

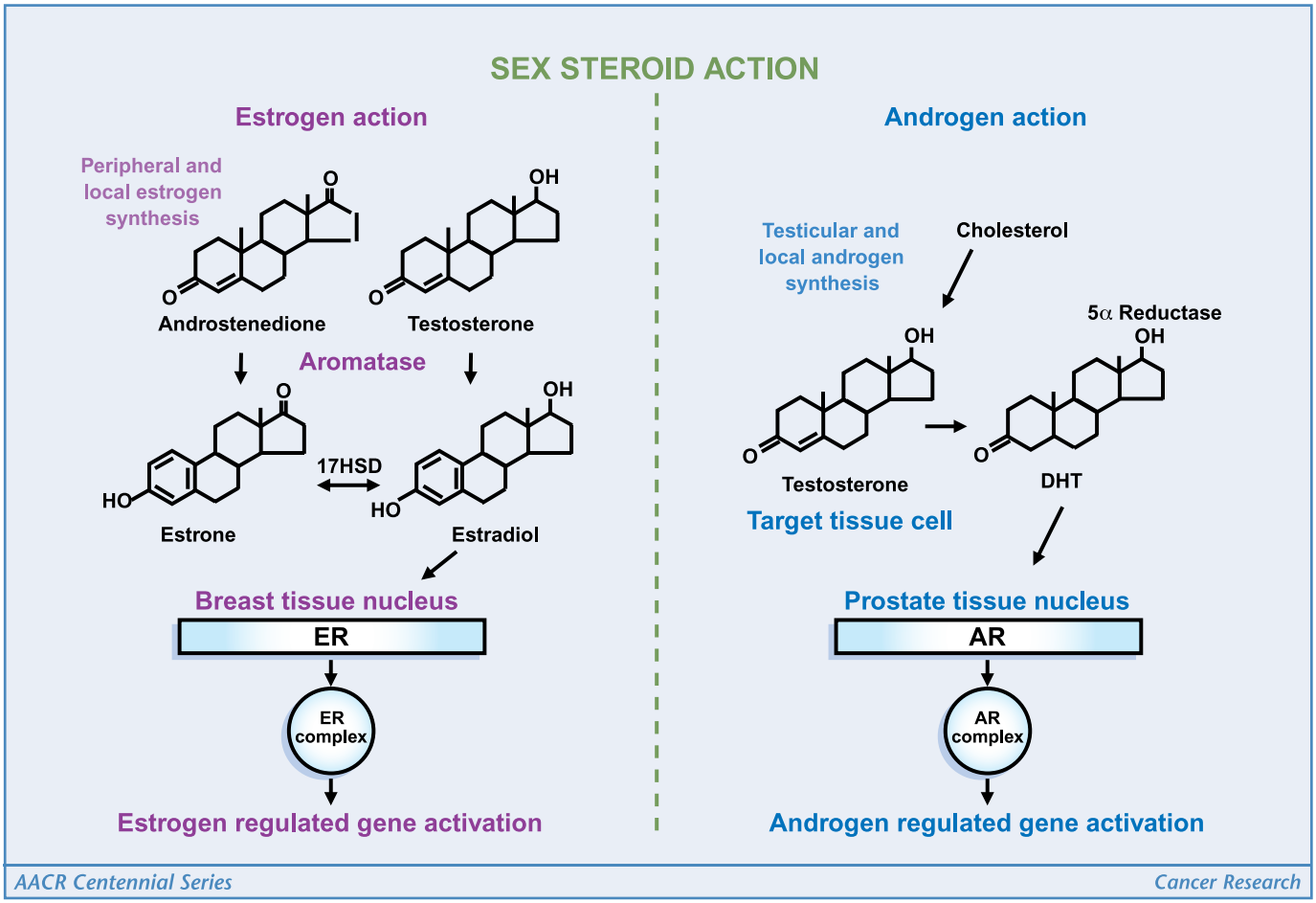

Figure 8. Comparison of the molecular mechanisms of estrogens and androgens in their respective target tissues. The transformations of the respective steroids are necessary for high binding affinity for their receptors, but the activation from prohormones occurs in different tissue sites relative to their target.

inhibitors have advanced to test their worth as chemopreventive agents (53).

A range of antiandrogenic drugs that competitively block the AR are available in clinical practice (114). Drug resistance to antiandrogen therapy parallels antiestrogen drug resistance (115), and following long-term antihormonal therapy with antiandrogens, androgen induces apoptosis in antiandrogen-resistant prostate cancer cells (116). Recent research has identified high local levels of androgen production as a major form of antihormonal drug resistance (117). As a result, a new therapeutic approach is the development of an inhibitor of androgens biosynthesis from cholesterol (Fig. 8) by blocking 17 hydroxylase/17,20 lyase. A promising compound abiraterone acetate (Fig. 4) is currently being evaluated in clinical trials (118). However, there is also a need to coadminister glucocorticoids so long term therapy must be monitored carefully.

The Successful Evolution of Targeted Antihormonal Therapy in the 20th Century and Beyond

The identification of the ER and subsequently the AR as the conduit for hormone-mediated development and growth in breast and prostate cancer, respectively, has had a profound effect on the approach to the treatment and prevention of cancers. These hormone-mediating molecules have proved to be the pathfinders for the development of targeted therapies that transformed the approach to cancer treatment away from the nonspecific cytotoxic chemotherapy approach during the 1950s to 1990s. As a result, there is current enthusiasm about the promise of individualized medicine and tumor-specific therapeutics (25, 119).

The effect of antihormonal therapy for breast cancer has been profound with improvements in patient survival, a menu of medicines is now available to suit individual patient needs and there is a decrease in national mortality rates in numerous countries (47). Additionally, there are now two SERMS (tamoxifen and raloxifene) available to reduce the incidence of breast cancer (67, 82). But progress in our understanding and application of SERMs is more than chemoprevention. The SERM concept (70) has spread to develop tissue-selective drugs for all members of the hormone receptor superfamily (25, 120). An enormous interest in developing selective glucocorticoid receptor modulators, selective progesterone receptor modulators, SARMs, and even agents to treat rheumatoid arthritis is an ongoing therapeutic outcome of translational research for the chemoprevention of breast cancer.

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No potential conflicts of interest were disclosed.

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References

1. Schinzinger A. Ueber carcinoma mammae [abstract]. 18th Congress of the German Society for Surgery Beilage zum Centralblatt fur Chirurgie 1889;16:55–6.
2. Beatson CT. On treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. Lancet 1896;2:162–5.
3. Boyd S. On oophorectomy in cancer of the breast. BMJ 1900;ii:1161–7.
4. Huggins C, Hodges CV. Studies on Prostatic Cancer. I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. Cancer Res 1941;1:293–7.
5. Lathrop AE, Loeb L. Further investigations on the origins of tumors in mice III on the part played by internal secretions in the spontaneous development of tumors. J Cancer Res 1916;1:1–19.
6. Allen E, Doisy EA. An ovarian hormone: preliminary reports on its localization, extraction and partial purification and action in test animals. JAMA 1923;81: 810–21.
7. Lacassagne A. Influence d'un facteur familial dans la production par la folliculine, de cancers mammaires chez la souris male. J Soc Biol 1933;114:427–9.
8. Lacassagne A. A comparative study of the carcinogenic action of certain oestrogenic hormones. Am J Cancer 1936;28:735–40.
9. Lacassagne A. Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer 1936;27:217–25.
10. Dodds EC, Goldberg L, Lawson W, Robinson R. Oestrogenic activity of certain synthetic compounds. Nature 1938;141:247–8.
11. Dodds EC, Lawson W, Noble RL. Biological effects of the synthetic oestrogenic substance 4: 4′dihydroxy-a: b-diethylstilbene. Lancet 1938;1:1389–91.
12. Robson JM, Schonberg A. Oestrous reactions including mating produced by triphenylethylene. Nature 1937; 140:196.
13. Haddow A, Watkinson JM, Paterson E. Influence of synthetic oestrogens upon advanced malignant disease. Br Med J 1944;2:393–8.
14. Haddow A. David A. Karnofsky memorial lecture. Thoughts on chemical therapy. Cancer 1970;26:737–54.
15. Huggins C. Control of cancers of man by endocrinologic methods; a review. Cancer Res 1956;16:825–30.
16. Smith OW. Diethylstilbestrol in the prevention and treatment of complications of pregnancy. Am J Obstet Gynecol 1948;56:821.
17. Herbst AL, Ulfelder H, Poskanzer DC. Adenocarcinoma of the vagina. N Engl J Med 1971;284:878–81.
18. Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res 1997;57: 3071–8.
19. Lippman M, Bolan G, Huff K. The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture. Cancer Res 1976;36:4595–601.
20. Shafie SM. Estrogen and the growth of breast cancer: new evidence suggests indirect action. Science 1980;209: 701–2.
21. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A 1986;83:2496–500.
22. Lerner LJ, Holthaus JF, Thompson CR. A non-steroidal estrogen antagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenylethanol. Endocrinology 1958;63:295–318.
23. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2003;2:205–13.
24. Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 1967;13:101–19.
25. Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 2008;44:30–8.
26. Jensen EV, Jacobson HI. Basic guides to the mechanism of estrogen action. Recent Prog Horm Res 1962;18:387–414.
27. Jensen EV. Antiestrogens. Recent Prog Horm Res 1962;18:418.
28. Toft D, Gorski J. A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc Natl Acad Sci U S A 1966;55:1574–81.
29. Toft D, Shyamala G, Gorski J. A receptor molecule for estrogens: studies using a cell-free system. Proc Natl Acad Sci U S A 1967;57:1740–3.
30. McGuire WL, Carbone PP, Sears ME, Escher GC. Estrogen receptors in human breast cancer: an overview. In: Estrogen Receptor in Human Breast Cancer. McGuire WL, Carbone PP, Volmer EP, editors. New York: Raven Press; 1975. p. 1–7.
31. Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. Clin Cancer Res 2003;9: 1980–9.
32. Baumler E. Paul Ehrlich, Scientist for Life. New York: Holmes & Meier; 1984.
33. Huggins C, Grand LC, Brillantes FP. Mammary cancer induced by a single feeding of polynuclear hydrocarbons and their suppression. Nature 1961;189:189–204.
34. Bittner JJ. Some possible effects of nursing on the mammary gland tumor incidence in mice. Science (New York) 1936;84:162.
35. Stewart TA, Pattengale PK, Leder P. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell 1984;38: 627–37.
36. Harper MJ, Walpole AL. Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. Nature 1966;212:87.
37. Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc 1987; 46:1870–4.
38. Klopper A, Hall M. New synthetic agent for the induction of ovulation. Preliminary trial in women. Br Med J 1971;1:152–4.
39. Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. Br J Cancer 1971;25:270–5.
40. Ward HW. Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J 1973;1: 13–4.
41. Welsch CW. Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. Cancer Res 1985;45:3415–43.
42. Russo J, Tay LK, Russo IH. Differentiation of the mammary gland and susceptibility to carcinogenesis. Breast Cancer Res Treat 1982;2:5–73.
43. Lieberman ME, Maurer RA, Gorski J. Estrogen control of prolactin synthesis *in vitro*. Proc Natl Acad Sci U S A 1978;75:5946–9.
44. Jordan VC, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. Adjuvant Ther Cancer 1979;2:19–26.
45. Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 1980;16:239–51.
46. EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 1998;354: 1451–67.
47. EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687–717.
48. Santen RJ, Lipton A. Successful medical adrenalectomy with amino-glutethimide. Role of altered drug metabolism. JAMA 1974;230:1661–5.
49. Schwarzel WC, Kruggel WG, Brodie HJ. Studies on the mechanism of estrogen biosynthesis. 8. The development of inhibitors of the enzyme system in human placenta. Endocrinology 1973;92:866–80.
50. Brodie AM, Schwarzel WC, Shaikh AA, Brodie HJ. The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. Endocrinology 1977; 100:1684–95.
51. Coombes CR, Goss P, Dowsett M, Gazet JC, Brodie A. 4-Hydroxyandostenedione in treatment of postmenopausal patients with advanced breast cancer. Lancet 1984;1:1237–9.
52. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007;72:7–25.
53. Goss P, Strasser K. Aromatase inhibitors in the treatment and prevention of breast cancer. J Clin Oncol 2001;19:881–94.
54. Howell A, Cuzick J, Baum M, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 2005;365:60–2.
55. Thurlimann B, Keshaviah A, Coates AS, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. N Engl J Med 2005;353:2747–57.
56. Lien EA, Solheim E, Kvinnsland S, Ueland PM. Identification of 4-hydroxy-N-desmethyltamoxifen as a metabolite of tamoxifen in human bile. Cancer Res 1988; 48:2304–8.
57. Goetz MP, Rae JM, Suman VJ, et al. Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. J Clin Oncol 2005;23:9312–8.
58. Jin Y, Desta Z, Stearns V, et al. CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. J Natl Cancer Inst 2005;97:30–9.
59. Sporn MB. Approaches to prevention of epithelial cancer during the preneoplastic period. Cancer Res 1976;36:2699–702.
60. Jordan VC. Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 1974;5:354.
61. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA- induced rat mammary carcinoma. Eur J Cancer 1976;12:419–24.
62. Cuzick J, Baum M. Tamoxifen and contralateral breast cancer [letter]. Lancet 1985;2:282.
63. Powles TJ, Hardy JR, Ashley SE, et al. A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. Br J Cancer 1989;60: 126–31.
64. Powles T, Eeles R, Ashley S, et al. Interim analysis of the incidence of breast cancer in the Royal Marsden

Hospital tamoxifen randomised chemoprevention trial [see comments]. Lancet 1998;352:98–101.
65. Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. J Natl Cancer Inst 2007;99:283–90.
66. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998;90:1371–88.
67. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 2005;97: 1652–62.
68. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 1988;48:812–5.
69. Han XL, Liehr JG. Induction of covalent DNA adducts in rodents by tamoxifen. Cancer Res 1992;52:1360–3.
70. Jordan VC. Selective estrogen receptor modulation: a personal perspective. Cancer Res 2001;61:5683–7.
71. Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 1983;32:1031–6.
72. Jones CD, Jevnikar MG, Pike AJ, et al. Antiestrogens. 2. Structure-activity studies in a series of 3-aroyl-2-arylbenzo[b]thiophene derivatives leading to [6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thien-3-yl] [4–2-(1-piperidinyl)ethoxy] -phenyl]methanone hydrochloride (LY156758), a remarkably effective estrogen antagonist with only minimal intrinsic estrogenicity. J Med Chem 1984;27:1057–66.
73. Buzdar AU, Marcus C, Holmes F, Hug V, Hortobagyi G. Phase II evaluation of Ly156758 in metastatic breast cancer. Oncology 1988;45:344–5.
74. Lewis JS, Jordan VC. Case Histories: Raloxifene. In: Comprehensive Medicinal Chemistry II 2006;8:103–21.
75. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987;10:31–5.
76. Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987;47:4020–4.
77. Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 1990;50:3189–92.
78. Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 1988;6:589–95.
79. Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. Cancer Res 1990;50:4177–89.
80. Ettinger B, Black DM, Mitlak BH, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators [see comments]. JAMA 1999;282:637–45. Erratum in: JAMA 1999;282:2124.
81. Cummings SR, Eckert S, Krueger KA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA 1999;281:2189–97.
82. Vogel VG, Costantino JP, Wickerham DL, et al. The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. JAMA 2006;295:2727–41.
83. Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 1994;63:451–86.
84. Nilsson S, Makela S, Treuter E, et al. Mechanisms of estrogen action. Physiol Rev 2001;81:1535–65.
85. Greene G, Fitch FW, Jensen EV. Monoclonal antibodies to estrophilin: probes for the study of estrogen receptors. Proc Natl Acad Sci U S A 1980;77:157–61.
86. Green S, Walter P, Kumar V, et al. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature (London) 1986;320:134–9.
87. Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J. Sequence and expression of human estrogen receptor complementary DNA. Science 1986; 231:1150–4.
88. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 1996;93: 5925–30.
89. Roger P, Sahla ME, Makela S, Gustafsson JA, Baldet P, Rochefort H. Decreased expression of estrogen receptor β protein in proliferative preinvasive mammary tumors. Cancer Res 2001;61:2537–41.
90. Paruthiyil S, Parmar H, Kerekatte V, Cunha GR, Firestone GL, Leitman DC. Estrogen receptor β inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. Cancer Res 2004;64:423–8.
91. McInerney EM, Weis KE, Sun J, Mosselman S, Katzenellenbogen BS. Transcription activation by the human estrogen receptor subtype β (ER β) studied with ER β and ER α receptor chimeras. Endocrinology 1998; 139:4513–22.
92. Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J, Nilsson S. Differential response of estrogen receptor α and estrogen receptor β to partial estrogen agonists/antagonists. Mol Pharmacol 1998;54: 105–12.
93. Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 1995; 270:1354–7.
94. Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev 2004;25:45–71.
95. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. The structural basis of estrogen receptor/co-activator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;95:927–37.
96. Brzozowski AM, Pike AC, Dauter Z, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997;389:753–8.
97. Wijayaratne AL, Nagel SC, Paige LA, et al. Comparative analyses of mechanistic differences among anti-estrogens. Endocrinology 1999;140:5828–40.
98. O'Malley BW. Little molecules with big goals. Science 2006;313:1749–50.
99. Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 2000;103:843–52.
100. Chen J, Kim J, Dalton JT. Discovery and therapeutic promise of selective androgen receptor modulators. Mol Interv 2005;5:173–88.
101. Gao W, Dalton JT. Expanding the therapeutic use of androgens via selective androgen receptor modulators (SARMs). Drug Discov Today 2007;12:241–8.
102. Narayanan R, Coss CC, Yepuru M, Kearbey JD, Miller DD, Dalton JT. Steroidal androgens and nonsteroidal, tissue-selective androgen receptor modulator, S-22, regulate androgen receptor function through distinct genomic and nongenomic signaling pathways. Mol Endocrinol 2008;22:2448–65.
103. Chang CY, Abdo J, Hartney T, McDonnell DP. Development of peptide antagonists for the androgen receptor using combinatorial peptide phage display. Mol Endocrinol 2005;19:2478–90.
104. Kazmin D, Prytkova T, Cook CE, et al. Linking ligand-induced alterations in androgen receptor structure to differential gene expression: a first step in the rational design of selective androgen receptor modulators. Mol Endocrinol 2006;20:1201–17.
105. Gaillard S, Grasfeder LL, Haeffele CL, et al. Receptor-selective coactivators as tools to define the biology of specific receptor-coactivator pairs. Mol Cell 2006;24:797–803.
106. Stein RA, Chang CY, Kazmin DA, et al. Estrogen-related receptor α is critical for the growth of estrogen receptor-negative breast cancer. Cancer Res 2008;68: 8805–12.
107. Sarwar N, Kim JS, Jiang J, et al. Phosphorylation of ERα at serine 118 in primary breast cancer and in tamoxifen-resistant tumours is indicative of a complex role for ERα phosphorylation in breast cancer progression. Endocr Relat Cancer 2006;13:851–61.
108. Thomas RS, Sarwar N, Phoenix F, Coombes RC, Ali S. Phosphorylation at serines 104 and 106 by Erk1/2 MAPK is important for estrogen receptor-α activity. J Mol Endocrinol 2008;40:173–84.
109. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 1988;48:5183–8187.
110. Yao K, Lee ES, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000;6:2028–36.
111. Jordan VC. The 38th David A. Karnofsky Lecture. The paradoxical actions of estrogen in breast cancer: survival or death? J Clin Oncol 2008;26:3073–82.
112. Huggins C, Dao TL. Adrenalectomy and oophorectomy in treatment of advanced carcinoma of the breast. J Am Med Assoc 1953;151:1388–94.
113. Thompson IM, Goodman PJ, Tangen CM, et al. The influence of finasteride on the development of prostate cancer. N Engl J Med 2003;349:215–24.
114. Kolvenbag GJCM, Furr BJA. Nonsteroidal Antiandrogens. Hormone Therapy in Breast and Prostate Cancer. In: Jordan VC, Furr BJA, editors. Totowa (NJ): Humana Press, Inc.; 2002. p. 347–68.
115. Scher HI, Sawyers CL. Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. J Clin Oncol 2005;23:8253–61.
116. Umekita Y, Hiipakka RA, Kokontis JM, Liao S. Human prostate tumor growth in athymic mice: inhibition by androgens and stimulation by finasteride. Proc Natl Acad Sci U S A 1996;93:11802–7.
117. Locke JA, Guns ES, Lubik AA, et al. Androgen levels increase by intratumoral *de novo* steroidogenesis during progression of castration-resistant prostate cancer. Cancer Res 2008;68:6407–15.
118. Attard G, Reid AH, Yap TA, et al. Phase I Clinical Trial of a Selective Inhibitor of CYP17, Abiraterone Acetate, Confirms That Castration-Resistant Prostate Cancer Commonly Remains Hormone Driven. J Clin Oncol 2008;28. Available from: http://jco/ascopubs.org/cgi/doi/10.1200/jco.2007.15.9749.
119. Strebhardt K, Ullrich A. Paul Ehrlich's magic bullet concept: 100 years of progress. Nat Rev 2008;8:473–80.
120. Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 2007;25:5815–24.
121. Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 1977;75: 305–16.

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Review

The St. Gallen Prize Lecture 2011: Evolution of long-term adjuvant anti-hormone therapy: consequences and opportunities

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SUMMARY

The successful translation of the scientific principles of targeting the breast tumour oestrogen receptor (ER) with the nonsteroidal anti-oestrogen tamoxifen and using extended durations (at least 5 years) of adjuvant therapy, dramatically increased patient survivorship and significantly enhanced a drop in national mortality rates from breast cancer. The principles are the same for the validation of aromatase inhibitors to treat post-menopausal patients but tamoxifen remains a cheap, life-saving medicine for the pre-menopausal patient. Results from the Oxford Overview Analysis illustrate the scientific principle of "longer is better" for adjuvant therapy in pre-menopausal patients. One year of adjuvant therapy is ineffective at preventing disease recurrence or reducing mortality, whereas five years of adjuvant tamoxifen reduces recurrence by 50% which is maintained for a further ten years after treatment stops. Mortality is reduced but the magnitude continues to increase to 30% over a 15-year period. With this clinical database, it is now possible to implement simple solutions to enhance survivorship. Compliance with long-term anti-hormone adjuvant therapy is critical. In this regard, the use of selective serotonin reuptake inhibitors (SSRIs) to reduce severe menopausal side effects may be inappropriate. It is known that SSRIs block the CYP2D6 enzyme that metabolically activates tamoxifen to its potent anti-oestrogenic metabolite, endoxifen. The selective norepinephrine reuptake inhibitor, venlafaxine, does not block CYP2D6, and may be a better choice. Nevertheless, even with perfect compliance, the relentless drive of the breast cancer cell to acquire resistance to therapy persists. The clinical application of long-term anti-hormonal therapy for the early treatment and prevention of breast cancer, focused laboratory research on the discovery of mechanisms involved in acquired anti-hormone resistance. Decades of laboratory study to reproduce clinical experience described not only the unique mechanism of selective ER modulator (SERM)-stimulated breast cancer growth, but also a new apoptotic biology of oestradiol action in breast cancer, following 5 years of anti-hormonal treatment. Oestradiol-induced apoptotic therapy is currently shown to be successful for the short-term treatment of metastatic ER positive breast cancer following exhaustive treatment with anti-hormones. The "oestrogen purge" concept is now being integrated into trials of long-term adjuvant anti-hormone therapy. The Study of Letrazole Extension (SOLE) trial employs "anti-hormonal drug holidays" so that a woman's own oestrogen may periodically purge and kill the nascent sensitized breast cancer cells that are developing. This is the translation of an idea first proposed at the 1992 St. Gallen Conference. Although tamoxifen is the first successful targeted therapy in cancer, the pioneering medicine is more than that. A study of the pharmacology of tamoxifen opened the door for a pioneering application in cancer chemoprevention and created a new drug group: the SERMs, with group members (raloxifene and lasofoxifene) approved for the treatment and prevention of osteoporosis with a simultaneous reduction of breast cancer risk. Thus, the combined strategies of long-term anti-hormone adjuvant therapy, targeted to the breast tumour ER, coupled with the expanding use of SERMs to prevent osteoporosis and prevent breast cancer as a beneficial side effect, have advanced patient survivorship significantly and promise to reduce breast cancer incidence.

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Introduction

Professor Hans-Joerg Senn asked me to cast light on future opportunities for improving adjuvant anti-hormone therapy that can be implemented or tested in clinical trial. This I will do, but first I will preface my remarks with a quote from Patrick Henry, the first elected Governor of Virginia, who said it best: *"I have but one lamp by which my feet are guided, and that is the lamp of experience. I know no way of judging of the future, but by the past."* In 1969, when I started my research on the pharmacology of non-steroidal anti-oestrogen, there was no tamoxifen (Fig. 1), only ICI 46,474, an effective anti-fertility agent in rats.[1] The compound had anti-oestrogenic properties, so I proposed[2] to enhance its clinical application from an orphaned drug, with modest efficacy in metastatic breast cancer, to a targeted anti-cancer agent for adjuvant therapy and chemoprevention. Tamoxifen became my lamp, and subsequent laboratory research results shed light on the future of successful and safe adjuvant anti-hormone therapy, a new drug group of selected estrogen receptor modulation (SERMs),[3] a lead compound in the SERMs raloxifene for clinical applications, the promise of multi-functional medicines, the unique qualities of acquired anti-hormone drug resistance and a new apoptotic biology of oestrogen in breast cancer (Fig. 1, Table 1).[4] Tamoxifen, a failed contraceptive in women, is now a pioneering medicine in oncology[1] and is listed as an essential medicine by the World Health Organization.

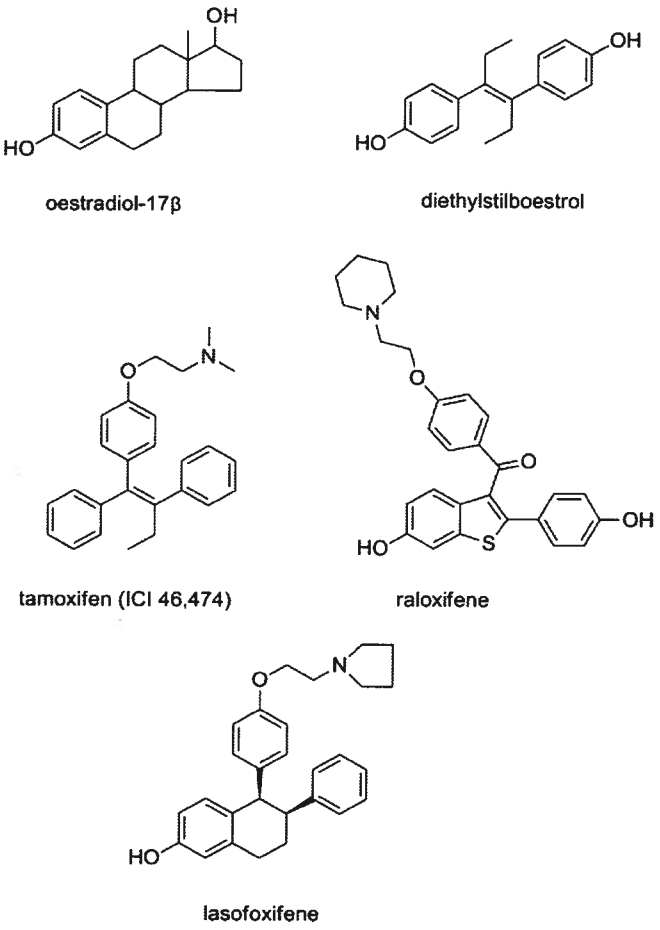

Fig. 1. The structure of medicines and compounds mentioned in the text. Oestradiol and diethylstilboestrol are oestrogens, whereas all others are selective oestrogen receptor modulators (SERMs) used in medicine for the treatment and chemoprevention of breast cancer (tamoxifen) or treatment and prevention of osteoporosis and the chemoprevention of breast cancer (raloxifene). The new SERM, lasofoxifene, is approved for the treatment and prevention of osteoporosis in the European Union.

The clinical validation[5,6] of the laboratory principles of targeting the breast tumour oestrogen-receptor (ER)[7] with long-term adjuvant antihormonal therapy (tamoxifen and oestrogen withdrawal)[8,9] using a long acting anti-oestrogen, metabolically activated to potent hydroxylated metabolites,[9–12] established a treatment strategy that continues to enhance the survivorship of millions of women world-wide. The key to success was the application of the first effective medicine to target the tumour through blocking oestrogen-stimulated growth at the ER, but coupled with the application of the counter-intuitive laboratory finding, that long-term adjuvant therapy would be superior to short-term therapy to control recurrence. The strategy succeeded, despite initial clinical findings that the tumour response to tamoxifen was not strongly correlated to ER status[13,14] and the legitimate concern that long-term therapy would precipitate early drug resistance. This concern was based on the fact that tamoxifen was only an effective treatment in unselected metastatic disease for about a year or two,[15] so why would extended or indefinite adjuvant tamoxifen treatment be effective at preventing recurrence in the adjuvant setting?

Clinical trials finally demonstrated that the laboratory principle of "longer was more effective at controlling recurrence" was correct.[5,6] The subsequent development of the aromatase (AIs)[16] expanded post-menopausal patient treatment options and reduced "oestrogen-like" side effects associated with tamoxifen, such as endometrial cancer and thromboembolic disorders.[17] There was also a modest improvement of disease-free survival compared with tamoxifen. The widespread acceptance of long-term antihormonal therapy as the standard of care and the intense and exhaustive examination of patient population databases, now permit questions to be addressed to improve patient survivorship. At a time of shrinking resources for biomedical research but expanding menus of purported targeted drugs to close one pathway or another, it is time to apply simple, basic rules that will make an impact immediately on enhancing survivorship. Only then is it prudent to fine tune the results from a position of strength, by interrogating the tumour biology with blockers of survival pathways.

Table 1
Decades of translational discovery. The development of scientific principles in the laboratory were translated to clinical trials ten years later and subsequently became the standards for clinic care for the treatment or chemoprevention of breast cancer, or in the case of the SERM, raloxifene, a treatment option for the treatment and prevention of osteoporosis with the prevention of breast carcinogenesis as a beneficial side effect.

Decade	Scientific principle	Clinical benefit
1970s	Long-term adjuvant tamoxifen therapy targeted to ER	–
	Foundation of chemoprevention with tamoxifen	–
1980s	Selective ER modulation	Survival benefits for long-term adjuvant tamoxifen
1990s	Evolution of drug resistance to hormones Anti-tumour actions of physiologic oestrogens	Chemoprevention with SERMs, tamoxifen and raloxifene
2000s	Oestrogen-induced apoptosis	Clinical translation of estrogen-induced apoptosis

Simple solutions to enhance survival

It seems obvious but it must be stated. The past 30 years of successful translational research is without value if an infrastructure does not exist to ensure that a patient's treatment is maintained when the medicine has proven value to aid survival from breast cancer. A medical team is available to support a patient's needs but there must be a refocus of the team to relearn basic principles: chronic therapy that requires years to provide benefit is worthless if the patients will not follow the regimen. This act will **dramatically** reduce their potential for survival. The fashion over the past four decades, for evidence based medicine, requires effective delivery. Significantly, delivery is a minor commitment compared to the effort behind discovering and proving the efficiency of a medicine in prospective clinical trials.

Based on the published evidence, several general principles are emerging about compliance. A recent analysis of anti-hormone therapy conducted in patients enrolled in the Kaiser Permanente of Northern California health system,[18] revealed that approximately 30% of all patients discontinued either AI or tamoxifen early but of those who did continue, 70% were fully adherent for up to 5 years. Thus, only 49% overall are adherent for the full course of adjuvant anti-hormonal therapy. Predictors of non-adherence were African-American race, lumpectomy, unknown tumour site, lymph node involvement and other co-morbidities. Adherence was associated with Asian/Pacific Island ethnicity, married, earlier years of diagnosis (tamoxifen era), prior chemotherapy, radiation therapy and longer prescription refills. These and similar findings[19,20] describe the extent of the problem but noncompliance with effective therapeutic agents also increases recurrence and mortality.[21–23]

Another significant finding of the Hershman study[18] was that young women under 40-years old were more likely to discontinue anti-hormone therapy. This group would be prescribed tamoxifen but reasons for stopping could be because the women chose to start a family or the menopausal side effects were too severe. In regard to the latter, many women have been routinely prescribed selective serotonin reuptake inhibitors (SSRIs) over the past decade to reduce menopausal side effects. Members of this drug group block the CYP2D6 enzyme that metabolically activates tamoxifen to the potent anti-oestrogen endoxifen, thereby (Fig. 2) impairing full drug benefit (Fig. 3).[24] However, it must be stressed that not all SSRIs have the same ability to block tamoxifen metabolism and as a result, studies that group all SSRIs together are not uniformly consistent with the hypothesis.[25,26] Nevertheless, the recent Canadian study of co-prescription of various SSRIs and the selective norepinephrine reuptake inhibitor (SNRI) venlafaxine does implicate paroxitene as increasing mortality during tamoxifen treatment and venlafaxine decreases mortality.[27] Overall, enhancing compliance and avoiding SSRIs that block CYP26D will significantly increase the chances of patient survival. That being said, the next issue to address is anti-hormone drug resistance.

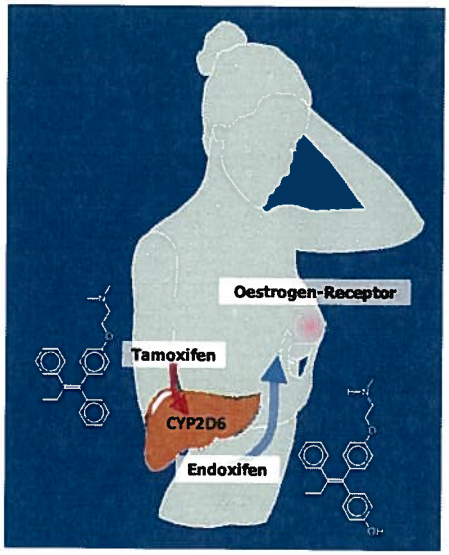

Fig. 2. The metabolic activation of tamoxifen with a low affinity to the tumour oestrogen receptor by the P_{450} enzyme CYP2D6 enzyme to endoxifen with a high affinity for the tumour oestrogen receptor.

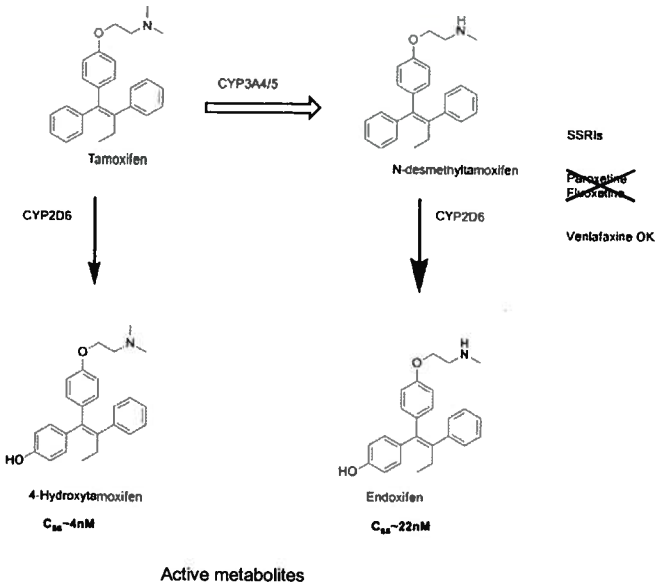

Fig. 3. The metabolism of tamoxifen to 4-hydroxytamoxifen, a metabolite with a high affinity for the oestrogen receptor. Tamoxifen's major metabolite is N-desmethyltamoxifen that has a similar binding affinity to the oestrogen receptors as tamoxifen. However, N-desmethyltamoxifen is metabolically activated to endoxifen, with a high binding affinity for the oestrogen receptor. The selective serotonin re-uptake inhibitors (SSRIs), paroxetine and fluoxetine block the metabolic activation of tamoxifen by blocking CYP2D6. Venlafaxine, a selective norepinephrine re-uptake inhibitor (SNRI), does not affect tamoxifen's metabolic activation, and therefore is the preferred choice to treat menopausal symptoms experienced with tamoxifen.

Anti-hormonal drug resistance can be manifest in two forms for the ER positive tumour: intrinsic resistance where the tumour does not respond at all to anti-hormone therapy, despite being ER positive, and acquired anti-hormone therapy where the tumour initially responds to anti-hormone therapy but then grows despite the continuing treatment. Much effort has focused on an understanding of the molecular mechanism of intrinsic anti-hormone resistance and it seems that cross-talk between growth factor receptors and the low levels of ER have essentially made the ER irrelevant for cell survival. No scientific advance has yet reversed intrinsic resistance and aided patients. In contrast, there have been significant advances in understanding acquired anti-hormone resistance in the laboratory and these emerging data have been translated to clinical practice.

The challenge: acquired drug resistance

Clinical experience with the successful application of long-term tamoxifen as an adjuvant therapy produced a clear survival advantage for patients.[28] Unselected patients treated for 5 years with adjuvant tamoxifen lived longer than patients in the non-treatment (placebo) arm but who were treated with tamoxifen at first recurrence as they had metastatic breast cancer. The clinical results with successful adjuvant tamoxifen therapy demonstrated[28] that our understanding of the development of drug resistance to tamoxifen treatment in ER positive disease was incorrect on

July 25, 1987 (the publication date of the Scottish MRC trial), but supported the principle of early treatment of micrometastatic disease. Also, it highlights the fact that resistance to tamoxifen for the treatment of metastatic disease occurs rapidly within 2 years, and this biology did not apply to an adjuvant application of tamoxifen. Despite the fact that the rat mammary carcinoma model demonstrates that earlier, longer treatment with an anti-oestrogen was a suitable clinical strategy,[8] there was no model of human diseases to test this hypothesis. However, in the mid-1980s, this was about to change. The ER positive breast cancer cell line MCF-7[29] exhibits oestradiol-stimulated tumor growth when transplanted into ovariectomized athymic mice. Tamoxifen blocks oestradiol-stimulated tumor growth but cannot maintain growth inhibition as ER positive tumors eventually grow despite tamoxifen treatment.[30] However, it seems that SERM and antihormonal resistance in breast cancer evolves and exposes a vulnerability in breast cancer that can be exploited in the clinic.[31]

The first transplantable model of tamoxifen resistance in breast cancer demonstrated that drug resistance to tamoxifen was unique.[32] Although tamoxifen can initially block oestradiol-stimulated growth of MCF-7 cells, resistant ER positive tumors can use either oestradiol or tamoxifen to *stimulate* tumor growth (Fig. 4). Tumours do not grow unless treated with tamoxifen or oestradiol so in the ovariectomized mouse, this is equivalent to the "non-oestrogen state" created by aromatase inhibitors. Tumours also do not grow if treated with the pure anti-oestrogen fulvestrant that destroys the ER.[33,34] This laboratory model replicates clinical experience with drug resistance to tamoxifen in metastatic breast cancer and explains why aromatase inhibitors or fulvestrant are effective second line treatments.[35,36] So, how does a study of the drug resistance to tamoxifen in the laboratory explain the effectiveness of 5 years of adjuvant tamoxifen to reduce recurrence rates in ER positive breast cancer to tamoxifen by fifty percent and continue to reduce mortality a decade after tamoxifen treatment is stopped? The answer is the evolution and reconfiguration of cell survival pathways that occurs in micrometastatic breast cancer during years of treatment.

Continuous retransplantation of successive generations of tamoxifen-stimulated MCF-7 tumor lines into athymic mice for more than 5 years results in a derived tumor line that does not respond to physiologic oestradiol with growth but rapid tumor regression through apoptotic cell death (Fig. 5).[37,38] These data were first presented at the St. Gallen meeting in 1992.[37] The concept offered at the time was that the ultimate and long lasting value of adjuvant tamoxifen therapy derives from stopping adjuvant tamoxifen when the woman's own oestrogen can now destroy the micrometastases that have been sensitized to

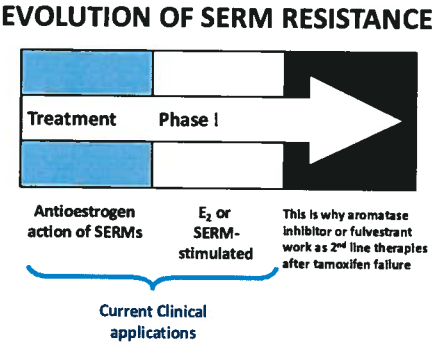

Fig. 4. The development of acquired antihormone resistance to selective oestrogen receptor modulators (SERMs) (tamoxifen or raloxifene). The unique feature of Phase I antihormone resistance is that oestrogen receptor positive breast tumours grow in response to either physiological oestradiol or the SERM. In the clinical setting (and laboratory models), an aromatase inhibitor (no oestrogen) or the pure anti-oestrogen, fulvestrant, that destroys the oestrogen receptor, stops the growth of Phase I resistant tumours to tamoxifen.[31]

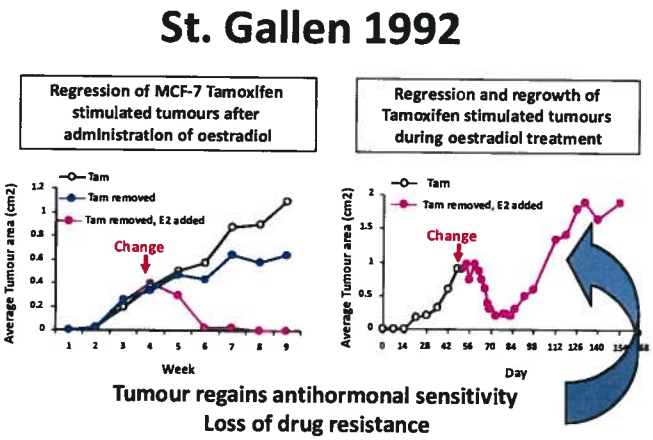

Fig. 5. Diagrammatic representation of the actions of physiologic oestradiol (E2) on the growth of small phase II MCF-7 tamoxifen resistant tumors in ovariectomized athymic mice. A larger tumour will regress with oestradiol treatment but will eventually display oestrogen-stimulated growth. If tumours are re-transplanted into a new generation of ovariectomized athymic mice and treated with oestradiol, tamoxifen will block oestrogen-stimulated tumour growth.[38] First presented in St. Gallen, 1993.[37]

oestrogen-induced apoptosis. The initial laboratory observations on low dose oestradiol-induced tumour regression were subsequently confirmed,[38] expanded[39–42] and translated successfully to clinical trial.[43,44] As a result, it is now possible to define the evolution of acquired anti-hormone therapy into a Treatment Phase where the anti-hormone blocks oestradiol stimulated tumour growth, Phase I when a SERM or oestradiol stimulates growth (or an aromatase inhibitor creates oestrogens independent growth) and Phase II when a SERM stimulates growth but physiological oestrogen provokes apoptosis either after stopping a SERM or after stopping an aromatase inhibitor (Fig. 6).

Thus, over the past four decades, general scientific principles have emerged and translated to clinical care for patients. The application of these principles of endocrine adjuvant therapy have benefited, and continued to benefit, millions of women worldwide, through a simple and cheap therapeutic intervention. We will now consider

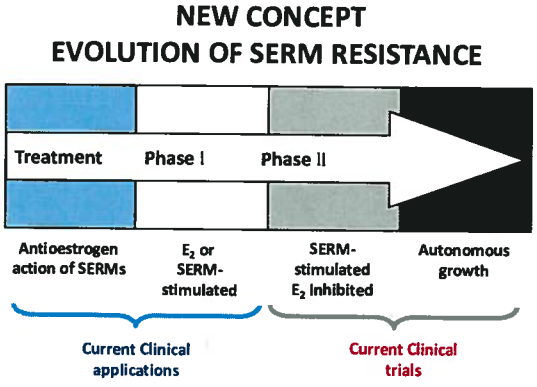

Fig. 6. The evolution of drug resistance to SERMs. Acquired resistance occurs during long-term treatment with a SERM and is evidenced by SERM-stimulated breast tumour growth. Tumours also continue to exploit oestrogen for growth when the SERM is stopped, so a dual signal transduction process develops. The aromatase inhibitors prevent tumour growth in SERM-resistant disease and fulvestrant that destroys the ER is also effective. This phase of drug resistance is referred to as Phase I resistance. Continued exposure to a SERM results in continued SERM-stimulated growth (Phase II), but eventually autonomous growth occurs that is unresponsive to fulvestrant or aromatase inhibitors. The event that distinguishes Phase I from Phase II acquired resistance is a remarkable switching mechanism that now causes apoptosis, rather than growth, with physiologic levels of oestrogen. A similar evolution occurs with aromatase inhibitor resistance from oestrogen independent growth with a transition to oestrogen-induced apoptosis. These distinct phases of laboratory drug resistance have their clinical parallels and this new knowledge is being integrated into the treatment plan.

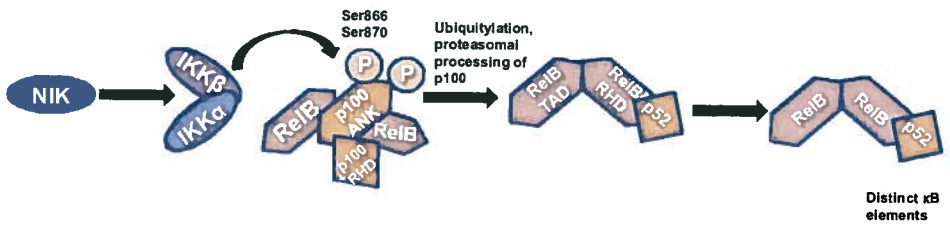

Fig. 7. The non-canonical pathway results in the activation of IKKα by NIK and phosphorylation of the NF-κB subunit. This process results in the conversion of p100 to p52. It is the p52-RelB heterodimers that target distinct κB elements on DNA. ANK: ankyrin-repeat motifs; NIK: NF-κB kinase; RelB: NF-κB family member; RHD: Rel-homology domain; TAD: transcriptional activation domain.

how emerging laboratory knowledge may reverse or at least hold Phase II resistance to enhance the longevity of the patient. We will, however then, revisit the clinical reality that increased tumour burden is a poor indicator of patient survival, so that the founding principles of our initial work, i.e. early treatment targeting the ER with long-term therapy[2] must be embraced by the clinical community.

Oestradiol-induced apoptosis under laboratory conditions

The administration of physiologic oestradiol to athymic mice implanted with phase II SERM (tamoxifen or raloxifene) resistant ER positive MCF-7 tumours[38,40,41,45] causes tumours to stop growing and/or rapidly regress. Similarly, the long-term oestrogen deprived clinical cell line MCF-7:5C[42,46] rapidly undergoes oestrogen-induced apoptosis both *in vitro* and *in vivo*. These laboratory observations are reminiscent of the pioneering studies of Sir Alexander Haddow FRS with his application of the first Chemical Therapy to successfully treat any cancer – high dose synthetic oestrogens to treat metastatic breast cancer.[47,48] He observed a 25% response rate but these were short-lasting.[47] The observation was made that no responses were observed close to menopausal but often dramatic responses occurred in women in their late 60s and 70s. By 1970, during the presentation of the Inaugural Karnofsky Award Lecture at the American Society of Clinical Oncology (ASCO)[48] (incidentally, when I was starting my PhD in Pharmacology at Leeds University) he stated: "... *the extraordinary extent of tumour regression observed in perhaps 1% of post-menopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us ...*".

Now we know that the responses Haddow observed occur because of oestrogen deprivation following the menopause. Longer oestrogen withdrawal after menopause was more effective at creating Phase II resistance in select patients, but high dose oestrogen therapy was necessary. Based on laboratory studies and clinical correlations, anti-hormone therapy does a better job in driving the rapid evolution to Phase II resistance and as a result, only physiological oestrogen is necessary to trigger apoptosis. Haddow's paradox that stood for 40 years now has clarity and we can start to offer treatment options to exploit the concept further.

Cell culture models provide a vehicle to examine, over time, oestrogen-induced apoptosis with the aim of pharmacologic modulation and the discovery of mechanisms that may have relevance for patient care. Through a knowledge of mechanisms, the elegant oestrogen trigger for naturally initiating tumour cell death may subsequently be exploited to other treatment scenarios. If we can decipher the process of ER-induced apoptosis from its current obscurity, this knowledge could be applied with the discovery of new drugs to trigger the mechanism without the involvement of ER. The ER is our current guide and light to find a new drug group.

We have undertaken an extensive examination of the actions of oestradiol on the growth (MCF-7), immediate apoptosis (MCF-7:5C) and delayed apoptosis (MCF-7:2A)[49] of our model cells using a 2-week time course of gene activity documented through mRNA analysis, using Agilent Gene Arrays. These studies were conducted in collaboration with Dr. Eric Ariazi and Dr. Heather Cunliffe. We extensively analyzed the gene time course, and completed gene segregation based on hierarchical pathway analysis. We found that MCF-7 and MCF-7:2A, our control cells, remained quiescent during the initial few days of oestradiol treatment (1nM) whereas the pre-apoptotic MCF-7:5C cells responded with a massive rise in the activation of inflammatory genes. Analysis of the sequence of events during the first few days of gene activation, we propose that apoptosis occurs in MCF-7:5C cells by the exploitation of the non-canonical pathway for NF-κB signal transduction (Fig. 7). Furthermore, we have mapped out the time-course activation of each caspase (except caspase 3 that is absent in MCF-7) and determined that caspase 4 is the first and controlling executioner to provoke programmed cell death. We have interrogated the apoptotic process with purported inhibitors of individual activated caspases to confirm our conclusion of the role of caspase 4. Blockade of caspase 4 blocks oestrogen-induced apoptosis.

Most importantly, the activation of inflammatory genes suggests that oestradiol-induced apoptosis could be inhibited or at least modulated by glucocorticoids. We have subsequently established that dexamethasone inhibits oestrogen-induced apoptosis in a concentration related manner. This novel observation may have important implications for the application of oestradiol-induced apoptosis for individualized patient care. Is it possible that the inadvertent administration of glucocorticoids during patient care could block oestrogen-induced apoptosis or that a patient's own glucocorticoids may also inhibit apoptosis, if patients are challenged with oestrogen following exhaustive anti-hormone therapy? The anti-glucocorticoid mifipristone (RU486) could potentially be used with oestrogen to block glucocorticoid action temporarily for a few weeks during low dose oestrogen administration to enhance apoptosis.

Examination of the Agilent gene array data confirmed our previous work[49] that elevated synthesis of glutathione is protecting MCF-7:2A cells from immediate apoptosis in response to oestrogen. Apoptosis appears to be retarded in MCF-7:2A cells but an activation of autophagy heralds an enhanced transcription of caspase 4 and then triggers oestrogen-induced apoptosis during the second week of oestradiol treatment. We have previously successfully used pharmacological inhibitors to test our hypothesis. Buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis,[49] enhances oestradiol-induced apoptosis from a slow event lasting 2 weeks to an immediate event. Unfortunately, BSO, though used extensively in clinical trial a decade or more ago, is no longer available to examine whether it is possible to enhance oestrogen-induced apoptosis in patients with select tumours.

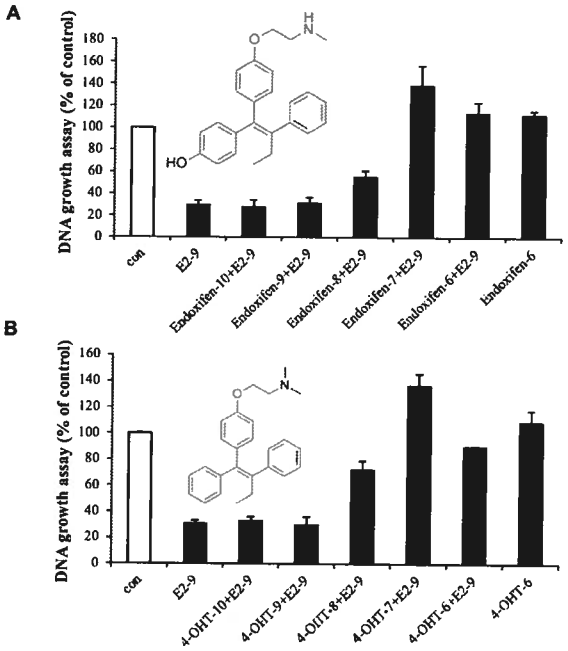

Fig. 8. The reversal of oestradiol-induced apoptosis (1nM) by increasing concentrations of 4-hydroxytamoxifen or endoxifen. This nonsteroidal antioestrogen effect highlights the ER dependence for oestradiol-induced apoptosis.

Thus far, our studies have described what happens, but the real question is how does the oestradiol/ER complex triggers apoptosis? Are there clues about the actual shape or structure of the oestrogen–ER complex that can be modulated and investigated further? The MCF-7:5C cells depend on a functioning ER for oestradiol-induced apoptosis. The pure anti-oestrogen fulvestrant binds to the ER and causes the rapid destruction of the protein complex. As a result, fulvestrant blocks oestradiol-induced apoptosis in a concentration related manner. Interestingly enough, the tamoxifen metabolites 4-hydroxytamoxifen (4OHTam) and endoxifen do not block or affect the autonomous growth of MCF-7:5C cells but do block the initiation of oestradiol-induced apoptosis. Herein lies a clue to the mechanism that triggers oestradiol-induced apoptosis (Fig. 8). X-Ray crystallographic studies of the ER ligand binding domain and the oestrogens, oestradiol and diethylstilboestrol (DES) and the SERMs 4OHTam[50] and raloxifene[51] provide a fascinating insight into oestrogen and anti-oestrogen action. The solution of the crystal structures demonstrate that the planar oestrogens are sealed within the ligand binding domain by helix 12 which then allows co-activators to bind to the activating function (AF)-2 site on the complex. This event amplifies oestrogen action through gene transcription. In contrast, the bulky side chain of the triphenylethylene 4OHTam and the benzothiophene raloxifene prevent helix 12 from sealing the hydrophobic ligand binding domain which prevents coactivator binding to AF-2. The promiscuous oestrogen-like activity of 4OHTam is explained by the inability of the anti-oestrogenic side chain to neutralize and shield the exposed aspartate at position 351 at the surface of the ligand binding domain. This exposed carboxylic acid communicates with AF-1 to induce oestrogen-like actions. Raloxifene completely blocks and neutralizes the aspartate at 351 and the raloxifene–ER complex does not activate AF-1. This hypothesis has been successfully interrogated with changes in the ligand and the aspartate at 351 to modulate the activation of a model oestrogen target gene Transforming Growth Factor α.[52–55] Overall, we concluded that activation of AF-1 by an exposed surface aspartate 351 confirms that helix 12 is not sealing the ligand binding domain so it can, therefore, communicate a signal to AF-1 to induce oestrogen-like gene activation. If aspartate 351 is masked under helix 12 with a planar oestrogen then AF-2 is activated and the communication between AF-1 and aspartate 351 is mute. These data and conclusions subsequently resulted in a reclassification of oestrogens into class 1 (planar) and class 2 (non-planar)[56] using a simple assay to determine whether helix 12 was locking the ligand into the hydrophobic ligand binding domain or not. However, the biological significance of this molecular insight was not apparent until recently.

Based on the fact that 4OHTam blocks oestradiol-induced apoptosis at the ER and the statement that the "bulky side chain" of 4OHTam altered the conformation of the ER preventing helix 12 from sealing the ligand binding domain,[50] we advanced the hypothesis that the "bulky side chain" of 4OHTam was the phenyl ring of the oestrogenic triphenylbut-1-ene not just the *para*-dimethylaminoethoxy group traditionally associated with anti-oestrogen action. Perhaps the phenyl ring of the triphenylbut-1-ene anti-oestrogen was stopping helix 12 from sealing the binding site? A series of triphenylethylenes (TPEs), previously known to be classified exclusively as oestrogens in rodent uterine weight and vagina cornification assays, was used to establish oestrogenic activity in MCF-7 breast cancer cells. All compounds were found to be full oestrogens in growth assays compared with oestradiol and DES and to fully-activate an ERE luciferase report ER gene system in MCF-7 cells.[57] In contrast, while oestradiol and DES will trigger apoptosis and cell death in MCF-7:5C cells within a week, the synthetic TPE "oestrogens" do not provoke massive apoptosis and indeed block oestradiol-induced apoptosis. Studies using the CHIP assay at the ERE site in the promoter region of the oestrogen responsive pS_2 gene demonstrate that whereas oestradiol E_2ER complex is recruited with the co-activator SRC3 in AF-2 neither 4OHTam nor the TPE ER complexes are recruited to the promoter.[58]

Overall, these data demonstrate that oestrogen-induced apoptosis is governed and programmed by the shape of the ER complex. As a consequence, shape governs coactivator binding at AF-2 and these events subsequently trigger apoptosis. A recent study[59] advances our initial oestrogen reclassification paper[56] and confirms, using a phage display library, that the shape of the ligand programs the external shape of the ER complex. A precise evaluation of the immediate early genes involved in the apoptotic response will describe the mechanism of the oestrogenic trigger for cell death. Exploitation of this knowledge may find applications in other disease states.

Oestrogen treatment: current clinical findings and translation to adjuvant therapy

The laboratory finding[37–39] that acquired resistance to anti-hormone therapy evolves and exposes a vulnerability of breast cancer cells to the apoptotic actions of physiological oestrogen, provides an important insight into potential therapeutic applications. As previously noted in this paper, the anti-tumour effect of physiological oestrogen is reminiscent of the early therapeutic use of high-dose oestrogen therapy for the treatment of metastatic breast cancer in post-menopausal women.[47] It was noted that the further from menopause patients were, the more likely there was to be a tumour response, but these responses never exceeded 30% in any given population.

It is now clear that the acute oestrogen deprivation caused by anti-hormones speeds up the molecular adaptation and reconfiguration of vulnerable survival pathways. The surviving populations of susceptible breast cancer cells also have increased sensitivity to oestrogen-induced apoptosis. Low-dose oestrogen therapy now becomes a clinically viable strategy with the prospect of reducing oestrogen-associated side effects.

The laboratory data generated and published in the 1990s proposed the clinical strategy of using low-dose oestrogen therapy

Table 2
The proof of principle for (a) high-dose oestrogen (DES, 15 mg daily) triggering tumour responses in patients with metastatic breast cancer following exhaustive antihormone therapy[43] or (b) a comparison of high-dose oestrogen (oestradiol, 30 mg daily) or low-dose oestrogen (oestradiol, 6 mg daily), producing similar clinical benefit rates following the failure of therapy with an aromatase inhibitor.[44]

(a) Response			
Complete	Partial	Stable disease	
4/32	6/32	2/32	
(b) Dose	No. of patients	Response	Clinical benefit
6 mg	34	10/34	29%
30 mg	32	9/32	28%

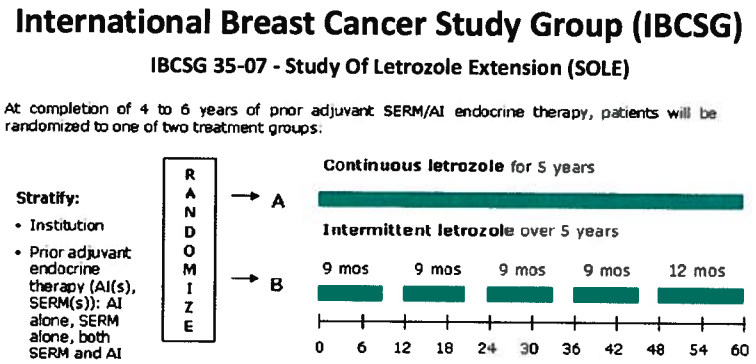

Fig. 9. The Schema for the Study of Letrozole Extension (SOLE) conducted by the International Breast Cancer Study Group (IBCSG 35-07). Patients randomized following five years of adjuvant antihormone therapy to letrozole continuously or intermittent letrozole (3-month drug holidays per year for 5 years). The rationale is that the woman's own oestrogen in the intermittent arm will trigger apoptosis in aromatase inhibitor resistant cells and reduce recurrence rates.

to "purge" breast cancer cells with Phase II-acquired anti-hormone resistance, but then the re-introduction of anti-hormone therapy would control oestradiol-stimulated tumour growth.[37,38] A European trial led by Dr. Per Lonning[43] recruited patients with metastatic breast cancer following exhaustive anti-hormone therapy to determine the effect of treatment with standard high-dose DES (5 mg tid). Results are summarized in Table 2a. Select patients responded well with one patient subsequently reported[60] being disease-free more than 10 years after first initiating a high-dose oestrogen "purge" therapy. "... "

In a follow-up study, Ellis[44] compared and contrasted high-dose (10 mg tid) and low-dose (1 mg tid) oestradiol therapy in patients who relapsed during adjuvant aromatase inhibitor therapy. Results are summarized in Table 2b. Results were not as impressive as in the Lonning study probably because patients did not receive "exhaustive" endocrine therapy prior to an oestrogen "purge". Nevertheless, the clinical trial confirms that low-dose oestrogen can produce similar clinical benefit when compared with high-dose oestrogen treatment but with fewer serious side effects.

Finally, there is further clinical evidence from the Women's Health Initiative (WHI) that oestrogen replacement therapy (ERT) alone causes a decrease rather than increase in the incidence of breast cancer[61], and a recent report from the Million Women Study in the UK demonstrates that oestrogen alone increases breast cancer incidence immediately following the menopause but if ERT is used more than 5 years after oestrogen exposure, oestrogen replacement therapy does not cause a rise in breast cancer incidence.[62] An overarching explanation for these apparently confusing clinical observations is clarified by our evolving molecular model to exploiting the role of oestradiol in the life and death of breast cancer cells.[63,64] We interpret these clinical findings based on the evolution of anti-hormone resistance as follows: breast cancer cells in an environment of oestrogen only grow in response to exogenous oestrogen, but following long-term oestrogen deprivation surviving breast cancer cells either die or at least do not develop into tumours.

The clinical and laboratory database also provides continuing support for the ongoing adjuvant Study of Letrozole Extension (SOLE) trial (Fig. 9). Patients who have completed 5 years of adjuvant therapy with tamoxifen, an AI or any sequence are then randomized to an AI continuously for 5 years or an AI with a drug holiday for 3 months a year. The trial seeks to exploit the hypothesis, advanced at the 1992 St. Gallen Meeting, that a woman's own oestrogen may act as an anti-tumour agent after adjuvant anti-hormone therapy is stopped. The SOLE trial proposes a rigorous test of the hypothesis under controlled conditions that promises to create a practical advantage for patients following drug holidays. Results from this trial coupled with the expanding molecular database concerning the modulation of oestrogen-induced apoptosis may result in the proposition of regularly purging patients for a week or two with ERT if decades of anti-hormone therapy are to become common place in order that the disease is held in check and prevented from recurring. The question is now – at what point is oestrogen intervention too late?

Fighting overwhelming cancer cell flexibility

The enemy is us, Haddow[48] in the Inaugural Karnofsky Lecture reasoned that it would not be possible to develop a cancer specific therapy in the same way Ehrlich had for syphilis, as cancer was our own cells. What he did not know was that the situation is worse than that. The replicative fidelity of normal cells replaces exactly what is lost, but in its own special place. Cytotoxic chemotherapy kills the patient by indiscriminately killing normal differentiated cells, and perhaps stem cells, so life saving repopulation for the host

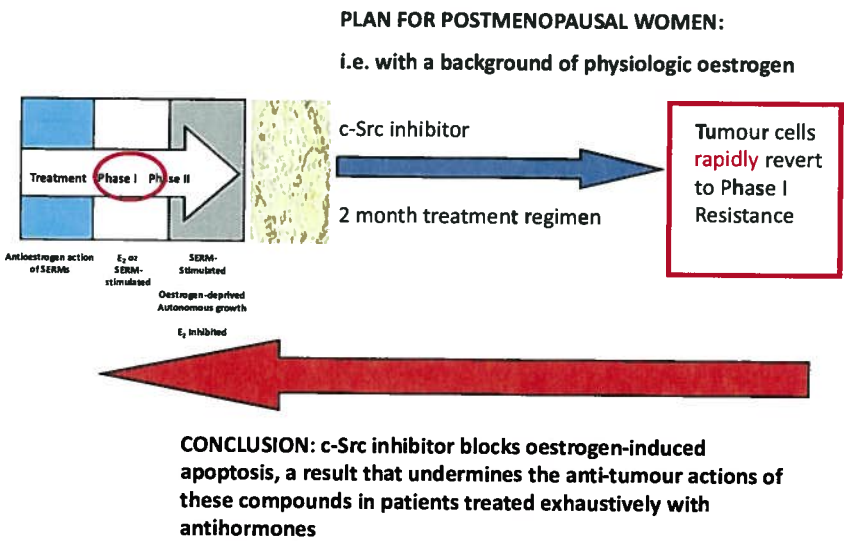

Fig. 10. The evolution of drug resistance and rapid alterations in cell populations if a c-Src inhibitor PP2 (5 μM) is incubated with MCF-7:5C cells in the presence of 1nM oestradiol for two months to mimic a clinical scenario of a postmenopausal woman who fails an aromatase inhibitor to block growth. Apoptosis from oestrogen is blocked and the cells revert to Phase I resistance, i.e. oestrogen and SERM-stimulated growth.

organism is impossible or too late. In contrast, human populations eventually adapt to external destructive forces such as fatal infectious diseases (plague, small pox, etc.) but individuals only survive through their preprogrammed nimble immunology. The survivors repopulate. And so it is with cancer at the cellular level within the body. However, immunology has not yet been proven to be of significance for breast cancer prevention. Haddow was right there – the enemy is us. The tumour at diagnosis has hundreds of mutations compared to the (purportedly) normal human genome.[65,66] This and activated oncogenes, or loss of tumour suppression genes, provides the random survival flexibility within the cancer cell population to adapt and eventually thrive in a hostile (cytotoxic) environment within a few months. The principle is a microscopic adaptation of simple Darwinian evolution that has played out over the millennia by animals on earth. Random mutations create a preferred trait that permits survival, while the non-adaptive species or population dies out. The situation with cancer only becomes worse through adaptive survival responses preprogrammed in the cancer stem cell. These cellular "spores" seek to expand and prepare for massive repopulation in an enforced anoxic environment. The clinician is confronted with a perverted microcosm of the struggle for life by cancer cells programmed to create infinite candidates in the quest for survival. The patient is overwhelmed by sheer numbers in the wrong places. This is the challenge of targeted molecular therapeutics but how to build rationally on the advances in survivorship achieved over the past 40-years in breast cancer?

The path to progress in drug development has not changed significantly during this time, despite our new knowledge of the disease. The administrative plan for drug evaluation is in place to protect citizens and provide safe and therapeutically proven medicines for clinical care. To market a new drug to treat breast cancer, a precise system must be followed to obtain government approval. Phase I clinical trials must offer the hope of potentially effective treatment to patients who have received all possible therapeutic options. The goal is to document dose limiting toxicities and at this stage of the disease, responses are a major bonus. Phase II trials focus on a cancer type of interest based on reasonable data from preclinical studies or an unanticipated response in Phase I trials. If a candidate is successful in Phase II trials, the drug is evaluated against or with the current standard of care. It should be emphasized that therapeutic results from Phase II trials with tamoxifen were not very dramatic, but Phase I data on toxicity for the patient was excellent compared with other therapies available. Only by targeting the ER in the tumour and applying long-term adjuvant therapy did patient survivorship increase. A discarded contraceptive became the "gold standard" for breast cancer therapy over a 30-year journey.[1]

With this background, how do we build on success? Today there are dozens of good potential targets and dozens of plausible candidates for each target. However, unlike the ER which was, it has turned out, the principle messenger to stimulate breast tumour growth in about 30% of tumours, other candidate targets are proving to be not the star but part of the chorus. In late stage disease, one pathway is blocked but others now compensate. Pathways to preserve cellular life can be essential in all cells, but a cancer cell specific pathway is the only key to success in cancer therapeutics.

Based on our current work investigating oestradiol-induced apoptosis of breast cancer cells with long-term acquired resistance, we proposed a hypothesis: can we block breast cancer cell survival mechanisms and enhance the chances that the cell must undergo apoptosis in response to oestradiol?

c-Src was the first identified oncogene in cancer and is said to be present in more than 70% of breast cancers.[67] It controls AKT and MAPK phosphorylation cascades as the intermediary from growth factor receptor activation. It would appear to be an ideal target to subvert cell survival; almost as good as the ER! We posed the question, that if we blocked c-Src in breast cancer cells resistant to aromatase inhibitors would we then enhance apoptosis? In other words, would we generate value for the cancer patient by increasing cell kill as we have previously found that c-Src inhibitors were completely ineffective in affecting growth of oestrogen stimulated MCF-7 cells, but had significant efficiency in blocking the growth of ER-negative MDA-MB-231 and oestrogen stimulated ER-positive T47D cells. More importantly, long-term oestrogen-deprived MCF-7 cells have elevated pSrc. As most ER-positive cancers are exhaustively treated with antihormones before Phase I/II testing and we were building on a known efficacy of estrogen therapy, the proposition appeared sound. Our model cell, MCF-7:5C, had elevated phospho c-Src and are targeted inhibitor PP2 completely blocked phosphorylation. However, a 2 month course of treatment of MCF-7:5C cells with physiological oestrogen levels (1nM) that would be present in a postmenopausal patient plus the c-Src inhibitor (5 microMolar), resulted in the blockade of oestrogen-induced apoptosis and the reversion of the cell population to

Phase 1 drug resistance (Fig. 10), i.e. estrogen or SERM-stimulated for growth. Within 2 months, the flexibility of cell populations had created no real advance that could realistically aid the patient.

Thus, as an illustration of the challenge we face for the application of logical targeted therapy, one could conclude the following: an expanding menu of targeted medicines is available for testing, but only select populations will respond. Testing a c-Src inhibitor in the incorrect stage of antihormone resistance or patient populations cannot be successful. This is the problem: the testing populations for registration may be inappropriate for a drug candidate that is magnificent in a neoadjuvant therapy naïve disease study. However, does this enhance registration? Unfortunately not.

We need practical strategies to aid communities to hold the development and death from breast cancer while we attempt to decipher the enormous complexity of pathways and permutations of targeted therapies. This conclusion brings us back to the second piece of translational research started in our laboratory in the 1970s – chemoprevention. Remarkably, the lamp of tamoxifen shed light on an alternative strategy to reduce cancer incidence and preempt the aforementioned Gordian Knot. Unfortunately, the initial strategy for the clinical application of chemoprevention requires the identification of high-risk populations to be treated with the pioneer tamoxifen. This approach is flawed. However, a public health strategy for an aging population that creates wellness for as long as possible is a laudable goal now within our grasp.

Tamoxifen is also about progress in chemoprevention

An extensive study of the pharmacology of tamoxifen[68] identified its ability to modulate oestrogen target tissues around the body; tamoxifen is anti-oestrogenic in the breast and oestrogen-like in bone and lowers circulating cholesterol.[69–72] Translational research also first identified the potential of tamoxifen to increase the risk of developing endometrial cancer during extended treatment schedules.[73–75] Tamoxifen blocks breast tumour growth and development but enhances endometrial cancer growth. As a result, new procedures were introduced for the gynecological monitoring of post-menopausal patients receiving long-term tamoxifen therapy. New agents, without endometrial problems, were needed for investigation. Knowledge of selective ER modulation by tamoxifen and also the pharmacology of the structurally-related failed breast cancer drug, raloxifene, led to the creation of a new drug group, the Selective ER Modulators (SERMs),[76] with the potential to treat and prevent multiple diseases in women ***and*** prevent breast cancer at the same time. The fact that raloxifene was less oestrogen-like than tamoxifen in the rodent uterus and less likely to increase the incidence of the endometrial cancer in patients[77,78] meant that safer compounds could be identified as chemopreventives for breast cancer but a new strategy to achieve the goal was essential. Benefits for a tiny, unidentifiable minority is unacceptable if the vast majority of women in a high risk population have side effects, some life-threatening. The road map for the pharmaceutical industry was clearly stated in 1990. *Is this the end of the possible applications for anti-oestrogens? Certainly not! We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer.*[79]

Raloxifene pioneered the concept in the clinic confirming the prediction that the prevention of breast cancer would occur during the treatment and prevention of osteoporosis in high risk post-menopausal women[80] with no increase in endometrial cancer. Today, the prediction that SERMs could control multiple diseases in women following the menopause is poised to become a reality. Lasofoxifene (Fig. 1) is approved in the European Union for the prevention and treatment of osteoporosis which simultaneously decreases the incidence of breast cancer, strokes and myocardial infarction, but without increasing endometrial cancer risk.[81] Lasofoxifene is more than one hundred times more potent than raloxifene and the aforementioned strategy[79] to improve women's health in aging populations is the new face of chemoprevention in breast cancer – treat the majority of women for major diseases like osteoporosis and coronary heart disease and prevent breast cancer as a beneficial side effect. The saving in health care costs by **not** paying for the treatment of breast cancer in tens of thousands of women **without** breast cancer will be considerable, but admittedly hard to quantitate.

Raloxifene is not only available in the United States of America for the treatment and prevention of osteoporosis but also for reduction of the incidence for breast cancer in post-menopausal, high-risk women.[82] However, the SERM must be given indefinitely to remain effective in both diseases.[83] In contrast, tamoxifen remains effective for decades after the limited treatment period of 5 years is stopped.[84,85] As mentioned previously, the key to understanding this fact probably resides in the laboratory study of drug resistance to SERMs and aromatase inhibitors and the development of a cellular susceptibility to oestrogen-induced apoptosis. The fact that the same tumour responsiveness to raloxifene appears to be retarded in clinical practice suggests that the known poor pharmacokinetics and bioavailability of raloxifene is not able to rapidly produce an "anti-oestrogenic" state around the nascent tumour like tamoxifen. This may explain the reduced performance of raloxifene against tamoxifen in the STAR trial, following the cessation of 5-years of treatment.[83]

Summary and closing thoughts

Over the past 40 years, we have witnessed a dramatic improvement in the survivorship of the majority of patients with a diagnosis of ER positive breast cancer. The SERM tamoxifen pioneered the process. Translational research has added further cheap and effective targeted anti-hormonal therapies to the physician's armamentarium that are proven to be of benefit in randomized adjuvant clinical trials world-wide. Not only has therapy been improved substantially over the past 40 years, from the time in the early 1970s when there was stated to be little prospect of successful survival advances with "endocrine therapy", but also the parallel path of chemoprevention has been pioneered successfully with the same SERM tamoxifen. This SERM heralded a new era of general medicine where a family of SERMs would allow women to expect to reduce their risk of fractures but prevent breast cancer at the same time. This was only a laboratory concept 20-years ago[79,86] but it seems obvious that with an aging population that seeks to remain active for as long as possible, that the SERMs will play their part in reducing the incidence of breast cancer if used wisely in the post-menopausal population.

The lessons learned with the lamp-light of the pioneer tamoxifen are now established principles in cancer therapeutics. The principles are: aim at the target (ER), start therapy as early as possible (i.e. as few lymph nodes as possible involved), long therapy is preferable to shorter therapy, compliance with the medicine is essential and drug interaction with SSRIs to stop menopausal side effect in a few should be avoided. Conforming to these principles aids patients' survival. The light from the lamp also taught us

what we did not know. Firstly tamoxifen is a selective modulator of ER action around a woman's body and heralded a new drug group (the SERMs) that prevent osteoporosis and prevent breast cancer as a beneficial side effect. This avoids the need to find the exact women who would benefit from chemoprevention using the Gail Model.[87] Secondly, drug resistance evolves so that oestradiol becomes an apoptotic trigger. Further studies solved the concern expressed by Haddow in 1970 that the mechanism of oestrogen-induced apoptosis was a mystery. Now oestrogen therapy has a niche application in patient care.

During the past 40 years, the mosaic of endocrine adjuvant therapy and chemoprevention with SERMs has been clarified by effective translational research.[4,88] The next challenge for a generation of "omic" scientists is to prioritize the opportunities in molecular therapeutics based on this solid start, so as to advance and individualize treatment.

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Conflict of interest statement

The authors have no conflict of interest to declare.

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References

1. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2003;**2**:205–13.
2. Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 2008;**44**:30–8.
3. Jordan VC. Selective estrogen receptor modulation: a personal perspective. *Cancer Res* 2001;**61**:5683–7.
4. Jordan VC. The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer – survival or death? *J Clin Oncol* 2008;**26**:3073–82.
5. EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;**351**:1451–67.
6. EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005;**365**:1687–717.
7. Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *Eur J Cancer* 1975;**11**:205–6.
8. Jordan V, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. *Adjuvant Ther Cancer* 1979;**2**:19–26.
9. Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 1980;**16**:239–51.
10. Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* 1977;**75**:305–16.
11. Jordan VC, Dix CJ, Naylor KE, Prestwich G, Rowsby L. Nonsteroidal antiestrogens: their biological effects and potential mechanisms of action. *J Toxicol Environ Health* 1978;**4**:363–90.
12. Allen KE, Clark ER, Jordan VC. Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure–activity relationships. *Br J Pharmacol* 1980;**71**:83–91.
13. NATO. Controlled trial of tamoxifen as adjuvant agent in management of early breast cancer. Interim analysis at four years by Nolvadex Adjuvant Trial Organisation. *Lancet* 1983;**1**:257–61.
14. NATO. Controlled trial of tamoxifen as single adjuvant agent in management of early breast cancer. Analysis at six years by Nolvadex Adjuvant Trial Organisation. *Lancet* 1985;**1**:836–40.
15. Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. *Br J Cancer* 1971;**25**:270–5.
16. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* 2007;**72**:7–25.
17. Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, Forbes JF, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* 2005;**365**:60–2.
18. Hershman DL, Kushi LH, Shao T, Buono D, Kershenbaum A, Tsai WY, et al. Early discontinuation and nonadherence to adjuvant hormonal therapy in a cohort of 8,769 early-stage breast cancer patients. *J Clin Oncol* 2010;**28**:4120–8.
19. Partridge AH, LaFountain A, Mayer E, Taylor BS, Winer E, Asnis-Alibozek A. Adherence to initial adjuvant anastrozole therapy among women with early-stage breast cancer. *J Clin Oncol* 2008;**26**:556–62.
20. Lash TL, Fox MP, Westrup JL, Fink AK, Silliman RA. Adherence to tamoxifen over the five-year course. *Breast Cancer Res Treat* 2006;**99**:215–20.
21. McCowan C, Shearer J, Donnan PT, Dewar JA, Crilly M, Thompson AM, et al. Cohort study examining tamoxifen adherence and its relationship to mortality in women with breast cancer. *Br J Cancer* 2008;**99**:1763–8.
22. Geiger AM, Thwin SS, Lash TL, Buist DS, Prout MN, Wei F, et al. Recurrences and second primary breast cancers in older women with initial early-stage disease. *Cancer* 2007;**109**:966–74.
23. Yood MU, Owusu C, Buist DS, Geiger AM, Field TS, Thwin SS, et al. Mortality impact of less-than-standard therapy in older breast cancer patients. *J Am Coll Surg* 2008;**206**:66–75.
24. Brauch H, Jordan VC. Targeting of tamoxifen to enhance antitumour action for the treatment and prevention of breast cancer: the 'personalised' approach? *Eur J Cancer* 2009;**45**:2274–83.
25. Dezentje VO, van Blijderveen NJ, Gelderblom H, Putter H, van Herk-Sukel MP, Casparie MK, et al. Effect of concomitant CYP2D6 inhibitor use and tamoxifen adherence on breast cancer recurrence in early-stage breast cancer. *J Clin Oncol* 2010;**28**:2423–9.
26. Lash TL, Cronin-Fenton D, Ahern TP, Rosenberg CL, Lunetta KL, Silliman RA, et al. Breast cancer recurrence risk related to concurrent use of SSRI antidepressants and tamoxifen. *Acta Oncol* 2010;**49**:305–12.
27. Kelly CM, Juurlink DN, Gomes T, Duong-Hua M, Pritchard KI, Austin PC, et al. Selective serotonin reuptake inhibitors and breast cancer mortality in women receiving tamoxifen: a population based cohort study. *BMJ* 2010;**340**:c693.
28. SCTO. Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee, Scottish Cancer Trials Office (MRC), Edinburgh. *Lancet* 1987;**2**:171–5.
29. Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Res* 1997;**57**:3071–8.
30. Osborne CK, Coronado EB, Robinson JP. Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol* 1987;**23**:1189–96.
31. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;**5**:207–13.
32. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 1988;**48**:5183–7.
33. Gottardis MM, Jiang SY, Jeng MH, Jordan VC. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. *Cancer Res* 1989;**49**:4090–3.
34. Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. *Cancer Res* 1991;**51**:3867–73.
35. Osborne CK, Pippen J, Jones SE, Parker LM, Ellis M, Come S, et al. Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. *J Clin Oncol* 2002; **20**:3386–95.
36. Howell A, Robertson JF, Quaresma Albano J, Aschermannova A, Mauriac L, Kleeberg UR, et al. Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. *J Clin Oncol* 2002;**20**:3396–403.
37. Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res* 1993;**127**:23–33.
38. Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;**6**:2028–36.
39. Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *J Natl Cancer Inst* 2001;**93**:1714–23.

40. Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, et al. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. *J Natl Cancer Inst* 2003;**95**:1586–97.
41. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* 2003;**95**:1597–608.
42. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 2005;**97**:1746–59.
43. Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jorgensen LM, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 2001;**67**:111–6.
44. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *JAMA* 2009;**302**:774–80.
45. Balaburski GM, Dardes RC, Johnson M, Haddad B, Zhu F, Ross EA, et al. Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. *Int J Oncol* 2010;**37**:387–98.
46. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 2005;**94**:131–41.
47. Haddow A, Watkinson JM, Paterson E, Koller PC. Influence of synthetic oestrogens on advanced malignant disease. *Br Med J* 1944;**2**:393–8.
48. Haddow A. David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer* 1970;**26**:737–54.
49. Lewis-Wambi JS, Kim HR, Wambi C, Patel R, Pyle JR, Klein-Szanto AJ, et al. Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* 2008;**10**:R104.
50. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 1998;**95**:927–37.
51. Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 1997;**389**:753–8.
52. Levenson AS, Jordan VC. The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. *Cancer Res* 1998;**58**:1872–5.
53. MacGregor Schafer J, Liu H, Bentrem DJ, Zapf JW, Jordan VC. Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. *Cancer Res* 2000;**60**:5097–105.
54. Liu H, Lee ES, Deb Los Reyes A, Zapf JW, Jordan VC. Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor alpha complex. *Cancer Res* 2001;**61**:3632–9.
55. Liu H, Park WC, Bentrem DJ, McKian KP, Reyes Ade L, Loweth JA, et al. Structure-function relationships of the raloxifene-estrogen receptor-alpha complex for regulating transforming growth factor-alpha expression in breast cancer cells. *J Biol Chem* 2002;**277**:9189–98.
56. Jordan VC, Schafer JM, Levenson AS, Liu H, Pease KM, Simons LA, et al. Molecular classification of estrogens. *Cancer Res* 2001;**61**:6619–23.
57. Maximov PY, Myers CB, Curpan RF, Lewis-Wambi JS, Jordan VC. Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. *J Med Chem* 2010;**53**:3273–83.
58. Sengupta S, Sharma CG, Jordan VC. Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells. *Horm Mol Biol Clin Investig* 2010;**2**:235–43.
59. Bourgoin-Voillard S, Gallo D, Laios I, Cleeren A, Bali LE, Jacquot Y, et al. Capacity of type I and II ligands to confer to estrogen receptor alpha an appropriate conformation for the recruitment of coactivators containing a LxxLL motif – Relationship with the regulation of receptor level and ERE-dependent transcription in MCF-7 cells. *Biochem Pharmacol* 2010;**79**:746–57.
60. Lonning PE. Additive endocrine therapy for advanced breast cancer – back to the future. *Acta Oncol* 2009;**48**:1092–101.
61. Stefanick ML, Anderson GL, Margolis KL, Hendrix SL, Rodabough RJ, Paskett ED, et al. Effects of conjugated equine estrogens on breast cancer and mammography screening in postmenopausal women with hysterectomy. *JAMA* 2006;**295**:1647–57.
62. Beral V, Reeves G, Bull D, Green J. Breast cancer risk in relation to the interval between menopause and starting hormone therapy. *J Natl Cancer Inst* 2011;**103**:296–305.
63. Lewis-Wambi JS, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? *Breast Cancer Res* 2009;**11**:206.
64. Maximov PY, Lewis-Wambi JS, Jordan VC. The paradox of oestradiol-induced breast cancer cell growth and apoptosis. *Curr Signal Transduct Ther* 2009;**4**:88–102.
65. Berger MF, Lawrence MS, Demichelis F, Drier Y, Cibulskis K, Sivachenko AY, et al. The genomic complexity of primary human prostate cancer. *Nature* 2011;**470**:214–20.
66. Pfeifer GP, Hainaut P. Next-generation sequencing: emerging lessons on the origins of human cancer. *Curr Opin Oncol* 2011;**23**:62–8.
67. Ottenhoff-Kalff AE, Rijksen G, van Beurden EA, Hennipman A, Michels AA, Staal GE. Characterization of protein tyrosine kinases from human breast cancer: involvement of the c-Src oncogene product. *Cancer Res* 1992;**52**:4773–8.
68. Jordan VC. Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. *Br J Pharmacol* 2006;**147**(Suppl 1):S269–76.
69. Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. *Fed Proc* 1987;**46**:1870–4.
70. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 1987;**10**:31–5.
71. Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, et al. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med* 1992;**326**:852–6.
72. Love RR, Wiebe DA, Newcomb PA, Cameron L, Leventhal H, Jordan VC, et al. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Ann Intern Med* 1991;**115**:860–4.
73. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 1988;**48**:812–5.
74. Fornander T, Cedermark B, Mattsson A, Skoog L, Theve T, Askergren J, et al. Adjuvant tamoxifen in early breast-cancer – occurrence of new primary cancers. *Lancet* 1989;**1**:117–20.
75. Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 1998;**90**:1371–88.
76. Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr Rev* 2004;**25**:45–71.
77. Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. *Life Sci* 1983;**32**:1031–6.
78. Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. *Cancer Res* 1990;**50**:3189–92.
79. Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. *Cancer Res* 1990;**50**:4177–89.
80. Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* 1999;**281**:2189–97.
81. Cummings SR, Ensrud K, Delmas PD, LaCroix AZ, Vukicevic S, Reid DM, et al. Lasofoxifene in postmenopausal women with osteoporosis. *N Engl J Med* 2010;**362**:686–96.
82. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* 2006;**295**:2727–41.
83. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. *Cancer Prev Res (Phila)* 2010;**3**:696–706.
84. Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* 2005;**97**:1652–62.
85. Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. *J Natl Cancer Inst* 2007;**99**:283–90.
86. Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 1988;**6**:589–95.
87. Gail MH, Brinton LA, Byar DP, Corle DK, Green SB, Schairer C, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst* 1989;**81**:1879–86.
88. Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. *Cancer Res* 2009;**69**:1243–54.

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Scientific rationale for postmenopause delay in the use of conjugated equine estrogens among postmenopausal women that causes reduction in breast cancer incidence and mortality

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Abstract

High-dose synthetic estrogens were the first successful chemical therapy used in the treatment of metastatic breast cancer in postmenopausal women, and this approach became the standard of care in postmenopausal women with metastatic breast cancer between the 1950s and the end of the 1970s. The most recent analysis of the Women's Health Initiative estrogen-alone trial in hysterectomized women revealed a persistently significant decrease in the incidence of breast cancer and breast cancer mortality. Although estrogens are known to induce the proliferation of breast cancer cells, we have shown that physiologic concentrations induce apoptosis in breast cancer cells with long-term estrogen deprivation. We have developed laboratory models that illustrate the new biology of estrogen-induced apoptosis or growth to explain the effects of estrogen therapy. The key to the success of estrogen therapy lies in a sufficient period of withdrawal of physiologic estrogens (5-10 y) and the subsequent regrowth of nascent breast tumor cells that survive under estrogen-deprived conditions. These nascent tumors are now vulnerable to estrogen-induced apoptosis.

Key Words: Breast cancer – Estradiol – Women's Health Initiative – Apoptosis.

Despite the extensive progress made in the management of breast cancer, it still remains the most common cause of cancer and the second leading cause of cancer deaths in women in the United States. An estimated 230,480 new cases of invasive breast cancer and an estimated 57,650 cases of breast carcinoma in situ were projected to occur in 2011.[1] In addition, approximately 39,520 women were expected to die of breast cancer in 2011.[1] Multiple risk factors for breast cancer have been established, and estrogen is a key growth stimulus in the development and progression of the disease. Beatson[2] in 1896 provided the first medical evidence of the estrogen dependency of breast cancer. The conclusion that a woman's ovaries provided the fuel that maintained breast cancer was based on the observed remission of advanced breast tumors in a premenopausal woman who underwent bilateral oophorectomy. Boyd[3] surveyed all known cases in 1900 and concluded a 30% response rate of breast cancer to any anti–hormone therapy (HT)—a figure that has stood the test of time. Animal models provided further evidence on the role of estrogens in breast cancer growth. Lathrop and Loeb[4] in 1916 observed a decrease in the occurrence of mammary carcinomas in castrated immature female mice. Estrogen, an ovarian hormone, was subsequently extracted and purified, and it induced vaginal cornification in ovariectomized mice.[5] This advancement elucidated the biological properties of synthetic estrogens using ovariectomized mice, therefore establishing a connection between the mitogenic potential of estrogens and breast cancer. The strategy of targeting the estrogen receptor (ER) has led to the discovery of endocrine therapies that either block estrogen action by using

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selective ER modulators (SERMs) or deprive the ER of estrogens by using aromatase inhibitors (AIs).[6] Anti-HTs remain the gold standard of care in the treatment and prevention of ER-positive breast cancer.[7]

WOMEN'S HEALTH INITIATIVE: RISKS AND BENEFITS

The use of HT continues to be a source of controversial debate. The Women's Health Initiative (WHI)[8] is a set of clinical studies designed to investigate and develop strategies for the prevention and control of common causes of morbidity and mortality in postmenopausal women. The WHI was initiated in 1991 with a tentative end date in 2007 to provide research findings on the effects of postmenopausal HT, calcium and vitamin D supplements, and diet modification on cardiovascular disease, osteoporosis, breast cancer, and colorectal cancer. The HT arm of the study includes a random assignment of 27,500 women either to placebo, estrogen plus progestin (HT), or estrogen alone (estrogen therapy [ET]; for hysterectomized women). The principal outcomes of the study were the incidences of coronary heart disease (CHD) and osteoporosis, with breast cancer as a potential adverse outcome.[8] To date, this is the largest, randomized, placebo-controlled trial that conducted parallel studies to assess the outcomes of combined HT and estrogen-alone therapy.[9]

Estrogen-plus-progestin therapy

Treatment with HT was associated with elevated overall risks. CHD is a leading cause of death in postmenopausal women, and previous animal studies have shown that estrogen treatment has the potential to prevent the development of coronary atherosclerosis.[10] Therefore, the results of the effect of HT on CHD were highly awaited. Women received conjugated equine estrogens (CEE) 0.625 mg/day plus medroxyprogesterone or placebo 2.5 mg/day. After a mean follow-up of 5.2 years, the trial was terminated because not only was the combination therapy not cardioprotective but HT also elevated the risk of CHD (hazard ratio [HR], 1.24; 95% CI, 1-1.54), which was most apparent at 1 year of therapy.[11] Furthermore, HT was associated with a doubled risk of venous thrombosis[12] and an increased risk of stroke,[13] and it did not confer protection against peripheral arterial disease,[14] dementia, and cognitive decline.[15] Combined HT increased total breast cancer (HR, 1.24; $P < 0.001$) and invasive breast cancer (HR, 1.24; $P = 0.003$) compared with placebo after 5 years of therapy.[16] The breast cancers in the group receiving HT were diagnosed initially at a slightly lower rate during the first 2 years of the study but subsequently increased throughout the intervention period. The elevated risk of breast cancer markedly declined soon after the cessation of combined HT.[17] Short-term use of HT was associated with a decrease in colorectal cancer cases when compared with placebo ($P = 0.0003$), but no protective effect against colorectal cancer mortality was observed during the 8-year intervention period and the follow-up period.[18] Although HT did not increase lung cancer rates,[19] more women from the combined therapy group died of lung cancer, in particular from non–small cell lung cancer. In addition, there was no significant difference in the incidences of endometrial cancer and ovarian cancer in both treatment arms.[20] The benefits of HT include a significantly decreased incidence of bone fractures.[21] Seven hundred thirty-three women (8.6%) in the estrogen-plus-progestin group and 896 women (11.1%) in the placebo group developed a fracture (HR, 0.76; 95% CI, 0.69-0.83). Total hip bone mineral density increased by 3.7% after 3 years of therapy with HT compared with 0.14% in the control group ($P < 0.001$). Current recommendations[22] include the use of individualized HT. HT can be initiated around the time of menopause to treat menopause-related symptoms and to prevent osteoporosis in high-risk women. Treatment should be considered in conjunction with personal risk factors, such as risk of venous thrombosis, CHD, stroke, and breast cancer.

Estrogen-alone treatment

Between 1993 and 1998, 10,739 postmenopausal women aged 50 to 79 years who had had a hysterectomy were treated with 0.625 mg of either CEE or placebo.[23] Despite the early termination of the combined hormone trial, the WHI ET study continued under careful scrutiny. However, in February 2004, the National Institutes of Health decided to terminate the intervention phase of the trial before the scheduled closeout interval from October 2004 to March 2005. The primary outcomes of the trial were the rate of CHD, the incidence of invasive breast cancer, and the incidences of stroke, pulmonary embolism, colorectal cancer, hip fractures, and death from other causes. After a mean follow-up of 6.8 years, no significant effect of ET on CHD rates was observed compared with placebo. During the active intervention period, a reduction in coronary events occurred in women assigned to ET (HR, 0.95; 95% CI, 0.79-1.16).[24] The reduction was more significant in women aged 50 to 59 years (HR, 0.63; 95% CI, 0.36-1.08). However, a 39% increase in the incidence of stroke was observed in the ET group ($P = 0.07$), whereas the risk of venous thromboembolism (VTE), including deep venous thrombosis and pulmonary embolism, increased by 33% in the ET arm, but only the increased rate of deep venous thrombosis was statistically significant ($P = 0.03$).[23] The increased risk for VTE was most apparent in the first 2 years, and the increased risk was less than that observed for the estrogen-plus-progestin study.[25] Therefore, ET provided no overall protection against cardiovascular disease in healthy postmenopausal women. Interestingly, invasive breast cancer was diagnosed at a 23% lower rate in the ET group (26 vs 33 per 10,000 person-years); however, this did not reach statistical significance ($P = 0.06$).[23] No statistical differences in colorectal cancer rates or total cancer rates were observed. The major positive finding in the ET trial in 2004 was a 30% to 39% reduction in the rates of fractures (HR, 0.70; 95% CI, 0.63-0.79). In addition, ET did not significantly affect overall mortality rate or cause-specific mortality. Results from the final analysis of the WHI ET trial[26] showed that a persistent decrease in the risk of breast cancer was associated with

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ET and was 0.27% per year compared with 0.35% per year in the placebo arm, reaching statistical significance (HR, 0.77; 95% CI, 0.62-0.95) after a median follow-up of 11.8 years. There was no difference between intervention HR and post-intervention HR ($P = 0.76$). Breast cancer risk reduction in the ET arm was most apparent in women without benign breast disease ($P = 0.01$) or a family history of breast cancer ($P = 0.02$). Breast cancer mortality was reduced in the ET group (six deaths, 0.009% per year) compared with controls (16 deaths, 0.024% per year; HR, 0.37; 95% CI, 0.13-0.91; $P = 0.03$). Fewer women in the ET group died of any cause after a breast cancer diagnosis than did women in the placebo arm ($P = 0.04$). Although breast cancer rates and mortality were lower for women who received ET, beneficial effects are yet to be determined in high-risk groups, and adverse effects of stroke and VTE remain problematic. HT seemed to have more risks, and the only clinical benefit was the reduction of osteoporosis, whereas ET, in addition to fracture prevention, decreased the incidence of and mortality from breast cancer. The question that needs to be addressed is whether it is possible to decipher the paradox that HT and ET produce completely different biological results (ie, HT increases, whereas ET reduces, the incidence of breast cancer). If clarity is possible, perhaps this knowledge can be used appropriately to help women.

CHEMICAL THERAPY FOR THE TREATMENT OF BREAST CANCER

The first successful chemical therapy used to treat cancer was discovered by Sir Alexander Haddow, a British-born physician. Haddow[27] grew up in Broxburn, a small town 10 miles west of Edinburgh, Scotland. He became motivated to study medicine and biology after he was admitted to a hospital for a perforated appendix and had the marvelous opportunity to observe the daily visits of great Edinburgh surgeons who were inspired to make a difference in an era when public health and hygiene were far from being developed. Upon graduation from medical school, he assisted with routine investigation of infections from the entire southeast of Scotland. While studying bacterial colony formation, he realized its resemblance to the formation of chemical tumors in higher forms.[28] He went on to study the influence of carcinogenic substances on normal and malignant growth, as well as the drug resistance of cells to resultant tumors. Incidentally, he found that many carcinogenic hydrocarbons also retarded the growth of malignant tumors.[29] To elucidate the molecular mechanism of these compounds, we paid particular attention to the inhibitory action of synthetic estrogens. In that era, reviews of animal experiment showed that treatment of animals with estrogens induced carcinoma of certain organs such as the cervix, uterus, and breast. The paradoxical action of estrogens showing growth properties, induction of tumors, and growth-retarding effects in certain circumstances led to the first ever reported clinical trial[30] in 1944. Seventy-three women with advanced cancer were recruited to the study. Forty postmenopausal women with metastatic breast cancer and 30 cases of malignant disease in other organs received treatment with synthetic estrogens: triphenylchlorethylene, triphenylmethylethylene, or stilbestrol. Ten of 22 women with advanced breast cancer treated with triphenylchlorethylene showed significant regression of the tumors. Breast cancer patients treated with stilbestrol showed that 5 of 14 cases underwent a regression of tumors similarly noted with triphenylchlorethylene. Among four cases of breast cancer treated with triphenylmethylethylene, only one showed a favorable response. Thirty cases of advanced cancer—excluding breast cancer but including cancer of the skin, maxillary antrum, urinary bladder, ovary, and prostate, and leukemia—were treated with triphenylchlorethylene; only carcinomas of the prostate and bladder showed partial regression of the tumors. Data from the clinical study suggest that the success of ET in breast cancer was dependent on the menopausal state of the women. Haddow and David[31] stated, "When the various reports were assembled at the end of that time, it was fascinating to discover that rather general impression, not sufficiently strong from the relatively small numbers in any single group, became reinforced to the point of certainty; namely, the beneficial responses were three times more frequent in women over the age of 60 years than in those under that age; that estrogens may, on the contrary, accelerate the course of mammary cancer in younger women, and that their therapeutic use should be restricted to cases 5 years beyond the menopause. Here was an early and satisfying example of the advantages which may accrue from cooperative clinical trial." Therefore, the longer that a woman is postmenopausal, the greater is the probability of tumor regression in metastatic breast cancer. However, "…the extraordinary extent of tumor regression observed in perhaps 1% of postmenopausal cases (with estrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us…."[31] Therefore, at this point in 1970, the underlying mechanism of estrogen-induced tumor regression still remained unanswered.

TIME TO TREATMENT FAILURE AND TRANSITION TO TAMOXIFEN

In the 1960s, based on the data from clinical trials, high-dose stilbestrol became the mainstay of treatment in postmenopausal women with advanced breast cancer. However, the estrogen treatment was not without pitfalls. It was imperative that ET not be instituted until ovarian secretion has ceased in a woman. The overall objective remission rate for estrogen treatment in 407 women with advanced breast cancer was 31%.[32] The remission rate was associated with the increasing number of years after menopause (Table 1). The rate of regression was 9% in women who were less than 5 years postmenopausal, whereas the rate increased to 35% in women who have been postmenopausal for more than 5 years, corresponding with what was observed by Haddow and David.[31] A remarkable feature of ET observed in this setting was the "withdrawal response." Stoll[32] previously described that when tumor response to estrogen administration was lost, 30% of

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TABLE 1. *Objective response rates in postmenopausal women with metastatic breast cancer who are using high-dose estrogen therapy*

Age since menopause	Patients, n	Regression, %
0-5 y (postmenopausal)	63	9
>5 y (postmenopausal)	344	35

The 407 patients are divided in relation to menopause status.[32] The objective remission rate of breast cancer tumors was higher in women more than 5 years postmenopausal.

cases on treatment withdrawal underwent a second but shorter period of tumor remission, indicating that women can be palliated over many years by intermittent estrogen and subsequent withdrawal. The introduction of tamoxifen, a nonsteroidal antiestrogen, in the late 1970s revolutionized the clinical practice of endocrine treatment of ER-positive breast cancer.[33] The evidence supporting the antiestrogenic action of tamoxifen was based on its antitumor action using carcinogen-induced rat mammary tumor models[34,35] and subsequent athymic mice transplanted with human breast cancer cell lines.[36] The clinical efficacy of tamoxifen was first evaluated in women with late or recurrent carcinoma of the breast.[37] Results from this study were compared with unpublished data from breast cancer patients who were treated with diethylstilbestrol (DES) at the same hospital. Although response rates were similar, women from the DES arm experienced more severe adverse effects. Similarly, Ingle et al[38] directly compared the use of either tamoxifen or DES in the treatment of advanced breast cancer in postmenopausal women. Analysis of the study revealed that there was no statistically significant difference between the efficacies of both treatments; however, similar to the study by Cole et al,[37] toxicity was greater for the women receiving DES and was severe enough for some women who dropped out of the study. Based on these data, DES fell out of favor for the treatment of metastatic breast cancer, and tamoxifen became the preferred agent. Tamoxifen subsequently became the standard of care in the adjuvant treatment and prevention of breast cancer. Several clinical trials investigated the long-term benefits of adjuvant tamoxifen therapy. An overview[39] of 55 randomized trials that compared the use of adjuvant tamoxifen versus no tamoxifen in breast cancer patients worldwide revealed that the reduction in recurrence for 1-year, 2-year, and 5-year trials during about 10 years of follow-up were 21%, 29%, and 47%, respectively. A highly significant trend toward a greater effect, based on longer treatment, was observed. A corresponding reduction in mortality of 12%, 17%, and 26%, respectively, was observed, and this trend was also significant ($P = 0.003$). A subsequent report of the meta-analysis[40] showed that 5 years of adjuvant tamoxifen decreased the annual breast cancer mortality rate by 31% at 15-year follow-up, irrespective of the use of chemotherapy, age, progesterone receptor status, or other tumor characteristics in ER-positive breast cancer patients. Furthermore, the reduction observed at 5 years is significantly ($P < 0.00001$ for recurrence; $P = 0.01$ for breast cancer mortality) more effective when compared with 1 to 2 years of adjuvant tamoxifen. More recently, results from the Adjuvant Tamoxifen—Longer Against Shorter (ATLAS) trial[41] showed that 10 years of adjuvant treatment with tamoxifen produced a further reduction in breast cancer recurrence and mortality when compared with 5 years of tamoxifen therapy. It is perhaps instructive to point out that the main effect of the decrease in mortality with a decade of tamoxifen occurs in the decade after tamoxifen treatment is stopped. This further suggests the hypothesis originally proposed in the early 1990s—that a woman's own estrogen destroys the appropriately sensitive tamoxifen-resistant micrometastasis.[42] Thus, the study of the evolution of anti–hormone drug resistance to tamoxifen ironically provided an insight into the mechanism of estrogen-induced apoptosis studied today. Nevertheless, the current recommendation for the adjuvant endocrine treatment of ER-positive breast cancer is that tamoxifen be used as a first-line treatment in premenopausal or perimenopausal women but that postmenopausal women take AIs as a primary agent for 5 years or for 2 to 3 years after tamoxifen[43] for a total of 5 years of initial anti-HT. The latter is based on several studies where AIs have shown some superiority to tamoxifen as first-line agents in the treatment of postmenopausal women with breast cancer, as well as a significant reduction in endometrial cancer.[44-46] Furthermore, 5 years of AI therapy have been shown to be highly beneficial as an extended adjuvant treatment in postmenopausal women who had previously received 5 years of tamoxifen therapy, showing a 2.9% improvement in disease-free survival at 4 years (HR, 0.68; $P = 0.0001$) when compared with placebo.[47,48]

EVOLUTION OF ANTI–HORMONE DRUG RESISTANCE

Despite the ability of long-term adjuvant tamoxifen to improve survival, some women develop disease recurrence owing to acquired drug resistance. Early laboratory models were created to understand the development of drug resistance and subsequent deployment of second-line therapies. Treatment of ovariectomized athymic mice transplanted with ER-positive MCF-7 tumors with tamoxifen initially caused tumor regression, but subsequent regrowth of tumors occurred despite continuous tamoxifen treatment.[49] Retransplantation of the resistant tumors into athymic mice or rats led to tumor growth in response to tamoxifen and estradiol (E_2).[50] Evaluation of these tumors showed that the tamoxifen-stimulated tumors contained twice the ER content of E_2-induced tumors.[50,51] However, continuous treatment of transplanted MCF-7 tamoxifen-resistant tumors with either a pure antiestrogen or no treatment in nude mice results in no tumor growth.[52] Because AIs deprive the ER of estrogens and fulvestrant degrades the ER, the findings from these studies presaged the clinical use of these drugs as second-line agents after failure of tamoxifen treatment.[53] However, the early models of drug resistance to SERMs are based on short-term treatments and replicate the failure of tamoxifen after 1 or 2 years of treatment in advanced breast cancer, and this represents phase 1 SERM resistance. To mimic 5 years of adjuvant tamoxifen therapy for micrometastatic breast cancer,

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we created laboratory models to induce phase 2 resistance to SERMs by serially transplanting tamoxifen-stimulated MCF-7 tumors into tamoxifen-treated athymic mice for more than 5 years.[54] Interestingly, on stopping tamoxifen, the tamoxifen-stimulated MCF-7 tumors rapidly regressed in response to physiologic E_2, although about 50% of tumors regrew after E_2 treatment. The paradoxical E_2-induced apoptosis suggests that a woman's own estrogen may produce an antitumor effect on presensitized micrometastatic tumors after 5 years of adjuvant tamoxifen.[42] Failure of tumor regression after exhaustive anti-HT with a paradoxical E_2-inhibited growth (phase 3 resistance) indicates a potential treatment plan using E_2 as third-line endocrine therapy.[55] Tumors that regrow after E_2-induced apoptosis revert back to the original cancer phenotype and are again sensitive to the antitumor actions of tamoxifen or AIs.[54]

ET IN METASTATIC BREAST CANCER

In more recent years, the use of estrogens continues to show clinical benefit to postmenopausal women with advanced breast cancer in an estrogen-deprived setting. Lonning et al[56] treated with high-dose DES (5 mg TID) 32 women who had previously taken multiple endocrine therapies. Four women achieved complete response, whereas four women achieved partial response. In addition, five women had an objective response lasting more than 52 weeks, whereas two patients had stable disease for more than 6 months. Six patients dropped out of the study owing to severe adverse effects. However, one of the patients who had complete regression of cytologically confirmed chest wall relapse and 5 years of DES therapy remained disease-free for 10 years and 6 months after starting treatment. A long-term follow-up of the study of Ingle et al[38] that compared DES therapy to tamoxifen showed that the 5-year survival was 35% for DES and 16% for tamoxifen ($P = 0.039$).[57] However, DES treatment was associated with nausea, edema, and vaginal bleeding problems, whereas hot flushes were more commonly observed with tamoxifen. Another 2009 clinical study[58] reported findings on the treatment of postmenopausal women who had AI-resistant metastatic breast cancer with low-dose E_2 (6 mg) and high-dose E_2 (30 mg). Clinical benefit rates were 28% (95% CI, 18-41) and 29% (95% CI, 19-42) in the high-dose arm and low-dose arm, respectively, but adverse event rate was higher in the 30-mg group when compared with the 6-mg group. Six patients who were estrogen-responsive were retreated with AIs, among which two had partial response and one had stable disease. This indicates resensitization to estrogen deprivation and correlates with the hypothesis on SERM resistance.[54]

EXPERIMENTAL APPROACH TO DECIPHERING THE MECHANISM OF E_2-INDUCED APOPTOSIS

A novel cell model[59] was developed by our laboratory to address concerns on acquired resistance to long-term estrogen deprivation. An ER-positive/progesterone receptor–negative hormone-independent breast cancer cell line, MCF-7:5C (a variant clone of wild-type MCF-7 cells), was obtained by culturing MCF-7 cells continuously in estrogen-free media. Treatment with physiologic E_2 for 6 days caused a dramatic 90% reduction in the growth of MCF-7:5C cells.[60] The growth inhibition observed was confirmed by annexin V and 4′,6-diamidino-2-phenylindole staining to be apoptosis. Fulvestrant also reduced the growth of MCF-7:5C cells, but the growth inhibition was not caused by apoptosis.[61] Furthermore, these cells were resistant to 4-hydroxytamoxifen (4-OHT). The tumorigenic potential of MCF-7:5C cells was examined by injecting cells into ovariectomized athymic mice, and these cells were found to spontaneously grow into tumors in the absence of E_2.[61] In contrast, MCF-7:5C tumors in mice treated with E_2 regressed in a time-dependent manner and became undetectable after 8 weeks of treatment. Similarly, fulvestrant also decreased the growth of MCF-7:5C tumors, but the reduction was statistically significantly less when compared with that of E_2 ($P < 0.001$). MCF-7:2A cells[62,63]—another long-term estrogen-deprived cell line derived from MCF-7 cells—is more resistant to E_2-induced apoptosis. Based on clinical data showing that only about 30% of patients respond to estrogens after anti–hormone resistance, it seemed imperative to see whether E_2-induced apoptosis could be enhanced in anti–hormone-resistant cells. Overexpression of Bcl-2 elevates cellular glutathione (GSH) level, which is associated with increased resistance to chemotherapy apoptosis,[64,65] whereas restoration of apoptosis occurs in Bcl-2–expressing cells depleted of GSH.[66] MCF-7:2A cells express high levels of GSH synthetase and GSH peroxidase 2, which are involved in GSH synthesis.[67] Exposure of MCF-7:2A cells to a combination therapy of E_2 and buthionine sulfoximine (BSO; a GSH inhibitor) for 48 to 96 hours produced a sevenfold increase in apoptosis, whereas individual treatments had no significant effect on growth. The in vitro findings correlated with in vivo data from a mouse xenograft model in which daily administration of BSO either as a single agent or in combination with E_2 significantly decreased the tumor growth of MCF-7:2A cells. Thus, this provides a potential strategy for future clinical trials involving combination therapy with BSO and low-dose estrogen to improve response in patients with anti–hormone-resistant advanced breast cancer.[68]

CONJUGATED EQUINE ESTROGENS

Extensive progress in the production of estrogen preparations for commercial use was made by scientists at Wyeth Pharmaceuticals Canada (then Ayerst), who extracted conjugated estrogens from a pregnant horse's urine.[69] In 1942, US Food and Drug Administration approval[70] was obtained for the clinical use of CEE (premarin) for the treatment of menopausal symptoms and related conditions. There was an initial worldwide acceptance of CEE in the 1960s; however, increased risks of developing endometrial cancer led to a decline in prescriptions to postmenopausal women.[71,72] In the 1980s, a new surge of interest in the use of ET for the treatment of

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osteoporosis led to clinical studies of women receiving either estrogen-alone therapy or estrogen-plus-progestin therapy. Women on estrogen and progestin treatment had a lower incidence of endometrial cancer,[73,74] indicating that progestin blocked the proliferative effect of estrogens on the endometrial lining. As a result, CEE were approved for the treatment and prevention of osteoporosis; women with an intact uterus were given progestin in addition to estrogens. CEE are made up of conjugated estrogens, and the tablet consists of at least 10 estrogens (Fig. 1), including estrone (59.2%), equilin (26.9%), 17α-dihydroequilin (16.3%), 17α-estradiol (4.32%), 17β-dihydroequilin (1.76%), 17α-dihydroequilenin (1.76%), 17β-dihydroequilenin (3.36%), equilenin (2.4%), 17β-estradiol (0.8%), and $\Delta^{8,9}$-dehydroestrone (4.16%). Generic synthetic versions of CEE are not currently approved by the Food and Drug Administration based on inadequacies noted in their active ingredients, bioequivalence, safety, and effectiveness.[75]

EFFECT OF CEE ON BREAST CANCER CELLS

Long-term concentrations of estrogen-deprived MCF-7 breast cancer cells undergo apoptosis upon treatment with physiologic E_2.[61] Based on the preliminary results of the WHI CEE study, we decided to elucidate the biological properties of the main estrogens in CEE in two different models of breast cancer cells. Estrogens have been shown to regulate the growth of ER-positive MCF-7 breast cancer cells. To study the biological activity of the actual estrogens, namely, equilin, estrone, and equilenin, we tested their ability to induce proliferation in MCF-7:WS8 cells, which contain ER and have retained estrogen responsiveness for a sustained period of continuous cell culture.[76] MCF-7 cells were grown in estrogen-free media for 3 days and treated with various concentrations of equilin, estrone, and equilenin, and their effects were compared with E_2 (Fig. 2A). All three estrogens were able to induce the cell growth of MCF-7 cells to the maximal level as E_2 in a dose-dependent manner. Equilin and estrone induced cell proliferation with maximal stimulation occurring at 0.1 nM, whereas equilenin reached maximal stimulation at 1 nM as compared with 0.01 nM for E_2. Next, we investigated the growth properties of equilin, estrone, and equilenin in long-term estrogen-deprived MCF-7:5C cells in comparison with E_2. Figure 2B shows that equilin, estrone, and equilenin drastically inhibited the growth of MCF-7:5C cells at comparable concentrations to E_2. Maximal growth inhibition was achieved with E_2 at 0.1 M, whereas equilin and estrone/equilenin

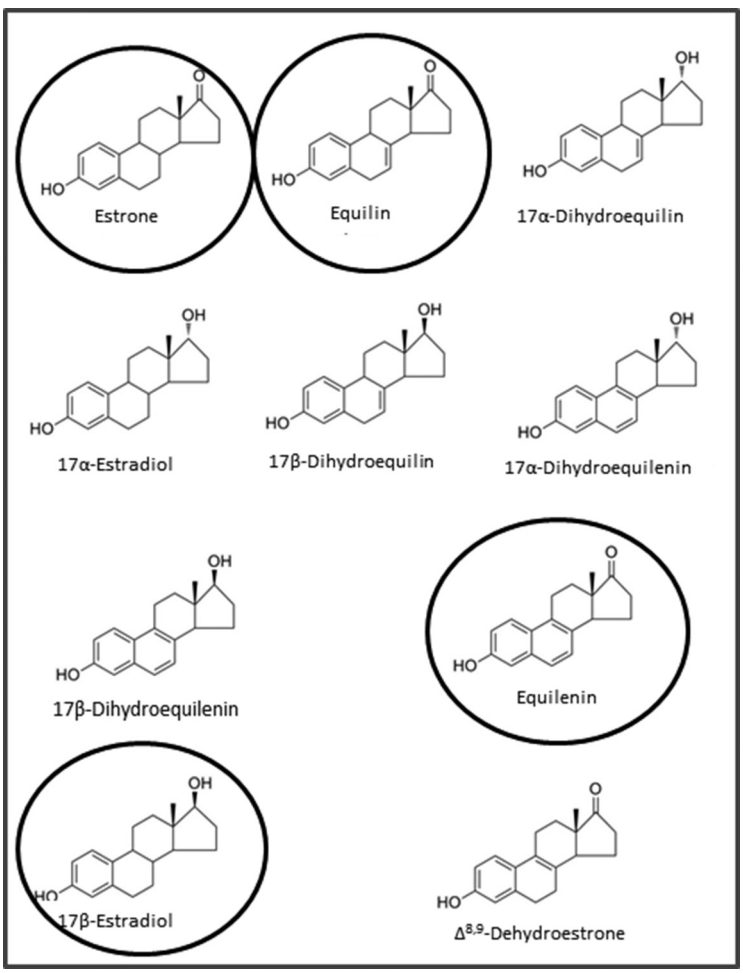

FIG. 1. Structures of the estrogenic constituents of premarin. Estradiol, equilin, estrone, and equilenin were used in our experimental studies.

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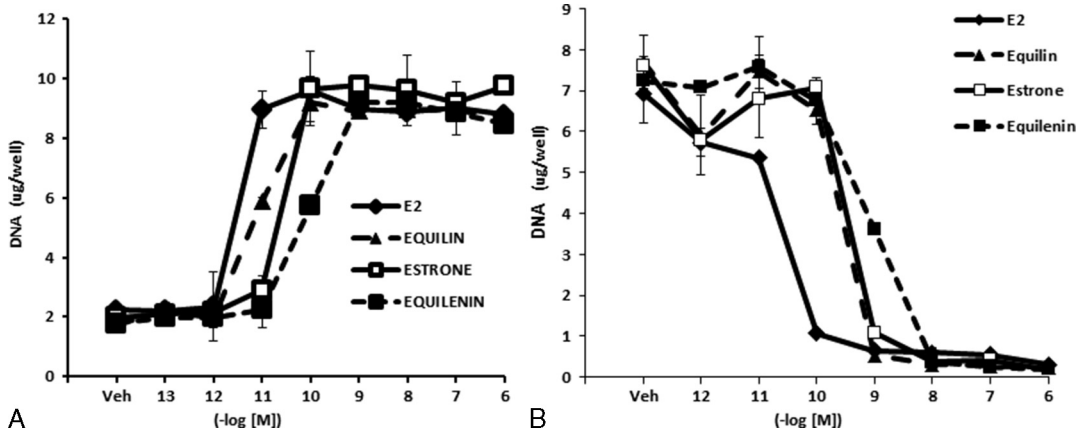

FIG. 2. Cell proliferation assay analysis of the biological properties of active steroids in conjugated equine estrogens in breast cancer cells. **A:** MCF-7 cells were grown in estradiol (E_2)–stripped media for 3 days; treated with various concentrations of E_2, equilin, estrone, and equilenin for 7 days; and compared with vehicle-treated cells (Veh; control). **B:** Equilin, estrone, and equilenin drastically inhibited the growth of MCF-7:5C cells similarly to E_2. The experiments were completed in triplicate and performed as previously described.[61]

reached maximal growth inhibition at 1 and 10 nM, respectively, after 7 days of treatment. To determine if the observed estrogen-induced growth inhibition of MCF-7:5C cells was caused by apoptosis, we used MCF-7:5C cells as controls, or E_2, equilin, estrone, or equilenin for 72 hours, and we measured apoptosis level using annexin V staining. E_2, equilin, estrone, and equilenin all showed increased apoptotic staining compared with control-treated cells (Fig. 3). The ability of conjugated estrogens to inhibit growth and to induce apoptosis in MCF-7:5C cells, and not parental MCF-7 cells, suggests that these biological properties are dependent on the duration of estrogen deprivation in breast cancer cells.

MOLECULAR MECHANISMS OF ESTROGEN-INDUCED APOPTOSIS

To decipher the precise series of events that precede estrogen-induced apoptosis, we interrogated differential gene expression in response to E_2 using Affymetrix-based microarray analysis.[63]

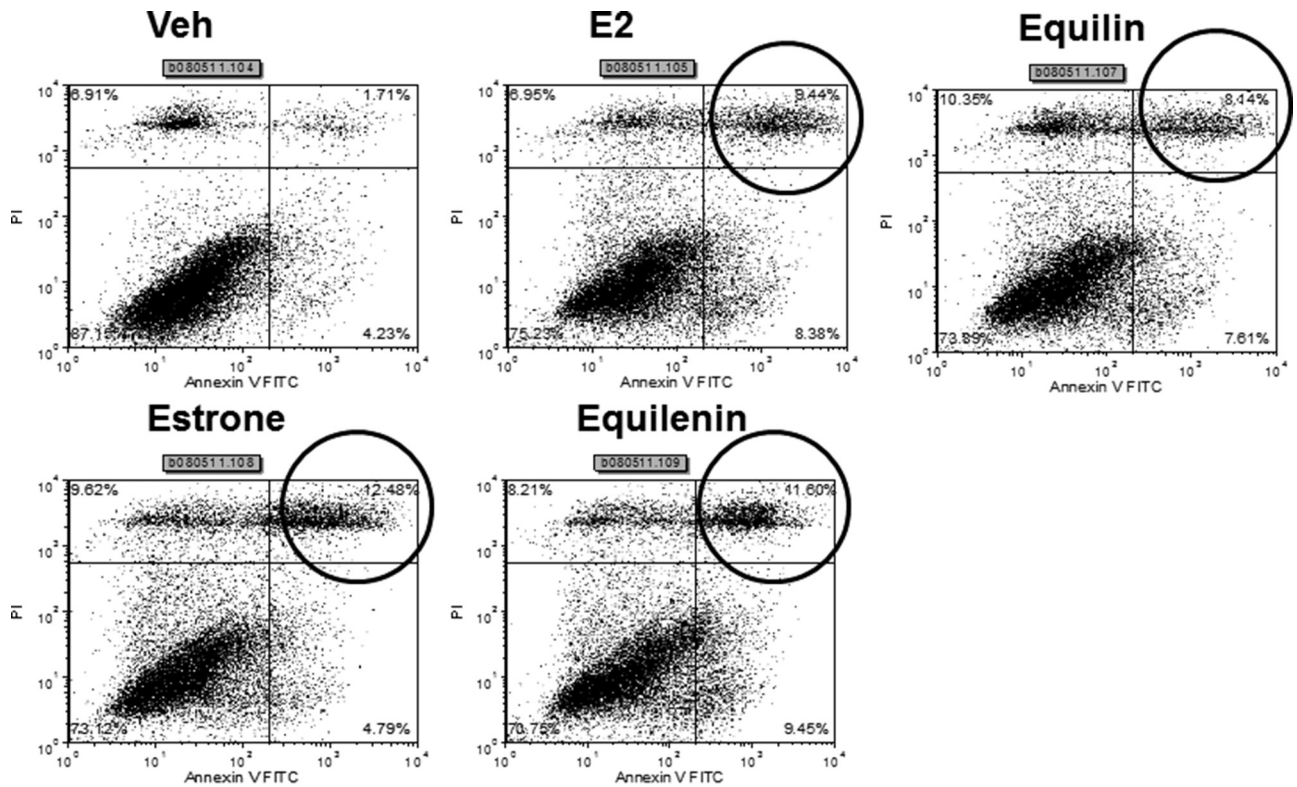

FIG. 3. Effects of estradiol (E_2) and active estrogens in conjugated equine estrogens on apoptosis in MCF-7:5C cells. MCF-7:5C cells were seeded in 100-mm plates and treated with vehicle-treated cells (Veh; control), 1 nM E_2, 1 nM equilin, 1 nM estrone, and 1 μM equilenin for 72 hours. Cells were stained with fluorescein isothiocyanate (FITC)–annexin V and propidium iodide (PI) and analyzed by flow cytometry as previously described.[61] The upper right box of Veh have less apoptotic cells (1.71%), whereas this fraction is increased for all estrogens (circled upper right-hand box).

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Specific genes were identified for MCF-7:5C, indicating that E_2 induced endoplasmic reticulum stress (ERS) and inflammatory stress responses that led to apoptosis. Identified ERS genes indicated that E_2 inhibited protein folding, leading to accumulation of unfolded proteins and widespread inhibition of protein translation with subsequent induction of cell death. In response to severe ERS, Bim (*B*cl-2–*i*nteracting *m*ediator of cell death; BCL211) was induced. Further evidence of the involvement of the mitochondrial pathway in E_2-induced apoptosis was reported by Lewis et al,[61] who showed increased expression of several proapoptotic proteins, including, Bax, Bak, Bim, Noxa, Puma, and p53, in E_2-treated MCF-7:5C cells. Reversal of the apoptotic effect of E_2 on these cells was observed with the blockade of Bax and Bim expression using short interfering RNAs. The involvement of the Fas/Fasl death signaling (extrinsic) pathway in the apoptotic effect of E_2 has been investigated. Osipo et al[77] demonstrated that E_2-induced regression of tamoxifen stimulated breast cancer tumors by activating the death receptor Fas and by suppressing the antiapoptotic/prosurvival factors nuclear factor-κB and HER2/neu. Similarly, the growth of raloxifene-resistant MCF-7 cells in vitro and in vivo was attenuated by E_2 by increasing Fas expression and by reducing nuclear factor-κB activity.[78] Studies are currently ongoing to determine the sequence of events that occur before E_2 induces apoptosis in MCF-7:5C cells.

The resolution of the crystal structure provided insight into the activation of the ER by E_2 and silencing by antiestrogens[79,80] and provides insight into the "trigger" mechanism for the ER complex. The shape that the ligands make with the ER is imperative to their ability to induce apoptosis in MCF-7:5C cells. E_2 is sealed within the hydrophobic pocket of the ligand binding domain of the ER by helix 12 and is bound by coactivators, leading to activation of apoptotic genes. On the other hand, 4-OHT pushes back helix 12 and prevents coactivator binding, and this may be responsible for its ability to block estrogen-induced apoptosis in MCF-7:5C cells. Knockdown of coactivator AIB1/SRC3 in MCF-7:5C cells led to a loss of the apoptosis-inducing effect of E_2, suggesting that AIB1 is a significant control hub of E_2 in apoptosis induced in these breast cancer cells.[81] Structure function studies show that the shape of estrogen[82] can modulate the shape of the estrogen-ER complex to induce apoptosis.[83] Hydroxylated triphenylethylenes, which are structurally similar to 4-OHT and have estrogenic properties in MCF-7 cells, have been shown to block E_2-induced apoptosis.[84] The antiestrogenic shape they make with the ER may be responsible

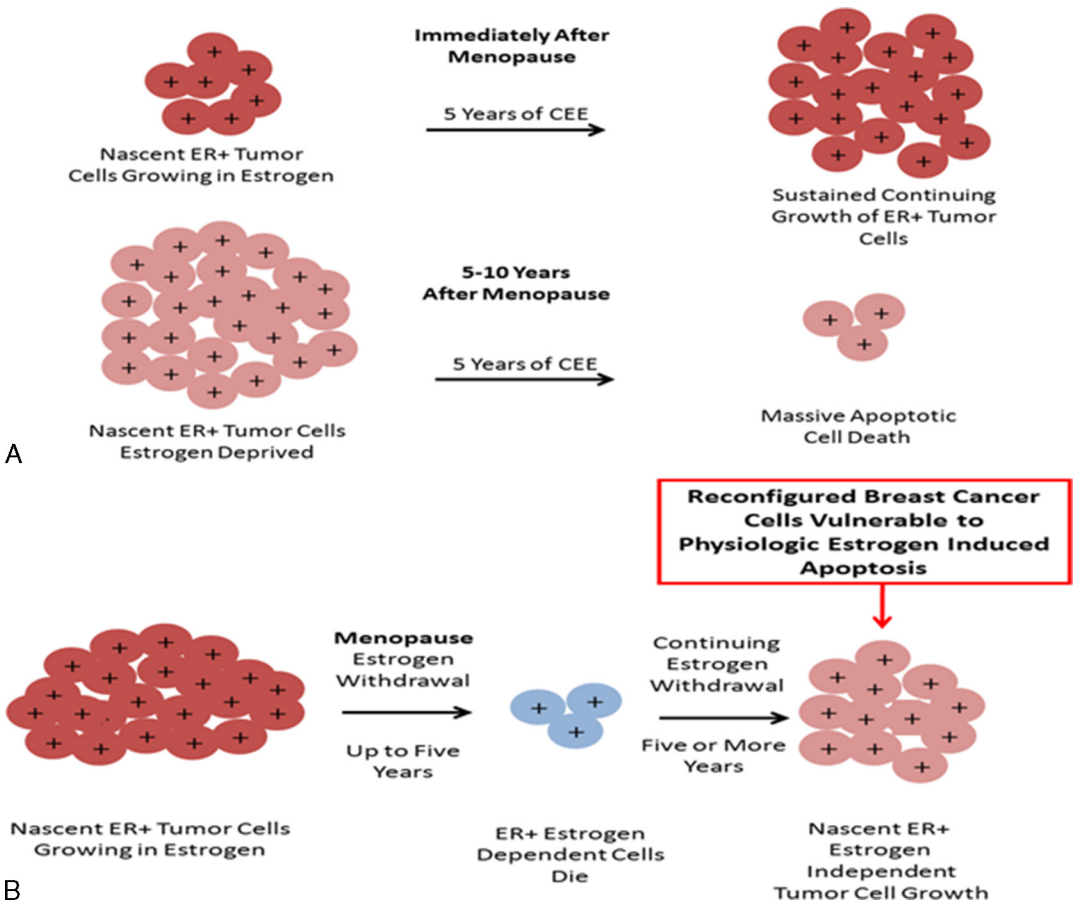

FIG. 4. The success of estrogen therapy is dependent on a woman's menopause status. **A:** Treatment of women with conjugated equine estrogens (CEE) immediately after menopause results in sustained growth of nascent estrogen receptor (ER)–positive tumors, whereas treatment 5 years after menopause causes apoptotic cell death. **B:** Estrogen withdrawal in postmenopausal women causes ER-positive cells to die, but some cells continue to grow independently of estrogen.

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for the delayed apoptotic effect of triphenylethylenes on MCF-7:5C cells. These pharmacologic studies are currently undergoing investigation and will be the focus of further reports.

DISCUSSION

Before the clinical use of anti-ET, high-dose estrogens were deemed to be effective in the induction of tumor regression in metastatic breast cancer.[30,32] In more recent times, ET shows significant clinical benefits on postmenopausal women who have undergone extensive antihormone treatment. Development of tamoxifen-stimulated tumors in athymic mice after a 5-year treatment with tamoxifen suggests that the development of anti–hormone resistance during years of treatment reconfigures the survival mechanism of breast cancer so that estrogen is no longer a potent mitogen that stimulates cell proliferation but rather becomes a death signal. Preclinical data clearly show that long-term estrogen deprivation of ER-positive MCF-7 breast cancers and subsequent treatment of cells with E_2 cause apoptosis of these cells. Creation of an estrogen-deprived environment either by withdrawal of estrogen treatment[32] or by exhaustive anti-HT increases the sensitivity of breast tumors to the ET, subsequently inducing tumor regression. Similarly, CEE alone reduce the incidence of breast cancer in hysterectomized postmenopausal women. This protective effect is not observed in women who receive addition progesterone therapy, suggesting that progestin may play a potential role in the increased breast cancer cases observed among postmenopausal women who received combined HT. To explain the aforementioned clinical data, laboratory studies show that estrogens in CEE were able to cause the proliferation of MCF-7 cells after these cells were grown in an estrogen-free medium for 3 days. This cell population is adapted to an environment rich in estrogen; thus, naturally, all cells grow with a "resupply" of natural steroidal estrogens. However, these same estrogens induce apoptosis to a similar extent as E_2 in MCF-7 cells that have been deprived of estrogen treatment for many years. The ability of ET to treat or prevent tumors is related to the menopause status of women and how long they have been physiologically deprived of estrogen. In the data by Stoll[32] (Table 1), the rate of remission of advanced breast cancer was significantly less in women who were less than 5 years postmenopausal (9%), and there was a 35% remission rate in women who were more than 5 years postmenopausal. It is important to stress that the majority of the women in the WHI CEE trial were older than 60 years, and the mean age at screening was 63.6 years. Here, the overall result was a reduction in breast cancer and mortality. There is a need for an "estrogen holiday" before starting ET. Induction of menopause in women gradually deprives the cells of estrogen. However, immediate treatment with estrogens may cause the growth of nascent ER-positive breast tumors that may increase breast cancer risk (Fig. 4A). The cells vulnerable to death with estrogens in CEE have been selected because estrogen deprivation at menopause causes estrogen-dependent nascent breast cancers to die, but not all die. Remaining cells that survive learn to grow without estrogen (Fig. 4B). These cells will continue to grow to produce breast cancer, unless exogenous estrogens induce apoptotic death. Therefore, 5 years of CEE treatment immediately after menopause will cause sustained continuing growth of ER-positive tumor cells. Because nascent ER-positive tumor cells have been estrogen-deprived in women who are 5 to 10 years postmenopausal, 5 years of CEE therapy induces massive apoptotic cell death, subsequent tumor cell death, and enhanced patient survival.

CONCLUSIONS

High-dose estrogen treatment is effective in causing tumor regression in metastatic breast cancer. The mechanism for this treatment was a paradox and was unknown for 60 years but is now being deciphered.[63] Objective tumor remission is observed in women who are more than 5 years postmenopausal.[30,32] ET administered to women in their late 60s causes a sustained decrease in breast cancer incidence and a decrease in mortality.[26] The question was "Why?" The key is the long-term estrogen deprivation of ER-positive breast cancer cells. We have created long-term estrogen deprivation breast cancer cell lines and, for the first time, have described the mechanism of estrogen-induced apoptosis. This new biology of estrogen-induced apoptosis can now be used to explain the effects of ET on reducing breast cancer incidence and mortality among women in their 60s.

REFERENCES

1. American Cancer Society. *Breast Cancer Facts & Figures 2011-2012*. Atlanta, GA: American Cancer Society Inc., 2011.
2. Beatson G. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. *Lancet* 1896;2:162-165.
3. Boyd S. On oophorectomy in cancer of the breast. *BMJ* 1900;2:1161-1167.
4. Lathrop AE, Loeb L. Further investigations on the origin of tumors in mice, 3: On the part played by internal secretion in the spontaneous development of tumors. *J Cancer Res* 1916;1:1-19.
5. Allen E, Doisy EA. An ovarian hormone: preliminary report on its localization, extraction and partial purification, and action in test animals. *JAMA* 1923;81:819-821.
6. Jordan VC, Brodie AMH. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* 2007;72:7-25.
7. Obiorah I, Jordan VC. Progress in endocrine approaches to the treatment and prevention of breast cancer. *Maturitas* 2011;70:315-321.
8. Design of the Women's Health Initiative clinical trial and observational study. The Women's Health Initiative Study Group. *Control Clin Trials* 1998;19:61-109.
9. Marjoribanks J, Farquhar C, Roberts H, Lethaby A. Long term hormone therapy for perimenopausal and postmenopausal women. *Cochrane Database Syst Rev* 2012;7:CD004143.
10. Karas RH. Animal models of the cardiovascular effects of exogenous hormones. *Am J Cardiol* 2002;90:22F-25F.
11. Manson JE, Hsia J, Johnson KC, et al. Estrogen plus progestin and the risk of coronary heart disease. *N Engl J Med* 2003;349:523-534.
12. Cushman M, Kuller, LH, Prentice, R, et al. Estrogen plus progestin and risk of venous thrombosis. *JAMA* 2004;292:1573-1580.
13. Wassertheil-Smoller S, Hendrix, S Limacher M, et al. Effect of estrogen plus progestin on stroke in postmenopausal women: the Women's Health Initiative: a randomized trial. *JAMA* 2003;289:2673-2684.
14. Hsia J, Criqui MH, Rodabough RJ, et al, for the Women's Health Initiative Investigators. Estrogen plus progestin and the risk of peripheral arterial disease. *Circulation* 2004;109:620-626.
15. Shumaker S, Legault C Rapp SR, et al. Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal

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women: the Women's Health Initiative Memory Study: a randomized controlled trial. *JAMA* 2003;289:2651-2662.
16. Chlebowski R, Hendrix SL, Langer RD, et al. Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative randomized trial. *JAMA* 2003; 289:3243-3253.
17. Chlebowski RT, Kuller LH, Prentice RL, et al. Breast cancer after use of estrogen plus progestin in postmenopausal women. *N Engl J Med* 2009;360:573-587.
18. Chlebowski RT, Wactawski-Wende J, Ritenbaugh C, et al. Estrogen plus progestin and colorectal cancer in postmenopausal women. *N Engl J Med* 2004;350:991-1004.
19. Chlebowski RT, Schwartz AG, Wakelee H, et al. Oestrogen plus progestin and lung cancer in postmenopausal women (Women's Health Initiative trial): a post-hoc analysis of a randomised controlled trial. *Lancet* 2009;374:1243-1251.
20. Anderson G, Judd HL, Kaunitz AM, et al. Effects of estrogen plus progestin on gynecologic cancers and associated diagnostic procedures: the Women's Health Initiative randomized trial. *JAMA* 2003;290:1739-1748.
21. Cauley J, Robbins J, Chen Z, et al. Effects of estrogen plus progestin on risk of fracture and bone mineral density: the Women's Health Initiative randomized trial. *JAMA* 2003;290:1729-1738.
22. The North American Menopause Society. The 2012 hormone therapy position statement of The North American Menopause Society. *Menopause* 2012;19:257-271.
23. Anderson GL, Limacher M, Assaf AR, et al. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *JAMA* 2004;291:1701-1712.
24. Hsia J, Langer RD, Manson JE, et al. Conjugated equine estrogens and coronary heart disease: the Women's Health Initiative. *Arch Intern Med* 2006;166:357-365.
25. Curb J, Prentice RL, Bray PF, et al. Venous thrombosis and conjugated equine estrogen in women without a uterus. *Arch Intern Med* 2006;166: 772-780.
26. Anderson GL, Chlebowski RT, Aragaki AK, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *Lancet Oncol* 2012;13: 476-486.
27. Haddow A. An autobiographical essay. *Cancer Res* 1974;34:3159-3164.
28. Haddow A. The influence of carcinogenic substances on sarcomata induced by the same and other compounds. *J Pathol Bacteriol* 1938;47: 581-591.
29. Haddow A. Influence of certain polycyclic hydrocarbons on the growth of the Jensen rat sarcoma. *Nature* 1935;136:868-869.
30. Haddow A, Watkinson JM, Patterson E. Influence of synthetic oestrogens upon advanced malignant disease. *BMJ* 1944;2:393-398.
31. Haddow A, David A. Karnosky Memorial Lecture: thoughts on chemical therapy. *Cancer Res* 1970;26:737-754.
32. Stoll B. Palliation by castration or by hormone administration. In: Stoll B, ed. *Breast Cancer Management Early and Late*. London, England: William Herman Medical Books Ltd., 1977:133-146.
33. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2003;2:205-213.
34. Jordan VC. Antitumor activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)–induced rat mammary carcinoma model. *J Steroid Biochem* 1974;5:354.
35. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. *Eur J Cancer* 1976;12: 419-424.
36. Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. *Fed Proc* 1987;46:1870-1874.
37. Cole M, Jones C, Todd I. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. *Br J Cancer* 1971;25:270-275.
38. Ingle J, Ahmann D, Green S, et al. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *N Engl J Med* 1981;304:16-21.
39. Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;351: 1451-1467.
40. Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005; 365:1687-1717.
41. Davies C, Pan H, Godwin J, et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor–positive breast cancer: ATLAS, a randomised trial [published online ahead of print December 4, 2012]. *Lancet* doi: 10.1016/S0140-6736(12)61963-1.
42. Wolf D, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res* 1993;127:23-33.
43. Burstein HJ, Prestrud AA, Seidenfeld J, et al. American Society of Clinical Oncology clinical practice guideline: update on adjuvant endocrine therapy for women with hormone receptor–positive breast cancer. *J Clin Oncol* 2010;28:3784-3796.
44. Cuzick J, Sestak I, Baum M, et al. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 10-year analysis of the ATAC trial. *Lancet Oncol* 2010;11:1135-1141.
45. Mouridsen H, Giobbie-Hurder A, Goldhirsch A, et al. Letrozole therapy alone or in sequence with tamoxifen in women with breast cancer. *N Engl J Med* 2009;361:766-776.
46. Jones S, Seynaeve C, Hasenburg A, et al. Results of the first planned analysis of the TEAM (Tamoxifen Exemestane Adjuvant Multinational) prospective randomized phase III trial in hormone sensitive postmenopausal early breast cancer. *Cancer Res* 2009;69:15.
47. Goss PE, Ingle JN, Martino S, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. *N Engl J Med* 2003;349:1793-1802.
48. Ingle JN, Tu D, Pater JL, et al. Intent-to-treat analysis of the placebo-controlled trial of letrozole for extended adjuvant therapy in early breast cancer: NCIC CTG MA.17. *Ann Oncol* 2008;19:877-882.
49. Osborne C, Coronado EB, Robinson JP. Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol* 1987;23:1189-1196.
50. Gottardis M, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term anti-estrogen administration. *Cancer Res* 1988;48:5183-5187.
51. Gottardis MM, Wagner RJ, Borden EC, Jordan VC. Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro. *Cancer Res* 1989;49:4765-4769.
52. Gottardis MM, Jiang S-Y, Jeng M-H, Jordan VC. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. *Cancer Res* 1989;49:4090-4093.
53. Howell A, Robertson JFR, Abram P, et al. Comparison of fulvestrant versus tamoxifen for the treatment of advanced breast cancer in postmenopausal women previously untreated with endocrine therapy: a multinational, double-blind, randomized trial. *J Clin Oncol* 2004;22:1605-1613.
54. Yao K, Lee E-S, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;6:2028-2036.
55. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;5:207-213.
56. Lonning P, Taylor P, Anker G, et al. High-dose estrogen treatment in post-menopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 2001;67:111-116.
57. Peethambaram P, Ingle J, Suman V, et al. Randomized trial of diethylstilbestrol vs. tamoxifen in postmenopausal women with metastatic breast cancer. An updated analysis. *Breast Cancer Res Treat* 1999; 54:117-122.
58. Ellis M, Gao F, Dehdashti F, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor–positive, aromatase inhibitor–resistant advanced breast cancer: a phase 2 randomized study. *JAMA* 2009;302:774-780.
59. Jiang SY, Wolf DM, Yingling JM, et al. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* 1992;90:77-86.
60. Lewis J, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 2005;94:131-141.
61. Lewis J, Meeke K, Osipo C, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 2005;97:1746-1759.
62. Pink JJ, Jiang S-Y, Fritsch M, Craig Jordan VC. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor–related protein. *Cancer Res* 1995;55: 2583-2590.

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63. Ariazi EA, Cunliffe HE, Lewis-Wambi JS, et al. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *Proc Natl Acad Sci* 2011;108:18879-18886.
64. Voehringer D. BCL-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity. *Free Radic Biol Med* 1999;27:945-950.
65. Meredith MJ, Cusick CL, Soltaninassab S, et al. Expression of Bcl-2 increases intracellular glutathione by inhibiting methionine-dependent GSH efflux. *Biochem Biophys Res Commun* 1998;248:458-463.
66. Rudin CM, Yang Z, Schumaker LM, et al. Inhibition of glutathione synthesis reverses Bcl-2–mediated cisplatin resistance. *Cancer Res* 2003; 63:312-318.
67. Lewis-Wambi J, Kim H, Wambi C, et al. Buthionine sulfoximine sensitizes anti–hormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* 2008;10:R104.
68. Lewis-Wambi J, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? *Breast Cancer Res* 2009;11:206.
69. Watkins E. *The estrogen elixir: a history of hormone replacement therapy in America*. Baltimore, MD: The John Hopkins University Press, 2007:10-132.
70. FDA. *FDA Backgrounder on Conjugated Estrogens*. Silver Spring, MD: US Food and Drug Administration Center for Drug Evaluation and Research, 2005.
71. Smith DC, Prentice R, Thompson DJ, Herrmann WL. Association of exogenous estrogen and endometrial carcinoma. *N Engl J Med* 1975;293: 1164-1167.
72. Ziel HK, Finkle WD. Increased risk of endometrial carcinoma among users of conjugated estrogens. *N Engl J Med* 1975;293:1167-1170.
73. Greenblatt R, Gambrell RD, Stoddard LD. The protective role of progesterone in the prevention of endometrial cancer. *Pathol Res Pract* 1982;174:297-318.
74. Gambrell R. The prevention of endometrial cancer in postmenopausal women with progestogens. *Maturitas* 1978;1:107-112.
75. FDA. *Conjugated Estrogens—Letter from Dr Janet Woodcock. Memorandum*, Silver Spring, MD: US Food and Drug Administration Center for Drug Evaluation and Research, 1997.
76. Soule HD, Vazquez J, Long A, et al. A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst* 1973; 51:1409-1416.
77. Osipo C, Gajdos C, Liu H, et al. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* 2003;95:1597-1608.
78. Liu H, Lee E, Gajdos C, et al. Apoptotic action of 17β-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. *J Natl Cancer Inst* 2003;95:1586-1597.
79. Brzozowski AM, Pike ACW, Dauter Z, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 1997;389: 753-758.
80. Shiau AK, Barstad D, Loria P, et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 1998;95:927-937.
81. Hu ZZ, Kagan BL, Ariazi EA, et al. Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. *PLoS One* 2011;6:e20410.
82. Jordan VC, Schafer JM, Levenson AS, et al. Molecular classification of estrogens. *Cancer Res* 2001;61:6619-6623.
83. Maximov PY, Myers CB, Curpan RF, et al. Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. *J Med Chem* 2010;53:3273-3283.
84. Maximov P, Sengupta S, Lewis-Wambi JS. The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: a hypothesis. *Horm Mol Biol Clin Invest* 2011;5:27-34.

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Four decades of discovery in breast cancer research and treatment – an interview with V. Craig Jordan

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ABSTRACT V. Craig Jordan is a pioneer in the molecular pharmacology and therapeutics of breast cancer. As a teenager, he wanted to develop drugs to treat cancer, but at the time in the 1960s, this was unfashionable. Nevertheless, he saw an opportunity and through his mentors, trained himself to re-invent a failed "morning-after pill" to become tamoxifen, the gold standard for the treatment and prevention of breast cancer. It is estimated that at least a million women worldwide are alive today because of the clinical application of Jordan's laboratory research. Throughout his career, he has always looked at "the good, the bad and the ugly" of tamoxifen. He was the first to raise concerns about the possibility of tamoxifen increasing endometrial cancer. He described selective estrogen receptor modulation (SERM) and he was the first to describe both the bone protective effects and the breast chemopreventive effects of raloxifene. Raloxifene did not increase endometrial cancer and is now used to prevent breast cancer and osteoporosis. The scientific strategy he introduced of using long term therapy for treatment and prevention caused him to study acquired drug resistance to SERMs. He made the paradoxical discovery that physiological estrogen can be used to treat and to prevent breast cancer once exhaustive antihormone resistance develops. His philosophy for his four decades of discovery has been to use the conversation between the laboratory and the clinic to improve women's health.

KEY WORDS: *tamoxifen, raloxifene, acquired antihormone resistance, estrogen, nonsteroidal antiestrogen, selective estrogen receptor modulator (SERM), estradiol-induced apoptosis*

The past is never dead. It is not even the past.
William Faulkner

Tamoxifen, originally classified as a nonsteroidal antiestrogen but now known as the first selective estrogen receptor modulator (SERM), is a pioneering medicine that for more than twenty years was the gold standard for the adjuvant treatment of breast cancer in pre and postmenopausal patients with estrogen receptor (ER)-positive tumors (Jordan, 2003). Millions of women continue to live longer and healthier lives because of tamoxifen treatment. Tamoxifen is also a pioneering medicine, as it is the first drug to be approved in the United States of America by the Food & Drug Administration (FDA) for the reduction of the incidence of breast cancer in high risk pre and postmenopausal women (Jordan, 2007).

Craig Jordan grew up with a passion for chemistry, but was specifically intrigued by the prospect of using organic chemistry to design drugs to treat cancer. At the age of thirteen, his mother allowed him to convert his bedroom into a chemistry laboratory, where he often got into difficulties during his experiments, either setting the curtains on fire as a rather over reactive experiment was being thrown out of the window, or destroying the lawn outside. However, he did convince his mother that by using the chemistry of fertilizers, he could re-grow the lawn again, but when he did, it came out an interesting shade of blue! Craig had a passion for teaching, and the chemistry and biology teachers at his school, Moseley Hall Grammar School in Cheadle, Cheshire, England allowed him to have a laboratory to teach biochemistry. It was these same teachers who convinced his parents that he should apply

Abbreviations used in this paper: AACR, American Association for Cancer Research; ASCO, American Society of Clinical Oncology; CEE, conjugated equine estrogen, DES, diethylstilbestrol; DMBA, dimethylbenzanthracene; EBCTCG, Early Breast Cancer Trialists' Collaborative Group; ECOG, Eastern Cooperative Oncology Group; ER, estrogen receptor; FDA, Food & Drug Administration; ICI, Imperial Chemical Industries; SERM, selective estrogen receptor modulator; STAR, Study of Tamoxifen and Raloxifene; TGFα, transforming growth factor alpha; WFEB, Worcester Foundation for Experimental Biology; WHI, Women's Health Initiative.

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Fig. 1. Before the ceremony for the degree of Doctor of Medicine *honoris causa* at Leeds University on the 18th of July, 2001. *Dr. Edward R. Clark, my PhD supervisor (1969-1972) (left) and Dr. Ronnie Kaye, Head of my degree course (1965-1969) (center), formally from the Department of Pharmacology, University of Leeds, England. I am on the right side with my signature glass of Burgundy.*

to university. By contrast, Craig was more content with the idea of becoming an organic chemistry technician at the research laboratories of Imperial Chemical Industries (ICI) near where he lived.

Craig was given an opportunity for interview at only one university (Leeds University, West Yorkshire, England), but he succeeded in convincing the two faculty interviewers, Dr. Ronnie Kaye and Dr. Edward Clark, that he should have a chance in the Pharmacology Department. Years later, Craig found out that the reason he was given an interview was that they had been intrigued at the Headmaster's letter, which stated the candidate was "an unusual young man" and then repeated the statement in capitals. On July 18 2001, Craig received the first honorary Doctor of Medicine degree from the University of Leeds for humanitarian research that has changed healthcare. The citation, presented by the Chancellor Lord Melvyn Bragg, starts: *"Craig Jordan is one of the most distinguished medical scientists of the last one hundred years."* He was delighted to be able to invite Drs. Clark and Kaye to the luncheon and the ceremony (Fig. 1). These were the two individuals who talent spotted Craig; Dr. Kaye was his tutor for his four years as an undergraduate, and Dr. Clark persuaded him to become a graduate student armed with the last available Medical Research Council studentship in the United Kingdom for the year 1969 (Fig. 2). Someone had declined their studentship, thus allowing Craig to do a Ph.D! Dr. Clark's project, that Craig found so attractive, was the prospect of extracting the estrogen receptor (ER) from the rodent uterus, purifying it and then crystallizing the ER protein with an estrogen and a nonsteroidal antiestrogen. The x-ray crystallography would be completed at the Astbury Department of Biophysics at the University of Leeds and all the work was estimated to take the three years of the scholarship. At that time, the nonsteroidal antiestrogens had failed to fulfill their promise in the pharmaceutical industry as "morning-after pills"; they were perfect in rats, but in women they did exactly the opposite and enhanced fertility by inducing ovulation.

The project in crystallizing the ER did not go as planned, so he rapidly changed his topic with a new title: "A study of the oestrogenic and anti-oestrogenic activities of some substituted triphenylethylenes and triphenylethanes" (Fig. 3). This was a good strategic research choice, as no one has yet succeeded in crystallizing the whole ER with either an estrogen or antiestrogen. But further difficulties were to arise in Craig's journey to a career in cancer research.

As a PhD student, Craig was talent spotted for an immediate tenure track faculty position because of his skill as a lecturer. He had no publications and his PhD topic was going nowhere. No one was recommending careers in failed contraceptives! During the interview with the University Committee charged with making the appointment, he was told that he would have to go to America to get his BTA (been to America) before he could start the job. First, however, he had to get a PhD, and to do that, it had to be examined. However, the University could find no one in the country qualified for the task. Sir Charles Dodds, the discoverer of the synthetic estrogen, diethylstilbestrol (DES), declined with regrets as he had not kept up with the literature for the past twenty years! But here is where luck and chance take control. He was in the right place at the right time and by meeting the right people, changed medicine.

Dr. Arthur Walpole was Head of the Fertility Control Program at ICI's Pharmaceuticals Division and a personal friend of the Chairman of Craig's Pharmacology Department. The University reluctantly accepted

Fig. 2. I always love dressing up! *The University of Leeds is my* alma mater, *and I have attended four ceremonies there:* **(A)** *Bachelor of Science, First Class Honours (1969),* **(B)** *Doctor of Philosophy (1973),* **(C)** *Doctor of Science, earned by examination. A select Committee evaluated my refereed publications to establish my contribution to Science (1985) and* **(D)** *Honorary Doctor of Medicine for humanitarian research (2001).*

Dr. Walpole (despite the fact that he was from industry!) to be Craig's examiner and he was also able to organize a two year visit to the Worcester Foundation for Experimental Biology (WFEB) in Shrewsbury, Massachusetts to study with Dr. Michael Harper on new methods of contraception. Harper and Walpole had completed all the early work on ICI 46,474 as a contraceptive at ICI Pharmaceuticals in the early 1960's. Craig vividly remembers the transatlantic telephone call with Dr. Harper: "Can you come in September?", "Will $12,000 a year be enough?" and "Will you work on prostaglandins?" "Yes, yes, yes" he replied and went off to the library to find out what prostaglandins were! But when he got to the WFEB in September 1972, he was told that Dr. Harper had gone to Geneva to be Head of Contraception Research at the World Health Organization. Craig was told to sit down, write up what he would do for the next two years and organize his own laboratory. He was now an independent investigator.

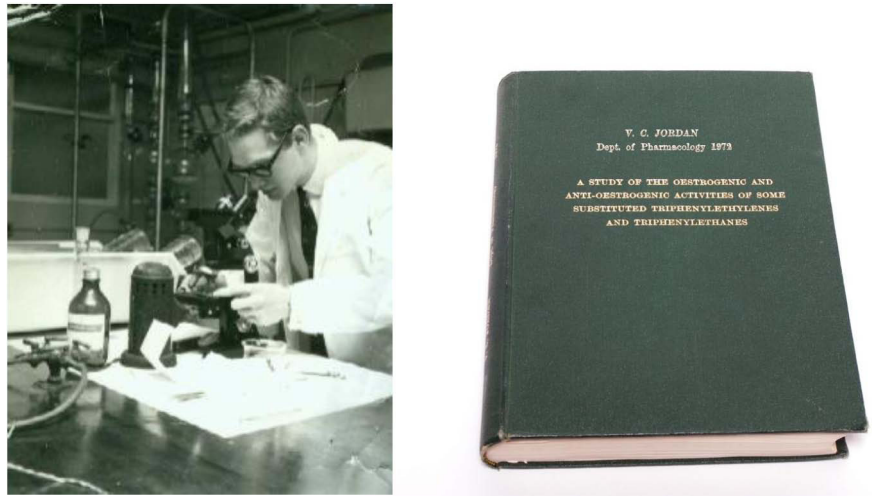

Fig. 3. My first publicity photograph during the time that I was a PhD student at the Department of Pharmacology, University of Leeds, England (1969-1972). *It was necessary as I had been selected as the Medical Research Council's student representative to the Nobel Prize Winner's Meeting in Lindau, Germany in 1972. I am examining cells from mouse vaginal smears; big science. Also shown is my PhD that nobody wanted to examine!*

A phone call to Dr. Walpole explained his dilemma at the WFEB but he felt that there was an opportunity for the failed morning-after pill, ICI 46,474 to be used for the treatment of breast cancer. This call was rewarded by Dr. Walpole arranging for funding and contacts with Ms. Lois Trench at ICI America for Craig to conduct the translational research on the drug that would become tamoxifen. As an independent Investigator, the research funding from ICI was an unrestricted research grant, but as Craig was not a cancer research scientist and he was at WFEB, the home of the oral contraceptive, what was the first step to be? Again, what's important is who you meet. After the National Cancer Act in 1971, the WFEB Director had made the decision to bring a cancer research specialist onto the Board of Scientific Advisors to help with future funding opportunities in hormones and cancer research. Dr. Elwood Jensen was the Director of the Ben May Laboratory for Cancer Research in Chicago, Illinois and was credited with the translational research where he described the ER in immature rat estrogen target tissues and then used this knowledge to propose a test for the hormone dependency of metastatic breast cancers. Simply stated, if the ER is absent in the tumor, the patient was unlikely to respond to endocrine ablation (oophorectomy, adrenalectomy or hypophysectomy), but if the tumor was ER-positive, there was a high probability that the tumor would respond to estrogen withdrawal. It was a practical test to avoid morbidity from unnecessary operations that require hospitalization.

Craig spent the day with Dr. Elwood Jensen in November 1972 and told him what he wanted to do with ICI 46,474. Craig subsequently traveled to the Ben May Laboratory for Cancer Research to be taught techniques of ER analysis and to learn all about the dimethylbenzanthracene (DMBA) rat mammary carcinoma model and then to Dr. Bill McGuire's laboratory in San Antonio, Texas to learn complementary analytical methods for the ER. Armed with these techniques and resources from ICI throughout the 1970s (his first decade of discovery), he created the laboratory principles of targeting the tumor ER and advocating the use of long term adjuvant tamoxifen therapy as the appropriate clinical strategy to save lives (Fig. 4) (Jordan and Koerner 1975; Jordan and Allen 1980). This proposition by Craig was not at all popular, as throughout the 1970s and 1980s in the United Kingdom, it was strongly believed there was no correlation between tamoxifen use and the presence of the ER in breast tumors. Additionally nobody was interested in a new antihormone therapy, as combination cytotoxic chemotherapy was king. It was going to cure cancer. However, Craig persevered and had the courage of his convictions that his laboratory research would save lives. As it turned out, tamoxifen has probably saved more lives than any other cancer therapeutic drug.

Craig also learned an important lesson at the WFEB around the time he was to leave and return to Leeds. A Senior Scientist at the WFEB, Dr. Eliahu Caspi, invited Craig to his office for an interview to explore the possibility of Craig staying at the WFEB. Craig recalls this was a very frightening experience, for Dr. Caspi had a no-nonsense personality, judged people and said what he thought. He stated that he had been asked to evaluate my C.V., as everybody was of the opinion that I would be a useful asset at the WFEB. He stared at Craig across the desk and said, *"You don't have a C.V., as you have no publications."* After the initial shock, Craig responded, *"But I haven't discovered anything yet."* The advice Craig received was some of the best advice he had received thus far in his career. He was told *"to tell them the story so far and link together several related publications to create a theme."* Craig has done this ever since, creating the theme of tamoxifen. In 1998, with the release of the successful chemoprevention trial with tamoxifen, Craig was referred to as the "Father of Tamoxifen" by the *Chicago Tribune*, a title that has stuck to this day.

Although many people published using tamoxifen in their studies as a laboratory tool or used it in the 1960s in reproduction research, Craig's focus from the outset was clear; the goal was to develop a medicine for the treatment and prevention of breast cancer (he conducted the first chemopreventive study in the laboratory in 1974 [Jordan, 1976], three years before the drug was approved by the FDA for the treatment of metastatic breast cancer in postmenopausal women). Craig stresses that but for the unrestricted support from ICI, meeting the right people and his uncompromising

Fig. 4. The Imperial Chemical Industries (ICI) Pharmaceuticals Meeting at King's College, Cambridge in the summer of 1977. *The goal of the meeting was physician education about research being done with tamoxifen. This was the first time I presented in public my ideas about targeting the tumor estrogen receptor and using long term treatment with tamoxifen as the best strategy to be applied to adjuvant therapy (Jordan V.C. ,1978.* Reviews on Endocrine-related Cancer *49-55). However, the major presentation that made everything change clinically was in Arizona in 1979 (Jordan, 1979). In the above picture, Michael Baum (right), was the Chair of the session at King's College and stated that they had plans to use two years of tamoxifen as an adjuvant therapy (on a hunch). Helen Stewart (left), was considering starting a pilot trial in Scotland using five years of adjuvant tamoxifen for the treatment of patients. For the placebo arm, patients would be treated with tamoxifen at first recurrence. If toxicity was acceptable, they would move forward to test the idea of early long term treatment or late treatment at first recurrence. Both trials showed survival advantages for long term adjuvant tamoxifen. The week after the King's College Meeting, I was at the University of Wisconsin at their Comprehensive Cancer Center to convince clinicians of the Eastern Cooperative Oncology Group (ECOG) that longer was going to be better. At the time, tamoxifen was not on the market in America but I was talent spotted by Paul Carbone, the Head of ECOG and the Director of the Comprehensive Cancer Center, to be recruited to the University of Wisconsin, Department of Human Oncology. Eventually, I would be the Director of their Breast Cancer Research and Treatment Program.*

determination (many referred to this at the time as poor career judgment), tamoxifen would probably not have happened. Scientists at ICI did not conduct any studies with the drug as an antitumor agent. Indeed, in late 1972, all of the data with ICI 46,474 was reviewed and the Research Director terminated clinical trials and stopped the development project. The Marketing Department had decided that a treatment for metastatic breast cancer was not going to generate sufficient revenue.

Arthur Walpole was towards the end of his career and chose to take early retirement, but only agreed to remain an employee if funds could be given to a young man he had met, Craig Jordan, who (as he did) wanted to turn ICI 46,474 into a drug to treat breast cancer. Walpole and Craig subsequently worked together on an ICI/University joint research scheme when Craig returned as Lecturer in the Department of Pharmacology at the University of Leeds in September 1974. Earlier in his career, Dr. Walpole was an accomplished cancer research scientist, but had not been allowed to work in this area by ICI because fertility control was considered to be potentially more lucrative (Jordan, 1988). Dr. Walpole died suddenly on July 2, 1977 before he could witness the success of Craig's laboratory strategy for the treatment and prevention of breast cancer.

The clinical development of tamoxifen was very progressive and validated all your assumptions. Could you tell us how you were involved in the clinical evaluation and how you convinced the company to invest in what may have been very challenging trials?

I think it's fair to say that this was not the real story, but the real story is unbelievable. I have always considered my research as being a conversation between the laboratory and the clinic, and I had the privilege of first introducing tamoxifen to clinical trials' organizations in America. My objective was to provide a scientific rationale for the clinical studies in treatment and prevention. My research and qualifications were required to obtain approval for tamoxifen as a medicine in both Japan and Germany, and I was delighted to be the only person invited from outside of ICI Pharmaceuticals to attend a celebration in 1977, of the Queen's Award for Technological Achievement for tamoxifen. The surprising part about the tamoxifen story is that although patents for the drug were obtained by ICI Pharmaceuticals around the world, in the mid- 1960's, these same patents were denied in the United States of America. Thus, all of the work I was completing on the antitumor actions of tamoxifen in the United States was done without patent protection for ICI. Looked at another way, it was clear that all the other pharmaceutical companies had no interest in the clinical development of tamoxifen, because either the drug was not going to work very well or not generate enough revenue. But it was my clinical strategy of long term adjuvant therapy that saved lives and made revenues (Jordan, 2008 a). Clinical testing went ahead and when the patents expired in the rest of the world, ICI was awarded the patent for the use of tamoxifen in the treatment of breast cancer in 1985, but back dated to the original patent application in 1965. Now, extended adjuvant therapy was the practical solution for effective treatment. Thus, for the next twenty years, ICI was able to generate enormous revenues in the United States, as tamoxifen was the standard of care for long term adjuvant tamoxifen therapy and the only game in town. This money catalyzed the advent of ICI marketing antiandrogens for prostate cancer and the aromatase inhibitors for breast cancer.

Watching your scientific activity since the beginning, you always seem fascinated by the development of small molecules since their conception up to their development. Is that what gives you much fun in your work?

I absolutely love experiments involving the structure function relationships of the antiestrogens. My basic scientific research has been to create models of gene modulation or replication to determine the structure of the ER antiestrogen complex that subsequently could be interrogated. This passion resulted in a whole series of publications focused on the modulation of the prolactin gene (Lieberman, *et al.*, 1983 a, b; Jordan and Lieberman, 1984) which then went through a metamorphosis to study the modulation of the SERM ER complex and the way that the ligand can interact with specific amino acids, thereby switching on or switching off the complex at target genes (Wolf and Jordan, 1994). We actually found the only natural mutation of the human ER in a laboratory model of tamoxifen-stimulated tumor growth. We engineered the mutant ER into ER-negative breast cancer cells and found it would make the antiestrogen, raloxifene, an estrogen at the transforming growth factor alpha (TGFα) target gene. For me, this was important as one amino acid in the ER could change the pharmacology of raloxifene. In other words, this provided a fascinating insight into the relationship of the antiestrogenic side chain and a specific amino acid at the surface of the ER protein (Levenson and Jordan, 1998; MacGregor-Schafer, *et al.*, 2000; *Liu et al.*, 2001, 2002).

Do you think that a drug may have a commercial future in the chemoprevention of cancer?

As you know, we have made enormous progress with advancing the failed breast cancer drug, raloxifene, and millions of women are now benefiting from its use for the treatment of osteoporosis, but with a reduction in breast cancer incidence at the same time. This is the practical reality of our early translational research completed at the University of Wisconsin in the second decade of discovery (1980s). The "Tamoxifen Team" discovered selective estrogen receptor modulation and tamoxifen and raloxifene were both now classified as SERMs (Jordan, 2001). But the realization that tamoxifen could not possibly have widespread use because it increases the risk (though this is very small) of endometrial cancer in postmenopausal women (Gottardis *et al.*, 1988), naturally guided us to our new SERM strategy in the late 1980s. We discovered that SERMs maintain bone density (Jordan *et al.*, 1987) and therefore could potentially prevent osteoporosis with the beneficial antiestrogenic side effect of preventing breast cancer (Gottardis and Jordan, 1987). We had solid translational research, as we had found that tamoxifen built bone both in the laboratory (Jordan *et al.*, 1987) and in clinical trial (Love *et al.*, 1992). Raloxifene has a better safety profile and does not increase the risk of endometrial cancer (Cummings *et al.*, 1999), but it does not reduce the risk of coronary heart disease. I think the new SERM, lasofoxifene (Cummings *et al.*, 2010), is very good, as it prevents osteoporosis, breast cancer, coronary heart disease and strokes, but without an increase of endometrial cancer. The problem is how to advance in a crowded market with low budgets for marketing. Lasofoxifene is approved but not marketed in the European Union.

No molecule targeting estrogen receptor has, to date, proved to be more efficient than tamoxifen in patients despite the development of a number of promising compounds. How do you explain that? Was it a choice of the pharmaceutical industry because of the cost of the development of such a compound?

The issue with tamoxifen is unique. It was clearly lucky that tamoxifen had an acceptable toxicology profile for the treatment of cancer. It came onto the market at a time when the standard of care was combination cytotoxic chemotherapy, so tamoxifen looked good to patients. Tamoxifen was not supposed to succeed, but advanced from strength to strength for twenty years. However, things change very rapidly in the arena of patient preference. In the early 1990s, when tamoxifen was being considered for testing as a chemopreventive and the specter of endometrial cancer translated from the laboratory (Gottardis *et al.*, 1988) to clinical practice, this was clearly not good news for well women. Worse still, tamoxifen was found to produce DNA adducts in rat liver and initiate rat liver hepatocarcinogensis (Jordan, 1995). Although liver tumors did not translate to clinical practice, this did not lessen concern, as the drug ended up with a black box label as a human carcinogen. Timing is everything with discovery and competitors could never catch up with clinical testing, despite the fact they may have been safer. We will never know.

To demonstrate that natural or synthetic molecules can prevent the occurrence of cancer is long and expensive. This raises the question of the life of the patents but also the natural molecules, which may not be patentable. Do you think there may be solutions to these problems?

I think it's currently impossible to find a solution to this dilemma. Clearly, the pharmaceutical industry will never advance with twenty year studies because the patents will run out. But here is a controversial point: the success of health care has now created the situation of increased longevity, so that drugs that enhance survival through prevention can only make matters worse. What is society to do? How does society find the resources to support an aging population?

You have developed recently a very provocative approach using estrogens for the treatment of breast cancers. This can be considered as a paradoxical use of estrogens? Could you explain to us a little bit about that.

The third and fourth decades have been a wonderful surprise in our journey of discovery. We posed the question (based upon the clinical acceptance of long term antihormonal therapy (Jordan, 2008 a) as the most appropriate adjuvant treatment for breast cancer): what is the mechanism and the timeframe for acquired antihormone resistance? Our first model clearly showed something unique as far as drug resistance is concerned—SERM-stimulated growth, something that is not seen with any other drug in cancer therapy (Gottardis and Jordan, 1988). This form of resistance occurred within a year or two and was consistent with the development of acquired resistance to tamoxifen in metastatic breast cancer. However, here was the dilemma: this model did not replicate the outstanding success observed with five years of adjuvant tamoxifen treatment (Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2011). In fact, five years of treatment continues to enhance decreases in mortality for more than a decade once tamoxifen is stopped. By a series of lucky accidents, one of my students (Doug Wolf) discovered that physiologic estrogen could cause dramatic tumor regression after five years of tamoxifen treatment, i.e. serial transplantation of tamoxifen-resistant tumors into generations of

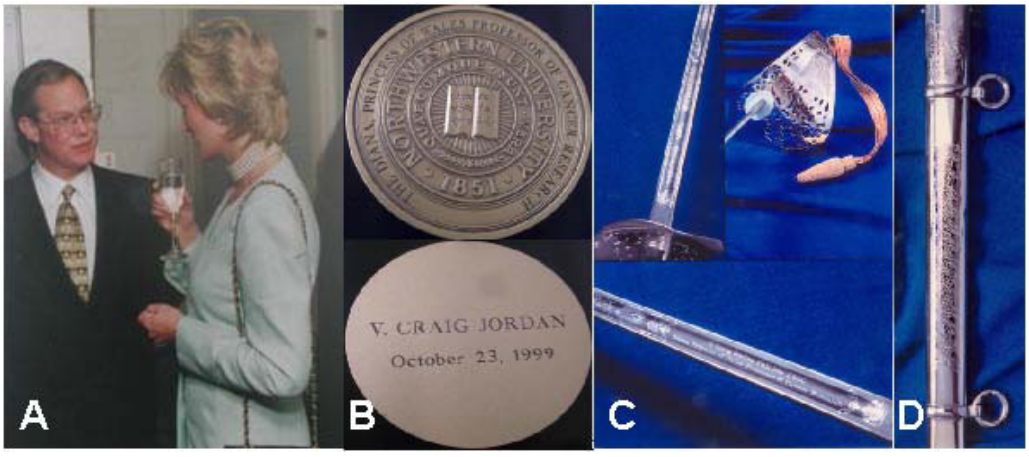

Fig. 5. The Diana, Princess of Wales Chair of Cancer Research. *In June 1996, Diana, the Princess of Wales visited Chicago for three days and we first met* **(A)** *at the evening reception at the home of the President of Northwestern University, Henry Bienen. The Chair was anonymously endowed at the Robert H. Lurie Comprehensive Cancer Center after Diana's untimely death on August 31, 1997. I was inaugurated on October 23, 1999, being presented with a unique Professorial medal* **(B)** *with copies being sent to her sons Prince William and Harry and also kept by my daughters, Helen and Alexandra. My students presented me with an engraved sword* **(C)** *to commemorate the event and their names and the dates of the award of their PhD degrees are engraved on the scabbard* **(D)**.

tamoxifen-treated mice (Wolf and Jordan, 1993). This discovery reminded me of the words of Sir Alexander Haddow, FRS in 1970 during the Inaugural Karnofsky Lecture at the American Society of Clinical Oncology (ASCO): "…the extraordinary extent of tumour regression observed in perhaps 1% of post-menopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us…" (Haddow, 1970). It is now clear that aggressive estrogen deprivation with aromatase inhibitors or SERMs can rapidly re-configure breast cancer cells through an evolution of drug resistance, which exposes a vulnerability that could not be anticipated—physiological estrogen induced apoptosis (Yao, 2000; Lewis *et al.*, 2005). When Haddow did his original work using high dose DES for the treatment of metastatic breast cancer in women during their late sixties and seventies, the best therapeutic results occurred the further away the patient was from the menopause. Antihormone therapy accelerates all of that in breast cancer, so physiologic estrogen can initiate the same triggering mechanism. Indeed, this is possibly the same mechanism that is occurring in the Women's Health Initiative (WHI) by conjugated equine estrogen (CEE) alone actually produces a decrease in the incidence of breast cancer in hysterectomized postmenopausal women (La Croix *et al.*, 2011). What is particularly interesting about these data is the six years of monitoring after CEE is stopped, there is a continued reduction in the incidence of breast cancer, i.e. the estrogen has destroyed the nascent breast cancer cells in the ducts (Jordan and Ford, 2011). Our current laboratory work is focused entirely on deciphering the molecular mechanism of estrogen-induced apoptosis (Ariazi, in press). In this way, we may find the vulnerability triggered by the ER estrogen complex for cellular destruction; that vulnerable site in the cancer cell may be the next target for a new class of selective anticancer agents applicable to sites other than breast cancer.

Your contributions to medicine have received a lot of recognition (Table 1) but how does one become the "Diana, Princess of Wales Professor of Cancer Research"?!

Life is all about chance meetings. In the mid-1990s, I was invited to organize a Breast Cancer Symposium in Chicago, and Diana was my Keynote Speaker (Fig. 5). She came on a three day visit to Northwestern University and the Robert H. Lurie Comprehensive Cancer Center. Naturally, it was a very special time and when she left to return to London, we agreed to correspond and I sent her copies of my books on tamoxifen. There was even talk of a return trip for either her or Prince William or Prince Harry, to open one of our new research buildings. Regrettably, everything changed with her untimely death in a tragic car accident in Paris on August 31, 1997. An anonymous donation was subsequently made to the Robert H. Lurie Comprehensive Cancer Center, and with letters from Lady Sarah McCorquodale, (her sister) and the Earl Spencer (her brother), it was agreed that I would hold a Professorship at

TABLE 1

AWARDS & HONORS

St. Gallen International Breast Cancer Prize	2011
Elected to the National Academy of Sciences, USA (Fig. 6)	2009
Elected Fellow of the Academy of Medical Sciences (UK equivalent of Inst.of Medicine in the US)	2009
Elected Fellow of the Society of Biology (UK)	2009
Honorary Doctor of Medicine Degree, University of Crete, Greece	2009
39th David A. Karnofsky Award, ASCO	2008
Honorary Fellowship of the Royal Society of Medicine (Fig. 7)	2008
Honorary Member of the Royal Pharmaceutical Society of Great Britain	2008
Gregory Pincus Award and Medal, Worcester Foundation for Biomedical Research, U. Mass	2007
American Cancer Society Award for Chemoprevention, ASCO	2006
Honorary Doctor of Science Degree, University of Bradford, England	2005
Alfred G. Knudson Jr. Chair in Basic Science, Fox Chase Cancer Center	2004
3rd George and Christine Sosnovsky Award in Cancer Therapy, Royal Society of Chemistry	2003
The Kettering Prize, General Motors Cancer Research Foundation	2003
Officer of the Most Excellent Order of the British Empire (OBE) Services to International Breast Cancer Research	2002
American Cancer Society Medal of Honor	2002
Inaugural Dorothy P. Landon AACR Prize in Translational Research	2002
Bristol Myers Squibb Award for Distinguished Achievement in Cancer Research	2001
Honorary Doctor of Medicine Degree, University of Leeds	2001
European Institute of Oncology Breast Cancer Therapy Award	2001
Honorary Doctor of Science Degree, University of Massachusetts	2001
Honorary Faculty Fellowship Award, University College, Dublin	2000
Diana, Princess of Wales Professor of Cancer Research, Robert H. Lurie Comprehensive Cancer Center	1999

Northwestern University in her name. Essentially, it was my British citizenship, a British medicine (tamoxifen), and our meeting and correspondence that was important to the family. On October 23, 1999, the Professorship was conferred on me by Henry Bienen, the President of Northwestern University and over a two day period, there was a Symposium in my honor by my former PhD students and during the celebration dinner, attended by representatives from the British Embassy, Barry Furr (the Chief Scientist from ICI), family, friends and colleagues, my students presented me with an engraved sword (Fig. 5) with each of the dates of their Ph.D engraved on the scabbard as battle honors—very moving!

You have contributed more than 600 research and review papers to the literature with more than 23,000 citations and an h-index of 80. If you had to select ten of your research papers and three reviews, which would they be and why?

Jordan V.C. (1976). *Eur J Cancer* 12: 419-424. Literally my first cancer research paper with tamoxifen that was rejected in 1974, but with kind and generous comments from one of the reviewers. I persevered and eventually this was one of the papers from my work used to justify the chemoprevention trials.

Jordan V.C. and Allen K.E. (1980). *Eur J Cancer* 16: 239-251. The paper makes three points: 1. this is the first refereed article that longer treatment is going to be better than shorter treatment; 2. our discovery of 4-hydroxytamoxifen's pharmacology indicating it to be a potent antiestrogen with a binding affinity for ER equivalent to estradiols (Jordan *et al*., 1977), naturally made us think that this would be a more powerful anticancer agent—not true, it cleared too quickly and 3. finally, we stated that antiestrogen treatment followed by estrogen deprivation would be a good strategy for people—true.

Gottardis M.M., *et al.,*1988). *Cancer Res* 48: 812-815. This was the paper that warned the clinical community that tamoxifen could potentially increase the incidence of endometrial cancer in patients—true.

Gottardis M.M. and Jordan V.C. (1988). *Cancer Res* 48: 5183-5187. This was the first report that acquired drug resistance with tamoxifen was unique and stimulated by SERMs—true.

Love R.R., *et al.,*1992). *New Engl J Med* 326: 852-856. This was the randomized clinical trial based on our laboratory evidence and subsequently those of others that tamoxifen would maintain bone density in people. This paper opened the door to raloxifene.

Levenson A.S. and Jordan V.C. (1998). *Cancer Res* 58: 1872-1875. A clean demonstration that a mutant ER found in a tamoxifen-stimulated tumor by a previous PhD student (Doug Wolf) could change an antiestrogen to an estrogen. This could be done by a natural process.

Cummings S.R., *et al.,*1999). *JAMA* 281: 2189-2197. Proof of principle that the concept we first articulated back in the late 1980s that you could develop a SERM to prevent osteoporosis and prevent breast cancer at the same time—true.

Yao K., *et al.,*2000). *Clin Cancer Res* 6: 2028-2036. The first refereed publication to demonstrate that drug resistance to tamoxifen evolves and exposes a vulnerability to permit physiologic estrogen to cause tumor regression. Subsequently translated to the clinic—true.

Vogel V.G., *et al.,*2006). The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA*. 295: 2727-2741. Two discarded drugs from the pharmaceutical industry that were re-invented in the same pharmacology laboratory to become the pioneering chemopreventive agents and FDA-approved—true.

Vogel V.G., *et al.,*2010). *Cancer Prev Res* 3: 696-706. A follow-up of the trial several years after stopping SERM treatment, confirmed the predictions of one of my PhD students (Marco Gottardis) in 1987 that tamoxifen would be the better chemopreventive in the long term.

I've always viewed an invitation to write a review article from a journal as a wonderful opportunity to project your personality, express your views and most importantly, reach out to young scientists and graduate students as theirs is the future. Here are my three choices:

Jordan V.C. (1984). *Pharm Rev* 36: 245-276. This was my first major review when I first came to America. No one had really treated the topic as an issue in pharmacology, as all of the previous reviews in the 1960s and 1970s were about the control of fertility. I wanted a summary of the mechanisms of action of antiestrogens. It was all of our knowledge up to that point (423 citations).

Jordan V.C. (2006). *Br J Pharmacol* 147: S269-S276. I was thrilled to be asked by the British Pharmacological Society to write the story of my research in a Special Issue of our Journal. I got

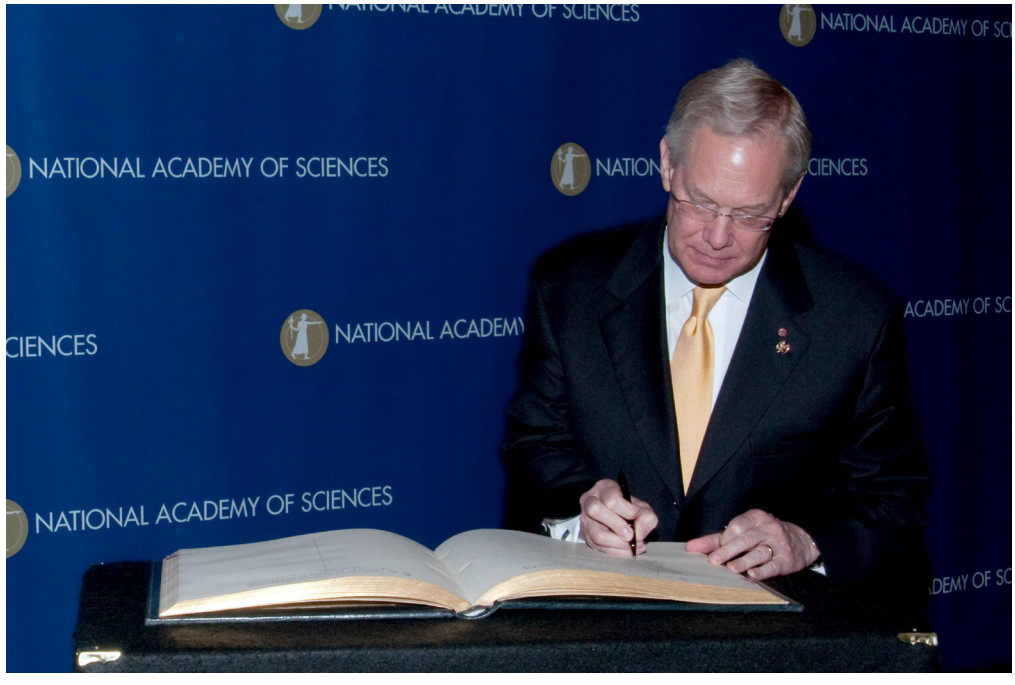

Fig. 6. Signing the "Great Book" of Members of the National Academy of the Sciences USA *during the Induction Ceremony on April 24, 2010.*

wonderful feedback from students.

Jordan V.C. (2009). *Cancer Res*. 69: 1243-1254. I was proud to be asked by the American Association for Cancer Research (AACR) to contribute a review of progress in hormone dependent tumors as a part of a series to celebrate the 100th anniversary of AACR.

I see that you received the David A. Karnofsky Award in 2008 from ASCO, but it is stated in the regulations for the Award that it is given in "recognition of innovative clinical research and developments that have changed the way oncologists think about the general practice of oncology." You are a laboratory scientist and not a clinician; didn't this surprise you?

When I received the telephone call from the Chair of the Awards Committee, Gabriel Hortobagyi, I was absolutely dumbfounded, because naturally, I knew I was not a clinician! All previous recipients were clinicians. This is ASCO's highest award, and I was being asked to join the legends of clinical practice. For the first fifteen minutes of my conversation with Gabriel, I examined with him every reason why I should not be their recipient. After fifteen minutes, he became exasperated and said, "Is this a yes, I accept?" I accepted the honor. Apparently, I learned, the reason the Committee selected my work was because as a laboratory scientist and a pharmacologist, I had always been present at clinical breast cancer meetings over the decades, putting forward my point-of-view in cancer treatment with SERMs. For me, the promise of life was the most important goal. But safety was essential. The involvement I had every day with the clinical evaluation of tamoxifen (Love *et al*., 1992), followed by leadership positions for the evaluation of raloxifene (Cummings *et al*., 1999), and then as the Scientific Chair of the Study of Tamoxifen and Raloxifene (STAR) (Vogel *et al*., 2006, 2010) allowed me to deploy the knowledge generated by my "Tamoxifen Team" over decades to save lives and advance women's health (Jordan, 2008 b). Please remember that when I started this improbable and unlikely journey at the beginning of the 1970s, cancer therapeutics with a targeted agent, chemoprevention, and the drug group, SERMs (or even tamoxifen for that matter!) did not exist. Cancer research was not recommended as a career for the pharmacologist and the pharmacologist would not knowingly venture into women's health. All of the revenues in the pharmaceutical industry were derived from heart drugs and drugs that affected the central nervous system (e.g. tranquilizers, etc.).

When I was starting the research for my PhD at Leeds University, Sir Alexander Haddow, FRS in the Inaugural Karnofsky Lecture (Haddow, 1970), was dismayed at the prospect for cancer therapeutics. Unlike the success noted with antibiotics for the treatment of different infectious diseases, there were no laboratory tests to establish whether a chemotherapy would be effective or not. The physician just had to give it to the patient and see if it worked! Haddow was also not convinced that a cancer-specific drug could be developed because cancer was self. In Haddow's Karnofsky Lecture publication, there was one glimmer of hope: Haddow had used the first chemical therapy to treat any cancer, i.e. high dose estrogen to treat metastatic breast cancer in women in their late sixties and seventies. He observed that some of the responses just melted the tumors away. But he was dismayed that the mechanisms had remained elusive. I am pleased to say that we have now solved the question surrounding the mechanism of estrogen-induced apoptosis (Ariazi, in press).

Fig. 7. Honorary Fellowship of the Royal Society of Medicine awarded by Professor Ilora Finlay, Baroness Finlay of Llandaff, President of the Royal Society of Medicine (2008). *This honor is awarded to individuals of international standing who have eminently distinguished themselves in the service of medicine and the fields which influence it. The Society permits, at most, 100 people into this elite group at any one time. In 2008 there were only 89 Honorary Fellows worldwide. In 2009, I received the Jephcott Medal from the Royal Society of Medicine, and in 2010, I was elected as the President of the Royal Society of Medicine Foundation in North America.*

It is fair to say that the work that has evolved and developed on the treatment and prevention of breast cancer over the past four decades has changed our outlook and replaced pessimism with hope. The first decade of discovery was essential to move forward in the field (Jordan, 2008 a). It has not only been possible to create change in medical practice, but the laboratory principles all translated to patient care to save or at least extend lives. That is what pharmacology is.

In closing, I must end where we began. I have thanked Drs. Kaye and Clark (Fig. 1) many times for the opportunity they gave me with a place at Leeds University. The reply I received was usually "we were only doing our job." Good words to remember and live by.

Acknowledgements

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References

ARIAZI E.A., CUNLIFFE H.E., LEWIS-WAMBI J.S., SLIFKER M.J., WILLIS A.L., RAMOS P., TAPIA C., KIM H.R., YERRUM S., SHARMA C.G.N., NICOLAS E., BALAGURUNATHAN Y., ROSS E.A. and JORDAN V.C. (in press). Estrogen Induces Apoptosis in Estrogen Deprivation-resistant Breast Cancer via Stress Responses as Identified by Global Gene Expression. *Proc Natl Acad Sci USA*.

CUMMINGS S.R., ECKERT S., KRUEGER K.A., GRADY D., POWLES T.J., CAULEY J.A., NORTON L., NICKELSEN T., BJARNASON N.H., MORROW M., LIPPMAN M.E., BLACK D., GLUSMAN J.E., COSTA A. and JORDAN V.C. (1999). The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. *JAMA* 281: 2189-2197.

CUMMINGS S.R., ENSRUD K., DELMAS P.D., REID D.M., GOLDSTEIN S., SRIRAM U., LEE A., THOMPSON J., ARMSTRONG R.A., THOMPSON D.D., POWLES T., ZANCHETTA J., KENDLER D., NEVEN P. and EASTELL R. (2010). Lasofoxifene in postmenopausal women with osteoporosis. *New Engl J Med* 362: 686-696.

EARLY BREAST CANCER TRIALISTS' COLLABORATIVE GROUP (EBCTCG). (2011). Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* 378: 771-784.

GOTTARDIS M.M. and JORDAN V.C. (1987). The antitumor actions of keoxifene (raloxifene) and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* 47: 4020-4024.

GOTTARDIS M.M. and JORDAN V.C. (1988). Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 48: 5183-5187.

GOTTARDIS M.M., ROBINSON S.P., SATYASWAROOP P.G. and JORDAN V.C. (1988). Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res*. 48: 812-815.

HADDOW A. (1970). David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer* 26: 737-754.

JORDAN V.C. and KOERNER S. (1975). Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *Eur J Cancer* 11: 205-206.

JORDAN V.C. (1976). Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. *Eur J Cancer.* 12: 419-424.

JORDAN V.C, COLLINS M.M., ROWSBY L. and PRESTWICH G. (1977). A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* 75: 305-316.

JORDAN V.C. (1978). Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. *Rev on Endocr Relat Cancer* 49-55.

JORDAN V.C., DIX C.J. and ALLEN K.E. (1979). The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In *Adjuvant Therapy of Cancer II* (Eds. S.E. Salmon and S. E. Jones). Grune & Stratton Inc., Philadelphia, pp. 19-26.

JORDAN V.C. and ALLEN K.E. (1980). Evaluation of the antitumour activity of the nonsteroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 16: 239-251.

JORDAN V.C. and LIEBERMAN M.E. (1984). Estrogen-stimulated prolactin synthesis *in vitro* classification of agonists, partial agonist and antagonist actions based on structure. *Mol Pharm* 26: 279-285.

JORDAN V.C. (1984). Biochemical pharmacology of antiestrogen action. *Pharm Rev* 36: 245-276.

JORDAN V.C., PHELPS E. and LINDGREN J.U. (1987). Effects of antiestrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 10: 31-35.

JORDAN V.C. (1988). The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. *Breast Cancer Res Treat* 11: 197-209.

JORDAN V.C. (1995). What if tamoxifen (ICI 46,474) had been found to produce liver tumors in rats in 1973? *Ann Oncol* 6: 29-43.

JORDAN V.C. (2001). Selective Estrogen Receptor Modulation: a personal perspective. (Perspectives in Cancer Research). *Cancer Res* 61: 5683-5687.

JORDAN V.C. (2003). Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2: 205-213.

JORDAN V.C. (2006). 75th Anniversary Edition British Journal of Pharmacology Special Issue. Tamoxifen (ICI 46,474) as a targeted therapy to treat and prevent breast cancer. *Br J Pharmacol* 147: S269-S276.

JORDAN V.C. (2007). Chemoprevention of Breast Cancer with Selective Oestrogen Receptor Modulators. *Nat Rev Cancer* 7: 46-53.

JORDAN V.C. (2008 a). Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 44: 30-38.

JORDAN V.C. (2008 b). The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer--survival or death? *J Clin Oncol* 26: 3073-3082.

JORDAN V.C. (2009). A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. *Cancer Res* 69: 1243-1254.

JORDAN V.C. and FORD L.S. (2011). Paradoxical Clinical Effect of Estrogen on Breast Cancer Risk: A "New" Biology of Estrogen-Induced Apoptosis. *Cancer Prev Res* 4: 633-637.

LA CROIX A.Z., CHLEBOWSKI R.T., MANSON J.E., ARAGAKI A.K., JOHNSON K.C., MARTIN L., MARGOLIS K.L., STEFANICK M.L., BRZYSKI R., CURB J.D., HOWARD B.V., LEWIS C.E. and WACTAWSKI-WENDE, J. (2011). Health outcomes after stopping conjugated equines estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. *JAMA* 205: 1305-1314.

LEVENSON A.S. and JORDAN V.C. (1998). The key to the antiestrogenic mechanism of raloxifene is Amino Acid 351 (Asp) in the estrogen receptor. *Cancer Res* 58: 1872-1875.

LEWIS J.S., MEEKE K., OSIPO C., ROSS E.A., KIDAWI N., LI Y., BELL E., CHANDEL N.S. and JORDAN V.C. (2005). Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 97: 1746-1759.

LIEBERMAN M.E., JORDAN V.C., FRITSCH M., SANTOS M.A. and GORSKI J. (1983 a). Direct and reversible inhibition of estradiol-stimulated prolactin synthesis by antiestrogens *in vitro. J Biol Chem* 258: 4734-4740.

LIEBERMAN M.E., GORSKI J. and JORDAN V.C. (1983 b). An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens *in vitro. J Biol Chem* 258: 4741-4745.

LIU H., LEE E.S., DE LOS REYES A., ZAPF J.W. and JORDAN V.C. (2001). Silencing and reactivation of the selective estrogen receptor modulator (SERM)-ER alpha complex. *Cancer Res* 61: 3632-3639.

LIU H., PARK W., BENTREM D.J., MCKIAN K.P., DE LOS REYES A., MACGREGOR-SCHAFER J., ZAPF J. and JORDAN V.C. (2002). Structure-function relationships of the raloxifene-estrogen receptor alpha complex for regulating transforming growth factor alpha expression in breast cancer cells. *J Biol Chem* 277:9189-9198.

LOVE R.R., MAZESS R.B., BARDEN H.S., EPSTEIN S., NEWCOMB P.A., JORDAN V.C., CARBONE P.P. and DEMETS D.L. (1992). Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *New Engl J Med* 326: 852-856.

MACGREGOR-SCHAFER J.I., LIU H., BENTREM D., ZAPF J. And JORDAN V.C. (2000). Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex by substituting glycine for aspartate at amino acid 351. *Cancer Res* 60: 5097-5105.

VOGEL V.G., COSTANTINO J.P., WICKERHAM D.L., CRONIN W.M., CECCHINI R.S., ATKINS J.N., BEVERS T.B., FEHRENBACHER L., PAJON E.R., WADE J.L., ROBIDOUX A., MARGOLESE R.G., JAMES J., LIPPMAN S.M., RUNOWICZ C.D., GANZ P.A., REIS S.E., MCCASKILL-STEVENS W., FORD L.G., JORDAN V.C. and WOLMARK N. (2006). The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA* 295: 2727-2741.

VOGEL V.G., COSTANTINO J.P., WICKERHAM D.L., CRONIN W.M., CECCHINI R.S., ATKINS J.N., BEVERS T.B., FEHRENBACHER L., PAJON E.R., WADE J.L., ROBIDOUX A., MARGOLESE R.G., JAMES J., RUNOWICZ C.D., GANZ P.A., REIS S.E., MCCASKILL-STEVENS W., FORD L.G., JORDAN V.C. and WOLMARK N. (2010). Update of the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing Breast Cancer. *Cancer Prev Res* 3: 696-706.

WOLF D.M. and JORDAN V.C. (1993). A laboratory model to explain the survival advantage observed in patients taking adjuvent tamoxifen therapy. *Recent Results Cancer Res* 127: 23-33.

WOLF D.M. and JORDAN V.C. (1994). The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. *Breast Cancer Res Treat* 31: 129-138.

YAO K., LEE E.S., BENTREM D.J., ENGLAND G., SCHAFER J.I.M., O'REGAN R. and JORDAN V.C. (2000). Antitumor action of physiologic estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 6: 2028-2036.

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R Didier and Y Croisille.
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Profile of V. Craig Jordan

In the mid-1970s, breast cancer survival rates were dismal. Researchers hoped to find a drug capable of thwarting the disease, but the prospects were few and far between. In a laboratory on the campus of the Worcester Foundation for Experimental Biology in Shrewsbury, Massachusetts, a group of experimental rats were dying from breast cancer. A researcher gave them a triphenyl ethylene—a purported antiestrogen—with the slim hope that it would slow progression of the disease. The cancer disappeared (1). Within a few years, a clinical trial of the drug was launched among women suffering from breast cancer. The women's tumors, just as those in the rats, shrank. By 1978, the US Food and Drug Administration had approved a triphenyl ethylene-based drug known as tamoxifen for the treatment of late-stage breast cancer (2).

Today, tamoxifen is a resounding success. By the numbers, breast cancer mortality rates held steady from 1975 to 1990 but declined by almost 20% from 1990 to 2000. Two-thirds of that decline is attributable to adding tamoxifen to the chemotherapy regimen already used to treat breast cancer. Among breast cancer survivors taking tamoxifen for 5 years, the standard dosage for the drug, mortality declined by nearly 40% (3, 4). The researcher who cured the rats, V. Craig Jordan, is now known as the "Father of Tamoxifen."

Since discovering tamoxifen's potential to prevent breast cancer more than three decades ago, Jordan, a 2009 inductee to the National Academy of Sciences, has devoted his career to understanding the characteristics of the drug—its benefits, pitfalls, and other applications. Thanks to that work, it is now known that tamoxifen and similar drugs act as both estrogen inhibitors and estrogens, depending on where they travel inside the body. Collectively, the drugs are referred to as selective estrogen receptor modulators, or SERMs. SERMs are now routinely prescribed to treat not just breast cancer but other estrogenic disorders, such as osteoporosis. Jordan says there is hope of someday using this same class of drugs to reduce the devastation of coronary heart disease.

In his Inaugural Article, Jordan returns to the topic of breast cancer to explain a paradox in the literature that has plagued scientists for decades. From his own work, Jordan knew that tamoxifen's antiestrogenic properties stopped the growth of breast cancer. However, in the 1940s, another researcher by the name of Alexander Haddow showed that giving postmenopausal women estrogen also caused the disease to grind to a halt (5). Now

V. Craig Jordan.

Jordan has explained how estrogen can both promote and prevent breast cancer. "We have solved a 70-year mystery," he says (6).

Early Childhood

Jordan, the Alfred G. Knudson Jr. Chair in Cancer Research at the Fox Chase Cancer Center in Philadelphia, Pennsylvania, was born in New Braunfels, Texas, in 1947 but moved to the United Kingdom as an infant with his British parents. Growing up, Jordan developed a deep, almost singular, infatuation with chemistry—the origins of which he cannot recall. At age 13, his mother let him build a chemistry laboratory in his bedroom, a prescient if costly decision.

"There were always fires in the bedroom and bombs going off in the back of the garden," Jordan says, recounting an experiment with sodium chlorate that went horribly awry. Rather than blow up the house, Jordan chucked the whole, smoldering mess out the window—creating a crater-sized gap where grass once grew. "My parents were furious," Jordan says. Telling them not to worry, Jordan reseeded the lawn and added some copper sulfate to expedite the growing process. "The grass did grow back," Jordan says, "but it was blue."

However, where Jordan excelled in chemistry, he foundered in other "lesser" subjects. "I thought plants were stupid," he says. By age 16, when he needed to pass five subject examinations to continue his education, Jordan only passed three, forcing his mother to beg the headmaster to let him retake the tests in a few months. Luckily, he passed.

By then, Jordan had become a tutor to his peers, teaching them the basics of chemistry, pharmacology, and biochemistry. Seeing that talent, a teacher by the name of Charles Bescoby convinced Jordan and his parents that he should not go to work as a technician at nearby Imperial Chemical Industries (ICI) Pharmaceuticals as he had long planned, but to university. Jordan received admission to the University of Leeds and graduated with a degree in pharmacology in 1969. He stayed on for another 3 years to receive his doctorate in the same subject, by then convinced that his future lay in developing a drug to treat cancer—a monumental chemistry challenge that appealed to Jordan's intellect.

ICI 46,474

However, Jordan's path to becoming a cancer drug expert was roundabout. At Leeds, he had extensively studied triphenyl ethylenes, the active compound in a drug that ICI had once believed would become the world's first-ever "morning-after pill" (7).

Going by the code name ICI 46,474, the drug had been shown to block estrogen from reaching the uterus in rats. However, hopes were dashed when a clinical trial in humans found that more women got pregnant when taking the drug than not (8). Jordan was studying to see just how that drug worked in the body—a complex, voluminous project. When he went to defend his thesis in 1972, the university had no experts on staff capable of grasping Jordan's thesis. So they called in Arthur Walpole, a researcher at ICI. Walpole held the patent on ICI 46,474 and was thus well placed to make sense of Jordan's opus.

After that chance encounter, Walpole helped Jordan line up a postdoctoral fellowship at the Worcester Foundation for Experimental Biology. He was to work with endocrinologist Michael Harper to develop new contraception methods. By the time Jordan arrived in Massachusetts, though, Harper had accepted another job, and the Worcester Foundation told Jordan to set up his own laboratory for 2 years. "I was on my own," Jordan says, with no idea of what to research. So he called Walpole, and the two men discussed turning ICI 46,474 into a drug to treat breast cancer.

This is a Profile of a recently elected member of the National Academy of Sciences to accompany the member's Inaugural Article on page 18879.

Despite the failure of ICI 46,474 as a morning-after pill, Walpole and Jordan knew that the drug had antiestrogenic properties. Although breast cancer has different causes, for most women it arises when estrogen binds to receptor sites in breast cancer cells, allowing them to proliferate. A drug capable of binding to and inactivating those receptors might just thwart the spread of the disease, Walpole theorized. As a contraception researcher, Walpole had no opportunity to research that idea. So he handed the project over to Jordan.

In the early 1970s, Jordan induced rats to develop mammary (breast) cancer and confirmed that the tumors needed estrogen hormones to survive. When the rats were given ICI 46,474 the tumors shrank—a situation only mirrored in rats whose ovaries had been removed (1). ICI 46,474, he concluded, held promise as a drug to treat and prevent breast cancer in women with estrogen receptor sites in their breast cancer cells.

The idea that a drug could prevent breast cancer, however, remained controversial. Jordan's paper was initially rejected before being accepted by the *European Journal of Cancer Research* in 1976. By then Jordan had completed his postdoctoral work in Massachusetts and become a full-time lecturer in pharmacology at his alma mater, the University of Leeds.

At Leeds, Jordan began studying how long tamoxifen should be administered in women with breast cancer. Using a rat tumor model, he showed that treatments shorter than a few years ultimately failed and the rats went on to develop tumors, whereas administering tamoxifen for longer periods thwarted the progression of the disease (9, 10). Today, the standard tamoxifen treatment extends over 5 years (11). Jordan's research eventually prompted ICI to launch clinical trials into the use of tamoxifen as drug to treat breast cancer. "Tamoxifen slowly became hot," Jordan says.

Two Faces of Tamoxifen

With tamoxifen poised for widespread rollout, however, Jordan began to worry that long-term estrogen deprivation through tamoxifen might trigger unforeseen side effects. Estrogen, he explains, is a double-edged sword for women. Although implicated in breast cancer, the hormone is also critical for the development of the cardiovascular system and bones. Jordan wondered whether long-term estrogen deprivation would lead to osteoporosis or heart disease. In 1980, he relocated to the University of Wisconsin, Madison, and started his own laboratory to research the health implications of using tamoxifen long term.

After finding that long-term tamoxifen use actually seemed to lessen the incidence of osteoporosis and heart disease in rodents (12), Jordan and colleagues launched a 2-year study of 140 postmenopausal women with a history of breast cancer. Half the women were treated with tamoxifen, whereas the other half received a placebo. As with the rodents, the researchers found that tamoxifen lowered cholesterol in women receiving the drug after 3 months and that such positive effects persisted for years (13). Similarly, bone density increased in women receiving tamoxifen but decreased in women receiving placebo (14).

Collectively, Jordan's research suggested that tamoxifen and another related drug, raloxifene, were not antiestrogenic everywhere in the body as previously assumed, but were selective estrogens and antiestrogens (SERMs). "It turns out that different tissues interpret the drugs' signal in different ways," Jordan says. "So, paradoxically, tamoxifen and raloxifene built bones."

Raloxifene is now widely prescribed to postmenopausal women in danger of developing osteoporosis (15). Estimates suggest that raloxifene use has inadvertently protected thousands of female users from developing breast cancer (16). The fact that women taking the drug report a lower incidence of breast cancer than the general population is just a "beneficial side effect," Jordan says (17).

Not all side effects of SERMs were desirable, however. In 1988, Jordan, working with then graduate student Marco Gottardis, showed that tamoxifen promoted the growth of endometrial tumors in women (18). However, subsequent research made clear that the benefits of using tamoxifen for the treatment of breast cancer far outweighed the risk of developing endometrial cancer. Today, tamoxifen is estimated to save approximately 30 times more women than it harms (19).

Interestingly, raloxifene did not promote the development of endometrial cancer, suggesting that it may be preferable to tamoxifen. However, *Cancer Prevention Results* published a 2010 update of a five-year study comparing the long-term health outcomes of women receiving tamoxifen with women receiving raloxifene. Although participants in both groups had equal outcomes after 41 months of treatment, tamoxifen emerged as the more effective weapon against the recurrence of breast cancer when that time frame doubled. Specifically, raloxifene was shown to be less than 80% as effective as tamoxifen (20, 21).

Estrogen as Cancer Killer

Despite all his headway into revealing tamoxifen's secrets, an issue that niggled at Jordan throughout his career has been that of resistance. If tamoxifen required 5 years to adequately treat breast cancer, would that give cancer cells too much time to find a new way to undermine the drug?

As early as the mid-1980s, Gottardis developed a tamoxifen-resistant human tumor in mice. He further showed that such tumors could be transplanted into future generations of mice and kept alive with tamoxifen treatment (22). Long-term tamoxifen use in humans, Jordan says, "seemed like a recipe for disaster."

Critical to Jordan's thinking, however, was the belief that estrogen blockers are required to thwart the growth of breast cancer. However, the theory did not hold up. Reports dating back to the 1940s showed that giving breast cancer patients estrogen also seemed to stop growth of the disease. In fact, before the emergence of tamoxifen, estrogen was routinely administered to postmenopausal women—or those no longer producing estrogen on their own—to combat the disease. Approximately 30% of patients responded favorably to the treatment.

More strikingly, when Doug Wolf, a graduate student in Jordan's laboratory in the 1990s, transplanted tamoxifen-resistant tumors from mouse to mouse and treated the animals with estrogen, he found that the tumors shrank (23, 24). "Estrogen didn't stimulate the growth of these tumors anymore. It killed them. They just melted away," Jordan recalls. "But we still didn't know the underlying mechanism of how that happened."

So Jordan set out to find out how the same hormone responsible for activating breast cancer also kills it off. He ultimately hopes to develop a new treatment approach for breast cancer patients who have grown resistant to tamoxifen. In his Inaugural Article, Jordan evaluates genetic changes to estrogen-starved breast cancer cells during the first week of estrogen therapy. The changes were striking, he says. The endoplasmic reticulum, or internal structure of the cell, quickly grew inflamed, triggering the cell's death. Moreover, Jordan found that cancer cell death occurred with relatively low doses of estrogen (6).

"In bodies that have been starved of estrogen, the hormone comes back as a jet fuel," Jordan says. That fuel overwhelms the estrogen receptor in breast cancer cells, causing them to invoke the "death signal." Jordan's finding suggests that it might make sense to treat women with tamoxifen-resistant tumors or those several years beyond menopause with low doses of estrogen. In estrogen-starved women, "The dramatic cell kill I get with estrogen is better than anything I saw with tamoxifen," Jordan says.

Living Legend

Growing up, Jordan says he did not have typical kid hobbies. Besides tinkering in his bedroom laboratory, he says, he loved ancient history. "I went on archaeological digs when I was a teenager in England," Jordan recalls.

His fondness for historical precedent, he says, is critical to his success as a pharmacologist. For the better part of a century, he says, nobody could understand why estrogen killed breast cancer in a certain subset of women. However, Jordan remembered Haddow's research from the 1940s and his graduate student's serendipitous finding with tamoxifen-resistant tumors from the 1990s. "I believe that we're all part of this continuum. We're in a relay race and we've got to know where we've come from to show us where we're going," he says.

That focus has earned Jordan innumerable awards, but the honor for which Jordan remains most proud is one bestowed upon him by Northwestern University and the family of the late Diana, Princess of Wales. A longtime supporter of women's health initiatives, Princess Diana came to Chicago to support a symposium hosted by *People* magazine on women's health and breast cancer. Jordan organized the event, and the two became friends. When Princess Diana died in a car accident in 1997, her family suggested establishing a professorship in her honor, earning Jordan the title Diana Princess of Wales Professor of Cancer Research at Northwestern University.

Chance encounters and obsession, Jordan says only half in jest, are key to his success. "Early on, I developed key concepts, and like a dog with a bone I never let those concepts go."

Sujata Gupta, *Freelance Science Writer*

1. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. *Eur J Cancer* 12:419–424.
2. Kiang DT, Kennedy BJ (1977) Tamoxifen (antiestrogen) therapy in advanced breast cancer. *Ann Intern Med* 87: 687–690.
3. Jordan VC (2008) Tamoxifen: Catalyst for the change to targeted therapy. *Eur J Cancer* 44:30–38.
4. Berry DA, et al. (2006) Modeling the impact of treatment and screening on U.S. breast cancer mortality: A Bayesian approach. *J Natl Cancer Inst Monogr* 36: 30–36.
5. Haddow A (1970) David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer* 26:737–754.
6. Ariazi EA, et al. (2011) Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *Proc Natl Acad Sci USA* 108:18879–18886.
7. Harper MJK, Walpole AL (1967) A new derivative of triphenylethylene: Effect on implantation and mode of action in rats. *J Reprod Fertil* 13:101–119.
8. Klopper A, Hall M (1971) New synthetic agent for the induction of ovulation: Preliminary trials in women. *BMJ* 1:152–154.
9. Jordan VC, Dix CJ, Allen KE (1979) The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. *Adjuvant Therapy of Cancer II*, eds Salmon SE, Jones SE (Grune & Stratton, Phildelphia), pp 19–26.
10. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 16:239–251.
11. Davies C, et al.; Early Breast Cancer Trialists' Collaborative Group (EBCTCG) (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: Patient-level meta-analysis of randomised trials. *Lancet* 378:771–784.
12. Jordan VC, Phelps E, Lindgren JU (1987) Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 10:31–35.
13. Love RR, et al. (1990) Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. *J Natl Cancer Inst* 82:1327–1332.
14. Love RR, et al. (1992) Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med* 326:852–856.
15. Ettinger B, et al.; Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators (1999) Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: Results from a 3-year randomized clinical trial. *JAMA* 282:637–645.
16. Jordan VC (2006) Optimising endocrine approaches for the chemoprevention of breast cancer beyond the Study of Tamoxifen and Raloxifene (STAR) trial. *Eur J Cancer* 42:2909–2913.
17. Cummings SR, et al. (1999) The effect of raloxifene on risk of breast cancer in postmenopausal women: Results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* 281:2189–2197.
18. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 48:812–815.
19. Early Breast Cancer Trialists' Collaborative Group (EBCTCG) (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. *Lancet* 365:1687–1717.
20. Vogel VG, et al. (2006) Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: The NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* 295: 2727–2741.
21. Vogel VG, et al. (2010) Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. *Cancer Prev Res (Phila)* 3:696–706.
22. Gottardis MM, Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 48:5183–5187.
23. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvent tamoxifen therapy.*Recent Results in Cancer Research* (Springer-Verlag, Heidelberg) Vol 127, pp 23–33.
24. Yao K, et al. (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 6: 2028–2036.

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Tamoxifen as the first targeted long-term adjuvant therapy for breast cancer

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Abstract

Tamoxifen is an unlikely pioneering medicine in medical oncology. Nevertheless, the medicine has continued to surprise us, perform, and save lives for the past 40 years. Unlike any other medicine in oncology, it is used to treat all stages of breast cancer, ductal carcinoma *in situ*, and male breast cancer and pioneered the use of chemoprevention by reducing the incidence of breast cancer in women at high risk and induces ovulation in subfertile women! The impact of tamoxifen is ubiquitous. However, the power to save lives from this unlikely success story came from the first laboratory studies which defined that 'longer was going to be better' when tamoxifen was being considered as an adjuvant therapy. This is that success story, with a focus on the interdependent components of: excellence in drug discovery, investment in self-selecting young investigators, a conversation with *Nature*, a conversation between the laboratory and the clinic, and the creation of the Oxford Overview Analysis. Each of these factors was essential to propel the progress of tamoxifen to evolve as an essential part of the fabric of society.

Key Words
- breast
- endocrine therapy

Endocrine-Related Cancer
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Introduction

'Science is adventure, discovery, new horizons, insight into our world, a means of predicting the future and enormous power to help others' (Hoagland 1990).

– Mahlon Hoagland, MD, Director, Worcester Foundation for Experimental Biology (1970–1985).

Tamoxifen (Imperial Chemical Industries (ICI) 46 474; Harper & Walpole 1967, Cole *et al.* 1971, Klopper & Hall 1971) is an old medicine with origins unlikely to predict pioneer or breakthrough status (Jordan 2003, 2006, Maximov *et al.* 2013). I was the least likely schoolboy to go to university (University of Leeds) but subsequently selected a career path 'to help develop a drug to treat cancer' (Poirot 2011). At the time, this was not a popular or even reasonable career path as treatments were primitive and invariably unsuccessful (except for childhood leukemia). Tamoxifen and I became the 'odd couple', but nobody cared in the 1970s, as combination cytotoxic chemotherapy was predicted to cure cancer. Be that as it may, tamoxifen slowly 'arrived' and advanced on the clinical scene in the 1970s but only as an orphan drug after all but being abandoned by the pharmaceutical industry. This old medicine never went away and continues to provide surprises (Davies *et al.* 2013, The aTTom Collaborative Group 2013). Through the application of experimental science in cancer therapeutics (I was, and remain, a pharmacologist first), questions were asked, but *Nature*'s replies were unanticipated. However, *Nature* does not lie, and if the controls are correct, and it is

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reproducible, then one in compelled to re-evaluate the implications for medicine. The science of tamoxifen became 'a means of predicting the future and enormous power to help others' (Hoagland 1990). This is that story.

In 1977, I presented an invited lecture at a medical symposium held by ICI Pharmaceuticals Division at King's College, Cambridge. I described a new strategy to treat breast cancer (Jordan 1978). This was to use tamoxifen, a palliative agent then used in the final stages of breast cancer as a long-term adjuvant therapy, but this was not the fashion. Already adjuvant therapy with cytotoxic chemotherapy was showing promise (Fisher *et al*. 1975, Bonadonna *et al*. 1976) on the way to cures. The clinical strategy was considered sound. The primary tumor is first removed with a mastectomy, then nonspecific cytotoxic chemotherapy is given for many months afterwards to destroy the micrometastases scattered unseen around the patient's body. Destruction of micrometastases would produce cures.

During the 1970s, I was supported by both ICI Pharmaceuticals Division and the Yorkshire Cancer Research campaign to explore the mechanism of action and clinical opportunities for ICI's orphan drug tamoxifen (Jordan 2006). Tamoxifen, a nonsteroidal antiestrogen, was no better than high-dose estrogen or androgen therapy (Cole *et al*. 1971, Ward 1973, Morgan *et al*. 1976, Ingle *et al*. 1981) as a treatment for metastatic breast cancer and was available as a palliative therapy in the UK and other countries (except the USA) to treat metastatic breast cancer in postmenopausal women. Only 'fewer side effects', and higher cost, separated tamoxifen from the other 'hormone therapies' (Cole *et al*. 1971, Ward 1973, Ingle *et al*. 1981). No cures were anticipated as the 'hormone therapies', as they were then called, were only effective in 30% of patients for a year or two. The medicine would not be approved in the USA for the treatment of metastatic breast cancer until December 1977 and chances for economic success for ICI Pharmaceuticals Division were hovering just above zero.

The experimental results I presented (Jordan 1978) at the medical symposium at King's College demonstrated that long-term tamoxifen treatment was superior to short-term treatment in suppressing rat mammary tumorigenesis (Fig. 1). At the time, numerous adjuvant trials of 1-year adjuvant tamoxifen were proposed for the simple reason that tamoxifen treatment only controlled breast cancer for a year (Hubay *et al*. 1980, Ribeiro & Palmer 1983, Ludwig Breast Cancer Study Group 1984, Cummings *et al*. 1985, Ribeiro & Swindell 1985, Rose *et al*. 1985). The new concept presented presaged any clinical trials of more than

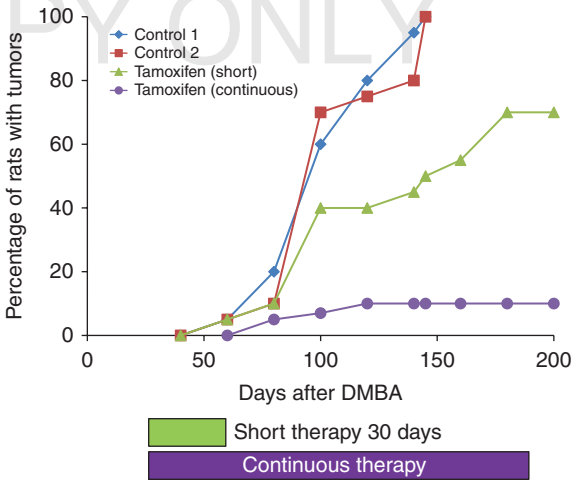

Figure 1
The use of the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model to demonstrate that longer or continuous therapy with daily tamoxifen (50 μg s.c. injection) was superior at preventing the appearance of mammary tumors when compared with short therapy of 30 days. Female Sprague–Dawley rats that are 50 days old were each given 20 mg DMBA by gavage in 2 ml peanut oil. In nontreated control groups of 20 animals, all rats had multiple palpable tumors by 150 days. The model design for therapy groups first administered DMBA at 50 days of age but the 30-day or continuous treatment was delayed for 30 days to permit initiation and promotion of mammary carcinogenesis to occur. The goal was to establish whether a short 30-day course of tamoxifen (estimated to be equivalent to 1 year of adjuvant tamoxifen in patients) could destroy the deranged microscopic cancer cells in the mammary glands or whether continuous therapy was required for complete tumor control and suppression. Continuous therapy is necessary. The strategy was to use tamoxifen only in patients with ER-positive tumors (Jordan & Koerner 1975) and use continuous therapy. This new strategy was first reported at the medical symposium held by ICI Pharmaceuticals Division at King's College, Cambridge, September 1977.

1 year of adjuvant tamoxifen and proposed that an appropriate clinical strategy for adjuvant tamoxifen treatment would be for extended or indefinite tamoxifen administration. My catch phrase at medical meetings was 'tamoxifen forever'. However, the proposal was immediately controversial. Attendees at the conference (Fig. 2) challenged the fidelity of the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model I was using, as it did not replicate human micrometastatic dissemination. Worse still, 'your strategy is dangerous!' It was universally known by the clinical community that tamoxifen would only be effective for <2 years in one-third of patients when used to treat metastatic disease in postmenopausal women. 'You're proposing we give long term or indefinite adjuvant tamoxifen to women, some of whom are already cured, so you can prevent a recurrence. Your treatment strategy may, infact, encourage premature

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Figure 2
The participants of the medical symposium held by ICI Pharmaceuticals Division at King's College, Cambridge, September 1977. The author (top) presented the new strategy; Prof. Michael Baum (right) was the session chair and leader of the proposed NATO trial that was planned to advance the current 1-year adjuvant tamoxifen trials to a 2-year treatment period. Helen Stewart (left) was in the audience and had plans to compare placebo and tamoxifen at first recurrence with 5 years of immediate adjuvant tamoxifen in the Scottish trial. Both trials (the NATO and Scottish trials) were to demonstrate, for the first time, survival advantages of adjuvant tamoxifen used for longer than 1 year.

drug resistance and we will have wasted a valuable palliative drug by using it too soon'.

Immediately after the King's College meeting, in October 1977, I had been invited to visit the University of Wisconsin Clinical Cancer Center in Madison by Paul Carbone (Director) and Doug Tormey (Head of the breast program) to spend several months doing collaborative research. I presented my ideas about long-term adjuvant tamoxifen therapy – a new strategy with a drug that was not yet on the market in the USA! I was immediately offered a job at the Cancer Center and asked to move to Madison. Doug Tormey, based on my lecture, decided to continue his patients on indefinite tamoxifen (Tormey & Jordan 1984, Tormey *et al.* 1987) and an Eastern Cooperative Oncology Group adjuvant protocol of indefinite tamoxifen was subsequently approved (Falkson *et al.* 1990). But first, I spent a year designing and creating the Ludwig Institute for Cancer Research in Bern, Switzerland. I was provided with a large travel budget as I was asked to quality control estrogen receptor (ER) assays (Jordan *et al.* 1983) for the Ludwig Adjuvant Tamoxifen Trials (regrettably only 1 year, Ludwig Breast Cancer Study Group (1984)). As a gamble, I decided to submit an abstract to the Adjuvant Therapy of Cancer II meeting in Tucson, Arizona, organized by the late Syd Salmon and Steve Jones. We now had much more data to support the proposal to use long-term tamoxifen as a long-term adjuvant therapy and I hoped, maybe, I would be lucky and get my abstract accepted for presentation. Imagine my surprise to find myself in the opening session sandwiched between the clinical greats of cancer research. The talk went well and was quickly published (Jordan *et al.* 1979) for global distribution to the clinical community. At the meeting, I was able to enlarge my circle of colleagues in clinical breast cancer research but one 'premonition' is worthy of mention. After my talk, Lois Trench, whom we will meet again later, turned to her colleagues in the marketing department of ICI Americas and exclaimed 'you have no idea what Dr Jordan has just announced with his talk on indefinite adjuvant tamoxifen. This will be a blockbuster!' And so it was. Those first animal experiments provided a scientific justification and road map for all subsequent long-term adjuvant clinical trials with tamoxifen that were to show unanticipated large survival advantages for patients (EBCTCG 1998 and EBCTCG 2005) and consistent decreases in death rates from breast cancer in national statistics (Peto *et al.* 2000, Berry *et al.* 2005).

There is no better example of the value of long-term adjuvant tamoxifen therapy than the recent reports of the Adjuvant Tamoxifen Longer Against Shorter (ATLAS; Davies *et al.* 2013) and adjuvant tamoxifen treatment offers more (aTTom; The aTTom Collaborative Group 2013). Until these trials of 10 years of adjuvant tamoxifen, it was well established that 5 years of tamoxifen is dramatically superior to no treatment (Davies *et al.* 2011), but ATLAS and aTTom compare 5 vs 10 years of tamoxifen. The conclusion is that 10 years of adjuvant tamoxifen cause a superior decrease in mortality than 5 years of adjuvant tamoxifen (Davies *et al.* 2013). However, the question that must be raised is why mortality only decreases for the 10-year treatment group in the decade after tamoxifen is stopped? To seek the answer to this paradox, that should not occur with a

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palliative nonsteroidal antiestrogen that blocks ER-mediated estrogen-stimulated growth of micrometastases (no drug, no action!), we have to return to the origins of tamoxifen, follow the interconnected events in translational research, and identify the factors that allowed tamoxifen to triumph.

In retrospect, the essential components to achieve the full potential of tamoxifen in the clinic were: a commitment to excellence in drug discovery, investment in a young self-selecting investigator, keeping an open mind with the conversation with *Nature*, and maintaining an active conversation between the laboratory and the clinical investigators. Laid over all of these essentials was the creation of the recurrent Oxford Overview Analyses of adjuvant trials by Sir Richard Peto and his team. This process formed the fundamental foundation to create the ATLAS trial based on firm clinical evidence and acts as a continuing catalyst to provide scientific support for aTTom. Finally, there is another dimension best described as seeing an opportunity, being in the right place at the right time and be willing to train yourself to be talent-spotted. Slightly different circumstances or a different decision or meeting can change everything: the play of professional chance. 'Sliding Doors', starring Gwyneth Paltrow and John Hannah, is an excellent film based on the premise that by just missing or catching a tube train in London, a life can be altered forever. The film then portrays two parallel lives to the conclusion. In the spirit of 'Sliding Doors', I will retell the progression of the aforementioned interconnecting components that created the tamoxifen of today.

A commitment to excellence in drug discovery

Following the chance discovery of the first nonsteroidal antiestrogen ethamoxytriphetol (MER25) by Lerner *et al.* (1958) at the William S Merrell company in Cincinnati and the finding that there was post-coital antifertility activity in laboratory animals (Segal & Nelson 1958), numerous companies immediately began synthesizing and screening for suitable compounds for use as 'morning-after pills'. Contraceptive research was the 'hot' topic and fashion in the wake of the approval of the oral steroid contraceptive 'to regulate the menstrual cycle' in 1960. A range of nonsteroidal compounds became available but one, clomiphene, induced ovulation in women – it guaranteed what it was planned to prevent! Clomiphene (Greenblatt *et al.* 1961) subsequently found sustained use in medicine for the induction of ovulation after a 5-day course, in subfertile women. However, clomiphene increases desmosterol levels, which is associated with cataract formation (Avigan *et al.* 1960, Laughlin & Carey 1962), and there was no further development for long-term therapy.

Drs Mike Harper and Arthur Walpole (Fig. 3) tested the antifertility properties of a range of compounds related to clomiphene at ICI Pharmaceuticals laboratory at Alderley Park near Macclesfield, Cheshire. The compounds were made by a talented organic chemist, Dr Dora Richardson (Fig. 3). Compound ICI 46 474, the antiestrogenic *trans* isomer of a substituted triphenylethylene, did not increase desmosterol (Harper & Walpole 1967) but like clomiphene was also found to induce ovulation (Klopper & Hall 1971). By coincidence, I was a summer student working in the nascent cancer research laboratory opposite Dr Walpole's fertility control laboratory in 1967. Alderley Park is just ten miles from my home where I grew up in Cheshire. Walpole was the Head of the Fertility Control Program at ICI Pharmaceuticals Division but was subsequently to play an essential role to ensure the successful development of ICI 46 474 as a cancer treatment. This was because Walpole had long-standing interest in cancer research (Jordan 1988) though he was required to work in what was judged to be the more fertile field of contraception. I was to meet Walpole again 5 years later in 1972, but this time he was the examiner of my PhD thesis entitled 'A study of the structure function relationship of substituted triphenylethylenes and triphenylethanes'.

Self-selecting young investigator

I started my lifelong 'love affair' with triphenylethylenes in 1969 when I chose to accept a PhD project to crystallize and study the X-ray crystallography of the ER complex liganded with an estrogen and antiestrogen. Jack Gorski (Toft & Gorski 1966, Toft *et al.* 1967) had just published a series of papers in the *PNAS* showing that the ER (ESR1) protein could easily be extracted from rat uteri. My PhD supervisor, in the Department of Pharmacology at Leeds University, was Dr Edward (Ted) Clark, a brilliant and exciting lecturer in medicinal chemistry with encyclopedic knowledge and a long-standing interest in estrogens. 'It will be simple' he said. 'You will extract and purify the rat uterine ER and crystallize it with an estrogen and an antiestrogen and do the X-ray crystallography up the road at the Astbury Department of Biophysics'. Well that did not work (the whole ER complex has yet to be crystallized!) and I switched to study the structure–function relationships of triphenylethylene antiestrogens – the failed contraceptives. Although this would prove to be a sound

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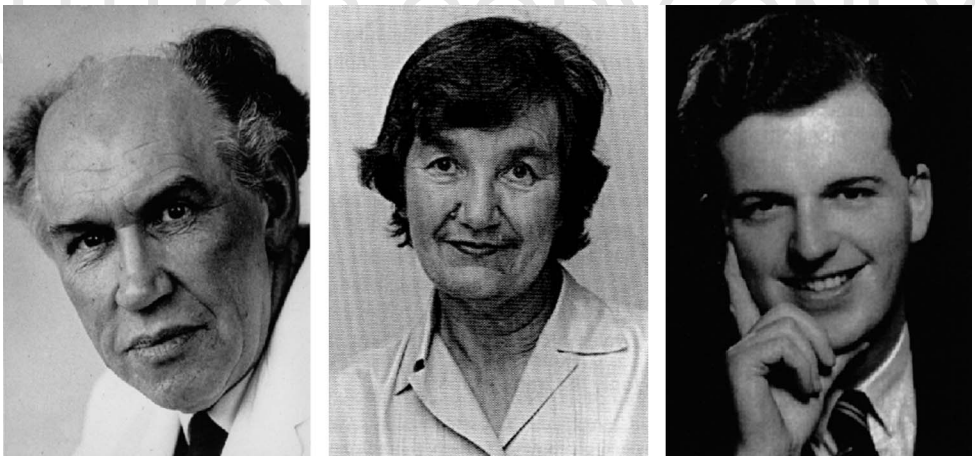

Figure 3

The principal players in the discovery of ICI 46 474 at ICI Pharmaceuticals Division, Cheshire, UK in the 1960s that eventually evolved into tamoxifen a decade later. Arthur Walpole (Walop; left) was the head of the Fertility Control Program tasked with the mission to discover safer compounds to 'regulate the sexual cycle'. Dora Richardson (center) was the team organic chemist who synthesized all of the isomers of the triphenylethylene derivatives that would be tested as antifertility agents in rats by Mike Harper and the team reproductive endocrinologist. Arthur Walpole would be the author's PhD examiner, scientific supporter, and administrative link to ICI until his untimely death on 2nd July 1977. Dora Richardson would provide the metabolites of tamoxifen to the author to be tested as anticancer agents and Mike Harper would offer the author a 2-year BTA (Been–To–America) at the Worcester Foundation, MA. Each individual was generous with important opportunities, investment, and support for a young investigator starting their adventure to investigate 'failed morning-after pills' as important future therapeutic agents in women's health.

foundation for a future, at the time no one was recommending careers in failed contraceptives!

During the 3 years of my PhD studies (1969–1972), armed with a Medical Research Council scholarship, I was talent-spotted by Prof. Michael Barrett, the new Chair of Pharmacology (a cardiovascular pharmacologist from ICI Pharmaceuticals Division) appointed in 1970. As an undergraduate, I had created, organized, and led our student society, named the Medean Society after the sorceress Medea who created magic potions to protect Jason (of Argonaut fame) from death as he completed his impossible tasks to retrieve the Golden Fleece. She was, it seems, the first to create effective chemopreventive agents!

Prof. Barrett recognized that I had talent for organization in science and, as a graduate student, I chose to create lectures for parent teacher organizations in the Leeds area schools on drug abuse. I strongly believed in public service, as reinvestment in the community was important to 'pay back' the investment of my free education. These lectures were also presented, at Prof. Barrett's insistence, to the undergraduates as I was also closely connected with the Leeds City Police Drug Squad as an advisor. Thirdly, Prof. Barrett was aware that I had been talent-spotted to be on the advisory staff for the Deputy Chief Scientist (Army) and one of my duties was to present drug abuse lectures for Army units throughout the country. In this role, I was Reserve Army Officer. I was focused on the perils of drug abuse and worked with the police. As a PhD student, I was researching the regulation of the sexual cycle with pharmacological agents, and I was an Army Officer advisor to the Deputy Chief Scientist (Army). In USA (1972–1974), I would often be asked to give talks in the community (the English accent went over well!), so I would preface my talks by stating that my career was based on drugs, sex and violence (with apologies to the 'sex, drugs and rock and roll' in the sixties; I was, however, actually a drummer in a rock band as a teenager!).

In 1972, Prof. Barrett now saw potential in me as a new staff member in his new Department of Pharmacology. I found myself as a prospective lecturer in Pharmacology, but first I had to complete my PhD in 'failed contraceptives'. During my interview for the lecturer's job, it was stated and required that I should spend 2 years in USA to acquire new scientific skills and return to invest the new knowledge back in Leeds University following my BTA (Been to America).

Prof. Barrett and the administration were, however, challenged to find an examiner for my PhD on 'failed contraceptives'. All approaches were declined – nobody cared as this was a topic considered of no significance. Prof. Barrett turned to his former colleague at ICI Pharmaceuticals Division, Dr Arthur Walpole, head of the Fertility Control Program to be my external examiner.

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The university administration was initially resistant to having 'someone from industry' as an examiner; but, fortunately for me and, perhaps, the future of tamoxifen, the administration finally agreed. Indirectly, the door had opened for the development of ICI 46 464, the failed contraceptive to evolve into the 'gold standard' tamoxifen for the adjuvant treatment of breast cancer.

Dr Mike Harper, the reproductive endocrinologist at ICI Pharmaceuticals Division who had completed all of the biology of ICI 46 474, was Mike Barrett's friend but was now heading a research program at the Worcester Foundation for Experimental Biology in Massachusetts. I remember my transatlantic telephone call with Mike Harper. 'Can you come in September, will $12 000 a year be OK, and will you work on prostaglandins?' 'Yes, yes, yes' I replied and went off to the library to find out what prostaglandins were! My examination with Arthur Walpole went well, but I had not anticipated that our lives would be intertwined for the remaining years of his life. I now found myself off to USA for 2 years as a Visiting Scientist (1972–1974).

I arrived at the Worcester Foundation, the home of the oral contraceptive, with the invitation and plan to work with Mike Harper on a 'once-a-month contraceptive'. However, when I arrived I found he had accepted a job as Head of Reproduction at the World Health Organization in Geneva, Switzerland. I was told I could do any research I liked for the next 2 years as long as some of it involved prostaglandins. I was confronted with a daunting task as a brand-new PhD graduate – start my own laboratory as an independent investigator, find my own funds, and hire and train a technician. Her name was Susan Koerner and she was spectacular. She was included as an author on my early papers.

I had always wanted to be involved in the discovery and development of drugs to treat cancer, so perhaps here was my opportunity. I was a pharmacologist, but do what you know and all I knew about was triphenylethylenes and the ER so a phone call to Arthur Walpole gained his support to aid in turning ICI 46 474 into a prospective breast cancer drug. What I did not know at that time was that ICI Pharmaceuticals Divisions had reviewed all the clinical data on ICI 46 474 in March 1972, and the decision was made to stop development for clinical use as there was no financial reward to be accrued for the treatment of metastatic breast cancer or as another inducer of ovulation (Jordan 2006). Arthur Walpole had tendered his resignation and sought early retirement. He would, however, remain at Alderley Park if ICI 46 474 was advanced for approval for clinical use as an orphan drug for the treatment of metastatic breast cancer and the induction of ovulation. 'Sliding Doors' occured for me in September 1972 with Mike Harper going to Geneva and me calling Arthur Walpole. Walpole supported me to receive an unrestricted research grant from ICI Americas and introduced me to the lady who became my lifelong friend – Lois Trench. She was the new drug monitor in charge of developing ICI 46 474 in the USA and she succeeded. She recruited me as the scientific consultant for ICI Americas to advocate tamoxifen to clinical trials groups (ECOG and the NSABP) for clinical testing. I returned to Leeds University in September 1974 as a lecturer in pharmacology with much work to accomplish. I had omitted to publish my work and had to catch up. Remember: if you do not publish, it never happened and you cannot claim the credit (only in your mind!).

Investment in young investigators

In 1974, Dr Roy Cotton was the clinician in charge of the development of Nolvadex (ICI 46 474, tamoxifen) for ICI Pharmaceuticals Division. He was my contact person with an agenda to devise a way for the Clinical Department to support my work at Leeds. He was inspirational and through his innovation advanced tamoxifen to become a 'pioneering medicine'. He devised a way for 'flexible support' that had minimal cost for ICI Pharmaceuticals Division or his clinical budget, but was to create a foundation for a blockbuster medicine for women's health. Roy Cotton provided hundreds of rats from Alderley Park stocks in Cheshire for my work at Leeds University. He arranged for continuous supplies of rats to be chauffeured to Leeds Medical School every week between 1975 and 1978 to complete dozens of experiments on the mechanism of action of tamoxifen, metabolism, the strategy to deploy tamoxifen as the first chemopreventive, and as the first targeted long-term antiestrogenic adjuvant therapy. The paper entitled 'Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy' (Jordan 1978) was the first to propose publically that 'longer was better than shorter adjuvant therapy' published in the *Reviews of Endocrine-Related Cancer*. The Yorkshire Cancer Research Campaign also provided essential support to this young investigator, without which we could not have supported our staff and students and bought essential equipment that demonstrated tamoxifen bound to the ER (Jordan & Prestwich 1977). Strange as this seems today, the ER was an unpopular and unproven mechanism of tamoxifen action for the clinical

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community in the UK and for the next 10 years, the ER assay we use today was not accepted in the 1970s–1980s in the UK. The good news was that instead of doing an ER assay, every breast cancer patient received tamoxifen anyway, and as a result untold numbers of lives were saved with tamoxifen from the beginning.

Conversation with Nature

In 1975, Marc Lippman published (Lippman & Bolan 1975) that tamoxifen was a competitive inhibitor of estrogen-stimulated growth of MCF7 breast cancer cells. Lois Trench in USA had provided me with a selection of frozen breast cancers to measure ER, and in 1975 we showed that tamoxifen blocks estradiol binding to the human tumor ER (Jordan & Koerner 1975). Now back at Leeds, I was refining another publication, started at the Worcester Foundation (Jordan 1974) that tamoxifen prevented rat mammary carcinogenesis (Jordan 1976*b*). At that time, chemoprevention of breast cancer was a 'forlorn hope'. Indeed, Michael Sporn had only just invented the new word (Sporn *et al*. 1976). I decided instead to turn to the issue of adjuvant therapy with tamoxifen. Marc Lippman stated in a line of his paper (Lippman & Bolan 1975) that high doses of tamoxifen were tumoricidal for MCF7 cells, so we decided to put it to the test *in vivo*.

When I was at the Worcester Foundation, I spent a day (and dinner) with the late Elwood Jensen, the then Director of the Ben May Laboratory for Cancer Research in Chicago, when he visited the Foundation in September 1972. He was a new member of the Scientific Advisory Board for the Foundation, appointed by Mahlon Hoagland, the new Director in 1970. I accepted Elwood's offer to go to Chicago in the summer of 1973 to learn ER assay techniques and the DMBA-induced rat mammary carcinogen model. Both techniques were essential for the job to be completed, to find new and novel clinical strategies for tamoxifen.

Back at Leeds some 3 years later, I devised a model that, in my naïve view, would replicate adjuvant therapy with tamoxifen despite the fact that it was not a real model of human disease. There was no real model, so there was no choice but to use what was available. My reasoning was as follows. If DMBA was administered to 50-day-old Sprague–Dawley rats, then all animals would develop tumors within 150 days. I planned two strategies initially: give the DMBA at 50 days of age and then treat daily with increasing doses of tamoxifen starting 30 days after DMBA but only for 1 month. A month in a rat's life is about a year for a human: what was proposed for current adjuvant trials with tamoxifen (Hubay *et al*. 1980, Ribeiro & Palmer 1983, Ludwig Breast Cancer Study Group 1984, Cummings *et al*. 1985, Ribeiro & Swindell 1985, Rose *et al*. 1985). The results show that there was a delay in tumorigenesis but then tumors appeared later with at least one tumor per rat (Jordan & Allen 1980, Jordan 1983). However, there was a clue as the higher the daily dose, the larger the delay in tumorigenesis. As it was known that tamoxifen had a long biological half-life (Fromson *et al*. 1973*a*,*b*), then I reasoned that tumorigenesis proceeded only after the drug was cleared following short-term treatment. We tried another approach, earlier or later after DMBA – earlier was better to prevent tumorigenesis (Jordan *et al*. 1979). So if the drug needs to be there to prevent the microfoci of deranged rat mammary epithelial cells from growing into tumors, then is long-term tamoxifen treatment superior to short-term therapy? The results showed that indefinite tamoxifen vs shorter tamoxifen are shown in Fig. 1 (Jordan 1978, Jordan *et al*. 1979, Jordan 1983). We had asked the question of what is the best way to give 'adjuvant tamoxifen' in the DMBA model and we did not get back the answer we expected but it was a consistent answer. No drug, no antiestrogen action – long-term therapy was the way to go. Conversion of the rat model to clinical practice: 5 or more years of adjuvant tamoxifen would be a superior adjuvant strategy than the planned 1-year of treatment.

Neither did we get the answer we anticipated when we tested the potent metabolite of tamoxifen 4-hydroxy-tamoxifen (Jordan *et al*. 1977) in the same model against tamoxifen (Jordan & Allen 1980). We had initially discovered that tamoxifen could be metabolically activated by 4-hydroxytamoxifen in our collaboration with ICI Pharmaceuticals Division, but I agreed to a delay in my publications for a year (Jordan *et al*. 1977) while ICI Pharmaceuticals Division sought to patent the metabolites. It was anticipated that there was little likelihood of successful development of tamoxifen to a financially rewarding product so there had been no need to follow protocol, waste time and money, to patent the metabolites. I was told years later, that the clinical staff at the beginning of the 1970s was told not to spend too much time on tamoxifen!

We tested the better antiestrogen, 4-hydroxy-tamoxifen, just in case we had found a better breast cancer drug. However, it turned out to be a less effective antitumor agent than tamoxifen in our model (Jordan & Allen 1980). The hydroxylated metabolite was cleared too quickly, simple pharmacology. Tamoxifen can be detected for up to 6 weeks after treatment stops. So it seems that tamoxifen maintained a supply of the active metabolite

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as the potent drug but the less potent parent acts as the depot that saturates a patient's body. Nevertheless, the metabolite experiments with 4-hydroxytamoxifen again showed that longer was better than shorter (Jordan & Allen 1980). Keep the drug there constantly: no drug present, no action. This was the principle that we advanced to the clinical community starting that day at King's College, Cambridge, in 1977.

A conversation between the laboratory and the clinic

My love for chemistry was always focused on what organic chemistry can do to create medicines to defeat disease. That for me was the guiding principle first created by Prof. Paul Ehrlich at the dawn of the 20th century when he created the first chemical therapy (chemotherapy) to cure syphilis (Baumler 1984). I seized upon the principle with alacrity in my teens with the desire to find molecules to treat cancer. This was pharmacology and 'failed contraceptives' were both my 'Sliding Doors' and my opportunity. But unless you train yourself and learn to be ready to seize the opportunity, it will vanish as quickly as it appeared. It is a moment in time governed by factors that you cannot control but determination and discipline will aid your quest for success. In my case, the topic was definitely not fashionable so nobody cared or very few. The 'few' were happy amateurs who wanted to contribute to human health when the majority considered 'another hormone therapy' a waste of time and resources. In my case, it was said I had poor career judgment because more than once the topic would crop up that if tamoxifen failed, then I would have nothing. It is true that tamoxifen would most certainly fail today as tamoxifen was unexpectedly proven to cause liver cancer in rats in the early 1990s (Greaves *et al*. 1993). This was some 20 years after clinical use started! Testing of the toxicology of an agent for cancer treatment is trivial but, for a medicine for healthy women (chemoprevention), the rules rightly change and major long-term toxicity testing occurs. No company today would develop tamoxifen knowing it caused cancer. But *Nature* gave the right answer if you were a rat (Greaves *et al*. 1993) and the right answer for women in the invaluable overview analyses that show no increase in liver cancer (Early Breast Cancer Trialists' Collaborative Group 1992, 2005).

Because pharmacology is about '*the enormous power to help others*', I chose to move my career into clinical cancer research through clinical cancer centers in the United States. The opportunities to learn and contribute to oncology at the University of Wisconsin Comprehensive Cancer Center are a tribute to Paul Carbone, Doug Tormey, and David Rose each making my recruitment happen. I chose to train myself. Actually, it was Lois Trench who initiated all of the process back in 1977 and funded studies through ICI Americas for me to travel to Madison for 3 months to see if I could be recruited. ICI Pharmaceutical Division also deserves the credit for encouraging my career development into clinical research. They provided a decade of support to my laboratory (1973–1983), to pay staff, students' scholarships (Clive Dix rose rapidly to be Research Director for Glaxo, and Anna Tate Riegel is an endowed Prof. in Oncology at Georgetown), laboratory supplies, 'free rats', and most importantly Arthur Walpole did not take early retirement but remained at ICI Pharmaceuticals Division as my link for my University of Leeds/ICI Pharmaceuticals Division Joint Research Scheme until his untimely death on 20th July 1977. He never saw the success of tamoxifen; but, our connection made the possibility of success a certainty (this is, however, only the wisdom of hindsight!).

At the King College meeting around this time, I met Prof. Michael Baum (Fig. 2) who was now to chair my session and introduce me. In the discussion of my paper, he mentioned that he had arbitrarily planned to use 2 years of adjuvant tamoxifen, thereby advancing ahead of the numerous 1-year trials (Hubay *et al*. 1980, Ribeiro & Palmer 1983, Ludwig Breast Cancer Study Group 1984, Cummings *et al*. 1985, Ribeiro & Swindell 1985, Rose *et al*. 1985). Bernie Fisher in USA planned to do the same and advance to 2 years following the NSABP symposium in Key Biscayne Florida organized by Lois Trench in 1976. I gave the pharmacology of tamoxifen talk (Jordan 1976*a*), but I promised ICI Pharmaceuticals Division that I would not speak about 'metabolites'! Tamoxifen, as I mentioned earlier, was not to be FDA approved until December 1977 in USA, so that step was a priority for the company and I strongly believed this was also a priority for women's health with breast cancer.

Michael Baum and John Patterson, now the clinician responsible for tamoxifen, taking over from Roy Cotton, worked to come up with an imaginative acronym for this group's adjuvant 2-year trial to be sponsored by ICI Pharmaceuticals Division. It was called the NATO group to make American clinicians think it was an American trial and read the results. The acronym stands for 'Nolvadex Adjuvant Trial Organization' and the NATO group has the distinction of being the first to detect a survival advantage for patients taking adjuvant tamoxifen (Baum *et al*. 1983, Nolvadex Adjuvant Trial Organisation 1983).

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Helen Stewart (Fig. 2) was in the audience at King's College in 1977. As it turned out, she would be running what was to be known as the Scottish trial led by Sir Patrick Forest and sponsored by the Medical Research Council (the same group who sponsored my PhD at Leeds University 'failed contraceptives'; I will forever be grateful as their investment really paid off!). The Scottish trialists were in the process of deciding whether patients could tolerate 5 years of tamoxifen. If so, their trial was then to start accruing patients to be randomized to 5 years of adjuvant tamoxifen or placebo and tamoxifen at first recurrence. Their results were published on 25th July 1987 (Scottish Cancer Trials Office (MRC) 1987) (coincidentally my birthday!) with significant survival advantages for early tamoxifen vs later use of tamoxifen upon recurrence. The animal studies therefore were '*a means of predicting the future*' when presented at King's College a decade earlier. For me, the '*power to help others*' was important as I subsequently traveled to speak at literally hundreds of clinical meetings worldwide. The clinical colleagues who became lifelong friends are too numerous to list but those close friends and colleagues in breast cancer research, Bill McGuire, Monica Morrow, and Gabriel Hortobagyi, deserve special recognition here for the part each was to play in my life.

By the mid 1980s, clinical trials slowly started to demonstrate some benefits of tamoxifen but, in the main, the trials were too small to declare 'breakthrough' as 'hormone therapy' was not curing everyone – chemotherapy would do that. Well perhaps but now enter the meta-analysis.

The Oxford Overview Analysis

Dr Craig Henderson tells the story of the first overview analysis (Henderson 1999). The overview was conducted by Sir Richard Peto, Sir Rory Collins, Richard Gray, and the team from the Clinical Trials Unit, Oxford University, in 1984. There were two main camps of randomized trials: the Europeans were cautious about the toxicity of cytotoxic chemotherapy and the American skeptical that a palliative 'hormone therapy' could aid survival. The results presented in a hotel at the Heathrow Airport in the mid-1980s showed that chemotherapy or tamoxifen improved disease-free survival and overall survival to about the same extent but in premenopausal and postmenopausal patients respectively. Since then, analyses have occurred in 1990 and 1995 and at regular intervals thereafter to this day. The value of seeing an analysis of all the data permitted the prevention trials with tamoxifen to advance as inhibition of contralateral breast cancer in adjuvant tamoxifen trials was consistently at 50% and safety with endometrial cancer in postmenopausal women was much less significant than feared. Also, the concern about tamoxifen-induced rat hepatocarcinogenesis was not translated to human treatment trials. The trends observed with 1, 2, and 5 years of adjuvant tamoxifen predicted 'even more' was going to be better. There would have been no ATLAS trial or a focus on unanticipated outcomes without the overview analysis. *Nature* was also to tell us something unanticipated about decreasing mortality with tamoxifen. If tamoxifen is classified as a nonsteroidal antiestrogen that blocks estrogen-stimulated growth of micrometastases as a cytostatic agent, then why does stopping tamoxifen at 5 years not cause recurrence? No drug, no effect. Instead it causes a continuing decrease in mortality after stopping the antiestrogen. We know that stopping tamoxifen too soon, i.e. at 1 or 2 years, regrettably reduces the numbers of lives saved. But why?

The legacy of long-term adjuvant tamoxifen

The full story of tamoxifen has recently been told (Maximov *et al*. 2013). Through study of the pharmacology of tamoxifen, its metabolites, and its ubiquitous use for the treatment and prevention of breast cancer, several other significant advances in therapeutics and women's health have occurred.

The introduction of long-term adjuvant tamoxifen therapy mandated an examination of the development of acquired resistance to tamoxifen in the laboratory. At the time, in the mid 1980s, there were some cell culture studies of resistance, but the finding that opened the door to understand the evolution of acquired resistance to tamoxifen treatment was the transplantable model of acquired resistance in athymic mice (Gottardis & Jordan 1988, Gottardis *et al*. 1989). These studies also lead to the discovery that tamoxifen could control the growth of breast cancer but causes the growth of pre-existing endometrial cancer (Gottardis *et al*. 1988). Different tissues responded to tamoxifen in different ways: in the breast it was an antiestrogen; but in the bones, endometrium, and the regulation of circulating cholesterol, estrogenic actions were predominant (Lerner & Jordan 1990, Jordan 2001). These observations gave the medicine selective ER modulators (SERMs). There were no SERMs in 1990, only tamoxifen was classified as a nonsteroidal antiestrogen to treat breast cancer (Jordan 1984). Today, there are numerous SERMs (tamoxifen, raloxifene, bazedoxifene,

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toremifene, ospemifene, and lasofoxifene) for a wide variety of indications. That story has recently been told (Jordan 2013).

An understanding of the evolution of acquired resistance to tamoxifen (Yao *et al.* 2000, Jordan 2004, 2008) also led to the discovery of the new biology of estrogen-induced apoptosis that not only has clinical applications to treat antihormone-resistant breast cancer (Ellis *et al.* 2009) and explain how estrogen replacement therapy can reduce the incidence of breast cancer in long-term estrogen-deprived (>10 years after menopause) women (Anderson *et al.* 2012), but also can explain the reason why tamoxifen therapy for >5 years can dramatically reduce mortality after stopping therapy. The woman's own estrogen may destroy selected and vulnerable clonal micrometastases (Wolf & Jordan 1993).

The idea that longer therapy with adjuvant tamoxifen in patients with ER-positive breast cancer was not fashionable at the start. This is the way it is with most new concepts in any discipline. The clinical strategies of using 1 year of adjuvant tamoxifen (Hubay *et al.* 1980, Ribeiro & Palmer 1983, Ludwig Breast Cancer Study Group 1984, Cummings *et al.* 1985, Ribeiro & Swindell 1985, Rose *et al.* 1985) were clinically sound in the late 1970s because clinical experience using tamoxifen to treat metastatic breast cancer showed that treatment was successful in a minority of unselected cases for <2 years. Suggesting a treatment strategy for indefinite adjuvant tamoxifen treatment was destined to fail at 2 years – but it did not. I believe that the reason lies in the fact that metastatic disease is too established and can readily subvert the stress caused by preventing estrogen-stimulated growth. It is also a matter of bulk and vascularization that aid the survival of breast cancer cells in metastatic disease. But micrometastatic disease is apparently indolent and not well established but survives through slow and deliberate microscopic steps to select cells with acquired resistance that evolves very slowly through phases of resistance to reach unstable and vulnerable clonal populations over 5 years of treatment. It takes this long in the laboratory (Yao *et al.* 2000) and physiological estrogen will now cause rapid tumor regression (Wolf & Jordan 1993, Yao *et al.* 2000). But what if estrogen from the patient now triggers estrogen-induced apoptosis in the adjuvant tamoxifen trial of 5 years or more (Early Breast Cancer Trialists' Collaborative Group 1998, Jordan 2008, Davies *et al.* 2013)?

Is there direct evidence that the new biology of estrogen that causes apoptosis gives us profound mortality decreases after tamoxifen is stopped? Yes, I believe so. We know (Anderson *et al.* 2012) from the Women's Health Initiative estrogen-only trial that there is a profound decrease in the incidence of breast cancer and mortality for women treated with estrogen in their 1960s when compared with placebo. Estrogen kills estrogen-deprived occult cancer cells more than a decade after menopause (Obiorah & Jordan 2013). None of this science would have been revealed but for the fact that long-term adjuvant tamoxifen advanced from a laboratory concept in the late 1970s (Jordan 1978, Jordan *et al.* 1979), through clinical trials, to be enhanced as a reality by the Oxford Overview Analyses (Davies *et al.* 2011). Today, we have a successful clinical strategy with the results of ATLAS (Davies *et al.* 2013) and aTTom (The aTTom Collaborative Group 2013). Further lives are saved with a cheap effective medicine that never went away. The science of long-term adjuvant tamoxifen was indeed '*an adventure, discovery, new horizons, insights into our world, a means of predicting the future, and enormous power to help others*' (Hoagland 1990).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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References

Anderson GL, Chlebowski RT, Aragaki AK, Kuller LH, Manson JE, Gass M, Bluhm E, Connelly S, Hubbell FA, Lane D *et al.* 2012 Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *Lancet Oncology* **13** 476–486. (doi:10.1016/S1470-2045(12)70075-X)

Avigan J, Steinberg D, Vroman HE, Thompson MJ & Mosettig E 1960 Studies of cholesterol biosynthesis. I. The identification of desmosterol in serum and tissues of animals and man treated with MER-29. *Journal of Biological Chemistry* **235** 3123–3126.

Baum M, Brinkley DM, Dossett JA, McPherson K, Patterson JS, Rubens RD, Smiddy FG, Stoll BA, Wilson A, Lea JC *et al.* 1983 Improved survival

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among patients treated with adjuvant tamoxifen after mastectomy for early breast cancer. *Lancet* **2** 450. (doi:10.1016/S0140-6736(83)90406-3)

Baumler E 1984 In *Paul Ehrlich: Scientist for Life*. New York, NY, USA: Holmes & Meier Pub.

Berry DA, Cronin KA, Plevritis SK, Fryback DG, Clarke L, Zelen M, Mandelblatt JS, Yakovlev AY, Habbema JD & Feuer EJ 2005 Effect of screening and adjuvant therapy on mortality from breast cancer. *New England Journal of Medicine* **353** 1784–1792. (doi:10.1056/NEJMoa050518)

Bonadonna G, Brusamolino E, Valagussa P, Rossi A, Brugnatelli L, Brambilla C, De Lena M, Tancini G, Bajetta E, Musumeci R *et al.* 1976 Combination chemotherapy as an adjuvant treatment in operable breast cancer. *New England Journal of Medicine* **294** 405–410. (doi:10.1056/NEJM197602192940801)

Cole MP, Jones CT & Todd ID 1971 A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. *British Journal of Cancer* **25** 270–275. (doi:10.1038/bjc.1971.33)

Cummings FJ, Gray R, Davis TE, Tormey DC, Harris JE, Falkson G & Arseneau J 1985 Adjuvant tamoxifen treatment of elderly women with stage II breast cancer. A double-blind comparison with placebo. *Annals of Internal Medicine* **103** 324–329. (doi:10.7326/0003-4819-103-3-324)

Davies C, Godwin J, Gray R, Clarke M, Cutter D, Darby S, McGale P, Pan HC, Taylor C, Wang YC *et al.* 2011 Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* **378** 771–784. (doi:10.1016/S0140-6736(11)60993-8)

Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V, Abraham M, Alencar VH, Badran A, Bonfill X *et al.* 2013 Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. *Lancet* **381** 805–816. (doi:10.1016/S0140-6736(12)61963-1)

Early Breast Cancer Trialists' Collaborative Group 1992 Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. *Lancet* **339** 71–85.

Early Breast Cancer Trialists' Collaborative Group 1998 Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* **351** 1451–1467. (doi:10.1016/S0140-6736(97)11423-4)

Early Breast Cancer Trialists' Collaborative Group 2005 Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* **365** 1687–1717. (doi:10.1016/S0140-6736(05)66544-0)

Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A *et al.* 2009 Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *Journal of the American Medical Association* **302** 774–780. (doi:10.1001/jama.2009.1204)

Falkson HC, Gray R, Wolberg WH, Gillchrist KW, Harris JE, Tormey DC & Falkson G 1990 Adjuvant trial of 12 cycles of CMFPT followed by observation or continuous tamoxifen versus four cycles of CMFPT in postmenopausal women with breast cancer: an Eastern Cooperative Oncology Group phase III study. *Journal of Clinical Oncology* **8** 599–607.

Fisher B, Carbone P, Economou SG, Frelick R, Glass A, Lerner H, Redmond C, Zelen M, Band P, Katrych DL *et al.* 1975 L-phenylalanine mustard (L-PAM) in the management of primary breast cancer. A report of early findings. *New England Journal of Medicine* **292** 117–122. (doi:10.1056/NEJM197501162920301)

Fromson JM, Pearson S & Bramah S 1973*a* The metabolism of tamoxifen (I.C.I. 46,474). I. In laboratory animals. *Xenobiotica* **3** 693–709. (doi:10.3109/00498257309151594)

Fromson JM, Pearson S & Bramah S 1973*b* The metabolism of tamoxifen (I.C.I. 46,474). II. In female patients. *Xenobiotica* **3** 711–714. (doi:10.3109/00498257309151595)

Gottardis MM & Jordan VC 1988 Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Research* **48** 5183–5187.

Gottardis MM, Robinson SP, Satyaswaroop PG & Jordan VC 1988 Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Research* **48** 812–815.

Gottardis MM, Wagner RJ, Borden EC & Jordan VC 1989 Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth *in vivo* and *in vitro*. *Cancer Research* **49** 4765–4769.

Greaves P, Goonetilleke R, Nunn G, Topham J & Orton T 1993 Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. *Cancer Research* **53** 3919–3924.

Greenblatt RB, Barfield WE, Jungck EC & Ray AW 1961 Induction of ovulation with MRL/41. Preliminary report. *Journal of the American Medical Association* **178** 101–104. (doi:10.1001/jama.1961.03040410001001)

Harper MJ & Walpole AL 1967 A new derivative of triphenylethylene: effect on implantation and mode of action in rats. *Journal of Reproduction and Fertility* **13** 101–119. (doi:10.1530/jrf.0.0130101)

Henderson IC 1999 Early overviews of adjuvant tamoxifen therapy. In *Tamoxifen for the Treatment and Prevention of Breast Cancer*, pp 57–77. Ed VC Jordan. Melville, New York, NY, USA: PRR.

Hoagland M 1990 *Towards the habbit of truth The Commonwealth Fund Book Program*. New York, NY, USA: W. W. Norton & Company.

Hubay CA, Pearson OH, Marshall JS, Rhodes RS, DeBanne SM, Rosenblatt J, Mansour EG, Hermann RE, Jones JC, Flynn WJ *et al.* 1980 Adjuvant chemotherapy, antiestrogen therapy and immunotherapy for stage II breast cancer: 45-month follow-up of a prospective, randomized clinical trial. *Cancer* **46** 2805–2808. (doi:10.1002/1097-0142(19801215)46:12+<2805::AID-CNCR2820461413>3.0.CO;2-H)

Ingle JN, Ahmann DL, Green SJ, Edmonson JH, Bisel HF, Kvols LK, Nichols WC, Creagan ET, Hahn RG, Rubin J *et al.* 1981 Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *New England Journal of Medicine* **304** 16–21. (doi:10.1056/NEJM198101013040104)

Jordan VC 1974 Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethyl benzanthracene (DMBA)-induced rat mammary carcinoma model. *Journal of Steroid Biochemistry* **5** 354. (doi:10.1016/0022-4731(74)90388-4)

Jordan VC 1976*a* Antiestrogenic and antitumor properties of tamoxifen in laboratory animals. *Cancer Treatment Reports* **60** 1409–1419.

Jordan VC 1976*b* Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. *European Journal of Cancer* **12** 419–424. (doi:10.1016/0014-2964(76)90030-X)

Jordan VC 1978 Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. *Reviews on Endocrine-Related Cancer* (October supplement) 49–55.

Jordan VC 1983 Laboratory studies to develop general principles for the adjuvant treatment of breast cancer with antiestrogens: problems and potential for future clinical applications. *Breast Cancer Research and Treatment* **3** S73–S86. (doi:10.1007/BF01855131)

Jordan VC 1984 Biochemical pharmacology of antiestrogen action. *Pharmacological Reviews* **36** 245–276.

Jordan VC 1988 The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. *Breast Cancer Research and Treatment* **11** 197–209. (doi:10.1007/BF01807278)

Jordan VC 2001 Selective estrogen receptor modulation: a personal perspective. *Cancer Research* **61** 5683–5687.

Jordan VC 2003 Tamoxifen: a most unlikely pioneering medicine. *Nature Reviews. Drug Discovery* **2** 205–213. (doi:10.1038/nrd1031)

Jordan VC 2004 Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* **5** 207–213. (doi:10.1016/S1535-6108(04)00059-5)

Jordan VC 2006 Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. *British Journal of Pharmacology* **147** (Suppl 1) S269–S276.

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Jordan VC 2008 The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer–survival or death? *Journal of Clinical Oncology* **26** 3073–3082. (doi:10.1200/JCO.2008.17.5190)

Jordan VC 2013 *Estrogen action, selective estrogen receptor modulators and women's health: progress and promise*. London, UK: Imperial College Press.

Jordan VC & Allen KE 1980 Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *European Journal of Cancer* **16** 239–251. (doi:10.1016/0014-2964(80)90156-5)

Jordan VC & Koerner S 1975 Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *European Journal of Cancer* **11** 205–206. (doi:10.1016/0014-2964(75)90119-X)

Jordan VC & Prestwich G 1977 Binding of [3H]tamoxifen in rat uterine cytosols: a comparison of swinging bucket and vertical tube rotor sucrose density gradient analysis. *Molecular and Cellular Endocrinology* **8** 179–188. (doi:10.1016/0303-7207(77)90090-9)

Jordan VC, Collins MM, Rowsby L & Prestwich G 1977 A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *Journal of Endocrinology* **75** 305–316. (doi:10.1677/joe.0.0750305)

Jordan VC, Dix CJ & Allen KE 1979 The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In *Adjuvant Therapy II*, pp 19–26. Eds SE Salmon & SE Jones. New York, NY, USA: Grune & Stratton.

Jordan VC, Zava DT, Eppenburger U, Kiser A, Sebek S, Dowdle E, Krozowski Z, Bennett RC, Funder J, Holdaway IM *et al.* 1983 Reliability of steroid hormone receptor assays: an international study. *European Journal of Cancer & Clinical Oncology* **19** 357–363. (doi:10.1016/0277-5379(83)90133-5)

Klopper A & Hall M 1971 New synthetic agent for the induction of ovulation: preliminary trials in women. *BMJ* **1** 152–154. (doi:10.1136/bmj.1.5741.152)

Laughlin RC & Carey TF 1962 Cataracts in patients treated with triparanol. *Journal of the American Medical Association* **181** 339–340. (doi:10.1001/jama.1962.03050300059020a)

Lerner LJ & Jordan VC 1990 Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. *Cancer Research* **50** 4177–4189.

Lerner LJ, Holthaus FJ Jr & Thompson CR 1958 A non-steroidal estrogen antiagonist 1-(*p*-2-diethylaminoethoxyphenyl)-1-phenyl-2-*p*-methoxyphenyl ethanol. *Endocrinology* **63** 295–318. (doi:10.1210/endo-63-3-295)

Lippman ME & Bolan G 1975 Oestrogen-responsive human breast cancer in long term tissue culture. *Nature* **256** 592–593. (doi:10.1038/256592a0)

Ludwig Breast Cancer Study Group 1984 Randomised trial of chemo-endocrine therapy, endocrine therapy, and mastectomy alone in postmenopausal patients with operable breast cancer and axillary node metastasis. *Lancet* **1** 1256–1260.

Maximov PY, McDaniel RE & Jordan VC 2013 *Tamoxifen: pioneering medicine in breast cancer Milestones in Drug Therapy*. Basel, Switzerland: Springer.

Morgan LR Jr, Schein PS, Woolley PV, Hoth D, Macdonald J, Lippman M, Posey LE & Beazley RW 1976 Therapeutic use of tamoxifen in advanced breast cancer: correlation with biochemical parameters. *Cancer Treatment Reports* **60** 1437–1443.

Nolvadex Adjuvant Trial Organisation 1983 Controlled trial of tamoxifen as adjuvant agent in management of early breast cancer. Interim analysis at four years. *Lancet* **1** 257–261.

Obiorah I & Jordan VC 2013 Scientific rationale for postmenopause delay in the use of conjugated equine estrogens among postmenopausal women that causes reduction in breast cancer incidence and mortality. *Menopause* **20** 372–382.

Peto R, Boreham J, Clarke M, Davies C & Beral V 2000 UK and USA breast cancer deaths down 25% in year at ages 20–69 years. *Lancet* **35** 1822. (doi:10.1016/S0140-6736(00)02277-7)

Poirot M 2011 Four decades of discovery in breast cancer research and treatment – an interview with V. Craig Jordan. *International Journal of Developmental Biology* **55** 703–712. (doi:10.1387/ijdb.113418mp)

Ribeiro G & Palmer MK 1983 Adjuvant tamoxifen for operable carcinoma of the breast: report of clinical trial by the Christie Hospital and Holt Radium Institute. *BMJ* **286** 827–830. (doi:10.1136/bmj.286.6368.827)

Ribeiro G & Swindell R 1985 The Christie Hospital tamoxifen (Nolvadex) adjuvant trial for operable breast carcinoma – 7-yr results. *European Journal of Cancer & Clinical Oncology* **21** 897–900. (doi:10.1016/0277-5379(85)90104-X)

Rose C, Thorpe SM, Andersen KW, Pedersen BV, Mouridsen HT, Blichert-Toft M & Rasmussen BB 1985 Beneficial effect of adjuvant tamoxifen therapy in primary breast cancer patients with high oestrogen receptor values. *Lancet* **1** 16–19. (doi:10.1016/S0140-6736(85)90966-3)

Scottish Cancer Trials Office (MRC) 1987 Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee, Edinburgh. *Lancet* **2** 171–175.

Segal SJ & Nelson WO 1958 An orally active compound with anti-fertility effects in rats. *Proceedings of the Society for Experimental Biology and Medicine* **98** 431–436. (doi:10.3181/00379727-98-24068)

Sporn MB, Dunlop NM, Newton DL & Smith JM 1976 Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). *Federation Proceedings* **35** 1332–1338.

The aTTom Collaborative Group 2013 aTTom: long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years in 6,953 women with early breast cancer. *Journal of Clinical Oncology* **31** (Supplement 5).

Toft D & Gorski J 1966 A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *PNAS* **55** 1574–1581. (doi:10.1073/pnas.55.6.1574)

Toft D, Shyamala G & Gorski J 1967 A receptor molecule for estrogens: studies using a cell-free system. *PNAS* **57** 1740–1743. (doi:10.1073/pnas.57.6.1740)

Tormey DC & Jordan VC 1984 Long-term tamoxifen adjuvant therapy in node-positive breast cancer: a metabolic and pilot clinical study. *Breast Cancer Research and Treatment* **4** 297–302. (doi:10.1007/BF01806042)

Tormey DC, Rasmussen P & Jordan VC 1987 Long-term adjuvant tamoxifen study: clinical update. *Breast Cancer Research and Treatment* **9** 157–158. (doi:10.1007/BF01807370)

Ward HW 1973 Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. *BMJ* **1** 13–14. (doi:10.1136/bmj.1.5844.13)

Wolf DM & Jordan VC 1993 A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results in Cancer Research* **127** 23–33.

Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM & Jordan VC 2000 Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clinical Cancer Research* **6** 2028–2036.

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'Who do you think you are?'

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I grew up in Bramhall, Cheshire, in the 1950s. In 1953, my family and neighbors all watched the coronation of Queen Elizabeth II on the small black and white TV in our living room. I was 6 years old. Little did I imagine that my mother and I would visit the Queen at Buckingham Palace 50 years later when I would be given my OBE for services to International Breast Cancer Research.

My father and his three brothers were all born in Bramhall but educated traditionally away from their parents – with prep school in North Wales and the Leys School, Cambridge. By contrast, my mother's family has ancient roots dating back a 1000 years in Mottram St Andrew near Prestbury, Cheshire. They are Mottrams and three are recorded to be present at the Battle of Poitiers in 1356. They were elite horse archers as part of the Black Prince's personal bodyguard. My maternal grandfather had been an officer in the Cheshire Regiment in two world wars and his house in Wilmslow was an 'Aladdin's cave' of uniforms, war trophies, and weapons displayed or secreted everywhere. This was my family who gave me a strong sense of right and wrong and stressed the value of service to others and the nation. Nothing in this early upbringing would predict any path to progress in breast cancer research. My interests were Roman history, a passion for chemistry, training, organizing, leading, and teaching other children in our neighborhood. I was not interested in schoolwork at Moseley Hall Grammar School for boys in Cheadle; but my chemistry teacher Mr Anderson captured my imagination as did Mr Charles Bescoby, my biology teacher. We will meet Mr Bescoby later.

My father had bought me a chemistry set and an air rifle when I was 10 years old. By the time I was 12 years old, my mother had allowed me to turn my bedroom into a chemistry laboratory. Experiments would often get out of hand, so a fuming brew would be hurled out of the window onto the lawn below leaving the curtains ablaze. Naturally the lawn died but on one occasion I convinced my mother that I could use chemistry to grow it back. Yes, I was successful but the grass regrew an interesting shade of blue! Now for the air rifle.

I discovered I could knock down all the lupins with my air rifle, fired from my bedroom window. I remember overhearing a conversation between my mother and father as they inspected the damaged flowers, 'Look' my mother exclaimed 'some large insect has taken a chunk out of each stem and it has fallen over'. I was an excellent shot, just like my grandfather who was the musketry training officer for the Cheshire Regiment.

I had no significant career objectives at that time, but at 15 years of age I am not sure anyone has. Perhaps join the Army or perhaps become a chemistry technician at the nearby ICI Pharmaceuticals Division, Alderley Park. But then failure enters your life. I only got three 'O' levels; five were required to stay in school and get into the sixth form, so I was about to be turned out to get a job in the real world. But failure at a young age is good I believe, in retrospect (but definitely not at the time) as it sets, in some, resolve. It was my mother's unshakable support that created the resolve.

During the summer in 1963, I secured a technician's job in organic synthesis at ICI, Alderley Park, but they suggested I should stay in school. My mother and I visited Mr Armishaw, the headmaster at Moseley Hall to plead my case. He did not have a chance! She promised I would comply with the law and would quickly get two further O levels and would take A-level Chemistry, Zoology, and O-level physics as 'he already had a job offer from ICI to be a technician'. I was in the sixth form and I started the Zoology Club. I also chose to teach other boys university-level biochemistry and Mr Bescoby gave me a laboratory. Unexpectedly, Mr Bescoby suggested to my parents I should apply for university. I was, however, making plans to do something else – I was the drummer in a rock band! Late one night, it was the final straw for my mother

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'Decide: Drummer or University'. I often wondered what happened to the other band members in the Hollies (or was it Freddie and the Dreamers?).

Mr Bescoby was now to teach me a lesson in commitment to the lives of others that I never forgot. I practice his philosophy every day. I was offered one interview at one University; the University of Leeds, Department of Pharmacology. In those days, everyone had a face-to-face interview, and I was greeted by Dr Ronnie Kaye and Dr Ted Clark of the Pharmacology Department. Dr Clark asked about a certain organic chemical reaction and I was off 'Very interesting as this is the topic of my biochemistry talk next week at the Zoology Club'. They could not shut me up. These two men were to 'give me my one chance' because of the letter my Headmaster (actually Mr Bescoby) had written supporting my application. Years later, Dr Kaye was to tell me that it stated 'Craig Jordan is an unusual young man (then repeated in capitals) a VERY UNUSUAL YOUNG MAN'. 'We had to see for ourselves what this meant' he continued. I stress in my mentoring that every 'chance' is the opportunity to win the gold medal in the Olympic finals (Fig. 1).

Back at Moseley Hall, Mr Bescoby chose to enter me for the scholarship (S-Level) zoology examinations. DNA was the hot topic since Watson and Crick's publications in the 1950s. Now they had won the Nobel Prize in 1963 so it was certain this would be an exam question. Mr Bescoby gave me a book on DNA and tutored me for an hour twice a week. I passed the S-level zoology examination and got the A-level grades necessary to go to Leeds. I won the school prize in zoology but bought a chemistry book.

Figure 1
The occasion before me being awarded the first honorary degree in medicine at Leeds University for humanitarian research 2001. I was delighted that Dr Ted Clark (l), my PhD supervisor, and Dr Ronnie Kaye could both be present at the degree ceremony. Both of these faculty members in the Pharmacology Department at Leeds University interviewed me in 1964 and subsequently offered me a place to go to the university.

Years later, I dedicated my first molecular biology paper to Mr Bescoby. We were the first to stably transfect the estrogen receptor (ER) into an ER-negative breast cancer cell (Jiang SY & Jordan VC 1992 Growth regulation of estrogen receptor negative breast cancer cells transfected with cDNA's for estrogen receptor. *Journal of the National Cancer Institute* **84** 580–591). A couple of years after publication, his daughter located me to tell me of her father's death, but the pride he had at my scientific success. Apparently, he used me as his example of an 'ugly duckling that turned into a swan' to subsequent generations of boys at Moseley Hall. Recently, Prof. Richard Vaughan-Jones at Oxford, another former pupil at Moseley Hall, told me this. Mr Bescoby's daughter enclosed his obituary and I discovered that he was the leader of the Manchester Branch of the Communist Party. In his case, he wanted to rebuild a better world after the Second World War, but never once did he choose to persuade me, or even discuss his political views. An honest man and dedicated teacher. He changed my life and perhaps built a future for women's health.

Dr Kaye, as head of Special Studies Pharmacy, would be my supporter and tutor at Leeds. I took the challenge of Leeds University very seriously. I was learning to survive academically in the spring of 1966, but I was concerned that the special studies Pharmacy course in the Pharmacology Department would not equip me for a career in cancer therapeutics. I thought a change should occur to better position me for a career in drug discovery.

Dr Kaye agreed to arrange a meeting with Dr Mogey, the admissions tutor in Pharmacology. We three met in Dr Mogey's office. He was a scary Scots Irishman who frowned at me over his half-moon glasses 'Well, I have reviewed your record, but I don't think your good enough to transfer.' After a pause, that seemed forever following this hammer blow, I rose to my feel and announced 'I will come top in the class of 120 in 3rd year Organic Chemistry, I will get a first in biochemistry and I will pass my mandatory advanced level physics course.' I turned and narrowly avoided smashing his glass door as I exited. I did as I said but now chose to decline the offer to transfer. However, I was wrong about Dr Mogey.

Years later, I discovered that Dr Mogey wrote one of my confidential letters of recommendation to be his colleague and faculty member in Pharmacology. The letter went something like this 'I have known Craig Jordan express strong emotional opinions in response to personal criticism. He has now learned to channel his energy for achieving success'. The following year (1966), I was to receive an Ackroyd Scholarship from Leeds University.

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It transpired that Dr Mogey had nominated and supported me for this honor. An honest gentleman. I later discovered that Prof. Sir George Porter, President of the Royal Institution was a chemistry student at Leeds. He stated how proud he was to be an Ackroyd Scholar as it gave him confidence to succeed. This is why today I support the Jordan Prize in Medicinal Chemistry at Leeds University and the Jordan Prize and Trophy for the best Cadet annually in Leeds University Officer Training Corps (LUOTC). I have supported both since 1996 but why the OTC? Remember grandfather?

Growing up in the early 1960s, I learned to respect the commitment and tradition of service exemplified by the British Army. But my grandfather would say 'have nothing to do with the Army. I know, I have been in two world wars' but then he would say 'but when war comes, as it surely will, you had better be ready'. Well there was a challenge! My first stop on going to Leeds University in October 1965 was the OTC to sign on. But all was not what it seemed in my life.

I was born in New Braunfels, Texas, on July 25, 1947. My mother, a fire service officer, had met and subsequently married an American officer from Dallas in 1944 and they went back to Texas after the war. They divorced in 1950 with my mother and I returning to Cheshire. I never met or had any first-hand knowledge of my 'DNA Dad'. My stepfather had adopted me as his legal son, so I became a British citizen. In October 1965, I joined the LUOTC and started to learn to be an officer. But in spring 1966, just in the same week that Dr Mogey interviewed me for transfer to Pharmacology, the officer commanding the OTC called me to his office and stated 'You were born in America, so you cannot be an officer in the Army. I am sorry but there it is'. A terrible week for me at Leeds, but you fold or fight. It is a test of resolve. I thought about the roadblock in my future plans for 6 months and then acted. I secretly visited our family solicitor who eagerly agreed to challenge the Ministry of Defense *pro bono* by getting a ruling from the Home Office. The Home Office letter declared that the Ministry of Defense could not deny me access to a commission. My commission occurred following my interview with the commissioning board in the summer of 1969. I had a first-class honors degree and the last Medical Research Council (MRC) scholarship to support me crystallizing the ER with an estrogen or an antiestrogen to discover how 'failed contraceptives' worked. However, as I sat waiting outside for my name to be called, I overheard someone on the board state 'Well, I suppose we better interview him anyway!'. That did not sound too positive. It was time to prepare for the Olympic final against the board! After introductions, it was clear I was sinking fast. Then came the question 'Why do you want to be commissioned as an officer in the British Army'. Without hesitation I replied 'I have a first class honors degree and a MRC scholarship to do a doctorate. Should I be worthy of a commission I request to be sent on Regular Army courses so I can use my knowledge to the full'. I was awarded a commission in the infantry and was immediately sent to the Regular Army operations training course in Nuclear, Biological, and Chemical (NBC) Warfare Defense; today's 'weapons of mass destruction'. This was just as I was learning to ovariectomize mice with my new PhD supervisor Dr Ted Clark, the man who had interviewed me to be admitted to Leeds back in 1964. He was not thrilled!

This initiative, in 1970, started a chain of events over the next 30 years. High points were: talent spotted for recruitment into the Intelligence Corps, on the Staff of the Deputy Chief Scientist (Army), attached to US Army, training as a narcotics officer in America, Sandhurst, Foreign Armies study, talent spotted by the SAS, Regular Army Reserve Officer, SAS, a personal recommendation by General Sir Michael Rose (of Iranian Embassy Siege fame 1980) to join the SAS Regimental Association, and finally, a presentation from General Sir Richard Dannatt, Chief of the General Staff in recognition of my commitment to the training of future officers. At the start of this odyssey to enter my alternate universe, following top secret security clearance by MI5, I recall thinking 'here I am guarding the Holy Grail, when 4 years ago I was declared an *enemy of the state*'. But back to the real world?

I started my love affair with 'triphenylethylenes' in 1969. The story of how a 'failed contraceptive' ICI 46 474 was reinvented as tamoxifen is told in the accompanying article (Tamoxifen as the First Targeted Long-Term Adjuvant Therapy for Breast Cancer). The critical interdependent role of the individuals involved (Mike Baratt, Arthur Walpole, Mike Harper, Dora Richardson, Roy Cotton, Lois Trench) is important as each was committed to the success of tamoxifen and actively support my career development. But one individual, Dr Eliahu Caspi at the Worcester Foundation, gave me the best advice. In 1974, at the end of my Been to America (BTA) experience, when I was about to return to Leeds, I was called into Dr Caspi's office. His task was to evaluate my CV and decide whether to offer me a job. He stated abruptly 'But you don't have any publications. You are collaborating with lots of people but you have nothing on paper to evaluate!' After a stunned silence during my replay of my 'Mogey moment' in 1966, I replied 'but I haven't discovered anything yet'.

Endocrine-Related Cancer

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His advice was perfect: 'Tell them the story so far. Write papers that are connected so you become known for a theme and body of work'. My 2-year BTA subsequently resulted in more than a dozen publications and a career was begun. Years later, I was invited to be the inaugural Eliahu Caspi Memorial lecturer at the Worcester Foundation. His accomplished family told me of their father, a Polish Jew, being captured and held in a Russian Prisoner of War camp (the Germans invaded from the West, the Russians from the east). He survived and escaped to the Middle East where he witnessed the birth of Israel as a member of the Hagana (early Israeli Defense Force) and then found his way to America. Following a PhD in Chemistry at Clark University, Worcester, he spent a distinguished career in steroid metabolism at the Worcester Foundation in Shrewsbury.

I found my way back to America in 1980 and the circle of my life started to close as the career opportunities opened. My father and grandfather both died in 1972 and my mother encouraged me to advance my career wherever that would be. By 1980, I accepted a job with Paul Carbone to go to the Wisconsin Clinical Cancer Centre. My mother decided to find my DNA relative 'Luther Trammel and his wife Lorraine in Houston who had helped us in 1950 following my mother's divorce from my 'DNA Dad'. She wrote to the library in Houston for addresses and candidates were offered. Luther and Lorraine replied. Luther was the son of one of the Hamilton sisters and I was the grandson of the other, through my 'DNA Dad'. In 1983, I drove my family to Houston to be greeted with the cry 'He has the Hamilton teeth'. Genetics in action!

At breakfast on the first morning in Houston, I was presented with a silver christening cup by Lorraine with the words 'I always knew you would sit around this table one day. I give this back to its rightful owner as it was given to me by your mother in 1950 as she left. It was the only thing she had of value and she wanted us to remember you'. This was now 33 years later! The cup is engraved Virgil Craig and was sent by my grandfather to commemorate my birth. Then Lorraine handed me an envelope containing short stories. To me short stories meant children's stories so I did not give this much thought until years later. This story unfolds after hearing tales about 'the War' and my 'DNA Dad'. My mother met 'DNA Dad' before D-Day June 6, 1944. He returned from the momentous landing 4 months later and they married. He returned to his unit and was present at the Battle of the Bulge, even today America's largest battle. When I read the short stories I went cold. The notepaper was from a company Air Land International with offices in Washington (Silver Spring, MD), Ankara, Bangkok, Yemen, Jeddah, etc. (you have got the picture!). Clearly, he was working with some strange arm of the US government (enough said). All the stories were about fighting in Thailand's civil war and drug smuggling in the Golden Triangle (northern Thailand) – Special Forces and Narcotic, i.e. my two areas of expertise in law enforcement in the US/UK and NATO. I, myself, could have written any of these stories.

All my life I had believed it was the influence of my grandfather's DNA and my mother's nurture but it was natural selection by my mother for my 'DNA Dad' too. He died in 1992.

I feel the key to success in my career has been my ability to build and lead small independent teams. This was true in my unconventional 'alternate universe' and in my science career. My Tamoxifen Teams were created in half a dozen places around the world over 4 decades. We always started with an empty suite of laboratories, then hired and trained staff to forge a Team. The successive Tamoxifen Teams quickly turned ideas into lives saved and I am privileged to have aided the career development of the members. I have always tried to put back what I myself received from my honest mentors. When asked later Dr Kaye would reply – 'I was only doing my job' Good words to live by. In 2013, the Tamoxifen Team was recognized on the Wall of Honour at the Royal Society of Medicine in London. This is my gift of respect for their excellent research accomplishments.

I am honored by the generosity of my academic colleagues on both sides of the Atlantic to be recognized by elections to prestigious professional societies. In the United Kingdom: fellowship of the Academy of Medical Sciences and honorary Fellowship of the Royal Society of Medicine. In the United States: member of the National Academy of Sciences, Fellow of the AACR Academy (http://www.aacr.org/home/scientists/aacr-academy/inaugural-class/v-craig-jordan.aspx), and ASCO's '50 Oncology Luminaries' (http://cancerprogress.net/node/2086).

The Tamoxifen Teams contributed a succession of new ideas that all translated from the laboratory to clinical practice: to use tamoxifen in the clinic with long-term adjuvant therapy, chemoprevention with tamoxifen, the selective ER modulators, the warning about an association between tamoxifen and endometrial cancer, raloxifene to prevent osteoporosis and prevent breast cancer at the same time, the evolution of acquired resistance to tamoxifen, and the new biology of estrogen-induced apoptosis.

These ideas all come out of simple and straightforward laboratory experiments dependent on the changing

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environment of the Tamoxifen Teams. Nevertheless, the ideas matured over time through resolve and persistence and especially through a supportive environment from academia, industry, and comprehensive cancer centers. Jules Verne wrote 'whatever one man is capable of conceiving (in my case SERMs), other men are capable of achieving.' That is multidisciplinary translational research; a.k.a. pharmacology. Much has changed since the barbaric days of breast cancer therapy in the 1960s. The story of the Tamoxifen Teams illustrates that progress is about people and passion. People make discoveries, people become advocates for change, and people change healthcare. An investment to provide opportunities for people works. Who are they? It is any young person willing to train themselves for that chance at the Olympic gold medal final and be self selected.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this profile.

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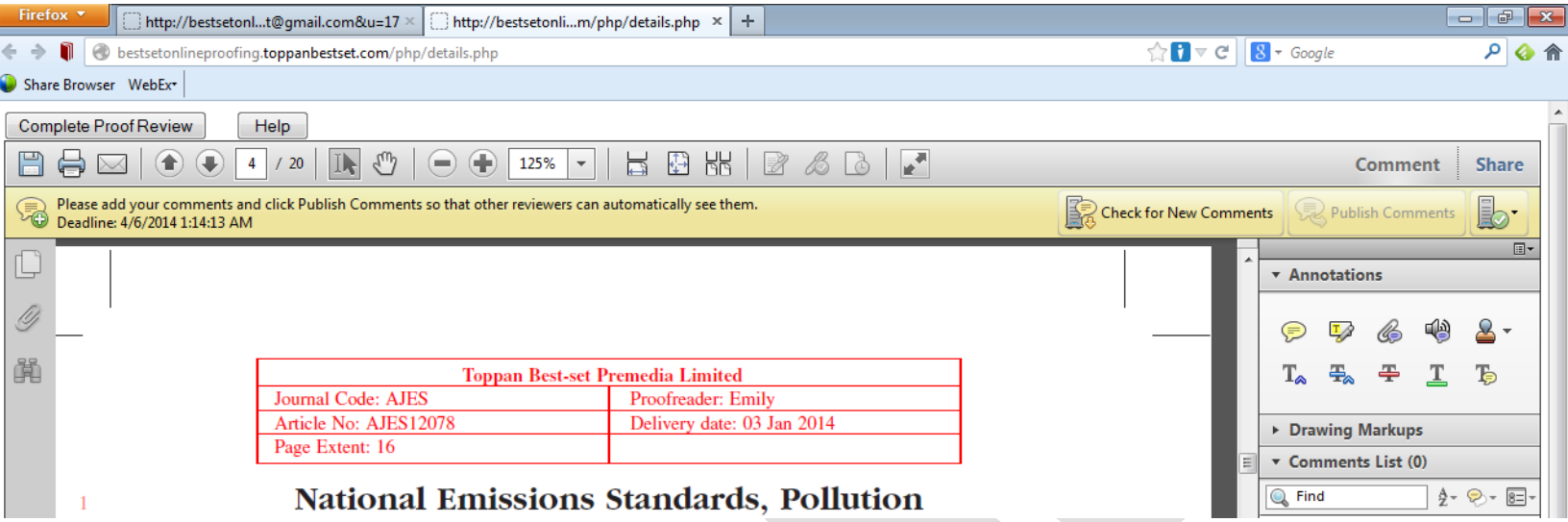

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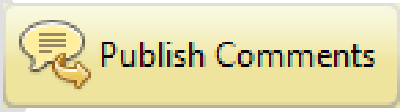

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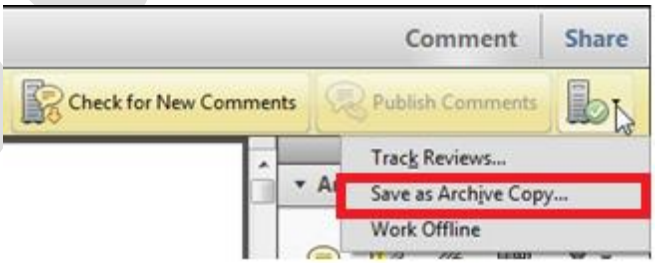

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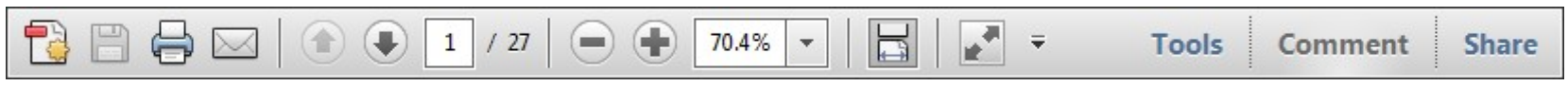

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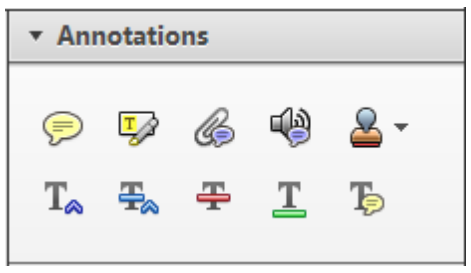

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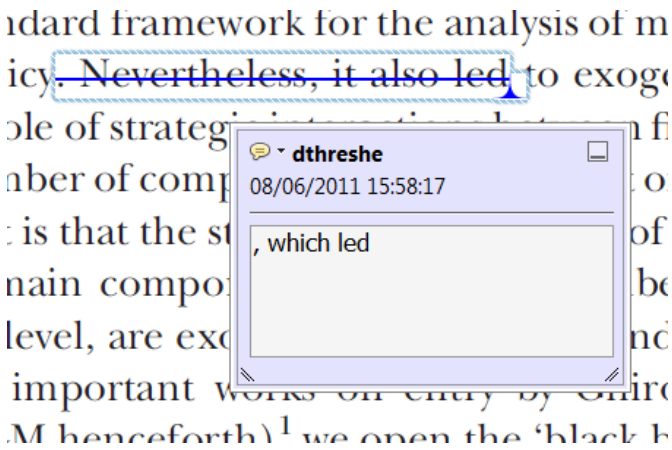

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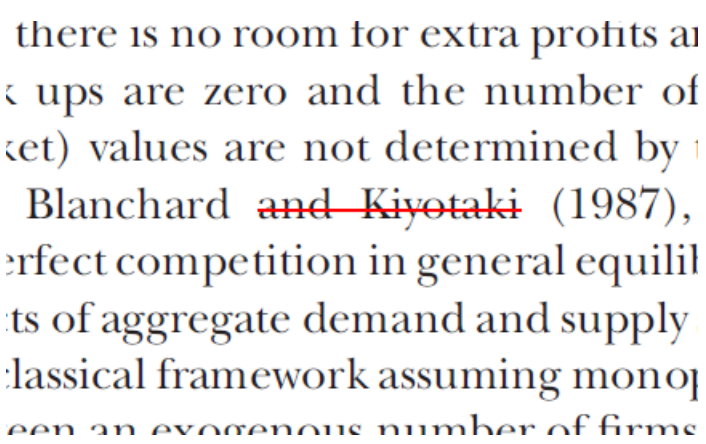

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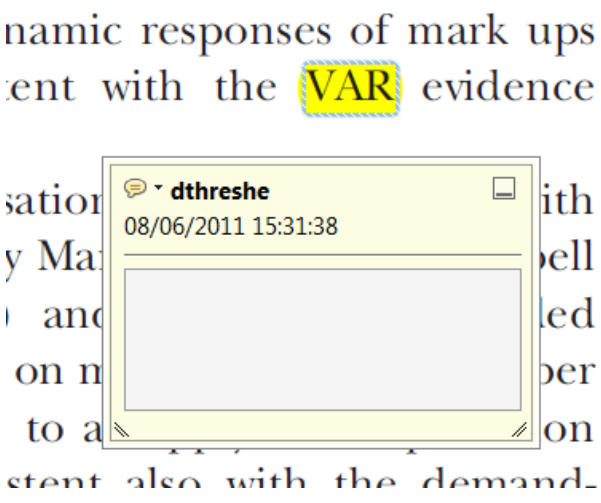

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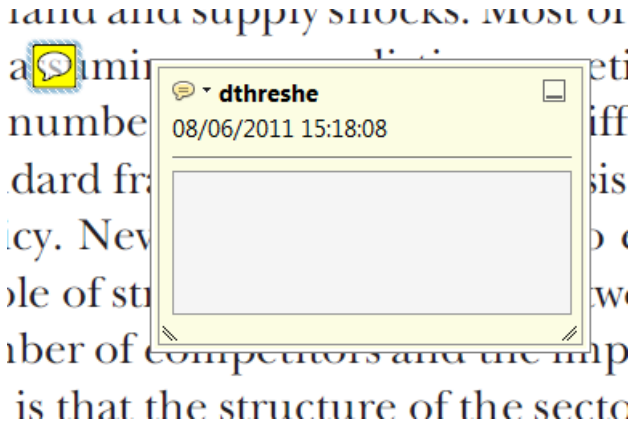

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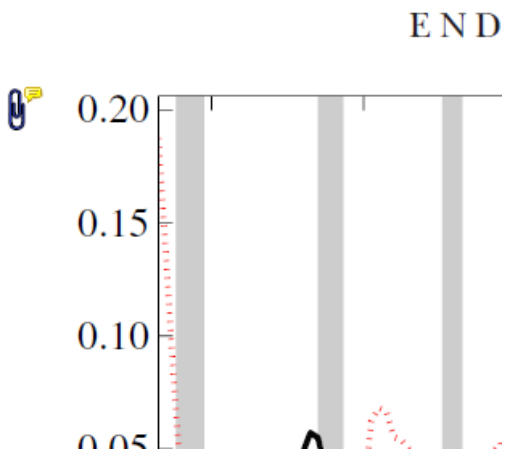

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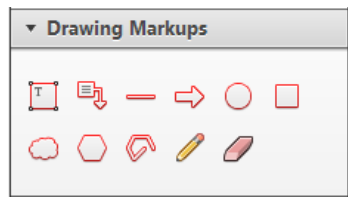

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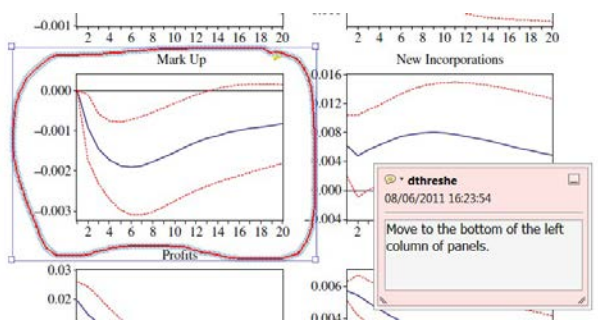

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Historical perspective: proven value of translational research with appropriate animal models to advance breast cancer treatment and save lives: the tamoxifen tale

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Introduction

Our current healthcare system is the beneficiary of the landmark successes of earlier pioneers who struggled, but persevered, to save lives. In the 19th century, two individuals stand out. Dr Louis Pasteur, a PhD basic scientist who luckily was 'encouraged' to conduct applied research and saved a life. Professor Paul Erhlich, a medically qualified research pathologist and winner of the Nobel Prize for antitoxin research, would create a model for successful synthetic drug development that would save thousands of lives. In my generation, it was my friend and supporter Sir James Black, Nobel laureate, who would advance the selectivity implied by receptor theory to treat patients for long periods with pathological conditions. Infectious diseases were cured within months but chronic heart disease, elevated blood pressure and gastric acid secretion were stabilized for years. Lives were saved and the practice of medicine changed to become evidence based. The key to success throughout was the creation and use of appropriate animal models.

In this article, I will focus on the essential aspects of animal models in the unanticipated development of an orphan medicine tamoxifen, used initially to treat late stage breast cancer. The results from the animal models taught the medical profession how to use tamoxifen effectively to save lives, how to detect life-threatening side effects, or provided clues about a new group of medicines that now have multiple applications in women's health. But first, what did our pioneers do and how did they do it?

A perspective on pioneers

Dr Louis Pasteur had already had a prestigious career studying crystal structure, inventing 'pasteurization' for milk and wine to stop spoilage and a vaccine to protect sheep from anthrax, when he turned his attention to the fatal disease rabies. He used a rabbit model to attenuate the rabies virus and a dog model to test the vaccine [1]. His initial goal was to develop an experimental vaccine for study in animals until the fateful day the mother of 9-year-old Joseph Meister pleaded with Pasteur to save her son from a slow and painful death. He had been severely bitten by a rabid dog and death was assured. The unexpected arrival of the young Joseph Meister at that moment was critical, as Pasteur had recently revised his method to prepare attenuated rabies virus and the strategy to treat dogs to protect them from rabies. Pasteur found through his earlier experiments that passing the virus through monkeys, was not optimal and he selected passage through rabbits and collected the infected spinal cords. He fixed them by drying inside flasks protected from moisture. Two weeks of drying reduced the extracted virus to

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become harmless to dogs who were now immune to rabies once inoculated with preparations of increasing virulence based on less dessication time. The young Meister was injected over a period of 11 days with a total of 13 injections of increasing rabies virulence. He escaped certain death. Following Pasteur's death and burial in the crypt of the Pasteur Institute in Paris, Joseph Meister became the faithful custodian to this medical pioneer's memory until the German occupation of Paris in 1940. It is said he chose suicide rather than surrender the keys to the crypt and the memorial to the scientist who saved his life and changed medicine.

Professor Paul Ehrlich devised the drug discovery and development system used today [2]. Earlier in his career as a pathologist he was fascinated to find that organic dyes would specifically bind to bacterial and not human tissue. This gave him the clue to devise chemical therapy. Ehrlich's primary interest was vaccines and antitoxins for which he received the Nobel Prize. Ehrlich believed in the fidelity of the immune system to neutralize and destroy infectious disease. However with the expansion of European colonial interests into Africa came new challenges. It became obvious that the immune system could not kill tropical diseases such as malaria and sleeping sickness whose cause was protozoal. The immune system was overwhelmed by the sheer bulk of the infectious agent. Ehrlich stated 'an attempt must be made to kill the parasites within the body by chemical agents. In other words chemical agents must be used when serum therapy is impossible. French scientists Alphonse Laurier (awarded the Nobel prize for the discovery of the causative agent of malaria) and Mesnel found they could transfer trypanasomes from mouse to mouse to replicate human disease. Progression of the disease could be monitored through blood tests.

Ehrlich used the model to show that dyes could be 'parasitotropic' in mice. Trypan red could cure infected mice. However, when Ehrlich identified the nitrogen-containing azo group in trypan red this brought him to organic arsenicals. An arsenical para-aminophenyl arsenic acid (atoxyl) was marketed already but the compound was ineffective in their model. They had discovered arsenical resistance. A fortunate series of scientific advances in microbiology in 1905 occurred with the chance observation by others, that syphilis was associated with spirochetes that occupied a position between protozoans and bacteria. This was followed by the validation of animal models by scientists in Italy in 1906. At this point Ehrlich appears to have integrated a study of syphilis and a study of resistance to trypanosomes to arsenicals into his laboratory strategic plan. The key to success for the eventual discovery of compound 606 (Salvarsan), through methodical structure activity relationship, was the recruitment of Sahachiro Hata from Tokyo to screen all the compounds in the appropriate models of human disease. Salvarsan was discovered in June 1909. Following toxicology in animals, clinical trials were conducted with the drug manufactured by the Hoechst Company in Germany. Another deadly infectious disease was cured and thousands lived.

Sir James Black (of β-adrenoceptor blocker fame) [3] worked in the laboratories of Imperial Chemical Industries (ICI) Pharmaceuticals Division, Alderley Park, near Macclesfield, Cheshire. He had left ICI by the time I was a summer student at ICI in 1967. Alderley Park was 10 miles from my home and I was then an undergraduate in the Pharmacology Department at Leeds University, keen to do research in cancer drug discovery. There was none of significance then at ICI but the cell biologist Dr Steven Carter (of cytochalasin B fame) [4] was looking at the effects of compounds on mouse cancer cells in culture. It was a start! Coincidentally, the Head of the Fertility control programme, Dr Arthur Walpole had his laboratory opposite Dr Carter's. He had just published a paper [5] on the effects of ICI 46 474 as a 'morning after pill' in rats – but nobody cared! We will meet ICI 46 474 (tamoxifen) later.

Although this was a prescient meeting with Dr Walpole as he would later be the examiner of my PhD on 'failed morning after pills' at Leeds in 1972, the critical players at the start of our tale were being assembled. I met Dr Michael Barrett (of atenolol fame) [6] whose laboratory was next to Dr Carter's at ICI. He had taken over the β-adrenoceptor blocker programme after Jim Black left. Dr Barrett was to talent spot me for a faculty position at Leeds when he became the Professor of Pharmacology in 1970.

Also at ICI in the summer of 1967, I had the privilege to meet Dr James Raventos who was studying gastric acid secretion in dogs with histamine. Jim later told me that the known antihistamines did not block histamine stimulated gastric acid secretion in the dog model. Based on Jim's pioneering studies on the regulation of accelerated cardiac function and arrhythmias in the dog model with his new β-adrenoceptor blockers, he reasoned that the 'antihistamine anomaly' must be because there was a second subtle histamine receptor modulating mechanism [3] – and so it was. The H_2-receptor blockers were born at Smith, Kline and French and long term treatment with H_2–receptor blocker 'antacids' was possible as was β-adrenoceptor blocker treatment for heart conditions before.

Regulations for the safety of medicines

Pasteur, Ehrlich and Black each chose not to conform to the dogma that disease and death were inevitable. Each chose to question Nature through experimental animal models. Their persistence was translated to patient care. However, success in one area of therapeutics demands regulations imposed by society on claims in other areas thereby preventing Charlatans peddling 'cures' that are neither evidenced based nor safe. The elected representatives of the people in our democratic society are charged

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with the responsibility to enact laws and regulations that ensure the safety of any new medicine. A strict protocol of appropriate animal toxicology is enforced by acts of government to prevent unanticipated injury or deaths.

It is not necessary to expand further as the concepts of safety and the documented worth of a medicine for patient care should be obvious to all. Nevertheless, a couple of examples will be given to illustrate instances when an inadequate system of protection can fail or a warning model appears to do so.

It is axiomatic that one should always err on the side of caution with safety and side effects of medicines. Thalidomide taught us that lesson so why was there no caution? The reason that the tragedy occurred was that there was no legal requirement to test for teratogenicity in the 1950s [7]. The toxicology concern was first raised by observation in humans [8]. Tragically, the value of thalidomide was seen to be in the control of nausea in pregnant women during the first trimester [9], exactly when limb development is occurring in the foetus. It is now known that thalidomide can stop blood vessel formation and limb formation is particularly vulnerable. Now there is rigorous teratogenesis testing of medicines to be used in women of childbearing age. It is important to note that thalidomide used in a cancer context, to treat a fatal disease, can produce improvements in multiple myeloma deployed as an anti-angiogenic agent.

The thalidomide tragedy and introduction of teratogenic testing is why women taking the anti-oestrogen tamoxifen during their childbearing years to treat breast cancer, must use barrier contraception to prevent pregnancy. However, there was an apparent anomaly in the toxicology testing of tamoxifen when it transitioned from cancer therapy with a requirement for only liberal toxicity testing for a fatal disease, to a chemopreventive in disease-free women only at risk for breast cancer. This toxicological surprise in rats given tamoxifen for years was hepatocellular carcinoma that was first reported [10] nearly 20 years after tamoxifen had been used by perhaps a million women worldwide.

Tamoxifen was discovered to be a rat liver carcinogen at high doses given for a lifetime [10] but increases in human hepatocellular carcinoma were not noted either in the 1990s [11, 12] when the toxicological issue was raised initially or indeed now [13]. Millions of women have benefited from tamoxifen with its long term use. However, tamoxifen would not have been knowingly developed by any company had the toxicological knowledge been available at the beginning of the tamoxifen tale in 1973 [14]. Without the success of tamoxifen as a lifesaving medicine there were no agents waiting as the 'first reserve' anti-oestrogen – nobody cared. Without the success of tamoxifen, there would have been no financial incentive to develop aromatase inhibitors [15] and there would have been no selective oestrogen receptor modulators (SERMs) [16, 17]. So this would seem to argue against animal testing? Certainly not. The toxicological requirements for an anticancer therapy to delay a fatal disease are rightly less draconian than for the treatment of a subject with an infection or no life-threatening disease. The fact the rat liver carcinogenesis was discovered after 20 years of tamoxifen use, allowed the clinical and toxicological community also to evaluate 'real world' experience in women [11, 12] No increase in liver cancer was noted. Scientists were able to determine that the rat is particularly susceptible with its metabolism of tamoxifen to producing a carcinogen but the human rapidly repairs DNA damage [18]. The system for protecting human safety for the introduction of an unknown drug to prevent a disease worked with appropriate toxicology testing in animals. Cancer patients lived because of appropriate testing and risk management for treatment of a fatal disease.

The early years of the tamoxifen tale

Cancer therapeutics and cancer prevention are a particular challenge as we seek to destroy renegade cells that are 'self'. Ehrlich chose to explore the development of appropriate animal models of human disease to address cancer chemical therapy (chemotherapy) at the dawn of the 20th century. In the year before he died in 1916 he declared 'I have wasted 15 years of my life on experimental cancer research' [19].

The banner of progress in therapeutics was picked up in the 1940s using a process of translational research i.e. first validation of an antitumour response in animal models and then a clinical trial. Sir Alexander Haddow FRS discovered [20] that high dose synthetic oestrogen treatment could produce a 30% response rate in breast cancer patients more than 5 years after their menopause [21]. This was the first chemical therapy to treat any cancer successfully and was proven in clinical trials. However, high dose oestrogen treatment is a paradox as all other approaches before the Haddow breakthrough caused regression of breast cancer by endocrine ablation (oophorectomy, adrenelectomy), i.e. taking away oestrogen just as tamoxifen blocks oestrogen from stimulating tumour growth. High dose oestrogen therapy remained the treatment of choice for breast cancer after the menopause for the next 30 years until the introduction of tamoxifen (1973 UK, 1977 USA) with fewer side effects [22, 23]. The only randomized trial [23] of high dose oestrogen *vs*. tamoxifen in unselected (no oestrogen receptor (ER) selection) postmenopausal patients with metastatic breast cancer was quite small with 74 patients and 69 patients, respectively. Response rates were both about 30% and disease control was similar over a 2 year period. Only the increased side effects noted with high dose oestrogen led the authors to recommend tamoxifen [23].

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It is interesting to note that all the early events in the story of breast cancer 'chemical therapy' are actually connected. Haddow's experimental oestrogens were synthesized by ICI [20]. Walpole was specifically interested in cancer research. He tried unsuccessfully to discover why only some tumours responded to oestrogen therapy [24] and successfully developed an early 'chemotherapy' [25] but was directed to create a safer 'morning after pill'. The discovery by scientists in America that synthetic oestrogens could be converted to anti-oestrogens through the skill of the medicinal chemist [26] that were also effective 'morning after pills' in the rat which could potentially now create another 'blockbuster' in the wake of the success of oral contraceptives. My connection with the anti-oestrogen research team at ICI throughout the 1970s has recently been told [27] and the clinical development of tamoxifen explained [28, 29]. However, tamoxifen is not about a single medicine but the pioneer in a group of medicines now called SERMs.

Forty years ago there were no SERMs, today there are five but with a sixth, lasofoxifene, approved in the European Union a few years ago. This approval has lapsed (Figure 1). The SERMs were predicted to treat multiple diseases in post-menopausal women simultaneously [26]. The currently approved SERMs treat breast cancer, prevent breast cancer, prevent osteoporosis and preparations prevent menopausal symptoms including dyspareunia. The general outline of the development of the two principal SERMs, tamoxifen and raloxifene, are illustrated and explained in Figures 2 and 3 and a current view of the molecular mechanism of action illustrated for target sites in Figure 4. These stories have been explained recently in detail [30, 31]. However, none of the SERM story would have occurred but for the appropriate use of animal models to guide clinical trials, to understand patient safety and finally to define a new biology of oestrogen-induced apoptosis. This cascade of knowledge answered the question 'how can oestrogen stimulate breast cancer growth (which is the basis of all successful anti-oestrogenic therapy for the past 40 years [32]) but also cause apoptosis as a breast cancer therapy [22, 23]'. It is animal models that aided the understanding of 'Haddow's paradox' [21] that oestrogen can kill correctly prepared breast cancer cells. That knowledge and the molecular mechanism again have clinical significance.

The role of appropriate animal models in breast cancer research to save lives

In 1972, just 2 months after my successful PhD examination with Dr Arthur Walpole on the topic of 'failed morning after pills' entitled *A study of the oestrogenic and anti-oestrogenic activities of some substituted triphenylethylenes and triphenylethanes*, I found myself at the Worcester

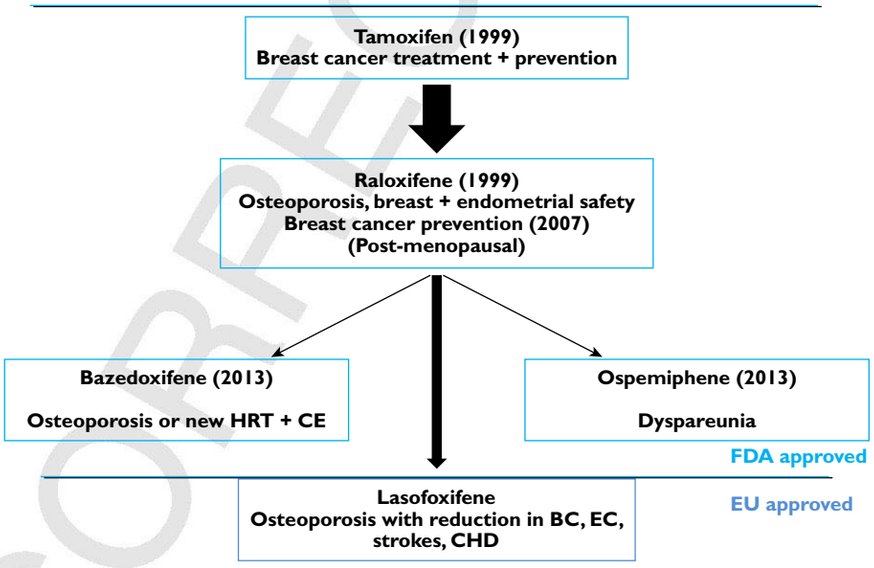

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Figure 1

The approvals of individual selective oestrogens receptor modulators (SERMs) in the United States of America through the evaluation system of the Food and Drug Adminisration (FDA). Approvals were specifically for indications at the highest level of toxicologic safety for women without disease but as a new hormone replacement therapy with conjugated oestrogen (HRT + CE) to prevent disease i.e. chemoprevention of osteoporosis, breast cancer (BC), menopausal symptoms or dyspareunia. One SERM, lasofoxifene, was approved for use in the European Union (EU) but was never launched or marketed despite the fact that clinical trials demonstrated a reduction in breast cancer (BC), osteoporosis fracture, strokes, endometrial cancer (EC) and coronary heart disease (CHD) [92]

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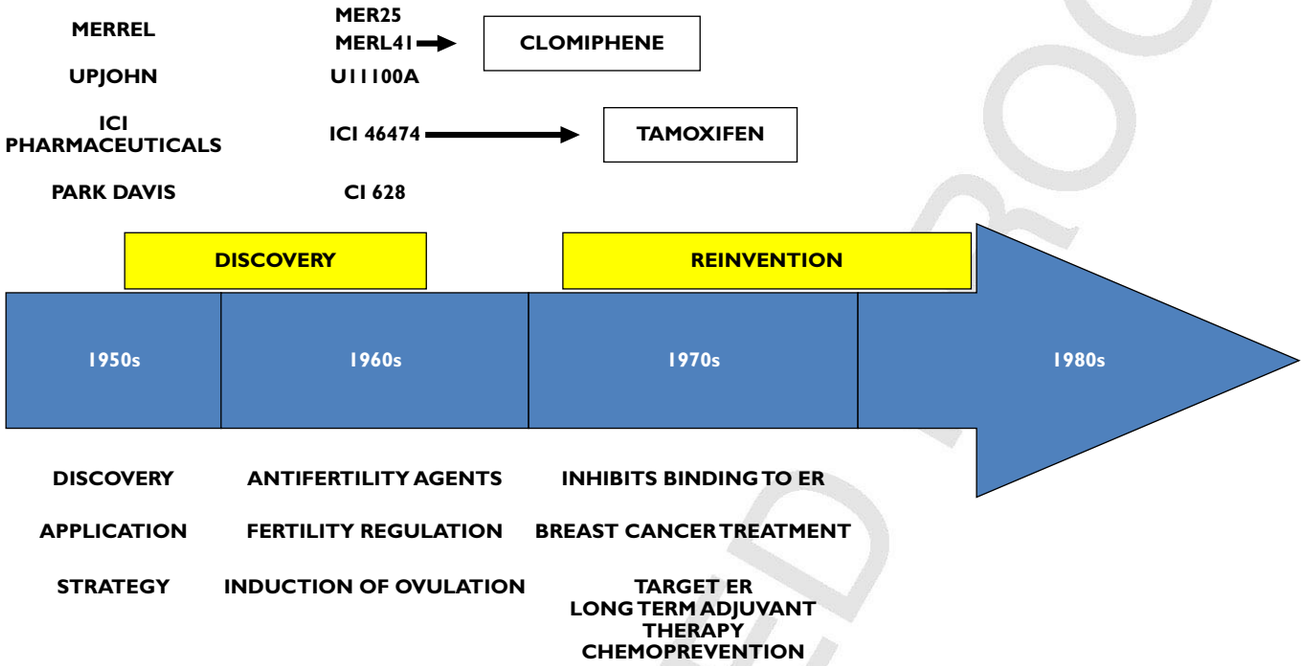

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Figure 2

A trickle to tamoxifen (ICI 46 474). During the 1960s, a number of triphenylethylene derivatives were discovered that were excellent novel post-coital antifertility agents in rats but induced ovulation in subfertile women (clomiphene and tamoxifen) [26]. Tamoxifen moved forward as a palliative treatment for metastatic breast cancer, only after being all but abandoned as a commercially viable enterprise. It was then rescued as an orphan drug in 1972 [93]. Laboratory models informed about possible applications as a long term adjuvant therapy or as a chemopreventive agent [27]. Clinical trials demonstrated major survival advantages for women with ER positive breast cancer who received long term (>5 years) tamoxifen therapy and tamoxifen was tested and was the first medicine to be approved for the reduction of breast cancer in high risk women [93]

Foundation for Experimental Biology in Shrewsbury, Massachusetts, USA. I discovered I was to be an independent investigator working in the 'home of the oral contraceptive' but I chose to explore the possibility with ICI of contributing to the development of their orphan drug ICI 46 474 (but not yet tamoxifen). During the time I was at the Worcester Foundation (1972–1974) there were only two clinical reports [22, 33] of the use of tamoxifen to treat breast cancer, but these were not randomized trials, there was no correlation between tumour ER and endocrine ablation, that was to be published in 1975 [34], and there was no mention of tamoxifen as it was not used in this context. A correlation between response and tumour ER was noted later [35, 36]. Adjuvant tamoxifen therapy and chemoprevention were not on the clinical landscape. There was much to do at the beginning to develop a rationale to advance a 'failed morning after pill'. They funded my research proposal but how to start. I needed to train myself to find a model to evaluate and quantify the antitumour effect of ICI 46 474. No laboratory antitumour studies had been done by ICI (or anyone else) but as Ehrlich had taught an 'appropriate animal model of human disease was necessary' to convince the clinical cancer community to conduct clinical trials. The prowess of ICI 46 474 as an effective 'morning after pill in rats' would not suffice!

I found my model in Chicago at the Ben May Cancer Research Laboratories of the University of Chicago. I visited at the invitation of the Director, the late Dr Elwood V. Jensen in the spring of 1973. I learned the 'Jensen method' of measuring the tumour ER, an enormous improvement over my 'Heath Robinson' approach alone in the basement of Leeds University Old Medical School during my PhD. I learned the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model [37] and had the good fortune to meet and talk with Professor Charles Huggins, the former director of the Ben May laboratory for Cancer Research and Nobel Laureate for his work on hormone dependent prostate cancer. This readily reproducible mammary tumour model is hormone (ovarian) dependent for growth and the tumours contained the ER [38]. It was the only appropriate model. For the next decade this model would be my medium to propose targeting the ER positive tumour [39] with long term adjuvant tamoxifen therapy [40–42] or use the animal model in the first step towards chemoprevention of breast cancer [43, 44]. All of this would occur at the

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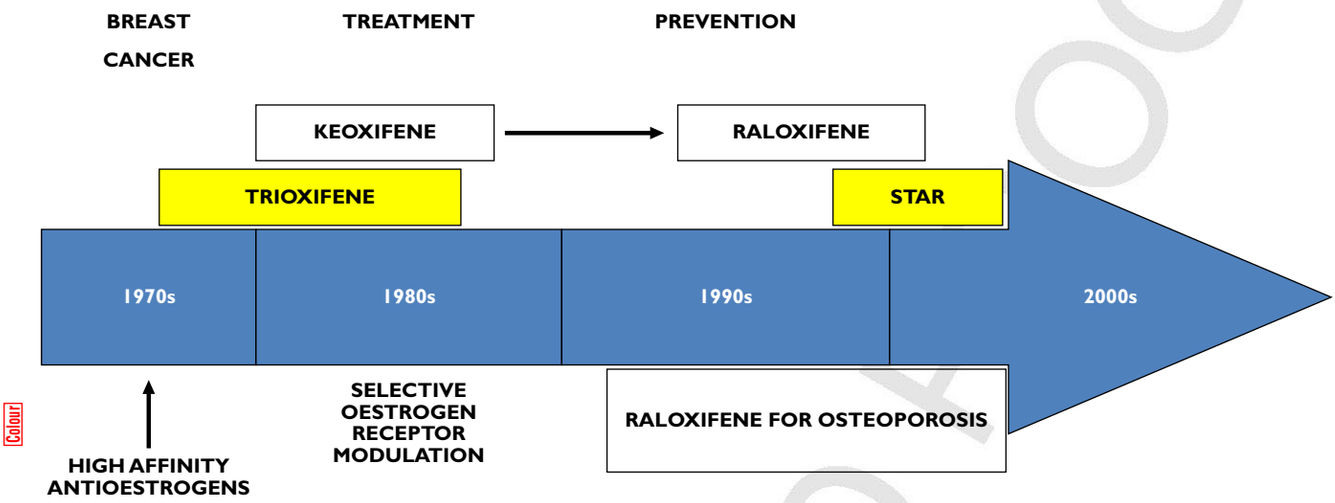

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Figure 3

Rush to raloxifene. The success of tamoxifen for the treatment of breast cancer created potential opportunities to develop drugs to correct toxicological issues of concern i.e. the increase in endometrial cancer. Trioxifene was developed as a potential competitor for tamoxifen but failed to demonstrate either increased efficacy in the treatment of metastatic breast cancer or decrease in serious side effects. In the wake of the discovery that tamoxifen was metabolically activated to 4-hydroxytamoxifen with high binding affinity for the ER (Figure 2) [70, 94] a compound LY156758 or keoxifene was developed that had high binding affinity for the ER and did not have oestrogen-like activity in the uterus [95]. Keoxifene failed to become a breast cancer therapy and was abandoned in 1987. However, the discovery that keoxifene prevented bone loss and mammary cancer in rats [51, 75] ultimately resulted in the resurrection of the molecule as raloxifene. The clinical testing resulted in the approval of raloxifene to treat and prevent osteoporosis in post-menopausal women in 1997 and for the reduction of the incidence in breast cancer in high risk post-menopausal women in 2006. This was the Study of Tamoxifen and Raloxifene (STAR). Unlike tamoxifen, raloxifene does not increase the incidence of endometrial cancer [78]

Worcester Foundation (1972–1974) and at the Department of Pharmacology of the University of Leeds (1974–1979). The next dimension in discovery for therapeutics would occur in the 1980s at the University of Wisconsin Clinical Cancer Center (Madison) (1980–1993) in the United States.

The nu/nu athymic mouse model was found to be immune deficient so human tumours could be transplanted and therapies studied to seek cures for cancer [45]. Of particular interest to my new embryonic tamoxifen team in the Department of Human Oncology at the Clinical Cancer Center were the observations that the ER positive human breast cancer cell line MCF-7 [46, 47] inoculated into the axillary mammary fat pad was able to grow into tumours with oestrogen treatment [48, 49]. Furthermore, tamoxifen prevented oestrogenic-stimulated tumour growth [50]. Here was the new model we needed.

Marco Gottardis was an extremely talented technician conducting animal studies in the Department of Human Oncology in the Cancer Center. He accepted my invitation to become a graduate student in my laboratory. His work and publications changed therapeutics and medical care multiple times as he expertly used carcinogen-induced rat mammary tumour models [51] and established our colony of MCF-7 tumour bearing athymic mice [52]. The latter model revolutionized our understanding of acquired resistance to long term tamoxifen therapy [53] and what to do about it in the clinic [54]. The athymic mouse model would provide the leads to the target site specificity of 'non-steroidal anti-oestrogens' [55, 56]. Harper & Walpole [57] had discovered the unusual species specificity to ICI 46 474. The triphenylethylene was classified as an oestrogen in the mouse vagina and this classification was confirmed by Terenius in immature micee with uterine weight tests [58]. ICI 46 474 was classified as an anti-oestrogen in the rat with partial agonist uterine action [5]. However the fact that ICI 46 474 (tamoxifen) acted as an anti-oestrogen to block oestrogen stimulated tumour cell growth in athymic mice [55] was a first clue that tamoxifen was tissue, not species, specific. The development of this observation in different target tissues would give the insight into a new group of medicines in women's health, the SERMs that switch on or switch off oestrogen target sites around the body [59]. This is a fascinating story in molecular pharmacology as the interpretation of the two known ERs, i.e. α and β with different coregulators and receptor processing at different gene promoters, can produce agonist or antagonist action. This multifaceted decision network is summarized in Figure 4. Marco is now the Vice President and Prostate Cancer Disease Area

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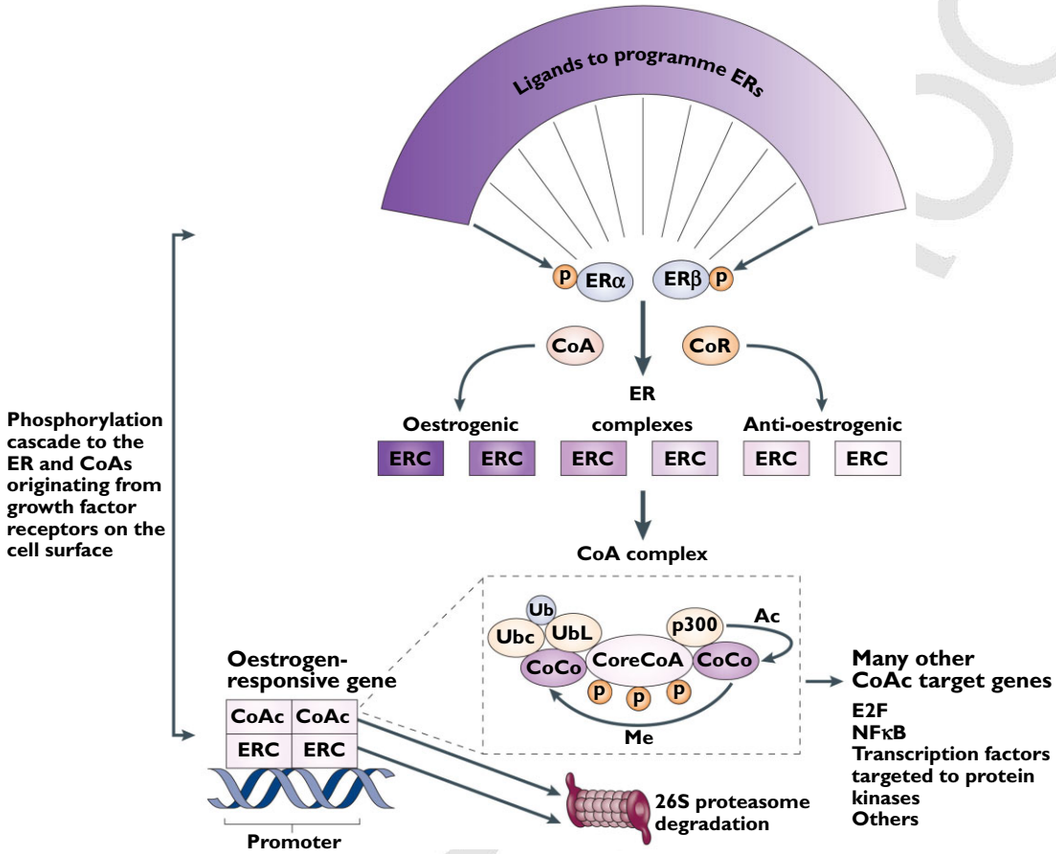

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Figure 4

The oestrogen target tissue decision network for selective oestrogen receptor modulation. The shape of the ligands that bind to the oestrogen receptors (ERs) α and β programmes the complex to become an oestrogenic or anti-oestrogenic signal. The context of the ER complex (ERC) can influence the expression of the response through the numbers of co-repressors (CoR) or co-activators (CoA). In simple terms, a site with few CoAs or high levels of CoRs might be a dominant anti-oestrogenic site. However, the expression of oestrogenic action is not simply the binding of the receptor complex to the promoter of the oestrogen-responsive gene, but a dynamic process of CoA complex assembly and destruction. A core CoA, for example, steroid receptor coactivator protein 3 (SRC3), and the ERC are influenced by phosphorylation cascades that phosphorylate target sites on both complexes. The core CoA then assembles an activated multiprotein complex containing specific co-co-activators (CoCo) that might include p300, each of which has a specific enzymatic activity to be activated later. The CoA complex (CoAc) binds to the ERC at the oestrogen-responsive gene promoter to switch on transcription. The CoCo proteins then perform methylation (Me) or acetylation (Ac) to activate dissociation of the complex. Simultaneously, ubiquitiylation by the bound ubiquitin-conjugating enzyme (Ubc) targets ubiquitin ligase (UbL) destruction of protein members of the complex through the 26S proteasome. The ERs are also ubiquitylated and destroyed in the 26S proteasome. Therefore, a regimented cycle of assembly, activation and destruction occurs on the basis of the preprogrammed ER complex. However, the co-activator, specifically SRC3, has ubiquitous action and can further modulate or amplify the ligand-activated trigger through many modulating genes that can consolidate and increase the stimulatory response of the ERC in a tissue. Therefore, the target tissue is programmed to express a spectrum of responses between full oestrogen action and anti-oestrogen action on the basis of the shape of the ligand and the sophistication of the tissue-modulating network. NFkB, nuclear factor kB. Reprinted with permission from the Nature Publishing Group, Jordan [96]

Stronghold Leader for the Oncology Therapeutic Area at Janssen Research and Development, LLC in New York.

It would be another graduate student, Doug Wolf who would have the transplantable model of acquired resistance to tamoxifen passed to him! He would discover that after retransplantation of the tumours for years into successive generations of tamoxifen-treated athymic mice, that physiological oestrogen could make tumours melt away [60]. This serendipitous discovery at Wisconsin would be developed fully [61] at the Robert H. Lurie Comprehensive Cancer Center at Northwestern University, Chicago (1993–2005) by surgical residents, medical oncology fellows or scientists: Kathy Yao, Gale England, Eun-Sook Lee, David Bentrem, Ruth O'Regan, Rita Dardes, Jennifer MacGregor, Hong Liu, Clodia Osipo, Debra Tonnetti and Joan Lewis all co-operated and achieved successes [61–67]. Our tamoxifen teams have remained an essential balance of clinical and laboratory expertise to ensure we never lose sight of the goal – improving

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cancer care. Doug Wolf is now the Senior Director Oncology regional medical research specialist at Pfizer.

I will illustrate the translational aspects of our tamoxifen tale by our tamoxifen teams over the decades with the following examples of successful translational research outcomes.

An appropriate strategy for the adjuvant antihormone treatment of breast cancer

Laboratory model

The DMBA-induced rat mammary carcinoma model has been examined extensively by hundreds of investigators [38] but the main hypothesis to be tested in our studies was that longer treatment starting when animals had only occult disease following DMBA administration was superior to short term therapy [40–42]. The secondary hypothesis to be addressed was that only ER positive disease would respond as tamoxifen and metabolites blocked the binding of [^{3}H]-oestradiol to tumour ER [39, 68–70].

Clinical translation

The overviews of clinical trials conducted every 5 years at Oxford demonstrated that only patients with an ER positive primary tumour responded to adjuvant tamoxifen and longer therapy (5 years) was superior to either 1 or 2 years of adjuvant tamoxifen [12, 13]. There was a 50% decrease in recurrence rates and a 30% decrease in mortality. Maybe a million lives were saved.

Tamoxifen and target site specific anticancer action

Laboratory model

Athymic mice were transplanted with an ER positive breast cancer and an ER positive endometrial cancer and treated with oestrogen to stimulate growth. Tamoxifen was administered to determine whether the anti-oestrogen controlled the growth of both breast and endometrial cancer. Breast cancer was controlled but endometrial cancers grew dramatically [56].

Clinical translation

Marco Gottardis and I presented these data prior to publication to staff at ICI Pharmaceuticals Division, Alderley Park. In 1987, I presented the results and my concerns at a medical conference organized during the celebration of the 900th anniversary of the first university in the world, the University of Bologna, Italy. As a result of my lecture, Dr Leonard Hardell wrote a letter to the Lancet [71] and I replied appealing for results from a large prospective clinical trial [72]. The database from Fornander and colleagues [73] demonstrated that longer tamoxifen (5 years) caused the detection of more endometrial cancer than shorter (2 years) of adjuvant tamoxifen. The report also confirmed that the incidence of new primary breast cancers was reduced by tamoxifen but endometrial cancer incidence went up. I replied [74]. Medical practice changed with new package inserts and gynaecologists became involved as part of the breast cancer patient care team. The whole process of translational research to clinical practice took 2 years and almost certainly saved lives.

The discovery of SERM action

Laboratory model

In the 1980s, as a prelude to chemoprevention, we rigorously investigated the fascinating target site specific actions of tamoxifen. Human breast tumours implanted in athymic mice did not grow [55] with tamoxifen despite the fact that tamoxifen is an oestrogen in the mouse [5]. But oestrogen is needed to maintain bone, so what would the value be of preventing a few breast cancers in a thousand post-menopausal women per year if hundreds of women subsequently developed osteoporosis? To our surprise both tamoxifen and raloxifene (an abandoned breast cancer drug called keoxifene) both maintained ovariectomized rat bone density [75] and prevented carcinogen-induced mammary cancers in a rat model [51]. Tamoxifen was better than raloxifene at suppressing mammary tumour appearance [51]. This is because tamoxifen has a long biological half-life producing optimal tumour suppression whereas raloxifene is a polyphenolic compound that is rapidly cleared and short acting.

The SERM concept applied to clinical practice was proposed in the last paragraph of the Cain Memorial Lecture in 1990 [26]. This roadmap for industry is reproduced in the last section, Retrospective and conclusions.

Clinical translation

The animal study of rat bone density with tamoxifen translated to building bone in post-menopausal women [76]. Raloxifene was approved to prevent osteoporosis but prevented breast cancer at the same time [77]. The chemoprevention trial Study of Tamoxifen and Raloxifene (STAR) showed that both SERMs were able to prevent breast cancer in high risk post-menopausal women by 50% during treatment [78] but after therapy stopped at 5 years tamoxifen maintained chemoprevention of breast cancer but raloxifene did not [79]. These clinical results echoed our laboratory study in animals 20 years earlier [51]. Raloxifene is recommended to be taken indefinitely to maintain chemoprevention of breast cancer. Perhaps hundreds of thousands of lives have been improved.

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The evolution of acquired resistance to tamoxifen

Laboratory model

The serial retransplantation of MCF-7 breast tumours with acquired resistance to tamoxifen into tamoxifen treated mice passes through two phases: Phase I acquired resistance occurs in the ER+ tumour within 1–2 years of tamoxifen treatment. Acquired resistant tumours are characterized as being stimulated to grow with either physiologic oestrogen or tamoxifen [53]. No oestrogen or tamoxifen treatment or treatment with a pure anti-oestrogen stops tumour growth [54]. Phase II acquired resistance develops with retransplantation after 3–5 years, but now tamoxifen stimulates tumour growth but physiological oestrogen causes tumour regression [61].

Clinical outcome

Low dose oestrogen causes a 30% benefit rate after a woman's tumour becomes resistant to long term adjuvant aromatase inhibitor treatment [80]. Most provocatively, the new science of oestrogen-induced apoptosis could be the reason for dramatic decreases in mortality after adjuvant tamoxifen is stopped. Recent data demonstrate that 10 years of tamoxifen is superior to 5 years of tamoxifen [81] but mortality is decreased by 50% compared with historical no treatment data but only in the decade after 10 years of tamoxifen is stopped. Oestrogen-induced apoptosis is also offered as the reason [82] mortality decreases with oestrogen alone treatment as hormone replacement therapy in 60 year old post-menopausal women following a decade of oestrogen deprivation following menopause. It may be that this research strategy leads to new and safer hormone replacement therapy for post-menopausal women.

Retrospective and conclusions

Looking back at this point in our tale, it can be predicted that this will not be the end at all, but the beginning of a new phase of a conversation with Nature. The outcomes of that conversation may determine the next advance in therapeutics.

What started out with a desire to contribute to the development of a medicine to treat cancer seemed, on reflection now, a forlorn hope 40 years ago [27] but I did not realize that at the time (fortunately)! The formula for a successful outcome in my quest to contribute, depended on two principal factors: a willingness to learn new laboratory techniques using relevant animal models that turned out to have significance for translational research in therapeutics and the willingness of innovative and committed individuals in industry and Yorkshire Cancer Research to invest in a young investigator [27]. This was followed by the generosity of a philanthropic organization, the Lynn Sage Breast Cancer Foundation in Chicago, who raised a million dollars a year for a decade for my tamoxifen team to define and understand the new science oestrogen-induced apoptosis [61–67].

As a pharmacologist, I defined my goal – use models to discover mechanisms and develop new medicines. Animal models were the key to that success. At the start, the use of long term adjuvant tamoxifen therapy was counterintuitive to the clinical community. Tamoxifen was only effective in controlling the growth of metastatic breast cancer for a year or two [22, 23] so it would be dangerous at worst, and unwise at best, to extend adjuvant tamoxifen beyond a year. But micrometastases are clearly different from larger metastatic lesions and a different pharmacology pertains. Perhaps millions of women benefited. There was no clinical understanding of the relevance of the mixed oestrogenic/anti-oestrogenic effects of tamoxifen. In the clinical lectures, I called it the 'oestrogenic tickle of tamoxifen'. The laboratory finding that tamoxifen selectively blocks oestrogen stimulated breast tumour growth but enhances the growth of pre-existing occult endometrial cancer changed all that [56]. Medical practice changed, gynaecologists were involved in breast cancer patient care and major medical problems were avoided that could have killed the patient without appropriate pre-emptive action. A cluster of consistent findings [51, 52, 55, 56, 75] by my tamoxifen team at Wisconsin (1980–1993) resulted in the group of medicines, the SERMs.

The idea that a 'non-steroidal anti-oestrogen' could switch on or switch off oestrogen target sites around the body could not have been anticipated without animal models to demonstrate antitumour action in the rat mammary gland [43, 44, 51] but oestrogen-like activity in ovariectomized rat bone [75]. This led to a road map for industry [26] as stated in my Bruce F. Cain Award and Memorial Lecture in 1989:

'Is this the end of the possible applications for anti-oestrogens? Certainly not. We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be post-menopausal women in general, thereby avoiding

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the requirement to select a high risk group to prevent breast cancer'.

The declaration resulted in a whole new drug group that overall, aids women's health. Millions of women continue to benefit.

Lastly, the creation of an animal model of acquired tamoxifen resistance of breast cancer informed us about the unique nature of tamoxifen-stimulated tumour growth. However, the then disheartening fact that this tumour model could not be transferred to cell culture, but demanded constant retransplantation into subsequent generations of tamoxifen treated athymic mice, opened the door to a discovery. Little did we suspect at the beginning, that this routine, labour-intensive procedure, would cause the tumours to evolve through continuing selection pressure over the years. Acquired resistance changed after a couple of years. Tamoxifen treatment caused acquired resistance with either tamoxifen or oestogen-stimulated growth. At 3–5 years of transplantation now the new tamoxifen resistant cell population responded to physiologic oestrogen with tumour regression. It is possible that a woman's own oestrogen does exactly the same to execute prepared micropopulations of tamoxifen resistant cells after 5 years of adjuvant tamoxifen stops [60]. Based on the original animal studies demonstrating the evolution of acquired resistance to tamoxifen, subsequent cellular models were used to decipher the molecular events involved in oestrogen-induced apoptosis [65, 83–86]. This knowledge became pre-positioned in the refereed literature so that the paradoxical finding of fewer breast cancers reported in the oestrogen alone clinical trial of the WHI with a reduction of mortality were understood. Select women lived [82] but the finding that a combination of oestrogen plus a synthetic progestin, which causes an increase in breast cancer incidence, now demands understanding. Resolution of mechanisms and the creation of a safer hormone replacement therapy that prevents breast cancer may indeed be the next chapter of the tamoxifen tale that affects the lives of millions of women worldwide.

However, it would be, perhaps, misleading to imply that human disease can always be modelled successfully with animal equivalents. There is, for example, no animal model for human breast cancer that faithfully replicates outcomes. Focusing on the pharmacology of tamoxifen, but bearing in mind this is just the tip of the iceberg of all medicines, a number of uncertainties and problems persist. To be successful as a therapeutic agent, the medicine must be taken for perhaps a decade or more as a treatment or as a chemopreventive agent in high risk women. Regrettably, and predictably, one of the major side effects of tamoxifen that reduces compliance is menopausal symptoms, particularly hot flushes. Decreases in compliance result in lives lost [87]. These are no satisfactory laboratory models to predict this in the clinic. Nevertheless, changes in patient care may be possible. A new combination of the SERM bazedoxifene plus conjugated oestrogen has recently been approved by the Food and Drug Administration in America for the treatment of osteoporosis or menopausal symptoms [88]. It seems that the oestrogen can win in the brain to ameliorate menopausal symptoms, but the SERM prevents oestrogen induced breast and endometrial cancer. The combination has an additive effect in bone, an effect first noted with both tamoxifen and raloxifene in animals [75]! Metabolism and pharmacodynamics remain a challenge in the two way conversation between laboratory animal results and clinical trials. Although algorithms are available, to model dosage modifications in animals is often not precise. Additionally blood concentrations and metabolites are not consistent between human and other species [89]. One long running controversy has been the genotyping of patients for CYP2D6 that governs the available levels of endoxifen in tamoxifen treated patients. The technical issues have recently been reviewed [90] but the simple theory that only higher levels of metabolically produced endoxifen will produce optimal results, can really only be addressed in cell culture. Animal modelling is not possible [89]. However, cell culture only provides data on a transient moment in the life of tumour cells and not the shifting adaptive populations that evolve over years of treatment.

As a science, our exploration evolves by trial and error as we meet each new challenge in selective toxicity. In cancer research there has been in the past decade, a huge shift to genetically engineered mice to answer the question 'is this gene significant?' At the other extreme is the continuous sequencing of human tumour types to discover patterns and vulnerabilities. However, human tumour data are a single 'snapshot' but what human cancer is, is a relentless journey of immense possibilities to overwhelm the human host. This remains hard to model if we subscribe to the mantra that every tumour is different and that only personalized medicine is the way of the future. Tamoxifen with its target of the tumour ER was the first personalized medicine in cancer. Now we have the challenge of navigating out of the Pandora's box we opened.

Professor Paul Ehrlich chose to view the selective targeting of a chemical therapy to cure disease as the search for the 'Magic Bullet'. Tamoxifen can, in retrospect, be viewed as the discovery of a 'Magic Machine Gun', as no other chemical therapy for cancer is used to treat all stages of breast cancer, ductal carcinoma *in situ* (DCIS), male breast cancer and can be used for the prevention of breast cancer, all by targeting the ER [91], but the ER target around a patient's body can be switched on or switched off selectively by tamoxifen. So, a search for new medicines gave us SERMs. Broad improvements in women's health by selective modulation of the same target in different tissues was an unanticipated consequence of 'anti-oestrogenic' treatment. Appropriate animal models significantly

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advanced health for millions of women to live longer and healthier lives. Mothers see their children grow up, children experience the affection of a grandmother.

Competing Interests

There are no competing interests to declare.

This article is dedicated to my late friend and supporter Sir James Black FRS. The inspiration to create this article occurred when the American Society of Clinical Oncology chose to select my contributions in translational research to be one of the 50 Oncology Luminaries http://cancerprogress.net/node/2086 and, simultaneously, Ms Elodie Picard, a veterinary student from Brussels enquired about my views on the use of animal models in medical research. This is the result. I thank my generations of members of my tamoxifen teams who used laboratory models to transform ideas into lives saved. I thank Fadeke Agboke and Russell McDaniel for their invaluable assistance with this manuscript. This work (VCJ) was supported by the Department of Defense Breast Program under Award number W81XWH-06-1-0590 Center of Excellence; the Susan G Komen For The Cure Foundation under Award number SAC100009; GHUCCTS CTSA (Grant # UL1RR031975) and the Lombardi Comprehensive Cancer Center Support Grant (CCSG) Core Grant NIH P30 CA051008. The content is solely the responsibility of the author and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health. Additionally, the views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

REFERENCES

1 Geison GL. The Private Science of Louis Pasteur, First edn. ••: Princeton University Press, 1995.

2 Baumler E. Paul Ehrlich: Scientist for Life, First edn. ••: Holmes & Meier Pub, 1984; 288.

3 Black J. Drugs from emasculated hormones: the principle of syntopic antagonism. Science 1989; 245: 486–93.

4 Carter SB. Effects of cytochalasins on mammalian cells. Nature 1967; 213: 261–4.

5 Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 1967; 13: 101–19.

6 Barrett AM. Cardiac beta-adrenoceptor blockade: the quest for selectivity. J Pharmacol 1985; 16: (Suppl 2): 95–108.

7 Emanuel M, Rawlins M, Duff G, Breckenridge A. Thalidomide and its sequelae. Lancet 2012; 380: 781–3.

8 McBride WG. Thalidomide and congenital abnormalities. Lancet 1961; ••: 1358.

9 Dally A. Thalidomide: was the tragedy preventable? Lancet 1998; 351: 1197–9.

10 Greaves P, Goonetilleke R, Nunn G, Topham J, Orton T. Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. Cancer Res 1993; 53: 3919–24.

11 Early Breast Cancer Trialists' Collaborative Group. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Lancet 1992; 339: 71–85.

12 Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 1998; 351: 1451–67.

13 Davies C, Godwin J, Gray R, Clarke M, Cutter D, Darby S, McGale P, Pan HC, Taylor C, Wang YC, Dowsett M, Ingle J, Peto R. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 2011; 378: 771–84.

14 Jordan VC. What if tamoxifen (ICI 46,474) had been found to produce rat liver tumors in 1973? A personal perspective. Ann Oncol 1995; 6: 29–34.

15 Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007; 72: 7–25.

16 Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 1. Receptor interactions. J Med Chem 2003; 46: 883–908.

17 Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. J Med Chem 2003; 46: 1081–111.

18 Phillips DH. Understanding the genotoxicity of tamoxifen? Carcinogenesis 2001; 22: 839–49.

19 Schrek R. Fashions in cancer research. In: The Year Book of Pathology and Clinical Pathology, ed. •• ••. Chicago: The Year Book Publishers, 1959; 26–39.

20 Haddow A, Watkinson JM, Paterson E, Koller PC. Influence of synthetic oestrogens on advanced malignant disease. Br Med J 1944; 2: 393–8.

21 Haddow A, David A. Karnofsky memorial lecture. Thoughts on chemical therapy. Cancer 1970; 26: 737–54.

22 Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. Br J Cancer 1971; 25: 270–5.

23 Ingle JN, Ahmann DL, Green SJ, Edmonson JH, Bisel HF, Kvols LK, Nichols WC, Creagan ET, Hahn RG, Rubin J, Frytak S. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. N Engl J Med 1981; 304: 16–21.

24 Walpole AL, Paterson E. Synthetic oestrogens in mammary cancer. Lancet 1949; 2: 783–6.

25 Jordan VC. The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. Breast Cancer Res Treat 1988; 11: 197–209.

UNCORRECTED PROOF

26 Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 1990; 50: 4177–89.

27 Jordan VC. Tamoxifen as the first targeted long term adjuvant therapy for breast cancer. Endocr Relat Cancer 2014; ••: ••–••. [10]

28 Jordan VC. Fourteenth Gaddum Memorial Lecture. A current view of tamoxifen for the treatment and prevention of breast cancer. Br J Pharmacol 1993; 110: 507–17.

29 Jordan VC. Tamoxifen (ICI 46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 2006; 147 (Suppl. 1): S269–276.

30 Maximov PY, McDaniel RE, Jordan VC. Tamoxifen: pioneering medicine in breast cancer. In: Milestones in Drug Therapy, ed. •• ••. Basel: Springer, 2013; ••–••. [11]

31 Jordan VC, ed. Estrogen Action, Selective Estrogen Receptor Modulators and Women's Health: Progress and Promise. London: Imperial College Press, 2013.

32 Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 2009; 69: 1243–54.

33 Ward HW. Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J 1973; 1: 13–4.

34 McGuire WL, Carbone PP, Vollmer EP, eds. Estrogen Receptors in Human Breast Cancer. ••: Raven Press, 1975. [12]

35 Morgan LR, Jr, Schein PS, Woolley PV, Hoth D, Macdonald J, Lippman M, Posey LE, Beazley RW. Therapeutic use of tamoxifen in advanced breast cancer: correlation with biochemical parameters. Cancer Treat Rep 1976; 60: 1437–43.

36 Kiang DT, Kennedy BJ. Tamoxifen (antiestrogen) therapy in advanced breast cancer. Ann Intern Med 1977; 87: 687–90.

37 Huggins C, Grand LC, Brillantes FP. Mammary cancer induced by a single feeding of polymucular hydrocarbons, and its suppression. Nature 1961; 189: 204–7.

38 Welsch CW. Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. Cancer Res 1985; 45: 3415–43.

39 Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 1975; 11: 205–6.

40 Jordan VC. Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. Reviews on Endocrine-related Cancer. 1978; •• (October Supplement): 49–55. [13]

41 Jordan VC, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In: Adjuvant Therapy of Cancer II, eds Salmon S, Jones S. New York: Grune & Stratton Inc., 1979; 19–26.

42 Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 1980; 16: 239–51.

43 Jordan VC. Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethyl benzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 1974; 5: 354.

44 Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 1976; 12: 419–24.

45 Giovanella BC, Stehlin JS, Jr, Williams LJ, Jr, Lee SS, Shepard RC. Heterotransplantation of human cancers into nude mice: a model system for human cancer chemotherapy. Cancer 1978; 42: 2269–81.

46 Soule HD, Vazguez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 1973; 51: 1409–16.

47 Brooks SC, Locke ER, Soule HD. Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J Biol Chem 1973; 248: 6251–3.

48 Soule HD, McGrath CM. Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice. Cancer Lett 1980; 10: 177–89.

49 Shafie SM, Grantham FH. Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. J Natl Cancer Inst 1981; 67: 51–6.

50 Osborne CK, Hobbs K, Clark GM. Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res 1985; 45: 584–90.

51 Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987; 47: 4020–4.

52 Gottardis MM, Robinson SP, Jordan VC. Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumoristatic action of tamoxifen. J Steroid Biochem 1988; 30: 311–4.

53 Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 1988; 48: 5183–7.

54 Gottardis MM, Jiang SY, Jeng MH, Jordan VC. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res 1989; 49: 4090–3.

55 Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc 1987; 46: 1870–4.

56 Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 1988; 48: 812–5.

57 Harper MJ, Walpole AL. Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. Nature 1966; 212: 87.

58 Terenius L. Structure-activity relationships of anti-oestrogens with regard to interaction with 17-beta-oestradiol in the mouse uterus and vagina. Acta Endocrinol (Copenh) 1971; 66: 431–47.

UNCORRECTED PROOF

59 Jordan VC. Selective estrogen receptor modulation: a personal perspective. Cancer Res 2001; 61: 5683–7.

60 Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 1993; 127: 23–33.

61 Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, Jordan VC. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000; 6: 2028–36.

62 Chisamore MJ, Ahmed Y, Bentrem DJ, Jordan VC, Tonetti DA. Novel antitumor effect of estradiol in athymic mice injected with a T47D breast cancer cell line overexpressing protein kinase C alpha. Clin Cancer Res 2001; 7: 3156–65.

63 Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, Loweth J, McKian K, De Los Reyes A, Wing L, Jordan VC. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*. J Natl Cancer Inst 2003; 95: 1586–97.

64 Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 2003; 95: 1597–608.

65 Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, Jordan VC. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 2005; 97: 1746–59.

66 O'Regan RM, Gajdos C, Dardes RC, De Los Reyes A, Park W, Rademaker AW, Jordan VC. Effects of raloxifene after tamoxifen on breast and endometrial tumor growth in athymic mice. J Natl Cancer Inst 2002; 94: 274–83.

67 O'Regan RM, Osipo C, Ariazi E, Lee ES, Meeke K, Morris C, Bertucci A, Sarker MA, Grigg R, Jordan VC. Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. Clin Cancer Res 2006; 12: 2255–63.

68 Jordan VC, Dowse LJ. Tamoxifen as an anti-tumour agent: effect on oestrogen binding. J Endocrinol 1976; 68: 297–303.

69 Jordan VC, Jaspan T. Tamoxifen as an anti-tumour agent: oestrogen binding as a predictive test for tumour response. J Endocrinol 1976; 68: 453–60.

70 Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 1977; 75: 305–16.

71 Hardell L. Tamoxifen as risk factor for carcinoma of corpus uteri. Lancet 1988; 2: 563.

72 Jordan VC. Tamoxifen and endometrial cancer. Lancet 1988; 2: 1019.

73 Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, Skoog L, Somell A, Theve T, Wilking N *et al*. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1989; 1: 117–20.

74 Jordan VC. Tamoxifen and endometrial cancer. Lancet 1989; 1: 733–4.

75 Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987; 10: 31–5.

76 Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, Carbone PP, DeMets DL. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 1992; 326: 852–6.

77 Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, Norton L, Nickelsen T, Bjarnason NH, Morrow M, Lippman ME, Black D, Glusman JE, Costa A, Jordan VC. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple outcomes of raloxifene evaluation. JAMA 1999; 281: 2189–97.

78 Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER Jr, Wade JL 3rd, Robidoux A, Margolese RG, James J, Lippman SM, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 2006; 295: 2727–41.

79 Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER, Wade JL 3rd, Robidoux A, Margolese RG, James J, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N. Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: preventing breast cancer. Cancer Prev Res 2010; 3: 696–706.

80 Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A, Jamalabadi-Majidi S, Crowder R, Siegel BA. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 2009; 302: 774–80.

81 Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V, Abraham M, Medeiros Alencar VH, Badran A, Bonfill X, Bradbury J, Clarke M, Collins R, Davis SR, Delmestri A, Forbes JF, Haddad P, Hou MF, Inbar M, Khaled H, Kielanowska J, Kwan WH, Mathew BS, Mittra I, Muller B, Nicolucci A, Peralta O, Pernas F, Petruzelka L, Pienkowski T, Radhika R, Rajan B, Rubach MT, Tort S, Urrutia G, Valentini M, Wang Y, Peto R. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. Lancet 2013; 381: 805–16.

82 Anderson GL, Chlebowski RT, Aragaki AK, Kuller LH, Manson JE, Gass M, Bluhm E, Connelly S, Hubbell FA, Lane D, Martin L, Ockene J, Rohan T, Schenken R, Wactawski-Wende J. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. Lancet Oncol 2012; 13: 476–86.

83 Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, Tapia C, Kim HR, Yerrum S, Sharma CG, Nicolas E,

UNCORRECTED PROOF

Balagurunathan Y, Ross EA, Jordan VC. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci U S A 2011; 108: 18879–86.

84 Fan P, Griffith OL, Agboke FA, Anur P, Zou X, McDaniel RE, Creswell K, Kim SH, Katzenellenbogen JA, Gray JW, Jordan VC. c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. Cancer Res 2013; 73: 4510–20.

85 Fan P, Agboke FA, McDaniel RE, Sweeney EE, Zou X, Creswell K, Jordan VC. Inhibition of c-Src blocks oestrogen-induced apoptosis and restores oestrogen-stimulated growth in long-term oestrogen-deprived breast cancer cells. Eur J Cancer 2014; 50: 457–68.

86 Obiorah I, Sengupta S, Curpan R, Jordan VC. Defining the conformation of the estrogen receptor complex that controls estrogen-induced apoptosis in breast cancer. Mol Pharmacol 2014; 85: 789–99.

87 McCowan C, Wang S, Thompson AM, Makubate B, Petrie DJ. The value of high adherence to tamoxifen in women with breast cancer: a community-based cohort study. Br J Cancer 2013; 109: 1172–80.

88 Jordan VC. A(nother) scientific strategy to prevent breast cancer in postmenopausal women by enhancing estrogen-induced apoptosis? Menopause 2014; ••: ••–••.

89 Maximov PY, McDaniel RE, Fernandes DJ, Korostyshevskiy VR, Bhatta P, Muerdter T, Flockhart D, Jordan VC. Laboratory examination of CYP2D6 relevance in premenopausal breast cancer patients treated with tamoxifen. Br J Pharmacol. Submitted.

90 Brauch H, Schwab M. Prediction of tamoxifen outcome by genetic variation of CYP2D6 in post-menopausal women with early breast cancer. Br J Clin Pharmacol 2014; 77: 695–703.

91 Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. Clin Cancer Res 2003; 9: 1980–9.

92 Cummings SR, Ensrud K, Delmas PD, LaCroix AZ, Vukicevic S, Reid DM, Goldstein S, Sriram U, Lee A, Thompson J, Armstrong RA, Thompson DD, Powles T, Zanchetta J, Kendler D, Neven P, Eastell R. Lasofoxifene in postmenopausal women with osteoporosis. N Engl J Med 2010; 362: 686–96.

93 Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2003; 2: 205–13.

94 Allen KE, Clark ER, Jordan VC. Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 1980; 71: 83–91.

95 Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 1983; 32: 1031–6.

96 Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nat Rev Cancer 2007; 7: 46–53.

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SERMs for the treatment and prevention of breast cancer

Ramona F. Swaby · Catherine G. N. Sharma · V. Craig Jordan

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Abstract Tamoxifen and raloxifene are both selective estrogen receptor modulators (SERMs). The medicines can block estrogen mediated breast cancer growth and development but will also maintain bone density in postmenopausal women and lower circulating cholesterol. Tamoxifen has remained the antihormonal therapy of choice for the treatment of ER positive breast cancer for the last 30 years. However, although adjuvant tamoxifen produces profound increases in disease-free and overall survival in patients with ER positive breast cancer, concerns about drug resistance, blood clots and endometrial cancer have resulted in a change to the use of aromatase inhibitors for the treatment of postmenopausal women. Nevertheless, tamoxifen remains the antihormonal treatment of choice for premenopausal women with ER positive breast cancer and for risk reduction in premenopausal women who are at high risk for developing breast cancer. The risk of endometrial cancer and thromboembolic disorders during tamoxifen therapy is not elevated in premenopausal women. It is important to note that aromatase inhibitors or raloxifene should not be used in premenopausal women. Raloxifene is used to prevent osteoporosis in postmenopausal women and, unlike tamoxifen, does not increase the risk of endometrial cancer. However, raloxifene does reduce breast cancer risk by 50–70% in both low risk and high risk postmenopausal women. Comparisons of raloxifene with tamoxifen show equal efficacy as a chemopreventive for breast cancer but there is a reduction in thromboembolic disorders, fewer endometrial cancers, hysterectomies, cataracts and cataract surgeries in women taking raloxifene. Overall, SERMs continue to fulfill their promise as appropriate medicines that target specific populations for the treatment and prevention of breast cancer.

Keywords Tamoxifen · Raloxifene · Estrogen receptor · Selective estrogen receptor modulator · Osteoporosis · Endometrial cancer

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1 Introduction

Schinzinger [1, 2] first proposed, whereas Beatson [3, 4] first reported, performing oophorectomy for the treatment of metastatic breast cancer in 1896. It has now become accepted that ovarian hormones, particularly estrogen, are central to the development of breast cancer. Laboratory evidence identified estrogen as the trophic hormone in estrogen target tissues (e.g. the uterus and some breast cancers) [5] so naturally "anti-estrogen" therapy became a central theme for the treatment and now prevention of breast cancer [6] One medicine, tamoxifen [7], originally classified as a nonsteroidal antiestrogen [8] but now reclassified as a selective estrogen receptor modulator (SERM) [9] has proved to be a pioneering intervention that not only produced dramatic survival advantages when used as an adjuvant therapy [10] but also became the first chemopreventive for any cancer [11, 12]. However, the recognition of SERM action [13, 14] actually opened the door to new opportunities in therapeutics and advanced the idea of multifunctional medicines to address a number of prevention issues pertinent to postmenopausal women's health. Osteoporosis is a major health care problem but emerging information about the inappropriateness of long-

term hormone replacement therapy (HRT) to prevent osteoporosis has acted as a catalyst for the development of new, safer SERMs.

In the United States alone, approximately 90 million prescriptions for HRT were dispensed annually from 1999 through 2002 [15]. Indeed, records suggest that hormonal replacement therapy was the most commonly prescribed medicine in the world during the late 1990s and early 2000s [16]. Despite epidemiologic data suggesting the overwhelming benefits of HRT, data regarding hormonal therapy use and breast cancer incidence were unconfirmed. Therefore, as part of the Women's Health Initiative (WHI), a large randomized controlled primary prevention trial to determine the risk benefit ratio of HRT in postmenopausal women was undertaken. In July of 2002, the principal results from the WHI study examining the effects of HRT were reported [17]. In this trial in which approximately 16,000 women were treated either with estrogen/progesterone combination HRT or placebo, an approximately 26% increase in the incidence of breast cancer was detected among the women treated with HRT. This data was subsequently confirmed and extended in the Million Women Study [18]. The Million Women Study, while not a randomized prospective clinical trial, followed cohorts of post-menopausal women during the same time frame as the WHI and collected information about their use of HRT. These cohorts were followed for cancer incidence and any death due to breast cancer. The overall conclusion was that users of HRT were more likely than never users of HRT to develop breast cancer and die from it [18]. The profound excess of new breast cancers that accumulated populations of women taking 5 or 10 years of HRT is illustrated in Fig. 1. As soon as these data were reported, the use of HRT dropped dramatically both within the United States and in Europe [15, 19].

Recently, a 7% decrease in the age-adjusted incidence of breast cancer has been observed from 2002–2003 [20]. This decline, not attributable to changes in mammography screening, represents a decline of approximately 14,000 breast cancer cases in the United States in 2003 when compared with 2002. The effect was found to be important for women age 50 or greater and specifically, statistically significant for women aged 50–74. Most importantly, this effect was essentially confined to hormonally responsive breast cancers. While these data do not speak to the initiation and development of breast cancers, the time course suggests that estrogen may play a role in propagating sub-clinical ER breast cancers that in a less estrogenic environment may have remained sub-clinical and/or eliminated through the body's usual tumor surveillance system. Clearly it would be advantageous to have targeted specific agents to treat and ultimately prevent breast cancer.

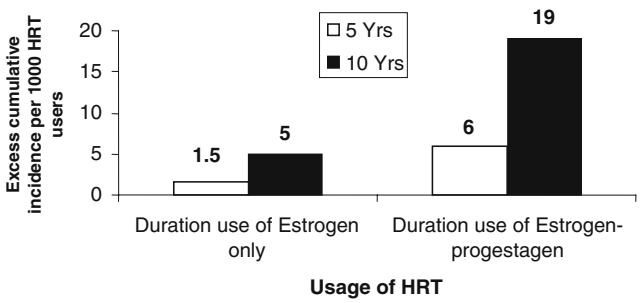

Fig. 1 Diagramatic representation of the estimated cumulative incidence of breast cancer in 1,000 postmenopausal women taking hormone replacement therapy (HRT) in excess of the incidence observed in women not taking HRT. The Million Women's Study [18] compared and contrasted women using estrogen only preparations for 5 or 10 years with those women taking a combination of estrogen and a progestin for the same period

The story of SERM recognition and development [21, 22] has its origins in the study of tamoxifen (ICI 46,474) a drug originally discovered at the laboratories of ICI Pharmaceuticals Division, UK, in their fertility control program [23] as a potential post coital contraceptive. The drug failed in its primary application but slowly succeeded in a secondary application as a treatment for breast cancer [7, 24].

2 Tamoxifen, the first SERM

Tamoxifen is a pioneering medicine [7] because it became one of the first targeted treatments for cancer where the treatment strategy used today translated from the laboratory to clinical practice. The pharmacology of tamoxifen was studied extensively in animal models of mammary carcinogenesis to explore appropriate strategies to enhance disease control in patients. Tamoxifen was found to inhibit binding of estrogen to the ER mammary carcinomas both *in vitro* and *in vivo* [25–27]. *In vitro*, tamoxifen was demonstrated to have low affinity for the estrogen receptor [28], however, tamoxifen acts as a prodrug and is rapidly converted in the liver to a metabolite with high affinity to block the ER [29]. Tamoxifen, as well as its active metabolites, achieve stable, steady-state levels within the serum that remain constant during treatment ranging from months to years (over 7 years) [30].

An examination of tamoxifen treatment during the early stages of tumorigenesis in the rat mammary carcinoma model demonstrated that longer rather than shorter durations of tamoxifen would be necessary to use as a strategy for the adjuvant treatment of breast cancer [31–33] However, there was initial concern that long-term adjuvant tamoxifen would cause premature drug resistance. Nevertheless, clinical trail strategies eventually explored the optimal duration for tamoxifen therapy. It is now possible to assess the value of the idea of targeting tamoxifen to treat

women with ER positive tumors with long-term therapy. The Oxford Overview Analysis has established treatment trends based on the results from worldwide randomized clinical trials.

When the Overview analyses were first initiated, tamoxifen was the only universally used antihormonal agent. With no other competition, tamoxifen became the "gold standard" and established the principles of tumor targeting and identified the appropriate treatment strategy to aid survivorship [10, 34–36].

- Five years of adjuvant tamoxifen enhances disease free survival. There is a 50% decrease in recurrences observed in ER positive patients 15 years after diagnosis.
- Five years of adjuvant tamoxifen enhances survival with a decrease in mortality 15 years after diagnosis.
- Adjuvant tamoxifen does not provide an increase in disease free or overall survival in ER negative breast cancer.
- Five years of adjuvant tamoxifen alone is effective in premenopausal women with ER positive breast cancer; tamoxifen is ineffective in ER negative breast cancer.
- The benefits of tamoxifen in lives saved from breast cancer, far outweighs concerns about an increased incidence of endometrial cancer in postmenopausal women.
- Tamoxifen does not increase the incidence of second cancers other than endometrial cancer.
- No non-cancer related overall survival advantage is noted with tamoxifen when given as adjuvant therapy.

The Overview analysis process is now being applied to the numerous new aromatase inhibitors [6] that are being compared to tamoxifen directly, after a few years of tamoxifen or after a full five years of tamoxifen (Fig. 2). As a group, the aromatase inhibitors are superior to tamoxifen with improved overall survival and a reduced incidence of estrogen-like side effects.

Once antihormonal therapy had started to achieve optimal success in the treatment of node positive and node negative disease during the last decade, the trend for clinical research during the 1990s was to build on the successes of SERMs as treatments for disease so that breast incidence could be reduced in specific populations of women.

3 Tamoxifen and primary prevention

Early laboratory observations [37, 38] plus the finding that tamoxifen decreases contralateral breast cancer by 50% when the drug is used as an adjuvant therapy [39], made tamoxifen the agent of choice for evaluation as a chemo-preventive agent. A series of clinical trials aimed at primary

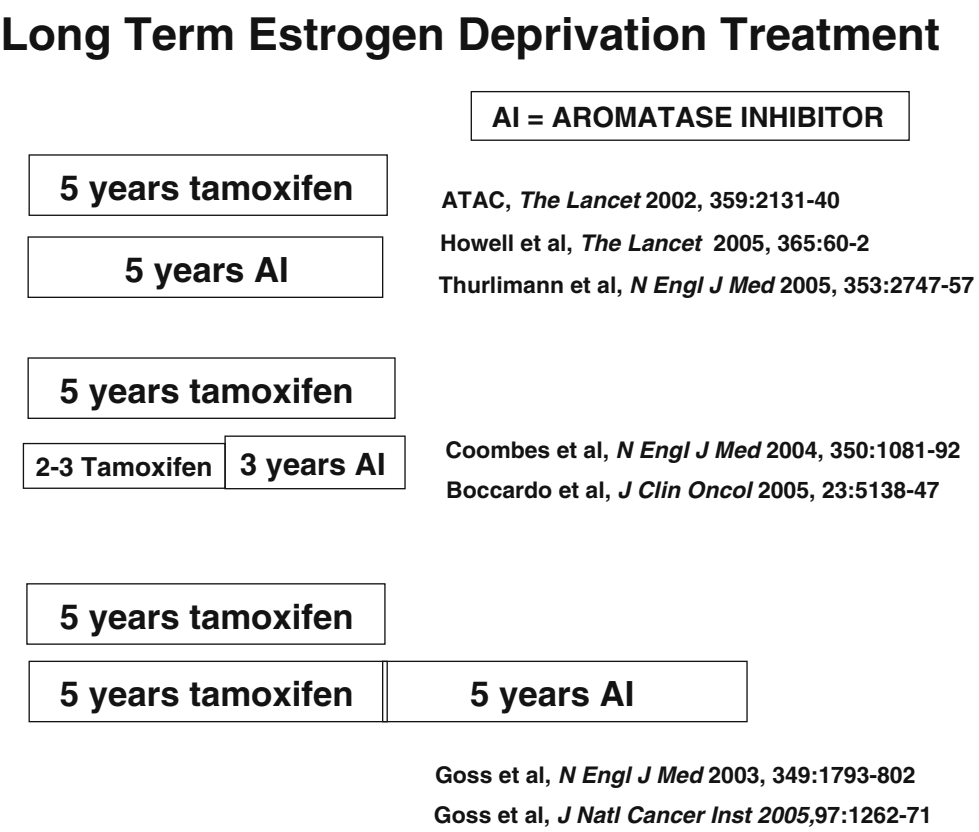

Fig. 2 A comparison of the strategies used to compare and contrast the therapeutic efficacy and side effects of various aromatase inhibitors with adjuvant tamoxifen in populations of postmenopausal women diagnosed with ER positive breast cancer

breast cancer prevention established tamoxifen as the first drug to be approved for risk reduction of any cancer. The trials have been compared and contrasted [40] so only the conclusions will be considered after presenting the two main studies.

The National Surgical Adjuvant Breast and Bowel Project (NSABP) initiated the Breast Cancer Prevention Trial (P-1) in 1993 [11]. Approximately 13,000 pre and postmenopausal women were recruited because they were at high risk for developing breast cancer either due to age close to the peak incidence age of breast cancer, a high Gail score [41], or that had a history of lobular carcinoma *in situ*. The volunteers were randomized to receive placebo or 5 years of tamoxifen at the previously established daily dose of 20 mg/day. Tamoxifen produced a 49% (two-sided $p<0.0001$) decrease in the development of invasive breast cancers and a 50% (two-sided $p<0.002$) decrease in the development of non-invasive breast cancers. This effect was restricted to ER positive tumors (a 69% reduction), with no effect on the development of ER negative tumors [11]. The NSABP P-1 clinical trial was important in that it once again confirmed the requirement of the ER in a tumor for tamoxifen to be effective. The NSABP P-1 Trial, of all the prevention clinical trials, was the only one that did not incorporate the use of HRT in either of the trial arms. Allowing for the use of HRT in other prevention clinical trials may explain the blunted efficacy results when compared to the NSABP P-1 trial.

The International Breast Cancer Intervention Study (IBIS-I) was an international phase III chemoprevention trial comparing tamoxifen vs. placebo [42]. This trial enrolled approximately 7,000 pre-and post-menopausal women recruited on several continents. Their age was between 35–70 years prospectively determined to be at increased risk for breast cancer development [42]. Risk factors for breast cancer included at least a two-fold relative risk for patients ages 45–70 years, a four-fold relative risk for ages 40–44 and an approximately ten-fold relative risk for ages 35–39. Therefore, almost all participants (97%) had a family history of breast cancer. Approximately one-third of all patients used HRT while being treated on this clinical trial. At a median follow-up of 50 months, a 32% reduction in the development of breast cancers was documented (69 vs. 101, $p=0.01$). The risk reduction was demonstrated among the occurrence of both invasive (25% reduction, 64 vs. 85) and non-invasive breast cancers (69% reduction, 5 vs. 16), although these subset analyses did not achieve statistical significance. There was no reduction in the occurrence of ER negative breast cancers.

Taken together, the above data supports the conclusion that tamoxifen lowers the risk of developing ER-positive breast cancer in patients without a personal history of breast cancer, but that are at higher risk for the development of breast cancer due to genetic and/or other established risk factors. The prevention of breast cancer comes at the expense of well documented side effects, including an approximately 2–5 fold increase in uterine cancer [12, 42], and an approximately 2–3 fold increase in thromboembolic disease but only in postmenopausal women. In addition to increased menopausal symptoms, vaginal discharge and ocular abnormalities occur with tamoxifen These definitive clinical trial data suggest that chemoprevention with tamoxifen should focus on high risk premenopausal women [43]. It is anticipated, based on the Overview Analysis [36] 5 years of treatment will be followed by continuing protection for the following 10 years. Naturally, once tamoxifen treatment is stopped, menopausal symptoms will stop but the problem is whether women will wish to tolerate 5 years of tamoxifen. Solutions to the problem of compliance have focused on the selective serotonin reuptake inhibitors (SSRIs) (Fig. 3) but recent studies of the metabolism of tamoxifen have revealed important lessons that can potentially refine current chemoprevention strategies.

4 Refining treatment and prevention with tamoxifen

Alterations in the cytochrome P450 system impact upon tamoxifen metabolism and its efficacy. Tamoxifen metabolites have been recognized to have antiestrogenic activity [44, 45]. More recently, the cytochrome P450 2D6 (CYP2D6) metabolic pathway was shown to be important in the production of the tamoxifen metabolite, 4-hydroxy-N-desmethyl-tamoxifen (endoxifen (Fig. 4)). Endoxifen has similar potency to 4-hydroxy tamoxifen [46], but an approximately ten-fold higher circulating concentration than 4-hydroxy-tamoxifen [45]. Therefore, if cytochrome CYP2D6 is metabolically inactivated due to genetic variants of this particular phenotype or through inhibition of the 2D6 enzyme from use of concomitant medications that inhibit CYP2D6, tamoxifen cannot be metabolized to its active metabolites, resulting in diminished efficacy. Jin et al. [47] examined plasma endoxifen concentrations in healthy women 4 months of beginning adjuvant tamoxifen therapy. Endoxifen concentrations in the blood were found to be statistically significantly lower in patients with a CYP2D6 homozygous or heterozygous variant genotype when compared to homozygous wild-type genotype. Similarly low concentrations of endoxifen were also identified within this same cohort of patients among subjects using concomitant potent inhibitors of CYP2D6 such as paroxetine (Fig. 3). Such diminished endoxifen levels have recently been demonstrated to correlate with worse clinical outcome [48]. SSRIs are commonly prescribed to women taking tamoxifen for the treatment of associated hot flushes but the SSRIs range from potent to mild inhibitors of the

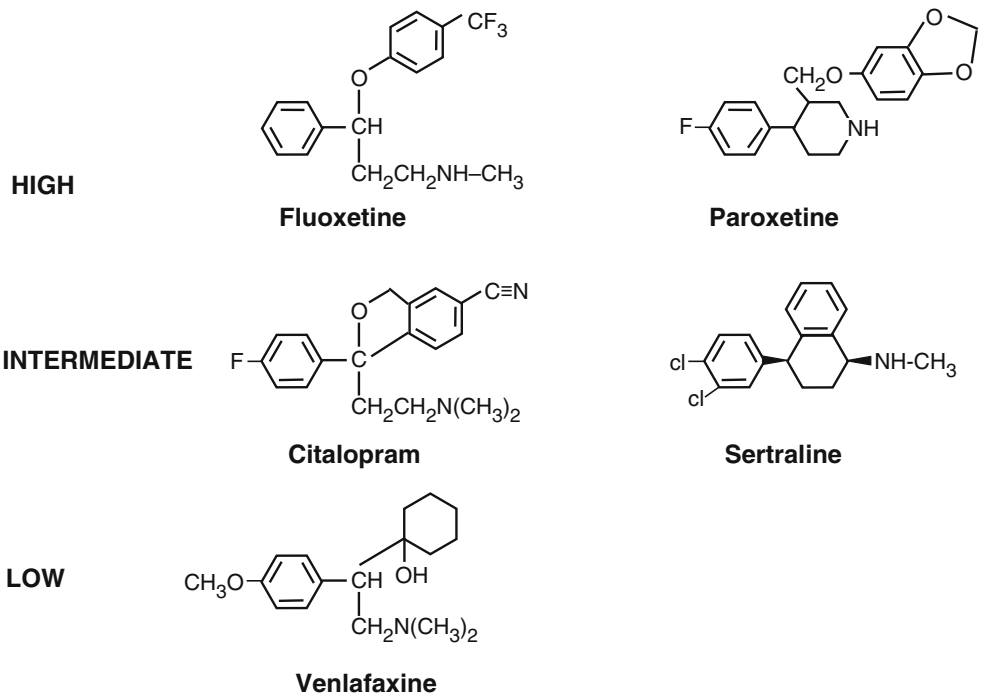

Fig. 3 The classification of selective serotonin reuptake inhibitors (*SSRIs*) used for the relief of hot flashes in women being treated with tamoxifen. The SSRIs have high, intermediate or low affinity for the CYP2D6 gene that metabolizes tamoxifen or N-desmethyl-tamoxifen to 4-hydroxytamoxifen or endoxifen respectively (See Fig. 4)

CYP2D6 cytochrome enzymes (Fig. 3). To determine whether this knowledge has clinical relevance, a retrospective analysis was performed on a North Central Cancer Treatment Group (NCCTG) randomized phase III clinical trial [48]. In this trial, postmenopausal women with ER-positive breast cancer were originally randomized to adjuvant treatment with either tamoxifen for 5 years or tamoxifen for 5 years followed by an additional year of fluoxymestrone (NCCTG 89-30-52). Paraffin embedded tumor samples from the tamoxifen only arm were genotyped for CYP2D6 wildtype and polymorphisms. Additionally, utilizing chart review, use of SSRIs was also evaluated with respect to relapse-free survival (RFS), disease-free survival (DFS) and overall survival (OS). In a multivariate analysis, patients homozygous for CYP2D6 variant (CYP2D6*4/*4) trended towards worse RFS (HR, 1.85; P=0.176) and DFS (HR, 1.86; P=0.089), without affecting OS (HR 1.12; P=0.780) compared to patients heterozygous for the CYP2D6 variant (CYP2D6 *4/4) or had wild-type CYP2D6 (CYP2D6 4/4). Additionally, the symptoms of moderate and severe hot flashes segregated with patients who were found to have the CYP2D6 *4/*4 homozygous gene polymorphism [48].When these data (NCCTG 89-30-52) were re-analyzed to include evaluation of concomitant CYP2D6 inhibitor use, multivariate analysis revealed that patients with significantly decreased tamoxifen metabolism due to either homozygous CYP2D6 *4/*4 variant genotype or due to concomitant use of an extensive CYP2D6 inhibitor, had a statistically significantly worse RFS (HR, adj=1.71, p=0.017) with a statistically significant risk of breast cancer relapse (HR 3.12, p=0.007) [49]. This suggests that in order to individualize therapy for premenopausal women with ER-positive early stage breast cancers, tamoxifen might be best for patients homozygous wildtype for CYP2D6 genotype and for those not requiring SSRI's for the treatment of hot flashes. Alternatively, Venlafaxine, which has low interaction with CYP2D6, could be used to control hot flashes. Alternative therapies such as the newer aromatase inhibitors might be considered, for example, for postmenopausal patients with diminished endoxifen metabolism either due to CYP2D6 genotyping or need for utilizing SSRIs for hot flush symptom management [50].

5 Recognition of selective estrogen receptor modulations

The recognition of SERM action and the realization that nonsteroidal antiestrogens were, in fact, target site specific estrogens and antiestrogens arose from the pharmacological evaluation of tamoxifen during the transition from breast cancer treatment to chemoprevention in the mid 1980s. It was reasoned that if estrogen was beneficial for maintaining bone density in postmenopausal women then perhaps the long-term administration of tamoxifen to women without cancer might prevent breast cancer but accelerate the development of osteoporosis. However, the finding that tamoxifen and the related compound raloxifene (then known as keoxifene) would prevent bone loss in ovariectomized rats [51–53] at doses that would prevent rat mammary carcinogenesis [32, 54] changed that perspective. More importantly, the simultaneous findings that tamoxifen

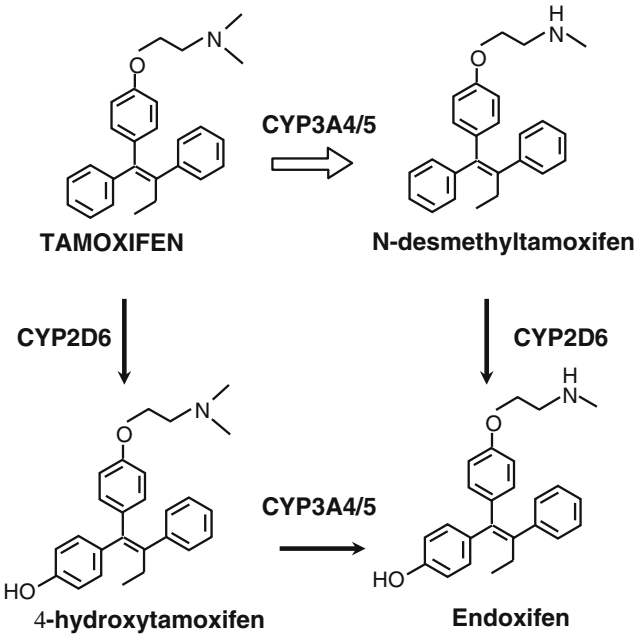

Fig. 4 The principal metabolites of tamoxifen observed in the serum of women receiving adjuvant tamoxifen therapy. Demethylation occurs through CYPA3/4 and aromatic hydroxylation through CYP2D6. The SSRIs (Fig. 3) block the metabolic activation of tamoxifen by binding to CYP2D6

could prevent estrogen-stimulated breast cancer growth but, at the same time, enhance the growth of the uterus or endometrial cancer [55, 56] rapidly translated to clinical practice with the finding that postmenopausal patients being treated with tamoxifen had an increased risk of developing endometrial cancer [57, 58]. This translational research resulted in gynecologists becoming involved in cancer care and safety procedures were established to avoid the progression of endometrial carcinoma stimulated to grow by tamoxifen. It was also reasoned that SERMs had opposing action in the uterus and breast and this translated to patients, why not translate the possibility of using SERMs to prevent breast cancer by treating osteoporosis?

6 The concept

A plan to prevent breast cancer as a public health initiative was initially described at the First International Chemoprevention meeting in New York in 1987. It is reasonable to simply state the proposal, published from the 1987 meeting and subsequently refined and presented at the annual meeting of the American Association for Cancer Research in San Francisco in 1989.

"The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused upon the identification of a population of high risk women to test "chemopreventive" agents. But, are resources being used less than optimally? An alternative would be to seize upon the developing clues provided by an extensive clinical investigation of available antiestrogens. Could analogs be developed to treat osteoporosis or even retard the development of atherosclerosis? If this proved to be true then a majority of women in general would be treated for these conditions as soon as menopause occurred. Should the agent also retain antibreast tumor actions then it might be expected to act as a chemosuppressive on all developing breast cancers if these have an evolution from hormone dependent to hormone independent disease. A bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century [13]." The concept was refined by 1990 [14] "We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer." This concept is exactly what has been translated to clinical practice [59, 60]: use a SERM (raloxifene) to treat osteoporosis and reduce the incidence of breast cancer as a beneficial side effect.

7 The SERM concept into practice

The Multiple Outcomes of Raloxifene (MORE) clinical trial was a multicenter, randomized, placebo controlled clinical trial utilizing raloxifene or placebo for the prevention of osteoporosis as its primary endpoint [59, 61, 62]. One of the multiple outcomes evaluated in this clinical trial was the secondary endpoint of breast cancer incidence. Therefore, post-menopausal women who met the criteria for diagnosis of osteoporosis were randomized in a 2:1 ratio to treatment with either of two doses of raloxifene—60 or 120 mg, or placebo. This population was an older population as the mean age of participants was approximately 66 years of age with over 80% aged 60 or older. Approximately 12% of trial subjects reported a first-degree relative with breast cancer. Additionally, approximately 29% of women reported previous HRT use at baseline and approximately 12% of women used HRT while being treated. In this population, raloxifene use was associated

with a 72% reduction in the incidence of invasive breast cancer (RR=0.28, 95% CI 0.17, 0.46) without significant impact on the incidence on *in situ* disease (nine vs. five cases for raloxifene and placebo, respectively, RR=0.90, 95% CI=0.30, 2.69). Of note, raloxifene had not effect upon the incidence of invasive estrogen receptor negative tumors (RR 1.13, 95% CI 0.35, 3.66).

In the Continuing Outcomes Relevant to Evista (CORE) trials, the chemopreventive effect of raloxifene were substantiated. This trial was essentially an extension of the MORE trial above for an additional 4 years of evaluation of the effect of extended raloxifene therapy [60]. Patients initially assigned to either 60 or 120 mg of treatment with raloxifene after the 4 years of the MORE trial were offered to continue raloxifene therapy with 60 mg of raloxifene (with the exception of patients still enrolled in the CORE trial assigned to 120 mg of raloxifene, i.e. less than 4 years of treatment). Similarly, patients initially assigned to the placebo arm of the MORE trial were continued on placebo. During the additional 4 years of evaluation, the continued use of raloxifene was associated with an approximately 59% reduction in the incidence of invasive breast cancer when compared to placebo (HR= 0.41, 95% CI −0.24–0.71) and a 66% reduction in the incidence of ER positive breast cancers (HR=0.34, 95% CI=0.18–0.66). Again, no protective effect was demonstrated in the development of ER negative breast cancers or *in situ* breast cancer. Over the 8 year period of evaluation from both the MORE data as well as the CORE data, raloxifene was demonstrated to reduce newly diagnosed invasive breast cancers by approximately 66% in total, when compared to placebo (HR=0.34, 95% CI= 0.22–0.50). This translated into an approximately 76% reduction in the relative occurrence of ER positive breast cancers (HR=0.24, 95% CI −0.15) with no resulting effect on ER negative breast cancers and *in situ* breast cancers, essentially providing confirmation of the earlier MORE trial results.

Based on analysis from the MORE trial evaluating cardiovascular risk, the Raloxifene Use for The Heart (Ruth) trial was undertaken with prevention of cardiac events and incidence of new breast cancer diagnosis as the primary objectives [63]. Approximately, 10,000 post-menopausal women with diagnosed coronary heart disease (CHD) or who were determined to be at risk for the development of CHD due to known risk factors such as diabetes mellitus, tobacco smoking and hypertension were randomized to treatment with either raloxifene 60 mg or placebo. Although raloxifene demonstrated no significant benefit for preventing primary coronary events in this patient population, (HR=0.95, 95% CI=0.84–1.07), a reduction in the development of invasive breast cancer was demonstrated. Once again, raloxifene use of approximately 5 years was associated with a 44% reduction (HR= 0.56, 95% CI=0.38–0.83) in the incidence of invasive breast cancer with treatment effect limited to ER positive breast cancers only. It is worthy to note that in this trial, analysis of breast cancer risk was performed and the preventative effect of raloxifene was also limited to patients at higher risk for developing breast cancer with a Gail score of 1.66 or higher. Most importantly, there was no increase in the risk of endometrial cancer confirming preclinical reports that raloxifene was substantially less effective than tamoxifen at stimulating endometrial cancer growth [64]. The final evaluation of raloxifene that will be presented is the chemoprevention of breast cancer growth determined in high risk postmenopausal women. The comparator medicine was tamoxifen.

8 Raloxifene and primary prevention

Patients were recruited into the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) trial from July 1, 1999 through November 4, 2004 [65]. This clinical trial randomizing patients to treatment with either tamoxifen or raloxifene for the primary prevention of breast cancer enrolled postmenopausal patients between the ages 35 and older, deemed to be at higher risk for the development of a first invasive breast cancer (the study primary endpoint) with either a 5 year predicted breast cancer risk of 1.66% based on the Gail model, or a previous history of lobular carcinoma *in situ* (LCIS) treated by local excision alone. It is worth noting that 19% of participants reported a family history of breast cancer in two or more first-degree relatives, and more than 71% reported a history of invasive breast cancer in one or more first-degree relative. Therefore, the mean predicted 5-year risk of developing breast cancer among the study population was 4.03% (SD, 2.17%). The primary endpoint of this randomized, double-blinded trial was the development of a first invasive breast cancer. Secondary endpoints also prospectively analyzed include, *in situ* breast cancer, endometrial cancer, all other cancers, cardiovascular disease, stroke, pulmonary embolism, DVT, transient ischemic attack, osteoporotic fracture, cataracts, death, and quality of life. The data was reported at a median follow-up time of 3.9 years. Both raloxifene and tamoxifen were equally effective at preventing the development of a first invasive breast cancer (RR 1.02; 95% CI, 0.82–1.28, p= 0.96). However, although not statistically significant, tamoxifen was better at preventing the occurrence of *in situ* breast cancers (57 vs. 80 for tamoxifen and raloxifene, respectively, p=0.052). This result is somewhat curious since the same mechanisms that would prevent an invasive breast cancer from developing could be expected to prevent

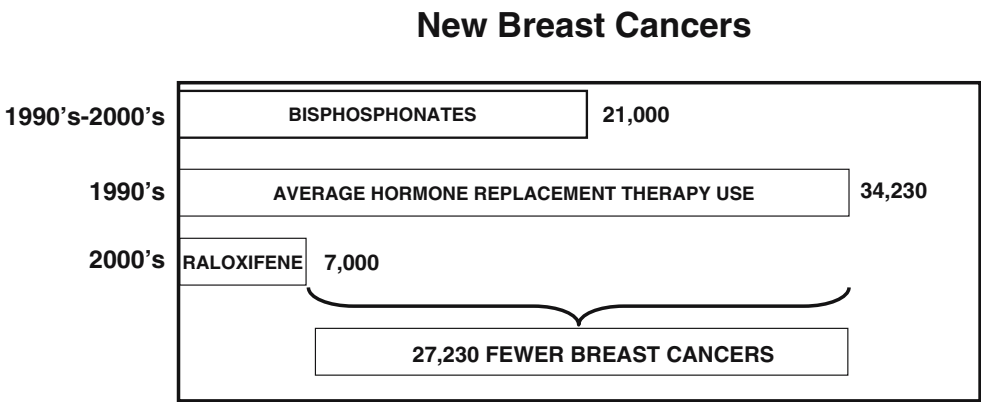

Fig. 5 An estimation of breast cancer incidence in a population of 500,000 postmenopausal women with the same risk for osteoporotic fractures as participants in the CORE trial [60] treated for a 10 year period with a bisphosphonate, hormone replacement therapy (HRT) based on the average breast cancer risk between the Women's Health Initiative [17] and the Million Women's Study [18] or currently with raloxifene. The overall change in prescribing practices from the former practice of using HRT to prevent osteoporosis as the standard treatment to the current practice of prescribing raloxifene would be anticipated to produce a net decrease of 27,230 breast cancers. (Reprinted with permission from the European Journal of Cancer [43])

in situ breast cancers. However, this finding has been reported previously in both the MORE and CORE studies where raloxifene did not appear to reduce the risk of non-invasive breast cancers, although both studies had small numbers of total events. This trial confirmed that raloxifene was less stimulatory for the uterus with less uterine hyperplasia (RR, 0.16; 95% CI, 0.09–0.29) and although there were more reported cases of uterine cancer with tamoxifen (36 vs. 23 cases), this did not reach statistical significance (RR. 0.62; 95% CI, 0.35–1.08). Higher rates of thromboembolic disease were reported for tamoxifen with 30% less events occurring in the raloxifene treated subjects

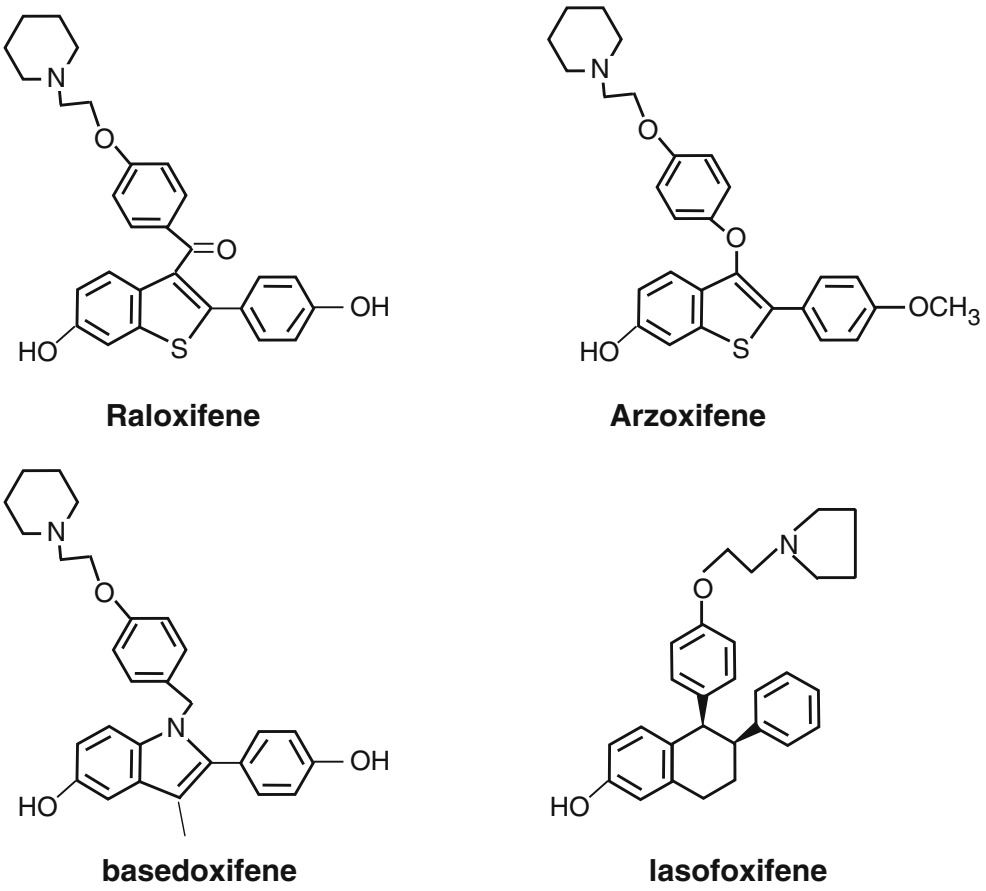

Fig. 6 A comparison of the structure of raloxifene with newer SERMs under development for the prevention of osteoporosis but with the potential to reduce the incidence of breast cancer as a beneficial side effect. Arzoxifene has a longer biological half life than raloxifene. Basedoxifene [74] and lasofoxifene [75] are two SERMs completing evaluation for the treatment of osteoporosis with the expectation that breast cancer incidence will be reduced

(RR, 0.70; 95% CI, 0.54–0.91). Additionally, higher rates of both cataract development (p=0.002) and patients undergoing cataract surgery (p=0.03) were higher in the tamoxifen arms. No difference in the rates of cardiovascular disease endpoints were reported. Interestingly, numerically there were higher numbers of unrelated cancers reported in the raloxifene arm. However, the overall numbers were small and the confidence intervals were wide suggesting that chance cannot be excluded as a possible cause. This clinical trial has now provided clinicians and post-menopausal patients with two viable options for primary prevention of breast cancer.

9 Direct and indirect approaches to chemoprevention

SERMs have proved to be valuable chemopreventive therapies to reduce the risk of breast cancer in both premenopausal (tamoxifen) and postmenopausal (tamoxifen and raloxifene) high risk women [66]. The approach to prevent the development of disease can be described as the direct approach for breast cancer chemoprevention. However, the changing fashion in restricting the application of HRT because of the definitive evidence that HRT increases the global incidence of breast cancer [18], and a decrease in HRT users will undoubtedly result in a fall in the incidence of breast cancer. If the availability of raloxifene to substitute for HRT for the prevention of osteoporosis is added into the equation, causing a reduction in breast cancer risk, then the SERMs will have gone some way in advancing the goal of reducing breast cancer incidence and mortality. The hypothetical benefits of the progress made in the past two decades in the chemoprevention of breast cancer are shown in Fig. 5. However, raloxifene is not an optimal drug for the prevention of breast cancer and osteoporosis. There are problems with both drug absorption and rapid Phase II metabolism [67]. In response, newer SERMS are now positioned (Fig. 6) to complete testing for the prevention of osteoporosis [68] and it is anticipated that they will also be a reduction in breast cancer incidence.

In closing, it is perhaps pertinent to state the current changes in the options for women's health that have occurred with the introduction of SERMs. Two decades ago, the concept [13] that SERMs could be useful multifunctional medicines has now become a clinically validated reality. During the past decade, there have been important changes in the evolution of ideas about women's health. HRT does not provide an easy solution to prevent coronary heart disease, osteoporosis and Alzheimer's disease. The WHI [17, 69–72] and the Million Women's Study [18] have defined the price to be paid with no decreases in coronary heart disease in the elderly, increases in breast cancer and modest but significant increases in Alzheimer's disease. There are suitable alternatives to the prevention of osteoporosis using bisphosphonates [73] but this intervention does not affect breast cancer or coronary heart disease. Statins have proven to be effective in retarding the development of arteriosclerosis and coronary heart disease. There is, however, no firm prospective evidence that these medicines reduce the incidence of breast cancer. In contrast, SERMs such as raloxifene can reduce the risk of osteoporosis and breast cancer. Admittedly raloxifene did not fulfill the promise to reduce the risk of coronary heart disease in the Raloxifene use for the Heart (RUTH) trial [63] but it is fair to say that the menu of medicines now available to prevent diseases that develop after menopause have steadily improved the prospects retarding disease development over the past 20 years.

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References

1. Schinzinger A. Ueber carcinoma mammae [abstract] 18th Congress of the German Society for Surgery. Beilage zum Centralblatt fur Chirurgie 1889;16:55–6.
2. Schinzinger A. Ueber carcinoma mammae [abstract].18th Kongress, Berlin, April 24–27, 1889. Berlin (Germany): Hirschwald. Verhandlungen der Deutschen Gesellschaft fur Chirurgie 1889;28.
3. Beatson GT. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. Lancet 1896;2:104–7.
4. Beatson CT. On treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. Lancet 1896;2:162–5.
5. Jensen EV, Jacobson HI. Basic guides to the mechanism of estrogen action. Recent Progr Hormone Res 1962;18:387–414.
6. Jordan VC, Brodie AMH. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007;72:7–25.
7. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nature Reviews Drug Discovery 2003;2:205–13.
8. Jordan VC. Biochemical pharmacology of antiestrogen action. Pharmacol Rev 1984;36:245–76.
9. Jordan VC. Selective estrogen receptor modulation: a personal perspective. Cancer Res 2001;61:5683–7.
10. EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 1998;354:1451–67.
11. Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998;90:1371–88.

12. Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 2005;97:1652–62.
13. Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 1988;6:589–95.
14. Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. Cancer Res 1990;50:4177–89.
15. Hersh AL, Stefanick ML, Stafford RS. National use of postmenopausal hormone therapy. JAMA 2004;291:47–53.
16. Riggs BL, Hartmann LC. Selective Estrogen-Receptor Modulators—Mechanisms of Action and Application to Clinical Practice. N Engl J Med 2003;348:618–29.
17. Writing Group for the Women's Health Initiative Investigators, Rossouw JEAG, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women. JAMA 2002;288:321–32.
18. Million Women Study Collaborators. Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet 2003;362:419–27.
19. Faber A, Bouvy ML, Loskamp L, van de Berg PB, Egberts ACG, de Jong-van den Berg LTW. Dramatic change in prescribing of hormonal replacement therapy in the Netherlands after publication of the Million Women Study: a follow-up study. Br J Clin Pharmacol 2005;60:641–7.
20. Ravdin PM, Cronin KA, Howlander N, Chlebowski RT, Berry DA. A sharp decrease in breast cancer incidence in the United States in 2003. Breast Cancer Res and Treatment Breast Cancer Research & Treatment 100 Supplement 1,S6:59, 2003.
21. Jordan VC. The science of selective estrogen receptor modulators: concept to clinical practice. Clin Cancer Res 2006;12:5010–3.
22. Jordan VC. SERMs: meeting the promise of multifunctional medicines. (Commentary). J Natl Cancer Inst 2007;99:350–6.
23. Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 1967;13:101–19.
24. Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. an early clinical appraisal of ICI 46474. Br J Cancer 1971;25:270–5.
25. Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 1975;11:205–6.
26. Nicholson RI, Golder MP. The effect of synthetic anti-oestrogens on the growth and biochemistry of rat mammary tumours. Eur J Cancer 1975;11:571–9.
27. Jordan VC, Jaspan T. Tamoxifen as an antitumour agent: oestrogen binding as a predictive test for tumour response. J Endocrinol 1976;68:453–60.
28. Skidmore J, Walpole AL, Woodburn J. Effect of some triphenylethylenes on oestradiol binding *in vitro* to macromolecules from uterus and anterior pituitary. J Endocrinol 1972;52:289–98.
29. Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 1977;75:305–16.
30. Langan-Fahey SM, Tormey DC, Jordan VC. Tamoxifen metabolites in patients on long-term adjuvant therapy for breast cancer. Eur J Cancer 1990;26:883–8.
31. Jordan VC, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. Adjuv Ther Cancer 1979;2:19–26.
32. Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 1980;16:239–51.
33. Jordan VC, Allen KE, Dix CJ. Pharmacology of tamoxifen in laboratory animals. Cancer Treat Rep 1980;64:745–59.
34. EBCTCG. Effects of adjuvant tamoxifen and of cytotoxic therapy on mortality in early breast cancer. An overview of 61 randomized trials among 28,896 women. N Engl J Med 1988;319:1681–92.
35. EBCTCG. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Lancet 1992;339:1–15.
36. EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687–717.
37. Jordan VC. Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 1974;5:354.
38. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. Eur J Cancer 1976;12:419–24.
39. Cuzick J, Baum M. Tamoxifen and contralateral breast cancer [letter]. Lancet 1985;2:282.
40. Cuzick J, Powles T, Veronesi U, Forbes J, Edwards R, Ashley S, et al. Overview of the main outcomes in breast-cancer prevention trials. Lancet 2003;361:296–300.
41. Gail MH, Brinton LA, Byar DP, Corle DK, Green SB, Schairer C, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. J Natl Cancer Inst 1989;81:1879–86.
42. IBIS Investigators. First results from the International Breast Study: a randomized prevention trial. Lancet 2002;360:817–24.
43. Jordan VC. Optimising endocrine approaches for the chemoprevention of breast cancer. Beyond the Study of Tamoxifen and Raloxifene (STAR) Trial. Eur J Cancer 2006;42:2909–13.
44. Furr BJ, Jordan VC. The pharmacology and clinical uses of tamoxifen. Pharmacol Ther 1984;25:127–205.
45. Stearns V, Johnson MD, Rae JM, Morocho A, Novielli A, Bhargava P, et al. Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. J Natl Cancer Inst 2003;95:1758–64.
46. Johnson MD, Zuo H, Lee KH, Trebley JP, Rae JM, Weatherman RV, et al. Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. Breast Cancer Res Treat 2004;85:151–9.
47. Jin Y, Desta Z, Stearns V, Ward B, Ho H, Lee KH, et al. CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. JNCI 2005;97:30–9.
48. Goetz MP, Rae JM, Suman VJ, Safgren SL, Ames MM, Visscher DW, et al. Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. J Clin Oncol 2005;23:9312–8.
49. Goetz MP, Knox SK, Suman VJ, Rae JM, Safgren SL, Ames MM, et al. The impact of cytochrome P450 2D6 metabolism in women receiving adjuvant tamoxifen. Breast Cancer Res Treat 2007;101:113–21.
50. Garber K. Tamoxifen pharmacogenetics moves closer to reality. J Natl Cancer Inst 2005;97:412–3.
51. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987;10:31–5.
52. Turner RT, Wakley GK, Hannon KS, Bell NH. Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. J Bone Miner Res 1987;2:449–56.
53. Turner RT, Wakley GK, Hannon KS, Bell NH. Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. Endocrinology 1988;122:1146–50.

54. Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987;47:4020–4.
55. Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc 1987;46:1870–4.
56. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 1988;48:812–5.
57. Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1989;1: 117–20.
58. Fisher B, Costantino JP, Redmond CK, Fisher ER, Wickerham DL, Cronin WM. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14 [see comments]. J Natl Cancer Inst 1994;86:527–37.
59. Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA 1999;281:2189–97.
60. Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, et al. For the CORE investigators continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 2004;96:1751–61.
61. Cauley JA, Norton L, Lippman ME, Eckert S, Krueger KA, Purdie DW, et al. Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial. Multiple outcomes of raloxifene evaluation. Breast Cancer Res Treat 2001;65:125–34.
62. Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators [see comments] [published erratum appears in JAMA 1999 Dec 8;282(22):2124]. JAMA 1999;282:637–45.
63. Barrett-Connor E, Mosca L, Collins P, Geiger MJ, Grady D, Kornitzer M, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 2006;355:125–37.
64. Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 1990;50:3189–92.
65. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. JAMA 2006;295:2727–41.
66. Jordan VC. Chemoprevention of breast cancer with selective estrogen-receptor modulators. Nat Rev, Cancer 2007;7:46–53.
67. Jordan VC. Beyond raloxifene for the prevention of osteoporosis and breast cancer. Br J Pharmacol. 2007;150:3–4.
68. Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. J Med Chem 2003;46:1081–111.
69. Espeland MA, Rapp SR, Shumaker SA, Brunner R, Manson JE, Sherwin BB, et al. Conjugated equine estrogens and global cognitive function in postmenopausal women: Women's Health Initiative Memory Study. JAMA 2004;291:2959–68.
70. Shumaker SA, Legault C, Kuller L, Rapp SR, Thal L, Lane DS, et al. Conjugated equine estrogens and incidence of probable dementia and mild cognitive impairment in postmenopausal women: Women's Health Initiative Memory Study. JAMA 2004;291:2947–58.
71. Shumaker SA, Legault C, Rapp SR, Thal L, Wallace RB, Ockene JK, et al. Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the women's health initiative memory study: a randomized controlled trial. JAMA 2003;289:2651–62.
72. Wassertheil-Smoller S, Hendrix SL, Limacher M, Heiss G, Kooperberg C, Baird A, et al. Effect of estrogen plus progestin on stroke in postmenopausal women: the Women's Health Initiative: a randomized trial. JAMA 2003;289:2673–84.
73. Licata AA. Discovery, clinical development, and therapeutic uses of bisphosphonates. Ann Pharmacother 2005;39:668–77.
74. Miller CP, Collini MD, Tran BD, Harris HA, Kharode YP, Marzolf JT, et al. Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. J Med Chem 2001;44:1654–7.
75. Rosati RL, Da Silva Jardine P, Cameron KO, Thompson DD, Ke HZ, Toler SM, et al. Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryltetrahydronaphthalene. J Med Chem 1998;41:2928–31.

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Nuclear receptor research historically was built upon natural and synthetic modulators of the steroid receptors as a basis. Therefore the steroid skeleton was chosen as the core element of the cover illustration.

Today nuclear receptors represent one of the most important classes of drug targets. Modern drug finding methods and technologies like computational chemistry (molecular modelling picture in A-ring of steroid skeleton), molecular and cell biology (B-ring), medicinal chemistry for the preparation of steroidal and nonsteroidal modulators (C-ring) and structural biology (D-ring) have to be used in close collaboration to find novel and improved treatments for severe diseases in specific tissues (background of the graphics).

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Preface

With volumes on *G-Protein-Coupled Receptors* and *Voltage-Gated Ion Channels* we have started to edit volumes dedicated to important target classes. Here we introduce the third book following this concept. Eckhard Ottow and Hilmar Weinmann contribute a volume focusing on *Nuclear Receptors as Drug Targets*.

Nuclear receptors are a large superfamily of transcription factors involved in important physiological functions such as the control of embryonic development, organ physiology, cell differentiation and homeostasis. They play an important role in metabolism, homeostasis, growth and development, aging, and reproduction. Beyond normal physiology, nuclear receptors also play a role in many pathological processes, such as cancer, diabetes, rheumatoid arthritis, asthma and r hormone-resistance syndromes. Despite their long history these transcriptional regulators remain of great interest in modern drug discovery.

Nuclear receptors are soluble proteins that can bind to specific DNA-regulatory elements and act as cell-type- and promoter-specific regulators of transcription. In contrast to other transcription factors, the activity of nuclear receptors can be modulated by binding to the corresponding ligands – small lipophilic molecules that easily penetrate biological membranes. For a number of nuclear receptors, identified in recent years, no ligands are known. These so-called orphan receptors have attracted considerable interest since they could lead to the discovery of new endocrine-regulatory systems.

The target family of human nuclear receptors has a common evolutionary history as evidenced by their folding/sequence relationships and their common cellular function. Functions of nuclear receptors are highly complex and the pathways that are controlled by nuclear receptors are connected either mutually or with other partner proteins. Despite this complexity the nuclear receptor family has a long history of successful drug discovery. Recently, drug discovery in the field of nuclear receptors developed capabilities for profiling compounds within a setting much closer to the native physiological environment compared to previous studies. New technologies such as high-throughput methods in chemistry and structural biology, novel biochemical methods, and pathway analysis tools such as differential gene expression and proteomics will enable new discoveries finally leading to drugs with

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improved therapeutic profiles. A better understanding of the ligand-induced activities that produce tissue-selective beneficial effects should enable the development of safer drugs with minimized side-effects. Furthermore, ligand discovery for the remaining orphan receptors might hold great promise. Target validation and better definition of therapeutic relevance for the remaining orphan nuclear receptors should be possible by using new tool compounds. Thus, despite its long history the nuclear receptor target family still bears tremendous potential, and nuclear receptor drug discovery should lead to highly effective and specific drugs for the future treatment of a broad variety of human diseases.

The present volume comprehensively treats nuclear receptors from the medicinal chemistry point of view. In an excellent introductory chapter, Eckhard Ottow and Hilmar Weinmann give a historic perspective on nuclear receptors as modern drug targets. The second section is dedicated to basic concepts and new perspectives in nuclear receptor research. Then, a comprehensive section individually treats estrogen, progesterone, androgen, glucocorticoid, as well as vitamin D receptors, followed by several chapters focusing on orphan and other nuclear receptors. Modern tools for nuclear receptor research are covered in the final part of the volume.

The Series Editors are grateful to Eckhard Ottow and Hilmar Weinmann for their enthusiasm in organizing this volume and to work with such a fine selection of authors. Last, but not least, we thank the publisher Wiley-VCH, in particular Dr. Nicola Oberbeckmann-Winter and Dr. Frank Weinreich, for their valuable contributions to this project and to the entire series.

April 2008

Raimund Mannhold, Düsseldorf
Hugo Kubinyi, Weisenheim am Sand
Gerd Folkers, Zürich

A Personal Foreword

Nuclear receptors have been drug targets for decades and this has led to an enormous body of knowledge about this target class and the medicinal chemistry of its small-molecule modulators. Nuclear receptors are interesting targets because of their great importance for many biological processes, with great potential for the treatment of severe diseases such as cancer, coronary heart disease and diabetes.

A further very important fact why nuclear receptors are attractive drug targets is that they are usually 'drugable', which means that the likelihood of identifying small-molecule agonists and antagonists, suitable for oral application, is rather high. Therefore, it is not surprising that numerous natural and synthetic nuclear receptor ligands, many of them belonging to the steroid structural class, are on the market. The huge economic impact of nuclear receptor targeting drugs is demonstrated by their estimated share of 10–15% of the $400 billion global pharmaceutical market. In 2003, 34 of the top 200 most prescribed drugs were targeting nuclear receptors.

The nuclear receptor family contains a large group of transcription factors, with 48 members identified in the human genome. Despite the fact that this figure is relatively small compared to the kinase or G-protein-coupled receptor target families, nuclear receptors form a very fascinating group which is still far from being completely understood in terms of its biological relevance or its modulation and control by natural and synthetic ligands.

Given such a strong motivation, this volume of *Methods and Principles in Medicinal Chemistry* attempts to present an overview on the various aspects of modern nuclear receptors research and its wide-ranging applications.

The book covers a broad spectrum of topics, ranging from pioneering research in the field of classical steroid hormones to very recently discovered orphan receptors and their modulators. State-of-the-art technologies are also discussed in the individual chapters that help to develop a deeper insight into the biochemical and pharmacological principles underlying the biological function of nuclear receptors.

In the introductory chapter a very brief overview on historic developments in nuclear receptor drug discovery from the pioneering experiments up to our current knowledge is given by Eckhard Ottow and Hilmar Weinmann.

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A field which is still far from being completely understood on a molecular level is the nuclear receptor–cofactor interaction. Luc Brunsveld, B. Vaz and S. Möcklinghoff give an introduction to our current knowledge in this field and explain why these interactions might be attractive targets for more selective modulators.

In the first chapter on classical steroid hormones Ross V. Weatherman brings more light to a special aspect of nuclear receptor regulatory systems by untangling the estrogen receptor web. Pharmacological use of a great variety of different chemotypes as subtype selective estrogens is summarized extensively by Gerrit H. Veenemann. The trilogy on the estrogen receptor is completed by V. Craig Jordan and Eric A. Ariazi who review estrogen receptors as therapeutic targets in breast cancer.

Klaus Schöllkopf and Norbert Schmees give an overview of the biology of the progesterone receptor and of the development of research in the field of steroidal progestins and recent trends in discovery of nonsteroidal selective progesterone receptor modulators.

Progesterone receptor antagonists and their potential clinical applications (e.g. in uterine myoma, endometriosis, breast, ovarian or uterine cancer, or as potential contraceptives) are discussed by Irving M. Spitz.

Androgens play an important role in male physiology due to their essential roles in male sexual differentiation, maintenance of muscle and bone mass, prostate growth, and spermatogenesis through the action of the androgen receptor. Michael L. Mohler, Casey E. Bohl, Ramesh Narayanan, Yali He, Dong Jin Hwang, James T. Dalton and Duane D. Miller review the various chemotypes of nonsteroidal tissue selective androgen receptor modulators and their clinical use (e.g. as prostate cancer treatments).

The following chapter by Heike Schäcke, Khusru Asadullah, Markus Berger and Hartmut Rehwinkel is dedicated to the glucocorticoid receptor as a target for classic and novel antiinflammatory therapy and novel glucocorticoid receptor ligands. The introduction of glucocorticoid therapy revolutionized antiinflammatory therapy. Fifty years after their initial clinical use, glucocorticoids are still the most important and frequently prescribed class of antiinflammatory drugs for various inflammatory disorders. Despite the many beneficial effects of classical glucocorticoids, however, their limitations and disadvantages seriously handicap their successful use as antiinflammatory agents. One goal of current research efforts therefore is to develop novel antiinflammatory strategies with more selective compounds, which are greatly needed.

Similarly, calcitriols, the vitamin D receptor agonists, are very well established for antiinflammatory treatment in daily clinical practice. Ekkehard May, Andreas Steinmeyer, Khusru Asadullah and Ulrich Zügel discuss the molecular and cellular principles of vitamin D action and drug discovery efforts for new vitamin D receptor modulators.

Peroxisome proliferator-activated receptors are ligand-activated receptors which regulate a number of genes involved in nutrient metabolism and energy homeostasis, and thus have served as drug targets for the treatment of metabolic diseases. Anne Reifel Miller and Alan M. Warshawsky review peroxisome proliferator-activated receptor γ modulation for the treatment of type 2 diabetes.

Retinoids are a class of polyisoprenoids that are derived by oxidative cleavage of β-carotenes of plant origin to yield vitamin A (retinol). They are essential for embryonic development and play important physiological functions, particularly in the brain and reproductive system, by regulating organogenesis, organ homeostasis, and cell growth, differentiation and apoptosis. The naturally occurring and synthetic retinoids are currently the subject of intense biological interest stimulated by the discovery of retinoid nuclear receptors and the realization of these compounds as nonsteroidal small-molecule hormones. Most retinoids that are currently used in dermatology and in oncology were discovered by chemical modifications on the basis of vitamin A structure and by biological evaluations in suitable pharmacological models. Vincent C. O. Njar provides the reader with a thorough overview of retinoids that are in clinical use.

Nuclear receptors also play an important role as drug targets in cardiovascular diseases. Peter Kolkhof, Lars Bärfacker, Alexander Hillisch, Helmut Haning and Stefan Schäfer review the mineralocorticoid receptor, peroxisome proliferator-activated receptorα and thyroid hormone receptors in detail based upon their therapeutic value, and additionally vitamin D receptor, retinoic acid receptors and retinoid X receptors as well as liver X receptors are covered with a special focus on their role in cardiovascular diseases.

The NR4A subfamily of receptors and their modulators is the topic of a chapter contributed by Henri Mattes. The NR4A subfamily of nuclear receptors has been implicated in Parkinson's disease, schizophrenia, manic depression, atherogenesis, Alzheimer's disease, rheumatoid arthritis, cancer and apoptosis. Therefore, there is currently great interest in the identification of selective modulators that may help to elucidate the mode of action of the NR4A subfamily.

Christoph Handschin describes the role of nuclear receptors, more specifically the pregnane X receptor and the constitutive androstane receptor, in the induction of drug metabolism and detoxification of drugs and other xenobiotics.

Nuclear receptor targeted screening libraries and chemogenomics approaches are a relatively recent field of research enabled by progress in cheminformatics as well as structural biology and combinatorial chemistry. J. Mestres summarizes the current knowledge and applications in this field of nuclear receptor research efforts.

As described in the individual chapters of this book, newly discovered receptors, recent insights into the effects of classical nuclear receptor action together with new technologies to explore their molecular mechanisms and to identify new modulators with finely tuned properties led to tremendous new interest in nuclear receptor research and fascinating novel results. The editors hope that the readers will share some of the excitement of this highly active field of research and the exciting emerging possibilities for the development of novel drug candidates.

We found work on this book both stimulating and thrilling, and we would like to acknowledge very much the great enthusiasm of all chapter authors in supporting this project and contributing their high-quality manuscripts within a tough schedule. Our acknowledgements go further to Dr. Frank Weinreich, Dr. Nicola Oberbeckmann-Winter and the whole staff of Wiley-VCH for their extremely professional support in the production of this monograph. Finally, we are

extremely thankful to the Editors of the series *Methods and Principles in Medicinal Chemistry*, Hugo Kubinyi, Raimund Mannhold and Gerd Folkers, for triggering this project and for giving us the opportunity to bring together this volume on *Nuclear Receptors as Drug Targets*.

May 2008

Eckhard Ottow
Hilmar Weinmann

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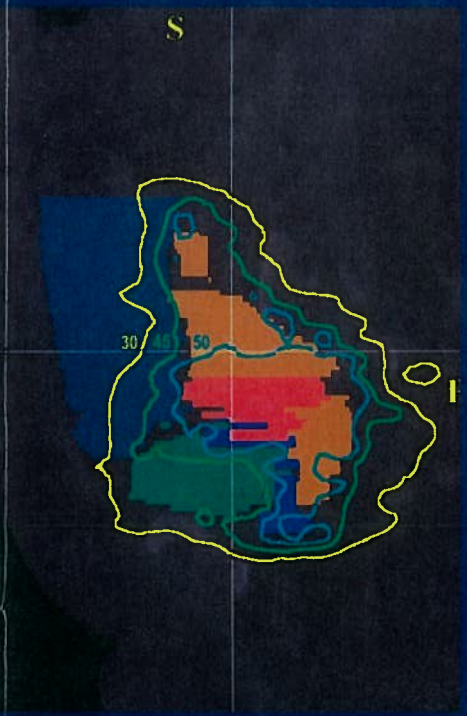

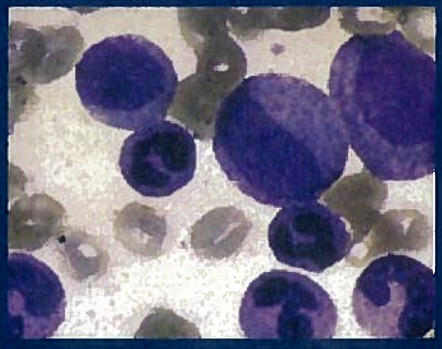

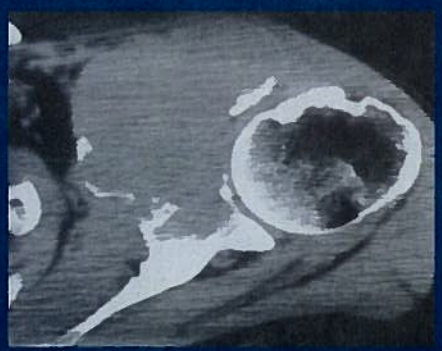

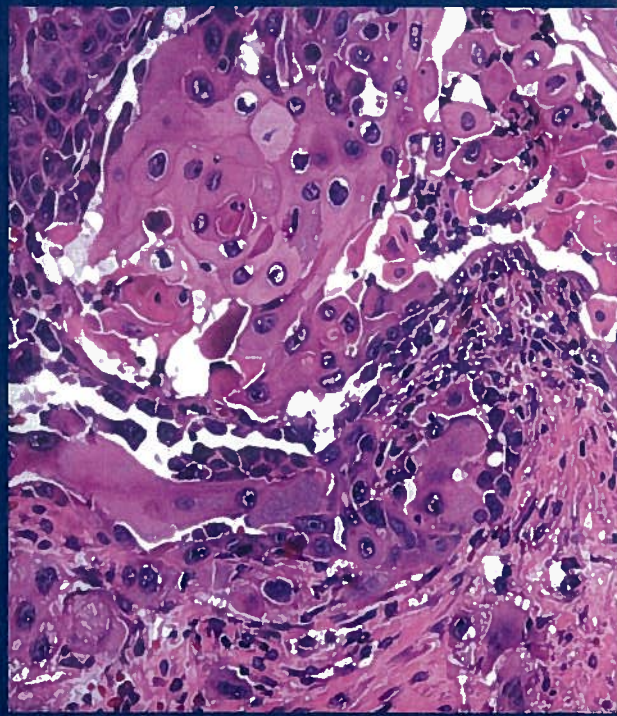

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The consummate reference source for medical oncologists, radiation oncologists, internists, surgical oncologists, and others who treat cancer patients, **Cancer Medicine 8** covers the ever-widening sphere of current cancer knowledge and clinical practice.

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III PRINCIPLES OF CANCER DIAGNOSIS
IV PRINCIPLES OF THERAPEUTIC MODALITIES
V PRINCIPLES OF MULTIDISCIPLINARY MANAGEMENT

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58 Antiestrogens, Progestins, and Aromatase Inhibitors

Aman U. Buzdar, MD ■ Shaheenah Dawood, MBBCh, MRCP (UK)MPH ■
Harold A. Harvey, MD ■ V. Craig Jordan, OBE, PhD, DSc

Introduction

The importance of the reproductive endocrine system in breast cancer treatment began to be appreciated at the turn of nineteenth century. It was around this time that it was realized that approximately one-third of premenopausal women with advanced breast cancer would respond to oophorectomy.[1] However, it was only when the estrogen receptor (ER) was discovered that it was possible to fully appreciate the mechanisms underlying the activity of ovarian ablation and other associated treatments for breast cancer such as ovarian irradiation, adrenalectomy, and hypophysectomy.[2] Research into the both the estrogen and progesterone pathways not only provided a deeper understanding of the underlying mechanism of the carcinogenic pathway involved in the development of breast cancer but also allowed identification of potential targets for therapeutic intervention.

This chapter discusses recent advances in the molecular biology and physiology underlying the ER and progesterone receptor (PR) pathways and potential targets for intervention. In addition it examines and compares the pharmacology and efficacy of the different endocrine agents used in the management of both early and advanced stage breast cancer (Fig. 58-1).

Biology Progestin Production and Action

Progestins are involved in the regulation of development and differentiation, proliferation, apoptosis, and metabolism in many target tissues with broad implications in neoplasia. In addition progestins serve as precursors to the estrogens, androgens, and adrenocortical steroids. Some of the progestin effects on target tissues are mediated by transcription, whereas other effects are more rapid and do not involve direct transcriptional effects. Progestins (Fig. 58-1A) include the naturally occurring hormone progesterone, 17α-acetoxyprogesterone derivatives in the pregnane series, 19-nortestosterone derivatives (estranes), and norgestrel and related compounds in the gonane series. In humans progesterone is the most important progestin.

Synthesis and Sites of Production

Progesterone is produced early in the scheme of the synthetic pathway involving the conversion of cholesterol to androgens, progestins, and estrogens. After menopause, in the absence of hormone replacement, the adrenal gland becomes the principal source of progestins (through the conversion of pregnenolone) as well as other sex steroids. In the premenopausal woman, progesterone is principally derived from the corpus luteum of the ovary, but in pregnancy after the eighth week of gestation, placental progesterone production greatly exceeds ovarian-derived progesterone. The placental trophoblast is the dominant cell responsible for progesterone production by the placenta. The development of a secretory endometrium in which the blastocyst can implant requires progesterone. Progesterone levels of 25 ng/mL are usual in the luteal phase of the menstrual cycle, where as levels up to 150 ng/mL are seen in late pregnancy.

Mechanism of Action

Progesterone functions in ribonucleic acid (RNA) transcription regulation through a complex series of interactions that is initiated by binding of the hormone to its cognate receptor. There are two isoforms of PR known as PR-A and PR-B that have distinct biological activities. PR-B has been shown to mediate the stimulatory activities of progesterone while PR-A functions to inhibit the action of PR-B as well as other steroid receptors.[3–5] Both isoforms are encoded by a single gene and their ratios vary in reproductive tissues as a consequence of developmental status, hormonal levels and tissue type. Both isoforms of PR contain AF-1 and AF-2 transactivation domains; PR-B contains an additional AF-3 domain, which contributes to its cell- and promoter-specific activity. The ligand for both isoforms of PR is identical.

In the absence of the hormone, PR is found in the nucleus in an inactive monomeric state associated with a complex of heat shock proteins (HSP-90, 70, 60, and 40) and is transcriptionally inactive.[6,7] Binding of progesterone to PR results in the dissociation of the heat shock proteins leading to the formation of receptor–ligand homodimers that remain localized in the nucleus and bind to highly selective progesterone response elements (PRE) located on target genes.[8] It is important to note that target cells must distinguish not only progesterone from other steroids present in small amounts, but also must distinguish progesterone from other hydrophobic molecules that are frequently found in 100-fold or greater excess. Such a high degree of discrimination is limited to differentiated cells that possess PR proteins and activatable PREs in their genome.[8,9] The next step in the process is the transcriptional activation by PR which results from the interaction with a number of coactivators including steroid receptor coactivator 1 (SRC-1), transcription intermediary factor 2, and retinoic acid coactivator 3, among others.[8–11] The SRC-1 interacts with the N-terminal AF-1 and the C-terminal AF-2 of the PR. This serves to emphasize that SRC-1 function to synergize the ligand-independent amino terminal AF-1 with the ligand-responsive carboxyl terminal AF-2 of the PR. The PR-coactivator complex then interacts further with additional proteins that have histone acetylase activity that causes chromatin remodeling serving to increase accessibility of transcriptional proteins to the promoter target.[10]

Physiologic Actions

Progestins are involved in a number of benign physiologic changes ranging from differentiated secretory activity to edematous changes in stromal tissues of the breast. They are also have implications on neoplastic processes being associated with both a decreased risk of endometrial neoplasms and a slightly increased risk of breast neoplasms when used in conjunction with estrogen replacement for menopause. Progestins have a critical role in the support of the products of conception: the differentiation of the endometrium and the promotion of the secretory phase of the endometrium; the maturation and cornification of the vaginal mucosal epithelium; the suppression of ovulation; the inhibition of gonadotropin release; the proliferation of breast epithelium and the induction of secretory activity in breast epithelium; and a natriuretic effect on the kidneys. A number of these biologic effects of progesterone are seen only in concert with priming of the target tissues with estrogen, whereas other effects appear to be interrelated with the actions of other steroid hormones, peptide hormones, and/or growth factors. Both progesterone and

estrogen influence the response when superpharmacologic doses are used. Possible interactions between progesterone, estrogen, and growth factors must be taken into account when constructing treatment strategies.

Synthetic Progestins

Synthetic progestins are derivatives of the steroid structure of either progesterone or testosterone. The synthetic progestins most often encountered include 17-hydroxyprogesterone, medroxyprogesterone (Provera), medroxyprogesterone acetate, megestrol acetate (Megace), norethindrone, norethindrone enanthate, norethindrone acetate, norethynodrel, norgestrel, desogestrel, and gestodene. The two most widely used synthetic progestins are medroxyprogesterone acetate (MPA) and megestrol acetate (MA), which differs only by a single bond at C6-C7. MPA can be administered either as an oral or intramuscular preparation, while MA is administered as an oral preparation.

Synthetic progestins are used most frequently in contraception, in conjunction with estrogen in postmenopausal hormone replacement therapy (HRT) and in the endocrine treatment of uterine and breast cancer. In postmenopausal HRT, progestins are added to estrogen replacement principally to minimize the risk of uterine cancer associated with estrogen-only therapy, although the effects of adding progestins to estrogen on the risk of breast cancer have become of increasing concern.[12] In premenopausal women, progestins and anti-progestins are used predominantly in contraceptive preparations.[13] Progestins are often used alone in selected women with climacteric symptoms who are advised not to take estrogens.

Metastatic Breast Cancers

Progestins and PRs have been studied extensively in cancerous human breast tissue. Patients whose tumors are PR positive have a higher probability of responding to endocrine therapy (not necessarily progestins) and in most series show a somewhat better prognosis with respect to both survival and disease-free interval.[14] Both MPA and MA have been shown to produce similar reductions in serum estrogens levels and produce responses of approximately 30% in patients with metastatic breast cancer.[15] The principal progestin used for metastatic breast cancer has been MA. The response of metastatic breast cancer to MA is predicted not only by the presence of ERs and/or PR but also by the observation of an objective response to previous hormonal therapy. Randomized studies have shown comparable efficacy of progestins

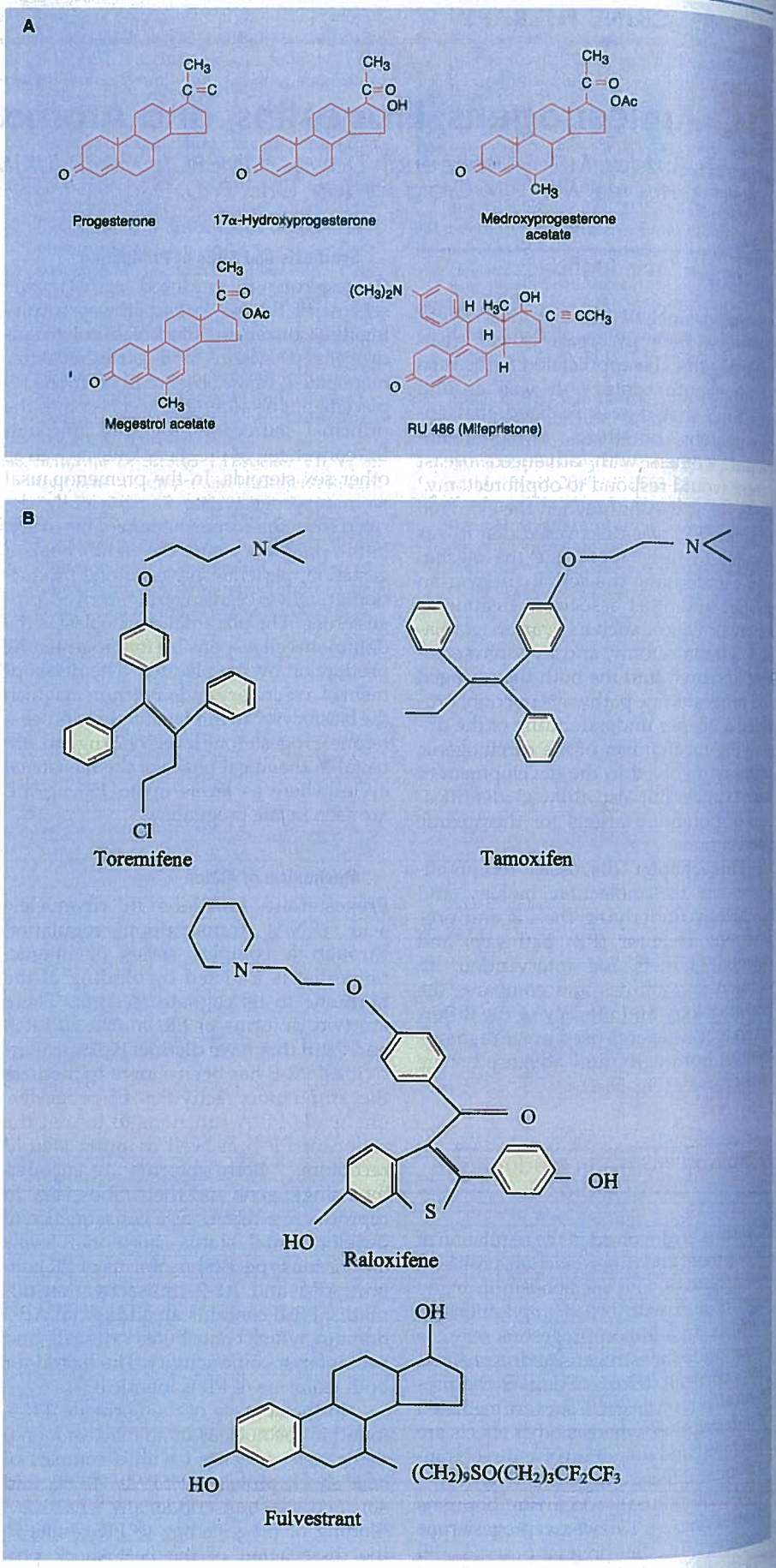

Figure 58-1 Endocrine agents for treatment or prevention of breast cancer. (A) Progestins (B) antiestrogens. (*Continued*)

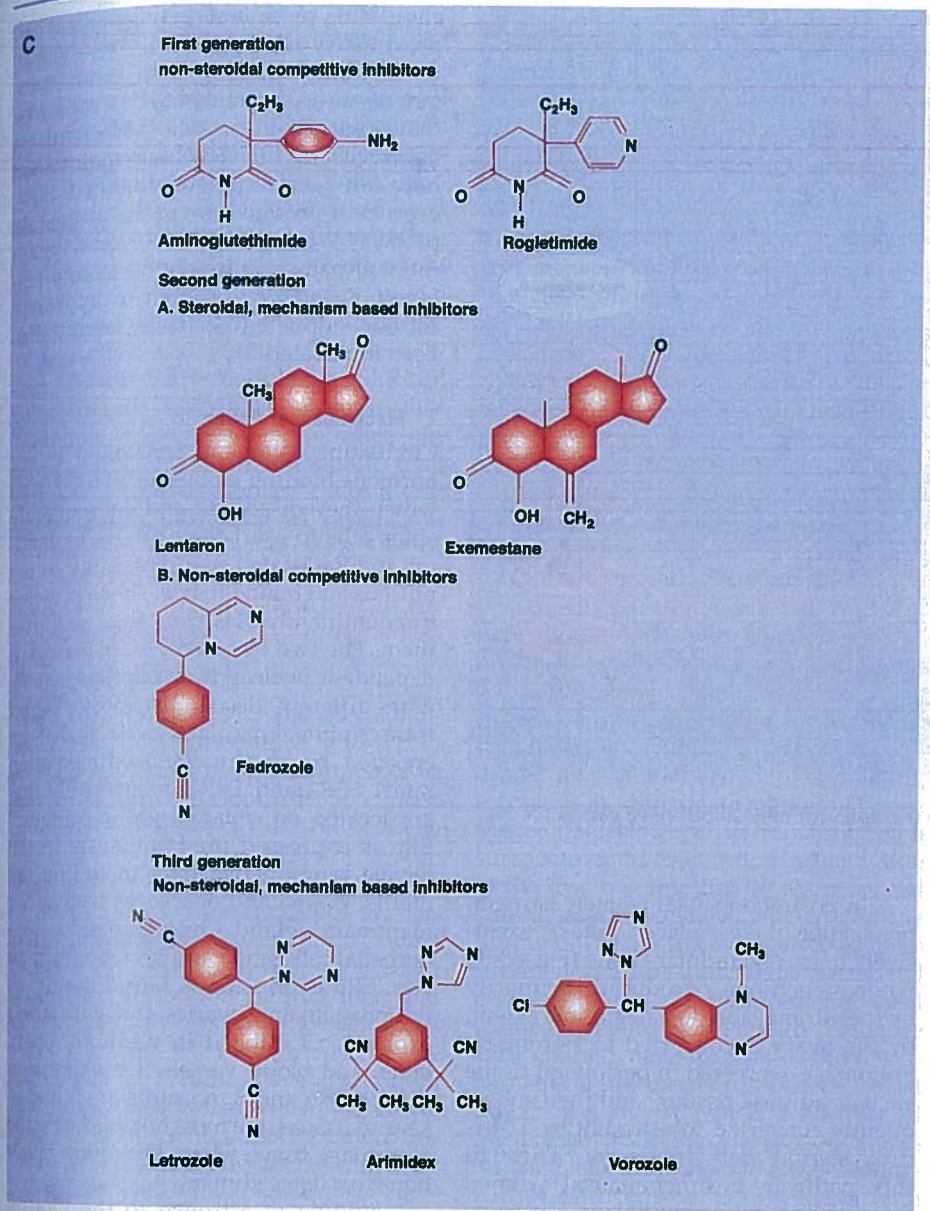

Figure 58-1 (*Continued*) (C) Aromatase inhibitors.

to tamoxifen, aminoglutethemide and aromatase inhibitors in the second and subsequent lines of treatment of metastatic breast cancer.[15,16] Currently progestin therapy for hormone receptor positive metastatic breast cancer is used principally after disease progression has been observed following use of selective ER modulators (eg, tamoxifen), aromatase inhibitors, and fulvestrant. As such a trial of progestins is commonly used as the third or subsequent line of therapy in patients with hormone receptor positive metastatic breast cancer (Fig. 58-2).

Uterine Cancer

The most common adverse effect of progestin is weight gain which occurs as results of increased appetite and fluid retention. Its appetite stimulating effect has frequently been used to treat cancer induced cachexia. Other reported side effects include hot flashes, sweating, vaginal bleeding, nausea, dyspnea, thromboembolism and rare cardiovascular events such as heart failure. Various dosing regimens of MA and MPA have been studied with a possible dose response effect observed. The recommended dose of MA is 160 mg/day and that of MPA is at least 400-500 mg/day.[17]

Progestins are also used in the treatment of endometrial carcinoma.[18] When diagnosed, adenocarcinoma of the uterus is cured by local therapy in 80% of cases. In the event of recurrence, exogenous progestin is an effective treatment in a significant fraction of cases: more than 30% of patients with recurrent disease demonstrate an objective response to exogenous progestins. ER and PR can be measured in these tumors, and the presence of these receptors correlates with differentiation of the tumor, prognosis for the patient, and response to progestins. The duration of response is not predicted by the presence of a receptor and varies from months to years. Tumors that lack ER and PR respond objectively to progestins in fewer than 10% of cases.

Anti-progestins

Anti-progestins have wide and varied therapeutic applications including uses as contraceptives, to induce labor and treatment of breast cancer, endometriosis, uterine leiomyomas and meningiomas.[19] The oldest and most widely used anti-progestin is RU 38486 or mifepristone that is a derivative of the 19-norprogestin norethindrone containing a dimethyl-aminophenol substituent at the 11β-position.[13,20] This compound is effectively absorbed orally and appears to bind with PR with high affinity and to effect altered co-regulatory protein interaction after binding. It has been shown to have both antagonist and some agonist activity and is thus considered to be a PR modulator. Together with prostaglandins it is used for the termination of early pregnancy.[20]

Biology of Estrogen Production and Action

A number of naturally occurring endogenous estrogens are produced in women with the most potent for both ERα- and β-mediated actions being estradiol followed by estrone and estriol. All three contain a phenolic A ring with a hydroxyl group at carbon 3 and a β-OH or ketone in position 17 of ring D with the phenolic A ring being the principle structural feature responsible for their selective high affinity binding to both ERs.

The principle role of naturally occurring estrogens is to modulate cell growth by causing an increase in stimulatory growth factors (eg, transforming growth factor-alpha [TGF-α]) and a decrease in inhibitory growth factors (eg, TGF-β).[21] These growth factors are thought to initiate, or prevent, progress through the cell cycle by interaction with their respective membrane receptors, with the regulatory mechanism functioning as an autocrine loop. There are also paracrine (cell-cell) influences of growth factors (eg, insulin-like growth factors-1 [IGF-1]) that can play a role in modulating the replication of epithelial cells.

Biosynthetic Pathway

The aromatase enzyme complex is located in the endoplasmic reticulum and consists of a cytochrome P450 hemoprotein (P-450 AROM, aromatase), and the flavoprotein nicotinamide adenine dinucleotide phosphate (NADPH) that is common to most cells types and whose

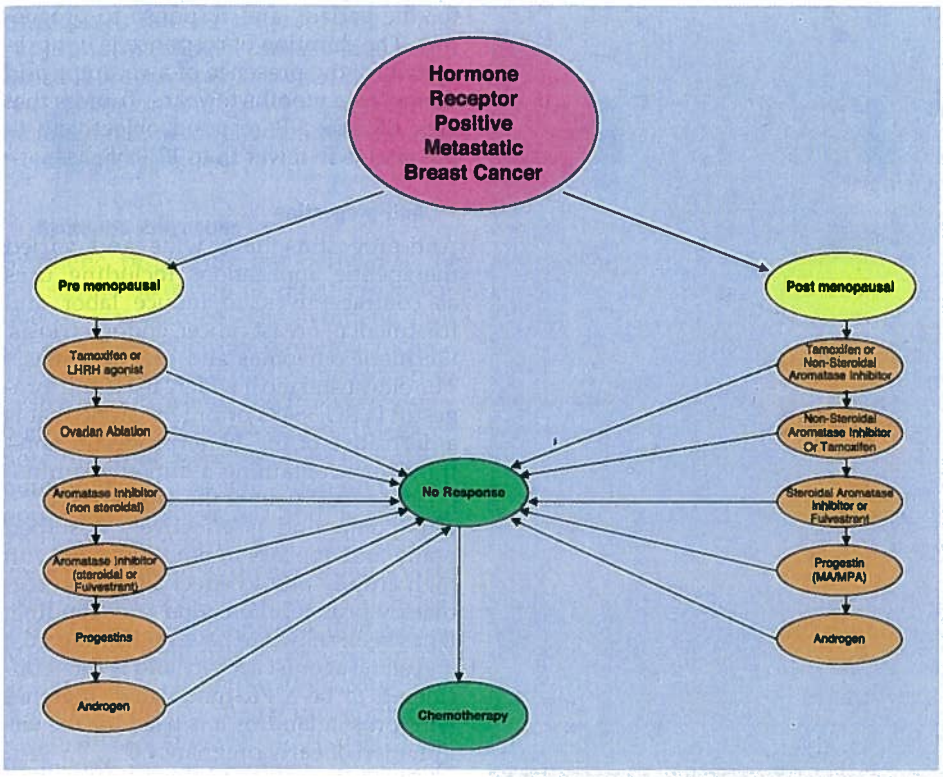

Figure 58-2 Schema of sequential endocrine therapies for metastatic breast cancer.

principle function is to donate electrons to cytochrome P450.[22] The principle enzyme involved in the conversion of androstenedione to estrone in the estrogen biosynthetic pathway is aromatase, a product of the CYP 19 gene that encodes a polypeptide of 503 amino acids with a molecular weight of 55 kilodaltons (KDa). Aromatase catalyzes three separate steroid hydroxylations involved in the conversion of androstenedione to estrone. The first two give rise to 19-hydroxy and 19-aldehyde structures, and the third, although still controversial, probably involves the C-19 methyl group with release of formic acid.[23]

Sites of Production

A number of tissues have the capacity to express aromatase and hence synthesize estrogens and these include the ovary, placenta, hypothalamus, liver, muscle, adipose tissue and malignant breast tumor tissue.[24]

In premenopausal women, the ovary is the most important site of aromatase and estrogen production. Luteinizing hormone (LH) controls production of androstenedione by the theca cell compartment, while follicle stimulating hormone (FSH) upregulates aromatase expression in granulose cells. Acting in concert, LH stimulates production of the substrate for aromatase; whereas, FSH increases the amount of aromatase so that estradiol production can increase by 8 to 10 fold at the time of ovulation.

In postmenopausal women estrogen production takes place almost exclusively in extraglandular tissue (Fig. 58-3). Androstenedione, produced primarily by the adrenal and, to a negligible extent, by the ovary is converted to estrone by aromatase expressed in peripheral tissue such as adipose tissue,[25] and then subsequently converted to estradiol by 17-hydroxysteriod dehydrogenase. Through this pathway postmenopausal women produce approximately 100 mg of estrone per day, with higher levels observed in obese women.[26] A fraction of estrone is also converted to estradiol to produce circulating plasma concentrations of approximately 10-20 pg/mL.

Estradiol levels in human breast tumor tissue are estimated to be 4 to 6 times that observed in plasma.[27] Mechanisms by which such high levels are maintained have not been completely defined: however, local production via the aromatase pathway is most likely involved with additional pathways involving steroid sulfatase, an enzyme known to hydrolyse estrone sulphate to estrone, having also been implicated.[28]

Mechanism of Action

Circulating estrogens are bound to sex hormone binding globulins (SHBG) from which they dissociate and subsequently enter cells to exert their effects by binding to ERs located predominantly in the nucleus and bound to heat shock proteins (predominantly Hsp90) that stabilize them. The two ERsα and β are estrogen-dependent nuclear transcription factors, with different tissue distributions and transcriptional regulatory effects that are encoded by erythrocyte sedimentation rate 1 ESR1 and ESR2, respectively that are located on separate chromosomes.[29] ERα is expressed predominantly in the female reproductive tract including the uterus, vagina and ovaries as well as the mammary gland, hypothalamus, endothelial cells, and vascular smooth muscles. ERβ expression is found mainly in the prostate and ovaries and with lower expression exhibited in the lung, brain, bone, and blood vessels. Co expression of both ERα and β receptors are found in several tissues, the most notable being the mammary tissue where they form either homo- or heterodimers.

Binding of estrogen to the ERs results in a conformational change in the receptor, leading to the release of ER from the stabilizing proteins. The estrogen-ER

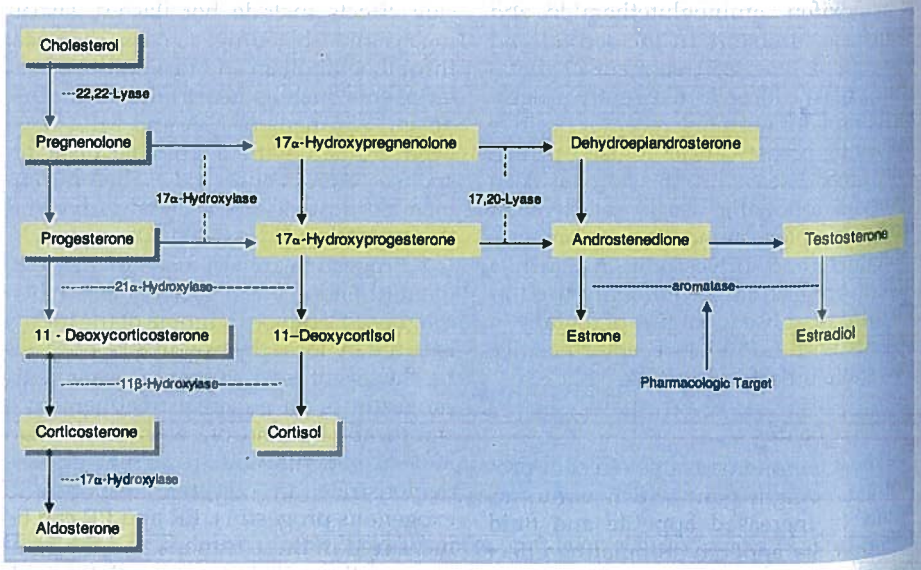

Figure 58-3 Steroid synthesis pathways and aromatase inhibitors target site.

homodimeric complex then binds to a specific sequence of nucleotides called estrogen response elements (ERE) that are located in the promoter region of various genes. This binding interaction also involves a number of nuclear proteins, co regulators as well as other components of the transcription machinery. Thus the genomic effects of estrogen are mainly the result of proteins synthesized from the regulation of transcription of a responsive gene. Two main modalities of treatment, the selective ER modulators (selective ER modulators [SERMS] tamoxifen or raloxifene) or aromatase inhibitors (exemestane, anastrozole, letrozole,[30] have been developed to treat estrogen responsive breast cancers that either target preventing its production or inhibiting its interaction with ERs for clinical purposes, the principal target for antihormonal therapeutic action in the breast tumor is ERα.[2]

Carcinogenic Effects

The major concern with the use of synthetic estrogens either alone or as a part of the preparation of oral contraceptives has been the development of cancer. Studies that reported the link between the intake of diethylstilbesterol (a synthetic estrogen) during the first trimester of pregnancy and the incidence of clear cell vaginal and cervical adenocarcinoma in later life of the offspring exposed in utero, established for the first time that developmental exposure to estrogens was associated with an increase in human cancer.[31,32] Studies have also shown that unopposed estrogen as part of the HRT in postmenopausal women increased the risk of endometrial cancer by 5 to 15 fold[33] with the increased risk prevented by the addition of a progestin.[34]

The relation of breast cancer risk and HRT in postmenopausal women has also been reported by two large trials. The Women's Health Initiative (WHI) was a large prospective trial that randomized women to either placebo or HRT. The investigators reported an increase in total risk of breast cancer of 24% among women who took an estrogen-progestin combination and a decrease of 23% among women without a uterus who took estrogen only compared to women who took placebo.[12,35] The Million Women Study (MWS) was a large cohort study that reported an increased relative risk of invasive breast cancer among women who did and did not take HRT.[36] Among women who took an estrogen-progestin combination the increased relative risk of invasive breast cancer was 2, and that for women who took estrogen alone was reported as 1.3.

Epidemiological studies have also linked high levels of natural estrogens to the development of cancer. High levels of natural estrogens are observed in women who are overweight. Among postmenopausal women who had never received HRT in the WHI those who were heavier (body mass index [BMI] >31.1) had an elevated risk of breast cancer compared to slimmer women (BMI < 22.6), relative risk [RR] 2.52; 95% CI 1.62-3.93).[33] High levels of natural estrogens were also found to be associated in a case-cohort study involving women with who had never received exogenous estrogens.[37] The investigators reported that compared to women with the lowest levels of circulating estradiol those with the highest levels (≥ 6.83 pmol/L or 1.9 pg/mL) had a RR of 3.6 (95% CI 1.3-10.0).

Bone mineral density has also been shown to be a surrogate marker of estrogen exposure. A high endogenous estrogen concentration has been reported to be associated with greater bone mineral density in elderly women.[38] Postmenopausal women with higher bone mineral densities have also been shown to have a higher incidence of breast cancer.[39] Such studies serve to indicate the potential benefit of circulating estrogens as a surrogate marker of breast cancer risk. However its reliability as a surrogate marker is controversial due to the low baseline line levels observed among postmenopausal women and the timing of circulating estrogen level measurement with relation to the menstrual cycle being important in premenopausal women. Regardless, enough evidence exist connecting estrogens to the development of hormone responsive breast cancer that has spawned a number of prevention trials that have used agents targeted either at blocking the production of estrogens or its interaction with its receptor.

SERMS and Antiestrogens

The first indication of the role of hormones in the development of breast cancer occurred more than a century ago when in 1896 Beatson observed that remission could be induced by removal of the ovaries in a subset of breast cancer patients.[1] Although not originally understood the observed effects occurred as a result of eliminating the primary source of estrogen in premenopausal women. This was confirmed in preclinical studies that demonstrated estradiol to promote proliferation of ER-positive breast cancer cells in culture[40] and numerous epidemiological studies that have linked estrogens to breast cancer risk.[35–39] With the realization of the important role estrogen played in the development and progression of breast cancer two groups of drugs were developed to counteract the action of estrogens. The first group essentially prevented the interaction of estrogen to its receptor and included the SERMS and antiesrtogens. SERMS including tamoxifen, raloxifene, and toremifene display unusual tissue selective pharmacology having estrogen agonist properties in some tissues (bone, liver, and cardiovascular system), estrogen antagonist properties in other tissues (brain and breast) and mixed agonist/antagonist estrogen properties in the uterus (Fig. 58-1B).[41] The antiestrogens which include fulvestrant are distinguished from SERMS in that they are uniformly estrogen antagonists. The second group of drugs blocks the production of estrogen by blocking the action of the aromatase enzyme and is known as aroamatase inhibitors (Fig. 58-1C). In this section we will review the various SERMS and antiestrogens used in clinical practice for the treatment and prevention of hormone receptor positive breast cancers.

Tamoxifen

Mode of Action ■ Tamoxifen is a nonsteroidal triphenylethylene compound[42] that exerts its effects by competitively inhibiting the binding of estradiol to ER thereby negating the stimulatory effects of estrogen causing the cell to be held at the G1 phase of the replication cycle.[43] Tamoxifen is an estrogen antagonist in the breast and an estrogen agonist in the endometrium and bone, and it is this balance in biological properties that is the key to the current strategies for the use of tamoxifen.

Clinical Pharmacology ■ The high therapeutic index of tamoxifen has permitted wide variations in dosage with schedules and dosage of treatment varying depending on the country and its initial clinical trials that evaluated efficacy of this drug. Schedules of 10 mg twice daily or 20 mg once daily are recommended in the United States, although 10 mg three times daily and 20 mg twice daily have been used in other countries.

Tamoxifen is administered orally and is rapidly absorbed, achieving a steady state serum levels within 4-6 weeks, and subsequently metabolized to N-desmethyltamoxifen (major metabolite) and 4-hydroxytamoxfien (minor metabolite) both of which have the potential to be further metabolized to 4-hydroxy-N-desmethyltamoxifen (minor metabolite).[44] Tamoxifen has a long serum half-life of 7 days, and the metabolite N-desmethyltamoxifen has an even longer half life of 14 days.[45] These long serum half lives are probably why a withdrawal response has not been routinely documented when tamoxifen therapy is

discontinued. Although no clinical cases of teratogenesis has been documented with tamoxifen it is not recommended in pregnant women. Furthermore tamoxifen is known to cause ovarian stimulation in premenopausal women with ovulatory cycles and thus women taking tamoxifen who are at risk of getting pregnant should be counseled about various contraceptive options.[46]

Tamoxifen in Advanced Breast Cancer ■ Tamoxifen is an endocrine option for metastatic disease in postmenopausal women and those with ER-positive disease are more likely to benefit from this therapy.[47] Correlation of clinical response and ER status indicates that approximately 48% of patients with ER-positive disease achieve partial or complete responses, whereas only 13% of patients with ER-negative disease exhibit some form of response with endocrine therapy indicating the selective efficacy of endocrine therapy among patients with hormone receptor positive disease. More recent data suggests that aromatase inhibitors are a better option as first line treatment for this cohort of patients so long as resistance to the drug has not developed. This will be discussed in further detail in the section "Aromatase Inhibitors."

Tamoxifen is a first line endocrine therapy in premenopausal women with advanced breast cancer. In this group of patients small randomized clinical trials have demonstrated that tamoxifen produces a response rate and overall survival similar to what is seen after oophorectomy.[48] However, with the development of effective LH-releasing hormone (LHRH) agonists such as goserelin (Zoladex), which acts to reduce ovarian steroidogenesis by preventing LH release from the pituitary gland, the combination of goserelin and tamoxifen has become established as an effective therapeutic option.[49] Hence recent guidelines have suggested that the use of LHRH agonists and tamoxifen alone or in combination are appropriate therapeutic options for women for premenopausal women with advanced metastatic hormone receptor positive disease[50] (Fig. 58-3).

Tamoxifen in the Adjuvant Setting ■ Many randomized trials have addressed the question of tamoxifen efficacy in the adjuvant setting among women with early stage breast cancer. An overview and meta-analysis of the results from 145,000 women with early stage breast cancer who were randomized to 194 trials of adjuvant systemic therapy (chemotherapy and/or hormonal therapy) were recently updated by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG).[51] The EBCTCG reported that 5-years of adjuvant tamoxifen among women with ER-positive disease resulted in reduction in the annual death rate by 31% regardless of age, PR status, menopausal status, or use of chemotherapy with benefits persisting up to 15-years of follow-up. In this report, 1-year of tamoxifen conferred little benefit; 5-years of tamoxifen was significantly more effective than 2-years, still requiring long term follow-up for assessing the benefit of more than 5-years of adjuvant tamoxifen treatment. However, results from the B-14 trial, a National Surgical Adjuvant Breast and Bowel Project (NSABP), in which women with lymph node negative ER-positive disease still in remission after receiving 5-years of tamoxifen were re randomized to receive either placebo or more prolonged therapy with tamoxifen have not shown any advantage from prolonged tamoxifen treatment through 7-years of follow-up.[52] Indeed, the longer duration of tamoxifen use was associated with shorter disease free survival (DFS) compared to the group who had stopped taking tamoxifen after 5-years (78% vs 82%, $p = .03$). Preliminary results from the Ajduvant Tamoxifen Longer Against Shorter (ATLAS) and Adjuvant Tamoxifen Treatment offer More (aTTom) trials, two large prospective trials that have randomized women with early stage breast cancer who completed five years of tamoxifen to either another five years tamoxifen, indicated a reduced risk of recurrence in the group that received continued tamoxifen beyond five years. Further follow-up is required to reliably assess the effects of tamoxifen on survival outcomes (both disease free and overall) as well as on any potentially associated side effects.[53,54] At present time, with the available evidence, current recommendations are that no more than five years tamoxifen therapy be offered as adjuvant therapy.[50]

Tamoxifen and Chemotherapy ■ In the latest update of the EBCTCG that addition of anthracyline based polychemotherapy regimens was reported to be associated with annual reduction of mortality of 38% and 20% among women aged <50 years and 50-69 years, respectively. The question however is whether the addition of an endocrine agent such as tamoxifen could further add to this benefit among women with ER-positive breast tumors. In the EBCTCG among 3330 women with ER-positive or ER unknown tumors 28.1% of women who received only chemotherapy experienced a recurrence compared to 17.5% who received chemotherapy and 5-years of tamoxifen with the difference being statistically significant.[51] Similarly among women with ER-positive or ER unknown breast tumors those who were less than 50 years of age and received chemotherapy and tamoxifen the EBCTCG reported a recurrence rate ratio of 0.64 (SE 0.08) and annual breast cancer mortality ratio of 0.65 (SE 0.10) compared to those who received tamoxifen alone with similar trends observed among women in the 50 to 69 years age group. When the sequence of chemotherapy and tamoxifen was explored among women 50 to 69 years of age a recurrence rate ratio and annual breast cancer mortality rate ratio of 0.80 (SE 0.03) and 0.90 (SE 0.03) among women treated with chemotherapy with tamoxifen compared to those who received tamoxifen alone with a recurrence rate ratio and annual breast cancer mortality rate ratio of 0.77 (SE 0.08) and 0.80 (SE 0.10) among women treated with chemotherapy followed by tamoxifen compared to those who received tamoxifen alone.[51] Therefore chemotherapy alone is not enough in women with ER-positive tumors with the clear data that the addition of tamoxifen is important. The data also indicate that sequential hormonal therapy maybe the better option. This is further strengthened by recent results from the South West Oncology Study Group (SWOG) 8814 study that reported improved disease free and overall survival outcomes when tamoxifen was given sequentially following cyclophosphamide, doxorubicin and 5-fluorouracil (CAF) compared with concurrent administration or tamoxifen alone.[55]

Prevention of Breast Cancer ■ Observations that long-term tamoxifen therapy reduced the incidence and risk of contralateral breast cancer in women with early stage breast cancer fueled interest in exploring the effect of tamoxifen in preventing the occurrence of breast cancer. An overview of the main outcomes from the five main breast cancer prevention trials, covering more than 28,000 patients, has shown that tamoxifen produced a 38% reduction in breast cancer incidence ($p < .0001$).[56] There was no effect on ER-negative disease ($p = .21$), but ER-positive cancers were reduced by 48% ($p < .0001$). However, endometrial cancer rates were increased (consensus RR 2.4; $p = .0005$) in patients receiving preventive tamoxifen, as were venous thromboembolic events (RR 1.9; $p < .0001$). As a result, tamoxifen cannot be used as a true preventive because the timing of the event is unknown and the unrestricted use of tamoxifen in young women of reproductive age would be unwise. An exception to this is when tamoxifen is used for the reduction of breast cancer risk in high-risk women, for which it is the first medicine to be approved by the U.S. Food and Drug Administration. Thus use of tamoxifen in preventive setting should be individualized to a woman's

risk of developing breast cancer. As such women with a prior history of ductal carcinoma in situ, lobular carcinoma in situ, atypical hyperplasia or those with a deleterious mutation of ($BRCA_1$) or $BRCA_2$ are considered to be at higher risk of developing breast cancer and represent an ideal cohort to target where benefit out weights risk of adverse events associated with tamoxifen.[57–59]

Raloxifene

Raloxifene is a benzothiophene second generation SERM that is FDA approved for the treatment and prevention of osteoporosis and for the reduction of risk of invasive breast carcinoma in postmenopausal women with either osteoporosis or who are at high risk for invasive breast cancer respectively.[60–63] This agent has no significant anti-tumor activity in the metastatic setting and is not approved for the treatment of advanced metastatic breast cancer. Four large prospective trials have reported on the efficacy of raloxifene as a chemopreventive agent for breast cancer. The Multiple Outcomes and of Raloxifene Evaluation (MORE) trial that randomized 7705 postmenopausal women with osteoporosis to receive either raloxifene or placebo, whose primary end point was development of a fracture, was the first major trial to suggest raloxifene as a potential agent for chemoprevention of breast cancer.[60] In this trial following 4 years of treatment raloxifene reduced the risk of ER-positive invasive breast cancer by 84% (RR 0.16; 95% CI 0.09, 0.30). The Continuing Outcomes Relevant to Evista (CORE) trial was an extension of the MORE trial to examine the effect of four additional years of raloxifene therapy on the incidence of invasive breast cancer in women in MORE who agreed to continue on the trial.[61] Combining the 8 years of follow up of both the MORE and CORE trials the investigators reported that the incidences of invasive breast cancer overall and ER-positive invasive breast cancer were reduced by 66% (hazard ratio [HR] 0.34; 95% CI 0.22-0.50) and 76% (HR 0.24; 95% CI 0.15-0.40), respectively, in the raloxifene group compared with the placebo group. The goal of the Raloxifene Use for the Heart (RUTH) trial was to investigate the effect of raloxifene on the incidence of coronary events and breast cancer in 10,101 postmenopausal women.[62] The investigators found reductions in breast cancer similar in size to that seen for tamoxifen in other studies. The NSABP P-2 trial was a prospective, double-blinded, randomized clinical trial that compared the efficacy and safety of tamoxifen on the risk of developing invasive breast cancer in a cohort of 19,747 postmenopausal women.[63] The investigators reported similar efficacy of tamoxifen compared to raloxifene in reducing the risk of invasive breast cancer (RR 1.02; 95% CI 0.82-1.28). In terms of side effects compared to tamoxifen, raloxifene had fewer gynecological and thromboembolic events. Interestingly, raloxifene reduced the risk of invasive breast cancer but had no effect on the incidence of ductal carcinoma in situ.

Toremifene

Toremifene (Fareston) is a structural derivative of tamoxifen with similar antiestrogenic and estrogenic properties demonstrated in laboratory animals. In general, toremifene is highly protein bound, which could explain its long serum half-life. Toremifene is less potent than tamoxifen, and consequently, clinical studies have evaluated doses of toremifene up to 240 mg/day. Toremifene is cross-resistant with tamoxifen, but clinical trials have shown that it exhibits a similar efficacy and side effect profile to tamoxifen, and so may be used as an alternative to treat advanced breast cancer.[64,65] At this time, there is insufficient data to recommend its use in the adjuvant setting.

Trilostane

Trilostane (Modrenal) is an antiadrenal drug that is usually used for short-term adrenal suppression in the treatment of Cushing syndrome. However, trilostane's ability to modify the binding of estrogen to the ER has prompted interest in its potential to block breast tumor cell proliferation. A meta-analysis of several small studies investigating the use of trilostane in postmenopausal women with advanced breast cancer reported clinical benefit with this agent, and further trials are needed to evaluate its worth as an endocrine therapy for advanced breast cancer.[66]

Fulvestrant

Fulvestrant (Faslodex) is an antiesrtogen with no agonist properties and unlike tamoxifen has the following mechanism of action: it binds, blocks, and increases degradation of ER protein, leading to an inhibition of estrogen signaling through the ER together with dramatic loss of cellular ER levels, and is also associated with a significant reduction in PgR) expression.[67,68] A prospective, combined analysis of two phase 3 trials comparing fulvestrant (250 mg intramuscular injection once monthly) to anastrozole in a cohort of postmenopausal women with advanced breast cancer who had progressed on prior tamoxifen therapy indicated that, after a median follow-up of 15.1 months, fulvestrant was well tolerated and was at least as effective as anastrozole (median times to progression [TTP] were 5.5 months vs 4.1 months) with a similar and acceptable adverse event profile.[69] Subsequently fulvestrant as first line treatment of advanced breast cancer was compared to tamoxifen in a randomized clinical trial. At a median follow up of 14.5 months no significant difference for the primary end point of time to progression between the two groups was observed.[70] In the setting of a phase 3 randomized clinical trial fulvestrant has also been compared to exemestane for the treatment of postmenopausal women with hormone receptor positive advanced breast cancer who had progressed or recurred on a non steroidal aromatase inhibitor.[71] In this study overall response rate (7.4% vs 6.7%; $p = .736$) and time to treatment progression (3.7 months in both groups) were similar between the fulvestrane and exemestane groups suggesting that fulvestrant was no more effective than a steroidal aromatase inhibitor among women who had progressed on a non-steroidal aromatase inhibitor. Since fulvestrant can take 3 to 6 months to reach steady state plasma levels at the 250 mg/month dose (approved dosing schedule) there are currently ongoing trials that are evaluating the standard dosing regimen to a loading dosing schedule. Currently the fulvestrant is approved for the treatment of hormone receptor positive metastatic breast cancer in postmenopausal that had progressed on prior tamoxifen therapy.[72]

Side Effects of SERMS and Antiestrogens

Side effects related to the SERMS and antiesrtogens develop mainly as a result of the blockage of the stimulatory function of estrogen on a variety of tissues. The most frequent side effect encountered is hot flashes, night sweats and vaginal dryness similar to that seen in women undergoing menopause. Other less frequent but important side effects pertain to the bone, blood vessels and carcinogenic effects.

Osteoporosis ■ Estrogen is important in maintaining bone health in premenopausal women with HRT and often recommended to prevent the development of osteoporosis in postmenopausal women. Long-term administration of an antiestrogen has the potential to cause premature osteoporosis in premenopausal women. However, due to the partial estrogen agonist function of SERMS clinical studies have shown tamoxifen therapy to be not associated with a reduction of bone density[73] and raloxifene is an approved treatment for osteoporosis.[61]

Coronary Heart Disease ■ Estrogen lowers low-density lipoprotein (LDL) cholesterol

levels and raises high-density lipoprotein (HDL) cholesterol levels and thus prolonged administration of an antiestrogen could produce a population at risk of premature coronary heart disease. However, the estrogen-like effects of tamoxifen has been shown to lower the circulating levels of cholesterol in female patients[74,75] with clinical studies reporting tamoxifen to be associated with either a significant or trend in reduction of risk of coronary heart disease.[76] Raloxifene has also been shown to reduce serum cholesterol levels[77]; however, has not been shown in a large randomized clinical trial to reduce the risk of coronary heart disease.[62]

Thromboembolism ■ A number of studies have demonstrated an association between the use of tamoxifen and subsequent thromboembolic episodes in both the treatment and preventive setting.[56,78] This is comparable with increases noted with HRT or raloxifene.[79] Patients with a known history of thromboembolic disorders should be carefully evaluated before a decision is made to use long-term tamoxifen therapy.

Endometrial Tumors ■ Research has demonstrated that increases in endometrial thickness, hyperplasia, and fibroids may follow treatment with tamoxifen.[80] Endometrial thickening is associated with the stromal component of the uterus rather than the epithelial component.[81] Clinical trials evaluating the efficacy of tamoxifen in the treatment and prevention of breast cancer have demonstrated an increased risk of endometrial tumors including carcinomas, and to a smaller extent, sarcomas.[82,83] Endometrial carcinoma that develops on tamoxifen therapy is not of high grade and as such is not associated with poor prognosis while endometrial sarcomas are generally associated with a poorer prognosis, seemingly because of less favorable histology and higher stage.[83] Thus when monitoring patients on tamoxifen treatment all cases of abnormal vaginal bleeding should be followed up with a gynecologic examination and an endometrial biopsy. It is important to note that this increased risk is restricted to postmenopausal women; premenopausal women are not at an increased risk of endometrial cancer. When raloxifene was directly compared to tamoxifen in the prevention of breast cancer (STAR trial), 36 cases of endometrial cancer were observed in the tamoxifen group compared to 23 cases in the raloxifene group (RR 0.62; 95% CI 0.35-1.08).[63]

Other Side Effects ■ Antiestrogens and SERMS have also been associated with ophthalmic side defects such cataracts and retinal changes.[84,85] Preclinical studies have also demonstrated tamoxifen to cause carcinogenesis in the liver; however an increase in human hepatocelluar carcinoma has not been demonstrated.[86]

Aromatase Inhibitors

In contrast to SERMs and antiesrtogens, aromatase inhibitors work by blocking the enzyme complex responsible for the final step in estrogen biosynthetic pathway and is thereby essentially preventing the production of the ER substrate. Moreover unlike tamoxifen aromatase inhibitors have no partial estrogen agonist function. Despite the ovaries being a rich source of aromatase, aromatase inhibitors are unable to sufficiently suppress ovarian estrogen production to postmenopausal levels, which may be due to compensatory rise in gonadotrophins which maintains adequate estrogen production, despite the presence of the inhibitor. In contrast aromatase inhibitors have been shown to adequately suppress estrogen production in postmenopausal women.

Aromatase inhibitors are classified into first-, second-, and third-generation aromatase inhibitors according to the specificity and potency with which they inhibit the aromatase enzyme (Fig. 58-1C). They are further subclassified according to their mechanism of action into steroidal (irreversible, type 1) and nonsteroidal (reversible, type 2) inhibitors. Type 1 inhibitors, including formestane and exemestane, function by irreversibly inhibiting the aromatase enzyme by covalently binding to it, resulting in permanent inactivation that persists even after discontinuation of the drug until the peripheral tissues synthesize new enzymes. Type 2 inhibitors, including anastrozole, letrozole and fadrozoel, in contrast bind reversibly to the active site of the aromatase enzyme and prevent product formation only as long as the inhibitor occupies the catalytic site. In this section we will focus on the newer third-generation aromatase inhibitors letrozole, anastrozole and exemestane that are in common clinical practice today. These aromatase inhibitors have challenged tamoxifen as the gold standard and are now the preferred first line treatment of postmenopausal women with hormone responsive breast cancer in either the early or advanced setting.

First-Generation Aromatase Inhibitors

Aminoglutethimide, a derivative of the sedative agent glutethimide, was initially introduced is an inhibitor of cytochrome P-450 N-mediated steroid hydroxylations.[87] The effects of this compound, however, are rather nonspecific because the drug affects a number of hydroxylation steps in the metabolic conversion of cholesterol to active steroid products, and overall, the use of aminoglutethimide plus glucocorticoid in women with breast cancer produces results similar to those expected from other forms of endocrine therapy. Side effects observed with standard doses of aminoglutethimide (1000 mg/day) include drug rash, fever, and lethargy.[87] With the development of more selective second- and third- generation aromatase inhibitors, aminoglutethimide is now rarely used for the treatment of breast cancer.

Second-Generation Aromatase Inhibitors

The two second-generation aromatase inhibitors on the market are fadrozole and formestane. Fadrozole (4-[5,6,7,8-tetrahydroimidazo-(1,5-a)-pyridin-5yl] benzonitrile)), a type 2 inhibitor, is a potent inhibitor of aromatase. Two large multicenter phase 3 trials have compared fadrozole with MA in patients who had received only tamoxifen as prior hormone therapy. No significant differences were observed between the two treatment arms of the trials with respect to time to treatment progression, overall response rate, response duration, or overall survival.[88] When compared to tamoxifen as a first line treatment among postmenopausal women with advanced breast cancer similar efficacy was observed between the two agents with fadrozole having a better tolerability profile.[89] Toxicity attributed to fadrozole is mild and consists mainly of nausea, anorexia, fatigue, and hot flashes. Fadrozole represents a major improvement over aminoglutethimide and the drug is approved in Japan for the treatment of patients with breast cancer.

Formestane (4-hydroxyandrostenedione, Lentaron), a type 1 inhibitor, is given by intramuscular injection and is thus associated with in-site reactions. It has been tested in clinical trials as second line treatment for postmenopausal women with metastatic disease and demonstrated similar efficacy to mestrol acetate among those who had progressed on tamoxifen[90] with clinical benefit also demonstrated in patients who have progressed on nonsteroidal aromatase inhibitors.[91]

Third-Generation Aromatase Inhibitors

Third generation aromatase inhibitors have now become the standard treatment for postmenopausal women with either advanced or early stage hormone responsive breast cancer having demonstrated superior efficacy and tolerability compared to tamoxifen. Third generation aromatoase inhibitors include exemestane, letrozole, and anastrazole.

Exemestane (6-methylene-androsta-1,4-diene-3,17-dione, Aromasin®), a

type 1 aromatase inhibitor, is an orally administered analog of the natural substrate androstenedione. It is rapidly absorbed from the gastrointestinal tract, reaching maximum plasma levels after 2 h and has been shown to lower estrogen levels more effectively than formestane. Single-dose administration of 25 mg/day inhibits aromatase activity by 97.9% and lowers plasma estrone and estradiol levels by about 90%.[92] The FDA approved dosing regimen for exemestane is 25 mg once daily.

Anastrozole (Arimidex), a type 2 inhibitor, is a potent and selective benzyltriazole derivative absorbed rapidly after oral administration with maximal plasma concentration occurring after 2 h, steady state plasma concentrations achieved after 7 days and has an elimination half-life in humans of approximately 32.2 h.[93] Anastrozole at doses of 1 or 10 mg administered once daily for 28 days has been shown to reduce total body aromatization by 96.7% and 98.1%, respectively. The FDA approved dosing regimen of anastrozole is 1 mg once daily.

Letrozole (4,4'-[(1H-1,2,4-triazol-1-yl) methylene] bis-benzonitrile, Femara), a type 2 inhibitor, is a highly potent inhibitor of aromatase in vitro, in vivo in animals and in humans, and is associated with greater suppression of estrogen than is achieved with other aromatase inhibitors. When administered orally to adult female rats at a dose of 1 mg/L/day for 14 days, letrozole decreased uterine weight to that observed after a surgical ovariectomy.[94] Clinical studies in normal healthy volunteers, as well as dose-seeking phase 1 trials in postmenopausal women with advanced breast cancer, showed that letrozole in a dose as little as 0.25 mg/day PO caused maximal suppression of plasma and urinary estrogens.[95] The FDA approved and recommended dosing regimen for letrozole is 2.5 mg once a day.

Vorozole (R83842; R76713; 6-[(S)4-Chlorophenyl)-1H-1,2,4-triazol-1-ylmethyl]-1-methyl-1H-benzotriazole) represents another specific type 2 aromatase inhibitor that has shown little toxicity in animal studies. However, despite results from phase 3 studies that have demonstrated the clinical efficacy of vorazole in postmenopausal women with metastatic disease,[96] this drug has been withdrawn from further clinical development.

Treatment of Metastatic Breast Cancer ■ Preclinical studies have shown aromatase inhibitors to be effective after initial treatment with tamoxifen.[97] Following demonstrated efficacy in phase 2 trials, a number of phase 3 trials have evaluated the efficacy of third generation aromatase (letrozole, anasztrozole, and exemestane) inhibitors as a second line agent compared to MA in the treatment of postmenopausal women with metastatic breast cancer who had previously been treated with tamoxifen (Table 58-1). In a cohort of 769 postmenopausal women with metastatic breast cancer, exemestane produced a statistically significant increase in median duration of overall clinical benefit (60.1 vs 49 weeks, $p = .025$), median time to tumor progression and median survival compared to MA.[98] In a similar cohort of 764 women from two pivotal phase 3 trials, patients randomized to either anastrozole (1 mg/day PO) or anastrozole (10 mg/day PO) had estimated hazards of progression of 0.97 (97.5% CI 0.75-1.24) and 0.92 (97.5% CI 0.71-1.19) respectively compared to patients receiving megastrol acetate.[99] No statistically significant dose-response differences were observed between the 1 mg/day and 10 mg/day dosage. With subsequent follow-up 2-year survival was 56.1% for the group of patients receiving anastrozole (1 mg/day), compared with 46.3% for patients treated with MA.[100] Similarly the efficacy of letrozole was evaluated in a pivotal trial of 555 postmenopausal women with metastatic breast cancer that had progressed on tamoxifen.[101] Letrozole (2.5 mg/day) yielded overall response rates of 36% and 35%, respectively, compared with 27% and 33%, respectively, for letrozole (0.5 mg/day) and 32% for MA. The median duration of response for letrozole (2.5 mg/day) was 33 months, compared with 18 months for both MA and letrozole 0.5 mg/day. A trend in time to tumor progression and survival that favored letrozole 2.5 mg/day was also observed.

Following the success of third generation aromatase inhibitors in the second line treatment of postmenopausal women with metastatic breast cancer focus shifted to first line treatment of this cohort directly comparing these agents with tamoxifen (Table 58-2). In a phase 2 study comparing exemestane (25 mg/day) with tamoxifen (20 mg/day) as first-line treatment for metastatic disease patients receiving exemestane had better objective response rates (complete response plus partial response) and median duration of response compared to tamoxifen.[102] The study was subsequently extended into a phase 3 trial where exemestane was reported to be well tolerated and was associated with a significantly longer progression-free survival compared with tamoxifen (10.9 vs 6.7 months, respectively).[103] In a combined analysis of two pivotal phase 3 trials that involved 1021 postmenopausal women with metastatic breast cancer first line treatment of anastrozole at a dose of 1 mg/day was compared to tamoxifen.[104] At a median follow-up of 18.5 months for patients with hormone receptor-positive tumors (59.8% of patients), median time to progression was significantly superior in the group receiving anastrozole compared to those receiving tamoxifen (10.7 months vs 6.4 months, $p = .022$). Similarly a large, multicenter, double-blind, first-line phase 3 clinical trial in 907 postmenopausal women with locally advanced or metastatic breast cancer compared letrozole (2.5 mg/day) with tamoxifen (20 mg/ day).[105] At a median follow-up of 32 months time to progression (median, 9.4 vs 6.0 months, respectively; $p < .0001$), time to treatment failure (median, 9 vs 5.7 months, respectively;

Table 58-1 ■ Combined Data From Phase 3 Trials Comparing Anastrozole, Letrozole, and Exemestane With Megestrol Acetate in Postmenopausal Women Previously Treated With Tamoxifen

	Combined Data		Initial Trial		Second Trial		Initial Trial	
	Anastrozole	Megestrol Acetate	Letrozole	Megestrol Acetate	Letrozole	Megestrol Acetate	Exemestane	Megestrol Acetate
No. of patients	263	253	174	189	199	201	366	403
Objective response,[a] %	12.6	12.2	23.6	16.4	16.1	14.9	15	12
Clinical benefit,[b] %	42.2	40.3	34.5	31.7	26.7	23.4	37	35
Progression, %	57.4	59.3	53.4	56.1	51.3	50.7	48	53
Median TTP, months	5	5	5-6	5.5	3	3	5	5
Median duration of benefit, months	18.3	15.7	33	18	17.5	15.4	15	12
Median survival, months	28.7	21.5	25.3	21.5	28.6	26.2	NA	28

[a]Objective response = complete response + partial response. [b]Clinical benefit = complete response + partial response + stable disease for ≥ 6 months.
Abbreviations: NA, not available; TTP, time to progression.

Table 58-2 Efficacy Data From Trials Comparing Anastrozole, Letrozole, and Exemestane With Tamoxifen In the First-Line Treatment of Postmenopausal Women With Metastatic Breast Cancer

	Phase 2 Studies				Phase 3 Studies			
	Letrozole	Tamoxifen	Exemestane	Tamoxifen	Anastrozole[a]	Tamoxifen[a]	Anastrozole[b]	Tamoxifen[b]
No. of patients	453	454	61	59	170	182	340	453
Objective response, %	30	20[c]	41	14	21	17	33	30
Clinical benefit, %	49	38[c]	56	42	59	46[c]	56	56
TTP, months	9	6[c]	9	5	11	6[c]	8	8
TTF, months	9	6[c]	NR	NR	8	5	6	6

[a]North American Study. [b]European Study. [c]Difference is statistically significant.
Abbreviations: Nonprotocol analysis; NR, not recorded.

$p < .0001$) and overall objective response rate (32% vs 21%, respectively; $p = .0002$) were all reported to be significantly superior in the group receiving letrozole compared to tamoxifen. Median overall survival was 34 months for letrozole and 30 months for tamoxifen.

Current guidelines recommend the use of tamoxifen as first line therapy in premenopausal women with metastatic breast cancer.[50] Aromatase inhibitors are recommended as first line therapy of advanced breast cancer in postmenopausal women[50] (Fig. 58-3).

Adjuvant Studies ■ With the efficacy of third generation aromatase inhibitors established in the treatment of postmenopausal women with hormone responsive metastatic breast cancer focus then shifted to determine their efficacy in the adjuvant treatment of early stage breast cancer. With the recognized increased risk of endometrial carcinoma and thromboembolic events associated with the use of tamoxifen, aromatase inhibitors provided a reasonable alternative. Adjuvant studies evaluating third generation aromatase inhibitors have explored their efficacy both as upfront adjuvant treatment and following a course of adjuvant tamoxifen (Table 58-3). The following section will review results of the major trials that have explored these issues.

Upfront Treatment of Early Disease ■ The "Arimidex," Tamoxifen, Alone or in Combination (ATAC) trial is a randomized, double-blind study of 9366 postmenopausal women with early stage breast cancer that was designed to compare the efficacy and tolerability of 5 years of treatment with tamoxifen with that of anastrozole versus the combination of anastrozole and tamoxifen.[106] A planned analysis at a median follow-up of 33 months resulted in the combination arm being discontinued due to lack of superior efficacy or tolerability to tamoxifen alone. The primary endpoint for this study was DFS and secondary endpoints were time to recurrence (TTR), incidence of new contralateral breast cancer, time to distant recurrence (TTDR), and overall survival (OS). At a median follow-up of 100 months among women with hormone receptor positive disease DFS was significantly superior in the anastrazole group compared to the tamoxifen group (HR 0.85; 95% CI 0.76-0.94; $p = .003$) as were TTR, TTDR and incidence of contralateral breast cancer. No significant difference was noted in OS between the two groups (HR 0.97; 95% CI 0.86-1.11; $p = .7$).[107] Fracture rates were higher in the anastrozole group; however the risk of fracture was similar between the two groups once the endocrine therapy was discontinued. No difference in cardio vascular morbidity or mortality was noted between the two groups. There were significantly fewer cases of stroke in the anastrozole group compared to tamoxifen. Tamoxifen was noted to be associated with more hot flashes, endometrial cancers and thromboembolic events.[106]

The Breast International Group 1-98 (BIG 1-98) trial randomized 8,028 postmenopausal women with newly diagnosed hormone receptor-positive breast cancer. BIG 1-98 included two primary adjuvant arms comparing 5 years of letrozole with 5 years of tamoxifen, and two sequential treatment arms comparing 2 years of letrozole followed by 3 years of tamoxifen and 2 years of tamoxifen fol-

Table 58-3 Efficacy Data From Studies of Aromatase Inhibitors In the Adjuvant Treatment of Postmenopausal Women With Early Breast Cancer

	Initial Therapy	Post-Tamoxifen Therapy		
	ATAC Trial	MA 17 Trial	IES Trial	ITA Trial
	Anastrozole vs Tamoxifen	Letrozole vs Placebo	Exemestane vs Tamoxifen	Anastrozole vs Tamoxifen
Median follow-up:	68 months	2.4 years	30.6 months	24 months
No. of patients	9366	5187	4742	426
	DFS HR 0.87 (95% CI 0.78, 0.97; $p = .01$)	Death, recurrence or CLBC HR 0.61 (95% CI 0.47, 0.79; $p \leq .001$)	Risk of recurrence HR 0.68 (95% CI 0.56, 0.82; $p = .00005$)	Death HR 0.18 (95% CI 0.02, 1.57; $p = .07$)
	TTP HR 0.79 (95% CI 0.70, 0.90; $p = .0005$)	Recurrence or CLBC HR 0.57 (95% CI 0.43, 0.75; $p = .00008$)	Survival free of distant metastases HR 0.66 (95% CI 0.52, 0.83; $p = .0004$)	Relapse HR 0.36 (95% CI 0.17, 0.75; $p = .006$)
	CLCB OR 0.58 (95% CI 0.38, 0.88; $p = .01$)		CLBC HR 0.44 (95% CI 0.20, 0.98; $p = .04$)	

Abbreviations: CI, confidence intervals; CLBC, contralateral breast cancer; DFS, disease-free survival; HR, hazard ratio; OR, odds ratio; TTP, time to progression.

lowed by 3 years of letrozole.[108] Current analysis compared all patients who initially received letrozole to those that initially received tamoxifen with a primary end point of DFS. At a median follow-up of 51 months 352 DFS events among 2463 women receiving letrozole and 418 events among 2459 women receiving tamoxifen resulting in an 18% reduction in the risk of an event (HR 0.82; 95% CI, 0.71-0.95; $p = .007$) among women receiving letrozole compared to those receiving tamoxifen was observed.[109] There was a significantly greater incidence of bone fractures in patients receiving letrozole compared with those receiving tamoxifen (OR 1.44; p .0006). Patients in the tamoxifen group had significantly more grade 3-5 thromboembolic events (OR 0.38; $p < .0001$) than patients in the letrozole group.[108] There was a non significant trend towards an increased in cardiovascular events (myocardial infarction and cardiac deaths) in the letrozole group as compared to the tamoxifen group. At present time there are no data regarding direct comparison of these two aromatase inhibitors to accurately discern differences in safety and efficacy profiles between the different third generation aromatase inhibitors.

Post-Tamoxifen Treatment ■ The second issue to be explored was the sequential use of a third generation aromatase inhibitor following a period of adjuvant treatment of tamoxifen among postmenopausal women with early stage breast cancer. The MA 17 trial was a randomized, double-blind, extended adjuvant, placebo-controlled trial in 5187 postmenopausal women who had received 5 years of adjuvant treatment with tamoxifen with DFS as the primary end point.[110] Due to superior efficacy observed in the letrozole group compared to the placebo group at the first interim analysis the study was prematurely terminated. In an updated analysis, at a median follow up of 30 months the letrozole group had a significantly longer DFS compared to the placebo group (HR 0.58; 95% CI 0.45-0.76; $p < .001$).[111] Letrozole was found to be well tolerated and was associated with a lower incidence of vaginal bleeding and an increase in hot flashes, arthritis, arthralgia, and myalgia, compared with placebo. After premature termination of the study the group who were in the placebo of arm of the trial was offered letrozole. Women who elected to take letrozole did so at median of 2.8 years from completion of tamoxifen treatment with a recent update reporting improved DFS among those who elected to take letrozole compared to those who did not.[112]

The Intergroup Exemestane Study (IES) examined the efficacy and safety of exemestane therapy after 2 to 3 years of adjuvant tamoxifen therapy. The trial enrolled 4742 patients who were randomly assigned to continue with tamoxifen, or to switch to exemestane for the remainder of the 5-year treatment period. At a median follow-up of 30.6 months the data indicate that switching to exemestane was associated with a significant improvement in disease-free survival compared with continuing with tamoxifen (HR 0.68; 95% CI 0.56-0.82; $p < .001$).[113] At a median follow-up of 55.7 months switching to exemestane resulted in a 24% improvement in DFS (HR 0.76; 95% CI 0.66-0.88; $p = .0001$) and a 15% improvement in overall survival (HR 0.85; 95% CI 0.71-1.02; $p = .08$) as compared to the tamoxifen group.[114] The NSABP B-33 trial, similar to the MA17 trial, was a randomized trial that evaluated 5 years of exemestane to 5 years of placebo following the completion of 5-years of tamoxifen.[115] Due to the results of the MA-17 study the NASBP B-33 study was terminated early and unblinded. Despite a premature closure and cross over of patients an improvement in 4-year DFS was observed among the original cohort who received exemestane compared to the placebo group (91% vs 89%; HR 0.68; $p = .07$).[115]

Several other trials have also evaluated the sequential administration of aromatase inhibitors following tamoxifen therapy. The Italian Tamoxifen Anastrozole (ITA) and Arimdex Nolvadex 95/Austrian Breast cancer Study Group 8 (ARNO 95/ABCSG) have shown that switching to anastrazole after 2-3 years of tamoxifen significantly reduced the risk of recurrence.[116,117] In the ABCSG trial 6a postmenopausal women were randomized to either anastrozole or placebo following five years of adjuvant tamoxifen therapy.[118] At a median follow-up of 62.3 months women in the anastrozole group had a 38% risk reduction in recurrence compared to placebo (HR 0.62; 95% CI 0.40-0.96, $p = .031$).

Based on the evidence presented above, the author recommends that aromatase inhibitors be offered upfront as adjuvant therapy among postmenopausal women with hormone receptor positive early stage breast cancer (Fig. 58-4). The highest risk of recurrence is within the first 2 to 3 years of diagnosis. Despite no demonstrated overall survival benefit demonstrated at this time, the use of aromatase inhibitors have superior DFS rates compared to tamoxifen which with longer follow up may translate into a survival benefit. However, it is also important to note that current guidelines of adjuvant endocrine therapy for postmenopausal women recommend an aromatase inhibitor as either initial therapy or after an initial period of treatment with tamoxifen,[119] with the decision based on the risks and benefits of each agent for an individual patient. Longer term follow-up of the adjuvant trials described above are awaited to better define long-term efficacy results, side effect profile and delineate duration of required treatment for an aromatase inhibitor used in the adjuvant setting.

Neoadjuvant Studies ■ Neoadjuvant treatment is intended to downstage a tumor before primary locoregional therapy with surgery, thus allowing breast conserving surgery in a greater number of patients, or making surgery possible in cases that were considered inoperable. As the third generation aromatase inhibitors have demonstrated efficacy in the

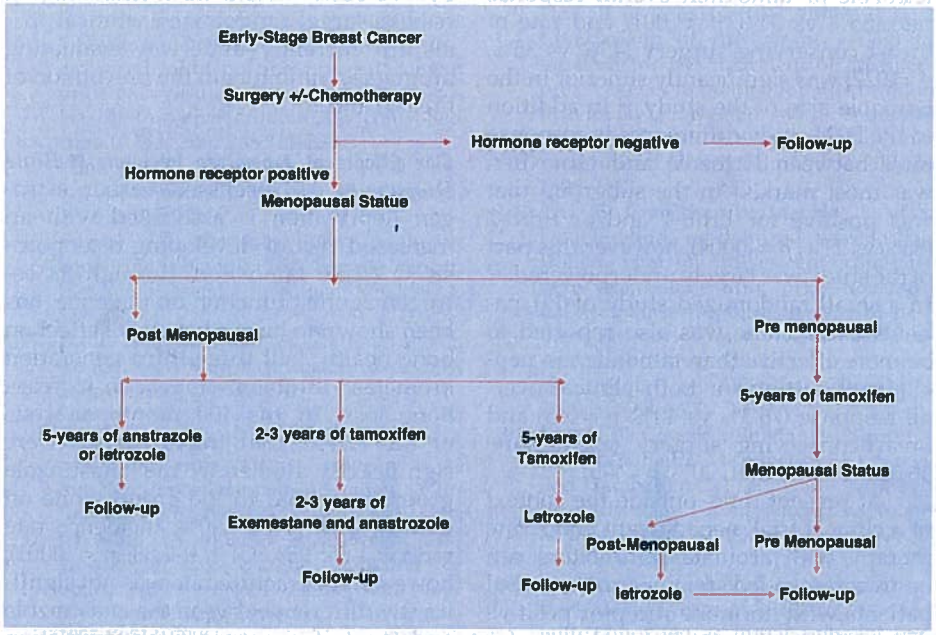

Figure 58-4 ■ Schema of systemic adjuvant therapy for postmenopausal women with hormone receptor positive disease.

treatment of advanced and early disease, it was important to examine the benefits these agents may bring to the pre operative setting.

Compared to neoadjuvant chemotherapy trials the neoadjuvant studies exploring the effect of endocrine therapy in the treatment of breast cancer have been smaller. Several trials have compared neoadjuvant tamoxifen to a third generation aromatase inhibitor postmenopausal women with hormone receptor positive breast cancer.[120–124] The Immediate Preoperative Arimidex, Tamoxifen, or Combined with Tamoxifen (IMPACT) trial compared 3 months of preoperative anastrazole with tamoxifen or a combination of both in 330 postmenopausal women with hormone receptor positive breast cancer.[36]No difference in overall response was noted in three groups (37% vs 36% vs 39%). However among the 124 women considered to require a mastectomy at baseline 46% treated with anastrozole were considered to be candidates for breast conserving surgery by their surgeon, compared with 22% receiving tamoxifen ($p = .03$).[120] Similarly in the PReOperative Arimidex Compared with Tamoxifen (PROACT) trial that randomized 451 postmenopausal women to either 3 months of neoadjuvant anastrozole or tamoxifen with or without chemotherapy reported that among the hormone therapy only group of patients who needed mastectomy, at baseline there was an improvement in breast conservation rates (43.0%) of patients receiving anastrozole compared to 30.8% receiving tamoxifen ($p = .04$).[121]

In the P024 study where 337 postmenopausal women were randomly assigned to either 4 months of neoadjuvant letrozole or tamoxifen overall response rate (55% vs 36%, $p < .001$) and rate of breast conserving surgery (45% vs 35%, $p = .022$) was significantly superior in the letrozole arm of the study.[122] In addition in the P024 study differences in response rates between letrozole and tamoxifen was most marked in the subgroup that was positive for ErbB-1 and/or ErbB-2 (88% vs 21%, $p = .0004$), however this part of the study was largely underpowered.[123] In a small randomized study of 151 patients exemestane was also reported to be more effective than tamoxifen in neoadjuvant setting for both clinical overall response (76.3% vs 40%; $p = .05$) and breast-conserving surgery (exemestane 36.8% vs tamoxifen 20%; $p = .05$).[124]

At present time, outside the context of a clinical trial, neoadjuvant endocrine therapy with aromatase inhibitors can be recommended for postmenopausal patients with hormone receptor positive disease who may not be candidates for preoperative chemotherapy because of associated existing comorbidity.[125] Several questions still remain to be addressed by future clinical trials including the appropriate duration of neoadjuvant endocrine therapy.

Chemoprevention of Breast Cancer ■ As discussed earlier the side effect profile of tamoxifen restricts its use in the prevention of breast cancer. Furthermore trials looking at tamoxifen and raloxifene in the prevention setting reduced the incidence of ER positive breast cancers by about 50% and thus looking for an agent that would reduce the incidence further is important. Aromatase inhibitors, with its proven efficacy in the treatment of hormone receptor positive breast cancers are a viable option. In the ATAC trial[107] at a median follow-up of 100 months, it was observed that the incidence of hormone receptor positive contralateral breast cancers was significantly lower in the anastrazole group compared to the tamoxifen group (HR 0.60; 95% CI 0.42-0.85; $p = .004$). Similarly in the BIG 1-98 trial[108,109] fewer cases of contralateral breast cancer were identified in the letrozole group than in the tamoxifen group (16 vs 27 cases). In the IES study[114] at a median follow-up of 55.7 months 17 cases of contralateral breast cancer were observed in the exemestane group compared to 35 cases in the tamoxifen group (HR 0.56; 95% CI 0.33-0.98; $p = .04$).

These trials data suggest that the third generation aromatase inhibitors can reduce the incidence of contralateral breast cancer by approximately ~40-50% above and beyond that observed by tamoxifen. By extrapolation this would be imply that these aromatase inhibitors, on their own, would reduce the incidence of ER positive contralateral breast cancer by ~70-80%.[126] With such encouraging results large randomized clinical trials are currently underway evaluating aromatase inhibitors in the prevention of breast cancer.

Side Effects of Aromatase Inhibitors ■ ***Bone Disease*** ■ As discussed earlier estrogen deprivation is associated with an increased risk of developing osteoporosis.[127] While tamoxifen, through its estrogen agonist function on the bone, has been shown to have a beneficial effect on bone health,[73] all three third generation aromatase inhibitors appear to increase bone loss. In the 100 month analysis of the ATAC trial fracture rates were significantly higher in the anastrazole group compared to the group while on therapy (2.93% vs 1.90%; incidence rate ratio = 1.55; 95% CI 1.31-1.83; $p < .0001$); however, the fracture rate was not significantly different between the anastrazole and tamoxifen groups after completion of 5 years treatment (1.56% vs 1.51%; incidence rate ratio = 1.03, 95% CI 0.81-1.31, $p = .79$).[105] In BIG 1-98 trial at a median follow-up of 51 months the 8.6% of patients in the letrozole group experienced a fracture compared to 5.8% of patients in the tamoxifen group with the difference being statistically significant ($p < .001$).[109] Similar observations were also made in the IES trial where at a median follow-up of 55.7 months a significantly increased fracture rate of 7% was observed in the exemestane group compared to 5% in the tamoxifen group (OR 1.45; 95% CI 1.13-1.87; $p = .003$.[128] One method of preventing or reversing bone loss associated with aromatase inhibitors would be to use bisphosphonates. In the integrated analysis of two randomized Zometa-Femara Adjuvant Synergy Trials (Z-FAST and ZO-FAST) 1667 patients that were receiving adjuvant letrozole received either upfront zoledronic acid or received it only when bone mineral density decreased to below –2.[129] At month 12 patients the upfront group had lumbar spine bone mineral density that was 5.2% higher than the group of patients who received delayed zoledronic acid. Longer follow up will be needed to determine its effect on the bone fractures.

Cardiovascular Disease ■ Postmenopausal women with breast cancer may be at a higher risk of cardiovascular events due to their age, menopausal status, associated co morbid conditions and exposure to chemotherapeutic agents used in the treatment of breast cancer. As described earlier, tamoxifen, through its estrogen agonist function, has been shown to have a lipid lowering effect that has translated into modest reductions in cardiovascular events.[76] Anastrozole has not been shown to appreciably alter lipid profiles,[130,131] and in the adjuvant setting myocardial infarctions experienced by women taking anastrozole was similar compared to the group taking tamoxifen.[107] In the BIG 1-98 trial at a median follow-up of 51 months women in the letrozole group experienced a higher low grade cholesterol elevation and cardiovascular events (other than ischemic heart disease and cardiac failure) compared to women in the tamoxifen group.[109] The higher low grade cholesterol elevation in the letrozole group relative to that of patients in the tamoxifen group may be a reflection of the lipid lowering effect of tamoxifen as mentioned earlier. Studies with exemestane have shown that apart from a modest drop in HDL cholesterol,[132] exemestane has no appreciable effect on lipid levels. In the IES study, at a median follow-up of 55.7 months, among all patients the incidence of cardiovascular events (excluding thromboembolic events) did not seem to differ between the exemestane and tamoxifen groups with approximately 1.3% of exemestane-treated patients experiencing

a myocardial infarction compared to 0.8% of tamoxifen-treated patients (p = .08). Longer follow-up will be required to assess the cardiovascular effects of the clinically used third generation aromatase inhibitors. Women with breast cancer are in general at higher risk of developing a cardiovascular event due to a multitude of factors and as such, and should be monitored and managed appropriately.

Other Adverse Events ■ Other side effects commonly associated with aromatase inhibitors include arthralgias, vaginal dryness, and dysparuenia. Although treatment with aromatase inhibitors increases the risk of vasomotor symptoms and vaginal bleeding/discharge large trials have shown that the incidence of these events were lower compared to those on tamoxifen treatment.[106–118] Furthermore these trials also reported that the incidence of thromboembolic events and endometrial carcinoma were also lower in women taking an aromatase inhibitor compared to those taking tamoxifen.

Resistance ■ In metastatic disease, the sequential utilization of hormonal agents can produce long-term palliation of hormone-dependent breast cancer. Eventually, however, the problem of hormone resistance is encountered. The mechanisms by which tumors become resistant to hormones, in general, are only partially understood.[133] Refractoriness to therapy with aromatase inhibitors is related not to the failure of these agents to suppress estradiol levels, but rather because of alterations in other cellular components, such as the growth factor receptor pathways and ability of then tumors to grow in estrogen deprived environments.[134] Our increasing understanding of these processes, and the development of target-orientated therapies such as trastuzumab (Herceptin), are interesting areas of future research that should make novel therapeutic approaches available for the treatment of endocrine resistance.

Conclusion

In summary a number of endocrine agents are now available for the management of both early and advanced stage hormone response breast cancer, each unique in its mechanism of action targeting different points in the ER and PR pathways. Tamoxifen has been ubiquitous as the front-line therapy for the treatment of all stages of breast cancer, and remains the central choice for the treatment of premenopausal women. Among postmenopausal women the introduction of the non-cross resistant aromatase inhibitors has changed recommendations being now at the fore front of treatment of both early and advanced staged breast cancers. However several questions regarding the use of aromatase inhibitors still remain including duration of use in the adjuvant setting and sequence of use with tamoxifen. Moreover among the three third generation aromatase inhibitors there are not head to head comparisons to support the superiority either in efficacy or safety of one aromatase inhibitor over another. Further more the use of aromatase inhibitors are not without side effects and clinical trials are underway to examine methods of preventing bone loss which can have a significant impact on quality of life. Antiestrogen (Fulvestrant) progestins including MA and MPA are useful agents to try when resistance to tamoxifen and aromatase inhibitors has developed.

Lastly, as more hormonal therapies become available, and our understanding of the molecular pathways underpinning resistance increases,[41,135] it is essential that the optimal sequence of endocrine agents be established in the treatment of breast cancer. This may prolong the time during which endocrine therapies can be used, so postponing the time when cytotoxic chemotherapy becomes a necessary option.

Selected References

The complete reference list can be found at www.CANCERMEDICINE8.com

10. Collingwood TN, Urnov FD, Wolffe AP. Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. J Mol Endocrinol. 1999;23:255–275.
12. Rossouw JE, Anderson GL, Prentice RL, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA. 2002;288:321–333.
15. Lundgren S. Progestins in breast cancer treatment. A review. Acta Oncol. 1992;31:709–722.
16. Buzdar AU, Hortobagyi G. Update on endocrine therapy for breast cancer. [Review] [53 refs]. Clin Cancer Res. 1998;4: 527–534.
17. Haller DG, Glick JH. Progestational agents in advanced breast cancer: an overview. Semin Oncol. 1986;13:2–8.
46. Sherman BM, Chapler FK, Crickard K, Wycoff D. Endocrine consequences of continuous antiestrogen therapy with tamoxifen in premenopausal women. J Clin Invest. 1979;64:398–404.
47. Jaiyesimi IA, Buzdar AU, Decker DA, Hortobagyi GN. Use of tamoxifen for breast cancer: twenty-eight years later [see comments] [Review]. J Clin Oncol. 1995;13:513–529.
48. Ingle JN, Krook JE, Green SJ, et al. Randomized trial of bilateral oophorectomy versus tamoxifen in premenopausal women with metastatic breast cancer. J Clin Oncol. 1986;4:178–185.
49. Klijn JG, Blamey RW, Boccardo F, et al. Combined tamoxifen and luteinizing hormone-releasing hormone (LHRH) agonist versus LHRH agonist alone in premenopausal advanced breast cancer: a meta-analysis of four randomized trials. J Clin Oncol. 2001;19:343–353.
51. Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005;365:1687–1717.
52. Fisher B, Dignam J, Bryant J, Wolmark N. Five versus more than five years of tamoxifen for lymph node-negative breast cancer: updated findings from the National Surgical Adjuvant Breast and Bowel Project B-14 randomized trial [see comments]. J Natl Cancer Inst. 2001;93:684–690.
53. Peto R, Davies C, and on behalf of the ATLAS Collaboration. ATLAS (Adjuvant Tamoxifen, Longer Against Shorter): international randomized trial of 10 versus 5 years of adjuvant tamoxifen among 11 500 women preliminary results. 30th Annual San Antonio Breast Cancer Symposium. 2007, Abstract.
54. Gray RG, Rea DW, Handley K, et al. and aTTom Collaborators. aTTom (adjuvant TamoxifeN̈To offer more?): Randomized trial of 10 versus 5 years of adjuvant tamoxifen among 6,934 women with estrogen receptor-positive (ER+) or ER untested breast cancerN̈preliminary results [Abstract]. J Clin Oncol. May 20, 2008;26(suppl).
56. Cuzick J, Powles T, Veronesi U, et al. Overview of the main outcomes in breast-cancer prevention trials [Review] [9 refs]. Lancet. 2003;361:296–300.
63. Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA. 2006;295:2727–2741.
70. Howell A, Robertson JF, Abram P, et al. Comparison of fulvestrant versus tamoxifen for the treatment of advanced breast cancer in postmenopausal women previously untreated with endocrine therapy: a multinational, double-blind, randomized trial. J Clin Oncol. 2004;22: 1605–1613.
71. Chia S, Gradishar W, Mauriac L, et al. Double-blind, randomized placebo controlled trial of fulvestrant compared with exemestane after prior nonsteroidal aromatase inhibitor therapy in postmenopausal women with hormone receptor-positive, advanced breast cancer: results from EFECT. J Clin Oncol. 2008;26:1664–1670.
107. Forbes JF, Cuzick J, Buzdar A, Howell A, Tobias JS, Baum M. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 100-month analysis of the ATAC trial. Lancet Oncol. 2008;9:45–53.
109. Coates AS, Keshaviah A, Thurlimann B, et al. Five years of letrozole compared with tamoxifen as initial adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer:

Complete References

The complete reference list can be found at www.CANCERMEDICINE8.com

1. Beatson GT. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. *Lancet.* 1896;ii:104–107.
2. Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. [Review] [95 refs]. *Clin Cancer Res.* 2003;9:1980–1989.
3. Vegeto E, Shahbaz MM, Wen DX, Goldman ME, O'Malley BW, McDonnell DP. Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. *Mol Endocrinol.* 1993;7:1244–1255.
4. Giangrande PH, McDonnell DP. The A and B isoforms of the human progesterone receptor: two functionally different transcription factors encoded by a single gene. *Recent Prog Horm Res.* 1999;54:291–313.
5. McDonnell DP, Goldman ME. RU486 exerts antiestrogenic activities through a novel progesterone receptor A form-mediated mechanism. *J Biol Chem.* 1994;269:11945–11949.
6. Smith DF. Chaperones in progesterone receptor complexes. *Semin Cell Dev Biol.* 2000;11:45–52.
7. Kosano H, Stensgard B, Charlesworth MC, McMahon N, Toft D. The assembly of progesterone receptor-hsp90 complexes using purified proteins. *J Biol Chem.* 1998;273:32973–32979.
8. Nettles KW, Greene GL. Ligand control of coregulator recruitment to nuclear receptors. *Annu Rev Physiol.* 2005;67:309–333.
9. McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell.* 2002;108:465–474.
10. Collingwood TN, Urnov FD, Wolffe AP. Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. *J Mol Endocrinol.* 1999;23:255–275.
11. Edwards DP. Regulation of signal transduction pathways by estrogen and progesterone. *Annu Rev Physiol.* 2005;67:335–376.
12. Rossouw JE, Anderson GL, Prentice RL, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *JAMA.* 2002;288:321–333.
13. Baulieu EE. Contragestion and other clinical applications of RU 486, an antiprogesterone at the receptor. *Science.* 1989;245:1351–1357.
14. Conneely OM, Lydon JP, De MF, O'Malley BW. Reproductive functions of the progesterone receptor. *J Soc Gynecol Investig.* 2000;7:S25–S32.
15. Lundgren S. Progestins in breast cancer treatment. A review. *Acta Oncol.* 1992;31:709–722.
16. Buzdar AU, Hortobagyi G. Update on endocrine therapy for breast cancer. [Review] [53 refs]. *Clin Cancer Res.* 1998;4: 527–534.
17. Haller DG, Glick JH. Progestational agents in advanced breast cancer: an overview. *Semin Oncol.* 1986;13:2–8.
18. Lentz SS, Brady MF, Major FJ, Reid GC, Soper JT. High-dose megestrol acetate in advanced or recurrent endometrial carcinoma: a Gynecologic Oncology Group Study. *J Clin Oncol.* 1996;14:357–361.
19. Spitz IM, Chwalisz K. Progesterone receptor modulators and progesterone antagonists in women's health. *Steroids.* 2000;65:807–815.
20. Christin-Maitre S, Bouchard P, Spitz IM. Medical termination of pregnancy. *N Engl J Med.* 2000;342:946–956.

21. Dickson RB, Lippman ME. Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. *Endocr Rev.* 1987;8:29–43.

22. Simpson ER, Merrill JC, Hollub AJ, Graham-Lorence S, Mendelson CR. Regulation of estrogen biosynthesis by human adipose cells. *Endocr Rev.* 1989;10:136–148.

23. Fishman J, Hahn EF. The nature of the final oxidative step in the aromatization sequence. *Steroids.* 1987;50:339–345.

24. bul-Hajj YJ, Iverson R, Kiang DT. Aromatization of androgens by human breast cancer. *Steroids.* 1979;33:205–222.

25. Judd HL, Judd GE, Lucas WE, Yen SS. Endocrine function of the postmenopausal ovary: concentration of androgens and estrogens in ovarian and peripheral vein blood. *J Clin Endocrinol Metab.* 1974;39:1020–1024.

26. Kirschner MA, Schneider G, Ertel NH, Worton E. Obesity, androgens, estrogens, and cancer risk. *Cancer Res.* 1982;42:3281s–3285s.

27. Fishman J, Nisselbaum JS, Menendez-Botet CJ, Schwartz MK. Estrone and estradiol content in human breast tumors: relationship to estradiol receptors. *J Steroid Biochem.* 1977;8:893–896.

28. Santen RJ, Leszczynski D, Tilson-Mallet N, et al. Enzymatic control of estrogen production in human breast cancer: relative significance of aromatase versus sulfatase pathways. *Ann N Y Acad Sci.* 1986;464:126–137.

29. Hanstein B, Djahansouzi S, Dall P, Beckmann MW, Bender HG. Insights into the molecular biology of the estrogen receptor define novel therapeutic targets for breast cancer. *Eur J Endocrinol.* 2004;150:243–255.

30. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids.* 2007;72:7–25.

31. Greenwald P, Barlow JJ, Nasca PC, Burnett WS. Vaginal cancer after maternal treatment with synthetic estrogens. *N Engl J Med.* 1971;285:390–392.

32. Herbst AL, Ulfelder H, Poskanzer DC, Longo LD. Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. 1971. *Am J Obstet Gynecol.* 1999;181:1574–1575.

33. Shapiro S, Kelly JP, Rosenberg L, et al. Risk of localized and widespread endometrial cancer in relation to recent and discontinued use of conjugated estrogens. *N Engl J Med.* 1985;313:969–972.

34. Pike MC, Peters RK, Cozen W, et al. Estrogen-progestin replacement therapy and endometrial cancer [see comments]. *J Natl Cancer Inst.* 1997;89:1110–1116.

35. Anderson GL, Limacher M, Assaf AR, et al. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *JAMA.* 2004;291:1701–1712.

36. Beral V. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet.* 2003;362:419–427.

37. Cauley JA, Lucas FL, Kuller LH, Stone K, Browner W, Cummings SR. Elevated serum estradiol and testosterone concentrations are associated with a high risk for breast cancer. Study of Osteoporotic Fractures Research Group [see comments]. *Ann Intern Med.* 1999;130:270–277.

38. Cauley JA, Gutai JP, Sandler RB, LaPorte RE, Kuller LH, Sashin D. The relationship of endogenous estrogen to bone density and bone area in normal postmenopausal women. *Am J Epidemiol.* 1986;124:752–761.

39. Zhang Y, Kiel DP, Kreger BE, et al. Bone mass and the risk of breast cancer among postmenopausal women. *N Engl J Med.* 1997;336:611–617.

40. Lippman ME, Bolan G. Oestrogen-responsive human breast cancer in long term tissue culture. *Nature.* 1975;256:592–593.

41. Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. *J Clin Oncol.* 2007;25:5815–5824.

42. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov.* 2003;2:205–213.

43. Osborne CK, Boldt DH, Clark GM, Trent JM. Effects of tamoxifen on human breast cancer cell cycle kinetics: accumulation of cells in early G1 phase. *Cancer Res.* 1983;43:3583–3585.

44. Lien EA, Solheim E, Lea OA, Lundgren S, Kvinnsland S, Ueland PM. Distribution of 4-hydroxy-N-desmethyltamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. *Cancer Res.* 1989;49:2175–2183.

45. Patterson JS, Settatree RS, Adam HK, Kemp JV. Serum concentrations of tamoxifen and major metabolite during long-term nolvadex therapy, correlated with clinical response. *Eur J Cancer.* 1980;Suppl 1:89–92.

46. Sherman BM, Chapler FK, Crickard K, Wycoff D. Endocrine consequences of continuous antiestrogen therapy with tamoxifen in premenopausal women. *J Clin Invest.* 1979;64:398–404.

47. Jaiyesimi IA, Buzdar AU, Decker DA, Hortobagyi GN. Use of tamoxifen for breast cancer: twenty-eight years later [see comments] [Review]. *J Clin Oncol.* 1995;13:513–529.

48. Ingle JN, Krook JE, Green SJ, et al. Randomized trial of bilateral oophorectomy versus tamoxifen in premenopausal women with metastatic breast cancer. *J Clin Oncol.* 1986;4:178–185.

49. Klijn JG, Blamey RW, Boccardo F, et al. Combined tamoxifen and luteinizing hormone-releasing hormone (LHRH) agonist versus LHRH agonist alone in premenopausal advanced breast cancer: a meta-analysis of four randomized trials. *J Clin Oncol.* 2001;19:343–353.

50. National Comprehensive Cancer Network. *Breast Cancer*. Available at: http://www.nccn.org/professionals/physicians_gls/PDF/breast.pdf. Clinical Practice Guidelines in Oncology v.2. 2008.

51. Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet.* 2005;365:1687–1717.

52. Fisher B, Dignam J, Bryant J, Wolmark N. Five versus more than five years of tamoxifen for lymph node-negative breast cancer: updated findings from the National Surgical Adjuvant Breast and Bowel Project B-14 randomized trial [see comments]. *J Natl Cancer Inst.* 2001;93:684–690.

53. Peto R, Davies C, and on behalf of the ATLAS Collaboration. ATLAS (Adjuvant Tamoxifen, Longer Against Shorter): international randomized trial of 10 versus 5 years of adjuvant tamoxifen among 11 500 women preliminary results. *30th Annual San Antonio Breast Cancer Symposium.* 2007, Abstract.

54. Gray RG, Rea DW, Handley K, et al. and aTTom Collaborators. aTTom (adjuvant TamoxifenÑTo offer more?): Randomized trial of 10 versus 5 years of adjuvant tamoxifen among 6,934 women with estrogen receptor-positive (ER+) or ER untested breast cancerÑpreliminary results [Abstract]. *J Clin Oncol*. May 20, 2008;26(suppl).

55. Albain K, Barlow W, O'Malley F. Concurrent (CAFT) versus sequential (CAF-T) chemohormonal therapy (cyclophosphamide, doxorubicin, 5-fluorouracil, tamoxifen) versus T alone for postmenopausal, node-positive, estrogen (ER) and/or progesterone (PgR) receptor-positive breast cancer: mature outcomes and new biologic correlates on phase III intergroup trial 0100. *SWOG-881427th Annual San Antonio Breast Cancer Symposium*. 2004, Abstract.

56. Cuzick J, Powles T, Veronesi U, et al. Overview of the main outcomes in breast-cancer prevention trials [Review] [9 refs]. *Lancet.* 2003;361:296–300.

57. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst.* 1998;90:1371–1388.

58. Gail MH, Costantino JP, Bryant J, et al. Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer [published erratum appears in *J Natl Cancer Inst* Feb 2, 2000;92(3):275] [Review] [85 refs]. *J Natl Cancer Inst.* 1999;91:1829–1846.

59. King MC, Wieand S, Hale K, et al. Tamoxifen and breast cancer incidence among women with inherited mutations in BRCA1 and BRCA2: National Surgical Adjuvant Breast and Bowel Project (NSABP-P1) Breast Cancer Prevention Trial. *JAMA.* 2001;286:2251–2256.

60. Cauley JA, Norton L, Lippman ME, et al. Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial. Multiple outcomes of raloxifene evaluation. *Breast Cancer Res Treat.* 2001;65:125–134.

61. Martino S, Cauley JA, Barrett-Connor E, et al. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J Natl Cancer Inst.* 2004;96:1751–1761.

62. Barrett-Connor E, Mosca L, Collins P, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Engl J Med.* 2006;355:125–137.

63. Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA.* 2006;295:2727–2741.

64. Holli K, Valavaara R, Blanco G, et al. Safety and efficacy results of a randomized trial comparing adjuvant toremifene and tamoxifen in postmenopausal patients with node-positive breast cancer. Finnish Breast Cancer Group. *J Clin Oncol.* 2000;18:3487–3494.

65. Pyrhonen S, Ellmen J, Vuorinen J, et al. Meta-analysis of trials comparing toremifene with tamoxifen and factors predicting outcome of antiestrogen therapy in postmenopausal women with breast cancer. *Breast Cancer Res Treat.* 1999;56:133–143.

66. Leonard RCF, Bundred N, Buzdar A. Multi-center clinical trials of trilostane (Modrenal) for advanced breast cancer in post-menopausal women [Abstract]. *Breast Cancer Res Treat.* 2003;87(suppl 1).

67. Osborne CK, Coronado-Heinsohn EB, Hilsenbeck SG, et al. Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. *J Natl Cancer Inst.* 1995;87:746–750.

68. Robertson JFR, Nicholson RI, Bundred NJ. Comparison of the short-term biological effects of fulvestrant (ICI 182, 780) with tamoxifen in postmenopausal women with primary breast cancer [Abstract]. *Breast Cancer Res Treat*. 2000;59(99).

69. Robertson JF, Osborne CK, Howell A, et al. Fulvestrant versus anastrozole for the treatment of advanced breast carcinoma in postmenopausal women: a prospective combined analysis of two multicenter trials. *Cancer.* 2003;98:229–238.

70. Howell A, Robertson JF, Abram P, et al. Comparison of fulvestrant versus tamoxifen for the treatment of advanced breast cancer in postmenopausal women previously untreated with endocrine therapy: a multinational, double-blind, randomized trial. *J Clin Oncol.* 2004;22: 1605–1613.

71. Chia S, Gradishar W, Mauriac L, et al. Double-blind, randomized placebo controlled trial of fulvestrant compared with exemestane after prior nonsteroidal aromatase inhibitor therapy in postmenopausal women with hormone receptor-positive, advanced breast cancer: results from EFECT. *J Clin Oncol.* 2008;26:1664–1670.

72. Bross PF, Cohen MH, Williams GA, Pazdur R. FDA drug approval summaries: fulvestrant. *Oncologist.* 2002;7:477–480.

73. Love RR, Mazess RB, Tormey DC, Barden HS, Newcomb PA, Jordan VC. Bone mineral density in women with breast cancer treated with adjuvant tamoxifen for at least two years. *Breast Cancer Res Treat.* 1988;12:297–302.

74. Bertelli G, Pronzato P, Amoroso D, et al. Adjuvant tamoxifen in primary breast cancer: influence on plasma lipids and antithrombin III levels. *Breast Cancer Res Treat.* 1988;12:307–310.

75. Love RR, Wiebe DA, Feyzi JM, Newcomb PA, Chappell RJ. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women after 5 years of treatment. *J Natl Cancer Inst.* 1994;86:1534–1539.

76. Braithwaite RS, Chlebowski RT, Lau J, George S, Hess R, Col NF. Meta-analysis of vascular and neoplastic events associated with tamoxifen. *J Gen Intern Med.* 2003;18:937–947.

77. Walsh BW, Kuller LH, Wild RA, et al. Effects of raloxifene on serum lipids and coagulation factors in healthy postmenopausal women [see comments]. *JAMA.* 1998;279:1445–1451.

78. Deitcher SR, Gomes MP. The risk of venous thromboembolic disease associated with adjuvant hormone therapy for breast carcinoma: a systematic review. *Cancer.* 2004;101:439–449.

79. Kedar RP, Bourne TH, Powles TJ, et al. Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial [see comments]. *Lancet.* 1994;343:1318–1321.

80. Decensi A, Fontana V, Bruno S, Gustavino C, Gatteschi B, Costa A. Effect of tamoxifen on endometrial proliferation. *J Clin Oncol.* 1996;14:434–440.

81. Fornander T, Rutqvist LE, Cedermark B, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet.* 1989;1:117–120.

82. Fisher B, Costantino JP, Redmond CK, Fisher ER, Wickerham DL, Cronin WM. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. *J Natl Cancer Inst.* 1994;86:527–537.

83. Bergman L, Beelen ML, Gallee MP, Hollema H, Benraadt J, van Leeuwen FE. Risk and prognosis of endometrial cancer after tamoxifen for breast cancer. Comprehensive Cancer Centres' ALERT Group. Assessment of Liver and Endometrial cancer Risk following Tamoxifen. *Lancet.* 2000;356:881–887.

84. Paganini-Hill A, Clark LJ. Eye problems in breast cancer patients treated with tamoxifen. *Breast Cancer Res Treat.* 2000;60:167–172.

85. Kaiser-Kupfer MI, Lippman ME. Tamoxifen retinopathy. *Cancer Treat Rep.* 1978;62:315–320.

86. Williams GM, Iatropoulos MJ, Djordjevic MV, Kaltenberg OP. The triphenylethylene drug tamoxifen is a strong liver carcinogen in the rat. *Carcinogenesis.* 1993;14:315–317.

87. Cocconi G. First generation aromatase inhibitorsÑaminoglutethimide and testololactone [Review]. *Breast Cancer Res Treat.* 1994;30:57–80.

88. Buzdar AU, Smith R, Vogel C, et al. Fadrozole HCL (CGS-16949A) versus megestrol acetate treatment of postmenopausal patients with metastatic breast carcinoma: results of two randomized double blind controlled multiinstitutional trials. *Cancer.* 1996;77:2503–2513.

89. Thurlimann B, Beretta K, Bacchi M, et al. First-line fadrozole HCI (CGS 16949A) versus tamoxifen in postmenopausal women with advanced breast cancer. Prospective randomised trial of the Swiss Group for Clinical Cancer Research SAKK 20/88 [see comments]. *Ann Oncol.* 1996;7:471–479.

90. Freue M, Kjaer M, Boni C, et al. Open comparative trial of formestane versus megestrol acetate in postmenopausal patients with advanced breast cancer previously treated with tamoxifen. *Breast.* 2000;9:9–16.

91. Carlini P, Frassoldati A, De Marco S, et al. Formestane, a steroidal aromatase inhibitor after failure of non-steroidal aromatase inhibitors (anastrozole and letrozole): is a clinical benefit still achievable? *Ann Oncol.* 2001;12:1539–1543.

92. Lonning PE. Pharmacology and clinical experience with exemestane. *Expert Opin Investig Drugs.* 2000;9:1897–1905.

93. Koberle D, Thurlimann B. Anastrozole: pharmacological and clinical profile in postmenopausal women with breast cancer. *Expert Rev Anticancer Ther.* 2001;1:169–176.

94. Bhatnagar AS, Hausler A, Schieweck K, Lang M, Bowman R. Highly selective inhibition of estrogen biosynthesis by CGS 20267, a new non-steroidal aromatase inhibitor. *J Steroid Biochem Mol Biol.* 1990;37:1021–1027.

95. Klein KO, Demers LM, Santner SJ, Baron J, Cutler GB, Jr, Santen RJ. Use of ultrasensitive recombinant cell bioassay to measure estrogen levels in women with breast cancer receiving the aromatase inhibitor, letrozole. *J Clin Endocrinol Metab.* 1995;80:2658–2660.

96. Goss PE, Winer EP, Tannock IF, Schwartz LH. Randomized phase III trial comparing the new potent and selective third-generation aromatase inhibitor vorozole with megestrol acetate in postmenopausal advanced breast cancer patients. North American Vorozole Study Group. *J Clin Oncol.* 1999;17:52–63.

97. Yue W, Santen RJ. Aromatase inhibitors: rationale for use following antiestrogen therapy. *Semin Oncol.* 1996;23:21–27.

98. Kaufmann M, Bajetta E, Dirix LY, et al. Exemestane is superior to megestrol acetate after tamoxifen failure in postmenopausal women with advanced breast cancer: results of a phase III randomized double-blind trial. The Exemestane Study Group. *J Clin Oncol.* 2000;18:1399–1411.

99. Buzdar A, Jonat W, Howell A, et al. Anastrozole, a potent and selective aromatase inhibitor, versus megestrol acetate in postmenopausal women with advanced breast cancer: results of overview analysis of two phase III trials. Arimidex Study Group. *J Clin Oncol.* 1996;14:2000–2011.

100. Buzdar AU, Jonat W, Howell A, et al. Anastrozole versus megestrol acetate in the treatment of postmenopausal women with advanced breast carcinoma: results of a survival update based on a combined analysis of data from two mature phase III trials. Arimidex Study Group. *Cancer.* 1998;83:1142–1152.

101. Dombernowsky P, Smith I, Falkson G, et al. Letrozole, a new oral aromatase inhibitor for advanced breast cancer: double-blind randomized trial showing a dose effect and improved efficacy and tolerability compared with megestrol acetate [see comments]. *J Clin Oncol.* 1998;16:453–461.

102. Paridaens R, Dirix L, Lohrisch C, et al. Mature results of a randomized phase II multicenter study of exemestane versus tamoxifen as first-line hormone therapy for postmenopausal women with metastatic breast cancer. *Ann Oncol.* 2003;14:1391–1398.

103. Paridaens R, Therasse P, Dirix L. First-line hormonal treatment (HT) for metastatic breast cancer (MBC) with exemestane (E) or tamoxifen (T) in postmenopausal patients (pts)Ña randomized phase III trial of the EORTC Breast Group [Abstract]. *Proc Am Soc Clin Oncol.* 2004;23(6).

104. Bonneterre J, Buzdar A, Nabholtz JM, et al. Anastrozole is superior to tamoxifen as first-line therapy in hormone receptor positive advanced breast carcinoma. *Cancer.* 2001;92:2247–2258.

105. Mouridsen H, Gershanovich M, Sun Y, et al. Phase III study of letrozole versus tamoxifen as first-line therapy of advanced breast cancer in postmenopausal women: analysis of survival and update of efficacy from the International Letrozole Breast Cancer Group. *J Clin Oncol.* 2003;21:2101–2109.

106. Baum M, Budzar AU, Cuzick J, et al. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. *Lancet.* 2002;359:2131–2139.

107. Forbes JF, Cuzick J, Buzdar A, Howell A, Tobias JS, Baum M. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 100-month analysis of the ATAC trial. *Lancet Oncol.* 2008;9:45–53.

108. Thurlimann B, Keshaviah A, Coates AS, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med.* 2005;353:2747–2757.

109. Coates AS, Keshaviah A, Thurlimann B, et al. Five years of letrozole compared with tamoxifen as initial adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer: update of study BIG 1–98. *J Clin Oncol.* 2007;25:486–492.

110. Goss PE, Ingle JN, Martino S, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. *New Engl J Med.* 2003;349:1793–1802.

111. Goss PE, Ingle JN, Martino S, et al. Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17. *J Natl Cancer Inst.* 2005;97:1262–1271.

112. Goss PE, Ingle JN, Pater JL, et al. Late extended adjuvant treatment with letrozole improves outcome in women with early-stage breast cancer who complete 5 years of tamoxifen. *J Clin Oncol.* 2008;26:1948–1955.

113. Coombes RC, Hall E, Gibson LJ, et al. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. *New England Journal of Medicine*. 2004;350: 1081–1092.

114. Coombes RC, Kilburn LS, Snowdon CF, et al. Survival and safety of exemestane versus tamoxifen after 2–3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial. *Lancet.* 2007;369:559–570.

115. Mamounas EP, Jeong JH, Wickerham DL, et al. Benefit from exemestane as extended adjuvant therapy after 5 years of adjuvant tamoxifen: intention-to-treat analysis of the National Surgical Adjuvant Breast and Bowel Project B-33 trial. *J Clin Oncol.* 2008;26:1965–1971.

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Molecular Oncology

Causes of Cancer and Targets for Treatment

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to the clinicians whose trials turned medicines into therapies,
and to the patients whose participation in research is
essential and whose diseases we work to relieve.

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Preface

This book was conceived more than five years before its publication date. It was intended to provide a resource that summarized technology, biochemistry, molecular pathophysiology, and targeted therapeutics. As contributors were being recruited and chapters written the field that was being described changed at an accelerating pace. It is a tribute to scientific progress that volumes like this one are out-of-date as they are published, but books like this are not meant to contain the most current laboratory discovery or report the most recent FDA approval.

While this book was being written there have been major advances in molecular oncology. The Cancer Genome Atlas (cancergenome.nih.gov) has demonstrated the broad spectrum of mutations in an expanding list of cancers. DNA sequence analysis alone has demonstrated that as cancers grow, metastasize, and develop treatment resistance, individual tumor sites within a single patient evolve differently and demonstrate increasingly complex spectra of driver and passenger mutations. These findings alone strongly support the Darwinian view of tumor progression. The complexities of cellular dysregulation in cancer may arise from DNA sequence changes, but extend to other levels of gene regulation. During the writing of this book the role of micro RNAs (miRs) in cancer was elucidated. Aberrations in epigenetics such as DNA methylation and histone acetylation were demonstrated. Cancer drug development has also proceeded at increasing rates. In the period 2008–2012 there were 51 approvals of new drugs for cancer treatment by the US Food and Drug Administration. Many of these approvals resulted from impressive data in phase II trials that clearly demonstrated efficacy where no agents have worked before.

As we have assembled the contributions for this volume we have watched as more and more information is provided and accessed in electronic format, replacing the printed word. It is not hard to predict that younger generations of investigators will dispense entirely with books and access all information on electronic screens. Clearly a volume like this is meant to provide rapid reference when accessed from a shelf in someone's office. We can only hope that publishers evolve their business to transition compendia like this one to electronic format that scientists and trainees can transport with them for access anywhere.

We the editors took on the task of assembling this volume to provide background for active researchers, to provide meaningful lists of important citations that form the foundation of the molecular pathophysiology of cancer, and to define the context in which current investigation is pursued. This book is intended for students and professionals in academia and industry. Where electronic databases are non-discriminatory and web-based searches can be overwhelming in their download lists, volumes like this provide the perspective and judgment of experts who have spent a very long time in a path of study and therefore share their understanding and viewpoints that are missed in database or electronic literature searches. Volumes like this collect the experience and wisdom of the contributors and therefore provide value and perspective. As journal titles proliferate and the scientific literature expands, it is books like this that guide knowledge and help organize the work in a field into a comprehensible narrative. We hope you find these pages useful.

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Part 4 Pharmacologic targeting of oncogenic pathways

Chapter 83

Anti-estrogens and selective estrogen-receptor modulators

Ping Fan and V. Craig Jordan

Introduction, definitions and scope

The estrogen receptor (ER), including estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), mediates the biological effects of estrogen for the development and progression of breast cancer, and it serves as an important diagnostic and therapeutic target for prevention and treatment of breast cancer. Targeted estrogen-receptor therapy is the most successful strategy in breast cancer treatment and prevention. These endocrine therapies include aromatase inhibitors (AIs) indirectly targeting the ER that block the synthesis of estrogen from androgen in peripheral tissues and show efficacy in post-menopausal breast cancer patients. Another direct strategy is to use pure anti-estrogens (also called selective estrogen-receptor down-regulators, SERDs), such as fulvestrant, which have no agonist activity and cause degradation of the ER. Fulvestrant has been approved to treat advanced breast cancer after tamoxifen failure. The most widely used therapy for ER-positive breast cancer is selective estrogen receptor modulators (SERMs), which are synthetic molecules that bind to the ER and can modulate its transcriptional capabilities in different ways in diverse estrogen target tissues. Tamoxifen, the pioneering SERM, is extensively used for targeted therapy of ER-positive breast cancers, and is also approved as the first chemo-preventive agent for lowering breast cancer incidence in high-risk women. The therapeutic and preventive efficacy of tamoxifen was initially proven by a series of experiments in the laboratory that laid the foundation of its clinical use. Unfortunately, use of tamoxifen is associated with *de novo* and acquired resistance, and some undesirable side effects. The molecular study of resistance provides an opportunity to precisely understand the mechanism of action of SERMs, which may further help in designing new and improved SERMs. Clinical studies demonstrate that another SERM, raloxifene, which is primarily used to treat post-menopausal osteoporosis, is as effective as tamoxifen in preventing breast cancer in post-menopausal women, but with fewer side effects. Overall, these findings open a new horizon for SERMs as a class of drug which can not only be used for therapy and prevention of breast cancer, but also for various other diseases and disorders. We will provide a basic background of anti-estrogens, the current utility of the two pioneering SERMs tamoxifen and raloxifene, discuss in detail the putative mechanism of action of SERMs, and consider progress with new SERMs.

Clinical applications

Adjuvant endocrine therapy plays an important role in the management of hormone-receptor-positive breast cancer, and has increased life expectancy for millions of women. Progress in the strategic use of endocrine therapy for breast cancer has occurred through close co-operation between the laboratory and the clinic (1–4). The Brodies (3,4) advanced knowledge of the specific targeting of the CYP19 aromatase enzyme, with the identification and subsequent development of 4-hydroxyandrostenedione (5) as the first practical suicide inhibitor of the aromatase enzyme. The Brodies' contribution eventually became the catalyst to create a whole range of agents (e.g. anastrozole) targeted to the aromatase enzyme for the treatment of breast cancer in post-menopausal women (6). The clinical application of aromatase inhibitors (AIs) to block estrogen synthesis is therapeutically successful for the adjuvant treatment of breast cancer and is considered to be equivalent or superior to adjuvant tamoxifen treatment, with fewer side effects, such as endometrial cancers, hysterectomies, and blood clots (7–11). There are two classes of agents to prevent the CYP19 aromatase enzyme from synthesizing estrogen: competitive inhibitors (e.g. letrozole or anastrozole) and suicide inhibitors (e.g.exemestane; 12). Since the mechanism for AIs to treat ER-positive breast cancer is to deplete estrogen in post-menopausal patients, they do not increase the risk of endometrial cancer or blood clots, and may be a better choice for post-menopausal breast cancer patients than tamoxifen. However, AIs are not effective in pre-menopausal women with actively functioning ovaries, because AIs do not inhibit ovarian estrogen production. In addition, AIs lack the estrogenic protective function against cardiovascular diseases or osteoporosis. As a result, the side effects of AIs are mostly consistent with estrogen deprivation, with a greater incidence of bone loss and musculoskeletal symptoms, and probably higher risk of cardiovascular disease, suggested by adjuvant trials comparing AIs and tamoxifen (13). This indirect method of targeting the tumor ER is too

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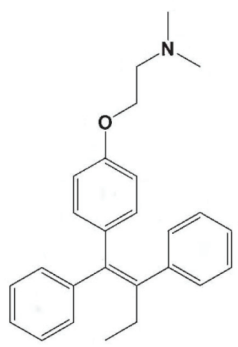
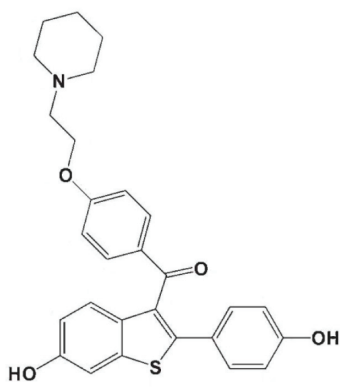
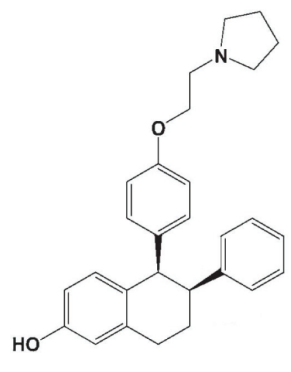


Tamoxifen

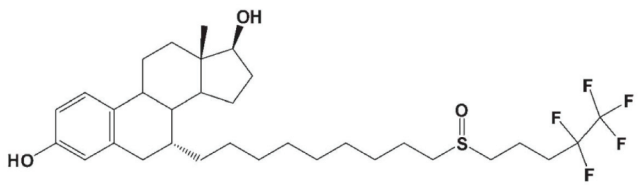


Raloxifene



Lasofoxifene

Figure 83.1 Structure of SERMs (tamoxifen, raloxifen, and lasofoxifene) and a pure antiestrogen (fulvestrant) named in the text.



Fulvestrant

large a topic to cover adequately in this chapter, so the interested reader is referred to the clinical and translational articles mentioned above for further information.

We have chosen instead to focus our chapter on compounds that target ER directly. Steroidal compounds that bind to the ER and cause rapid destruction of the complex are called "pure anti-estrogens" as they exhibit no estrogen-like actions at sites around the body. Fulvestrant (ICI 182,780; Figure 83.1) is a second-generation pure anti-estrogen that has fluorine atoms at the terminus of the 7α side chain to retard metabolism to estrogen. What appears to be unique about pure anti-estrogens is the observation that they provoke the rapid destruction of the ER in breast cancer cells in culture (14), mouse uterus (15), and breast tumors *in situ* (16). Fulvestrant has been approved to treat advanced breast cancer after tamoxifen failure, and a recent Phase III trial indicated that fulvestrant and the AI, exemestane, were equally effective, with a similar safety profile (17). The main side effects of concern with pure anti-estrogens increased risk of osteoporosis and coronary heart disease.

In contrast, SERMs are non-steroidal compounds that bind to the ER and modulate the signal-transduction pathway at different target sites around the. Tamoxifen (Figure 83.1), the pioneering SERM, has been used ubiquitously in clinical practice for the last 30 years for the treatment of breast cancer (18,19). In the early 1970s, a failed post-coital contraceptive, ICI 46,474, was reinvented as tamoxifen, the first targeted therapy for breast cancer (18). Tamoxifen is considered as the standard of care for the treatment of ER-positive breast cancer (20), and is credited with saving the lives of 400 000 breast cancer patients (2000 figure). Furthermore, adjuvant tamoxifen treatment can reduce the incidence of contralateral breast cancer (21). A five-year course (long-term) of tamoxifen treatment provides protection superior to one to two years of treatment, demonstrating that longer is better. This illustrates the translation of the earlier laboratory principle (22). Currently, five years of adjuvant tamoxifen is recommended to be optimal, since extending treatment beyond five years provides no further improvement (23–24). Tamoxifen is also the first approved drug for chemoprevention of breast cancer incidence in high-risk pre- and post-menopausal women, as scored by the Gail model (25). Clinical experiments show the efficacy of tamoxifen in reducing the incidence of breast cancer in women and that it continues to do so after therapy has been stopped in both pre- and post-menopausal women at high risk (26–28). Overall, there was a 38% reduction in the incidence of breast cancer for those who received tamoxifen, compared with placebo (29). The main limitation to the use of tamoxifen in the clinic relates to its side-effect profile with thromboembolic events and increased rates of endometrial cancer in post-menopausal women (29). The second-generation SERM, raloxifene (formerly called keoxifene; Figure 83.1), failed as a treatment for breast cancer, but is effective on maintaining bone density in post-menopausal women and decreasing circulating

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cholesterol (30–32). Most importantly, raloxifene is as effective as tamoxifen in preventing breast cancer without an increase in the risk of endometrial cancer (33–35). There was no statistically significant reduction in ER-negative or non-invasive cancers (36). Raloxifene has similar thromboembolic effects as tamoxifen.

Molecular mechanisms of SERM action

SERMs are synthetic compounds that target to estrogen receptors and exert estrogenic or anti-estrogenic activities in a tissue/cell-specific manner. The anti-tumor effects of SERMs are thought to be due to its anti-estrogenic activity, mediated by competitive inhibition of estrogen binding to estrogen receptors (37). As a consequence, tamoxifen inhibits the expression of estrogen-regulated genes, including growth factors and angiogenic factors secreted by the tumor that may stimulate growth by autocrine or paracrine mechanisms (38). SERMs are anti-estrogenic in the breast, but estrogen-like in the bones, and reduce circulating cholesterol levels. This discovery in the laboratory suggests the clinical application to simultaneously prevent osteoporosis, coronary heart disease, and breast cancer (25,39,40). However, SERMs also have different degrees of estrogenicity in the uterus. Tamoxifen exhibits partial agonistic activity thought to be associated with an increased risk of endometrial cancer, but raloxifene does not (41–46). Co-regulators are crucial in determining the final tissue outcome in terms of transcriptional activation or repression mediated by SERMs (47,48). It is more than a decade since the first steroid-receptor co-activator was first described (49). Now dozens of co-activator molecules are known, and also co-repressor molecules exist to prevent gene transcription by unliganded receptors (50,51). X-ray crystallography of the ligand-binding domains (LBD) of the ER liganded with either estrogens or anti-estrogens shows the potential of ligands to promote or prevent co-activator binding based on the shape of the estrogen– or anti-estrogen–ER complex (52,53). Evidence has accumulated that the broad spectrum of ligands that bind to the ER can create a broad range of ER complexes that are either fully estrogenic or anti-estrogenic at a particular target site (54). Thus, a mechanistic model of estrogen and anti-estrogen action has emerged based on the shape of the ligand, which programs the complex to adopt a particular shape that ultimately interacts with co-activators or co-repressors in target cells to determine the estrogenic or anti-estrogenic response, respectively. The three homologous members of the p160 SRC family (SRC1, SRC2, and SRC3) mediate the transcriptional functions of nuclear receptors and other transcription factors, and are the most studied of all the transcriptional co-activators (55). The relative abundance of SRC1 in uterine cells is responsible for the agonistic activity of tamoxifen, whereas in breast cancer cells, with low SRC1 levels, tamoxifen acts as an estrogen antagonist (41). However, raloxifene, another related SERM, does not recruit SRC-1, even in the uterine cells (41), suggesting that interaction with a specific ligand that elicits a unique conformation of the receptor is critical for the interaction of co-regulators. These observations further provide an explanation for the earlier studies, where tamoxifen has been reported to induce growth of endometrial cancer cells, but not of breast cancer cells in athymic mice (45) and also that the agonistic properties of raloxifene are less in endometrial cancer cells (46).

Drug resistance to SERMs

There are three types of resistance to SERMs based on the following mechanisms: metabolic resistance, intrinsic resistance, and acquired resistance (56).

Metabolic resistance

Tamoxifen efficacy depends on the formation of the clinically active metabolites 4-hydroxytamoxifen and endoxifen (Figure 83.2), which have a greater affinity for the estrogen receptor and ability to control cell proliferation, as compared to the parent drug (57). Metabolic resistance to tamoxifen is mostly related to CYP2D6, an enzyme product that metabolizes tamoxifen into its active forms 4-hydroxytamoxifen and endoxifen (58). This topic has recently been extensively reviewed (59,60) and will therefore be mentioned only briefly. The metabolic activation of tamoxifen occurs via demethylation to *N*-desmethyltamoxifen and subsequent transformation to the hydroxy metabolite endoxifen (61). Metabolic activation appears to be important for tamoxifen to acquire potent anti-estrogenic and anti-tumor activity. However, there are wide variations in the CYP2D6 enzyme within the population that can influence drug metabolism. The genotype of CYP2D6 has been shown in multiple clinical trials to be directly related to the outcome of tamoxifen use (62). However, the results are not always consistent, and currently several groups are seeking to resolve this inconsistency (63). For the present, the identification of patients for optimal long-term use of tamoxifen should exclude those high-risk women with a mutant CYP2D6 gene. In addition to the genotype of CYP2D6, it is important to consider that other drugs may interact with the enzyme system and block the metabolic activation of tamoxifen. Unfortunately, selective serotonin-reuptake inhibitors (SSRIs) that are used to relieve the menopausal side effects of tamoxifen are also metabolized by CYP2D6 and block the metabolic activation of tamoxifen (64). The proper choice of SSRI is therefore important, so as not to impair tamoxifen metabolism (63,64).

Intrinsic resistance

Approximately 30% ER-positive breast cancer patients do not respond to tamoxifen (65). This type of resistance is referred to as *de novo* resistance or intrinsic resistance. Historically, metastatic breast cancer that is ER- and PR-positive is approximately 80% responsive to endocrine therapy whereas tumors that are ER-positive but PR-negative are only 40% responsive to

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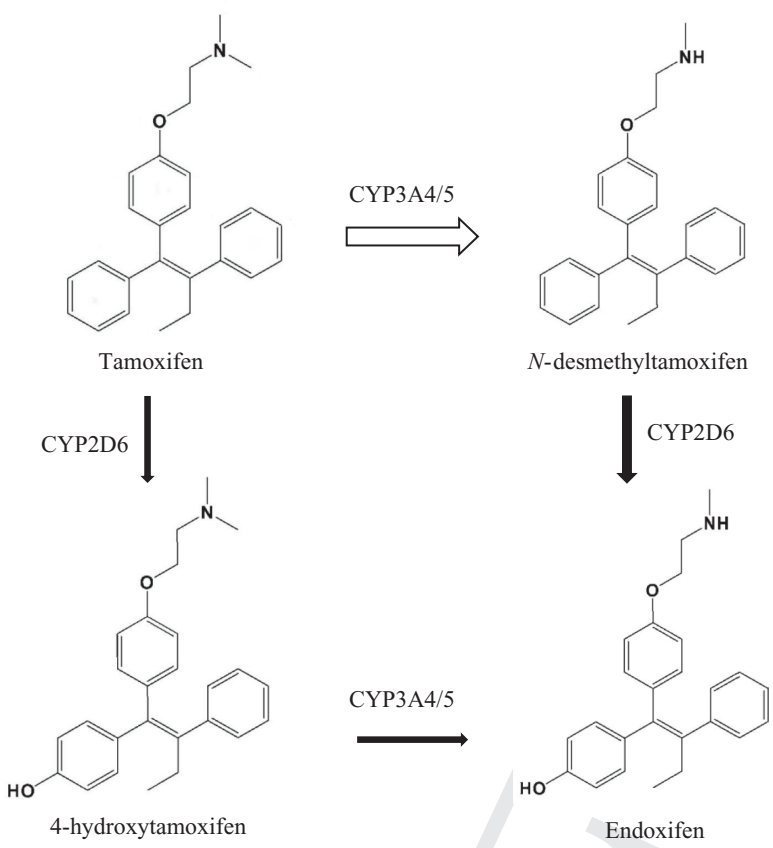

Figure 83.2 The metabolic activation of tamoxifen to phenolic metabolites that have a high binding activity for the human estrogen receptor. Both 4-hydroxytamoxifen and endoxifen are potent anti-estrogens *in vitro*.

anti-hormonal therapy (66,67). Although the mechanisms for this resistance are not yet fully clear, growth-factor signaling has been studied extensively and linked to SERMs resistance. We have known for about 20 years that enhanced growth-factor signaling via the human epidermal growth-factor receptor 1 (HER-1 or EGFR) pathway impairs estrogen induction of PR in breast cancer cells (68) and enhanced paracrine growth-factor stimulation undermines the effectiveness of anti-estrogen treatment at the ER (69,70). These earlier observations have recently been confirmed and extended using breast cancer cells artificially transfected with insulin-like growth-factor receptor and using large tumor databases (71,72). Tumor-cell drug resistance to tamoxifen develops very quickly (eight weeks) in athymic mice with HER-2/*neu* engineered MCF-7 cells (73) compared with the natural process of more than six months (74). The ligand-independent activation of the ER by MAPKs, which themselves are phosphorylated and thereby activated by HER-2 signaling in such tumors, may contribute to resistance (75–77). The cumulative data on the role of co-regulators in ER function suggest that they are important contributors to endocrine therapy such as tamoxifen (78). Tamoxifen acts as an agonist in experimentally engineered breast cancer cells with high levels of the HER-2/*neu* growth-factor receptor and the co-activator SRC3 (AIB1; 79). Tumors with a relatively high abundance of AIB1, especially those with enhanced HER-2 signaling that can activate AIB1, are also less responsive to tamoxifen therapy (80,81). AIB1 might be a predictor marker for tamoxifen ineffectiveness in ER-positive, HER2-positive, and PR-negative breast cancer. On the other hand, low expression of the ER co-repressor NcoR is associated with shorter relapse-free survival in breast cancer patients who only received tamoxifen after surgery (82).

Acquired resistance

Tamoxifen remains an effective choice as endocrine therapy for ER-positive breast cancer. However, acquisition of resistance to all forms of treatments is inevitable and a major clinical concern. Breast cancer patients who initially respond to tamoxifen later develop "acquired resistance" that is characterized by tamoxifen-stimulated growth. This can be replicated in the laboratory with MCF-7 xenograft tumors implanted in ovariectomized athymic mice (74). The laboratory model is consistent with the clinic observation that aromatase inhibitor or fulvestrant are equally effective after the failure of tamoxifen treatment (83,84). It therefore appears that the ER remains fully functional in the laboratory model of acquired tamoxifen resistance. In clinical studies, only 17–28% patients with acquired tamoxifen resistance have a loss of ER function (85,86), and it is more likely that acquired resistance is associated with the stimulation of other growth/survival pathways (87). It is commonly accepted that activation of growth-factor receptor

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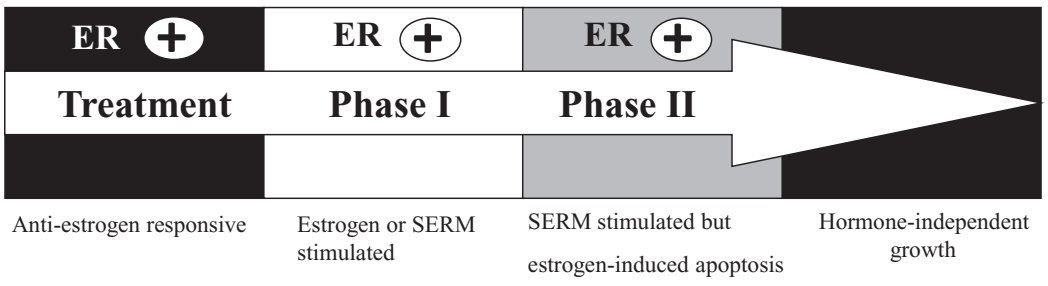

Figure 83.3 The evolution of resistance to selective ER modulators (SERMs: tamoxifen or raloxifene) in long-term therapy. Phase I acquired resistance develops after a year or two of therapy in the laboratory and during treatment of ER-positive metastatic breast cancer. Phase II acquired resistance occurs after five years of SERM treatment in the laboratory or potentially during five years adjuvant tamoxifen therapy for ER-positive breast cancer.

pathways, especially the EGFR family, is responsible for both *de novo* and acquired tamoxifen resistance (88–91). The ERα and EGFR pathways exert their biological functions through their own receptors and via cross-talk with each other (88,89). Activation of EGFR increases ligand-independent and ligand-dependent transcriptional activity of ERα (89,90). Recent data have shown that non-receptor tyrosine-kinase c-Src is activated and promoted cellular invasion and motility in acquired tamoxifen-resistant breast cancer cells (88,92,93). Furthermore, c-Src was shown to be functionally critical in mediating tamoxifen resistance, since blocking its activity has been shown to reverse tamoxifen resistance (88).

Laboratory observations show that acquired tamoxifen-resistant breast cancer cells/tumors respond differently to estrogen, and three phases of tamoxifen-resistance have been described (Figure 83.3), which depend on the length of tamoxifen exposure (94).Tumors with Phase I resistance are stimulated by estrogen and tamoxifen, but inhibited by AIs and fulvestrant; tumors with Phase II resistance are stimulated by tamoxifen, but are inhibited by estrogen, due to apoptosis (94). The laboratory models suggest a new treatment strategy, in which limited duration, low-dose estrogen can be used to purge Phase-II-resistant breast cancer cells so that the tumors will be responsive to anti-estrogen therapy again. Remarkably, drug resistance evolves and the survival signaling pathways in tamoxifen-resistant tumors become reorganized so that instead of estrogen being a survival signal, physiologic estrogen now inhibits tumor growth (95). This discovery provides an invaluable insight into the evolution of drug resistance to SERMS (96) and this knowledge is now being used to justify clinical trials of estrogen therapy following long-term anti-hormone therapy (97–99).

All these discoveries about the mechanisms of resistance provide therapeutic targets for SERMs resistance, which encourage combination of SERMs with other compounds to either overcome SERMs resistance or to postpone acquired resistance (88,91,100,101).

Challenges and new SERMs

The successful clinical application of tamoxifen in medicine has resulted in the investigation of numerous related molecules to develop the "ideal SERM" (94). An ideal SERM would decrease the incidence of osteoporosis, coronary heart disease, hot flashes, and breast cancer without increasing the risk of blood clots and endometrial cancer. Major efforts are therefore being directed to make new SERMs with a better therapeutic profile and fewer side effects. The development of dozens of SERMs has been discontinued due to ineffectiveness for human disease or severe side effects, but several new SERMs are under active investigation with great potential in breast cancer treatment and/or prevention, alone or in combination with other type of drugs, which has been extensively reviewed (102–104). However, a new compound, lasofoxifene, has been reported to have high binding affinity to the ER and have potent activity in preserving bone density. Lasofoxifene also exerts potential cardioprotective effects, but lacks increasing endometrial cancer risk (104,105).

Conclusions

Over the past 30 years, the rigorous investigation of the pharmacology of tamoxifen facilitated its ubiquitous use for the targeted treatment of breast cancer and chemoprevention, and pioneered the exploration of selective estrogen receptor modulators (SERMs; 106). This new concept subsequently served as the prototype for the development of raloxifene, a failed breast cancer drug, for the prevention of osteoporosis and breast cancer without tamoxifen's utertrophic side-effects in post-menopausal women. Numerous SERMs are currently under development not only for use in breast cancer, but also for use in osteoporosis and coronary heart disease.

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References

1. Jordan VC. Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. Journal of Steroid Biochemistry 1974;5:354.
2. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA- induced rat mammary carcinoma. European Journal of Cancer 1976;12:419–24.
3. Schwarzel WC, Kruggel WG, Brodie HJ. Studies on the mechanism of estrogen biosynthesis: the development of inhibitors of the enzyme system in human placenta. Endocrinology 1973; 92:866–80.
4. Brodie AM, Schwarzel WC, Shaikh AA, Brodie HJ. The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. Endocrinology 1977;100:1684–95.
5. Coombes CR, Goss P, Dowsett M, Gazet JC, Brodie A. 4-Hydroxy andostenedione in treatment of postmenopausal patients with advanced breast cancer. Lancet 1984;1: 1237–9.
6. Goss P, Strasser K. Aromatase inhibitors in the treatment and prevention of breast cancer. Journal of Clinical Oncology 2001;19:881–94.
7. Thurlimann B, Keshaviah A, Coates AS, *et al.* A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. New England Journal of Medicine 2005;353:2747–57.
8. Howell A, Cuzick J, Baum M, *et al.* Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 2005;365:60–2.
9. Goss PE, Ingle JN, Martino S, *et al.* Randomized trial of letrozole followingtamoxifen as extended adjuvant therapy inreceptor-positive breast cancer: updated findings from NCIC CTG MA. 17. Journal of the National Cancer Institute 2005;97: 1262–71.
10. Bonneterre J, Thurlimann B, Robertson JFR, *et al.* Anastrozole versus tamoxifen as first-line therapy for advanced breast cancer in 668 postmenopausal women: results of the Tamoxifen or Arimidex Randomized Group Efficacy and Tolerability Study. Journal of Clinical Oncology 2000;18:3748–57.
11. Coombes RC, Hall E, Gibson LJ, *et al.* The Intergroup Exemestane Study, a randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. New England Journal of Medicine 2004;350:1081–92.
12. Jordan VC, Brodie AMH. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007;72:7–25.
13. Perez EA. Safety profiles of tamoxifen and the aromatase inhibitors in adjuvant therapy of hormone-responsive early breast cancer. Annals of Oncology 2007;18:viii26–35.
14. Dauvois S, Danielian PS, White R, Parker MG. Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover. Proceedings of the National Academy of Sciences USA 1992;89: 4037–41.
15. Gibson MK, Nemmers LA, Beckman WC, *et al.* The mechanism of ICI 164,384 antiestrogenicity involves rapid loss of estrogen receptor in uterine tissue. Endocrinology 1991;129: 2000–10.
16. DeFriend DJ, Howell A, Nicholson RI, *et al.* Investigation of a new pure antiestrogen (ICI 182780) in women with primary breast cancer. Cancer Research 1994;54:408–14.
17. Chia S, Gradishar W, Mauriac L, *et al.* Double-blind, randomized placebo controlled trial of fulvestrant compared with exemestane after prior nonsteroidal aromatase inhibitor therapy in postmenopausal women with hormone receptor-positive, advanced breast cancer: results from EFECT. Journal of Clinical Oncology 2008;26:1664–70.
18. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nature Reviews Drug Discovery 2003;2:205–13.
19. Veronesi U, Boyle P, Goldhirsch A, Orecchia R, Viale G. Breast cancer. Lancet 2005;365:1727–41.
20. Strasser-Weippl K, Goss PE. Advances in adjuvant hormonal therapy for postmenopausal women. Journal of Clinical Oncology 2005;23:1751–9.
21. Cuzick J, Baum M. Tamoxifen and contralateral breast cancer [letter]. Lancet 1985;2:282.
22. Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. European Journal of Cancer 2008;44:30–8.
23. Fisher B, Dignam J, Bryant J, *et al.* Five versus more than five years of tamoxifen therapy for breast cancer patients with negative lymph nodes and estrogen receptor-positive tumors. Journal of the National Cancer Institute 1996;88:1529–42.
24. Fisher B, Dignam J, Bryant J, Wolmark N. Five versus more than five years of tamoxifen for lymph node negative breast cancer: updated findings from the National Surgical Adjuvant Breast and Bowel Project B-14 Randomized Trial. Journal of the National Cancer Institute 2001;93:684–90.
25. Jordan VC, Gapstur S, Morrow M. Selective estrogen receptor modulation and reduction in risk of breast cancer, osteoporosis, and coronary heart disease. Journal of the National Cancer Institute 2001;93:1449–57.
26. Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. Journal of the National Cancer Institute 2007;99:283–90.
27. Fisher B, Costantino JP, Wickerham DL, *et al.* Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. Journal of the National Cancer Institute 1998;90: 1371–88.
28. Fisher B, Costantino JP, Wickerham DL, *et al.* Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. Journal of the National Cancer Institute 2005;97: 1636–7.
29. Cuzick J, Powles T, Veronesi U, *et al.* Overview of the main outcomes in breast cancer prevention trials. Lancet 2003;361:296–300.
30. Jordan VC. The rise of raloxifene and the fall of invasive breast cancer. Journal of the National Cancer Institute 2008 18;100:831–3.

PROOF

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CUUK2316-83 CUUK2316/Gelmann ISBN: 978 0 521 87662 9 August 2, 2013 9:33

31. Morrow M, Jordan VC. The current status of breast cancer chemoprevention: a star is born. Journal of Surgical Oncology 2007; 95:4–5.
32. Cummings SR, Eckert S, Krueger KA, *et al.* The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial, Multiple Outcomes of Raloxifene Evaluation. Journal of the American Medical Association 1999;281:2189–97.
33. Ettinger B, Black DM, Mitlak BH, *et al.* Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators [see comments]. Journal of the American Medical Association 1999;282:637–45. Erratum in: Journal of the American Medical Association 1999;282:2124.
34. Vogel VG, Costantino JP, Wickerham DL, *et al.* The Study of Tamoxifen and Raloxifene (STAR): report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. Journal of the American Medical Association 2006;295:2727–41. Erratum in: Journal of the American Medical Association 2006;296:2926. Journal of the American Medical Association 2007;298:973.
35. DeMichele A, Troxel AB, Berlin JA, *et al.* Impact of raloxifene or tamoxifen use on endometrial cancer risk: a population-based case-control study. Journal of Clinical Oncology 2008;26: 4151–9.
36. Martino S, Cauley JA, Barrett-Connor E, *et al.* Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. Journal of the National Cancer Institute 2004; 96:1751–61.
37. Jordan VC. Tamoxifen (ICI 46,474) as a targeted therapy to treat and prevent breast cancer. British Journal of Pharmacology 2006;147:S269–76.
38. Osborne CK. Tamoxifen in the treatment of breast cancer. New England Journal of Medicine 1998;339:1609–18.
39. Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Research 1990;50: 4177–89.
40. Jordan VC. SERMs: meeting the promise of multifunctional medicines. Journal of the National Cancer Institute 2007;99:350–6.
41. Shang Y, Brown M. Molecular determinants for the tissue specificity of SERMs. Science 2002;295:2465–8.
42. O'Regan RM, Cisneros A, England, GM, *et al.* Effects of the antiestrogens tamoxifen, toremifene, and ICI 182,780 on endometrial cancer growth. Journal of the National Cancer Institute 1998; 90:1552–8.
43. Grese TA, Sluka JP, Bryant HU, *et al.* Molecular determinants of tissue selectivity in estrogen receptor modulators. Proceedings of the National Academy of Sciences USA 1997;94:14105–10.
44. Fornander T, Rutqvist LE, Cedermark B, *et al.* Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1989;1: 117–20.
45. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Research 1988;48: 812–15.
46. Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Research 1990; 50:3189–92.
47. O'Malley BW, Kumar R. Nuclear receptor coregulators in cancer biology. Cancer Research 2009;69: 8217–22.
48. O'Malley BW. Little molecules with big goals. Science 2006;313:1749–50.
49. Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 1995;270: 1354–7.
50. Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocrine Reviews 2004;25:45–71.
51. Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 2000;103: 843–52.
52. Shiau AK, Barstad D, Loria PM, *et al.* The structural basis of estrogen receptor/co-activator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;95:927–37.
53. Brzozowski AM, Pike AC, Dauter Z, *et al.* Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997;389:753–8.
54. Wijayaratne AL, Nagel SC, Paige LA, *et al.* Comparative analyses of mechanistic differences among antiestrogens. Endocrinology 1999;140: 5828–40.
55. Xu J, Wu RC, O'Malley BW. Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nature Reviews Cancer 2009;9:615–30.
56. Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. Journal of Clinical Oncology 2007;25:5815–24.
57. Jordan VC. New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer. Steroids 2007;72:829–42.
58. Jin Y, Desta Z, Stearns V, *et al.* CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. Journal of the National Cancer Institute 2005;97:30–9.
59. Weinshilboum R. Pharmacogenomics of endocrine therapy in breast cancer. Advances in Experimental Medicine and Biology 2008;630:220–31.
60. Brauch H, Jordan VC. Targeting of tamoxifen to enhance antitumour action for the treatment and prevention of breast cancer: the "personalised" approach? European Journal of Cancer 2009;45:2274–83.
61. Stearns V, Johnson MD, Rae JM, *et al.* Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. Journal of the National Cancer Institute 2003; 95:1758–64.
62. Schroth W, Goetz MP, Hamann U, *et al.* Association between CYP2D6 polymorphisms and outcomes among women with early stage breast cancer treated with tamoxifen. Journal of the

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American Medical Association 2009; 302:1429–36.

63. Flockhart D. CYP2D6 genotyping and the pharmacogenetics of tamoxifen. Clinical Advances in Hematology and Oncology 2008;6:493–4.
64. Borges S, Desta Z, Li L, *et al.* Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: Implication for optimization of breast cancer treatment. Clinical Pharmacology and Therapeutics 2006;80:61–74.
65. Harvell DM, Richer JK, Singh M, *et al.* Estrogen regulated gene expression in response to neoadjuvant endocrine therapy of breast cancers: tamoxifen agonist effects dominate in the presence of an aromatase inhibitor. Breast Cancer Research and Treatment 2008;112:489–501.
66. Bloom ND, Tobin EH, Schreibman B, Degenshein GA. The role of progesterone receptor in the management of advanced breast cancer. Cancer 1980;45:2992–7.
67. Osborne CK, Yochmowitz MG, Knight WA 3rd, McGuire WL. The value of estrogen and progesterone receptors in the treatment of breast cancer. Cancer 1980;46:2884–8.
68. Cormier EM, Wolf MF, Jordan VC. Decrease in estradiol-stimulated progesterone receptor production in MCF-7 cells by epidermal growth factor and possible clinical implication for paracrine-regulated breast cancer growth. Cancer Research 1989;49: 576–80.
69. Cormier EM, Jordan VC. Contrasting ability of antiestrogens to inhibit MCF-7 growth stimulated by estradiol or epidermal growth factor. European Journal of Cancer and Clinical Oncology 1989;25:57–63.
70. Robinson SP, Jordan VC. The paracrine stimulation of MCF-7 cells by MDA-MB-231 cells: possible role in antiestrogen failure. European Journal of Cancer and Clinical Oncology 1989: 25:493–7.
71. Cui X, Zhang P, Deng W, *et al.* Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/ Akt/mammalian target of rapamycin pathway: Progesterone receptor as a potential indicator of growth factor activity in breast cancer. Molecular Endocrinology 2003;17:575–88.
72. Arpino G, Weiss H, Lee AV, *et al.* Estrogen receptor-positive, progesterone receptor-negative breast cancer: Association with growth factor receptor expression and tamoxifen resistance. Journal of the National Cancer Institute 2005;97: 1254–61.
73. Benz CC, Scott GK, Sarup JC, *et al.* Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Research and Treatment 1992;24:85–95.
74. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Research 1988;48:5183–7.
75. Kato S, Endoh H, Masuhiro Y, *et al.* Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 1995;270:1491–4.
76. Bunone G, Briand PA, Miksicek RJ, Picard D. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO Journal 1996;15:2174–83.
77. Pietras RJ, Arboleda J, Reese DM, *et al.* HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene 1995;10:2435–46.
78. Osborne CK, Bardou V, Hopp TA, *et al.* Role of the estrogen receptor coactivator AIB1 (SRC3) and HER2/neu in tamoxifen resistance in breast cancer. Journal of the National Cancer Institute 2003;95:353–61.
79. Shou J, Massarweh S, Osborne CK, *et al.* Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. Journal of the National Cancer Institute 2004;96:926–35.
80. Smith CL, Nawaz Z, O'Malley BW. Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Molecular Endocrinology 1997;11:657–66.
81. Takimoto GS, Graham JD, Jackson TA, *et al.* Tamoxifen resistant breast cancer: coregulators determine the direction of transcription by antagonist-occupied steroid receptors. Journal of Steroid Biochemistry and Molecular Biology 1999;69:45–50.
82. Girault I, Lerebours F, Amarir S, *et al.* Expression analysis of estrogen receptor alpha coregulators in breast carcinoma: evidence that NCOR1 expression is predictive of the response to tamoxifen. Clinical Cancer Research 2003;9:1259–66.
83. Osborne CK, Pippen J, Jones SE, *et al.* Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. Journal of Clinical Oncology 2002;20:3386–95.
84. Howell A, Robertson JF, Quaresma Albano J, *et al.* Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. Journal of Clinical Oncology 2002;20:3396–403.
85. Johnston SR, Saccani-Jotti G, Smith IE, *et al.* Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. Cancer Research 1995;55:3331–8.
86. Gutierrez MC, Detre S, Johnston S, *et al.* Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. Journal of Clinical Oncology 2005;23:2469–76.
87. Normanno N, Di Maio M, De Maio E, *et al.* Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. Endocrine-Related Cancer 2005;12: 721–47.
88. Fan P, Wang J, Santen RJ, Yue W. Long-term treatment with tamoxifen facilitates translocation of estrogen receptor alpha out of the nucleus and enhances its interaction with EGFR in MCF-7 breast cancer cells. Cancer Research 2007;67:1352–60.
89. Nicholson RI, Gee JMW. Oestrogen and growth factor cross-talk and endocrine insensitivity and acquired resistance in breast cancer. British Journal of Cancer 2000;82:501–13.

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CUUK2316-83 CUUK2316/Gelmann ISBN: 978 0 521 87662 9 August 2, 2013 9:33

90. Long B, McKibben BM, Lynch M, van den Berg HW. Changes in epidermal growth factor receptor expression and response to ligand associated with acquired tamoxifen resistance or oestrogen independence in the ZR-75–1 human breast cancer cell line. British Journal of Cancer 1992;65: 865–9.
91. Massarweh S, Osborne CK, Creighton CJ, *et al*. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. Cancer Research 2008;68: 826–33.
92. Hiscox S, Morgan L, Green TP, *et al*. Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. Breast Cancer Research and Treatment 2006; 97:263–74.
93. Zhao Y, Planas-Silva MD. Mislocalizaion of cell-cell adhesion complexes in tamoxifen-resistant breast cancer cells with elevated c-Src tyrosine kinase activity. Cancer Letters 2009;275: 204–12.
94. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. Cancer Cell 2004;5:207–13.
95. Yao K, Lee ES, Bentrem DJ, *et al*. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clinical Cancer Research 2000;6:2028–36.
96. Jordan VC. The 38th David A. Karnofsky Lecture. The paradoxical actions of estrogen in breast cancer: survival or death? Journal of Clinical Oncology 2008;26:3073–82.
97. Jordan VC, Lewis JS, Osipo C, Cheng D. The apoptotic action of estrogen following exhaustive antihormonal therapy: a new clinical treatment strategy. Breast 2005;14:624–30.
98. Lønning PE, Taylor PD, Anker G, *et al*. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Research and Treatment 2001;67:111–6.
99. Ellis MJ, Gao F, Dehdashti F, *et al*. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. Journal of the American Medical Association 2009;302: 774–80.
100. Nicholson RI, Hutcheson IR, Hiscox SE, *et al*. Growth factor signalling and resistance to selective oestrogen receptor modulators and pure anti-oestrogens: the use of anti-growth factor therapies to treat or delay endocrine resistance in breast cancer. Endocrine-Related Cancer 2005;12:S29–36.
101. Hiscox S, Jordan NJ, Smith C, *et al*. Dual targeting of SRC and ER prevents acquired antihormone resistance in breast cancer cells. Breast Cancer Research and Treatment 2009;115: 57–67.
102. Peng J, Sengupta S, Jordan VC. Potential of selective estrogen receptor modulators as treatments and preventives of breast cancer. Anticancer Agents in Medicinal Chemistry 2009;9: 481–99.
103. Ariazi EA, Jordan VC. Estrogen receptors as therapeutic targets in breast cancer. In: Ottow E, Weinmann H, editors. *Nuclear receptors as drug targets*. Weinheim: Wiley-VCH; 2008:127–99.
104. Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. Journal of Medicinal Chemistry 2003;46:1081–111.
105. Cummings SR, Ensrud K, Delmas PD, *et al*. Lasofoxifene in postmenopausal women with osteoporosis. New England Journal of Medicine 2010;362:686–96.
106. Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Research 2009;69:1243–54.

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OPINION

Chemoprevention of breast cancer with selective oestrogen-receptor modulators

V. Craig Jordan

Abstract | Twenty years ago, a new therapeutic dimension was conceived that not only had the potential to treat and prevent osteoporosis, but to prevent breast and endometrial cancer at the same time. As osteoporosis was known to be caused by oestrogen withdrawal after menopause, whereas breast and endometrial cancer are caused by unopposed oestrogen action, the new tissue-selective oestrogens and anti-oestrogens, or selective oestrogen-receptor modulators (SERMs), had to recruit new networks to activate or suppress target tissues selectively. New medicines now promise to provide chemoprevention strategies for women at risk for the development of many diseases.

Despite recent advances in the targeted treatment of cancer, there is clearly a benefit to preventing the development of cancer in the first place. Fewer patients being treated for cancers would reduce the burden on health-care systems, and enable increasing numbers of people to live healthier more productive lives. Less than 10 years ago, the promise of chemoprevention became a clinical reality. Tamoxifen, a compound originally called a nonsteroidal anti-oestrogen but now referred to as a selective oestrogen-receptor modulator (SERM)[1], was shown to reduce the incidence of breast cancer in both pre and postmenopausal women at high risk[2]. In this case, high risk is defined by a validated computer model that assesses the reproductive and family history of individuals to estimate the likelihood of developing breast cancer during the next 5 years or as a lifetime risk[3,4]. Tamoxifen, which was already a successful breast cancer drug targeted to block the oestrogen receptor (ER) and prevent oestrogen-stimulated breast tumour growth[1], became the first drug available for use in the United States to reduce breast cancer incidence in high-risk premenopausal and postmenopausal women. However, the recognition that SERMs are oestrogen-like in bone but anti-oestrogenic in the breast created the possibility that both osteoporosis and breast cancer risk could be reduced[5,6]. This strategy of preventing osteoporosis in postmenopausal women at the same time as reducing breast cancer risk is now clinically validated. Raloxifene is the first SERM approved for the treatment and prevention of osteoporosis that also reduces breast cancer incidence[7]. However, the recent completion of the Study of Tamoxifen and Raloxifene (STAR)[8] that evaluated the benefits and side effects of these two SERMs for the reduction of breast cancer incidence in high-risk postmenopausal women now provides an opportunity to assess the molecular biology and pharmacology of SERMs as multifunctional medicines. New initiatives in the understanding of female physiology and disease will enable the correct SERM to be used for the appropriate at-risk women and facilitate the development of new and improved agents for further applications in health care.

The recognition of selective modulation of the ER in the laboratory[9] created a new drug group with several therapeutic possibilities (BOX 1). The evidence that supported translational research and the initiatiation of clinical investigations was based on a re-examination of the drug group referred to as non-steroidal anti-oestrogens. Tamoxifen, the pioneering agent[1], was described as both a partial oestrogen agonist and antagonist in the rat uterus, but was in fact classified as a full oestrogen in the mouse uterus and vagina. These were important biological facts with which to get a clearer picture of the target-site-specific actions of SERMs. Four main pieces of laboratory evidence converged to establish that the SERM concept was a class effect: first, ER-positive breast cancer cells inoculated into athymic (immune deficient) mice grew into tumours in response to oestradiol but not in response to tamoxifen, despite the fact that oestradiol and tamoxifen increased mouse uterine weight[10]; second, raloxifene (then known as LY156758 or keoxifene) was less oestrogenic than tamoxifen in the rodent uterus[11], but both raloxifene and tamoxifen maintained bone density in ovariectomized rats[12] (oestrogenic action) and prevented rat mammary carcinogenesis (anti-oestrogenic action)[13]; third, tamoxifen blocked oestradiol-induced ER-positive breast tumour growth in athymic mice, but ER-positive endometrial carcinomas grew rapidly[14]; and fourth, raloxifene was less effective than tamoxifen at promoting endometrial cancer growth[15].

The general conclusion was that tamoxifen and raloxifene could selectively switch on or switch off the ER at sites around the body depending on the tissue. These laboratory conclusions not only translated to patients, but also provided a strategy for the development of new tissue-selective drugs to simultaneously prevent osteoporosis and reduce the risk of breast cancer[5,6].

Clinical evaluation of SERMs

Tamoxifen, the prototypical SERM, is available in the United States for the reduction of breast cancer incidence in high-risk premenopausal and postmenopausal women. Tamoxifen produces about a 50% decrease in breast cancer incidence[2,16], which is consistent with the 50% decrease in contralateral breast cancer noted in adjuvant therapy

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studies[17]. Nevertheless, the main concern about using tamoxifen is an increase in blood clots and endometrial cancer (BOX 2). Both of these side effects of tamoxifen are linked to its oestrogen-like properties in postmenopausal women, who have a low-oestrogen environment. Unfortunately, what is not emphasized is the favourable risk–benefit ratio for tamoxifen in pre-menopausal women because there is no significant increase in either blood clots or endometrial cancer compared with placebo controls[2,4,16]. Indeed, in countries where tamoxifen is cheap there is potentially a benefit to using tamoxifen as a chemopreventive in very high-risk premenopausal women[18] as a health-care policy. In the United States, the Gail score[3] is a population-based model to estimate the 5 year lifetime risk of breast cancer, and defines high risk as 1.67 or above. Very high risk would be a Gail score of 3.5 or above[18].

Raloxifene must now be considered to be the first multifunctional SERM. The medicine has successfully been tested for the treatment and prevention of osteoporosis[19], and is available in many countries for that indication. An evaluation of breast cancer incidence in women treated with raloxifene for the prevention of osteoporosis[7] shows a 75% decrease in invasive breast cancer, and, as with tamoxifen, only the ER-positive disease is reduced. These data emphasize the fact that SERMs target the ER-mediated growth mechanism[20]. However, because the use of any medicine to prevent osteoporosis requires years of treatment, the original 4 year trial, referred to as the Multiple Outcomes for Raloxifene Evaluation (MORE)[7,19], was extended to 8 years as the Continuing Outcomes Relative to Evista (CORE) trial. The breast cancer endpoint remained at about 65% inhibition of invasive breast cancer[21]. These data validate the original hypothesis that a non-steroidal anti-oestrogen in the same class as tamoxifen could be used not only to prevent osteoporosis but also to prevent breast cancer as a beneficial side effect[5,6].

The MORE trial data provided the rationale for the National Surgical Adjuvant Breast and Bowel Project (NSABP) to test raloxifene (60 mg a day) against tamoxifen (20 mg a day) to reduce breast cancer incidence in high-risk postmenopausal women in the STAR trial. In addition, on the basis of the ability of raloxifene to lower circulating cholesterol (that is, low density lipoprotein cholesterol)[22], the study Raloxifene use for the Heart (RUTH) was initiated as a placebo-controlled trial to determine whether the incidence of coronary heart disease could be reduced.

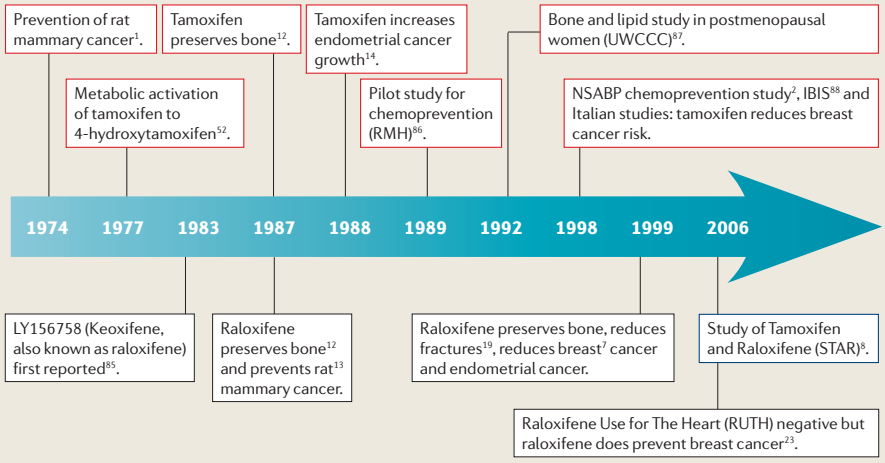
The STAR trial showed equivalence between tamoxifen and raloxifene for reducing the risk of invasive breast cancer in high-risk postmenopausal women[8]. There were fewer cases of thromboembolic disorders, cataracts, cataract surgery, endometrial cancer and hysterectomy in patients who took raloxifene compared with tamoxifen. However, there seemed to be fewer cases of non-invasive breast cancer (ductal carcinoma *in situ* and lobular carcinoma *in situ*)[8] in the tamoxifen-treated group than the raloxifene-treated group, although the difference did not reach statistical significance. This might be associated with the reduced biological half life of raloxifene compared with tamoxifen (see below). Nevertheless, raloxifene is a safer agent than tamoxifen for use as a chemopreventive in high-risk postmenopausal women.

The RUTH trial failed to show a benefit in reducing deaths or hospitalizations for coronary heart disease[23]. Clearly, the presumed benefit from raloxifene based on lipid lowering is incorrect in practice. However, the placebo-controlled trial did again show that raloxifene could significantly reduce the incidence of invasive breast cancer and not increase the risk of endometrial cancer[23].

Therefore, the clinical evaluation of SERMs as chemopreventives for ER-positive breast cancer has shown that the concept is valid. However, the clinical use of these agents naturally aroused curiosity about how a simple model of oestrogen and anti-oestrogen action could explain the SERM phenomenon. The generally accepted principle in the 1980s was that oestrogens bound to the ER to initiate the transcription of genes and increase cell division and/or reduce cell death. A non-steroidal anti-oestrogen blocked the ER and some or all of the actions of oestradiol. The old model clearly could not cope with the new biology of the SERMs.

Box 1 | **Development of tamoxifen and raloxifene for chemoprevention**

The recognition and development of selective oestrogen-receptor modulators culminated in the Study of Tamoxifen and Raloxifene (STAR)[8] clinical trial. This journey has taken about 30 years. The laboratory finding that tamoxifen prevented rat mammary carcinogenesis and was metabolically activated to 4-hydroxytamoxifen[52] (FIG. 2) provided an important lead for renewed structure activity relationship studies[1] that resulted in the description of raloxifene (LY156758, originally called keoxifene)[85]. The 'anti-oestrogen' failed its application as a breast cancer drug, but through the recognition of the bone-preserving properties of both tamoxifen and LY156758 (REF. 12), keoxifene was reinvented as raloxifene in the 1990s and successfully tested as a treatment and preventive for osteoporosis in postmenopausal women. The evolution of tamoxifen from a successful treatment for breast cancer to become the first agent to be used to prevent the development of cancer occurred throughout the 1980s and 1990s. The selective oestrogenic and anti-oestrogenic actions of tamoxifen in bone[12] and endometrial cancer[14] translated from the laboratory in the 1980s to clinical practice in the 1990s[84]. Pilot chemoprevention studies occurred first at the Royal Marsden Hospital (RMH), UK[86], and studies on human bone density[87] at the University of Wisconsin Clinical Cancer Center (UWCCC), US. It is known that tamoxifen and raloxifene are both equivalent for preventing fractures and reducing the incidence of breast cancer[8], but raloxifene does not increase the risk of endometrial cancer[7,23], which tamoxifen was found to do in the National Surgical Adjuvant Breast and Bowel Project (NSABP)[2] and the International Breast Intervention Study (IBIS)[88]. Red outline denotes tamoxifen, black outline raloxifene, and blue outline both tamoxifen and raloxifene.



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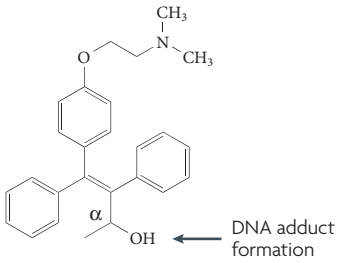
Box 2 | **Endometrial cancer and SERMs**



The laboratory finding that tamoxifen encourages the growth of human endometrial cancer but blocks oestrogen-stimulated growth of breast cancer transplanted to the same athymic mouse[14] translated to clinical practice[84,89]. It is estimated that tamoxifen causes a 4–5-fold increase in the detection of endometrial cancers in postmenopausal women, but not in premenopausal women. The risk of developing endometrial cancer and dying is small compared with the lives saved during the treatment of breast cancer, but the concern in high-risk populations of the possibility of developing endometrial cancer during tamoxifen treatment is a significant factor in women declining chemoprevention. Raloxifene is less oestrogenic in the rodent uterus than tamoxifen, and does not increase the risk of endometrial cancer in postmenopausal women[7,8,23].

Mechanisms

Unopposed oestrogen treatment is associated with a higher incidence of endometrial cancer in postmenopausal women. Recent studies suggest an association between oestrogen, endometrial cancer and the *SULT1A1*2* allele that impairs sulphation so that oestrogen undergoes reduced local phase II metabolism[90] (for an explanation of phase II metabolism, see the Molecular Pharmacology of SERMs (selective oestrogen-receptor modulators) section of this Perspective). Tamoxifen can induce growth in endometrial cancer cells under laboratory conditions[14], and as such could increase the detection of growing pre-existing disease in postmenopausal women[91]. The increase in gynaecological symptoms (such as bleeding) caused by tamoxifen might, in fact, lead to increased detection of pre-existing disease because patients are examined more rigorously. Tamoxifen-stimulated growth seems to result from the gene *PAX2* (paired box gene 2) becoming hypomethylated in endometrial cancer, and both oestrogen and tamoxifen can increase the proliferation and growth of endometrial tumours through an oestrogen receptor-α and PAX2-mediated mechanism[92]. Nevertheless, based on the fact that tamoxifen is a complete carcinogen in the rat liver, owing to the formation of DNA adducts by α-hydroxytamoxifen (see figure), considerable effort has focused on identifying DNA adducts in humans[93,94]. No unifying molecular mechanism of causation is universally accepted. However, recent reports about the direct carcinogenic activity of oestrogen for the causation of breast cancer[95,96] might have some relevance to events in the uterus.

Molecular mechanisms of SERM action

There are two ERs, referred to as ERα and ERβ[24,26]. The receptor proteins are encoded on different chromosomes and have homology as members of the steroid receptor superfamily, but there are distinct patterns of distribution and distinct and subtle differences in structure and ligand binding affinity[27]. An additional dimension that might be significant for tissue modulation is the ratio of ERα to ERβ at a target site. A high ERα–ERβ ratio correlates well with very high levels of cellular proliferation, whereas the predominance of functional ERβ over ERα correlates with low levels of proliferation[28–31]. The ratio of ERs in normal and neoplastic breast tissue might be an important factor for the long-term success of chemoprevention with SERMs.

There are functional differences between ERα and ERβ that can be traced to the differences in the activating function 1 (AF1) domain located in the N terminus of the ER. The amino-acid homology of AF1 is poorly conserved (only 20%). By contrast, the AF2 region located at the C terminus of the ligand-binding domain differs by only one amino acid — D545 in ERα and N496 in ERβ. As the AF1 and AF2 regions are crucial for interaction with other co-regulatory proteins and gene transcription, the structural differences between them provides a clue about the potential functional differences between ERα and ERβ. Studies that used chimeras of ERα and ERβ by switching the AF1 regions showed that this region contributes to the cell-specific and promoter-specific differences in transcriptional activity. In general, SERMs can partially activate engineered genes regulated by an oestrogen-response element through ERα but not ERβ[32–34]. By contrast, 4-hydroxytamoxifen and raloxifene can stimulate activating protein-1 (AP1)-regulated reporter genes with both ERα and ERβ in a cell-dependent fashion[35].

The simple model for oestrogen action, with either ERα or ERβ controlling oestrogen-regulated events, has now evolved into a fascinating mix of protein partners that have the potential to modulate gene transcription (FIG. 1). It is more than a decade since the first steroid receptor coactivator protein (SRC1) was first described[36]. Now dozens of co-activator molecules are known, and co-repressor molecules also exist to prevent gene transcription by unliganded receptors[37].

Naturally, the finding that there are two ERs has resulted in the synthesis of a range of receptor-specific ligands to switch a particular receptor on or off[38]. But, it is the external shape of the resulting complex that becomes the catalyst for changing the response to a SERM in a tissue target. Kraichely and co-workers[39] used new agonists for ERα and ERβ to detect subtle quantitative differences in their interaction with members of the SRC family (SRC 1, 2 and 3), but the molecular biology of SERM action is far more complex.

It is reasonable to ask how does the ligand programme the receptor complex to interact with other proteins? X-ray crystallography of the ligand-binding domains of the ER bound to either oestrogens or anti-oestrogens shows the potential of ligands to promote co-activator binding or prevent co-activator binding on the basis of the shape of the oestrogen or anti-oestrogen receptor complex[40,41]. Evidence has accumulated that the broad spectrum of ligands that bind to the OER can create a broad range of OER complexes that are either fully oestrogenic or anti-oestrogenic at a particular target site[42]. Therefore, a mechanistic model of oestrogen action and anti-oestrogen action (FIG. 1) has emerged based on the shape of the ligand that programmes the complex to adopt a particular shape that ultimately interacts with co-activators or co-repressors in target cells to determine the oestrogenic or anti-oestrogenic response, respecitvely. But how the response is initiated?

Not surprisingly, the co-activator model of steroid hormone action has increased in complexity, thereby amplifying the molecular mechanisms of modulation. It seems that co-activators are not simply protein partners that connect one site to another in a complex[43]. The co-activators actively participate in modifying the activity of the complex. The post translational modification of co-activators through many kinase pathways initiated by cell surface growth factor receptors (for example, epidermal growth factor receptor, insulin-like growth factor receptor 1 and ERBB2, also known as HER2) can result in a dynamic model of steroid hormone action. The core co-activator (such as SRC3) (FIG. 1) first recruits a specific set of co-co-activators (such as p300 and ubiquitin-conjugating ligases) under the direction of many protein remodellers (for example, the peptidyl-prolyl isomerase PIN1, heat shock

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proteins and proteasome ATPases) to form a multi-protein co-activator complex that interacts with the phosphorylated ER at the specific gene-promoter site[43]. Most importantly, the proteins assembled by the core co-activator as the core co-activated complex have individual enzymatic activities to acetylate or methylate adjacent proteins. This results in the dissociation of the complex and simultaneous tagging with activated ubiquitin. The activated ubiquitin is transferred to the ubiquitin-conjugating enzyme that interacts with the ubiquitin ligase, which has already identified its protein target. Several cycles of the reaction can polyubiquitylate a substrate, that is, ER or a co-activator, to either be activated further (Lys63 linkage) or degraded by the 26S proteasome (Lys48 linkage)[44], depending on the ubiquitin–ubiquitin linkage.

Therefore, for effective gene transcription programmed and targeted by the shape and phosphorylation status of the ER and co-activators, a dynamic and cyclic process of remodelling capacity is required for transcriptional assembly[45] that is immediately followed by the routine destruction of transcription complexes by the proteasome. Oestrogen and SERM receptor complexes have different accumulation patterns in the target cell nucleus[46,47], primarily because the relative rates of destruction of the complexes are different[48].

These fundamental mechanisms[43,49] can also be applied to the potential development of drug resistance to tamoxifen in breast cancer. Model systems have shown the conversion of the tamoxifen–ER complex from an anti-oestrogenic to an oestrogenic signal in an environment of increased phosphorylation caused by the overexpression of the ERBB2 cell-surface receptor and an increase in the SRC3 (AIB1) co-activation pool[50,51]. SRC3 probably increases independent of ERBB2 increases. However, the increased level of this co-activator and its increased phosphorylation state derived from an activated ERBB2 phosphorylation pattern will increase the oestrogen-like activity of tamoxifen at the ER. Clearly, issues of SERM action at target tissues and the eventual development of drug resistance in breast cancer will converge as the duration of SERM use extends from a few years to at least a decade.

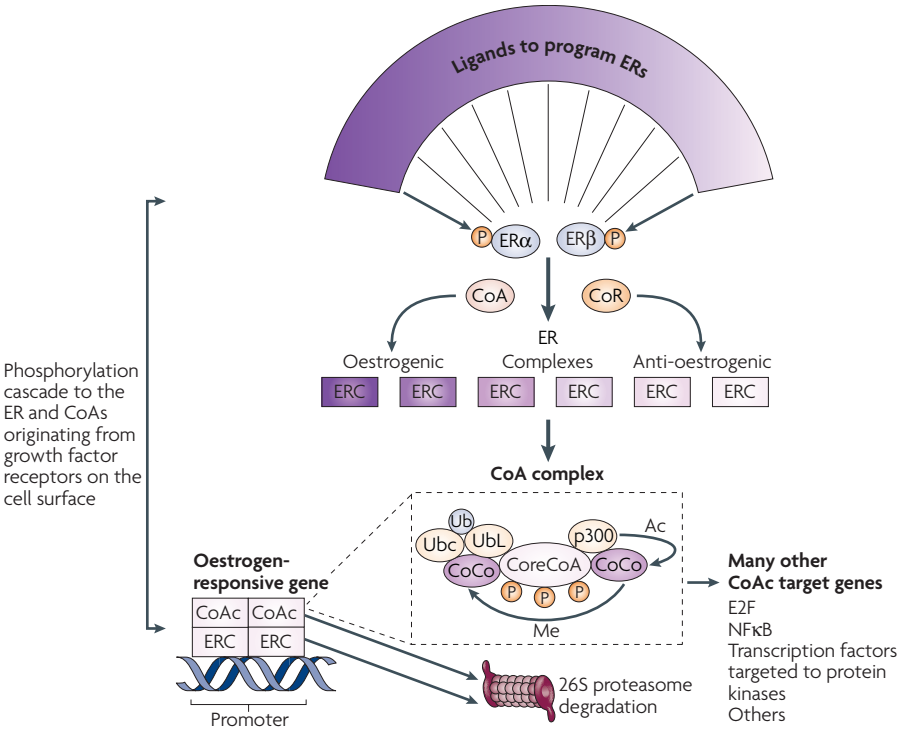

Figure 1 | **Molecular networks potentially influence the expression of SERM action in a target tissue.** The shape of the ligands that bind to the oestrogen receptors (ERs)α and β programmes the complex to become an oestrogenic or anti-oestrogenic signal. The context of the ER complex (ERC) can influence the expression of the response through the numbers of co-repressors (CoR) or co-activators (CoA). In simple terms, a site with few CoAs or high levels of CoRs might be a dominant anti-oestrogenic site. However, the expression of oestrogenic action is not simply the binding of the receptor complex to the promoter of the oestrogen-responsive gene, but a dynamic process of CoA complex assembly and destruction[43]. A core CoA, for example, steroid receptor coactivator protein 3 (SRC3), and the ERC are influenced by phosphorylation cascades that phosphorylate target sites on both complexes. The core CoA then assembles an activated multiprotein complex containing specific co-co-activators (CoCo) that might include p300, each of which has a specific enzymatic activity to be activated later. The CoA complex (CoAc) binds to the ERC at the oestrogen-responsive gene promoter to switch on transcription. The CoCo proteins then perform methylation (Me) or acetylation (Ac) to activate dissociation of the complex. Simultaneously, ubiquitiylation by the bound ubiquitin-conjugating enzyme (Ubc) targets ubiquitin ligase (UbL) destruction of protein members of the complex through the 26S proteasome. The ERs are also ubiquitylated and destroyed in the 26S proteasome. Therefore, a regimented cycle of assembly, activation and destruction occurs on the basis of the preprogrammed ER complex[43]. However, the co-activator, specifically SRC3, has ubiquitous action and can further modulate or amplify the ligand-activated trigger through many modulating genes[101] that can consolidate and increase the stimulatory response of the ERC in a tissue. Therefore, the target tissue is programmed to express a spectrum of responses between full oestrogen action and anti-oestrogen action on the basis of the shape of the ligand and the sophistication of the tissue-modulating network. NFκB, nuclear factor κB.

Molecular pharmacology of SERMs

The metabolism, pharmacogenomics and pharmacokinetics of SERMs continue to present challenges. Just when everything seems to be straightforward, old drugs create unanticipated surprises. Initially, there was little pharmacological information or interest in the metabolism of tamoxifen in animals or man; this was not a main requirement to register a drug to treat advanced breast cancer in the 1970s[1]. The situation remained the same during the 1980s, a time when tamoxifen was about to become the standard of care as the adjuvant anti-hormonal treatment of ER-positive breast cancer, and studies were planned to evaluate the worth of tamoxifen to prevent breast cancer in high-risk women[1]. At that time, it was accepted that tamoxifen was either metabolically activated to 4-hydroxytamoxifen[52,53], a minor metabolite with high binding affinity for the ER but with a short half life[54], or demethylated to *N*-desmethyltamoxifen, a compound with low binding affinity for the ER but a long biological half life (FIG. 2). *N*-Desmethyltamoxifen was thought to be further demethylated to desdimethyltamoxifen, and subsequently deaminated to the weakly anti-oestrogenic glycol derivative

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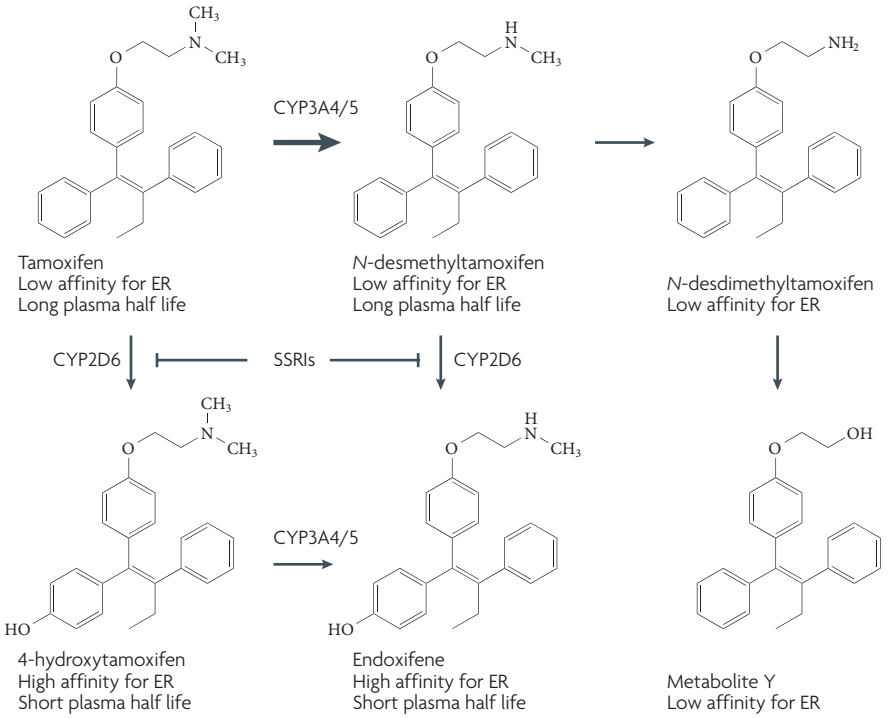

Figure 2 | **The metabolism of tamoxifen in humans.** Circulating drug levels of tamoxifen (20 mg a day) reach a steady state in about 3–4 weeks. The principal route of tamoxifen metabolism is through CYP3A4/5 (cytochrome P450, family 3, subfamily A, polypeptide 4/5) demethylation to *N*-desmethyltamoxifen, which has twice the serum half life as tamoxifen, and levels are usually 50–100% higher than those of tamoxifen. *N*-Desmethyltamoxifen is further demethylated to desdimethyltamoxifen and then deaminated to metabolite Y, a glycol derivative. Tamoxifen and *N*-desmethyltamoxifen are both para-hydroxylated by CYP2D6 to form 4-hydroxytamoxifen and endoxifene, respectively. Levels of endoxifene are twice those seen for 4-hydroxytamoxifen, which in turn are about 5% of those observed for tamoxifen. Specific selective serotonin reuptake inhibitors (SSRIs) (BOX 3) bind to CYP2D6 to prevent the hydroxylation of tamoxifen and *N*-desmethyltamoxifen. This prevents the metabolic activation of tamoxifen. ER, oestrogen receptor.

of tamoxifen referred to as metabolite Y[55]. These anti-oestrogenic metabolites deactivate the ER, but based on concentrations in SERM and affinity, all were considered to have a role in blocking oestrogen action.

The ubiquitous application of tamoxifen as a long-term, well-tolerated treatment for breast cancer during the past decade, and its use as a preventive in high-risk women, resulted in the close examination of symptom management, especially hot flashes, to increase compliance. Selective serotonin reuptake inhibitors (SSRIs) (BOX 3) are effective at controlling the hot flashes experienced by up to 45% of patients treated with tamoxifen. However, the recent identification and characterization[56–58] of the high-affinity metabolite of tamoxifen, 4-hydroxy-*N*-desmethyltamoxifen (endoxifene), and the finding that endoxifene levels are reduced by the co-administration of SSRIs[59–61], is an important observation that has potential therapeutic implications. It follows that as SSRIs block CYP2D6 (cytochrome P450, family 2, subfamily D, polypeptide 6), therefore inhibiting the metabolism of tamoxifen to endoxifene, then the efficacy of tamoxifen as an anticancer agent (treatment or chemopreventive) could be impaired by either the ubiquitous use of SSRIs to prevent hot flashes or the administration of tamoxifen to women with a defect in the CYP2D6 enzyme that no longer converts tamoxifen to endoxifene. Preliminary evidence suggests that this might be the case[61,62].

Knowledge of the metabolic activation of tamoxifen to hydroxylated metabolites with high affinity for the ER created an opportunity to design the high affinity SERMs, raloxifene, basedoxifene and lasofoxifene (BOX 4). However, the pharmacokinetics and pharmacodynamics of these polyphenolic compounds make it difficult to deliver sufficient concentrations to the breast tissue of healthy women. Raloxifene and other SERM members that are benzothiophene derivatives are short acting[13,63,64]. However, raloxifene has a plasma elimination half-life of about 27 hours, which apparently results from reversible phase II metabolism, which conjugates the polyphenolic drugs before their excretion as sulphates and glucuronides.

There seem to be two aspects to be considered for a polyphenolic SERM to be an effective chemopreventive for breast cancer. First, raloxifene is conjugated by the human intestinal enzymes UDP- glucuronosyltransferase 1A8 (UGT1A8) and UGT1A10 (REF. 65), but it is the dynamic relationship between absorption, phase II metabolism and excretion in the intestine[66] that controls the 2% bioavailability of raloxifene[64]. The second aspect for consideration is the retention of raloxifene in the target tissue. This depends on local sulphation, which inactivates the SERM before diffusion out of the tissue. Here again, there are disparities in the efficacy of multiple-sulphation enzymes (sulphotransferases, SULTs) to terminate the bioactivity of raloxifene in a target site. For example, 4-hydroxytamoxifen[52] is only sulphated by three of seven SULT isoforms, whereas raloxifene is sulphated by all seven[67]. In addition, SULT1E1, which sulphates raloxifene in endometrial tissue, is only expressed in the secretory phase[67] of the menstrual cycle after ovulation. An alternative to designing phase II metabolism-insensitive SERMs is to create a long-acting SERM with protected phenolic groups that require metabolic activation. Arzoxifene (BOX 4) is a methoxy derivative of raloxifene[68] that is superior to raloxifene as a chemopreventive for rat mammary carcinoma[68]. The SERM shows activity for the treatment of ER-positive metastatic breast cancer, but in two trials a low dose (20 mg a day) was superior to a high dose (50 mg a day)[69,70]. Arzoxifene is completing evaluation as a treatment and preventive for osteoporosis, and trials to determine its efficacy for the prevention of breast and endometrial cancer are eagerly awaited.

Other chemoprevention strategies

If oestrogen is responsible for the development and growth of breast cancer, it is only natural to consider that a 'no oestrogen' state would be the ultimate chemopreventive for breast cancer. The current agents of choice to prevent oestrogen synthesis in postmenopausal women are referred to as aromatase inhibitors. These drugs block the conversion of steroidal precursors, androstenedione and testosterone, to oestrogens by the CYP19 aromatase enzyme in a woman's body fat,

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as well as breast epithelial and stromal cells. Several different aromatase inhibitors are available (for example, anastrozole, letrozole and exemestane), and have shown increased superiority against tamoxifen for the treatment of breast cancer. Aromatase inhibitors have few side effects, specifically blood clots or endometrial cancer, when studies[71–75] have compared and contrasted their efficacy and safety against tamoxifen for the adjuvant treatment of breast cancer in postmenopausal women. Nevertheless, the fact that it is not possible to determine precisely whom to select for chemoprevention means that many postmenopausal women who will never develop breast cancer will be treated with aromatase inhibitors to benefit a few. In other words, based on the population of the STAR trial[8], if 1,000 very high-risk postmenopausal women were selected that might develop 10 breast cancers (8 of these would be ER-positive and receptive to therapy) during a year, then 992 women would be treated unnecessarily to prevent 8 ER-positive tumours. This is clearly inappropriate as a public-health policy.

Nevertheless, the use of aromatase inhibitors as chemopreventive agents in postmenopausal women is currently being evaluated in clinical trials. The scientific rationale is valid on the basis of the reduced incidence of contralateral breast cancer noted in trials of adjuvant therapy[71–75]. A trial by the NSABP is currently comparing raloxifene with the aromatase inhibitor letrozole, the International Breast Intervention Study 2 (IBIS-2) is comparing anastrozole with placebo[76], and the National Cancer Institute of Canada is comparing exemestane with placebo (MAP3)[77]. Apart from the inability to appropriately target high-risk women, there is a need to monitor bone density and institute supplemental treatment, where necessary, with bisphosphonates. Bisphosphonates are analogues of pyrophosphate that have their central oxygen atom replaced by carbon. They form a three-dimensional structure that chelates calcium in bone and prevents resorption[78]. At present, the high cost of aromatase inhibitors with associated monitoring might be too expensive for a routine government-sponsored health-care intervention.

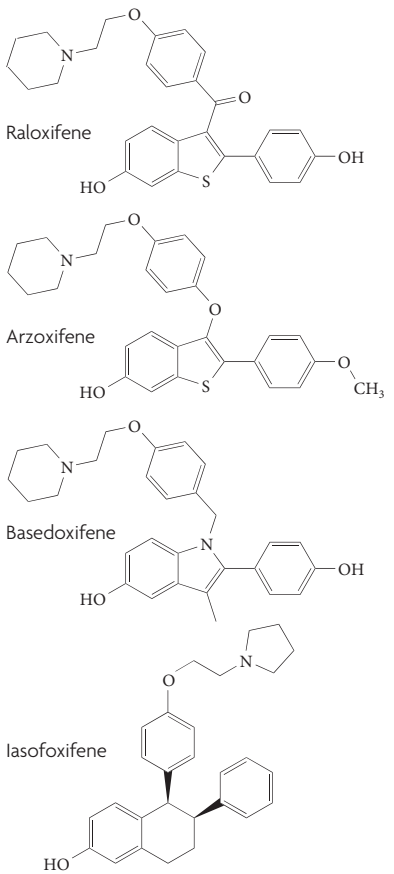
To avoid the concerns about a general decrease in circulating oestrogen produced by current aromatase inhibitors, there is interest in determining whether breast-specific inhibitors could be developed. The idea is to create a selective aromatase inhibitor to exploit the observation that several tissue-specific promoter regions have been identified upstream of the *CYP19* gene[79,80]. As an example of progress with exploiting the possibility of tissue selectivity, the orphan nuclear receptor liver receptor homolog-1 (LRH1) is a specific transcription activator of aromatase in breast pre-adipocytes[81]. Clearly LRH1 would be an interesting therapeutic target. It is known that prostaglandin E_2 is an important regulator of aromatase expression in breast cancer, and the non-steroidal anti-inflammatory drugs that inhibit COX1 and COX2 also suppress aromatase activity[82]. The authors of the study noted[82] that a COX2 inhibitor was particularly active. However, recently a new series of sulphonilide analogues has been reported that suppress aromatase activity independent of COX2 (REF. 83) in breast cancer cells. Clearly, there is enormous potential for drug development.

Box 3 | SSRIs

Selective serotonin reuptake inhibitors (SSRIs) can be used to treat depression associated with general medical illness, but more specifically hot flashes and associated menopausal symptoms. The SSRIs specifically target serotonin-containing neurons. Serotonin is synthesized from L-tryptophan by hydroxylation, and subsequently deaminated to 5-hydroxytryptamine (5HT) or serotonin. The release of 5HT causes an interaction with a wide range of 5HT receptors that are both autoinhibitory and stimulatory. The long-term use of SSRIs prevents the reuptake of 5HT from the synapse into the presynaptic serotonin neuron, where secondary deamination and inactivation occurs through monoamine oxidase. The inability to remove and destroy 5HT causes neuronal desensitization. Most importantly, some of the SSRIs (fluoxetine and paroxetine) are potent inhibitors of the CYP2D6 (cytochrome P450, family 2, subfamily D, polypeptide 6) enzyme, that along with CYP1A2 mediates hydroxylation of the aromatic ring on tamoxifen. Ongoing studies show that potent inhibitors of CYP2D6 in the SSRI family significantly decrease levels of the tamoxifen metabolite endoxifene[60,61]. Evidence is available that venlafaxine (Effexor) does not lower endoxifene levels, but these conclusions[61] need to be confirmed with a larger study.

Box 4 | New SERMs

Several new selective oestrogen-receptor modulators (SERMs) are being evaluated at present in clinical trials for the prevention of osteoporosis. Arzoxifene[68] and basedoxifene[97,98] bear a striking resemblance to the structure of raloxifene. By contrast, lasofoxifene[99,100] is a derivative of the non-steroidal anti-oestrogen nafoxidine that was tested as a breast cancer drug in the 1970s but was not pursued because most patients experienced light sensitivity[1]. The new molecule is the L-enantiomer that has 20 times the binding affinity for the oestrogen receptor (ER) as the D-enantiomer, and the L-enantiomer has twice the bioavailability of the D-enantiomer[99]. The difference is believed to be the result of enantioselective glucuronidation of the L-enantiomer. The fact that lasofoxifene and basedoxifene are polyphenolic compounds that are both susceptible to phase II metabolism might make the transition from treatment for osteoporosis to a widely used chemopreventive more challenging. If the SERMs are poorly bioavailable because of first-pass metabolism in the liver, then the medicine might still perform well as an anti-tumour agent in the low-oestrogen environment observed in osteoporotic postmenopausal women. By contrast, if the SERMs are used to prevent breast cancer in healthy postmenopausal women with high levels of body fat, this will create an environment of high circulating oestrogen levels. As a result, the low bioavailability will be unable to constantly block the breast from oestrogen action.



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Chemoprevention today

It is now possible to select women who will benefit from taking SERMs to reduce the risk of breast cancer. It should be obvious that as a group, the SERMs are more flexible than other drug classes such as the aromatase inhibitors, which are restricted to breast cancer in postmenopausal women. The reason for this is that constitutive oestrogen synthesis occurs in peripheral body fat in postmenopausal women, and this can be blocked by aromatase inhibitors. By contrast, oestrogen synthesis in the ovaries of premenopausal women is regulated by gonadotrophins during the menstrual cycle. Decreases in circulating oestrogen that could occur with an aromatase inhibitor are immediately increased through a compensatory increase of gonadotrophins. Decreases in circulating oestrogen are detected at the hypothalamo–pituitary axis, thereby activating the oestrogen-regulated negative feed–back loop for the increased secretion of gonadotrophins. Therefore, the action of a competitive inhibitor of aromatase is reversed. A future question is whether the efficacy of a suicide inhibitor such as exemestane would be superior to a SERM.

Knowledge of the beneficial risk–benefit ratio of tamoxifen in premenopausal women[4] must now be melded with knowledge that tamoxifen must be given for at least 5 years for effective chemoprevention in premenopausal women. This is the current standard of care. However, the increased incidence of side effects, such as menopausal symptoms of hot flashes, often becomes a real challenge to maintaining patient compliance. If the drug is stopped there will be no benefit. Unfortunately, if an inappropriate SSRI is prescribed to reduce hot flashes the efficacy of the chemoprevention strategy will be undermined, as the SSRI blocks endoxifene production by CYP2D6 (REFS 61,62) (BOX 3, FIG. 2). In addition, if a woman has a non-enzymatic variant of CYP2D6, then there is potentially no value to tamoxifen treatment. Advances in our knowledge of drug interactions and pharmacogenetics are proving to be valuable for targeting tamoxifen to the appropriate premenopausal woman.

Raloxifene now seems to offer real benefits in reducing the incidence of breast cancer in high-risk postmenopausal populations[8] or in women already receiving raloxifene for the prevention of osteoporosis[21]. Although aromatase inhibitors might, at a later date, offer advantages over raloxifene as a breast cancer preventive, the additional monitoring of patient bone density and intervention with a bisphosphonate will require additional medical resources.

Finally, it is important to stress that SERMs are not static, but as a drug group continue to evolve as new members are tested and introduced into clinical practice (BOX 4). Furthermore, the principles used[5,6] to create SERMs are now being applied to other members of the steroid receptor superfamily[37] so that the required target site effect of a particular hormone can be switched on or off. This advance holds the promise of an expanded menu of medicines to address the selective treatment and prevention of many diseases once thought to be impossible.

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1. Jordan, V. C. Tamoxifen: a most unlikely pioneering medicine. *Nature Rev. Drug Discovery* **2**, 205–213 (2003).
2. Fisher, B. *et al.* Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J. Natl Cancer Inst.* **90**, 1371–1388 (1998).
3. Gail, M. H. *et al.* Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J. Natl Cancer Inst.* **81**, 1879–1886 (1989).
4. Gail, M. H. *et al.* Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J. Natl Cancer Inst.* **91**, 1829–1846 (1999).
5. Jordan, V. C. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest.* **6**, 589–595 (1988).
6. Lerner, L. J. & Jordan, V. C. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res.* **50**, 4177–4189 (1990).
7. Cummings, S. R. *et al.* The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* **281**, 2189–2197 (1999).
8. Vogel, V. G. *et al.* The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA* **295**, 2727–2741 (2006).
9. Jordan, V. C. Selective estrogen receptor modulation: a personal perspective. *Cancer Res.* **61**, 5683–5687 (2001).
10. Jordan, V. C. & Robinson, S. P. Species-specific pharmacology of antiestrogens: role of metabolism. *Fed. Proc.* **46**, 1870–1874 (1987).
11. Black, L. J., Jones, C. D. & Falcone, J. F. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. *Life Sci.* **32**, 1031–1036 (1983).
12. Jordan, V. C., Phelps, E. & Lindgren, J. U. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res. Treat.* **10**, 31–35 (1987).
13. Gottardis, M. M. & Jordan, V. C. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res.* **47**, 4020–4024 (1987).
14. Gottardis, M. M., Robinson, S. P., Satyaswaroop, P. G. & Jordan, V. C. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res.* **48**, 812–815 (1988).
15. Gottardis, M. M., Ricchio, M. E., Satyaswaroop, P. G. & Jordan, V. C. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. *Cancer Res.* **50**, 3189–3192 (1990).
16. Fisher, B. *et al.* Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J. Natl Cancer Inst.* **97**, 1652–1662 (2005).
17. Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* **365**, 1687–1717 (2005).
18. Melnikow, J. *et al.* Chemoprevention: drug pricing and mortality: the case of tamoxifen. *Cancer* **107**, 950–958 (2006).
19. Ettinger, B. *et al.* Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. *JAMA* **282**, 637–645 (1999).
20. Jensen, E. V. & Jordan, V. C. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. *Clin. Cancer Res.* **9**, 1980–1989 (2003).
21. Martino, S. *et al.* For the CORE investigators. Continuing Outcomes Relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J. Natl Cancer Inst.* **96**, 1751–1761 (2004).
22. Walsh, B. W. *et al.* Effects of raloxifene on serum lipids and coagulation factors in healthy postmenopausal women. *JAMA* **279**, 1445–1451 (1998).
23. Barrett-Connor, E. *et al.* Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N. Engl J. Med.* **355**, 125–137 (2006).
24. Jensen, E. V. & Jacobson, H. I. Basic guides to the mechanism of estrogen action. *Recent Progr. Hormone Res.* **18**, 387–414 (1962).
25. Greene, G. L. *et al.* Sequence and expression of human estrogen receptor complementary DNA. *Science* **231**, 1150–1154 (1986).
26. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S. & Gustafsson, J. A. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl Acad. Sci. USA* **93**, 5925–5930 (1996).
27. Kuiper, G. G. *et al.* Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138**, 863–870 (1997).
28. Roger, P. *et al.* Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. *Cancer Res.* **61**, 2537–2541 (2001).
29. Shaaban, A. M. *et al.* Declining estrogen receptor-beta expression defines malignant progression of human breast neoplasia. *Am. J. Surg. Pathol.* **27**, 1502–1512 (2003).
30. Paruthiyil, S. *et al.* Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. *Cancer Res.* **64**, 423–428 (2004).
31. Acconcia, F. *et al.* Survival versus apoptotic 17beta-estradiol effect: role of ER alpha and ER beta activated non-genomic signaling. *J. Cell Physiol.* **203**, 193–201 (2005).
32. McInerney, E. M., Weis, K. E., Sun, J., Mosselman, S. & Katzenellenbogen, B. S. Transcription activation by the human estrogen receptor subtype beta (ER beta) studied with ER beta and ER alpha receptor chimeras. *Endocrinology* **139**, 4513–4522 (1998).
33. Barkhem, T. *et al.* Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. *Mol. Pharmacol.* **54**, 105–112 (1998).
34. Hall, J. M. & McDonnell, D. P. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* **140**, 5566–5578 (1999).
35. Paech, K. *et al.* Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. *Science* **277**, 1508–1510 (1997).
36. Onate, S. A., Tsai, S. Y., Tsai, M. J. & O'Malley, B. W. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270**, 1354–1357 (1995).
37. Smith, C. L. & O'Malley, B. W. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocrine Rev.* **25**, 45–71 (2004).
38. Jordan, V. C. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *J. Med. Chem.* **46**, 1081–1111 (2003).
39. Kraichely, D. M., Sun, J., Katzenellenbogen, J. A. & Katzenellenbogen, B. S. Conformational changes and coactivator recruitment by novel ligands for estrogen receptor-alpha and estrogen receptor-beta: correlations with biological character and distinct

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differences among SRC coactivator family members. *Endocrinology* **141**, 3534–3545 (2000).
40. Shiau, A. K. *et al*.. The structural basis of estrogen receptor/co-activator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**, 927–937 (1998).
41. Brzozowski, A. M. *et al*. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389**, 753–758 (1997).
42. Wijayaratne, A. L. *et al*. Comparative analyses of mechanistic differences among antiestrogens. *Endocrinology* **140**, 5828–5840 (1999).
43. Lonard, D. M. & O'Malley, B. W. The expanding cosmos of nuclear receptor coactivators. *Cell* **125**, 411–414 (2006).
44. Sun, Y. E3 Ubiquitin Ligases as Cancer Targets and Biomarkers. *Neoplasia* **8**, 645–654 (2006).
45. Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A. & Brown, M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* **103**, 843–852 (2000).
46. Jordan, V. C., Dix, C. J., Rowsby, L. & Prestwich, G. Studies on the mechanism of action of the nonsteroidal antioestrogen tamoxifen (I. C. I. **46**, 474) in the rat. *Mol. Cell. Endocrinol.* **7**, 177–192 (1977).
47. Horwitz, K. B. & McGuire, W. L. Nuclear mechanisms of estrogen action. Effects of estradiol and anti-estrogens on estrogen receptors and nuclear receptor processing. *J. Biol. Chem.* **253**, 8185–8191 (1978).
48. Wijayaratne, A. L. & McDonnell, D. P. The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. *J. Biol. Chem.* **276**, 35684–35692 (2001).
49. Wu, R. C., Smith, C. L. & O'Malley, B. W. Transcriptional regulation by steroid receptor coactivator phosphorylation. *Endocrine Rev.* **26**, 393–399 (2006).
50. Osborne, C. K. *et al*. Role of the estrogen receptor coactivator AIB1 (SRC3) and HER2/neu in tamoxifen resistance in breast cancer. *J. Natl Cancer Inst.* **95**, 353–361 (2003).
51. Shou, J. *et al*. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2–positive breast cancer. *J. Natl Cancer Inst.* **96**, 926–935 (2004).
52. Jordan, V. C., Collins, M. M., Rowsby, L. & Prestwich, G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J. Endocrinol.* **75**, 305–316 (1977).
53. Allen, K. E., Clark, E. R. & Jordan, V. C. Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. *Br. J. Pharmacol.* **71**, 83–91 (1980).
54. Jordan, V. C. & Allen, K. E. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur. J. Cancer* **16**, 239–251 (1980).
55. Jordan, V. C. *et al*. Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient during therapy for advanced breast cancer. *Cancer Res.* **43**, 1446–1450 (1983).
56. Johnson, M. D. *et al*. Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. *Breast Cancer Res. Treat.* **85**, 151–159 (2004).
57. Lim, Y. C., Desta, Z., Flockhart, D. A. & Skaar, T. C. Endoxifen (4-hydroxy-N-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen. *Cancer Chemother. Pharmacol.* **55**, 471–478 (2005).
58. Lim, Y. C. *et al*. Endoxifen, a secondary metabolite of tamoxifen, and 4-OH-tamoxifen induce similar changes in global gene expression patterns in MCF-7 breast cancer cells. *J. Pharmacol. Exp. Ther.* **318**, 503–512 (2006).
59. Stearns, V. *et al*. Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. *J. Natl Cancer Inst.* **95**, 1758–1764 (2003).
60. Jin, Y. *et al*. CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. *J. Natl Cancer Inst.* **97**, 30–39 (2005).
61. Borges, S. *et al*. Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. *Clin. Pharmacol. Ther.* **80**, 61–74 (2006).
62. Goetz, M. P. *et al*. Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. *J. Clin. Oncol.* **23**, 9312–9318 (2005).
63. Jordan, V. C. & Gosden, B. Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. *Endocrinology* **113**, 463–468 (1983).
64. Snyder, K. R., Sparano, N. & Malinowski, J. M. Raloxifene hydrochloride. *Am. J. Health Syst. Pharm.* **57**, 1669–1675 (2000).
65. Kemp, D. C., Fan, P. W. & Stevens, J. C. Characterization of raloxifene glucuronidation *in vitro*: contribution of intestinal metabolism to presystemic clearance. *Drug Metab. Dispos.* **30**, 694–700 (2002).
66. Jeong, E. J., Lin, H. & Hu, M. Disposition mechanisms of raloxifene in the human intestinal Caco-2 model. *J. Pharmacol. Exp. Ther.* **310**, 376–385 (2004).
67. Falany, J. L., Pilloff, D. E., Leyh, T. S. & Falany, C. N. Sulfation of raloxifene and 4-hydroxytamoxifen by human cytosolic sulfotransferases. *Drug Metab. Dispos.* **34**, 361–368 (2006).
68. Suh, N. *et al*. Arzoxifene, a new selective estrogen receptor modulator for chemoprevention of experimental breast cancer. *Cancer Res.* **61**, 8412–8415 (2001).
69. Baselga, J. *et al*. Randomized, double-blind multicenter trial comparing two doses of arzoxifene (LY353381) in hormone-sensitive advanced or metastatic breast cancer patients. *Annal. Oncol.* **14**, 1383–1390 (2003).
70. Buzdar, A. *et al*. Phase II, randomized double-blind study of two dose levels of arzoxifene in patients with locally advanced or metastatic breast cancer. *J. Clin. Oncol.* **21**, 1007–1014 (2003).
71. ATAC Trialists' Group. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomized trial. *Lancet* **359**, 2134–3139 (2002).
72. Goss, P. E. *et al*. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. *N. Engl J. Med.* **349**, 1–10 (2003).
73. Howell, A. *et al*. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* **365**, 60–62 (2005).
74. Coombes, R. C. *et al*. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. *N. Engl J. Med.* **350**, 1081–1092 (2004).
75. Thurlimann, B. *et al*. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N. Engl J. Med.* **353**, 2747–2757 (2005).
76. Goss, P. E. Breast cancer prevention--clinical trials strategies involving aromatase inhibitors. *J. Steroid Biochem. Mol. Biol.* **86**, 487–493 (2003).
77. Goss, P. E. & Strasser-Weippl, K. Prevention strategies with aromatase inhibitors. *Clin. Cancer Res.* **10**, 372S–379S (2004).
78. Licata, A. A. Discovery, clinical development, and therapeutic uses of bisphosphonates. *Ann. Pharmacother.* **39**, 668–677 (2005).
79. Simpson, E. R. *et al*. Tissue-specific promoters regulate aromatase cytochrome P450 expression. *J. Steroid Biochem. Mol. Biol.* **44**, 321–330 (1993).
80. Simpson, E., Clyne, C, Rubin, G. *et al*. Aromatase-a brief overview. *Annu. Rev. Physiol.* **64**, 93–127 (2002).
81. Safi, R. *et al*. Coactivation of liver receptor homologue-1 by peroxisome proliferator-activated receptor gamma coactivator-1alpha on aromatase promoter II and its inhibition by activated retinoid X receptor suggest a novel target for breast-specific antiestrogen therapy. *Cancer Res.* **65**, 11762–11770 (2005).
82. Diaz-Cruz, E. S., Shapiro, C. L. & Brueggemeier, R. W. Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells. *J. Clin. Endocrinol. Metab.* **90**, 2563–2570 (2005).
83. Su, B., Diaz-Cr, E. S., Landini, S. & Brueggemeier, R. W. Novel sulfonanilide analogues suppress aromatase expression and activity in breast cancer cells independent of COX-2 inhibition. *J. Med. Chem.* **49**, 1413–1419 (2006).
84. Fornander, T. *et al*. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* **1**, 117–120 (1989).
85. Black, L. J., Jones, C. D. & Falcone, J. F. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. *Life Sci.* **32**, 1031–1036 (1983).
86. Powles, T. J. *et al*. A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *Br. J. Cancer* **60**, 126–131 (1989).
87. Love, R. R. *et al*. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N. Engl. J. Med.* **326**, 852–856 (1992).
88. Cuzick, J. *et al*. First results from the International Breast Cancer Intervention Study (IBIS-I): a randomised prevention trial. *Lancet* **360**, 817–824 (2002).
89. Fisher, B. *et al*. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. *J. Natl Cancer Inst.* **86**, 527–537 (1994).
90. Rebbeck, T. R. *et al*. Estrogen sulfation genes, hormone replacement therapy, and endometrial cancer risk. *J. Natl Cancer Inst.* **98**, 1311–1320 (2006).
91. Horwitz, R. I., Feinstein, A. R., Horwitz, S. M. & Robboy, S. J. Necropsy diagnosis of endometrial cancer and detection-bias in case/control studies. *Lancet* **2**, 66–68 (1981).
92. Wu, H. *et al*. Hypomethylation-linked activation of PAX2 mediates tamoxifen-stimulated endometrial carcinogenesis. *Nature* **438**, 981–987 (2005).
93. Phillips, D. H., Hewer, A., Grover, P. L., Poon, G. K. & Carmichael, P. L. Tamoxifen does not form detectable DNA adducts in white blood cells of breast cancer patients. *Carcinogenesis* **17**, 1149–1152 (1996).
94. Phillips, D. H. Understanding the genotoxicity of tamoxifen? *Carcinogenesis* **22**, 839–849 (2001).
95. Cavalieri, E. *et al*. Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. *Biochim. Biophys. Acta* **1766**, 63–78 (2006).
96. Russo, J. *et al*. 17-Beta-estradiol induces transformation and tumorigenesis in human breast epithelial cells. *Faseb J.* **20**, 1622–1634 (2006).
97. Miller, C. P. *et al*. Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. *J. Med. Chem.* **44**, 1654–1657 (2001).
98. Greenberger, L. M. *et al*. A new antiestrogen, 2-(4-hydroxy-phenyl)-3-methyl-1-[4-(2-piperidin-1-yl-ethoxy)-benzyl]-1H-in dol-5-ol hydrochloride (ERA-923), inhibits the growth of tamoxifen-sensitive and-resistant tumors and is devoid of uterotropic effects in mice and rats. *Clin. Cancer Res.* **7**, 3166–3177 (2001).
99. Rosati, R. L. *et al*. Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryltetrahydronaphthalene. *J. Med. Chem.* **41**, 2928–2931 (1998).
100. Cohen, L. A. *et al*. Lasofoxifene, a novel selective estrogen receptor modulator with chemopreventive and therapeutic activity in the N-nitroso-N-methylurea-induced rat mammary tumor model. *Cancer Res.* **61**, 8683–8688 (2001).
101. O'Malley, B. W. Little molecules with big goals. *Science* **313**, 1749–1750 (2006).

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SERMs: Meeting the Promise of Multifunctional Medicines

V. Craig Jordan

The successful development and clinical evaluation of the selective estrogen receptor modulators in the Study of Tamoxifen and Raloxifene trial provides an occasion to reflect on the milestone that has been achieved and the potential for further progress in the chemoprevention of breast cancer. The evolution of tamoxifen from a successful treatment for breast cancer to the first chemopreventive for any cancer took two decades. Clinicians gained an enormous amount of experience with the use of tamoxifen as a treatment, and, as a result, there were few surprises in terms of efficacy or the side effect profile when the medicine was used to prevent breast cancer in high-risk women. In contrast, raloxifene emerged via the novel path of the evidence-based hypothesis that a drug targeted at one disease, osteoporosis, could also prevent breast cancer. Changes in health care strategies to implement chemoprevention take time, but the evidence now suggests that chemoprevention has become a reality in clinical practice.

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With declining investment in cancer research and reluctance by the pharmaceutical industry to address prevention, why must the chemoprevention of breast cancer remain a priority? The disease has a high incidence, with an estimated 1 million women worldwide diagnosed with breast cancer annually. Solid tumors are difficult to control, but it can be argued that substantial progress is being made with targeted therapies for breast cancer that save lives. Treatments targeting the tumor estrogen receptor (ER) (1) or the growth factor receptor HER2/neu (2) confer statistically significant survival advantages in clinical trials (3–7). In the case of tamoxifen, there is evidence that the drug has contributed to the reduction in national death rates from breast cancer (4). With the experience gained from the successful treatment of breast cancer with targeted antiestrogens, it should be obvious to the casual observer that the application of the same principle—in the right women at the right time—to prevent the development of the disease would provide much needed relief for a society burdened with an overextended and overexpensive health care system.

However, it would be naive to expect that the medical science community can prevent breast cancer at a single stroke. The lessons of the Study of Tamoxifen and Raloxifene (STAR) (8) trial show that progress is slow, often unpredictable, and, at least in the case of STAR trial, dependent on three factors: good ideas that translate from the laboratory to the clinic, fashions in research, and the development of a patenting strategy that ensures exclusivity for a company during clinical testing.

The practical application of molecular theory to the prevention of cancer requires collaborative teams from multiple disciplines to translate a concept into lives saved. Unfortunately, society has erected an artificial barrier to achieving success in chemoprevention. This is because in assessing the successfulness of a new treatment for breast cancer, the benchmark is "lives saved". In chemoprevention, it may take a generation to quantify "lives saved", but if the medicine is safe and there is a dramatic reduction in the incidence of breast cancer, it follows that the treatment must ultimately reduce the death rate from breast cancer. Most importantly, the medicine used for chemoprevention should have minimal side effects to ensure compliance. To achieve the goal of chemoprevention, prospective clinical trials must demonstrate advantages over current approved therapies or the traditional "wait and see" approach with routine screening.

Once the concept of chemoprevention becomes a reality and an agent is proven to reduce the risk of breast cancer, the cost–benefit ratio to the health care system must be advantageous. Only half of the women who develop breast cancer can be identified using the Gail model (9), and identification of specific women for intervention is based on large populations with only a small percentage of women developing the disease. At present, therefore, large numbers of women must be treated to benefit the few. As a result, the preventive treatment must be of low cost, highly effective, and without serious side effects if health care is to be improved, and these qualities are critical to widespread acceptance by national managed health care systems.

The feasibility of reducing breast cancer incidence in high-risk pre- and postmenopausal women has been established by the pioneering work of Fisher et al. and the National Surgical Adjuvant Breast and Bowel Project (NSABP) P-1 Study (10,11). Tamoxifen administered at a daily dose of 20 mg for 5 years reduced breast cancer incidence by about 50%. This milestone achievement in translational research was the result of a century of laboratory and clinical studies to understand the genesis of breast cancer (12). The STAR trial now establishes the practicality of chemoprevention of breast cancer, but the knockout blow has yet to be delivered. My purpose in this commentary is to explain how cancer research evolved to result in the STAR trial

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See "Notes" following "References."

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and to present options available to future progress in breast cancer chemoprevention.

Tamoxifen, the First Selective Estrogen Receptor Modulator

The story of the "reinvention" of tamoxifen—at one time a failed contraceptive discovered in the fertility control department at ICI Pharmaceuticals Division (now AstraZeneca)—as the first targeted therapy for the treatment of ER-positive breast cancer has recently been described (13,14). The scientific approach to cancer drug development used in the 1960s and 1970s is adequately summarized elsewhere (13), but the clinical focus at that time was directed exclusively to breast cancer therapy rather than chemoprevention. The scientific principles established in the laboratory—of targeting ER-positive tumors for treatment, while coupling the treatment with long-term adjuvant treatment regimens—produced a substantial increase in patient survivorship. It has been estimated that 500 000 women are alive today because of long-term adjuvant therapy being appropriately administered to patients with ER-positive tumors (13).

However, the transition of tamoxifen from a treatment to chemopreventive was already occurring in the 1970s and 1980s during the era of breast cancer treatment. The discovery that tamoxifen could prevent the initiation and promotion of rat mammary carcinogenesis (15–18), coupled with the clinical finding that tamoxifen treatment reduced the anticipated increase of contralateral breast cancer (19), acted as a catalyst for consideration of tamoxifen as a potential chemopreventive in women at elevated risk (20–22). The major obstacles to progress through the 1980s were in deciding whom to treat and when to deploy a chemopreventive agent, along with safety concerns. Up until the early 1980s, tamoxifen was classified as a nonsteroidal antiestrogen (23), which created a dilemma: if estrogen was essential to maintain bone density and could possibly protect women from coronary heart disease, how could women at risk for breast cancer be treated to reduce that risk if the result would be increases in osteoporotic fractures and deaths from coronary heart disease? The recognition of selective ER modulation during the 1980s ultimately propelled selective estrogen receptor modulators (SERMs) to center stage in efforts to improve women's health and to the testing of two of them in the STAR trial.

Selective Estrogen Receptor Modulation

The recognition of SERM activity in the target tissues of laboratory animals resulted in several successes in women's health. Paradoxically, while tamoxifen was being recognized as an "antiestrogen" that blocked breast or mammary cancer growth (15,16,24,25), it also was found to maintain bone density in ovariectomized rats (26–28). The concept of target tissue–specific effects of tamoxifen was extended further with the discovery that in the same athymic mouse tamoxifen prevented estrogen-stimulated human breast tumor growth while increasing uterine weight and stimulating the growth of endometrial carcinoma (29,30). This concept translated immediately to improvements in health care (31,32) with the observation that tamoxifen increased the risk of endometrial cancer but decreased the incidence of contralateral breast cancer in postmenopausal patients (33). This observation was subsequently confirmed in other randomized clinical trials using tamoxifen as a treatment (34), with the results that patient care was improved and gynecologists were involved in the health care of breast cancer patients. Most important, the new knowledge about the SERM action of tamoxifen allowed measures to be put in place to detect increases in early-stage, low-grade endometrial cancer in the NSABP P-1 chemoprevention trial (10,11).

Data showing that bone density was maintained in rats treated with tamoxifen (26) were used to support the evaluation of the actions of tamoxifen on postmenopausal bone density in the Wisconsin Tamoxifen Study. A secondary endpoint in this trial was the levels of circulating lipids because it had been established that tamoxifen reduces circulating cholesterol levels in the ovariectomized rat (35). In fact, the drug already possessed a patent in the United Kingdom for use as a hypocholesterolemic agent (13). The Wisconsin Tamoxifen Study demonstrated that tamoxifen maintained bone density in postmenopausal women (36) and reduced the circulating levels of low-density lipoprotein (LDL) cholesterol but did not reduce the levels of beneficial high-density lipoprotein cholesterol (37,38). Thus, there were grounds to conclude that bone density would be maintained in postmenopausal women treated with tamoxifen and to suggest that the drug might reduce the risk of coronary heart disease (39).

These predictions were addressed in the results of the NSABP P-1 study (10,11): tamoxifen reduced the risk of breast cancer by 50%, elevated the risk of endometrial cancer in postmenopausal women fivefold, and reduced (though not to a statistically significant extent) the incidence of fractures. However, it did not reduce the incidence of coronary heart disease. In the P-1 trial, tamoxifen also reduced the incidence of breast cancers in women with hyperplasia by 80% and reduced ductal carcinoma in situ by 50%. Tamoxifen also increased the risk of deep-vein thrombosis and pulmonary emboli, and these results were confirmed subsequently in the first International Breast Intervention Study, in which Cuzick et al. (40,41) found an increase in deep-vein thrombosis with tamoxifen and an increase in the death rate caused by pulmonary emboli as a result of elective surgical procedures. The results of the NSABP P-1 trial are summarized in Fig. 1, which shows the incidence of recorded endpoints in the control and treatment arms at 48 and 74 months of follow-up. Tamoxifen was approved by the US Food and Drug Administration (FDA) for reduction of the risk of breast cancer in high-risk women in 1998.

Raloxifene as a Multifunctional Medicine

The development of raloxifene to its current status as a treatment for osteoporosis and a preventive treatment for breast cancer is summarized in Fig. 2. The findings that the failed breast cancer drug keoxifene (LY156758) (42,43) could preserve bone density in ovariectomized rats (26), could prevent rat mammary carcinogenesis (44), and was less effective than tamoxifen in stimulating the growth of human endometrial carcinomas implanted into athymic mice (45) demonstrated that the target tissue–selective actions of tamoxifen (now called selective ER modulation) were common to other drugs in the group previously referred to as nonsteroidal antiestrogens. These laboratory findings resulted in the publication of a strategy by which nonsteroidal antiestrogens related to tamoxifen such as keoxifene could be used to simultaneously prevent osteoporosis in postmenopausal women and reduce the incidence of breast cancer in the general population (46,47). It was as a

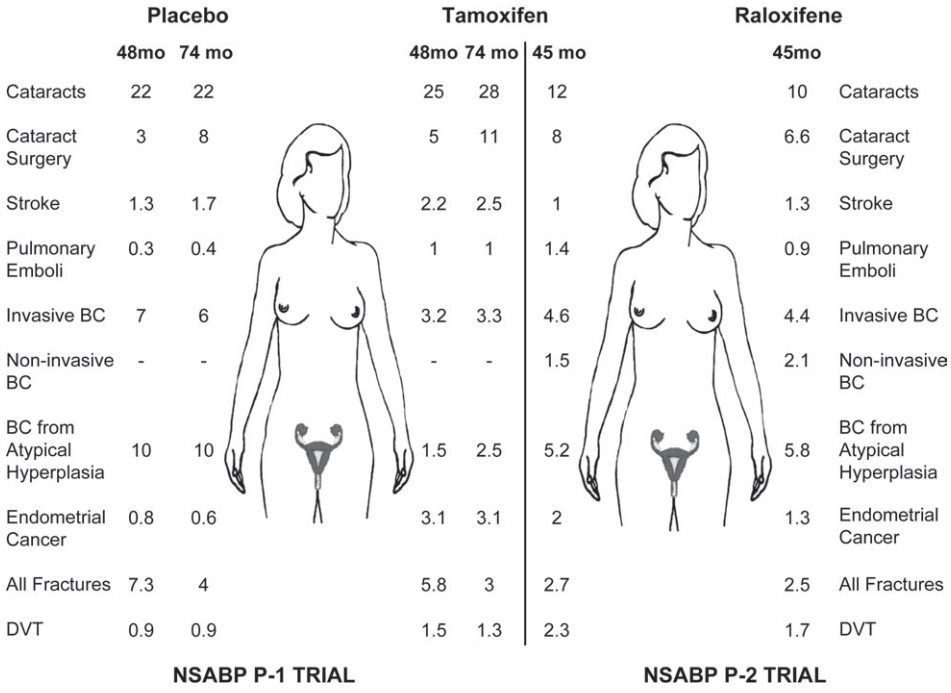

Fig. 1. Comparison of the incidence per 1000 women per year for the recorded endpoints in the National Surgical Adjuvant Breast and Bowel Project (NSABP) P-1 placebo-controlled trial of tamoxifen evaluated at 48 months (10) or 74 months (11) and the Study of Tamoxifen and Raloxifene (NSABP P-2) trial evaluated at 45 months (8). The incidence rates for the NSABP P-1 trial are for postmenopausal (age > 50 years) patients only. In the NSABP P-1 trial, the results for noninvasive breast cancer were reported in as the pooled results from pre- and postmenopausal women and grouped as a cumulated risk. As a result, these data are omitted for NSABP P-1. BC = breast cancer; DVT = deep-vein thrombosis.

result of this strategy, supported by the data from animal models (26,44,45), that keoxifene (LY156758) was reinvented as raloxifene (LY139481 HCL). When the previous laboratory findings that raloxifene maintained bone density in the rat, lowered circulating cholesterol, and possessed low activity as an estrogen in the rodent uterus were confirmed (48), clinical studies were initiated to evaluate raloxifene as a novel agent to preserve bone density in osteoporotic women.

Clinical trials demonstrated that raloxifene treatment maintained bone density in women at risk for osteoporosis (49) and reduced LDL cholesterol (50) to the same degree as tamoxifen (36,37). The success of the SERM concept was underscored by the observation that raloxifene reduced spinal fractures in women at high risk for such fractures (51) and reduced the risk of breast cancer by 75% without measurable increases in endometrial cancer (52,53). Raloxifene was approved by the FDA for the treatment and prevention of osteoporosis in 1998.

The confirmation that SERMs are multifunctional medicines suggests two opportunities for chemoprevention of breast cancer. One is based on an indirect approach, i.e., introducing a novel modality to prevent osteoporosis and reduce the risk of breast cancer as a beneficial side effect (46,47). The other is a direct approach, namely reducing the risk of breast cancer in women at elevated risk by treating them with raloxifene, the feasibility of which was determined by the STAR trial. Both approaches can now be evaluated.

The Indirect Approach to Breast Cancer Risk Reduction

It is estimated that half a million women are currently taking raloxifene for the treatment and prevention of osteoporosis (8). To preserve and build bone density, the medicine must be taken continuously, and in practice, the treatment regimen could last for 10 years or more. The pivotal antiosteoporosis trial—the Multiple Outcomes of Raloxifene Evaluation (MORE)—was extended for an additional 4 years as a safety vanguard study; this study evolved

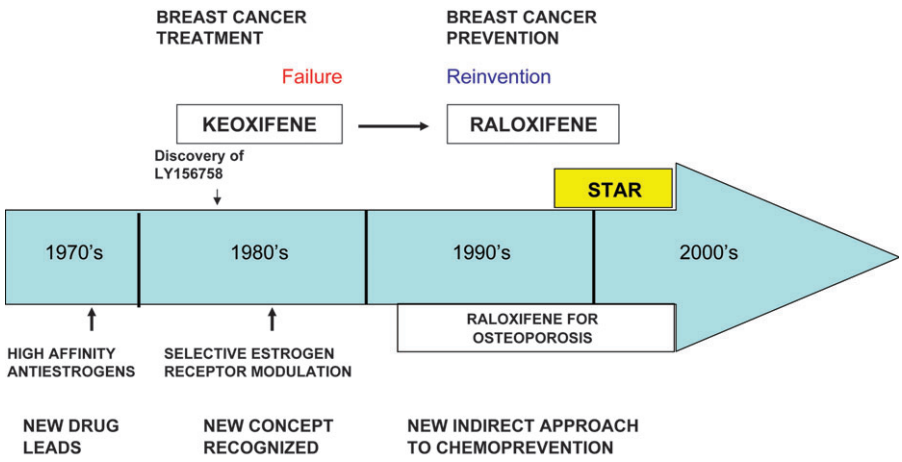

Fig. 2. The evolution of raloxifene (LY156758 or LY139481 HCL). In the late 1970s, tamoxifen was found to be metabolically activated to a hydroxylated metabolite with a high binding affinity for the estrogen receptor. This discovery (69) created a new lead in drug discovery that resulted in the description of LY156758 in 1983 (43). Concerns about using nonsteroidal antiestrogens as long-term treatments and preventives for breast cancer caused a closer examination of the pharmacology of nonsteroidal antiestrogens. Laboratory studies, conducted during 1986-90 (70,71), described the target site estrogenic and antiestrogenic effects of the compounds now referred to as selective estrogen receptor modulators (SERMs). Raloxifene was used unsuccessfully to treat breast cancer (late 1980s) and then reinvented in the early 1990s as a SERM to treat osteoporosis that produced a lower incidence of breast cancer (52). These data were the basis for the Study of Tamoxifen and Raloxifene (STAR) trial in which raloxifene was used as a chemopreventive for breast cancer (8).

into the Continuing Outcomes Relevant to Evista (CORE) study. The results from this long-term trial of raloxifene now provide an invaluable database to estimate reductions in age-related incidence of breast cancer. The breast cancer incidence rates among postmenopausal women at risk for osteoporosis have been estimated to be 1.4 and 4.2 cancers per 1000 women per year for women taking 60 mg raloxifene daily or placebo, respectively (54).

These data from the CORE trial (54) permit a rough calculation of the impact of raloxifene on public health. With hormone replacement therapy currently considered as a final option for the treatment and prevention of osteoporosis, the "at-risk" population is usually treated initially with a variety of formulations of bisphosphonates that have no impact on breast cancer incidence. Thus, based on the results of the CORE trial (54), if 500 000 postmenopausal women took bisphosphonates for 10 years to prevent osteoporosis, there would be an accumulation of 21 000 (500 000 women × 10 years × 4.2 breast cancers per 1000 women per year) breast cancers requiring surgery and adjuvant therapy with radiation, chemotherapy, and/or antihormone therapy. If these same women received raloxifene to prevent osteoporosis, there would be, based on current estimates, 7000 (500 000 women × 10 years × 1.4 breast cancers per 1000 women per year) breast cancers. Thus, there would be 14 000 fewer breast cancers and 14 000 fewer women who require surgery and adjuvant therapy, not to mention a 40% decrease in the number of fractures experienced by the 500 000 high-risk women (51). This advance in public health must be viewed as a clear success for the SERM concept.

Conclusions of the Study of Tamoxifen and Raloxifene

Building on this advance, the recent results of the STAR trial now promise to offer additional opportunities for successful use of SERMs to treat women in the general population, specifically women who are at elevated risk for breast cancer but are not osteoporotic. The STAR trial is part of an ongoing exploration of the potential of medicines to reduce breast cancer incidence in populations of women at high risk, and therefore, its results cannot be considered in isolation but must be assessed based on the prior experience with the NSABP P-1 trial. However, the NSABP P-1 and the STAR trials have important differences in their populations and designs. For example, the STAR trial participants are at a higher risk for breast cancer than the women who participated in the NSABP P-1 prevention trial (8,10). Both the STAR and NSABP P-1 trials recruited women ascertained to be at elevated risk using the Gail model (55), but the STAR trial only recruited postmenopausal women. (Raloxifene has not been evaluated appropriately in premenopausal women and should not be used to treat premenopausal women at risk for breast cancer.) Thus, only relative trends can be identified in lieu of exact comparisons.

The broad conclusions of the STAR (NSABP P-2) trial are summarized and compared with the two evaluations of the NSABP P-1 trial (10,11) in Fig. 1. The incidence rates of the various endpoints in the NSABP P-1 trial are only the results for women more than 50 years of age, and, therefore, valid comparisons can be made with incidence rates in the NSABP P-2 trial. However, the data pertaining to noninvasive breast cancer in the P-1 trial were not broken down into women above or below 50 years and were represented only as cumulative rates and not as an annual rate (10,11). These data are therefore omitted from Fig. 1. The most promising results of STAR trial are, first, that raloxifene is equivalent to tamoxifen at reducing the incidence of invasive breast cancer and, second, that it is associated with a lower incidence of endometrial cancer, endometrial hyperplasia, hysterectomies, cataracts and cataract surgery, and total thromboembolic events (pulmonary emboli or deep venous thromboses) than tamoxifen. The controversial aspect of the trial appears to be the failure of raloxifene to control completely the development of noninvasive breast cancer after 2 years of treatment (8).

Examination of the breast cancer endpoints of invasive and noninvasive diseases and comparision of STAR trial data with CORE/MORE trial data shows that raloxifene actually caused a reduction in invasive breast cancer by between 65% and 75% in osteoporotic women and not the estimated 50% decrease observed in STAR trial (8,52,54,). STAR trial results (8) cannot be compared with previous studies of noninvasive breast cancer (10) because there are no reported data on the effectiveness of treatment in postmenopausal women alone in these studies and numbers are too small in CORE/MORE trial (52,54) for valid comparisons. Possible explanations for the good but suboptimal performance of raloxifene in preventing breast cancer in STAR trial are the differing pharmacologies of raloxifene and tamoxifen and the different populations of CORE/MORE and STAR trials.

The pharmacologic properties of tamoxifen and the group of benzothiophene nonsteroidal antiestrogens that include raloxifene are very different. Tamoxifen exhibits more estrogen-like properties in the rodent uterus than do benzothiophene-related compounds (43,56), and the biologic properties of the tamoxifen–ER complex are more similar to the estrogen–ER complex than the complex formed by raloxifene binding (57,58). Studies in vivo demonstrate that raloxifene-like compounds have an extremely short duration of action compared with that of tamoxifen (44,59,60). This is because the polyphenolic benzothiophene derivatives have poor bioavailability (2%) and undergo rapid phase II metabolism in the intestines and liver (61,62). In contrast, 40% of tamoxifen absorption is from the gastrointestinal tract, and the drug has a long biologic half-life so that its levels persist for up to 6 weeks after therapy stops. Tamoxifen is also metabolized to active phenolic derivatives with high affinity for the estrogen–ER (23). Thus, compliance may be critical to maintain the optimal antiestrogenic actions of raloxifene and tamoxifen, but the effectiveness of tamoxifen, the suboptimal SERM with more estrogenic properties compared to raloxifene in target tissues, will be less dependent on optimal compliance. Failure to maintain adequate raloxifene levels in noncompliant STAR trial patients would allow for promotion of breast tumor growth by endogenous estrogen.

Raloxifene Use for the Heart

The demonstrated effectiveness of SERM treatments in lowering circulating cholesterol levels (37,38,50) and the presumed ability of hormone replacement therapy to lower the risk of coronary heart disease prompted the initiation of a prospective clinical trial to evaluate the ability of raloxifene to reduce the risk of coronary heart disease. The Raloxifene Use for the Heart (RUTH) trial (63) randomly assigned 10 101 postmenopausal women

with coronary heart disease or multiple risk factors for coronary heart disease to either 60 mg raloxifene (5044 women) or placebo (5057 women). The two primary outcomes, a coronary event (death, myocardial infarction, or hospitalization for an acute coronary syndrome) and invasive breast cancer, were evaluated after a median follow-up of 5.6 years. There was no evidence that raloxifene had a statistically significant effect on the risk of coronary heart disease (63) despite the previous tantalizing indications that both raloxifene (64) and tamoxifen (39) might have some benefit. The result, however, is consistent with conclusion of the Oxford Overview Analysis that tamoxifen does not improve survival from causes of death other than breast cancer (4). In contrast to what was observed in the MORE trial (52), death from stroke in the RUTH trial was elevated to a statistically significant extent in women taking raloxifene, with 59 deaths from stroke in those taking raloxifene compared to 39 deaths from stroke in controls (64).

Despite this apparent setback, the RUTH trial has provided additional important information about cancer incidence in a placebo-controlled trial of raloxifene. Endometrial cancer was not elevated in women treated with raloxifene. There were 21 endometrial cancers in 3900 nonhysterectomized women receiving raloxifene and 17 endometrial cancers in 3882 placebo-treated controls. These data clarify the results of the STAR trial where the numbers of endometrial cancers in women treated with tamoxifen increased but not to a statistically significant extent compared with the numbers among women treated with raloxifene. It is possible that the higher hysterectomy rate in women treated with tamoxifen resulted in a lower endometrial cancer rate.

The second planned outcome of the RUTH trial was the incidence of invasive breast cancer. The rate for the placebo-treated women was 2.7 per 1000 women per year, whereas the rate for raloxifene-treated women was 1.5 per 1000 per year. This 44% decrease in invasive breast cancer is consistent with the STAR trial but, again, not as impressive as that observed in the CORE/MORE trial (52,54).

Progress in Prevention

The success for the two SERMs, tamoxifen and raloxifene, has depended on good ideas based on effective translational research, changes in the fashions of research for the past 40 years, and the development of a patenting strategy that permits a company to test an idea during the period of exclusivity. The fashions in research changed from a focus on contraception in the 1950s and 1960s to breast cancer treatment in a period that extended from the 1970s through 1990s and finally to the current focus on chemoprevention of breast cancer and the prevention of osteoporosis. Preexisting ideas about the potential of breast cancer chemoprevention (20–22) and use of SERMs to prevent osteoporosis and breast cancer (46,47) flourished as opportunities for the broad applications of SERMs were advanced, but these advances only occurred because of delays in patenting that permitted commitment by the pharmaceutical industry. Tamoxifen was not patented for breast cancer treatment in the United States until 1985, despite the fact that FDA approval was obtained in December 1977 (13). Similarly, raloxifene was patented as a potential cancer treatment in the early 1980s, but the patent for osteoporosis did not occur until 1992 (65). It is unlikely that any progress in women's health and chemoprevention would have occurred without patent protection. But what of future progress? The academic community cannot advance women's health without optimal medicines to test. Despite the advances noted with tamoxifen and raloxifene, these were not optimal agents designed to perform the tasks they were called upon to perform. The truth is that there was nothing else available from the pharmaceutical industry.

For the moment, raloxifene is proving to be an important advance in chemoprevention because it is a multifunctional medicine that can target women at low risk for breast cancer with osteopenia and healthy women with a high risk of breast cancer. Nevertheless, new SERMs are necessary for clinical testing in postmenopausal women. The SERM concept (46) clearly works, but a long-acting SERM is required to replace raloxifene, a drug that does not appear to perform optimally in a high-estrogen environment. The long-acting drug arzoxifene is superior to raloxifene in laboratory studies for chemoprevention (66), but its development has been stalled because raloxifene has proved to be financially beneficial to treat and prevent osteoporosis.

And what of tamoxifen, the first SERM? Twenty years ago, tamoxifen was noted to increase the risk of endometrial cancer in postmenopausal women (33) but not in premenopausal women. Additionally, there are reasonable concerns about deep venous thromboses and pulmonary emboli, although these concerns do not extend to the premenopausal women who are at elevated risk for breast cancer (10). The future use of tamoxifen for chemoprevention may well be restricted to high-risk women who will develop breast cancer during their premenopausal years. The risk–benefit ratio for tamoxifen is favorable (67) in premenopausal women. However, perhaps more importantly, the antitumor actions of 5 years of adjuvant tamoxifen persist and increase for at least 10 years after treatment stops (4). The posttreatment protective effect of tamoxifen is noted in animal models (fewer tumors developed) (17), adjuvant clinical studies [decreased mortality (4) and decreased contralateral breast cancer] (3), and continuing decreases in primary breast cancer in the NSABP P-1 trial (11). A prevention strategy using tamoxifen in high-risk premenopausal women will continue to prevent tumor development after tamoxifen treatment is stopped and when side effects and quality-of-life issues disappear. However, it must be stressed that raloxifene and aromatase inhibitors cannot be used to block estrogen synthesis to reduce breast cancer risk in this patient population. Raloxifene has not been tested in premenopausal women, and the manufacturer recommends against this indication. Aromatase inhibitors are only effective in blocking the constitutive synthesis of estrogen in postmenopausal women. Ovarian estrogen synthesis in premenopausal women is regulated by a pituitary-controlled feedback system, so the blockade by an aromatase inhibitor is reversed by enhanced gonadtropin secretion. Therefore, tamoxifen remains the only proven intervention in premenopausal women.

Tamoxifen and raloxifene both specifically reduce the incidence of ER-positive breast cancer. However, as the testing of chemopreventive agents targeted to the ER progresses and evolves, cost-effectiveness issues are being addressed. A recent study by Melnikow et al. (68) illustrates the dilemma for health care management posed by the price of treatment. The authors concluded that tamoxifen-pricing differences between different health care

systems in the United States and Canada are important and that tamoxifen's use as a chemopreventive becomes cost-effective only for women at the highest risk in places where the cost of the drug is extremely low. The issue of cost-effectiveness is now even more timely as the cost of switching from tamoxifen to the more expensive aromatase inhibitors for the treatment of breast cancer has become a major issue for National Health Services in Europe. The next round of chemoprevention trials will compare SERMs with the aromatase inhibitors. The issue of osteoporosis induced by aromatase inhibitors remains a health care concern because the cost of treating large populations of women with expensive agents, monitoring them with dual energy x-ray absorptiometry, and providing them with supplementation with bisphosphonates and Vitamin D only to benefit the few may ultimately be an unreasonable public health care burden. In contrast, the proven promise of raloxifene, a safer SERM targeted specifically to women for the treatment and prevention of osteoporosis but one that also reduces the incidence of breast cancer, is a major first step in developing multifunctional medicines to improve health care.

References

(1) Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. Clin Cancer Res 2003;9:1980–9.

(2) Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 1987;235:177–82.

(3) Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 1998;354:1451–67.

(4) Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687–717.

(5) Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CEJ, Davidson NE, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med 2005;353:1673–84.

(6) Joensuu H, Kellokumpu-Lehtinen PL, Bono P, Alanko T, Kataja V, Asola R, et al. FinHer Study Investigators. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. N Engl J Med 2006;354:809–20.

(7) Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Herceptin Adjuvant Trial Study Team. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 2005;353:1659–72.

(8) Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. The Study of Tamoxifen and Raloxifene (STAR): report of the National Surgical Adjuvant Breast and Bowel Project P-2 trial. JAMA 2006;295:2727–41.

(9) Rockhill B, Spiegelman D, Byrne C, Hunter DJ, Colditz GA. Validation of the Gail et al. model of breast cancer risk prediction and implications for chemoprevention. J Natl Cancer Inst 2001;93:358–66.

(10) Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998;90:1371–88.

(11) Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 2005;97:1652–62.

(12) Milestone 5. (1915) Hormones and cancer. The enemy within. Comment uploaded: 1 April 2006. Available at: http://www.nature.com/milestones/milecancer/full/milecancer05.html. [Last accessed: February 1, 2007.]

(13) Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2003;2:205–13.

(14) Jordan VC. Tamoxifen (ICI 46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 2006;147:S269–76.

(15) Jordan VC. Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 1974;5:354.

(16) Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. Eur J Cancer 1976;12:419–24.

(17) Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 1980;16:239–51.

(18) Jordan VC, Allen KE, Dix CJ. Pharmacology of tamoxifen in laboratory animals. Cancer Treat Rep 1980;64:745–59.

(19) Cuzick J, Baum M. Tamoxifen and contralateral breast cancer [letter]. Lancet 1985;2:282.

(20) Carbone PP. Breast cancer 1984: role of tamoxifen. In: Jordan VC, editors. Estrogen/antiestrogen action and breast cancer therapy. Madison (WI): The University of Wisconsin Press; 1986. p. 483–97.

(21) Cuzick J, Wang DY, Bulbrook RD. The prevention of breast cancer. Lancet 1986;1:83–6.

(22) Powles TJ, Hardy JR, Ashley SE, Farrington GM, Cosgrove D, Davey JB, et al. A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. Br J Cancer 1989;60:126–31.

(23) Jordan VC. Biochemical pharmacology of antiestrogen action. Pharmacol Rev 1984;36:245–76.

(24) Lippman ME, Bolan G. Oestrogen-responsive human breast cancer in long term tissue culture. Nature 1975;256:592–3.

(25) Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. Br J Cancer 1971;25:270–5.

(26) Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987;10:31–5.

(27) Turner RT, Wakley GK, Hannon KS, Bell NH. Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. J Bone Miner Res 1987;2:449–56.

(28) Turner RT, Wakley GK, Hannon KS, Bell NH. Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. Endocrinology 1988;122:1146–50.

(29) Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc 1987;46:1870–4.

(30) Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 1988;48:812–5.

(31) Hardell L. Tamoxifen as risk factor for carcinoma of corpus uterus. Lancet 1988;2:563.

(32) Jordan VC. Tamoxifen and endometrial cancer [letter]. Lancet 1988;2:1019.

(33) Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1989;1:117–20.

(34) Fisher B, Costantino JP, Redmond CK, Fisher ER, Wickerham DL, Cronin WM. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. J Natl Cancer Inst 1994;86:527–37.

(35) Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 1967;13:101–19.

(36) Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, et al. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 1992;326:852–6.

(37) Love RR, Newcomb PA, Wiebe DA, Surawicz TS, Jordan VC, Carbone PP, et al. Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 1990;82:1327–32.

(38) Love RR, Wiebe DA, Newcomb PA, Cameron L, Leventhal H, Jordan VC, et al. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann Intern Med 1991;115:860–4.

(39) McDonald CC, Stewart HJ. Fatal myocardial infarction in the Scottish adjuvant tamoxifen trial. The Scottish Breast Cancer Committee. BMJ 1991;303:435–7.

(40) Cuzick J, Forbes J, Edwards R, Baum M, Cawthorn S, Coates A, et al. First results from the International Breast Cancer Intervention Study (IBIS-I): a randomised prevention trial. Lancet 2002;360:817–24.
(41) Cuzick J, Powles T, Veronesi U, Forbes J, Edwards R, Ashley S, et al. Overview of the main outcomes in breast-cancer prevention trials. Lancet 2003;361:296–300.
(42) Buzdar A, Marcus C, Holmes F, Hug V, Hortobagyi G. Phase II evaluation of Ly156758 in metastatic breast cancer. Oncology 1988;45: 344–5.
(43) Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 1983;32:1031–6.
(44) Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987;47:4020–4.
(45) Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 1990;50:3189–92.
(46) Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 1988;6:589–95.
(47) Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: eighth Cain memorial award lecture. Cancer Res 1990;50:4177–89.
(48) Black LJ, Sato M, Rowley ER, Magee DE, Bekele A, Williams DC, et al. Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 1994;93:63–9.
(49) Delmas PD, Bjarnason NH, Mitlak BH, Ravoux AC, Shah AS, Huster WJ, et al. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N Engl J Med 1997;337:1641–7.
(50) Walsh BW, Kuller LH, Wild RA, Paul S, Farmer M, Lawrence JB, et al. Effects of raloxifene on serum lipids and coagulation factors in healthy postmenopausal women. JAMA 1998;279:1445–51.
(51) Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators [erratum in JAMA 1999;282:2124]. JAMA 1999; 282:637–45.
(52) Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA 1999;281:2189–97.
(53) Cauley JA, Norton L, Lippman ME, Eckert S, Krueger KA, Purdie DW, et al. Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial. Multiple Outcomes of Raloxifene Evaluation. Breast Cancer Res Treat 2001;65:125–34.
(54) Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, et al. CORE Investigators. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 2004;96:1751–61.
(55) Gail MH, Brinton LA, Byar DP, Corle DK, Green SB, Schairer C, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. J Natl Cancer Inst 1989; 81:1879–86.
(56) Black LJ, Goode RL. Uterine bioassay of tamoxifen, trioxifene and a new estrogen antagonist (LY117018) in rats and mice. Life Sci 1980;26: 1453–8.
(57) Levenson AS, Jordan VC. The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. Cancer Res 1998;58:1872–5.
(58) MacGregor-Shafer JI, Liu H, Bentrem D, Zapf J, Jordan VC. Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex by substituting glycine for aspartate at amino acid 351. Cancer Res 2000;60:5097–105.
(59) Jordan VC, Gosden B. Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 1983;113:463–8.
(60) Jordan VC. Laboratory studies to develop general principles for the adjuvant treatment of breast cancer with antiestrogens: problems and potential for future clinical applications. Breast Cancer Res Treat 1983; 3(Suppl):S73–86.
(61) Snyder KR, Sparano N, Malinowski JM. Raloxifene hydrochloride. Am J Health Syst Pharm 2000;57:1669–75;quiz 1676–8.
(62) Kemp DC, Fan PW, Stevens JC. Characterization of raloxifene glucuronidation in vitro: contribution of intestinal metabolism to presystemic clearance. Drug Metab Dispos 2002;30:694–700.
(63) Barrett-Connor E, Mosca L, Collins P, Geiger MJ, Grady D, Kornitzer M, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 2006;355:125–37.
(64) Barrett-Connor E, Grady D, Sashegyi A, Anderson PW, Cox DA, Hoszowski K, et al. Raloxifene and cardiovascular events in osteoporotic postmenopausal women. JAMA 2002;287:847–57.
(65) Lewis JS, Jordan VC. Case histories: raloxifene. In: Comprehensive medicinal chemistry II. Vol 8. 2006:83–102.
(66) Suh N, Lamph WW, Glasebrook AL, Grese TA, Palkowitz AD, Williams CR, et al. Prevention and treatment of experimental breast cancer with the combination of a new selective estrogen receptor modulator,arzoxifene, and a new rexinoid, LG 100268. Clin Cancer Res 2002; 8:3270–5.
(67) Gail MH, Costantino JP, Bryant J, Croyle R, Freedman L, Helzlsouer K, et al. Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. J Natl Cancer Inst 1999;91:1829–46.
(68) Melnikow J, Kuenneth C, Helms LJ, Barnato A, Kuppermann M, Birch S, et al. Chemoprevention: drug pricing and mortality: the case of tamoxifen. Cancer 2006;107:950–8.
(69) Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 1977;75:305–16.
(70) Jordan VC. Selective estrogen receptor modulation: a personal perspective. Cancer Res 2001;61:5683–7.
(71) Jordan VC, Gapstur S, Morrow M. Selective estrogen receptor modulation and reduction in risk of breast cancer, osteoporosis, and coronary heart disease. J Natl Cancer Inst 2001;93:1449–57.

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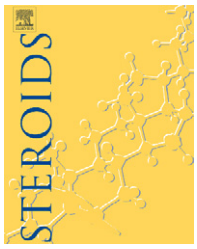

Review

New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer

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ABSTRACT

The metabolism of tamoxifen is being redefined in the light of several important pharmacological observations. Recent studies have identified 4-hydroxy *N*-desmethyltamoxifen (endoxifen) as an important metabolite of tamoxifen necessary for antitumor actions. The metabolite is formed through the enzymatic product of *CYP2D6* which also interacts with specific selective serotonin reuptake inhibitors (SSRIs) used to prevent the hot flashes observed in up to 45% of patients taking tamoxifen. Additionally, the finding that enzyme variants of *CYP2D6* do not promote the metabolism of tamoxifen to endoxifen means that significant numbers of women might not receive optimal benefit from tamoxifen treatment. Clearly these are particularly important issues not only for breast cancer treatment but also for selecting premenopausal women, at high risk for breast cancer, as candidates for chemoprevention using tamoxifen.

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1. Introduction

The aim of the body's biotransformation mechanisms is to prevent potentially toxic xenobiotic substances that include drugs, from damaging the body. That being the case, an orally active medicine must overcome numerous challenges to reach a target organ and produce the appropriate pharmacological effect at a receptor system. There is not one but several stages of biotransformation of a lipophilic drug such as tamoxifen that are designed to enhance the hydrophilic nature of the chemical so it can be rapidly eliminated. The stages of biotransformation are called phases I, II and III.

Phase I metabolism enhances the water solubility of a lipophilic chemical by hydroxylating an aromatic compound to become a phenol or hydrolyzing an esterified compound. These reactions are conducted by the family of cytochrome P_{450} enzymes referred to as CYP's. Phase II metabolism further increases the water solubility of the Phase I product by attaching highly water soluble entities. In the case of selective estrogen receptor modulators (SERMs) sugars (glucuronic acid) and salts (sulfates) are the most important conjugation products. In contrast, the phase III system is efflux pump molecules (also known as *p*-glycoproteins and multi-drug resistance transports protein) that exclude unmetabolized drugs from the epithelial cells of the intestinal tract immediately upon absorption.

In general terms, the ingested SERM must survive "first pass" metabolism from the intestine to the liver to have any chance of reaching target organs around the body. The general principles are illustrated in Fig. 1 where the SERM is biotransformed by CYPs in the intestinal wall and Phase II metabolism occurs via intestinal bacteria. A fraction of the administered dose is then absorbed into the hepatic portal vein and further biotransformed by phase I CYPs and/or glucuronidated or sulfated in phase II metabolism in the liver. By way of example, only 2% of the administered raloxifene survives and is bioavailable for systemic distribution [1].

2. Tamoxifen, the first SERM

The nonsteroidal antiestrogen tamoxifen (ICI 46,474 Nolvadex®) is a pioneering medicine [2] used to treat all stages of breast cancer in more than 120 countries throughout the world. The compound ICI 46,474 was discovered in the Fertility Control Program at Imperial Chemical Industries (ICI Pharmaceuticals Division, now AstraZeneca) in Alderley Park, Cheshire, England in the early 1960s [3–5]. The drug was found to be an extremely potent postcoital contraceptive in the rat [4,5]. Unfortunately, ICI 46,474 did not exhibit antifertility properties in women, in fact, quite the opposite, it induced ovulation [6,7]. As a result, the medicine was, at one time, marketed in the United Kingdom for the induction of ovulation in subfertile women with a functional hypothalamo-pituitary-ovarian axis.

There is a known link between estrogen and the initiation and growth of some breast cancers [8] so the nonsteroidal antiestrogen ICI 46,474 was tested as a potential treatment for advanced breast cancer in postmenopausal women. The antiestrogen produced response rates of 25–35% in unselected patients comparable to diethylstilbestrol and high dose androgen therapy, the standard endocrine therapies at the time [9,10]. However, fewer side effects were noted with tamoxifen [9,10]. As a result, the drug was approved as a palliative option for the hormonal treatment of breast cancer in the UK in 1973. There the story may have ended had not tamoxifen been reinvented as the first targeted therapy for breast cancer [2].

The seminal observations by Elwood Jensen that estrogen action is mediated by the estrogen receptor (ER) [11,12] in its target tissues (uterus, vagina, pituitary and breast tumors) opened the door to targeting tamoxifen to select patients with the ER in their metastatic tumor [13,14]. However, a strategic plan was developing to use tamoxifen in a broader range of patient populations. Laboratory studies conducted in the 1970s showed that tamoxifen blocked estrogen binding to the ER [15–17], should be used as a long-term adjuvant therapy to suppress tumor recurrence [18–20] and the drug also had potential as a chemopreventive agent [21,22].

Clinical studies subsequently confirmed that long-term adjuvant tamoxifen therapy, targeted to the patients with ER positive breast cancers, significantly decreased the death rate from the disease [23] and contributes to the current decline in death from breast cancer nationally [24]. Overall, the strategy of targeted long-term "antiestrogenic" [25] treatment for breast cancer has presaged the current fashion of targeting anticancer agents to other organ sites in the body.

Despite the fact that aromatase inhibitors show superiority over tamoxifen as adjuvant therapy in postmenopausal women [26–29], several issues have surfaced that have retained tamoxifen as a useful therapeutic agent worldwide. The medicine is extremely cheap compared to aromatase inhibitors so tamoxifen remains an essential anticancer agent in undeveloped countries or in countries with under-funded managed healthcare systems. Furthermore, tamoxifen is the only appropriate antiestrogenic therapy for premenopausal women whether they are being treated for breast cancer or whether chemoprevention is being considered [30]. For these reasons, new knowledge that can enhance the appropriate use of an established drug is of value to improve healthcare.

There are current initiatives to translate emerging knowledge on genetic variations in drug metabolism to target patient populations [31]. It is reasoned that by applying pharmacogenomic tests to specific patient populations, there will be fewer surprises with side effects, drug interactions, and a higher probability of increasing therapeutic effectiveness in the treatment or prevention of disease. The promise of practical progress is exemplified in this article using tamoxifen as the model drug.

Tamoxifen is a prodrug and can be metabolically activated to 4-hydroxytamoxifen [32–34] or alternatively can be metabolically routed via *N*-desmethyltamoxifen to 4-hydroxy-*N*-desmethyltamoxifen [35,36] (Fig. 2). The hydroxy metabolites of tamoxifen have a high binding affinity for the ER [32,37]. The finding that the enzyme produced by *CYP2D6* activates tamoxifen to hydroxylated metabolites 4-hydroxytamoxifen and endoxifen [38] has implications for cancer therapeutics. Women with enzyme variants that cannot make endoxifen may not have as successful an outcome

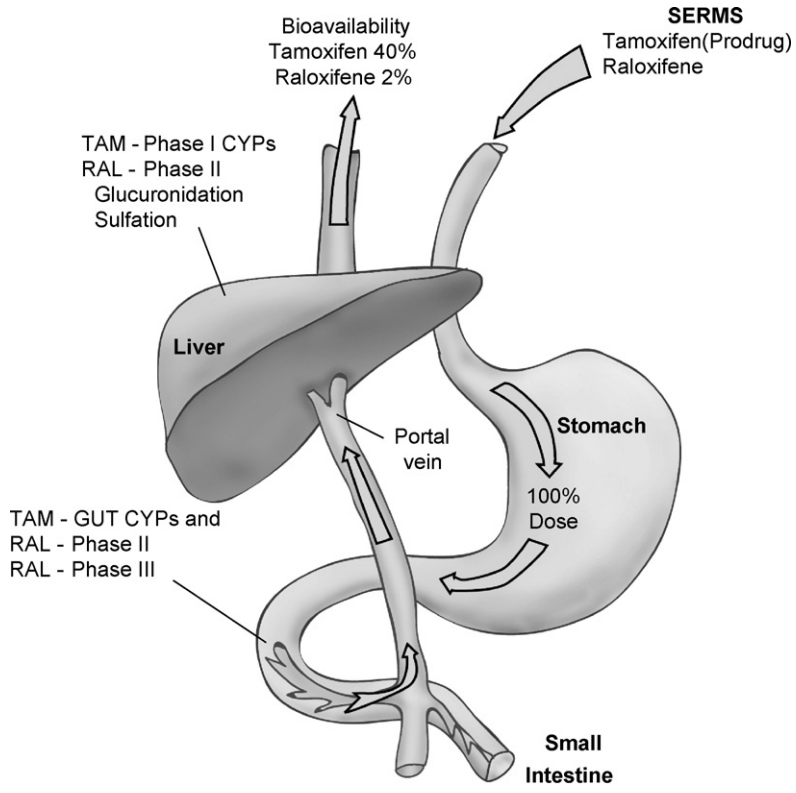

Fig. 1 – The stylized representation of the absorption of two selective estrogen receptor modulators (SERMS) tamoxifen (TAM) or raloxifene (RAL) into the circulation as bioactive molecules. The polyphenolic SERM raloxifene must transverse phase II and phase III obstacles in the gut and the liver to get into the general circulation. This results in very little of the ingested drug being bioavailable at target sites. In contrast, tamoxifen is extremely lipophilic and 98% protein bound to serum albumin. This extends the duration of action of tamoxifen because phase II metabolism to phenolic compounds is retarded.

with tamoxifen therapy. Alternatively, women who have a normal enzyme may make high levels of the potent antiestrogen endoxifen and experience hot flashes. As a result, these women may take selective serotonin reuptake inhibitors (SSRIs) to ameliorate hot flashes but there are potential pharmacological consequences to this strategy. Some of the SSRIs are metabolitically altered by the *CYP2D6* enzyme product [39]. It is therefore possible to envision a drug interaction whereby SSRIs block the metabolic activation of tamoxifen.

This article will describe the scientific twists and turns that tamoxifen and its metabolites have taken over the past 30 years. The story is naturally dependent on the fashions in therapeutic research at the time. What seems obvious to us as a successful research strategy today, with millions of women taking tamoxifen, was not so 30 years ago at the beginning when the clinical community and pharmaceutical industry did not see "antihormones" as a priority at all for drug development [25]. In 1972, tamoxifen was declared an orphan drug with no prospects [2].

3. Basic mechanisms of tamoxifen metabolism

The original survey of the putative metabolites of tamoxifen was conducted in the laboratories of ICI Pharmaceuticals Division and published in 1973 [40]. A number of hydroxylated metabolites were noted (Fig. 3) following the administration of ^{14}C labeled tamoxifen to various species (rat, mouse, monkey, and dog). The major route of excretion of radioactivity was in the feces. The rat and dog were used to show that up to 53% of the radioactivity derived from tamoxifen was excreted via the bile and up to 69% of this was reabsorbed via a enterohepatic recirculation until eventual elimination occurs [40]. The hydroxylated metabolites are excreted as glucuronides. However, no information about their biological activity was available until the finding that 4-hydroxytamoxifen had a binding affinity for the ER equivalent to 17β estradiol [32]. Similarly, 3,4-dihydroxytamoxifen (Fig. 3) bound to the human ER but interestingly enough, 3,4-dihydroxytamoxifen was not significantly estrogen-like in the rodent uterus despite being antiestrogenic [32].

Additional studies on the metabolism of tamoxifen in four women [41] identified 4-hydroxytamoxifen as the primary metabolite using a thin layer chromatographic technique to identify ^{14}C labeled metabolites. This assumption, coupled with the potent antiestrogenic actions of 4-hydroxytamoxifen [32] and the conclusion that it was an advantage, but not a requirement for tamoxifen to be metabolically activated [33,42] seemed to confirm the idea that 4-hydroxytamoxifen was the active metabolite that bound in rat estrogen target tissues to block estrogen action [34]. However, the origi-

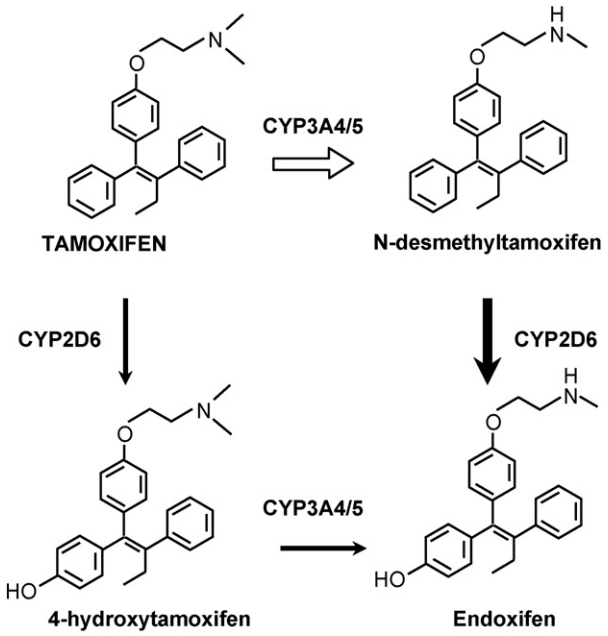

Fig. 2 – The metabolic activation of tamoxifen to phenolic metabolites that have a high binding activity for the human estrogen receptor. Both 4-hydroxytamoxifen and endoxifen are potent antiestrogens *in vitro*.

nal analytical methods used to identify 4-hydroxytamoxifen as the major metabolite in humans were flawed [43] and subsequent studies identified *N*-desmethyltamoxifen (Fig. 4) as the major metabolite circulating in human serum [44]. The metabolite was found to be further demethylated to *N*-desdimethyltamoxifen (metabolite Z) [45] and then deaminated to metabolite Y, a glycol derivative of tamoxifen [46,47]. The metabolites (Fig. 4) that are not hydroxylated at the 4 position of tamoxifen (equivalent to the three phenolic hydroxyl of estradiol) are all weak antiestrogens that would each contribute to the overall antitumor actions of tamoxifen at the ER based on their relative binding affinities for the ER and their actual concentrations locally.

At the end of the 1980s the identification of another metabolite tamoxifen 4-hydroxy *N*-desmethyltamoxifen in animals [48] and man [35,36] was anticipated but viewed as obvious and uninteresting. The one exception that was of interest was metabolite E (Fig. 3) identified in the dog [40]. This phenolic metabolite without the dimethylaminoethyl side chain is a full estrogen [47,49]. The dimethylaminoethoxy side chain of tamoxifen is necessary for antiestrogenic action [49].

It is not a simple task to study the actions of metabolites *in vivo*. Problems of pharmacokinetics, absorption and subsequent metabolism all conspire to confuse the interpretation of data. Studies *in vitro* using cell systems of estrogen target tissues were defined and refined in the early 1980s to create an understanding of the actual structure–function relationships of tamoxifen metabolites. Systems were developed to study the regulation of the prolactin gene in primary cultures of immature rat pituitary gland cells [42,50] or cell replication in ER positive breast cancer cells [51–54]. Overall, these models were used to describe the importance of a phenolic hydroxyl to tether a triphenylethylenes appropriately in the ligand-binding domain of the ER and to establish the appropriate positioning of an "antiestrogenic" side chain in the "antiestrogen region" of the ER [50] to modulate gene activation and growth [42,50,55–58]. These structure–function studies, that created hypothetical models of the ligand-ER/complex, were rapidly advanced with the first reports of the X-ray crystallography of the estrogen, 4-hydroxytamoxifen [59] or raloxifene ER [60] complexes. The ligand–receptor protein interaction

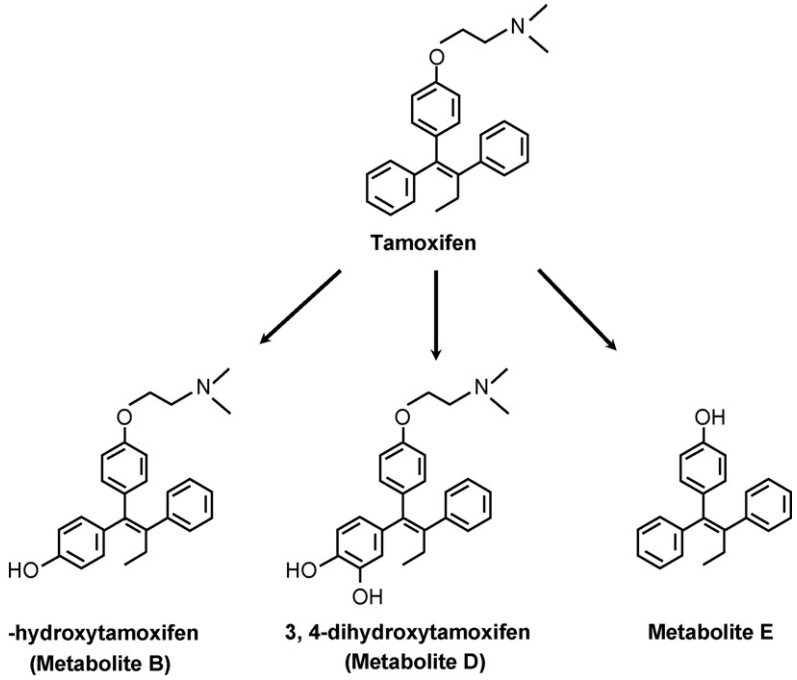

Fig. 3 – The original hydroxylated metabolites of tamoxifen noted in animals by Fromson et al. [40].

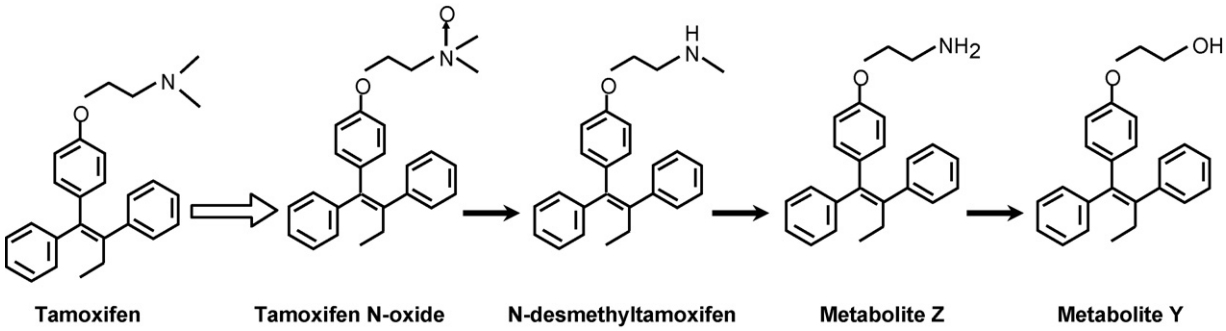

Fig. 4 – The serial metabolic demethylation and deamination of the antiestrogenic side chain of tamoxifen. Each of the metabolites is a weak antiestrogen with poor binding affinity for the estrogen receptor.

was subsequently interrogated by examining the interaction of the specific amino acid, asp 351 with the antiestrogenic side chain of the ligand [61]. A mutation was found as the dominant ER species in a tamoxifen-stimulated breast tumor grown in athymic mice [61,62]. The structure–function relationships studies, that modulated estrogen action at a transforming growth factor alpha gene target, demonstrated that the ligand shape would ultimately program the shape of the ER complex in a target tissue [30,63–65]. This concept is at the heart of metabolite pharmacology and is required to switch on and switch off target sites around the body. The other piece of the mechanism of SERMs puzzle that was eventually solved was the need for another player to partner with the ER complex. Coactivators [66] can enhance the estrogen-like effects of compounds at a target site [67]. However, in the early 1990s, the molecular and clinical use of this knowledge with the development and application of SERMs was in the future [68].

The urgent focus of translational research in the early 1990s was to discover why tamoxifen was a complete carcinogen in rat liver [69,70] and to determine whether there was a link between metabolism and the development of endometrial cancer noted in very small but significant numbers of postmenopausal women taking adjuvant tamoxifen [71,72].

All interest in the metabolism of tamoxifen focused on the production of DNA adducts [73] that were responsible for rat liver carcinogenesis and, at the time, believed to be potentially responsible for carcinogenesis in humans [74]. Although many candidates were described [75–78], the metabolite found to be responsible for the initiation of rat liver carcinogenesis is α-hydroxytamoxifen [79–83] (Fig. 5). α-Hydroxytamoxifen has been resolved into *R*-(+) and *S*-(−) enantiomers. Metabolism by rat liver microsomes gave equal amounts of the two forms, but in hepatocytes the *R* form gave 8× the level of DNA adducts as the *S* form. As both had the same chemical reactivity towards DNA, Osborne et al. [84] suggested that the *R* form was a better sulfotransferase substrate. This enzyme is believed to catalyze DNA adduct formation. Subsequently, Osborne et al. [85] conducted studies with alpha-hydroxy-*N*-desmethyltamoxifen; the *R*-(+) gave 10× the level of adducts in rat hepatocytes as the *S*-(−).

There were reasonable concerns that the hepatocarcinogenicity of tamoxifen in rats would eventually translate to humans but fortunately this is now known to be untrue [86]. The demonstration of carcinogenesis in the rat liver appears to be related to poor DNA repair mechanisms in the inbred strains of rats. In contrast, it appears that the absence of liver carcinogenesis in women exposed to tamoxifen [87] is believed to result from the sophisticated mechanisms of DNA repair inherent in humans cells.

It is clear from this background about the early development of tamoxifen and the fact that tamoxifen was considered to be such a safe drug in comparison to other cytotoxic agents

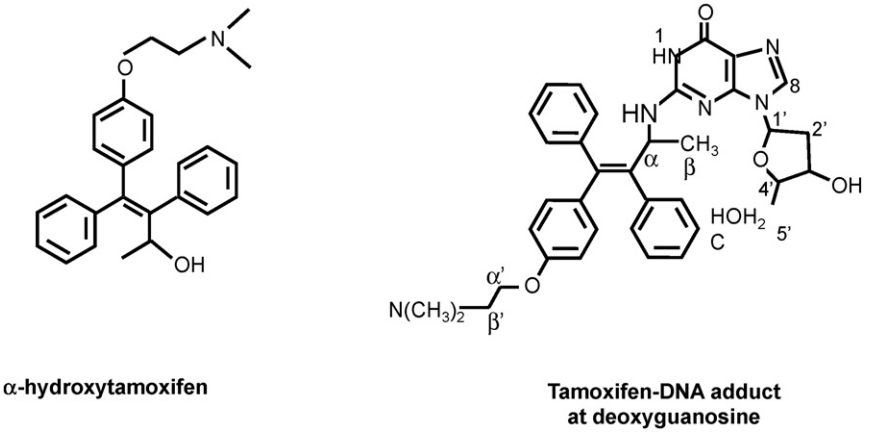

Fig. 5 – The putative metabolite of tamoxifen, α-hydroxytamoxifen that produces DNA adducts through covalent binding to deoxyguanosine.

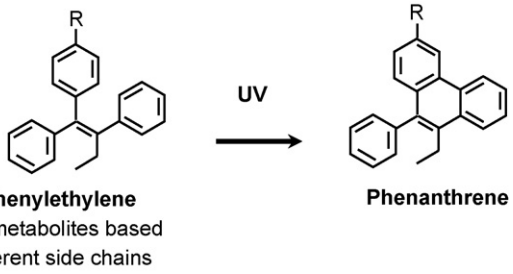

Fig. 6 – The UV activation of a triphenylethylenes to a florescent phenanthrene. This basic reaction is exploited in the detection of serum tamoxifen levels.

used in therapy during the 1970s and 1980s, that there was little enthusiasm for in-depth studies of tamoxifen metabolism. However, this perspective was to change in the 1990s with the widespread use of tamoxifen as the gold standard for the treatment and prevention of breast cancer. Questions needed to be addressed: (1) what happens to tamoxifen in patients? and (2) can improvements be made to the molecule?.

4. Clinical pharmacology

A number of analytical techniques are available to evaluate blood levels of tamoxifen and its metabolites once the drug is absorbed. The early method of thin layer chromatography, and the current method of high performance liquid chromatography (HPLC) both depend on the conversion of the triphenylethylenes to fluorescent phenanthrenes for their detection (Fig. 6). The original description of the reaction [88] was successfully adapted [89] to identify tamoxifen, *N*-desmethyltamoxifen and 4-hydroxytamoxifen in plasma samples.

Subsequent improvements were made [90] but the method significantly underestimated phenolic metabolites (4-hydroxytamoxifen) and had no internal standardization. In contrast, a method of post-column fluorescence activation [91] or preliminary purification from interfering substance using a Sep-Pack C18 cartridge (Waters Association, Milford, MA) [92] with internal standardization considerably improved accuracy. The detection of tamoxifen metabolites in serum was further improved by Lien et al. [93] and recently by Lee et al. [94] who adapted the methods [95,96] developed to perform "on line" extraction and post-column cyclization. Using this methodology the limits of detection for 4-hydroxy tamoxifen and endoxifen are 0.5 and 0.25 ng/ml, respectively [97]. Since there was such initial controversy about the identification of metabolites in patient serum, it is perhaps important to describe the validation of 4-hydroxydesmethyltamoxifen as a metabolite of tamoxifen in patients. Tamoxifen metabolites were investigated in a 57-year-old female patient receiving tamoxifen treatment [35]. Two major chromatographic peaks were identified in bile following treatment with β-glucuronidase. On major peak co-eluted with 4-hydroxytamoxifen but the second peak was proven to be 4-hydroxy-*N*-desmethyltamoxifen using (a) co-elution with an authentic standard on reversed-phase chromatography and formation of fluorescent derivative by cyclization; (b) the detection of a molecular ion $(M+1)+$ of 374 $m/2$ as determined by liquid chromatography–mass spectrometry; and (c) a fragmatogram identical to that of the authentic standard, obtained by mass spectrometry. Subsequent refinement of the technology improved detection for identification of 4-hydroxy-*N*-desmethyltamoxifen in human serum, tissues [36] and rat tissues [93].

Studies confirm that tamoxifen is 98% bound to serum albumin which ultimately creates a long biological half-life (plasma half-life 7 days) [93]. A single oral dose of 10 mg tamoxifen (half the daily dose) produces peak serum levels of 20–30 ng of tamoxifen/ml within 3–6 h but it must be stressed that patient variation is very large [98]. Nevertheless, continuous therapy with either 10 mg bid [98] or 20 mg bid [99] produces steady state levels within 4 weeks. Blood levels of tamoxifen can average around 150 ng/ml for 10 mg tamoxifen bid and 300 ng/ml for 20 mg tamoxifen bid. A strategy of using loading doses [98,100] to elevate blood levels rapidly has not produced any therapeutic benefit.

Overall, the results from the metabolic studies with tamoxifen during the 1970s and 1980s did not help clinicians to use tamoxifen more effectively. The structures of metabolites were in fact used as leads to create new molecules for clinical development.

5. Metabolic mimicry

The demonstration [32] that the class of compounds referred to as nonsteroidal antiestrogens were metabolically activated to compounds with high binding affinity for the ER created additional opportunities for the medicinal chemists within the pharmaceutical industry to develop new agents. This was particularly true once the nonsteroidal antiestrogens were recognized to be SERMs [101–103] and had applications not only for the treatment and prevention of breast cancer but also as potential agents to treat osteoporosis and coronary heart disease [104,105]. The reader is referred to other recent review articles to obtain further details of new medicines under investigation [104,105] but some current examples are worthy of note and will be mentioned briefly. Compounds of interest that have their structural origins as metabolites from nonsteroidal antiestrogens are summarized in Fig. 7. Raloxifene is an agent that originally was destined to be a drug to treat breast cancer but it failed in that application [106]. It appears that the pharmacokinetics and bioavailability of raloxifene are a challenge. Only about 2% of administered raloxifene is bioavailable [1] but despite this, the drug is known to have a long biological half-life of 27 h. The reason for this disparity is that raloxifene is a polyphenolic drug that can be glucuronidated and sulfated by bacteria in the gut so the drug cannot be absorbed [107,108]. This phase II metabolism in turn controls enterohepatic recirculation and ultimately impairs the drug from reaching and interacting with receptors in the target. This concern has been addressed with the development of the long-acting raloxifene derivative arzoxifene that is known to be superior to raloxifene as a chemopreventive in rat mammary carcinogenesis [109]. One of the phenolic groups (Fig. 7) is methylated to provide protection from phase II metabolism.

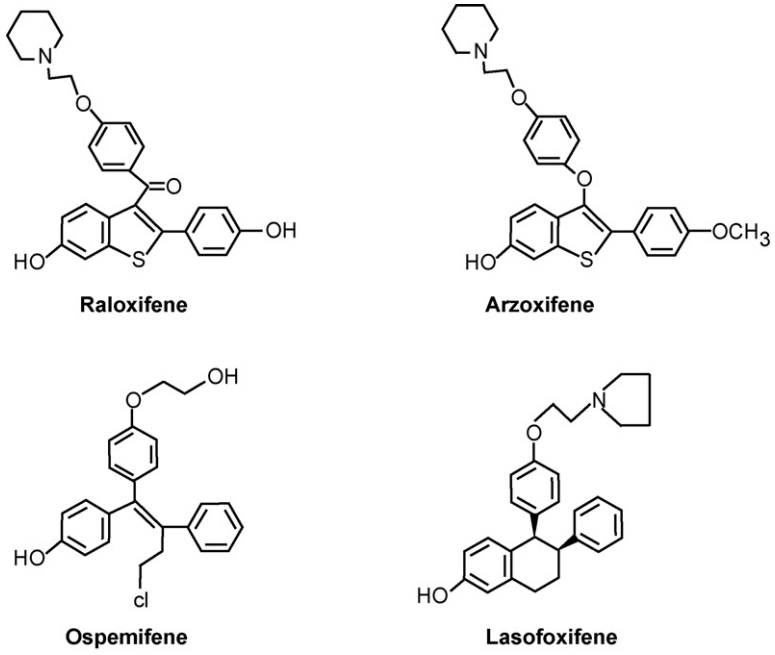

Fig. 7 – The formulae of SERMs that have been developed based on the knowledge of the metabolic activation of tamoxifen (and nafoxidine, see text) as well as the metabolism of the antiestrogen side chain of tamoxifen to a glycol.

Nevertheless, arzoxifene has not performed well as a treatment for breast cancer [110,111]; higher doses are less effective than lower doses. These data imply that effective absorption is impaired by phase III metabolism. That being said, the results of trials evaluating the effects of arzoxifene as a drug to treat osteoporosis, using lower doses, are eagerly awaited. Perhaps arzoxifene will be a better breast cancer preventive than a treatment.

Unfortunately, the bioavailability of phenolic drugs is also dependent on phase II metabolism to inactive conjugates in the target tissue. 4-Hydroxytamoxifen [32] is only sulfated by three of seven sulfotransferase isoforms whereas raloxifene is sulfated by all seven [112]. Maybe local phase II metabolism plays a role in neutralizing the antiestrogen action of raloxifene in the breast. Falany et al. [112] further report that SULT1E1, that sulfates raloxifene in the endometrium, is only expressed in the secretory phase. In contrast, 4-hydroxytamoxifen is sulfated at all stages of the uterine cycle.

Lasofoxifene is a diaryltetrahydronaphthalene derivative referred to as CP336156 [113] that has been reported to have high binding affinity for ER and have potent activity in preserving bone density in the rat [114,115]. The structure of CP336156 is reminiscent of the putative antiestrogenic metabolite of nafoxidine [116] that failed to become a breast cancer drug because of unacceptable side effects [117]. There are two diasteromeitric salts of the chemical shown in Fig. 7. CP336156 is the *l* enantiomer that has 20 times the binding affinity for the ER as the *d* enantiomer. Studies demonstrate that the *l* enantiomer had twice the bioavailability of the *d* enantiomer. The authors [113] ascribed the difference to enantioselective glucuronidation of the *d* isomer. An evaluation of CP336156 in the prevention and treatment of rat mammary tumors induced by *N*-nitroso-*N*-methylurea shows activity similar to that of tamoxifen [118].

Ospemifene or deaminohydroxytoremifene is related to metabolite Y formed by the deamination of tamoxifen [47]. Metabolite Y has a very low binding affinity for the ER [47,119] and has weak antiestrogenic properties compared with tamoxifen. Ospermifene is a known metabolite of toremifene (4 chlorotoremifene) but unlike tamoxifen, there is little carcinogenic potential in animals [120]. It is possible that the large chlorine atom on the 4 position of toremifene and ospermifene reduces α hydroxylation to the ultimate carcinogen related to α hydroxy tamoxifen (Fig. 6). Deaminohydroxytoremifene has very weak estrogenic and antiestrogenic properties *in vivo* [121] but demonstrates SERM activity in bone and lowers cholesterol. The compound is proposed to be used as a preventative for osteoporosis. Preliminary clinical data in healthy men and postmenopausal women demonstrate pharmacokinetics suitable for daily dosing between 25 and 200 mg [122]. Interestingly enough, unlike raloxifene, ospermifene has a strong estrogen-like action in the vagina but neither ospermifene nor raloxifene affect endometrial histology [123,124]. Overall, the goal of developing a bone specific agent is reasonable, but the key to commercial success will be the prospective demonstration of the prevention of breast and endometrial cancer as beneficial side effects. This remains a possibility based on prevention studies completed in the laboratory [125,126].

6. Tamoxifen metabolism today

A comprehensive evaluation of the sequential biotransformation of tamoxifen has been completed by Desta et al. [38]. They used human liver microsomes and experiments with specifically expressed human cytochrome P450's to identify the prominent enzymes involved in phase I metabolism. Their

results are summarized in Fig. 2 with the relevant *CYP* genes indicated for the metabolic transformations. The authors make a strong case that *N*-desmethyltamoxifen, the principal metabolite of tamoxifen that accumulates in the body, is converted to endoxifen by the enzymatic product of *CYP2D6*. The *CYP2D6* product is also important to produce the potent primary metabolite 4-hydroxytamoxifen but the metabolite can also be formed by the enzymatic products: *CYP2B6*, *CYP2C9*, *CY2C19* and *CYP3A4*.

The *CYP2D6* phenotype is defined as the metabolic ratio (MR) by dividing the concentration of an unchanged probe drug, known to be metabolized by the CYP2D6 gene product, by the concentration of the relevant metabolite at a specific time. These measurements have resulted in the division of the *CYP2D6* phenotype in four metabolic classes: poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM) and ultrarapid metabolizes (UM). Over 80 different single nucleotide polymorphisms have been identified but there are inconsistencies in the precise definitions of the ascribing a genotype to a phenotype [127,128]. Bradford [128] and Raimundo et al. [129] have described the frequency of common alleles for *CYP2D6*. Pertinent to the current discussion of tamoxifen metabolism, the *CYP2D6**4 allele [130] is estimated to have a frequency of 12–23% in Caucasians, 1.2–7% in black Africans and 0–2.8% in Asians [127,128]. A lower estimate of (<10%) of the PM phenotype is presented by Bernard et al. [131].

The molecular pharmacology of endoxifen has recently been reported [37,132,133]. Endoxifen and 4-hydroxytamoxifen were equally potent at inhibiting estrogen-stimulated growth of ER positive breast cancer cells MCF-7, T47D and BT474. Both metabolites are significantly superior *in vitro* to tamoxifen the parent drug. Additionally, the estrogen-responsive genes pS_2 and progesterone receptor were both blocked to an equivalent degree by endoxifen and 4-hydroxytamoxifen [132,133]. Lim et al. [133] have extended the comparison of endoxifen and 4-hydroxytamoxifen in MCF-7 cells by comparing and contrasting global gene regulation using the Affymetrix U133A Gene Chip Array. There were 4062 total genes that were either up or down regulated by estradiol whereas, in the presence of estradiol, 4-hydroxytamoxifen or endoxifen affected 2444 and 2390 genes, respectively. Overall, the authors [133] demonstrated good correlation between RTPCR and select genes from the microarray and concluded that the global effects of endoxifen and 4-hydroxytamoxifen were similar.

Stearns et al. [97] and Jin et al. [134] have confirmed and significantly extended Lien's original identification of endoxifen and observation [35,36] that there are usually higher circulating levels of endoxifen than 4-hydroxytamoxifen in patients receiving adjuvant tamoxifen therapy. However, Flockhart's group [97] have advanced the pharmacogenomics and drug interactins surrounding tamoxifen therapy that should be a consideration in the antihormonal treatment of breast cancer.

The ubiquitous use of tamoxifen for the treatment of node negative women [135] during the 1990s, the use of tamoxifen plus radiotherapy following lumpectomy for the treatment of ductal carcinoma in situ (DCIS) [136] as well as the option to use tamoxifen for chemoprevention in high risk pre- and postmenopausal women [137] enhanced awareness of the menopausal side effects experienced by women when taking tamoxifen. Up to 45% of women with hot flashes grade them as severe [137] therefore there have been efforts to improve quality of life. Treatments with the SSRIs are popular [97,138,139] (Fig. 8). The SSRIs are twice as effective as the "placebo" effect at reducing menopausal symptoms in randomized clinical trials [138–140], so there is naturally an increased usage of SSRIs with long-term tamoxifen treatment to maintain compliance. Unfortunately, the metabolism of tamoxifen to hydroxylated metabolites [141–143] and the metabolism of SSRIs [39,144–147] both occur via the *CYP2D6* gene product. Indeed Stearns et al. [97] showed that the SSRI inhibitor paroxetine reduced the levels of endoxifen during adjuvant tamoxifen

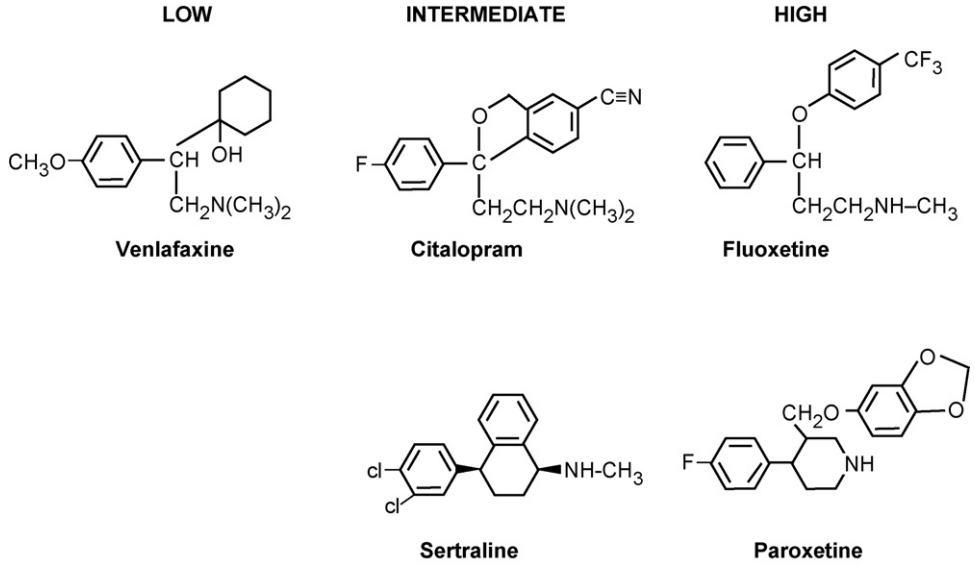

Fig. 8 – The structures of selective serotonin reuptake inhibitors (SSRIs) that have low intermediate or high affinity for the *CYP2D6* enzyme system. High affinity binders for *CYP2D6* block the metabolic activation of tamoxifen to endoxifen (Fig. 2).

therapy and endoxifen levels decrease by 64% in women with wild type *CYP2D6* enzyme. Patients were examined who were taking venlafaxine, sertraline, and paroxetine and compared with those women who were homozygotes for the *CYP2D6*4/*4* inactive genotype. Patients with the wild type gene who took the most potent inhibitor paroxetine had serum levels of endoxifen equivalent to the patients with the aberrant *CYP2D6* gene. In fact, the clinical data were consistent with the inhibition constants for the inhibition of *CYP2D6* by paroxetine (potent), fluoxetine, sertraline, citalopram (intermediate) and venlafaxine (weak) which are 0.05, 0.17, 1.5, 7 and 33 μmol/l, respectively.

The *CYP2D6* gene product that is fully functional (wild type) is classified as the *CYP2D6*1*. A large number of alleles are associated with no enzyme activity or reduced activity. Conversely, high metabolizers can have multiple copies of the *CYP2D6* allele [31]. A recent study by Borges et al. [148] continues to expand our understanding of the detrimental effect of *CYP2D6* variants plus concomitant administration of SSRIs on endoxifen levels. But, it is the clinical correlations with tumor responses and side effects that are starting to provide clues about the importance of pharmacogenomics for tamoxifen to be optimally effective as a breast cancer drug.

7. Clinical correlations

The significance of genotyping on clinical outcomes of a tamoxifen trial have been addressed using paraffin-embedded tumor blocks from a North Central Center Treatment Group (NCCTG) trial NCCTG 89-30-52 [149]. The postmenopausal women with ER positive tumors received 5 years of adjuvant tamoxifen therapy. The tumor blocks were used to determine *CY2D6* (*4 and *6) and *CYP3A5* (*3) and *17* buccal swabs were used to test the veracity of the tumor genotyping. The concordance rate for the buccal swabs was 100%. Overall, the *CYP3A5*3* variant was not associated with any adverse clinical outcomes but the women with the *CYP2D6*4/*4* genotype had a higher risk of disease relapse but a lower incidence of side effects such as hot flashes [149]. The implication is that tamoxifen must be converted to endoxifen, a more potent antiestrogen.

In a follow up study [150] using the same database established for trial NCCTG 89-30-52, patient records were screened to determine the extent of SSRI prescribing. The goal was to establish the combined effect of genotyping and SSRI inhibition of the *CYP2D6* enzyme. Overall, the authors [150] concluded that a mutated *CYP2D6* gene or the inadvertent use of SSRIs that inhibit the *CYP2D6* enzyme product are independent predictors of breast cancer outcomes for postmenopausal women with breast cancer taking tamoxifen. In a recent complimentary study, Mortimer et al. [151] demonstrated that hot flashes were a strong predictor of positive outcomes for adjuvant tamoxifen treatment.

Although all of the current emphasis has been on the biological effects of tamoxifen in patients with the *CYPD6*4* variant, studies of *CYP3A5* 1* and **3 1A1 *1* and *2* and *UGT2B15 ** and **2* have been undertaken and compared with carriers of *CYP2D6*4*. In contrast to the studies of Goetz et al. [149], patients who carry the *SULT1A1*1*, *CYP2D7*4* and *CYP3A5*3* alleles, and would be predicted to give rise to lower concentrations of metabolites with high affinity for the ER, might actually benefit from tamoxifen [152–155]. No differences were noted between genotypes *CYP2D6*, *SULT1A1* or *UGT 2B15* and tamoxifen treatment but Wegman et al. [155] claim that genetic variants of *CYP3A5* may predict response to tamoxifen. Clearly, reasons for the different conclusions need to be advanced. The hypothesis that variants of metabolizing enzymes can affect patient outcomes for the treatment of breast cancer must now be addressed in large populations and with prospective studies.

8. Conclusions

Overall, the study of tamoxifen metabolism has provided important clues which guided medicinal chemists to synthesize and develop new medicines. The study of metabolites has also provided valuable insight into the mechanism of action of SERMs at their target the ER. However, it is the recent research on the value of genotyping *CYPs* in breast cancer patients to improve response rates to tamoxifen therapy that is showing important promise. Genotyping patients for *CYP2D6* appears to be valuable to exclude the suboptimal use of tamoxifen in select individuals. Additionally, and perhaps more importantly, an effect of SSRIs on the blood levels of endoxifen has raised the possibility that the cheap and effective veteran tamoxifen could be targeted further to select populations of women to improve response rates. Avoiding SSRIs with a high affinity for *CYP2D6* gene product could improve tamoxifen's efficacy. Since tamoxifen is still the antihormonal treatment of choice for premenopausal patients and the only choice for breast cancer risk reduction in premenopausal women, then genotyping from buccal swabs appears to be a cheap and effective way of ensuring that tamoxifen is used to treat the appropriate woman.

It is necessary, however, to close on a note of caution. Very few patients have been studied to create definitive guidelines. That being said, the task of proving the value of these tantalizing clues and hypotheses is the responsibility of clinicians to organize prospective clinical trials or at least there must be investment in the further analysis of archival material from randomized trials. The value of committing resources to establish hypothesis as fact is clear. An important cheap medicine should potentially be given only to women who will benefit from it. Indeed, it may be the role of *CYP2D6* in tamoxifen metabolism that is creating the small but significant advantage of aromatase inhibitors versus tamoxifen in postmenopausal women [26,27]. Again, this can be tested as the tumor blocks and patient records could be reviewed to determine genotyping and whether SSRIs were used. It would be remarkable to discover that the pharmacology of tamoxifen is undermining activity rather than the current view that aromatase inhibitors were better medicines because they have, unlike the SERMs, no estrogen-like actions at the level of the tumor.

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REFERENCES

[1] Snyder KR, Sparano N, Malinowski JM. Raloxifene hydrochloride. Am J Health Syst Pharm 2000;57(18):1669–75, quiz 1676–1678.
[2] Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2003;2:205–13.
[3] Harper MJ, Walpole AL. Contrasting endocrine activities of *cis* and *trans* isomers in a series of substituted triphenylethylenes. Nature 1966;212(57):87.
[4] Harper MJ, Walpole AL. Mode of action of I.C.I. 46,474 in preventing implantation in rats. J Endocrinol 1967;37(1):83–92.
[5] Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 1967;13:101–19.
[6] Klopper A, Hall M. New synthetic agent for the induction of ovulation. Preliminary trial in women. Br Med J 1971;1:152–4.
[7] Williamson JG, Ellis JP. The induction of ovulation by tamoxifen. J Obstet Gynaecol Br Commonw 1973;80:844–7.
[8] Kennedy BJ. Hormone therapy for advanced breast cancer. Cancer 1965;18:1551–7.
[9] Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. Br J Cancer 1971;25:270–5.
[10] Ward HW. Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J 1973;1(844):13–4.
[11] Jensen EV, Jacobson HI. Basic guides to the mechanism of estrogen action. Recent Progr Hormone Res 1962;18:387–414.
[12] Jensen EV, Block GE, Smith S, Kyser K, DeSombre ER. Estrogen receptors and breast cancer response to adrenalectomy. Natl Cancer Inst Monogr 1971;34:55–70.
[13] Kiang DT, Kennedy BJ. Tamoxifen (antiestrogen) therapy in advanced breast cancer. Ann Intern Med 1977;87(6): 687–90.
[14] Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. Clin Cancer Res 2003;9:1980–9.
[15] Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 1975;11:205–6.
[16] Jordan VC, Dowse LJ. Tamoxifen as an anti-tumour agent: effect on oestrogen binding. J Endocrinol 1976;68(02):297–303.
[17] Jordan VC, Jaspan T. Tamoxifen as an antitumour agent: oestrogen binding as a predictive test for tumour response. J Endocrinol 1976;68:453–60.
[18] Jordan VC. Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. Rev Endocr Relat Cancer 1978;(Suppl):49–55.
[19] Jordan VC, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. Adjuvant Ther Cancer 1979;2:19–26.
[20] Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 1980;16:239–51.
[21] Jordan VC. Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 1974;5:354.
[22] Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. Eur J Cancer 1976;12:419–24.
[23] EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 1998;354:1451–67.
[24] EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687–717.
[25] Jordan VC, Brodie AMH. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007;72:7–25.
[26] Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, Forbes JF, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 2005;365:60–2.
[27] Thurlimann B, Keshaviah A, Coates AS, Mouridsen H, Mauriac L, Forbes JF, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. N Engl J Med 2005;353:2747–57.
[28] Goss PE, Ingle JN, Marino S, Robert NJ, Muss HB, Piccart MJ, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. N Engl J Med 2003;349:1–10.
[29] Coombes RC, Hall E, Gibson LJ, Paridaens R, Jassem J, Delozier T, et al. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. N Engl J Med 2004;350(11):1081–92.
[30] Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nat Rev Cancer 2007;7:46–53.
[31] Andersson T, Flockhart DA, Goldstein DB, Huang SM, Kroetz DL, Milos PM, et al. Drug-metabolizing enzymes: evidence for clinical utility of pharmacogenomic tests. Clin Pharmacol Ther 2005;78:559–81.
[32] Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 1977;75(2): 305–16.
[33] Allen KE, Clark ER, Jordan VC. Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure–activity relationships. Br J Pharmacol 1980;71(1):83–91.
[34] Borgna JL, Rochefort H. Hydroxylated metabolites of tamoxifen are formed in vivo and bound to estrogen receptor in target tissues. J Biol Chem 1981;256(2):859–68.
[35] Lien EA, Solheim E, Kvinnsland S, Ueland PM. Identification of 4-hydroxy-*N*-desmethyltamoxifen as a metabolite of tamoxifen in human bile. Cancer Res 1988;48(8):2304–8.
[36] Lien EA, Solheim E, Lea OA, Lundgren S, Kvinnsland S, Ueland PM. Distribution of 4-hydroxy-*N*-desmethyltamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. Cancer Res 1989;49(8):2175–83.
[37] Johnson MD, Zuo H, Lee KH, Trebley JP, Rae JM, Weatherman RV, et al. Pharmacological characterization of

4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. Breast Cancer Res Treat 2004;85(2):151–9.
[38] Desta Z, Ward BA, Soukhova NV, Flockhart DA. Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: prominent roles for CYP3A and CYP2D6. J Pharmacol Exp Ther 2004;310(3):1062–75.
[39] Crewe HK, Lennard MS, Tucker GT, Woods FR, Haddock RE. The effect of selective serotonin re-uptake inhibitors on cytochrome P4502D6 (CYP2D6) activity in human liver microsomes. Br J Clin Pharmacol 1992;34(3):262–5.
[40] Fromson JM, Pearson S, Bramah S. The metabolism of tamoxifen (I.C.I. 46,474). I. In laboratory animals. Xenobiotica 1973;3(11):693–709.
[41] Fromson JM, Pearson S, Bramah S. The metabolism of tamoxifen (ICI 46,474). Part II. In female patients. Xenobiotica 1973;3:711–3.
[42] Lieberman ME, Jordan VC, Fritsch M, Santos MA, Gorski J. Direct and reversible inhibition of estradiol-stimulated prolactin synthesis by antiestrogens in vitro. J Biol Chem 1983;258(8):4734–40.
[43] Adam HK, Gay MA, Moore RH. Measurement of tamoxifen in serum by thin layer densitometry. J Endocrinol 1980;84:35–41.
[44] Adam HK, Douglas EJ, Kemp JV. The metabolism of tamoxifen in humans. Biochem Pharmacol 1979;27:145–7.
[45] Kemp JV, Adam HK, Wakeling AE, Slater R. Identification and biological activity of tamoxifen metabolites in human serum. Biochem Pharmacol 1983;32:2045–52.
[46] Bain RR, Jordan VC. Identification of a new metabolite of tamoxifen in patient serum during breast cancer therapy. Biochem Pharmacol 1983;32(2):373–5.
[47] Jordan VC, Bain RR, Brown RR, Brown RR, Gosden B, Santos MA. Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient during therapy for advanced breast cancer. Cancer Res 1983;43:1446–50.
[48] Robinson SP, Langan-Fahey SM, Jordan VC. Implications of tamoxifen metabolism in the athymic mouse for the study of antitumor effects upon human breast cancer xenografts. Eur J Cancer Clin Oncol 1989;25(12):1769–76.
[49] Jordan VC, Gosden B. Importance of the alkylamino-ethoxy side chain for the estrogenic and antiestrogenic actions of tamoxifen and trioxifene in the immature rat uterus. Mol Cell Endocrinol 1982;27:291–306.
[50] Lieberman ME, Gorski J, Jordan VC. An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 1983;258(8):4741–5.
[51] Katzenellenbogen JA, Carlson KE, Katzenellenbogen BS. Facile geometric isomerization of phenolic nonsteroidal estrogens and antiestrogens: limitations to the interpretation of experiments characterizing the activity of individual isomers. J Steroid Biochem 1985;22:589–96.
[52] Katzenellenbogen BS, Norman MJ, Eckert RL, Peltz SW, Mangel WF. Bioactivities, estrogen receptor interactions, and plasminogen activator-inducing activities of tamoxifen and hydroxy-tamoxifen isomers in MCF-7 human breast cancer cells. Cancer Res 1984;44(1):112–9.
[53] Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 1986;83(8):2496–500.
[54] Murphy CS, Meisner LF, Wu SQ, Jordan VC. Short- and long-term estrogen deprivation of T47D human breast cancer cells in culture. Eur J Cancer Clin Oncol 1989;25(12):1777–88.
[55] Jordan VC, Lieberman ME, Cormier E, Koch R, Bagley JR, Ruenitz PC. Structural requirements for the pharmacological activity of nonsteroidal antiestrogens in vitro. Mol Pharmacol 1984;26(2):272–8.
[56] Jordan VC, Koch R, Mittal S, Schneider MR. Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. Br J Pharmacol 1986;87:217–23.
[57] Murphy CS, Langan-Fahey SM, McCague R, Jordan VC. Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. Mol Pharmacol 1990;38(5):737–43.
[58] Murphy CS, Parker CJ, McCague R, Jordan VC. Structure–activity relationships of nonisomerizable derivatives of tamoxifen: importance of hydroxyl group and side chain positioning for biological activity. Mol Pharmacol 1991;39(3):421–8.
[59] Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, et al. The structural basis of estrogen receptor/co-activator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;95:927–37.
[60] Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997;389(6652):753–8.
[61] Wolf DM, Jordan VC. The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 1994;31(1):129–38.
[62] Wolf DM, Jordan VC. Characterization of tamoxifen stimulated MCF-7 tumor variants grown in athymic mice. Breast Cancer Res Treat 1994;31(1):117–27.
[63] MacGregor-Shafer JI, Liu H, Bentrem D, Zapf J, Jordan VC. Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex by substituting glycine for aspartate at amino acid 351. Cancer Res 2000;60:5097–105.
[64] Levenson AS, Jordan VC. The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. Cancer Res 1998;58(9):1872–5.
[65] Liu H, Lee E-S, De Los Reyes A, Zapf JW, Jordan VC. Silencing and reactivation of the selective estrogen receptor modulator (SERM)–ER alpha complex. Cancer Res 2001;61:3632–9.
[66] Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 1995;270(5240):1354–7.
[67] Jordan VC, O'Malley BM. Selective estrogen receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol; in press.
[68] Jordan VC, Gapstur S, Morrow M. Selective estrogen receptor modulation and reduction in risk of breast cancer, osteoporosis, and coronary heart disease. J Natl Cancer Inst 2001;93(19):1449–57.
[69] Greaves P, Goonetilleke R, Nunn G, Topham J, Orton T. Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. Cancer Res 1993;53(17):3919–24.
[70] Hard GC, Iatropoulos MJ, Jordan K, Radi L, Kaltenberg OP, Imondi AR, et al. Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. Cancer Res 1993;53(19):4534–41.
[71] Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1989;1(8630):117–20.

[72] Fisher B, Costantino JP, Redmond CK, Fisher ER, Wickerham DL, Cronin WM. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. J Natl Cancer Inst 1994;86(7):527–37 [see comments].
[73] Han XL, Liehr JG. Induction of covalent DNA adducts in rodents by tamoxifen. Cancer Res 1992;52:1360–3.
[74] Rutqvist LE, Johansson H, Signomklao T, Johansson U, Fornander T, Wilking N. Adjuvant tamoxifen therapy for early stage breast cancer and second primary malignancies. J Natl Cancer Inst 1995;87:645–51.
[75] Styles JA, Davies A, Lim CK, De Matteis F, Stanley LA, White IN, et al. Genotoxicity of tamoxifen, tamoxifen epoxide and toremifene in human lymphoblastoid cells containing human cytochrome P450s. Carcinogenesis 1994;15(1): 5–9.
[76] Lim CK, Zhi-Xin Y, Lamb JH, Whihte INH, DeMatteis F, Smith LL. A comparative study of tamoxifen metabolism in female rat, mouse and human liver microsomes. Carcinogenesis 1994;15:589–93.
[77] Moorthy B, Sriram P, Pathak DN, Bodell WJ, Randerath K. Tamoxifen metabolic activation: comparison of DNA adducts formed by microsomal and chemical activation of tamoxifen and 4-hydroxytamoxifen with DNA adducts formed in vivo. Cancer Res 1996;56(1):53–7.
[78] Pongracz K, Pathak DN, Nakamura T, Burlingame AL, Bodell WJ. Activation of the tamoxifen derivative metabolite E to form DNA adducts: comparison with the adducts formed by microsomal activation of tamoxifen. Cancer Res 1995;55(14):3012–5.
[79] Potter GA, McCague R, Jarman M. A mechanistic hypothesis for DNA adduct formation by tamoxifen following hepatic oxidative metabolism. Carcinogenesis 1994;15(3): 439–42.
[80] Phillips DH, Carmichael PL, Hewer A, Cole KJ, Poon GK. Alpha-hydroxytamoxifen, a metabolite of tamoxifen with exceptionally high DNA-binding activity in rat hepatocytes. Cancer Res 1994;54(21):5518–22.
[81] Phillips DH, Potter GA, Horton MN, Hewer A, Crofton-Sleigh C, Jarman M, et al. Reduced genotoxicity of [D5-ethyl]-tamoxifen implicates alpha-hydroxylation of the ethyl group as a major pathway of tamoxifen activation to a liver carcinogen. Carcinogenesis 1994;15(8):1487–92.
[82] Osborne MR, Hewer A, Hardcastle IR, Carmichael PL, Phillips DH. Identification of the major tamoxifen-deoxyguanosine adduct formed in the liver DNA of rats treated with tamoxifen. Cancer Res 1996;56(1): 66–71.
[83] Phillips DH, Carmichael PL, Hewer A, Cole KJ, Hardcastle IR, Poon GK, et al. Activation of tamoxifen and its metabolite alpha-hydroxytamoxifen to DNA-binding products: comparisons between human, rat and mouse hepatocytes. Carcinogenesis 1996;17(1):89–94.
[84] Osborne MR, Hewer A, Phillips DH. Resolution of alpha-hydroxytamoxifen; R-isomer forms more DNA adducts in rat liver cells. Chem Res Toxicol 2001;14(7):888–93.
[85] Osborne MR, Hewer A, Phillips DH. Stereoselective metabolic activation of alpha-hydroxy-*N*-desmethyltamoxifen: the *R*-isomer forms more DNA adducts in rat liver cells. Chem Res Toxicol 2004;17(5):697–701.
[86] Phillips DH. Understanding the genotoxicity of tamoxifen? Carcinogenesis 2001;22(6):839–49.
[87] Jordan VC. What if tamoxifen (ICI 46,474) had been found to produce rat liver tumors in 1973? A personal perspective. Ann Oncol 1995;6(1):29–34.
[88] Mallory FB, Wood CS, Gordon JT. Photochemistry of stilbenes III. Some aspects of photocylisation to phenanthrenes. J Am Chem Soc 1986;86:3094–102.
[89] Mendenhall LW, Kobayashi H, Shih FML, Sternson LA, Higuchi T, Fabian C. Clinical analysis of tamoxifen, an antineoplastic agent, in plasma. Clin Chem 1978;24:1518–24.
[90] Golander Y, Sternson LA. Paired-ion chromatographis analysis of tamoxifen and two major metabolites in plasma. J Chromatogr 1980;181:41–9.
[91] Brown RR, Bain R, Jordan VC. Determination of tamoxifen and metabolites in human serum by high-performance liquid chromatography with post-column fluorescence activation. J Chromatogr 1983;272(2):351–8.
[92] Camaggi CM, Strocchi E, Canova N. High performance liquid chromatographic analysis or tamoxifen and major metabolites in human plasma. J Chromatogr 1983;275:436–42.
[93] Lien EA, Solheim E, Ueland PM. Distribution of tamoxifen and its metabolites in rat and human tissues during steady-state treatment. Cancer Res 1991;51:4837–44.
[94] Lee KH, Ward BA, Desta Z, Flockhart DA, Jones DR. Quantification of tamoxifen and three metabolites in plasma by high-performance liquid chromatography with fluorescence detection: application to a clinical trial. J Chromatogr B Analyt Technol Biomed Life Sci 2003;791:245–53.
[95] Fried KM, Wainer IW. Direct determination of tamoxifen and its four major metabolites in plasma using coupled column high-performance liquid chromatography. J Chromatogr B Biomed Appl 1994;655(2):261–8.
[96] Kikuta C, Schmid R. Specific high-performance liquid chromatographic analysis of tamoxifen and its major metabolites by “on-line” extraction and post-column photochemical reaction. J Pharm Biomed Anal 1989;7(3):329–37.
[97] Stearns V, Johnson MD, Rae JM, Morocho A, Novielli A, Bhargava P, et al. Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. J Natl Cancer Inst 2003;95(23):1758–64.
[98] Fabian C, Sternson L, Barnett M. Clinical pharmacology of tamoxifen in patients with breast cancer: comparison of traditional and loading dose schedules. Cancer Treat Rep 1980;64(6–7):765–73.
[99] Patterson JS, Settatree RS, Adam HK, Kemp JV. Serum concentrations of tamoxifen and major metabolite during long-term Nolvadex therapy, correlated with clinical response. Eur J Cancer 1980;Suppl 1:89–92.
[100] Wilkinson PM, Ribiero GG, Adama HK, Kemp JV, Patterson JS. Tamoxifen (Nolvadex) therapy—rationale for loading dose followed by maintenance dose for patients with metastatic breast cancer. Cancer Chemother Pharmacol 1982;10:33–5.
[101] Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 1988;6(5):589–95.
[102] Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. Cancer Res 1990;50:4177–89.
[103] Jordan VC. Selective estrogen receptor modulation: a personal perspective. Cancer Res 2001;61:5683–7.
[104] Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. J Med Chem 2003;46(7):1081–111.
[105] Ariazi EA, Ariazi JL, Cordera F, Jordan VC. Estrogen receptors as therapeutic targets in breast cancer. Curr Top Med Chem 2006;6:195–216.

[106] Buzdar A, Marcus C, Holmes F, Hug V, Hortobagyi G. Phase II evaluation of Ly156758 in metastatic breast cancer. Oncology 1988;45(5):344–5.
[107] Kemp DC, Fan PW, Stevens JC. Characterization of raloxifene glucuronidation in vitro: contribution of intestinal metabolism to presystemic clearance. Drug Metab Dispos 2002;30:694–700.
[108] Jeong EJ, Liu Y, Lin H, Hu M. Species- and disposition model-dependent metabolism of raloxifene in gut and liver: role of UGT1A10. Drug Metab Dispos 2005;33:785–94.
[109] Suh N, Lamph WW, Glasebrook AL, Grese TA, Palkowitz AD, Williams CR, et al. Prevention and treatment of experimental breast cancer with the combination of a new selective estrogen receptor modulator, arzoxifene, and a new rexinoid, LG 100268. Clin Cancer Res 2002;8(10):3270–5.
[110] Baselga J, Llombart-Cussac A, Bellet M, Guillem-Porta V, Enas N, Krejcy K, et al. Randomized, double-blind multicenter trial comparing two doses of arzoxifene (LY353381) in hormone-sensitive advanced or metastatic breast cancer patients. Ann Oncol 2003;14:1383–90.
[111] Buzdar A, O'Shaughnessy JA, Booser DJ, Pippen Jr JE, Jones SE, Munster PN, et al. Phase II, randomized double-blind study of two dose levels of arzoxifene in patients with locally advanced or metastatic breast cancer. J Clin Oncol 2003;21:1007–14.
[112] Falany JL, Pilloff DE, Leyh TS, Falany CN. Sulfation of raloxifene and 4-hydroxytamoxifen by human cytosolic sulfotransferases. Drug Metab Dispos 2006;34:361–8.
[113] Rosati RL, Da Silva Jardine P, Cameron KO, Thompson DD, Ke HZ, Toler SM, et al. Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryltetrahydronaphthalene. J Med Chem 1998;41(16):2928–31.
[114] Ke HZ, Paralkar VM, Grasser WA, Crawford DT, Qi H, Simmons HA, et al. Effects of CP-336,156, a new, nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. Endocrinology 1998;139(4):2068–76.
[115] Ke HZ, Qi H, Crawford DT, Chidsey-Frink KL, Simmons HA, Thompson DD. Lasofoxifene (CP-336,156), a selective estrogen receptor modulator, prevents bone loss induced by aging and orchidectomy in the adult rat. Endocrinology 2000;141(4):1338–44.
[116] Tatee T, Carlson KE, Katzenellenbogen JA, Robertson DW, Katzenellenbogen BS. Antiestrogens and antiestrogen metabolites: preparation of tritium-labeled (+/−)-*cis*-3-[*p*-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phenoxyl]-1.2-propanediol (U-23469) and characterization and synthesis of a biologically important metabolite. J Med Chem 1979;22(12):1509–17.
[117] Legha SS, Slavik M, Carter SK. Nafoxidine—an antiestrogen for the treatment of breast cancer. Cancer 1976;38(4):1535–41.
[118] Cohen LA, Pittman B, Wang CX, Aliaga C, Yu L, Moyer JD. Lasofoxifene, novel selective estrogen receptor modulator with chemopreventive and therapeutic activity in the *N*-nitroso-*N*-methylurea-induced rat mammary tumor model. Cancer Res 2001;61(24):8683–8.
[119] Robertson DW, Katzenellenbogen JA, Hayes JR, Katzenellenbogen BS. Antiestrogen basicity–activity relationships: a comparison of the estrogen receptor binding and antiuterotrophic potencies of several analogues of (Z)-1,2 dipheny1-1-[4-[2-(dimethyl-amino)ethoxy]phenyl]-1-butene (tamoxifen Nolvadex) having altered basicity. J Med Chem 1982;25:167–71.
[120] Hellmann-Blumberg U, Taras TL, Wurz GT, DeGregorio MW. Genotoxic effects of the novel mixed antiestrogen FC-1271a in comparison to tamoxifen and toremifene. Breast Cancer Res Treat 2000;60(1):63–70.
[121] Qu Q, Zheng H, Dahllund J, Laine A, Cockcroft N, Peng Z, et al. Selective estrogenic effects of a novel triphenylethylene compound, FC1271a, on bone, cholesterol level, and reproductive tissues in intact and ovariectomized rats. Endocrinology 2000;141(2):809–20.
[122] DeGregorio MW, Wurz GT, Taras TL, Erkkola RU, Halonen KH, Huupponen RK. Pharmacokinetics of (deaminohydroxy)toremifene in humans: a new, selective estrogen-receptor modulator. Eur J Clin Pharmacol 2000;56(6–7):469–75.
[123] Rutanen EM, Heikkinen J, Halonen K, Komi J, Lammintausta R, Ylikorkala O. Effects of ospemifene, a novel SERM, on hormones, genital tract, climacteric symptoms, and quality of life in postmenopausal women: a double-blind, randomized trial. Menopause 2003;10(5):433–9.
[124] Komi J, Lankinen KS, Harkonen P, DeGregorio MW, Voipio S, Kivinen S, et al. Effects of ospemifene and raloxifene on hormonal status, lipids, genital tract, and tolerability in postmenopausal women. Menopause 2005;12(2):202–9.
[125] Namba R, Young LJ, Maglione JE, McGoldrick ET, Liu S, Wurz GT, et al. Selective estrogen receptor modulators inhibit growth and progression of premalignant lesions in a mouse model of ductal carcinoma in situ. Breast Cancer Res 2005;7(6):R881–9.
[126] Wurz GT, Read KC, Marchisano-Karpman C, Gregg JP, Beckett LA, Yu Q, et al. Ospemifene inhibits the growth of dimethylbenzanthracene-induced mammary tumors in Sencar mice. J Steroid Biochem Mol Biol 2005;97(3):230–40.
[127] Beverage JN, Sissung TM, Sion AM, Danesi R, Figg WD. CYP2D6 polymorphisms and the impact on tamoxifen therapy. J Pharm Sci 2007;96(9):2224–31.
[128] Bradford LD. CYP2D6 allele frequency in European Caucasians, Asians, Africans and their descendants. Pharmacogenomics 2002;3(2):229–43.
[129] Raimundo S, Toscano C, Klein K, Fischer J, Griese EU, Eichelbaum M, et al. A novel intronic mutation, 2988G > A, with high predictivity for impaired function of cytochrome P450 2D6 in white subjects. Clin Pharmacol Ther 2004;76(2):128–38.
[130] Hanioka N, Kimura S, Meyer UA, Gonzalez FJ. The human CYP2D locus associated with a common genetic defect in drug oxidation: a G1934—a base change in intron 3 of a mutant CYP2D6 allele results in an aberrant 3′ splice recognition site. Am J Hum Genet 1990;47(6):994–1001.
[131] Bernard S, Neville KA, Nguyen AT, Flockhart DA. Interethnic differences in genetic polymorphisms of CYP2D6 in the U.S. population: clinical implications. Oncologist 2006;11(2):126–35.
[132] Lim YC, Desta Z, Flockhart DA, Skaar TC. Endoxifen (4-hydroxy-*N*-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen. Cancer Chemother Pharmacol 2005;55:471–8.
[133] Lim YC, Li L, Desta Z, Zhao Q, Rae JM, Flockhart DA, et al. Endoxifen, a secondary metabolite of tamoxifen, and 4-OH-tamoxifen induce similar changes in global gene expression patterns in MCF-7 breast cancer cells. J Pharmacol Exp Ther 2006;318(2):503–12.
[134] Jin Y, Desta Z, Stearns V, Ward B, Ho H, Lee KH, et al. CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. JNCI 2005;97:30–9.
[135] Fisher B, Costantino J, Redmond C, Poisson R, Bowman D, Couture J, et al. A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. N Engl J Med 1989;320(8):479–84.

[136] Fisher B, Dignam J, Wolmark N, Wickerham DL, Fisher ER, Mamounas E, et al. Tamoxifen in treatment of intraductal breast cancer: National Surgical Adjuvant Breast and Bowel Project B-24 randomised controlled trial. Lancet 1999;353(9169):1993–2000.
[137] Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998;90:1371–88.
[138] Loprinzi CL, Kugler JW, Sloan JA, Mailliard JA, LaVasseur BI, Barton DL, et al. Venlafaxine in management of hot flashes in survivors of breast cancer: a randomised controlled trial. Lancet 2000;356(9247):2059–63.
[139] Loprinzi CL, Sloan JA, Perez EA, Quella SK, Stella PJ, Mailliard JA, et al. Phase III evaluation of fluoxetine for treatment of hot flashes. J Clin Oncol 2002;20(6):1578–83.
[140] Stearns V, Beebe KL, Iyengar M, Dube E. Paroxetine controlled release in the treatment of menopausal hot flashes: a randomized controlled trial. JAMA 2003;289(21):2827–34.
[141] Dehal SS, Kupfer D. CYP2D6 catalyzes tamoxifen 4-hydroxylation in human liver. Cancer Res 1997;57(16):3402–6.
[142] Crewe HK, Ellis SW, Lennard MS, Tucker GT. Variable contribution of cytochromes P450 2D6, 2C9 and 3A4 to the 4-hydroxylation of tamoxifen by human liver microsomes. Biochem Pharmacol 1997;53(2):171–8.
[143] Coller JK, Krebsfaenger N, Klein K, Endrizzi K, Wolbold R, Lang T, et al. The influence of CYP2B6, CYP2C9 and CYP2D6 genotypes on the formation of the potent antioestrogen Z-4-hydroxy-tamoxifen in human liver. Br J Clin Pharmacol 2002;54(2):157–67.
[144] Lessard E, Yessine MA, Hamelin BA, Gauvin C, Labbe L, O'Hara G, et al. Diphenhydramine alters the disposition of venlafaxine through inhibition of CYP2D6 activity in humans. J Clin Psychopharmacol 2001;21(2):175–84.
[145] Albers LJ, Reist C, Vu RL, Fujimoto K, Ozdemir V, Helmeste D, et al. Effect of venlafaxine on imipramine metabolism. Psychiatry Res 2000;96(3):235–43.
[146] Yoon YR, Cha IJ, Shon JH, Kim KA, Cha YN, Jang IJ, et al. Relationship of paroxetine disposition to metoprolol metabolic ratio and CYP2D6*10 genotype of Korean subjects. Clin Pharmacol Ther 2000;67(5):567–76.
[147] Jeppesen U, Gram LF, Vistisen K, Loft S, Poulsen HE, Brosen K. Dose-dependent inhibition of CYP1A2, CYP2C19 and CYP2D6 by citalopram, fluoxetine, fluvoxamine and paroxetine. Eur J Clin Pharmacol 1996;51(1):73–8.
[148] Borges S, Desta Z, Li L, Skaar TC, Ward BA, Nguyen A, et al. Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. Clin Pharmacol Ther 2006;80(1):61–74.
[149] Goetz MP, Rae JM, Suman VJ, Safgren SL, Ames MM, Visscher DW, et al. Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. J Clin Oncol 2005;23(36): 9312–8.
[150] Goetz MP, Knox SK, Suman VJ, Rae JM, Safgren SL, Ames MM, et al. The impact of cytochrome P450 2D6 metabolism in women receiving adjuvant tamoxifen. Breast Cancer Res Treat 2007;101(1):113–21.
[151] Mortimer JE, Flatt SW, Parker BA, Gold EB, Wasserman L, Natarajan L, et al. Tamoxifen, hot flashes and recurrence in breast cancer. Breast Cancer Res Treat 2007, doi:10.1007/s10549-007-9162X.
[152] Nowell S, Sweeney C, Winters M, Stone A, Lang NP, Hutchins LF, et al. Association between sulfotransferase 1A1 genotype and survival of breast cancer patients receiving tamoxifen therapy. J Natl Cancer Inst 2002;94(21):1635–40.
[153] Nowell SA, Ahn J, Rae JM, Scheys JO, Trovato A, Sweeney C, et al. Association of genetic variation in tamoxifen-metabolizing enzymes with overall survival and recurrence of disease in breast cancer patients. Breast Cancer Res Treat 2005;91(3):249–58.
[154] Wegman PP, Wingren S. CYP2D6 variants and the prediction of tamoxifen response in randomized patients: author response. Breast Cancer Res 2005;7(6):E7.
[155] Wegman P, Elingarami S, Carstensen J, Stal O, Nordenskjold B, Wingren S. Genetic variants of CYP3A5, CYP2D6, SULT1A1, UGT2B15 and tamoxifen response in postmenopausal patients with breast cancer. Breast Cancer Res 2007;9(1):R7.

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Selective Estrogen-Receptor Modulators and Antihormonal Resistance in Breast Cancer

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ABSTRACT

Selective estrogen-receptor (ER) modulators (SERMs) are synthetic nonsteroidal compounds that switch on and switch off target sites throughout the body. Tamoxifen, the pioneering SERM, blocks estrogen action by binding to the ER in breast cancers. Tamoxifen has been used ubiquitously in clinical practice during the last 30 years for the treatment of breast cancer and is currently available to reduce the risk of breast cancer in high-risk women. Raloxifene maintains bone density (estrogen-like effect) in postmenopausal osteoporotic women, but at the same time reduces the incidence of breast cancer in both high- and low-risk (osteoporotic) postmenopausal women. Unlike tamoxifen, raloxifene does not increase the incidence of endometrial cancer. Clearly, the simple ER model of estrogen action can no longer be used to explain SERM action at different sites around the body. Instead, a new model has evolved on the basis of the discovery of protein partners that modulate estrogen action at distinct target sites. Coactivators are the principal players that assemble a complex of functional proteins around the ligand ER complex to initiate transcription of a target gene at its promoter site. A promiscuous SERM ER complex creates a stimulatory signal in growth factor receptor–rich breast or endometrial cancer cells. These events cause drug-resistant, SERM-stimulated growth. The sometimes surprising pharmacology of SERMs has resulted in a growing interest in the development of new selective medicines for other members of the nuclear receptor superfamily. This will allow the precise treatment of diseases that was previously considered impossible.

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INTRODUCTION

The estrogen receptor (ER) is the trigger[1] that initiates estrogen action in its target tissues (eg, uterus, vagina, and pituitary gland). The subsequent identification of the ER in some breast cancers created a mechanistic link to explain the hormonal dependence of some breast cancers.[2] Ultimately, this knowledge was used to reinvent a failed postcoital contraceptive, ICI 46474,[3] as tamoxifen, the first targeted antiestrogenic therapy for breast cancer.[4] The clinical strategy of targeting ER-positive breast tumors with long-term adjuvant therapy has saved hundreds of thousands of lives.[5] As a result, the evolving use of tamoxifen therapy during the last three decades has proved to be the cornerstone for the treatment and prevention of breast cancer.[6]

However, the recognition[7] that the "nonsteroidal antiestrogens" were, in fact, selective estrogens and antiestrogens at different target tissues around the body, created a new dimension in drug development and enhanced therapeutic possibilities. The selective estrogenic properties of tamoxifen and raloxifene maintained bone density[8] but the selective antiestrogenic properties prevented rat mammary carcinogenesis.[9] These laboratory data were used to develop an evidence-based therapeutic strategy[10,11] that has now become a clinical reality with the development of raloxifene. This second-generation selective ER modulator (SERM) prevents osteoporosis but also prevents breast cancer as a beneficial side effect.[12] With this significant advance in therapeutics, it has become clear that the action of SERMs at different target sites can no longer be explained by an ER model that simply turns estrogen action on or off. Other physiologic factors must be involved.

In this article, we will describe our evolving understanding of SERM action at its target sites. Although the ER complex is programmed by the shape of the SERM buried inside the receptor, it is the new protein players called coactivators and corepressors[13] that are now known to modulate and control the dynamics of the complex as it turns on or turns off subcellular signaling networks at target sites around the body. However, we believe it is important to state at the outset that although we have, by necessity, chosen to explain the molecular mechanism of SERMs to retain therapeutic relevance in oncology, we prefer to use the term steroid

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receptor modulators (SRMs) when considering mechanisms. The molecular biology of selective activity is clearly universal within the steroid receptor superfamily.[13] This fact has important therapeutic implications for future drug discovery.

MECHANISMS OF SELECTIVE RECEPTOR MODULATOR ACTION

Of the 48 members of the nuclear receptor (NR) family, approximately half have been determined to be regulatable by ligands.[14,15] The remaining molecules are regulated by signaling pathways that impart post-translational modifications to these endocrine/metabolic transcription factors. The nuclear receptors are signal-dependent transcription factors that have two main purposes: (1) to locate target genes by binding at specific DNA sequences (termed hormone response elements [HREs]) that are located at these genes; and then, (2) to recruit transcriptional coregulators to the gene.[16] Ligands can induce both activation and repression of target genes. NRs recruit coactivators to activate genes, and corepressors to repress genes.[17,18] These two functionally different classes of molecules comprise the totality of 285-member coregulator superfamily, most of which are coactivators. The general domain structure of coactivators is shown schematically in Figure 1, and a great deal of additional basic and clinical information is provided on the Nuclear Receptor Signaling Atlas Web site (www.nursa.org). Although the NR coregulators were identified only approximately 11 years ago,[19] they are generally accepted as the rate-limiting components of transcriptional control in mammals.

The molecular mechanisms by which distinct ligands can bind to the same nuclear receptor and yet exert tissue-specific actions, has been somewhat of a mystery until the last decade, when the contributions of basic receptor research have led to an enlightened viewpoint.[13] We now realize the complexities and the relative importance of the fundamental elements that factor into the equations for tissue-selective SRM actions. These elements are (1) receptor isoform subtypes; (2) ligand-induced conformations of the receptor; (3) precise sequence compositions of the HREs; (4) nuclear receptor coregulators (coactivators and corepressors), which are recruited by the active or inactive conformation of the receptor to the gene site; and (5) cell and signaling context. Although the coregulator recruitment is of paramount importance, under most conditions, all five of the preceding events can have a modulating influence on the actions of an SRM.

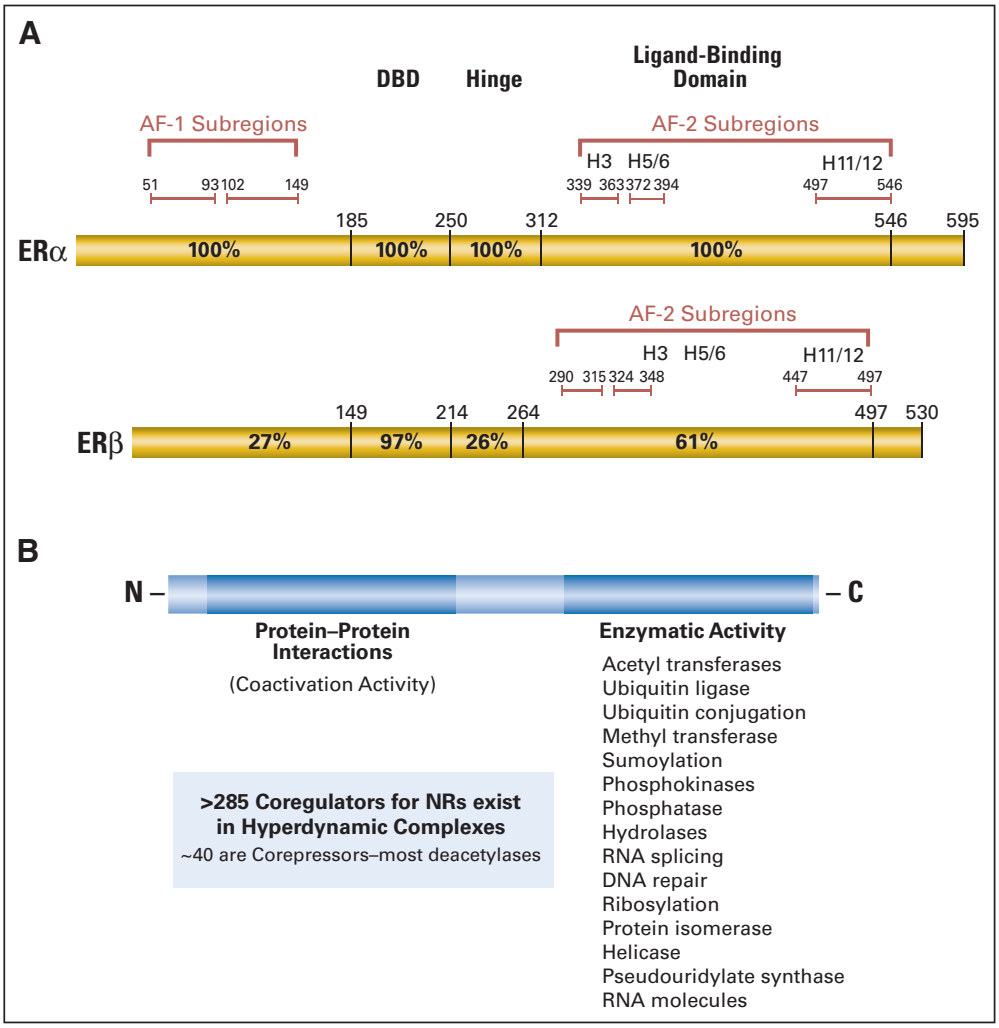

Fig 1. (A) The structure of the known estrogen receptors (ERs) with the identified (red) activating functions (AFs) that bind coactivators. Also identified is the DNA-binding domain (DBD) and the ligand binding domain. (B) A typical domain structure of a nuclear receptor (NR) coactivator is shown. There are two main domains: (1) a protein-protein interacting domain that binds other cocoactivators in the functional high molecular weight coactivator complex and (2) an enzymatic domain that either has intrinsic enzyme activity or binds a protein that has enzyme activity. Numerous enzyme activities have been demonstrated in the many coactivators discovered to date.

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RECEPTOR ISOFORMS

Multiple function-specific isoforms have been discovered for a number of receptors, including those for progesterone receptor (PR; PRa, PRb), ER (ERα, ERβ), and glucocorticoid receptor (GR; GRα, GRβ).[15] These isoforms have different primary structures and therefore beget different gene functions. Since the tissue concentration of receptor isoforms can vary in a tissue-specific manner, the functions of the cognate receptor ligand in a given tissue can vary also. Perhaps the ERα and the ERβ isoforms have the most contradictory functions, with ERα having a growth promoting action and ERβ having a growth-inhibitory action in certain tissues.[20] Consequently, the tissue-selective ratio of ERα/ERβ can provide a tissue-selective function.

LIGAND-INDUCED RECEPTOR CONFORMATION

For many years it was suspected that a transcription-inducing ligand acted simply by shifting the equilibrium of its cognate NR from an inactive to an active conformation. Two complimentary experimental approaches helped to clarify receptor-mediated modulations. A comprehensive pharmacologic evaluation of the structure function relationship of estrogens and antiestrogens both at an ER-regulated prolactin gene target[21,22] and by regulating breast cancer cell replication,[23] built up a hypothetical model of molecular modulation. The pharmacologic studies concluded the size and position of the "antiestrogenic" side chain of the then nonsteroidal antiestrogens controlled the folding of the ER at an antiestrogenic region of the ER.[21,24,25] Simply stated, the "crocodile" model proposed equilibrium mixtures of receptor jaws closed (estrogenic complex) or propped open by the ligand (partial estrogenic/antiestrogenic complex) to modulate gene function at target sites.[26,27] Complementary early biochemical studies utilized protease structural mapping and antibody epitope mapping techniques to demonstrate that progesterone and estrogen bound to their cognate receptors and induced a conformational alteration in the carboxy-terminal tail of the receptor, whereby the tail flipped back over the ligand pocket and the active form was stabilized.[28,29] It was the eventual x-ray crystallography of these molecules, however, that provided a more detailed picture of this model, whereby a c-terminal helix 12 was the lid that covered the ligand pocket and formed a landing platform for newly recruited coactivators (or corepressors).[30-32] The newly recruited coregulators then carry out all of the reactions required for the entire transcriptional process (discussed further herein). Different receptors binding to the same genetic sequence can recruit different coactivators and thereby provide quantitatively or qualitatively different gene responses (Fig 2). Similarly, different ligands occupying the same receptor at a gene site can induce different structural conformations in that receptor and lead to recruitment of different coactivators, and consequently, different gene expression patterns.

DNA BINDING ELEMENT (HRE) OF THE TARGET GENE

The precise composition of different genomic HREs in mammals varies. HREs are usually composed of short inverted or direct repeats of approximately 7 deoxynucleotides each. When minor variations in the receptor contact sequence occur, and in combination with other surrounding transcription factors, the receptor can be forced into an altered conformation that in turn recruits different coregulators and provides distinct functions for these genes, if they are expressed in that tissue.[33] This basic principle has been demonstrated, but it is unclear as to how often this is a significant factor in SRM actions. What is clear is that recruitment of the receptor complex to the HRE is cyclical with binding and destruction.[34]

NUCLEAR RECEPTOR COREGULATORS

Current opinions place the coregulators in the driving seat of tissue-specific actions of SRMs. The potency and selectivity for all subreactions of transcription reside in these coregulators, and thus, they are critically important for not only gene function, but also tissue-selective gene function. Currently there are approximately

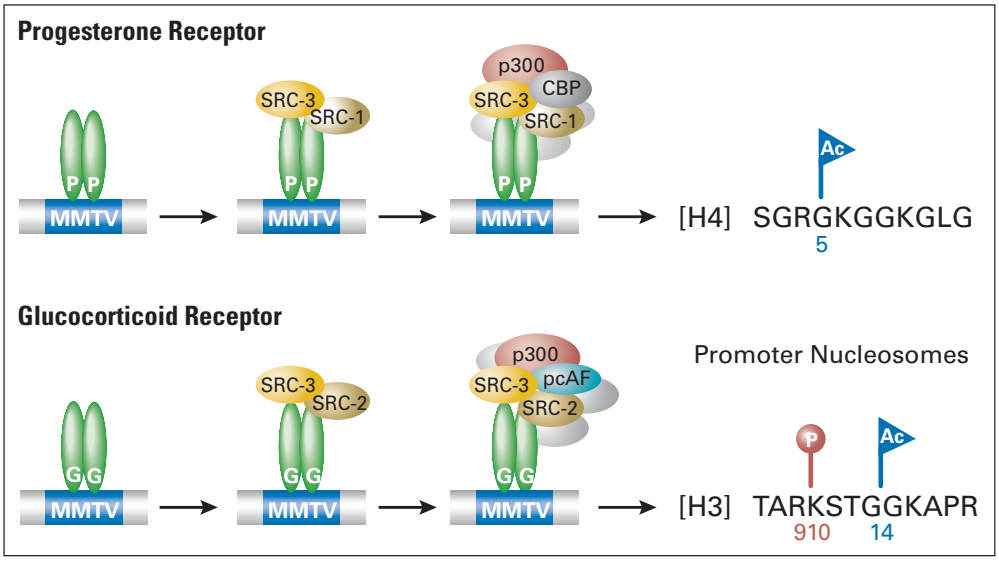

Fig 2. Differential recruitment of coactivators to same gene by receptors determine specific promoter chromatin modifications and transcription initiations. The top panel contains a schematic of the progesterone receptor bound to the hormone response element (HRE) of the *MMTV* gene. The bottom panel demonstrates that when the glucocorticoid receptor is bound to the same HRE of the *MMTV*, it accumulates different coactivator proteins. Each of the receptors induce different patterns of histone modifications and subsequent transcription. The panels illustrate that the ligand-bound receptor itself plays the dominant role in what coactivators are recruited, and thus can modify target gene transcription accordingly. Ac, acetylation; P, phosphorylation.

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285 NR coregulators, of which the vast majority are coactivators (approximately 40 are corepressor according to the Nuclear Receptor Signaling Atlas). Most occur in the majority of tissues, but at different individual concentrations in each tissue. Consequently, each tissue has a "quantitative finger print" of coactivators based on the relative concentrations of each molecule in that tissue.[16] This inherited complement of coregulators provides a basis for tissue-selective actions by a given NR.

Coregulators function as large, high-molecular weight complexes of approximately six to seven cocoregulator proteins.[17] Most of the coregulators are enzymes that participate in remodeling the local chromatin structure at the target promoter, initiating transcription by RNA polymerase, encouraging efficient elongation of RNA chain synthesis, regulating alternative RNA splicing, and, finally, destroying the active transcription factors at the promoter site. These series of substeps of transcription occur in rapid sequence (approximately 15 seconds apart) and are controlled by sequential occupation of the promoter by specific coregulator complexes that direct the transcriptional substep reactions.

For the most part, the coregulators are themselves regulated at the post-transcriptional level.[17] Their intracellular concentrations are determined by their proteasomal degradation rates. Levels are raised by inhibiting the rate of degradation, and vice versa for lowering levels. Traditional ubiquitin-mediated degradation occurs, as well as an ubiquitin-independent turnover by 11S cap proteins such as REGγ.[35] Degradation can be inhibited by post-translational modification of a coactivator at certain sites; alternatively, specific kinases can phosphorylate these sites to promote higher cellular levels of coactivator.

CELL AND SIGNALING CONTEXT

The cell context plays a role in selective gene responses to ligand because differentiation produces cells with specific available gene complements for expression. The cell also has a predetermined basal concentration of each of the coregulators and their cognate activating/inactivating enzymes, thereby establishing a threshold of available regulatory molecules. This cellular concentration of coregulators provides the potential for activity. For actual conversion to active functional molecules, however, the coregulators must be regulated by a variety of post-translational modifications, such as phosphorylation, ubiquitinylation, acetylation, SUMOylation, methylation, etc. In general, coactivators are activated by phosphorylations and monoubiquitylations.[36] Protein-protein interactions in the large coactivator complexes are regulated by acetylations and methylations. Coactivators are inactivated by SUMOylation and degraded after polyubiquitylations. These general rules often vary for a given coactivator. Considering the crucial role that post-translational modifications play in coactivator function, it is logical to assume that the roles of signaling pathways that contain these modifying enzymes also play important roles. Since the signaling pathways have certain cell specificities and are subject to environmental stimuli for their regulation, cell context can play a role in selective activities of SRMs.

OTHER REGULATORY INFLUENCES

Because equilibrium reactions are the basis for biology, the promotional and contradictory influences inherent to the cell can affect coregulator function and transcriptional potency. As discussed above, coregulator concentrations are subject to turnover by ubiquitin-dependent and ubiquitin-independent proteasomal degradation pathways, whose activities can be abrogated by certain counteracting kinases. Therefore the cell concentrations and activation of degradation pathways for coregulators can play a role in SRM actions. In addition, in vivo systemic metabolism and selective cellular uptake or metabolism of ligands can sometimes modify SRM activities.

WHAT ARE THE MOST IMPORTANT FACTORS FOR TISSUE-SPECIFIC SRM ACTIVITY?

The cell levels of activated coregulators are the primary determinant of tissue-specific SRM activity.[13] Having described herein the complete interacting equations and complexities of coregulator function, it remains that (1) the cellular complement of coregulators and (2) the cell and signaling context are the primary determinants of coregulator function. Consequently, they are the primary determinants of SRM functions.

SRMs are generally mixed antagonist/agonist ligands for receptors. When a receptor is occupied by a mixed antagonist/agonist ligand, the conformation generated in the receptor is neither purely antagonistic nor purely agonistic for activity. Rather, the conformation is intermediate for both functions (Fig 3). A pure agonist induces a receptor conformation that has a strong affinity for coactivators. A pure antagonist induces a receptor conformation that has a strong affinity for corepressors. The mixed antagonist/agonist ligand induces an intermediate conformation that, in turn, is intermediate in its affinity for both coactivators and corepressors. In other words, this receptor conformation is programmed by the local concentrations of activated coactivators and corepressors. The mechanism will obey the laws of physical chemistry. If the cellular concentration of preferred

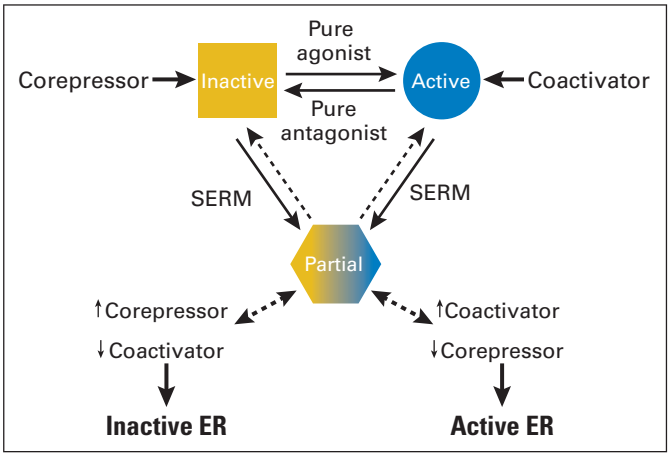

Fig 3. Hypothesis for tissue specific effects of selective estrogen-receptor modulators (SERMs). A schematic is shown for the contributions of coactivators and corepressors to the tissue-specific antagonist/agonist activities of a steroid receptor modulator (SRM). In the presence of a pure antagonist, a receptor is stabilized in the inactive conformational state and binds corepressor tightly. In the presence of a pure agonist, it conforms to a fully active conformation and binds coactivator tightly. In the presence of a mixed antagonist/agonist SRM, the receptor adopts an intermediate partial conformation that is neither fully inactive or fully active. In this intermediate conformation, the SRM-bound receptor is then even more subject to interactions with the relative intracellular concentrations of preferred coactivators or corepressors for its activity.

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coactivators is high (or corepressors low), then the receptor is forced into the active conformation by the excess of coactivators and receptor dependent gene expression takes place. If the cellular concentration of preferred corepressors is high (or coactivators low), then the receptor is forced into the inactive conformation by the excess of corepressors and receptor-dependent gene expression is shut down. Since activation of coregulators occurs by post-translational modifications, the status of the cell signaling pathways that produces these post-translational modifications is an overlying modulator of SRM activity.

With this background of the physiologic basis for SERM action, it is now appropriate to meld these emerging data with the current applications of SERMs in the clinic and the evolving ideas about drug resistance to SERMs.

CURRENT THERAPY WITH SERMs

The clinical application[5] of the laboratory strategy of long-term antihormonal therapy[37-39] as an adjuvant to treat breast cancer has now become the standard of care. Two approaches to antihormonal therapy have occurred during the last three decades: long-term treatment to block estrogen-stimulated growth at the level of the tumor ER[39] and, subsequently, the use of aromatase inhibitors to block estrogen biosynthesis in postmenopausal patients.[6] It is clear that the aromatase inhibitors offer advantages over tamoxifen as adjuvant treatments for postmenopausal patients; there are fewer adverse effects (blood clots and endometrial cancer), and aromatase inhibitors have a small but significant improved efficacy.[40,41] However, substantial numbers of postmenopausal patients continue to receive tamoxifen treatment either for economic reasons or because they are hysterectomized and at low risk for blood clots (low body mass index or they are athletically active). Postmenopausal women who have completed 2 to 5 years of adjuvant tamoxifen are also eligible for a further 5 years of antihormonal therapy with an aromatase inhibitor.[42-44] However, the veteran SERM tamoxifen is still the antihormonal treatment of choice for premenopausal patients and the antihormonal treatment for ductal carcinoma in situ (DCIS),[45] and remains the appropriate treatment to reduce breast cancer risk in premenopausal women at elevated risk.[46] It is important to stress that premenopausal women treated with tamoxifen do not experience elevations in endometrial cancer and blood clots, so the risk:benefit ratio is strongly in favor of tamoxifen treatment.[47]

The development of raloxifene[48] has created a new therapeutic dimension. Raloxifene is used either as a treatment and preventive for osteoporosis but with a quantifiable decrease in the incidence of breast cancer,[49,50] or as an agent for the reduction of breast cancer incidence in high-risk postmenopausal women.[51] The advantage of raloxifene as a SERM is that there are no increases in endometrial cancer[51,52] incidence previously noted with tamoxifen in postmenopausal women.[46,53]

The target site-specific actions of tamoxifen and raloxifene in breast and endometrial cancer were first noted in the laboratory,[54,55] but the question to be asked is why. On the basis of our earlier arguments about the mechanism of actions of SERMs, studies of the cellular context and coactivator content demonstrate the tissue-specific actions of tamoxifen and raloxifene in the uterine cancer cell.[56]

Overall, the SERM concept[10,11] clearly works in clinical practice, but the use of long-term SERM treatment regimens raises the important issue of the eventual development of drug resistance. Laboratory studies have already shown that long-term SERM treatment changes the pharmacology from an antiestrogen- to SERM-stimulated growth.[57,58] This acquired resistance is a topic of immediate clinical concern.

THE DIMENSION OF DRUG RESISTANCE TO SERMs

There are currently three possible mechanisms for drug resistance to tamoxifen. Either the patient can influence the effectiveness of tamoxifen via alterations in metabolism, or the ER-positive tumor is or can become refractory to treatment. These mechanisms are illustrated in Figure 4.

Metabolic Resistance

The metabolic activation of tamoxifen occurs via demethylation to *N*-desmethyltamoxifen and subsequently transformation to the hydroxy metabolite endoxifen.[59,60] This topic has recently been reviewed[61] and will therefore be mentioned only briefly. Metabolic activation appears to be important for tamoxifen to acquire potent antiestrogenic and antitumor activity. Although large-scale prospective clinical trials have not been completed to prove the hypothesis definitively in large populations, there is sufficient preliminary data to warrant further study. Extensive laboratory studies demonstrate[62] that endoxifen is formed by the CYP2D6 enzyme system. However, there are wide variations in the CYP2D6 enzyme in the population that can influence drug metabolism. The wild-type CYP2D6 enzyme is referred to as CYP2D61*, whereas CYP2D64*/4* is a null variant. It is estimated that approximately 10% of the population have CYP2D6 variants, so the case can be made that these patients should be considered for other antiestrogenic interventions (eg, aromatase inhibitors). Another dimension for consideration is the control of menopausal symptoms, especially hot flushes. If tamoxifen is a prodrug and needs to be converted to endoxifen to achieve maximal antitumor activity at the tumor ER, then these same patients may have severe hot flushes. The selective serotonin reuptake inhibitors (SSRIs) have been found to be of value to treat hot flushes. The widespread use of tamoxifen as a long-term adjuvant therapy, especially in premenopausal patients, has naturally increased SSRI use. Unfortunately, the SSRIs such as fluoxetine and paroxetine are potent inhibitors of the CYP2D6 enzyme.[63] Therefore, symptom treatment has the potential to undermine the efficacy of tamoxifen if the incorrect SSRI is employed. Venlafaxine has a very low affinity for the CYP2D6 enzyme system and may be the agent of choice for treatment of hot flushes.[63] It should, however, be pointed out that there is no substantial clinical evidence to support this conclusion. A larger body of prospective clinical data is required to confirm the admittedly compelling preliminary studies.

Intrinsic Resistance

A proportion of ER-positive tumors are intrinsically resistance to tamoxifen therapy. Historically, metastatic breast cancer that is ER and PR positive is approximately 80% responsive to antihormonal therapy (endocrine ablation or tamoxifen) whereas tumors that are ER positive but PR negative are only 40% responsive to antihormonal therapy.[64,65] We have known for about 20 years that enhanced growth factor signaling via the human epidermal growth factor receptor 1 (HER-1; EGFR) pathway impairs estrogen induction PR in breast cancer cells[66] and enhanced paracrine growth factor stimulation undermines that effectiveness of antiestrogen treatment at the ER.[67,68]

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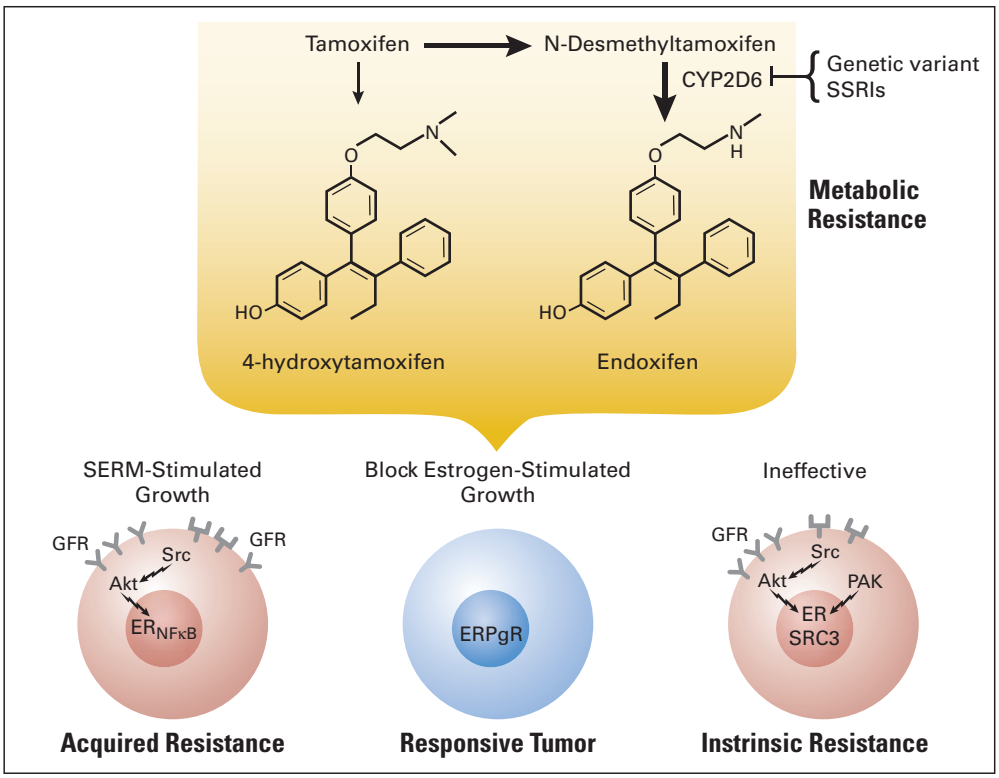

Fig 4. The possible types of drug resistance to the selective estrogen-receptor modulators (SERMs), particularly tamoxifen. Tamoxifen is a prodrug that needs to be metabolically activated by CYP2D6 to the active antiestrogen endoxifen. Mutation of CYP2D6 or the administration of specific selective serotonin reuptake inhibitors (SSRIs; eg, paroxetine or fluoxetine) to reduce hot flushes impairs metabolic activation and reduces the efficacy to tamoxifen. Tamoxifen and its hydroxylated metabolites are most effective at blocking estrogen-stimulated tumor growth if the cells contain both estrogen receptor (ER) and progesterone receptor (PgR). In contrast, tamoxifen is much less effective in controlling the growth of tumors, have high levels of membrane growth factor receptors (GFRs) that can activate phosphorylation cascades via Src, Akt and PAK. The ER and coactivator SRC3 could be targets for phosphorylation in these PgR-negative tumors. The tumor has intrinsic resistance to tamoxifen treatment. In contrast, tumors that initially respond to tamoxifen can acquire resistance to tamoxifen by increasing the level of GFR that phosphorylates Src and Akt. These SERM (tamoxifen)-stimulated tumors have increased nuclear levels of nuclear factor κ B (NFκB) but the tumors still rely on the ER for survival as second line treatments with either aromatase inhibitors (to block local estrogen production) or fulvestrant (to block the ER and cause preventative destruction) can result in the control of tumor growth.

These earlier observations have recently been confirmed and extended using breast cancer cells artificially transfected with insulin-like growth factor receptor[69] and using large tumor databases.[70] Tumor cell drug resistance to tamoxifen develops very quickly (8 weeks) in athymic mice with HER-2/*neu* engineered MCF-7 cells[71] compared with the natural process of more than 6 months.[57] Tamoxifen acts as an agonist in experimentally engineered breast cancer cells with high levels of the HER-2/*neu* growth factor receptor and the coactivator SRC3 (AIB1).[72]

In another approach, the possible connection between HER-2/*neu*, ER, PR and tamoxifen resistance has been evaluated in a tissue database linked to clinical outcomes. Intrinsic tamoxifen resistance is associated with HER-2/*neu*–, ER-positive, PR-negative tumors that have an increase in coactivator SRC3 (AIB1) levels.[73] Although the actual number is a small group of approximately 10% to 15% breast cancer patients, it does perhaps provide a clue to test who should avoid tamoxifen treatment.

The idea that growth factor receptor could be a predictor of SERM resistance has recently[74,75] been extrapolated to explain the reason for aromatase inhibitors being superior to tamoxifen as adjuvant therapy. A retrospective analysis[76] shows that patients with ER-positive, PR-negative tumors are more likely to respond to aromatase inhibitors than to tamoxifen. However, the conclusions, though attractive, require confirmation with prospective studies because of inconsistencies with the results from other direct trial databases comparing tamoxifen with an aromatase inhibitor and the recent reevaluation of the steroid receptor database in the original study of tamoxifen and anastrozole.[77]

Acquired Resistance

Laboratory studies show that the treatment of athymic mice implanted with ER-positive, PR-positive MCF-7 tumors with continuous tamoxifen will eventually develop tamoxifen-stimulated tumors that will grow in response to either tamoxifen or estradiol.[57] Either no treatment or treatment with the pure antiestrogen fulvestrant[57,78,79] results in no tumor growth. Because no treatment in the ovariectomized athymic mouse is equivalent to treatment with an aromatase inhibitor and fulvestrant destroys the ER,[80] one could conclude that tumor growth is prevented in the absence of a stimulatory signal transduction pathway. This hypothesis is consistent with the clinical observation that anastrozole and fulvestrant treatment are equivalent after the failure of tamoxifen therapy.[81,82]

Goss et al[42] demonstrated that patients with ER-positive tumors and treated for 5 years with tamoxifen continue to be responsive to subsequent treatment with 5 years of the aromatase inhibitor letrozole.[83] This result could be interpreted as the slow development of acquired resistance by the breast cancer micrometastases during 5 years of tamoxifen so that these patients respond to a non–cross-resistant therapy that prevents tumor growth by blocking the ability of the patient to synthesize estrogen. Thus, the use of letrozole after tamoxifen is incrementally building on the already established long-term antitumor effect of tamoxifen that lasts for at least 10 years after the cessation of adjuvant therapy.[5]

CONSEQUENCES OF LONG-TERM ANTIHORMONE THERAPY

Laboratory models of drug resistance should replicate the duration of SERM administration to patients. Most laboratory models of antihormone resistance are either engineered with stable transfection of the HER-2/*neu* gene into MCF-7 cells[72,84] or reflect the early development of resistance (SERM-stimulated growth)[57] to treatment. This later form of resistance is consistent with tamoxifen failure during the

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treatment of metastatic disease. Under these clinical circumstances, tamoxifen treatment is effective for approximately 1 year. This form of SERM resistance is referred to as phase I.[85] However, tamoxifen is used as an adjuvant therapy for 5 years,[86] and it is reasonable to suggest that raloxifene will need to be administered for 10 years or more to maintain effectiveness as an antiosteoporosis medicine. Current studies[49] show that up to 8 years of raloxifene reduces the majority of (65%) but not all ER-positive breast cancers. Some tumors must, therefore, become raloxifene resistant.

The repeated transplantation of MCF-7 breast tumors into successive generations of tamoxifen-treated ovariectomized athymic mice for more than 5 years replicates the exposure of tumor cells to adjuvant tamoxifen. This approach to study SERM resistance results in a continuing dependence on tamoxifen to produce growth, but cross-resistance with the SERMs toremifene and raloxifene develops[79,87,88] and a significant change in the response of tamoxifen or raloxifene resistant cells to physiologic estradiol.[87,89,90] The signaling pathways for estrogen no longer support growth, but initiate apoptosis by inducing fas receptor, rapidly reducing levels of HER-2/*neu* and reducing nuclear factor κ B (NFκB) levels.[91] This form of SERM resistance is referred to as phase II resistance.[85] As might be expected, the pure antiestrogen fulvestrant can completely prevent tumor growth in animals. Paradoxically, when combined with physiological estrogen, fulvestrant not only reverses the apoptotic actions of estrogen but also causes robust tumor growth.[91] The mechanism for this therapeutically relevant observation is unclear, but may involve a dramatic upregulation of HER-2 and HER-3[92] but may also involve the recently described ligand (estrogen, SERM, fulvestrant) activator G protein GPR30.[93] It is possible that this novel observation may have value to plan an appropriate strategy to use fulvestrant plus an aromatase inhibitor as a third-line endocrine therapy.[94] The widespread clinical use of aromatase inhibitors now brings up the question of the consequences of the long-term use of aromatase inhibitors as adjuvant therapies. There will be an eventual development of drug resistance.

Early studies of estrogen deprivation in cell culture demonstrated that cellular ER levels and spontaneous cell replication increase.[95,96] Subsequent studies demonstrated that the cells initially become supersensitized to the growth properties of minute quantities of estrogen,[97,98] but as the duration of estrogen deprivation is extended, the cells respond to estrogen with the initiation of apoptosis.[99] This observation[99] has been used to explain the earlier application of high-dose estrogen therapy to treat postmenopausal women with metastatic breast cancer.[100] However, estrogen-deprived cell lines only need very low concentrations of estrogen in the postmenopausal range (lnM) to initiate apoptosis.[101,102] Cell death occurs through an increase in proapoptotic genes[103] and can be enhanced by specifically reducing the synthesis of bcl-2.[104] These preclinical studies are being translated to clinical trials by destroying phase II antihormone-resistant breast cancer cells with limited low-dose estrogen therapy followed by maintenance with further treatment with an aromatase inhibitor treatment.[103]

An alternate approach to study the development of drug resistance to aromatase inhibitors in vivo utilizes ER-positive MCF-7 breast cancer cells stably transfected with the CYP19 aromatase enzyme gene.[105] The cells grow into tumors in athymic mice treated with the enzyme substrate androstenedione that is converted to estrone.[106] The model has been used effectively to examine the integration of SERM and aromatase inhibitor therapy and has effectively replicated the clinical experience.[107-110] Results not only clearly demonstrate the efficacy of aromatase inhibitors when compared with tamoxifen but also demonstrate the development of resistance to aromatase inhibitors.[111] Aromatase resistant tumors become more dependent on growth factor receptor pathways via mitogen-activated protein kinase.[112,113]

Overall, the basic knowledge of SERM action and the development of laboratory models of antihormonal resistance are proving invaluable to identify molecular targets for future advances in cancer therapeutics. Important clues about the pivotal role of SRCs in SERM drug resistance and tumor cell survival are already apparent. We predict that further progress in cancer cell biology will occur through an enhanced investment to understand the modulatory mechanisms of NRs and their coactivator partners. The new knowledge will create unanticipated opportunities to control cancer in the future.

FUTURE POTENTIAL FOR NEW SRM DEVELOPMENT

With the advent of this recent knowledge of the molecular mechanisms of action of transcriptional regulators such as NRs and coregulators, new insights to drug development are rapidly becoming available. The discovery of tamoxifen as a SERM and the successful development of additional SERMs such as raloxifene, have encouraged exploitation of the SERM concept[10,11] by pharmaceutical companies to discover additional new SRM ligands for other NRs. Some examples are selective progestin modulators (SPRMs)[114,115] that inhibit uterine cancer but are devoid of stimulatory action in the breast; selective androgen receptor modulators (SARMs)[116,117] that are anabolic for muscle and bone, but spare the prostate; selective glucocorticoid receptor modulators (SGRMs)[118] that are strongly anti-inflammatory but do not induce glucose intolerance and connective tissue destruction; and selective peroxisome proliferator-activated receptor γ (PPARγ) receptor modulators (SPARMs)[119-121] that promote insulin sensitivity.[13] All of the foregoing examples are under current development or are being tested in clinical trials. In the case of each of these SRMs, the molecular mechanisms and pathways for their efficacy described herein represent the guiding principles for their tissue-specific actions and represent a substantial health care return for the investment in basic mechanistic scientific research.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: V. Craig Jordan, Bert W. O'Malley
Administrative support: V. Craig Jordan
Provision of study materials or patients: Bert W. O'Malley
Collection and assembly of data: V. Craig Jordan, Bert W. O'Malley
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REFERENCES

1. Jensen EV, Jacobson HI: Basic guides to the mechanism of estrogen action. Recent Progr Hormone Res 18:387-414, 1962

2. McGuire WL, Carbone PP, Sears ME, et al: Estrogen receptors in human breast cancer: An overview, in McGuire WL, Carbone PP, Volmer EP (eds): Estrogen Receptor in Human Breast Cancer. New York, NY, Raven Press, 1975, pp 1-7

3. Harper MJ, Walpole AL: A new derivative of triphenylethylene: Effect on implantation and mode of action in rats. J Reprod Fertil 13:101-119, 1967

4. Jordan VC: Tamoxifen: A most unlikely pioneering medicine. Nat Rev Drug Discov 2:205-213, 2003

5. EBCTCG: Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. Lancet 365:1687-1717, 2005

6. Jordan VC, Brodie AMH: Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 72:7-25, 2007

7. Jordan VC: Selective estrogen receptor modulation: A personal perspective. Cancer Res 61:5683-5687, 2001

8. Jordan VC, Phelps E, Lindgren JU: Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10:31-35, 1987

9. Gottardis MM, Jordan VC: Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 47:4020-4024, 1987

10. Jordan VC: Chemosuppression of breast cancer with tamoxifen: Laboratory evidence and future clinical investigations. Cancer Invest 6:589-595, 1988

11. Lerner LJ, Jordan VC: The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. Cancer Res 50:4177-4189, 1990

12. Cummings SR, Eckert S, Krueger KA, et al: The effect of raloxifene on risk of breast cancer in postmenopausal women: Results from the MORE randomized trial—Multiple Outcomes of Raloxifene Evaluation. JAMA 281:2189-2197, 1999

13. Smith CL, O'Malley BW: Coregulator function: A key to understanding tissue specificity of selective receptor modulators. Endocr Rev 25:45-71, 2004

14. Mangelsdorf DJ, Thummel C, Beato M, et al: The nuclear receptor superfamily: The second decade. Cell 83:835-839, 1995

15. Tsai MJ, O'Malley BW: Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451-486, 1994

16. McKenna NJ, O'Malley BW: Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 108:465-474, 2002

17. Lonard DM, O'Malley BW: The expanding cosmos of nuclear receptor coactivators. Cell 125:411-414, 2006

18. Glass CK, Rosenfeld CS: The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev 14:121-141, 2000

19. Oñate SA, Tsai SY, Tsai MJ, et al: Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270:1354-1357, 1995

20. Weihua Z, Andersson S, Cheng G, et al: Update on estrogen signaling. FEBS Lett 546:17-24, 2003

21. Lieberman ME, Gorski J, Jordan VC: An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741-4745, 1983

22. Jordan VC, Lieberman ME: Estrogen-stimulated prolactin synthesis in vitro: Classification of agonist, partial agonist, and antagonist actions based on structure. Mol Pharmacol 26:279-285, 1984

23. Murphy CS, Langan-Fahey SM, McCague R, et al: Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. Mol Pharmacol 38:737-743, 1990

24. Wolf DM, Jordan VC: The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31:129-138, 1994

25. Levenson AS, Catherino WM, Jordan VC: Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. J Steroid Biochem Mol Biol 60:261-268, 1997

26. Jordan VC, Koch R, Mittal S, et al: Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: Modulation of prolactin synthesis in vitro. Br J Pharmacol 87:217-223, 1986

27. Jordan VC: Laboratory models of breast cancer to aid the elucidation of antiestrogen action. J Lab Clin Med 109:267-277, 1987

28. Allan GF, Leng X, Tsai SY, et al: Hormone and antihormone induce distinct conformational changes which are central to steroid receptor activation. J Biol Chem 267:19513-19520, 1992

29. Vegeto E, Allan GF, Schrader WT, et al: The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. Cell 69:703-713, 1992

30. Brzozowski AM, Pike AC, Dauter Z, et al: Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753-758, 1997

31. Shiau AK, Barstad D, Loria PM, et al: The structural basis of estrogen receptor/co-activator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927-937, 1998

32. Togashi M, Borngraeber S, Sandler B, et al: Conformational adaptation of nuclear receptor ligand binding domains to agonists: Potential for novel approaches to ligand design. J Steroid Biochem Mol Biol 93:127-137, 2005

33. Lefstin JA, Yamamoto KR: Allosteric effects of DNA on transcriptional regulators. Nature 392:885-888, 1998

34. Shang Y, Hu X, DiRenzo J, et al: Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 103:843-852, 2000

35. Li X, Lonard DM, Jung SY, et al: The SRC-3/AIB1 coactivator is degraded in a ubiquitin- and ATP-independent manner by the REGgamma proteasome. Cell 124:381-392, 2006

36. O'Malley BW: Little molecules with big goals. Science 313:1749-1750, 2006

37. Jordan VC, Allen KE: Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239-251, 1980

38. Jordan VC, Allen KE, Dix CJ: Pharmacology of tamoxifen in laboratory animals. Cancer Treat Rep 64:745-759, 1980

39. Jensen EV, Jordan VC: The estrogen receptor: A model for molecular medicine: The Dorothy P Landon AACR Prize for Translational Research. Clin Cancer Res 9:1980-1989, 2003

40. Howell A, Cuzick J, Baum M, et al: Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 365:60-62, 2005

41. Thürlimann B, Keshaviah A, Coates AS, et al: A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. N Engl J Med 353:2747-2757, 2005

42. Goss PE, Ingle JN, Martino S, et al: Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: Updated findings from NCIC CTG MA.17. J Natl Cancer Inst 97:1262-1271, 2005

43. Boccardo F, Rubagotti A, Puntoni M, et al: Switching to anastrozole versus continued tamoxifen treatment for early breast cancer: Preliminary results of the Italian Tamoxifen Anastrozole Trial. J Clin Oncol 23:5138-5147, 2005

44. Coombes RC, Hall E, Gibson LJ, et al: A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. N Engl J Med 350:1081-1092, 2004

45. Fisher B, Dignam J, Wolmark N, et al: Tamoxifen in treatment of intraductal breast cancer: National Surgical Adjuvant Breast and Bowel Project B-24 randomised controlled trial. Lancet 353:1993-2000, 1999

46. Fisher B, Costantino JP, Wickerham DL, et al: Tamoxifen for prevention of breast cancer: Report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90:1371-1388, 1998

47. Gail MH, Costantino JP, Bryant J, et al: Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. J Natl Cancer Inst 91:1829-1846, 1999

48. Lewis JS, Jordan VC: Case histories: Raloxifene, in Taylor J, Triggle D (eds): Comprehensive Medicinal Chemistry II, Volume 8. Oxford, United Kingdom, Elsevier Limited, 2007, pp 103-121

49. Martino S, Cauley JA, Barrett-Connor E, et al: For the CORE Investigators Continuing Outcomes Relevant to Evista: Breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 96:1751-1761, 2004

50. Jordan VC: Optimising endocrine approaches for the chemoprevention of breast cancer: Beyond the Study of Tamoxifen and Raloxifene (STAR) trial. Eur J Cancer 42:2909-2913, 2006

51. Vogel VG, Costantino JP, Wickerham DL, et al: The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. JAMA 295:2727-2741, 2006

52. Barrett-Connor E, Mosca L, Collins P, et al: Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 355:125-137, 2006

53. Fisher B, Costantino JP, Wickerham DL, et al: Tamoxifen for the prevention of breast cancer: Current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97:1652-1662, 2005

54. Gottardis MM, Robinson SP, Satyaswaroop PG, et al: Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48:812-815, 1988

55. Gottardis MM, Ricchio ME, Satyaswaroop PG, et al: Effect of steroidal and nonsteroidal antiestrogens

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on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 50:3189-3192, 1990

56. Shang Y, Brown M: Molecular determinants for the tissue specificity of SERMs. Science 295: 2465-2468, 2002

57. Gottardis MM, Jordan VC: Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 48:5183-8187, 1988

58. O'Regan RM, Osipo C, Ariazi E, et al: Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. Clin Cancer Res 12:2255-2263, 2006

59. Stearns V, Johnson MD, Rae JM, et al: Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. J Natl Cancer Inst 95:1758-1764, 2003

60. Jin Y, Desta Z, Stearns V, et al: CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. JNCI 97:30-39, 2005

61. Jordan VC: New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer. Steroids (in press)

62. Desta Z, Ward BA, Soukhova NV, et al: Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: Prominent roles for CYP3A and CYP2D6. J Pharmacol Exp Ther 310:1062-1075, 2004

63. Borges S, Desta Z, Li L, et al: Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: Implication for optimization of breast cancer treatment. Clin Pharmacol Ther 80:61-74, 2006

64. Bloom ND, Robin EH, Schreibman B, et al: The role of progesterone receptor in the management of advanced breast cancer. Cancer 45:2992-2997, 1980

65. Osborne CK, Yochmowitz MG, Knight WA, et al: The value of estrogen and progesterone receptors in the treatment of breast cancer. Cancer 46: 2884-2888, 1980

66. Cormier EM, Wolf MF, Jordan VC: Decrease in estradiol-stimulated progesterone receptor production in MCF-7 cells by epidermal growth factor and possible clinical implication for paracrine-regulated breast cancer growth. Cancer Res 49:576-580, 1989

67. Cormier EM, Jordan VC: Contrasting ability of antiestrogens to inhibit MCF-7 growth stimulated by estradiol or epidermal growth factor. Eur J Cancer Clin Oncol 25:57-63, 1989

68. Robinson SP, Jordan VC: The paracrine stimulation of MCF-7 cells by MDA-MB-231 cells: Possible role in antiestrogen failure. Eur J Cancer Clin Oncol 25:493-497, 1989

69. Cui X, Zhang P, Deng W, et al: Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: Progesterone receptor as a potential indicator of growth factor activity in breast cancer. Mol Endocrinol 17:575-588, 2003

70. Arpino G, Weiss H, Lee AV, et al: Estrogen receptor-positive, progesterone receptor-negative breast cancer: Association with growth factor receptor expression and tamoxifen resistance. J Natl Cancer Inst 97:1254-1261, 2005

71. Benz CC, Scott GK, Sarup JC, et al: Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res Treat 24:85-95, 1992

72. Shou J, Massarweh S, Osborne CK, et al: Mechanisms of tamoxifen resistance: Increased estrogen receptor-HER2/neu cross-talk in ER/HER2–positive breast cancer. J Natl Cancer Inst 96:926-935, 2004

73. Osborne CK, Bardou V, Hopp TA, et al: Role of the estrogen receptor coactivator AIB1 (SRC3) and HER2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst 95:353-361, 2003

74. Dowsett M, Harper-Wynne C, Boeddinghaus I, et al: HER-2 amplification impedes the antiproliferative effects of hormone therapy in estrogen receptor-positive primary breast cancer. Cancer Res 61:8452-8458, 2001

75. Ellis MJ, Coop A, Singh B, et al: Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: Evidence from a phase III randomized trial. J Clin Oncol 19:3808-3816, 2001

76. Dowsett M, Cuzick J, Wale C, et al: Retrospective analysis of time to recurrence in the ATAC trial according to hormone receptor status: An hypothesis-generating study. J Clin Oncol 23:7512-7517, 2005

77. Jordan VC, Gelber R: Problems with the progesterone receptor in practice? J Clin Oncol 25:1957-1959, 2007

78. Osborne CK, Coronado-Heinsohn EB, Hilsenbeck SG, et al: Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. J Natl Cancer Inst 87:746-750, 1995

79. Lee ES, MacGregor-Schafer JI, Yao K, et al: Cross resistance of triphenylethylene-type antiestrogen but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6:4893-4899, 2000

80. Wijayaratne AL, McDonnell DP: The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. J Biol Chem 276:35684-35692, 2001

81. Osborne CK, Pippen J, Jones SE, et al: Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: Results of a North American trial. J Clin Oncol 20:3386-3395, 2002

82. Howell A, Robertson JFR, Quaresma Albano J, et al: Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. J Clin Oncol 20:3396-3403, 2002

83. Goss PE, Ingle JN, Martino S, et al: Efficacy of letrozole extended adjuvant therapy according to estrogen receptor and progesterone receptor status on the primary tumor. J Clin Oncol 25:2006-2011, 2007

84. Massarweh S, Osborne CK, Jiang S, et al: Mechanisms of tumor regression and resistance to estrogen deprivation and fulvestrant in a model of estrogen receptor-positive, HER-2/neu-positive breast cancer. Cancer Res 66:8266-8273, 2006

85. Jordan VC: Selective estrogen receptor modulation: Concept and consequences in cancer. Cancer Cell 5:207-213, 2004

86. EBCTCG: Tamoxifen for early breast cancer: An overview of the randomised trials. Lancet 354: 1451-1467, 1998

87. Wolf DM, Jordan VC: A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127:23-33, 1993

88. O'Regan RM, Gajdos C, Dardes RC, et al: Effects of raloxifene after tamoxifen on breast and endometrial tumor growth in athymic mice. J Natl Cancer Inst 94:274-283, 2002

89. Yao K, Lee ES, Bentrem DJ, et al: Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6:2028-2036, 2000

90. Liu H, Lee ES, Gajdos C, et al: Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 95:1586-1597, 2003

91. Osipo C, Gajdos C, Liu H, et al: Paradoxical action of fulvestrant on estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 95:1597-1608, 2003

92. Osipo C, Meeke K, Cheng D, et al: Role for HER2/neu and HER3 in fulvestrant-resistant breast cancer. Int J Oncol 30:509-520, 2007

93. Prossnitz ER, Arterburn JB, Sklar LA: GPR30: A G protein-coupled receptor for estrogen. Mol Cell Endocrinol 265- 266:138-142, 2007

94. Jordan VC, Lewis JS, Osipo C, et al: The apoptotic action of estrogen following exhaustive antihormonal therapy: A new clinical treatment strategy. Breast 14:624-630, 2005

95. Katzenellenbogen BS, Kendra KL, Norman MJ, et al: Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. Cancer Res 47: 4355-4360, 1987

96. Welshons WV, Jordan VC: Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. Eur J Cancer 232:1935-1939, 1987

97. Berstein LM, Wang JP, Zheng H, et al: Long-term exposure to tamoxifen induces hypersensitivity to estradiol. Clin Cancer Res 10:1530-1534, 2004

98. Shim WS, Conaway M, Masamura S, et al: Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. Endocrinology 141: 396-405, 2000

99. Song RX, Mor G, Naftolin F, et al: Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 93:1714-1723, 2001

100. Haddow A, Watkinson JM, Paterson E: Influence of synthetic oestrogens upon advanced malignant disease. BMJ 2:393-398, 1944

101. Lewis JS, Osipo C, Meeke K, et al: Estrogen induced apoptosis in breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 94:131-141, 2005

102. Lewis JS, Meeke K, Osipo C, et al: Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. JNCI 97:1746-1759, 2005

103. Jordan VC, Lewis-Wambi J, Kim H, et al: Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients. Breast (in press)

104. Song RX, Zhang Z, Mor G, et al: Down-regulation of Bcl-2 enhances estrogen apoptotic action in long-term estradiol-depleted ER+ breast cancer cells. Apoptosis 10:667-678, 2005

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105. Zhou DJ, Pompon D, Chen SA: Stable expression of human aromatase complementary DNA in mammalian cells: A useful system for aromatase inhibitor screening. Cancer Res 50:6949-6954, 1990

106. Brodie AH, Jelovac D, Long B: The intratumoral aromatase model: Studies with aromatase inhibitors and antiestrogens. J Steroid Biochem Mol Biol 86:283-288, 2003

107. Brodie AH, Mouridsen HT: Applicability of the intratumor aromatase preclinical model to predict clinical trial results with endocrine therapy. Am J Clin Oncol 26:S17-26, 2003

108. Jelovac D, Macedo L, Handratta V, et al: Effects of exemestane and tamoxifen in a postmenopausal breast cancer model. Clin Cancer Res 10:7375-7381, 2004

109. Long BJ, Jelovac D, Handratta V, et al: Therapeutic strategies using the aromatase inhibitor letrozole and tamoxifen in a breast cancer model. J Natl Cancer Inst 96:456-465, 2004

110. Jelovac D, Macedo L, Goloubeva OG, et al: Additive antitumor effect of aromatase inhibitor letrozole and antiestrogen fulvestrant in a postmenopausal breast cancer model. Cancer Res 65: 5439-5444, 2005

111. Long BJ, Jelovac D, Thiantanawat A, et al: The effect of second-line antiestrogen therapy on breast tumor growth after first-line treatment with the aromatase inhibitor letrozole: Long-term studies using the intratumoral aromatase postmenopausal breast cancer model. Clin Cancer Res 8:2378-2388, 2002

112. Jelovac D, Sabnis G, Long BJ, et al: Activation of mitogen-activated protein kinase in xenografts and cells during prolonged treatment with aromatase inhibitor letrozole. Cancer Res 65:5380-5389, 2005

113. Sabnis GJ, Jelovac D, Long B, et al: The role of growth factor receptor pathways in human breast cancer cells adapted to long-term estrogen deprivation. Cancer Res 65:3903-3910, 2005

114. Chabbert-Buffet N, Meduri G, Bouchard P, et al: Selective progesterone receptor modulators and progesterone antagonists: Mechanisms of action and clinical applications. Hum Reprod Update 11: 293-307, 2005

115. Spitz IM: Progesterone receptor antagonists and selective progesterone receptor modulators (SPRMs). Semin Reprod Med 23:3-7, 2005

116. Gao W, Dalton JT: Expanding the therapeutic use of androgens via selective androgen receptor modulators (SARMs). Drug Discov Today 12:241-248, 2007

117. Trump RP, Blanc JB, Stewart EL, et al: Design and synthesis of an array of selective androgen receptor modulators. J Comb Chem 9:107-114, 2007

118. Kym PR, Kort ME, Coghlan MJ, et al: Nonsteroidal selective glucocorticoid modulators: The effect of C-10 substitution on receptor selectivity and functional potency of 5-allyl-2,5-dihydro-2,2,4-trimethyl-1H-[1]benzopyrano[3,4-f]quinolines. J Med Chem 46:1016-1030, 2003

119. Sporn MB, Suh N, Mangelsdorf DJ: Prospects for prevention and treatment of cancer with selective PPARgamma modulators (SPARMs). Trends Mol Med 7:395-400, 2001

120. Kopelovich L, Fay JR, Glazer RI, et al: Peroxisome proliferator-activated receptor modulators as potential chemopreventive agents. Mol Cancer Ther 1:357-363, 2002

121. Schupp M, Clemenz M, Gineste R, et al: Molecular characterization of new selective peroxisome proliferator-activated receptor gamma modulators with angiotensin receptor blocking activity. Diabetes 54:3442-3452, 2005

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Review

Tamoxifen: Catalyst for the change to targeted therapy

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ABSTRACT

In the early 1970s, a failed post-coital contraceptive, ICI 46,474, was reinvented as tamoxifen, the first targeted therapy for breast cancer. A cluster of papers published in the European Journal of Cancer described the idea of targeting tamoxifen to patients with oestrogen receptor positive tumours, and proposed the strategic value of using long-term tamoxifen therapy in an adjuvant setting with a consideration of the antitumour properties of the hydroxylated metabolites of tamoxifen. At the time, these laboratory results were slow to be embraced by the clinical community. Today, it is estimated that hundreds of thousands of breast cancer patients are alive today because of targeted long-term adjuvant tamoxifen therapy. Additionally, the first laboratory studies for the use of tamoxifen as a chemopreventive were published. Eventually, the worth of tamoxifen was tested as a chemopreventive and the drug is now known to have an excellent risk benefit ratio in high risk pre-menopausal women. Overall, the rigorous investigation of the pharmacology of tamoxifen facilitated tamoxifen's ubiquitous use for the targeted treatment of breast cancer, chemoprevention and pioneered the exploration of selective oestrogen receptor modulators (SERMs). This new concept subsequently heralded the development of raloxifene, a failed breast cancer drug, for the prevention of osteoporosis and breast cancer without the troublesome side-effect of endometrial cancer noted in post-menopausal women who take tamoxifen. Currently, the pharmaceutical industry is exploiting the SERM concept for all members of the nuclear receptor superfamily so that medicines can now be developed for diseases once thought impossible.

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1. Introduction

A new dynasty gives dominion over the ruling dynasty through perseverance and not by sudden action (Ibn Khaldun 14th Century Arab Historian) – and so it is with changes in the approach to cancer therapy. This article will focus specifically on a cluster of scientific papers[1–3] published in the European Journal of Cancer that presaged the dramatic changes that have occurred in the past 35 years in our approach to cancer therapy. To set the scene, it is first appropriate to describe the research and treatment philosophy for breast cancer before tamoxifen.

In the 1960s, the use of combination cytotoxic chemotherapy for the treatment of breast cancer had moved to centre stage in the wake of an abstract presented at the American Association for Cancer Research.[4] The cytotoxic 'cocktail' presented by Cooper, containing cyclophosphamide, methotrexate, 5 fluorouracil, vincristine and prednisone (CMFVP), produced a dramatic response rate of >80% in patients with advanced breast cancer. In the 1960s, there was every reason to believe that cancer would be curable if (1) the right drug combination could be found; (2) the patient could be kept alive through the aggressive high dose regimens; and (3) pa-

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tients could be treated with a low tumour burden. Cytotoxic chemotherapy became king and a new dynasty was established with the initiation of a lexicon of drug combinations and schedules and ultimately, bone marrow transplantation. The introduction of adjuvant therapy, as it turned out, would be essential for the successes we see today when the move occurred from cytotoxic chemotherapy to tamoxifen treatment. The initial hypothesis for the use of cytotoxic chemotherapy was reasonable and logical; adjuvant chemotherapy would destroy undetected micrometastases harboured around the patient's body after surgical removal of the primary tumour. The perfect result would be enhanced cures for women with breast cancer but the biology of breast cancer conspired to defeat the best attempts of oncologists to deploy non-specific cytotoxic chemotherapy effectively. The hypothesis was flawed.

It is the responsibility of each new generation to challenge the fashions in medicine created by the ruling dynasty. Progress by defying the dynasty can be profound and today we witness the results of an unlikely revolution in thinking that had its roots in the 1970s. Around the world, death rates from breast cancer are declining and patients are living longer, recurrence-free lives with less morbidity. Tamoxifen is an integral reason for current progress, but this was unanticipated in the 1970s. Thirty-five years ago it would have been unthinkable to suggest that 'hormone therapy' would enhance survivorship and that breast cancer risk reduction would now be a clinical reality.

Our knowledge of human oncogenes, an unknown idea in 1972 (C-src the first oncogene was described in 1976) now provides invaluable clues to exploit, selectively, the metabolic vulnerabilities in cancer. This knowledge is creating justifiable optimism by targeting the disease specifically with new agents. The current generation has witnessed the clinical (and economic!) success of agents like trastuzumab that targets gene amplified HER2-neu[5] in select breast cancers to produce disease control[6–8] not previously thought possible. However, the new era of individualised targeted medicines that promises 'to kill or prevent the cancer but not harm the patient' did not start with biotechnology.

The origins of targeted therapy started in the 1970s by challenging cytotoxic chemotherapy with an alternative approach to treatment which was achieved by adapting the pharmacological principles of drug receptor theory to cancer care. At that time, cancer research was considered to be a hopeless career choice, but a series of events put the right people in the right place at the right time to recognise a unique opportunity to advance cancer therapeutics. No advances occur in isolation; they build on the work of previous generations and in this case, by collegial interaction.

2. Tamoxifen (ICI 46,474) before targeting

ICI 46,474, the antioestrogenic *trans* isomer of a substituted triphenylethylene, was discovered in the laboratories of Imperial Chemical Industries (ICI) Ltd. Pharmaceuticals Division (now AstraZeneca). The team, Dora Richardson (Chemist), Michael J.K. Harper (Reproductive Endocrinologist) and Arthur L. Walpole (Head of Reproduction Research) was tasked with developing a post-coital contraceptive during the early 1960s based on the structural clues already published by other pharmaceutical companies. All of the studies conducted at ICI throughout the 1960s were focused on reproduction and the patent issued throughout the world (except the United States where the patent was denied for 20 years because the findings did not demonstrate innovation) stated 'the alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesteraemic activity'. Claims that the compounds could be used as a breast cancer treatment had to be removed from the patent applications in America as they were considered to be fantastic![9] More importantly, there was no evidence to back up the claim.

Walpole was not only interested in reproductive endocrinology but also cancer research and treatment.[10] The scientists at ICI had found an unusual species specificity with ICI 46,474; the compound was apparently a classical oestrogen in the mouse vagina but an antioestrogen in rat tests.[11,12] The question was what was the pharmacology of ICI 46,474 in humans: an oestrogen or an antioestrogen? Walpole advanced clinical testing of ICI 46,474 in both 'the control of hormone dependent tumours' and 'the regulation of the sexual cycle'. Clinical testing was initiated to evaluate activity to treat breast cancer at the Christie Hospital in Manchester and the Princess Margaret Hospital, Birmingham[13,14] and reproductive cycle studies proceeded elsewhere.[15] In 1972, all conclusions were reviewed by ICI Ltd. Pharmaceuticals Division in Alderley Park, Macclesfield, Cheshire. Unlike the results observed in the rat, ICI 46,474[16] was not a contraceptive in humans. The drug induced ovulation and could potentially be used as a profertility agent.[15] ICI 46,474 exhibited modest activity as a breast cancer therapy which was equivalent to historical controls treated with high dose oestrogens or androgens.[13] The advantage of tamoxifen, that was to be critical for future applications, was a low incidence of toxic side-effects. However, the decision by senior management was to abandon further development,[9,17] primarily because the financial return for co-marketing a breast cancer drug used by a limited number of patients for about a year for the palliation of metastatic breast cancer was too small and there was virtually no market for another agent to induce ovulation in subfertile women. Clomiphene was already the medicine of choice.[18]

Walpole responded by electing to take early retirement if ICI 46,474 did not get marketed. He was at the end of his scientific career and he truly believed that tamoxifen had promise if only further studies could be completed on the 'orphan drug'. But how would this occur? Walpole and I met in September, 1972, when he was the external examiner of my PhD entitled 'Structure function relationships of some triphenylethylenes and triphenylethanes' at the University of Leeds. Following this meeting, Walpole provided resources for me to conduct the scientific work that reinvented a failed contraceptive to become the first targeted therapy for the treatment and prevention of breast cancer. We collaborated until his untimely death in 1977.[10]

3. Foundations

In 1969, I was seduced by the idea of crystallising the oestrogen receptor (OER) with an oestrogen and a non-steroidal antioestrogen. My supervisor thought it would be a little uninteresting, but at least the project would be straightforward as Leeds had a premier X-ray crystallography department called the Astbury Department of Biophysics. The OER protein could be easily extracted from uteri,[19,20] but I quickly found that purification was not a simple task. I switched my PhD topic to study the pharmacology of non-steroidal antioestrogens. As it turned out, this was a good career choice as no one has yet succeeded in crystallising the whole liganded OER!

I wanted to develop drugs for cancer, but there were no opportunities to pursue this goal during my PhD. What made life more complicated in 1972 was the fact that the University could not find anyone to be my external examiner; no one cared about the pharmacology of failed contraceptives! Although administrators at the University protested against the choice of someone from industry, Arthur Walpole was eventually appointed as my examiner; a fortunate event that was subsequently to advance the clinical application of tamoxifen by establishing a scientific foundation through an investigation of its antitumour actions in the laboratory.

During the final year of my PhD, I was invited to stay at Leeds as a lecturer in Pharmacology. However, first I was required to go to the Worcester Foundation for Experimental Biology (now the Worcester Foundation for Biomedical Research, part of the University of Massachusetts Medical School) to work with Michael Harper, Walpole's former colleague at ICI. When I arrived in September 1972, Harper declared that he had accepted a job at the World Health Organisation in Geneva and that 'I could do anything I wanted for the next two years'.

Here was the opportunity I wanted. A phone call to Walpole at ICI secured his enthusiastic financial support to re-examine ICI 46,474 in the laboratory, but this time the focus would be its mechanism of action as an anticancer agent. I was made a consultant to introduce ICI 46,474 to clinical trials groups in American and Lois Trench, the drug monitor for Stuart Pharmaceuticals (ICI Americas in Wilmington, Delaware) coordinated all administrative details between 1972 and 1974 to get the project off the ground. But how to start?

Elwood V. Jensen, Director of the Ben May Research Laboratory was on the scientific advisory board for the Worcester Foundation in 1972 (Fig. 1). During his visit in late 1972, we spent time going over my thesis and I explained what I wanted to do with ICI 46,474. He generously invited me to Chicago the next year to learn sucrose density gradient analysis in order to study whether tamoxifen blocked oestradiol binding to the human and animal OER. I also learned how to induce mammary tumours in rats using dimethylbenzanthracene (DMBA) so that the mechanism of antitumour action of tamoxifen could be evaluated under controlled laboratory conditions. The DMBA model was the only model available at the time to study hormones and cancer. The work commenced at the Worcester Foundation in the summer of 1973 and by the end of the year, results were pouring out. Lois Trench secured human tumours for sucrose density gradient analysis, but I felt no pressure to publish as no one was really interested. Chemotherapy was king and no one anticipated that another 'hormone therapy' would be an advance. As a pharmacologist, I was just happy to be contributing to the development of an anticancer drug.

Fig. 1 – V. Craig Jordan and Elwood V. Jensen on the occasion of learning they were going to be the inaugural recipients of the Dorothy P. Landon/AACR Prize (2002) for Translational Research. This is the highest award presented by the AACR and recognised the seminal work for both of these scientists; Elwood Jensen identified OER as the mediator of oestrogen action in its target tissues and some breast tumours, and Craig Jordan's research that reinvented ICI 46,474 from being a failed contraceptive to the first targeted therapy for breast cancer as the drug tamoxifen.

Avoiding writing up my results could not last forever. Dr. Eliahu Caspi, a senior scientist at the Worcester Foundation, was directed to interview me to explore the possibility of me staying at the Worcester Foundation and not returning to Leeds. This was a surprise, but there was an even bigger surprise in store when he glared at me over his desk and announced 'that I did not have a CV because I had not any publications'. I announced I had not yet solved any problems so what was the point? And he proceeded to give me the best advice of my academic career up to that time. 'Tell them the story so far; each paper should take no longer than two weeks to write-up and link together a series of studies with a theme'. I have not stopped writing since; which brings me back to the three papers I eventually published in the European Journal of Cancer.[1–3]

4. Transition to targeting Tamoxifen (Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 1975;11:205–6)

Lars Terenius published two important papers in the European Journal of Cancer that described the action of nafoxidine for the treatment of DMBA-induced rat mammary tumours[21] and the ability of the first non-steroidal antioestrogen MER 25[22] to prevent rat mammary carcinogenesis.[23] These studies demonstrated 'proof of principle' for the application of antioestrogens to treat breast cancer, but neither compound showed any promise in the clinic because of serious toxic side-effects.[24,25] In fact, this was the consistent story for all of the antioestrogens, except for tamoxifen.

ICI, 46,474 was examined systematically in my laboratory to explore mechanisms and applications that could be exploited in the clinic. These studies were supported by ICI with unrestricted funds, first at the Worcester Foundation (1972–1974) and subsequently at the University of Leeds as a University Joint/Research Scheme (1974–1979). Most importantly, ICI arranged for thousands of rats to be chauffeured from Alderley Park to Leeds so I could complete my work. Those free rats, as it turned out, would be worth their weight in gold with the billions of pounds of profits earned with tamoxifen! Simultaneously, Rob Nicholson, at the Tenovus Institute in Cardiff started to use tamoxifen as a laboratory tool to investigate oestrogen and antioestrogen action in the DMBA-induced rat mammary tumour model. Again, these studies were published in the European Journal of Cancer.[26–28]

The studies I conducted in the laboratory initially focused on the ER as a therapeutic target. The questions that were addressed were 'can tamoxifen block oestrogen binding?' and 'is tamoxifen the active agent?' ICI 46,474 has a very low binding affinity for the ER and we used sucrose density gradient analysis to provide the first consistent evidence that tamoxifen blocks the binding of oestradiol to the human breast and endometrial cancer 8S oestrogen receptor.[1] We focused specifically on the role of the OER in tamoxifen action during the mid 1970s so that there would be a better understanding of tamoxifen action in its target tissues, the mammary tumour and uterus.[29–34]

At this time, we also made the observation that hydroxylated metabolites played an important role in the antioestrogenic and antitumour actions of tamoxifen.[35,36] We concluded that it was an advantage, but not a requirement, for tamoxifen to be metabolically activated to 4-hydroxytamoxifen. As a result of these studies, 4-hydroxytamoxifen became the standard laboratory tool to study the molecular biology of antioestrogen action *in vitro* and in 1998 was used to crystallise the ligand binding domain of the OER with an antioestrogenic molecule.[37] The key to this accomplishment was that 4-hydroxytamoxifen has about a 100× higher binding affinity for the OER than tamoxifen.

5. Tamoxifen for prevention? (Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. Eur J Cancer 1976;12:419–24)

In 1936, Profession Antoine Lacassagne suggested, based on his animal studies, that 'a therapeutic antagonist should be found to prevent the congestion of oestrone in the breast' so that breast cancer could be prevented.[38] Forty years later, the first experiment I was to complete with tamoxifen showed that just two injections of the 'antioestrogen' would almost completely prevent carcinogenesis in the rat mammary gland.[2,39] I concluded that the mechanism was most likely blocking oestrogen action at the level of the OER in the mammary tissue and nascent tumour. These and subsequent studies[40–42] provided the scientific foundation for the eventual examination of the worth of tamoxifen to prevent breast cancer in high risk pre- and post-menopausal women.[43–46] The key to tamoxifen's success in this application was a sustained duration of action and its ability to produce antitumour actions long after the therapy has stopped.[44,47]

6. Long-term adjuvant tamoxifen therapy (Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 1980;16:239–51)

In the 1970s, the initial clinical studies of tamoxifen were focused entirely on its application as a treatment for metastatic breast cancer. The efficacy of tamoxifen was the same as that of high dose oestrogen therapy (diethylstilboestrol 15 mg daily), but the advantage of tamoxifen was fewer serious side-effects.[13,48] The translation of the early laboratory findings with tamoxifen[1,2] to the treatment of advanced breast cancer showed an association between the efficacy of tamoxifen as an antitumour agent and OER status.[49] However, it was the transition from the use of tamoxifen as a palliative therapy to adjuvant therapy that was to have the greatest impact on survivorship and to establish tamoxifen as the gold standard for antihormonal therapy from 1980 to 2000.

The goal of adjuvant therapy is to destroy the micrometastases that have spread around the body at the time of primary surgery. Early results with chemotherapy were extremely promising[50,51] and some significant improvements were noted once the overview analysis of worldwide randomised clinical trials was analysed and published.[52] However, the use of tamoxifen in this application was less readily accepted

because of the belief that tamoxifen was only a palliative therapy. As a prelude to the application of tamoxifen as an adjuvant therapy, I introduced the antioestrogen first to the Eastern Cooperative Oncology Group (ECOG)[53,54] and subsequently to the National Surgical Breast and Bowel Project (NSABP).[55] Early adjuvant clinical trials selected one year of adjuvant therapy[56–60] because of the fact that tamoxifen was effective in unselected patients with advanced breast cancer for about one year and there was a sincere concern that longer therapy would induce pre-mature drug resistance. These beliefs were to change in the mid 1970s with the laboratory finding that long-term antihormonal therapy was more effective than short-term therapy.

Marc Lippman published an important paper in 1975 on the actions of tamoxifen in cell culture.[61] He demonstrated that oestradiol could reverse the action of tamoxifen to stop

Fig. 2 – Participants at a Breast Cancer Symposium in September 1977 at Kings College, Cambridge, England. The concept of extended adjuvant tamoxifen treatment was first proposed at this meeting. Clinical studies of a 1-year adjuvant tamoxifen were in place; regrettably, a decade later this approach was shown to produce little survival benefit for patients. In the insets (top), the author, who presented the new concept (bottom left); Professor Michael Baum, the session chairman who was about to launch the Nolvadex Adjuvant Trial Organization (NATO) 2-year adjuvant tamoxifen trial[95,96]; and (bottom right) Dr. Helen Stewart, who was a participant at the conference. She would initiate a pilot trial in 1978 and, led by Sir Patrick Forest, would later guide the full randomised Scottish Trial of 5 years' adjuvant tamoxifen treatment versus control in the 1980s.[97] Both clinical trials were later proven to produce survival advantages for patients. The concept of longer tamoxifen treatment producing more survival benefits for patients was eventually established indirectly by the Oxford Overview Analysis in 1992 and directly by the Swedish group led by Dr. Lars Rutqvist.[98]

cell replication and that tamoxifen could actually kill breast cancer cells at high concentrations. We decided to test the idea that tamoxifen was cytotoxic *in vivo* using the DMBA-induced rat mammary carcinoma model.

We reasoned that daily treatment with tamoxifen for a month in the rat would be equivalent to a year in a woman. Administration of DMBA (20 mg in 2 ml peanut oil po) to 50-day-old female Sprague–Dawley rats resulted in the development of multiple mammary tumours in all animals about 150 d later.[62] The experimental approach we used was to administer different daily doses of tamoxifen for a month starting one month after DMBA administration. This design was to allow carcinogenesis to proceed following DMBA administration so that we could assess the effectiveness of tamoxifen to destroy the microfoci of deranged cells in the mammary tissue. This was as close as one could get to an endocrine adjuvant model in the 1970s.

Tamoxifen was compared with 4-hydroxytamoxifen because we had found it was the most potent antioestrogen then known[31]; at least 10 times more potent than tamoxifen. We chose to test 4-hydroxytamoxifen because we anticipated that it would be a more potent antitumour agent than tamoxifen. To our surprise, not only was 4-hydroxytamoxifen not as effective as tamoxifen, but short-term tamoxifen was unable to 'cure' animals. High doses of tamoxifen were superior to low doses of tamoxifen in reducing tumour numbers and controlled tumour appearance, but all animals eventually developed at least one tumour. Clearly, there was a link between dose and anticancer action, but it was because higher doses were cleared from the body more slowly and not that the higher dose was more active. Tamoxifen was acting as a tumouristatic agent – the drug was effective as long as the drug was present to suppress tumour growth (Fig. 2).[3,63,64] We proved this concept experimentally by showing that antioestrogens were effective at controlling tumourigenesis as long as treatment was continued. Indeed, if tumours occurred during antioestrogen therapy, they would respond to a second antihormone therapy, in this case, oestrogen withdrawal following ovariectomy. We concluded '*It was clear that antioestrogens do not destroy all the foci of hormone dependent tumour cells and long courses of therapy or the use of antihormonal methods e.g. ovariectomy are essential to control tumour growth*'.[3] This notion lead to the idea that longer was going to be better as a strategy to employ for adjuvant tamoxifen therapy and provided a scientific foundation for the successful use of subsequent oestrogen deprivation, i.e. an aromatase inhibitor following 5 years of tamoxifen treatment.[65,66]

The overview analysis of randomised clinical trials that occurs about every five years at Oxford has really revolutionised clinical thinking. The publications summarise treatment progress through the clinical trials mechanism. The clinical proof that longer tamoxifen therapy is better than shorter tamoxifen therapy is most readily demonstrated in the OER positive pre-menopausal patients. One year of tamoxifen was ineffective, but 5 years produced a dramatic effect on disease-free survival and overall survival.[67] More importantly, tamoxifen produced a survival advantage for women, of a magnitude that would change the perception of endocrine agents as only palliative. The key to success was targeting women with the right tumour with the correct duration of treatment at the right stage.

7. Conclusion

What were the consequences of reinventing a failed contraceptive ICI46,474[16] to become tamoxifen, the first targeted agent for the treatment of breast cancer[9]? The laboratory strategy of targeting OER positive tumours[1] with long-term adjuvant therapy[3,64] ultimately resulted in the improved survivorship of hundreds of thousands of women [67,68] around the world. Indeed, the fact that tamoxifen is cheap and accessible to under-funded healthcare systems worldwide means that this form of targeted therapy continues to save lives. However, unlike the targeted therapies of today that usually have a single anticancer application, tamoxifen became the gold standard for the targeted therapy of all stages of breast cancer (including male breast cancer), the treatment of ductal carcinoma in situ,[69] a pioneering agent for the chemoprevention of breast cancer in high risk women[45,70,71] and the lead compound for the new drug group, the SERMs.[72–76]

The extensive laboratory studies of tamoxifen and the related non-steroidal antioestrogen LY156,758 (keoxifene) undertaken as a prelude to initiating major trials in breast cancer prevention, described the pharmacology of SERMs that switch on and switch off target sites throughout the body. As an example of the immediate translation of the discovery of SERM action, tamoxifen was noted to block breast cancer growth but enhances the growth of endometrial cancer growth under laboratory conditions.[77] This laboratory concept translated to improved clinical care through awareness that tamoxifen increased the incidence of endometrial cancer in post-menopausal women treated for breast cancer. In another example of the application of SERMs, a failed breast cancer drug, keoxifene, was reinvented[42,72,78] as raloxifene, the first SERM to be successfully used to treat osteoporosis with the beneficial side-effect of preventing breast cancer indirectly.[79,80] Following rigorous testing in clinical trials,[81] raloxifene is now also available to prevent breast cancer in high risk post-menopausal women. The overall result of 30 years of translational research in breast cancer prevention is that there are now two therapeutic options, tamoxifen and raloxifene, for women who choose to reduce their risk of breast cancer.[81,82] Thirty years ago there were no choices. Based on clinical testing, tamoxifen has a good risk benefit ratio in pre-menopausal women[83] and raloxifene has a better safety profile in post-menopausal women.[81] It should be stressed, however, that raloxifene cannot be used to reduce breast cancer risk in premenopausal women.

Perhaps of greater significance is the fact that tamoxifen has become a pioneering agent to initiate new investigations in therapeutics. A study of the pharmacology of tamoxifen has been the catalyst to study the pharmacogenomics of tamoxifen which is redefining healthcare.[84] It appears that the specific metabolism of tamoxifen to a hydroxylated metabolite endoxifen is important for anticancer actions. This topic has recently been reviewed.[85] Finally, the importance of understanding the unique pharmacology of tamoxifen can be placed in perspective. In retrospect, tamoxifen could, in fact, be viewed as the lead compound that was essential to initiate the synthesis of a broad range of new SERMs for the treatment

of diseases as diverse as osteoporosis[86–92] and rheumatoid arthritis[93,94] and the subsequent extrapolation of the SERM concept to all members of the nuclear receptor superfamily.[76] The advances documented with targeting tamoxifen now offer the promise of designing drugs to treat diseases previously thought to be impossible.

Conflict of interest statement

None declared.

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REFERENCES

1. Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *Eur J Cancer* 1975;**11**:205–6.
2. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. *Eur J Cancer* 1976;**12**:419–24.
3. Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 1980;**16**:239–51.
4. Cooper RG. Combination chemotherapy in hormone resistant breast cancer. *Proc AACR* 1963;**10**:15.
5. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science* 1987;**235**:177–82.
6. Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *New Engl J Med* 2005;**353**:1673–84.
7. Joensuu H, Kellokumpu-Lehtinen PL, Bono P, et al. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. *New Engl J Med* 2006;**354**:809–20.
8. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *New Engl J Med* 2005;**353**:1659–72.
9. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2003;**2**:205–13.
10. Jordan VC. The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. *Breast Cancer Res Treat* 1988;**11**(3):197–209.
11. Harper MJ, Walpole AL. Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. *Nature* 1966;**212**(57):87.
12. Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. *J Reprod Fertil* 1967;**13**:101–19.
13. Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. *Br J Cancer* 1971;**25**:270–5.
14. Ward HW. Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. *Br Med J* 1973;**1**(844):13–4.
15. Klopper A, Hall M. New synthetic agent for the induction of ovulation. Preliminary trial in women. *Br Med J* 1971;**1**:152–4.
16. Harper MJ, Walpole AL. Mode of action of I.C.I. 46,474 in preventing implantation in rats. *J Endocrinol* 1967;**37**(1):83–92.
17. Jordan VC. Tamoxifen (ICI 46,474) as a targeted therapy to treat and prevent breast cancer. *Brit J Pharmacol* 2006;**147**:S269–76.
18. Greenblatt RB, Roy S, Mahesh VB, Barfeld WE, Jungck EL. Induction of ovulation with clomiphene citrate. *Am J Obstet Gynecol* 1962;**84**:900–12.
19. Toft D, Gorski J. A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *Proc Natl Acad Sci USA* 1966;**55**(6):1574–81.
20. Toft D, Shyamala G, Gorski J. A receptor molecule for estrogens: studies using a cell-free system. *Proc Natl Acad Sci USA* 1967;**57**(6):1740–3.
21. Terenius L. Anti-oestrogens and breast cancer. *Eur J Cancer* 1971;**7**(1):57–64.
22. Lerner LJ, Holthaus JF, Thompson CR. A non-steroidal estrogen antagonist 1-(*p*-2-diethylaminoethoxyphenyl)-1-phenyl-2-*p*-methoxyphenylethanol. *Endocrinology* 1958;**63**:295–318.
23. Terenius L. Effect of anti-oestrogens on initiation of mammary cancer in the female rat. *Eur J Cancer* 1971;**7**:65–70.
24. Lerner LJ. The first non-steroidal antiestrogen-MER 25. In: Sutherland RL, Jordan VC, editors. *Nonsteroidal antioestrogens: molecular pharmacology and antitumour activity.* Sydney, Australia: Sydney Academic Press; 1981. p. 1–6.
25. Legha SS, Slavik M, Carter SK. Nafoxidine—an antiestrogen for the treatment of breast cancer. *Cancer* 1976;**38**(4):1535–41.
26. Nicholson RI, Golder MP. The effect of synthetic anti-oestrogens on the growth and biochemistry of rat mammary tumours. *Eur J Cancer* 1975;**11**(8):571–9.
27. Nicholson RI, Davies P, Griffiths K. Effects of oestradiol-17beta and tamoxifen on nuclear oestradiol-17beta receptors in DMBA-induced rat mammary tumours. *Eur J Cancer* 1977;**13**(3):201–8.
28. Nicholson RI, Golder MP, Davies P, Griffiths K. Effects of oestradiol-17beta and tamoxifen on total and accessible cytoplasmic oestradiol-17beta receptors in DMBA-induced rat mammary tumours. *Eur J Cancer* 1976;**12**(9):711–7.
29. Jordan VC, Koerner S. Tamoxifen as an anti-tumour agent: role of oestradiol and prolactin. *J Endocrinol* 1976;**68**(02):305–11.
30. Jordan VC, Jaspan T. Tamoxifen as an antitumour agent: oestrogen binding as a predictive test for tumour response. *J Endocrinol* 1976;**68**:453–60.
31. Jordan VC, Dowse LJ. Tamoxifen as an anti-tumour agent: effect on oestrogen binding. *J Endocrinol* 1976;**68**(02):297–303.
32. Jordan VC. Antiestrogenic and antitumor properties of tamoxifen in laboratory animals. *Cancer Treat Rep* 1976;**60**(10):1409–19.
33. Jordan VC, Dix CJ, Rowsby L, Prestwich G. Studies on the mechanism of action of the nonsteroidal antioestrogen tamoxifen (I.C.I. 46,474) in the rat. *Mol Cell Endocrinol* 1977;**7**(2):177–92.
34. Jordan VC, Prestwich G. Binding of [3H]tamoxifen in rat uterine cytosols: a comparison of swinging bucket and vertical tube rotor sucrose density gradient analysis. *Mol Cell Endocrinol* 1977;**8**(3):179–88.
35. Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* 1977;**75**(2):305–16.
36. Allen KE, Clark ER, Jordan VC. Evidence for the metabolic activation of non-steroidal antioestrogens: a study of

structure–activity relationships. *Br J Pharmacol* 1980;**71**(1):83–91.
37. Shiau AK, Barstad D, Loria PM, et al. The structural basis of estrogen receptor/co-activator recognition and the antagonism of this interaction by tamoxifen. *Cell* 1998;**95**:927–37.
38. Lacassagne A. Hormonal pathogenesis of adenocarcinoma of the breast. *Am J Cancer* 1936;**27**:217–25.
39. Jordan VC. Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. *J Steroid Biochem* 1974;**5**:354.
40. Jordan VC, Allen KE, Dix CJ. Pharmacology of tamoxifen in laboratory animals. *Cancer Treat Rep* 1980;**64**(6-7):745–59.
41. Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the *N*-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* 1987;**47**(15):4020–4.
42. Jordan VC, Lababidi MK, Mirecki DM. Anti-oestrogenic and anti-tumour properties of prolonged tamoxifen therapy in C3H/OUJ mice. *Eur J Cancer* 1990;**26**(6):718–21.
43. Powles TJ, Hardy JR, Ashley SE, et al. A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *Br J Cancer* 1989;**60**(1):126–31.
44. Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. *J Natl Cancer Inst* 2007;**99**(4):283–90.
45. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 1998;**90**:1371–88.
46. Veronesi U, Maisonneuve P, Costa A, et al. Prevention of breast cancer with tamoxifen: preliminary findings from the Italian randomised trial among hysterectomised women. Italian Tamoxifen Prevention Study. *Lancet* 1998;**352**(9122):93–7.
47. Cuzick J, Forbes JF, Sestak I, et al. Long-term results of tamoxifen prophylaxis for breast cancer—96-month follow-up of the randomized IBIS-I trial. *J Natl Cancer Inst* 2007;**99**(4):272–82.
48. Ingle JN, Ahmann DL, Green SJ, et al. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *New Engl J Med* 1981;**304**:16–21.
49. Kiang DT, Kennedy BJ. Tamoxifen (antiestrogen) therapy in advanced breast cancer. *Ann Intern Med* 1977;**87**(6):687–90.
50. Fisher B, Carbone PP, Economou SG, et al. L-Phenylalanine mustard (L-PAM) in the management of primary breast cancer. A report of early findings. *New Engl J Med* 1975;**292**:117–22.
51. Bonadonna G, Bursamolino GE, Valaguss P, et al. Combination chemotherapy as an adjuvant treatment in operable breast cancer. *New Engl J Med* 1976;**294**:405–10.
52. EBCTCG. Effects of adjuvant tamoxifen and of cytotoxic therapy on mortality in early breast cancer. An overview of 61 randomized trials among 28,896 women. *New Engl J Med* 1988;319(26):1681–92.
53. Jordan VC. The antioestrogen tamoxifen (ICI 46,474) as an antitumour agent. In: Proceedings of the eastern cooperative oncology group meeting, Miami, Florida; February 11–12, 1974.
54. Jordan VC. Tamoxifen mechanism of antitumour activity in animals and man. In: Proceedings of the eastern cooperative oncology group meeting, Jasper, Alberta; June 22–25, 1974.
55. Tamoxifen Workshop. In: Proceedings of the annual spring meeting of the primary breast cancer therapy group, Key Biscayne, Florida, April 28. *Cancer Treat Rep* 1976;60:1409–66.
56. Randomised trial of chemo-endocrine therapy, endocrine therapy, and mastectomy alone in postmenopausal patients with operable breast cancer and axillary node metastasis. Ludwig Breast Cancer Study Group. *Lancet* 1984;1(8389): 1256–60.
57. Pritchard KI, Meakin JW, Boyd NF, et al. A randomized trial of adjuvant tamoxifen in postmenopausal women with axillary node positive breast cancer. In: Jones SE, Salmon SE, editors. *Adjuvant therapy of breast cancer IV.* New York: Grune and Stratton; 1984. p. 339–48.
58. Rose C, Thorpe SM, Andersen KW, et al. Beneficial effect of adjuvant tamoxifen therapy in primary breast cancer patients with high oestrogen receptor values. *Lancet* 1985;**1**:16–29.
59. Ribeiro G, Palmer MK. Adjuvant tamoxifen for operable carcinoma of the breast: report of clinical trial by the Christie Hospital and Holt Radium Institute. *Br Med J (Clin Res Ed)* 1983;**286**(6368):827–30.
60. Ribeiro G, Swindell R. The Christie Hospital tamoxifen (Nolvadex) adjuvant trial for operable breast carcinoma—7-yr results. *Eur J Cancer Clin Oncol* 1985;**21**(8):897–900.
61. Lippman ME, Bolan G. Oestrogen-responsive human breast cancer in long term tissue culture. *Nature* 1975;**256**:592–3.
62. Huggins C, Grand LC, Brillantes FP. Mammary cancer induced by a single feeding of polynuclear hydrocarbons and their suppression. *Nature* 1961:189–204.
63. Jordan VC. Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. *Rev Endocr-Relat Cancer* 1978(October Suppl.):49–55.
64. Jordan VC, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. *Adjuvant Ther Cancer* 1979;**2**:19–26.
65. Goss PE, Ingle JN, Marino S, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. *New Engl J Med* 2003;**349**:1–10.
66. Goss PE, Ingle JN, Martino S, et al. Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17. *J Natl Cancer Inst* 2005;**97**:1262–71.
67. EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;354:1451–67.
68. EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005;365:1687–717.
69. Fisher B, Dignam J, Wolmark N, et al. Tamoxifen in treatment of intraductal breast cancer: National Surgical Adjuvant Breast and Bowel Project B-24 randomised controlled trial. *Lancet* 1999;**353**(9169):1993–2000.
70. Cuzick J, Powles T, Veronesi U, et al. Overview of the main outcomes in breast-cancer prevention trials. *Lancet* 2003;**361**:296–300.
71. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* 2005;**97**:1652–62.
72. Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 1988;**6**(5):589–95.
73. Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res* 1990;**50**:4177–89.
74. Jordan VC. Designer estrogens. *Sci Am* 1998;**279**(4):60–7.
75. Jordan VC. Selective estrogen receptor modulation: a personal perspective. *Cancer Res* 2001;**61**:5683–7.
76. Jordan VC, O'Malley BM. Selective estrogen receptor modulators and antihormonal resistance in breast cancer. *J Clin Oncol* 2007; in press, doi:10.1200/JCO.2007.11.3886.

77. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 1988;**48**:812–5.
78. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 1987;**10**(1):31–5.
79. Cummings SR, Eckert S, Krueger KA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* 1999;**281**:2189–97.
80. Jordan VC. Optimising endocrine approaches for the chemoprevention of breast cancer. Beyond the Study of Tamoxifen and Raloxifene (STAR) trial. *Eur J Cancer* 2006;**42**:2909–13.
81. Vogel VG, Costantino JP, Wickerham DL, et al. The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA* 2006;**295**:2727–41.
82. Jordan VC. Tamoxifen or raloxifene for breast cancer chemoprevention: a tale of two choices. *Cancer Epidemiol Biomar Prev* 2007;**16**:2207–9.
83. Gail MH, Costantino JP, Bryant J, et al. Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J Natl Cancer Inst* 1999;**91**(21):1829–46.
84. Goetz MP, Rae JM, Suman VJ, et al. Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. *J Clin Oncol* 2005;**23**(36):9312–8.
85. Jordan VC. New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer. *Steroids* 2007;**72**:829–42.
86. Renaud J, Bischoff SF, Buhl T, et al. Estrogen receptor modulators: identification and structure–activity relationships of potent ERalpha-selective tetrahydroisoquinoline ligands. *J Med Chem* 2003;**46**:2945–57.
87. Kim S, Wu JY, Birzin ET, et al. Estrogen receptor ligands. II. Discovery of benzoxathiins as potent, selective estrogen receptor alpha modulators. *J Med Chem* 2004;**47**:2171–5.
88. Renaud J, Bischoff SF, Buhl T, et al. Selective estrogen receptor modulators with conformationally restricted side chains. Synthesis and structure–activity relationship of Eralpha-selective tetrahydroisoquinoline ligands. *J Med Chem* 2005;**48**:364–79.
89. Pigeon P, Top S, Vessieres A, et al. Selective estrogen receptor modulators in the ruthenocene series. Synthesis and biological behavior. *J Med Chem* 2005;**48**:2814–21.
90. Gungor T, Chen Y, Golla R, et al. Synthesis and characterization of 3-arylquinazolinone and 3-arylquinazolinethione derivatives as selective estrogen receptor beta modulators. *J Med Chem* 2006;**49**(8): 2440–55.
91. Jain N, Kanojia RM, Xu J, et al. Novel chromene-derived selective estrogen receptor modulators useful for alleviating hot flushes and vaginal dryness. *J Med Chem* 2006;**49**(11):3056–9.
92. Qin Z, Kastrati I, Chandrasena RE, et al. Benzothiophene selective estrogen receptor modulators with modulated oxidative activity and receptor affinity. *J Med Chem* 2007;**50**:2682–92.
93. Steffan RJ, Matelan E, Ashwell MA, et al. Synthesis and activity of substituted 4-(indazol-3-yl)phenols as pathway-selective estrogen receptor ligands useful in the treatment of rheumatoid arthritis. *J Med Chem* 2004;**47**(26):6435–8.
94. Keith Jr JC, Albert LM, Leathurby Y, et al. The utility of pathway selective estrogen receptor ligands that inhibit nuclear factor-kappa B transcriptional activity in models of rheumatoid arthritis. *Arthritis Res Ther* 2005;**7**(3):R427–38.
95. NATO. Controlled trial of tamoxifen as adjuvant agent in management of early breast cancer. Interim analysis at four years by Nolvadex Adjuvant Trial Organisation. *Lancet* 1983;1(8319):257–61.
96. Baum M, Brinkley DM, Dossett JA, et al. Improved survival among patients treated with adjuvant tamoxifen after mastectomy for early breast cancer [letter]. *Lancet* 1983;**2**(8347):450.
97. Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee Scottish Cancer Trials Office (MRC), Edinburgh. *Lancet* 1987;2:171–5.
98. Randomized trial of two versus five years of adjuvant tamoxifen for postmenopausal early stage breast cancer. Swedish Breast Cancer Cooperative Group [see comments]. *J Natl Cancer Inst* 1996;88(21):1543–49.

Low-Dose Estrogen Therapy to Reverse Acquired Antihormonal Resistance in the Treatment of Breast Cancer

Ramona F. Swaby, V. Craig Jordan

Abstract

Estrogen is a potent stimulus for growth in its target organs: the uterus, vagina, and some estrogen receptor–positive breast cancers. However, estrogen is also able to control menopausal symptoms and maintain bone density in postmenopausal women. Until recently, there was also believed to be a link between estrogen and the prevention of cardiovascular disease. For these reasons, hormone replacement therapy (HRT) with an orally active estrogen and progesterone has been used routinely for more than 50 years to maintain physiologic homeostasis after menopause. Not surprisingly, HRT increases the risk of developing breast cancer. The link between estrogen and breast cancer growth served as the incentive to develop long-term tamoxifen therapy and, subsequently, the aromatase inhibitors (AIs) as successful "anti-estrogenic" treatments. Unfortunately, the consequence of exhaustive therapy is drug resistance. Laboratory studies have defined the evolution of tumor drug resistance to tamoxifen, raloxifene (used for breast and osteoporosis chemoprevention), and the AIs. Remarkably, the long-term exposure of breast cancers to antihormonal therapy also exposes a vulnerability that is being exploited in the clinic. Years of antihormonal therapy alters the cellular response mechanism to estrogen. Normally, estrogen is classified as a survival signal in breast cancer, but in sensitive antihormone-resistant cells, estrogen induces apoptosis. When resistant cells are killed, antihormonal therapy is once again effective. This new targeted approach to the treatment of metastatic breast cancer could open the door to novel approaches to treatment with drug combinations.

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Key words: Aromatase inhibitors, Diethylstilbestrol, Fulvestrant, Medroxyprogesterone acetate, Raloxifene

Introduction

Estrogen is essential for life. Without estrogen reproduction would be impossible. However, with the evolution of the human race and the development of functional societies has come the promise of an extended life through the control and, in some cases conquest, of disease.

The end of the 19th century was a period of important medical advances, with the introduction of vaccines and the start of the chemotherapeutic era for infectious diseases. The life expectancy for women was short: 48.3 years.[1] After a century of implementing public health advances with vaccination and antibacterial therapies, life expectancy for women in 2004 was 80.4 years.[2] This is true for all developed countries, but with success in public health comes new challenges for a population that is larger than ever before.

Cancer is essentially a disease of advancing years. Specifically, breast cancer is rare in women aged < 30 years (occurring in approximately 4 per 100,000 women) but increases dramatically during the next 40 years of life. The incidence of breast cancer in a population of women aged 70-75 years is 400 per 100,000 women per year. Although there is some emerging evidence that estrogen can cause transformation of breast or mammary cells,[3] there is evidence from prospective studies that the practice of prescribing hormone replacement therapy (HRT) to prevent osteoporosis and hypothetically to prevent aging has significantly increased breast cancer incidence.[4-6] A brief examination of why HRT became so fashionable and the current clinical concerns will serve as a physiologic background to address the rationale for the development of endocrine therapies (high dose), sex hormones, or antihormones for breast cancer treatment over the past 50 years.

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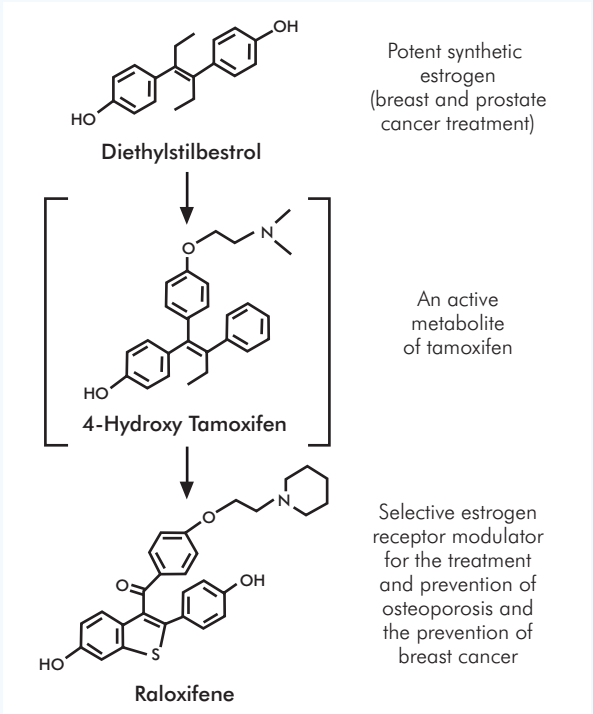
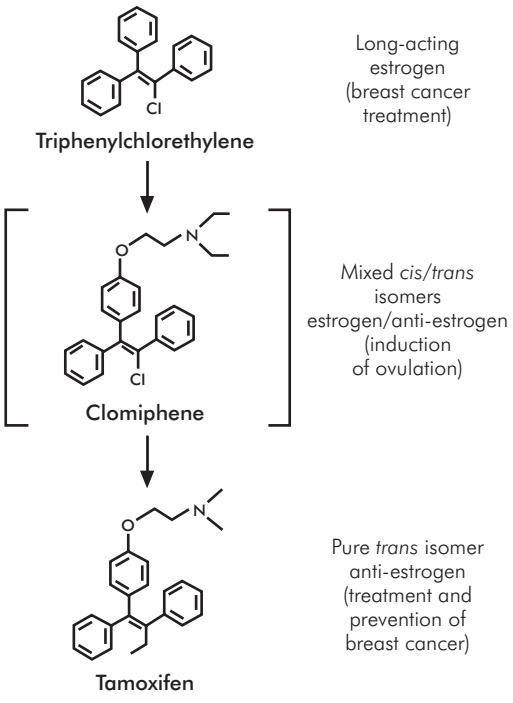
Figure 1 The Evolution in Structure-Function Relationships of Estrogens Based on Diethylstilbestrol



This potent estrogen has a high affinity for the ER and, historically, was used at high doses (15 mg daily) to treat breast and prostate cancer. Description of the metabolic activation of tamoxifen to 4-hydroxy tamoxifen[9,10] was the first clue that tamoxifen was a prodrug and needed to be converted to metabolites with a high binding affinity for ER. Raloxifene (formerly the failed breast cancer drug keoxifene[11]) was used with knowledge from previous structure-function studies to design an anti-estrogen with low uterotropic action but a high affinity for ER. The compound is a SERM used for the long-term treatment and prevention of osteoporosis and the prevention of breast cancer.[12,13] Raloxifene, unlike tamoxifen, has not been found to increase uterine hyperplasia or increase the incidence of endometrial cancer.[13,14]

Figure 2 The Evolution in Structure-Function Relationships of Anti-Estrogens Based on Triphenylchlorethylene[8]



The long-acting estrogen triphenylchlorethylene was used as treatment for breast cancer[15] and served as the basis for the discovery of clomiphene (a mixture of estrogenic and anti-estrogenic *cis* and *trans* isomers[19] used for the treatment of infertility.[20] Clomiphene was not used to treat breast cancer because of concerns about toxicity but served as the lead compound for the subsequent synthesis of tamoxifen, the pure *trans* isomer of a triphenylethylene,[21] that was eventually developed for the targeted treatment and prevention of breast cancer.[22]

Hormone Replacement Therapy

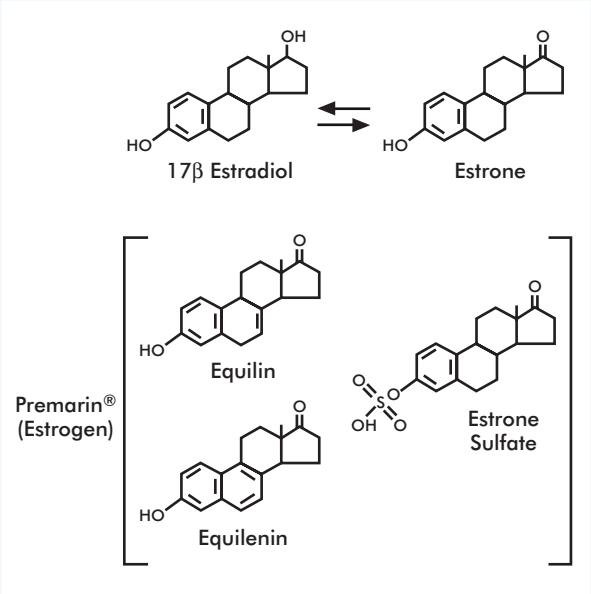
The initial goal for estrogen replacement was to ameliorate the menopausal symptoms that occurred when ovarian estrogen synthesis ceased. Subsequently, the focus was to maintain bone density or prevent increases in coronary heart disease in women later in life. Two approaches occurred to enhance and maintain the physiologic actions of estrogen past menopause.

Synthetic estrogens based on the structure of triphenylethylene or the very potent but shorter-acting diethylstilbestrol (DES; Figure 1),[7-14] were described in the literature, and they proved to be a cheap source of new medicines. High-dose synthetic estrogen administration was found to be effective in the treatment of breast and prostate cancer,[15] but even low doses of synthetic estrogens never really became accepted as HRT in postmenopausal women. Indeed, DES subsequently achieved notoriety as an estrogen supplement to prevent recurrent abortion, though children of treated mothers had a high incidence of clear-cell carcinoma of the vagina.[16,17] In contrast, the synthetic estrogens based on triphenylethylenes were subsequently to undergo a metamorphosis and be transformed into anti-estrogens used for the treatment of breast cancer (Figure 2).[8,15,18-22]

The estrogen (Figure 3) derived from pregnant mares (Premarin®) was initially used as an estrogen replacement therapy for postmenopausal women. However, it found that there was a 6-fold elevation in endometrial cancer.[23,24] The stimulatory action of estrogen in the uterus was neutralized by combining the orally active estrogen with the synthetic progesterone medroxyprogesterone acetate (MPA) known as Prem Pro®. This preparation was used by patients for up to a decade to prevent osteoporosis and menopausal symptoms, and it was also taken by many women in the belief it would prevent aging and coronary heart disease (CHD).

The actual link between HRT and breast cancer was addressed prospectively in 2 studies initiated during the 1990s. The Women's Health Initiative (WHI) recruited 16,608 women aged between 50 and 79 years who received conjugated equine estrogen 0.625 mg daily plus MPA 2.5 mg daily or placebo. Coronary heart disease with invasive breast cancer was the primary adverse outcome. The Million Women Study recruited 1,084,110 women aged 50-

Figure 3 The Natural Estrogens 17β Estradiol and Estrone Are Metabolically Interconverted in Women



The orally active estrogen preparation Premarin® is obtained by extracting pregnant mare's urine. The principal estrogen is estrone sulphate, which can be activated to estrone with sulphatase. Estrone in turn can then be converted by 17 hydroxysteroid dehydrogenase to the potent estrogen 17β estradiol. The other minor compounds in Premarin® are equilin and equilenin. Both are weak estrogens.

64 years to determine the effects of specific types of HRT on incidence and fatal breast cancer.

The WHI, with a mean follow-up of 5.2 years, was stopped prematurely because invasive breast cancer incidence exceeded the stopping boundary.[4] Overall, it was found that breast cancers were diagnosed in the women treated with HRT at a later stage compared with those who received placebo, possibly because there was an increase in mammographic density.[6] Overall, the study investigators did not find that HRT should be used to reduce the risk of CHD.[4] However, a recent subanalysis of younger women in the group indicates minor benefit.[25] The Million Women's Study concluded that HRT is associated with an increased risk of incidence of fatal breast cancer, particularly if the HRT was an estrogen/progesterone combination.[5] The authors estimated that, over the decade 1993-2003, HRT had increased the incidence of invasive breast cancer in the United Kingdom by an excess of 20,000 new cases.

It is interesting to note that with the publication of the WHI Study and the Million Women's Study in the first 5 years of the 21st century, there has been a significant decline in HRT prescriptions.[26-30] As a result, this has been associated with a drop in the incidence of breast cancer.[31] Thus, estrogen has a justified reputation as a potent stimulant of breast cancer development and growth. This reputation led to the development of anti-estrogenic targeted strategies to treat and prevent breast cancer.

Anti-Estrogenic Treatment Strategies

In the latter part of the 19th century, farmers in Scotland ovariectomized their farm animals to extend milk production. The observation had also been made that the histology of the lactating breast was similar to breast cancer. This knowledge was subsequently used by George Beaston to justify the oophorectomy of a young woman who had inoperable advanced metastatic breast cancer (MBC).[32] The woman responded dramatically, but further evaluation of the concept demonstrated that only 1 in 3 women would have effective disease control for about 1-3 years.[33] Nevertheless, the concept of endocrine ablation as a standard treatment for MBC was subsequently extended to postmenopausal women with the use of adrenalectomy and hypophysectomy.[34] The response rate (RR) remained at 30%, but it was not until the pioneering work of Jensen and Jacobson[35] and the identification of the estrogen receptor (ER) that progress was made in understanding estrogen-regulated growth mechanisms. The development of the ER assay used primarily to exclude women who would not respond to endocrine ablation was an important step forward in breast cancer treatment.[36,37] In other words, the presence of the ER in a breast tumor increased the probability that endocrine ablation would be successful. Because this was in the era before tamoxifen, it also suggested a use for the drug ICI-46474, discovered in the antifertility program at Imperial Chemical Industries (ICI) Pharmaceuticals Division (now AstraZeneca). The compound failed in its primary application as an antifertility agent[21] because, like clomiphene,[20] it induced ovulation in subfertile women.[38] The compound was found to have modest activity as a treatment for unselected breast cancer[39] but ICI-46474 was subsequently reinvented during the 1970s as a targeted therapy for breast cancer.[22] A scientific foundation was established in the laboratory for the treatment and prevention of breast cancer[40-42] by blocking estrogen action at the level of the ER.[43]

Coincidentally, another approach to controlling the growth of estrogen-stimulated breast cancer was also emerging in the 1970s, with the specific targeting of the aromatase enzyme CYP19, which converts androstenedione or testosterone into estrone or estradiol, respectively, in postmenopausal patients.[44] The first clinically useful specific AI was 4-hydroxyandrostenedione, which binds irreversibly to the active site of the enzyme.[45] There are now numerous AIs that bind irreversibly or competitively at the active site of the aromatase enzyme.

Transition to Tamoxifen

Before 1981, the standard of care for the palliative treatment of postmenopausal women with MBC included high-dose estrogen treatment.[34] Although the mechanism of action was unknown, treatment with DES was accepted as being among the most effective of the medical hormonal manipulations used, with expected RRs of approximately 36%. Other common hormonal approaches included "androgenization" with androgens (RR, 21%); high-dose progesterones, used as a single agent or

in combination with estrogen; and glucocorticosteroids as a means of chemical adrenalectomy to interrupt the hormonal feedback-stimulation axis. These additional hormonal therapies resulted in expected RRs ranging from 15% to 50%, with the lower figures being more realistic.

Also known as tamoxifen, ICI-46474 is a nonsteroidal anti-estrogen demonstrated in animal laboratory models to oppose the action of estrogens.[46] An early clinical appraisal of this agent was initially undertaken in 46 postmenopausal patients with MBC whose treatment had progressed after previous treatment with hormonal therapies.[39] Of the 46 patients treated with tamoxifen for ≥ 3 months, 10 patients (21%) demonstrated partial or complete response. Additionally, 17 patients (37%) experienced stable disease (SD), with some also experiencing response of visceral metastases. Tamoxifen was well tolerated, with few serious side effects. Hot flashes and nausea and vomiting were the most significant side effects, resulting in treatment discontinuation in a few (4%).

Based on this and other encouraging data,[47] a randomized clinical evaluation of tamoxifen and DES was undertaken.[48] A total of 151 postmenopausal women with MBC and measurable disease who might have been previously treated with chemotherapy, but had not been treated with previous hormonal therapies for metastatic disease, were randomized to treatment with tamoxifen (10 mg twice daily) or DES (5 mg 3 times daily). Treatment with DES (RR, 41%) resulted in higher RRs than treatment with tamoxifen (RR, 33%), but the difference was not statistically significant. Clinical benefit rates (complete response [CR] + partial response [PR] + SD) of 84% and 78% were also similar for tamoxifen and DES, respectively. Toxicity profiles favored tamoxifen, with significantly lower rates of nausea and vomiting, edema, and vaginal bleeding. Several smaller randomized trials also confirmed these findings.[49-51] No significant differences between estrogen preparations and tamoxifen with respect to reported RRs (ranging from 25% to 53%), clinical benefit rates, and/or duration of response were found. Because tamoxifen was associated with fewer side effects without loss of efficacy, it replaced DES as the first-line medical intervention of choice for postmenopausal women with MBC. Updated long-term follow-up analysis of > 14 years have confirmed the initial reported RRs.[52] However, of interest, with longer follow-up, 5-year survival is significantly superior (adjusted $P = .039$) for the patients treated with DES (35%) compared with those treated with tamoxifen (16%).

Tamoxifen dosing was modeled in the laboratory to show that early chronic dosing of rats was more important at preventing mammary cancer development than were larger interval doses.[21,53,54] These translational animal studies in vivo established the current standard use of long-term adjuvant anti-estrogen therapy chronically administered to prevent breast cancer recurrence. Five years of adjuvant tamoxifen is known to reduce the local recurrence and distant metastatic disease by approximately 50% in patients whose breast cancer is ER-positive.[55] Adjuvant tamoxifen also reduces the risk of breast cancer mortality by approximately one-third.

Long-Term Antihormonal Therapy

The scientific strategy[43] of targeting ER-positive breast tumors with long-term antihormonal therapy[54] has now reached its zenith. Long-term antihormonal adjuvant therapy is routine for patients with an ER-positive tumor, and several clinical facts are now clear. Five years of adjuvant tamoxifen therapy is now considered sufficient to provide long-term survival benefits for patients[56] and the antitumor effects of tamoxifen extend for ≥ 10 years after a 5-year course of adjuvant therapy.[55] Side effects in postmenopausal women using tamoxifen principally include increases in endometrial cancer risk and blood clots. Although the risk:benefit ratio is acceptable when tamoxifen is used as a therapy, this is not acceptable for postmenopausal women wishing to reduce the risk of breast cancer.[57-59]

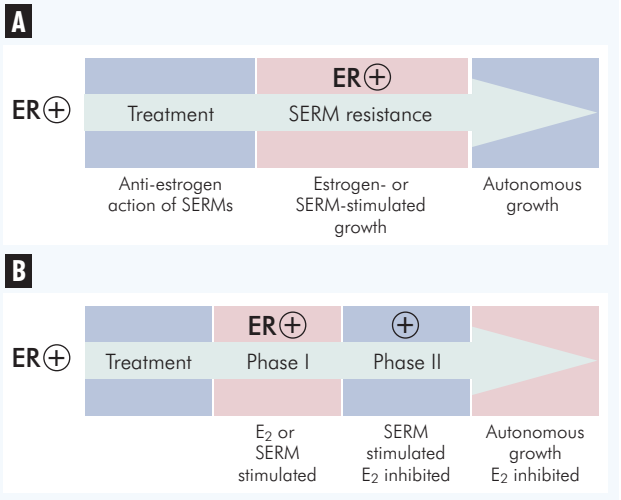
Aromatase inhibitors used for breast cancer treatment improve survival and reduce concerns about blood clots and endometrial cancer,[60-63] but there is a potential concern about osteoporosis that can be adequately addressed with bisphosphonate treatment for women with either osteopenia or osteoporosis. No results are as yet available for the use of AIs as chemopreventive agents, but the selective estrogen receptor modulator (SERM) raloxifene is available for the prevention of osteoporosis with, as predicted,[64-67] the prevention of breast cancer as a beneficial side effect.[12,68] The use of raloxifene for this indication by one-half million osteoporotic women has reduced breast cancer incidence by approximately 27,000 over 10 years.[69] Recently, the application of raloxifene has been extended to primary chemoprevention in high-risk postmenopausal women.[13]

Each of the applications of SERMs or AIs described above uses a 5-year treatment period. A small study demonstrated that longer-term tamoxifen extending to 10 years did not improve recurrence rates but did increase accumulated side effects.[70] In contrast, the application of a non–cross-resistant AI after 5 years of tamoxifen improves not only disease-free survival but also reduces the incidence of side effects and contralateral breast cancer.[71,72] Thus, the proposal[54] of using a SERM followed by estrogen deprivation has now become a clinical reality and long-term antihormonal therapy for the treatment and prevention of breast cancer is the standard of care. However, the ubiquitous application of antihormones in medicines now has consequences for breast cancer cells potentially exposed to estrogen deprivation for a decade. The treatment of antihormonal drug resistance is a challenge that needs to be addressed to develop inexpensive and effective future interventions.

Drug Resistance to Tamoxifen: Evolution from Benefit to Liability

With the advent of newer third-generation selective AIs, it is common practice for postmenopausal patients to be treated with tamoxifen followed by extended adjuvant anti-estrogen therapy with an AI, resulting in ≥ 5-10 years total of chronic,

Figure 4 The Evolution of Antihormonal Resistance in Breast Cancer



(A) The current clinical view of drug resistance to tamoxifen or any SERM. Long-term tamoxifen treatment eventually selects for tamoxifen-stimulated tumor growth. These tumors are recognized by responding to tamoxifen withdrawal[76] but also grow in response to physiologic correlation of estrogen. These observations are supported by laboratory studies.[74] This form of tamoxifen resistance forms the basis for the response of patients to AIs or fulvestrant after tamoxifen failure[77,78] and the basis of the success of extended antihormonal therapy with 5 years of tamoxifen followed by 5 years of an AI.[71] **(B)** The emerging laboratory view of drug resistance to SERM or AIs. Drug resistance evolves to a point where the tumor is exclusively dependent on the SERM (tamoxifen and raloxifene) or there is autonomous growth via the ER with long-term estrogen withdrawal. The biology of estrogen changes dramatically as the tumor cell evolves from phase I to phase II. Estrogen then becomes an inhibitory or apoptotic signal. These emerging new laboratory data have important implications for future clinical practice.

continuous anti-estrogen blockade.[71] However, anti-estrogen therapy is not able to prevent all recurrences, suggesting that, despite the presence of the ER, a majority of tumors become resistant. In fact, continuous extended-therapy tamoxifen has consequences for initially estrogen-responsive breast cancer cells. Here again, preclinical in vivo modeling has provided scientific insight. The estrogen-responsive, ER-positive breast cancer cell line MCF-7 has been successfully grown into tumors by inoculation into athymic mice.[73] Subsequent treatment with long-term tamoxifen has been used to mimic the effects of adjuvant therapy. Years of treatment are replicated by serially transplanting any growing tumors into tamoxifen-treated athymic, ovariectomized mice. Initially, tumors established in the presence of estrogen are suppressed from growth by tamoxifen, maintaining cytostatic activity without progressive increase in size for several months.[74] However, eventually, tamoxifen-stimulated tumors start to grow, but the tumors also grow in response to physiologic estradiol levels.[74] These characteristics are described as phase I SERM resistance, in which a SERM (eg, tamoxifen or raloxifene[75]) or estrogen can stimulate tumor growth in cells previously exposed to treatment with long-term tamoxifen or SERM therapy (Figure 4).[71,74,76-78] In the clinic, phase I tumor resistance is usually treated with an AI or fulvestrant to destroy the ER.[77,78]

A New Biology of Estrogen Action

If long-term tamoxifen-treated tumors continue to be passaged for 4-5 years to mimic adjuvant tamoxifen therapy, they acquire molecular changes associated with an unanticipated vulnerability. Selective ER modulator–stimulated growth is thought to be mediated by antiapoptotic pathways.[79,80] Unexpectedly, estrogen, rather than promoting growth of these long-term estrogen-deprived cells, now produces a tumoricidal effect.[81,82] To confirm this laboratory finding, fresh mice were "bitransplanted" with newly established MCF-7 tumor and long-term tamoxifen-resistant MCF-7 tumor within the same animal on different sides of the axillary region of the mammary fat pads. When treated with tamoxifen, the wild MCF-7 tumor did not grow in response to tamoxifen treatment, while the tamoxifen-resistant MCF-7 tumor grew. In contrast, estrogen stimulated the wild-type MCF-7 tumor to grow but the long-term-tamoxifen–resistant tumor did not grow. This suggested that the difference in response was not a result of difference in the host having an enhanced or altered response to estrogens and/or tamoxifen but rather a property inherent to the ER-positive breast cancer cells acquired in the setting of chronic estrogen deprivation over long periods of time.[82] These characteristics are described as phase II SERM resistance, in which ER-positive tumors are stimulated to grow by tamoxifen but killed by estrogen.

There is also another consequence of phase II SERM resistance. Fulvestrant, the pure anti-estrogen, is able to prevent phase II tumor growth after tamoxifen withdrawal, and the results are comparable to no treatment.[80] Again, these laboratory results are consistent with the clinical use of fulvestrant or an AI after the development of tamoxifen resistance.[77,78] However, the laboratory finding that physiologic estrogen plus fulvestrant causes robust tumor growth[80,83] raises the question of a negative drug interaction between fulvestrant and physiologic estradiol. The inhibitor actions of each agent are canceled out by the combination. Fulvestrant is not very active as a third-line agent, which raises the possibility that the estrogen already present in the postmenopausal woman might interfere in an unanticipated fashion with the inhibitory action of the pure anti-estrogen. Clinical studies are ongoing, examining the efficacy of a fulvestrant/AI combination.

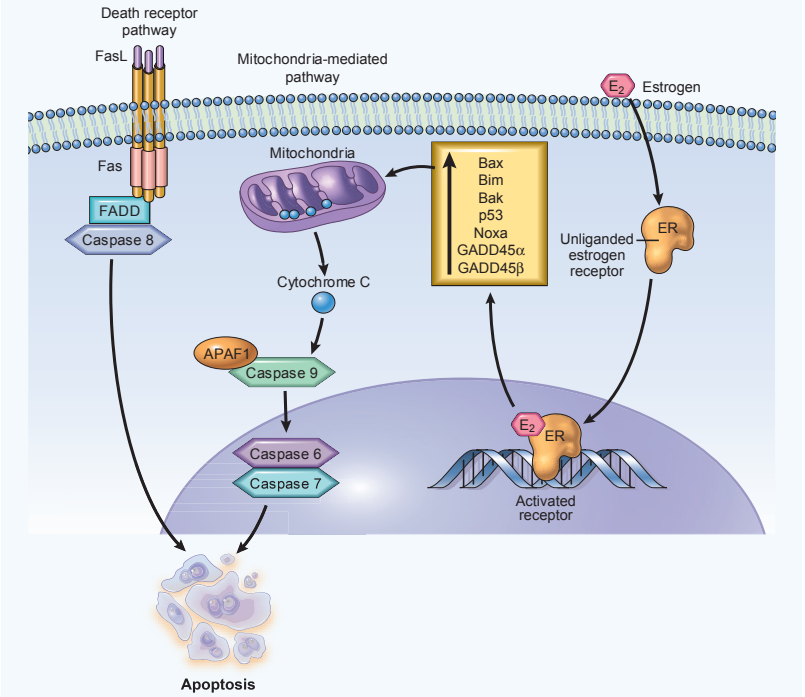
Overall, the recognition of the new biology of estrogen action observed after the development of long-term tamoxifen treatment raises the question of the global relevance of the observation to estrogen withdrawal after treatment with AIs, and the potential exploitation of the new knowledge of mechanisms can be identified.

Long-Term Estrogen Withdrawal Apoptotic Mechanisms

The increasing clinical use of AIs to reduce estrogen synthesis as a strategy to treat breast cancer has resulted in increased efforts to examine drug resistance to estrogen withdrawal rather than SERM action. Early stud-

ies growing MCF-7 breast cancer cells in estrogen-free media resulted in an increase in intracellular ER levels and spontaneous cell growth.[84,85,79] Several estrogen-independent clones were isolated for study,[86,87] and it was proposed that MCF-7 cells are hypersensitized to grow in extremely low levels of estrogen, ie, below the level that can be detected or further reduced.[88] However, Song and colleagues[89] observed that increasing concentrations of estradiol could increase apoptosis in estrogen-deprived cells by increasing the concentration of FASL that activates death receptor pathways. Thus, the original observations that phase II tamoxifen-resistant tumors could be treated with physiologic estrogen[81,82] were extended to AI–resistant cells. However, in contrast to the study by Song et al,[89] phase II tamoxifen-resistant tumors responded to increasing estrogen treatment by increasing the FAS receptor and decreasing HER2/*neu* and nuclear factor–κB associated with tumor regression.[80] Furthermore, MCF-7 cells kept for many years under estrogen-directed conditions using media containing stripped fetal bovine serum have produced rapid apoptosis via an intrinsic medium diverted at the mitochondrion.[90,91] However, Lewis et al[91] and Song and Santen[92] find that apoptosis is modulated through Bcl-2 or Bcl-$2X_L$. A representative schema based on the studies of Lewis and colleagues[91] is shown in Figure 5.[80,89,91]

Figure 5 The Sequence of Subcellular Events that Occur in Experimental Models During Estradiol-Induced Apoptosis in Breast Cancer



In some models, estradiol increases FasL,[89] but in others, Fas receptor increases, and there is a reduction in the survival signals from HER2/*neu* and NF-κB.[80] In contrast, Lewis et al[91] have described the actions of estradiol mediated through a mitochondrial mechanism.
Abbreviations: APAF1 = apoptotic peptidase activating factor–1; FADD = Fas-associated protein with death domain; GADD = growth arrest and DNA damage; NK-κB = nuclear factor–κB

It is also perhaps important to state that the new knowledge is emerging through re-examination of existing cell lines. In early publications, studying the effects of estrogen withdrawal, no estrogen-induced apoptosis was noted,[86,87] but by altering culture conditions or extending the period of estrogen exposure, apoptosis occurs.[90,93] Overall, the phenomenon observed with long-term estrogen withdrawal is similar to the phase II resistance of the model described for SERMs.

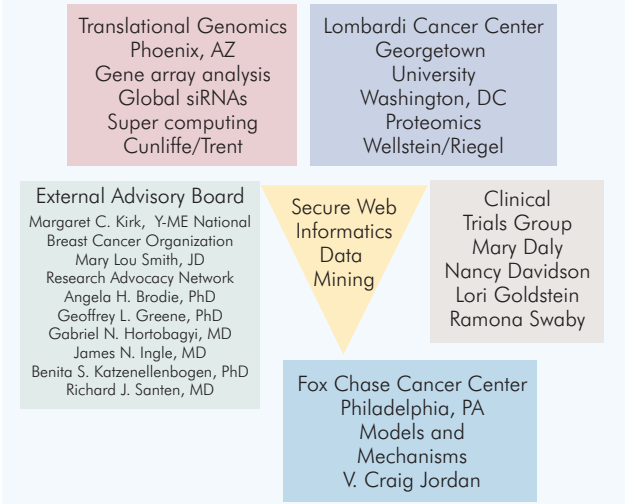
Clinical Clues

In the clinic, patients with ER-positive breast cancer are treated with exhaustive anti-estrogen therapies. However, over time and with sequential anti-estrogen therapy, anti-estrogen resistance can be expected to occur in as many as 50% of patients.[94] With each successive anti-estrogen treatment of such recurrent tumors, tumor response becomes less durable. Also, the combination of tamoxifen plus DES was no better than tamoxifen alone.[94] Lonning et al addressed the hypothesis that patients with ER-positive breast cancers who had been treated exhaustively with antihormonal therapy could potentially respond to high-dose estrogen therapy.[95] Thirty-two patients with advanced breast cancer previously exposed to between 2 and 10 (median, 4) endocrine treatments were treated with DES (5 mg 3 times daily). Therapy was well tolerated, but 4 patients terminated treatment within 2 weeks of starting, and another 2 patients stopped treatment before progress. One of these patients had SD for 15 weeks, and 1 had a PR for 39 weeks. Of the remainder, 4 patients obtained a CR, and 6 patients obtained a PR. Two patients had SD for 6 months, and 1 had SD for ≥ 1 year. Overall, these extremely encouraging preliminary studies with high-dose estrogen therapy are complemented by anecdotal reports of the effectiveness of low-dose estrogen treatment for women with endocrine-refractory breast cancer after exhaustive antihormonal therapy (Dr. James Ingle and Mr. Michael Dixon, personal communications, 2007). As a result, several clinical studies are currently under way (Drs. Matthew Ellis and Richard Santen, personal communications, 2007).

Estrogen-Induced Apoptosis: Clinical and Laboratory Correlations

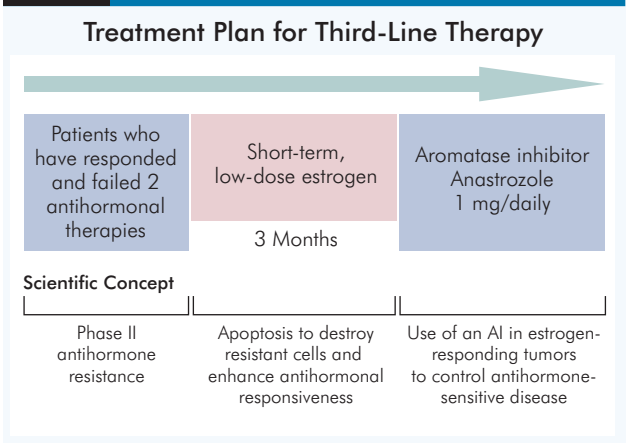
Based on the preclinical laboratory modeling, we have translated the new biology of estrogen action into a Department of Defense Center of Excellence grant with laboratory and clinical collaborators illustrated in Figure 6. Our goal is to define the pathways for estrogen induced survival and apoptosis in endocrine-responsive breast and endometrial cancer and use the emerging data-

Figure 6 Organization of Department of Defense Center of Excellence Grant Titled "A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis"



The model systems to study the survival and apoptosis induced with estrogen are being used for time course experiments at the Fox Chase Cancer Center. The materials are distributed to Translational Genomics for genomic analysis using comparative genomic hybridization, small interfering RNA (siRNA) analysis, or agilent gene array analysis, and the Vincent T. Lombardi Cancer Center is involved to conduct proteomics. All results are uploaded into a shared secure Web site for data processing and target identification by our informatics and biostatistics group. Each laboratory is able to validate emerging pathways and study individual genes of interest. Our program is integrated with a clinical trials program that provides patient samples for the validation of apoptotic or survival pathways.

Figure 7 Anticipated Treatment Plan for Third-Line Endocrine Therapy



Patients must have responded and experienced failure with 2 successive antihormonal therapies to be eligible for a course of low-dose estradiol therapy for 3 months. The anticipated RR is 30%,[95] and responding patients will be treated with anastrozole until relapse. Validation of the treatment plan via the Center of Excellence grant (Figure 5) will establish a platform to enhance RRs with apoptotic estrogen by integrating known inhibitors of tumor survival pathways into the 3-month low-dose estrogen debulking treatment plan. The overall goal is to increase RRs and maintain patients longer on antihormonal strategies before chemotherapy is required.

base to guide the interpretation and development of a series of clinical trials. The ultimate goal of our clinical trial design is illustrated in Figure 7[95] and currently consists of 2 separate but interconnected therapeutic estrogen trials.

In trial 1, "A Single-Arm Phase II Study of Pharmacologic-Dose Estrogen in Postmenopausal Women with Hormone Receptor–Positive Metastatic Breast Cancer After Failure of Sequential Endocrine Therapies," 88 patients who have clearly responded and failed ≥ 2 anti-estrogenic therapies will be treated for 12 weeks with 30 mg estradiol. Patients who respond or have SD will be treated subsequently with 1 mg anastrozole until disease progression. Serum and, where possible, recurrent tissue biopsies will be used to determine serum apoptotic markers (Apoptosense®) and target genes in tumor material as markers of apoptosis or tumor progression. These data will be compared and contrasted with the results obtained from preclinical studies using our cell and animal models.

In trial 2, "Reversal of Antiestrogen Resistance with Sequential Dose De-escalation of Pharmacologic Estrogen in a Single-Arm Phase II Study of Postmenopausal Women with Hormone Receptor–Positive Metastatic Breast Cancer After Failure of Sequential Endocrine Therapies," patients who have responded and subsequently failed 2 anti-estrogenic therapies will be treated as groups with successively lower doses of daily estradiol to determine the lowest dose necessary to produce an equivalent response to estradiol 30 mg in trial 1.

On completion of the integrated research program, several questions can be addressed to improve the treatment of patients with MBC:

(1) Can a select group of patients be identified from tumor markers or early serum apoptotic products who will respond to limited low-dose estradiol treatment and who will subsequently remain under disease control with anastrozole treatment?

(2) Can cell survival pathways be identified for tumors that do not respond to estradiol treatment?

(3) Can survival pathways be subverted to improve RRs to estradiol-induced apoptosis?

Conclusion

The development and extensive clinical application of long-term antihormonal therapy[44] has had consequences for the patient with the development of antihormonal drug resistance in some breast cancers.[96] However, with the development of drug resistance to exhaustive antihormonal therapy, a vulnerability of the cancer has been exposed. The recognition of the new biology of estrogen action that causes apoptosis in sensitive breast tumors now opens an unanticipated door of opportunity to exploit the findings to aid patients. Although the actual clinical responses might not be profound in unselected patient populations or in populations whose tumors do not have stage II breast cancer, our ability to decipher apoptotic mechanisms from laboratory models and eventually target patients appropriately might eventually have profound, positive effects for some

patients. The translational knowledge gained over the next few years might again provide unanticipated opportunities to exploit the discovery of "apoptotic triggers" for other forms of cancer.

It is perhaps pertinent to restate that, for 70 years, there has been an "ebb and flow" relationship in the role of estrogen in breast tumor homeostasis. We have illustrated in this review many of the changing fashions that have occurred in how estrogen is perceived as a benefit or a villain in women's health. The effects of modulating the ER system in the breast, at one time or another, have been dismissed because they are small or believed to be of no major consequence. Nevertheless, the small observations become accumulative. By way of example, it is important to recall that initial use of tamoxifen, a failed contraceptive, to treat unselected populations showed only modest responses for some patients with MBC.[22] Years later, after deciphering the target populations and translating the appropriate treatment strategies from the laboratory to the clinic, the drug became the gold standard for endocrine therapy[22] and was credited with improving the survival of hundreds of thousands of women.[55] The challenge for the future is to exploit the profound apoptotic action of estradiol as a lead to develop innovative new therapies for cancer.

Acknowledgements

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References

1. Table 27. Life expectancy at birth, at 65 years of age, and at 75 years of age, by race and sex: United States, selected years 1900-2004, pg 192. In: *National Center for Health Statistics Health, United States, 2007 With Chartbook on Trends in the Health of Americans.* Hyattsville, MD: 2007. Available at: http://www.cdc.gov/nchs/data/hus07.pdf#027. Accessed: April 17, 2008.
2. Minino AM, Heron MP, Murphy SL, et al. Deaths: final data for 2004. *National vital statistics reports, CDC* 2007; 55:19 Available at: http://www.cdc.gov/nchs/data/nvsr/nvsr55/nvsr55_19.pdf. Accessed: April 17, 2008.
3. Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. *N Engl J Med* 2006; 354:270-82.
4. Rossouw JE, Anderson GL, Prentice RL, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. *JAMA* 2002; 288:321-33.
5. Beral V. Breast cancer and hormone-replacement therapy in the Million Women Study [published erratum in: *Lancet* 2003; 362:1160]. *Lancet* 2003; 362:419-27.
6. Chlebowski RT, Hendrix SL, Langer RD, et al. Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. *JAMA* 2003; 289:3243-53.
7. Dodds EC, Goldberg L, Lawson W, et al. Oestrogenic activity of certain synthetic compounds. *Nature* 1938; 141:247-8.
8. Robson JM, Schonberg A, Fahim HA. Duration of action of natural and synthetic estrogens. *Nature* 1938; 142:292-3.
9. Jordan VC, Collins MM, Rowsby L, et al. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* 1977; 75:305-16.
10. Allen KE, Clark ER, Jordan VC. Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. *Br J Pharmacol* 1980; 71:83-91.
11. Buzdar A, Marcus C, Holmes F, et al. Phase II evaluation of Ly156758 in metastatic breast cancer. *Oncology* 1988; 45:344-5.
12. Cummings SR, Eckert S, Krueger KA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation [published erratum in: *JAMA* 1999; 282:2124]. *JAMA* 1999; 281:2189-97.
13. Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial [published errata in: *JAMA* 2006; 296:2926 and *JAMA* 2007; 298:973]. *JAMA* 2006; 295:2727-41.
14. Barrett-Connor E, Mosca L, Collins P, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Eng J Med* 2006; 355:125-37.
15. Haddow A, Watkinson JM, Paterson E. Influence of synthetic oestrogens upon advanced malignant disease. *Br Med J* 1944; 2:393-8.
16. Greenwald P, Barlow JJ, Nasca PC, et al. Vaginal cancer after maternal treatment with synthetic estrogens. *N Engl J Med* 1971; 285:390-2.
17. Herbst AL, Ulfelder H, Poskanzer DC. Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. *N Engl J Med* 1971; 284:878-81.
18. Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res* 1990; 50:4177-89.
19. Clark JH, Guthrie SC. Agonistic and antagonistic effects of clomiphene citrate and its isomer. *Biol Reprod* 1981; 25:667-72.
20. Greenblatt RB, Roy S, Mahesh VB, et al. Induction of ovulation. *Am J Obstet Gynecol* 1962; 84:900-12.
21. Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. *J Reprod Fertil* 1967; 13:101-19.
22. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2003; 2:205-13.
23. Smith DC, Prentice R, Thompson DJ, et al. Association of exogenous estrogen and endometrial carcinoma. *N Engl J Med* 1975; 293:1164-7.
24. Ziel HK, Finkle WD. Increased risk of endometrial carcinoma among users of conjugated estrogens. *N Engl J Med* 1975; 293:1167-70.
25. Rossouw JE, Prentice RL, Manson JE, et al. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause [published erratum in: *JAMA* 2008; 299:1426]. *JAMA* 2007; 297:1465-77.
26. Faber A, Bouvy ML, Loskamp L, et al. Dramatic change in prescribing of hormone replacement therapy in The Netherlands after publication of the Million Women Study: a follow-up study. *Br J Clin Pharmacol* 2005; 60:641-7.
27. de Jong-van den Berg LT, Faber A, van den Berg PB. HRT use in 2001 and 2004 in The Netherlands--a world of difference. *Maturitas* 2006; 54:193-7.
28. Usher C, Teeling M, Bennett K, et al. Effect of clinical trial publicity on HRT prescribing in Ireland. *Eur J Clin Pharmacol* 2006; 62:307-10.
29. Hing E, Brett KM. Changes in U.S. prescribing patterns of menopausal hormone therapy, 2001-2003. *Obstet Gynecol* 2006; 108:33-40.
30. Power ML, Schulkin J, Rossouw JE. Evolving practice patterns and attitudes toward hormone therapy of obstetrician-gynecologists. *Menopause* 2007; 14:20-8.
31. Ravdin PM, Cronin KA, Howlader N, et al. The decrease in breast-cancer incidence in 2003 in the United States. *N Engl J Med* 2007; 356:1670-4.
32. Beatson GT. On treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. *Lancet* 1896; 11:104-7.
33. Boyd S. On oophorectomy in cancer of the breast. *Br Med J* 1900; 2:1161-7.
34. Kennedy BJ. Hormone therapy for advanced breast cancer. *Cancer* 1965; 18:1551-7.
35. Jensen EV, Jacobson HI. Basic guides to the mechanism of estrogen action. *Recent Progr Horm Res* 1962; 18:387-414.
36. Jensen EV, Block GE, Smith S, et al. Estrogen receptors and breast cancer response to adrenalectomy. *Natl Cancer Inst Monogr* 1971; 34:55-70.
37. McGuire WL, Carbone PP, Sears ME, et al. Estrogen receptors in human breast cancer: an overview. In: McGuire WL, Carbone PP, Volmer EP, eds. *Estrogen Receptor in Human Breast Cancer.* New York: Raven Press; 1975:1-7.
38. Klopper A, Hall M. New synthetic agent for the induction of ovulation: preliminary trials in women. *Br Med J* 1971; 1:152-4.
39. Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late

breast cancer. An early clinical appraisal of ICI 46474. *Br J Cancer* 1971; 25:270-5.
40. Jordan VC, Dowse LJ. Tamoxifen as an anti-tumour agent: effect on oestrogen binding. *J Endocrinol* 1976; 68:297-303.
41. Jordan VC, Jaspan T. Tamoxifen as an anti-tumour agent: oestrogen binding as a predictive test for tumour response. *J Endocrinol* 1976; 68:453-60.
42. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA- induced rat mammary carcinomata. *Eur J Cancer* 1976; 12:419-24.
43. Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *Eur J Cancer* 1975; 11:205-6.
44. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* 2007; 72:7-25.
45. Coombes CR, Goss P, Dowsett M, et al. 4-hydroxyandostenedione in treatment of postmenopausal patients with advanced breast cancer. *Lancet* 1984; 2:1237-9.
46. Harper MJ, Walpole AL. Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. *Nature* 1966; 212:87.
47. Ward HW. Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. *Br Med J* 1973; 1:13-4.
48. Ingle JN, Ahmann DL, Green SJ, et al. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *N Engl J Med* 1981; 304:16-21.
49. Stewart HJ, Forrest AP, Gunn JM, et al. The tamoxifen trial - a double-blind comparison with stilboestrol in postmenopausal women with advanced breast cancer. *Eur J Cancer* 1980; suppl 1:83-8.
50. Beex L, Pieters G, Smals A, et al. Tamoxifen versus ethinyl estradiol in the treatment of postmenopausal women with advanced breast cancer. *Cancer Treat Rep* 1981; 65:179-85.
51. Matelski H, Greene R, Huberman M, et al. Randomized trial of estrogen vs. tamoxifen therapy for advanced breast cancer. *Am J Clin Oncol* 1985; 8:128-33.
52. Peethambaram PP, Ingle JN, Suman VJ, et al. Randomized trial of diethylstilbestrol vs. tamoxifen in postmenopausal women with metastatic breast cancer. An updated analysis. *Breast Cancer Res Treat* 1999; 54:117-22.
53. Jordan VC, Dix CJ, Allen KE. The effectiveness of long-term treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In: Salmon SE, Jones SE, eds. *Adjuvant Therapy of Cancer II*. New York: Grune and Stratton; 1979; 2:19-26.
54. Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 1980; 16:239-51.
55. Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005; 365:1687-717.
56. Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998; 351:1451-67.
57. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Nat Cancer Inst* 1998; 90:1371-88.
58. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Nat Cancer Inst* 2005; 97:1652-62.
59. Gail MH, Costantino JP, Bryant J, et al. Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer [published erratum in: *J Nat Cancer Inst* 2000; 92:375]. *J Nat Cancer Inst* 1999; 91:1829-46.
60. Howell A, Cuzick J, Baum M, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* 2005; 365:60-2.
61. Breast International Group (BIG) 1-98 Collaborative Group, Thürlimann B, Keshaviah A, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer [published erratum in: *N Engl J Med* 2006; 354:2200]. *N Engl J Med* 2005; 353:2747-57.
62. Coombes RC, Hall E, Gibson LJ, et al. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer [published errata in: *N Eng J Med* 2004; 351:2461 and *N Eng J Med* 2006; 355:1746]. *N Eng J Med* 2004; 350:1081-92.
63. Coates AS, Keshaviah A, Thürlimann B, et al. Five years of letrozole compared with tamoxifen as initial adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer: update of study BIG 1-98. *J Clin Oncol* 2007; 25:486-92.
64. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 1987; 10:31-5.
65. Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* 1987; 47:4020-4.
66. Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 1988; 6:589-95.
67. Ettinger B, Black DM, Mitlak BH, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators [published erratum in: *JAMA* 1999; 282:2124]. *JAMA* 1999; 282:637-45.
68. Martino S, Cauley JA, Barrett-Connor E, et al. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J Natl Cancer Inst* 2004; 96:1751-61.
69. Jordan VC. Optimising endocrine approaches for the chemoprevention of breast cancer beyond the Study of Tamoxifen and Raloxifene (STAR) trial. *Eur J Cancer* 2006; 42:2909-13.
70. Fisher B, Dignam J, Bryant J, et al. Five versus more than five years of tamoxifen for lymph node-negative breast cancer: updated findings from the National Surgical Adjuvant Breast and Bowel Project B-14 randomized trial. *J Natl Cancer Inst* 2001; 93:684-90.
71. Goss PE, Ingle JN, Marino S, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. *N Engl J Med* 2003; 349:1793-802.
72. Goss PE, Ingle JN, Martino S, et al. Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17. *J Natl Cancer Inst* 2005; 97:1262-71.
73. Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Res* 1997; 57:3071-8.
74. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 1988; 48:5183-7.
75. O'Regan RM, Osipo C, Ariazi E, et al. Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. *Clin Cancer Res* 2006; 12:2255-63.
76. Howell A, Dodwell DJ, Anderson H, et al. Response after withdrawal of tamoxifen and progestogens in advanced breast cancer. *Ann Oncol* 1992; 3:611-7.
77. Osborne CK, Pippen J, Jones SE, et al. Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. *J Clin Oncol* 2002; 20:3386-95.
78. Howell A, Robertson JF, Quaresma Albano J, et al. Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. *J Clin Oncol* 2002; 20:3396-403.
79. Gomez BP, Riggins RB, Shajahan AN, et al. Human X-Box binding protein-1 confers both estrogen independence and antiestrogen resistance in breast cancer cell lines. *FASEB J* 2007; 21:4013-27.
80. Osipo C, Gajdos C, Liu H, et al. Paradoxical action of fulvestrant on estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Nat Cancer Inst* 2003; 95:1597-607.
81. Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. In: *Recent Results in Cancer Research.* Heidelberg: Springer-Verlag; 1993:23-33.
82. Yao K, Lee ES, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000; 6:2028-36.
83. Osipo C, Meeke K, Cheng D, et al. Role for HER2/neu and HER3 in fulvestrant-resistant breast cancer. *Int J Oncol* 2007; 30:509-20.
84. Katzenellenbogen BS, Kendra KL, Norman MJ, et al. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res* 1987; 47:4355-60.
85. Welshons WV, Jordan VC. Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *European J Cancer* 1987; 232:1935-9.
86. Jiang SY, Wolf DM, Yingling JM, et al. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol and Cell Endo* 1992; 90:77-80.
87. Pink JJ, Jiang SY, Fritsch M, et al. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* 1995; 55:2583-90.
88. Masamura S, Santner SJ, Heitjan DF, et al. Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. *J Clin Endocrinol Metab* 1995; 80:2918-25.

89. Song RX, Mor G, Naftolin F, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *J Nat Cancer Inst* 2001; 93:1714-23.
90. Lewis JS, Osipo C, Meeke K, et al. Estrogen induced apoptosis in breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 2005; 94:131-41.
91. Lewis JS, Meeke K, Osipo C, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *JNCI* 2005; 97:1746-59.
92. Song RX, Santen RJ. Apoptotic action of estrogen. *Apoptosis* 2003; 8:55-60.
93. Jordan VC, Lewis-Wambi J, Kim H, et al. Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients. *Breast* 2007; 16(suppl 2):105-13.
94. Mouridsen HT, Salimtschik M, Dombernowsky P, et al. Therapeutic effect of tamoxifen versus combined tamoxifen and diethylstilboestrol in advanced breast cancer in postmenopausal women. *Eur J Cancer* 1980; suppl 1:107-10.
95. Lonning PE, Taylor PD, Anker G, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 2001; 67:111-6.
96. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004; 5:207-13.

Selective Estrogen Receptor Modulators and Phytoestrogens

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- breast cancer
- chemoprevention

Abstract

Scientific achievements in the last two decades have revolutionized the treatment and prevention of breast cancer. This is mainly because of targeted therapies and a better understanding of the relationship between estrogen, its receptor, and breast cancer. One of these discoveries is the use of synthetic selective estrogen modulators (SERMs) such as tamoxifen in the treatment strategy for estrogen receptor (ER)-positive breast cancer. Hundreds of thousands of lives have been saved because of this advance. Not only is tamoxifen used in the treatment strategy for patients who have breast cancer, but also for prevention in high-risk premenopausal women. Another synthetic SERM, raloxifene, which was initially used to prevent osteoporosis, is also as effective as tamoxifen for prevention in high-risk postmenopausal women. In certain regions of the world, particularly in Asia, a low incidence of breast cancer has been observed. These women have diets that are high in soy and low in fat, unlike the Western diet. Interest in the protective effects of soy derivatives has led to the research of phytoestrogens and metabolites of soy that are described by some as natural SERMs. As a result, many clinical questions have been raised as to whether phytoestrogens, which are also found in other natural foods, can protect against breast cancer. This article reviews the development and role of the more common SERMs, tamoxifen and raloxifene. In addition, this paper will also highlight the emerging studies on phytoestrogens and their similarity and dissimilarity to SERMs.

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Bibliography
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Introduction

Great strides have been made in the last 25 years in the fight against breast cancer. One of the more notable developments has been the search for ways to prevent cancer. The development of selective estrogen receptor modulators (SERMs) has been a significant step towards achieving that goal. Tamoxifen, an antiestrogen in the breast and the pioneering SERM, has been the gold standard, and often the only choice in many countries for the treatment of breast cancer [1]. It also became the first drug ever to be approved by the United States (US) Food and Drug Administration (FDA) for the chemoprevention of breast cancer in high-risk women [1]. This chapter will review the development of tamoxifen the prototypical SERM and its use and development as a chemopreventive agent. In addition this article will also highlight the emerging information regarding phytoestrogens that are being regarded by some as natural SERMs.

Background

By the turn of the 20th century it was known that oophorectomy in pre-menopausal women with metastatic breast cancer could cause regression of the disease [2], [3]. This showed a link between products produced by the ovaries and the growth of some breast cancers. The product was found to be estrogen [4]. In 1936, Professor Antoine Lascassagne hypothesized that breast cancer was caused by a special hereditary sensitivity to estrogen and suggested that the development of an estrogen antagonist could prevent disease [5]. Over twenty-five years later in 1962 Jensen and Jacobsen [6] described the estrogen receptor (ER) as the mediator of estrogen action, setting the stage for the manipulation of this receptor for multiple purposes [7].

Investigation of possible contraceptive agents led to the reinvention of ICI 46474, a failed contraceptive agent, to become tamoxifen, the first targeted anti-cancer agent. The study of tamoxifen

in the laboratory led to the finding that it inhibited the growth of ER-positive breast cancer cells *in vitro* [8]. In addition, animal studies showed that tamoxifen prevented rat mammary carcinogenesis [9], [10] but had a stimulatory effect on rat uterine weight [11]. The actions of non-steroidal antiestrogens were clearly not wholly explainable as estrogen agonists or antagonists and a model to describe their unique actions led to the development of the SERM concept [12], [13], [14].

What are SERMs?

SERMs are synthetic non-steroidal agents that bind to the ER and produce a change in the biological activity of the receptor depending on the tissue type. The primary target site for SERMs, the ER, is a nuclear receptor. To fully understand the unique nature of SERMs the actions of estrogen on the body must be revisited. Estrogen in premenopausal women is primarily produced by the ovaries. There are multiple target sites for estrogen and it has various actions throughout the body. Estrogens decrease cholesterol levels by lowering the circulating low-density lipoproteins (LDL). Its actions also include maintenance of bone density in postmenopausal women, and hormonal regulation, and control of the menstrual cycle in premenopausal women. These actions are summarized in **Fig. 1**. In contrast, the effect of SERMs depends on the target sites and is shown in **Fig. 2**.

A pure estrogen agonist would be one that stimulates the positive action of estrogen at all its targets. Conversely, a pure antagonist would inhibit all the actions of estrogen at all of its target sites. In contrast, SERMs have partial agonist and antagonist properties depending on the target site hence their uniqueness.

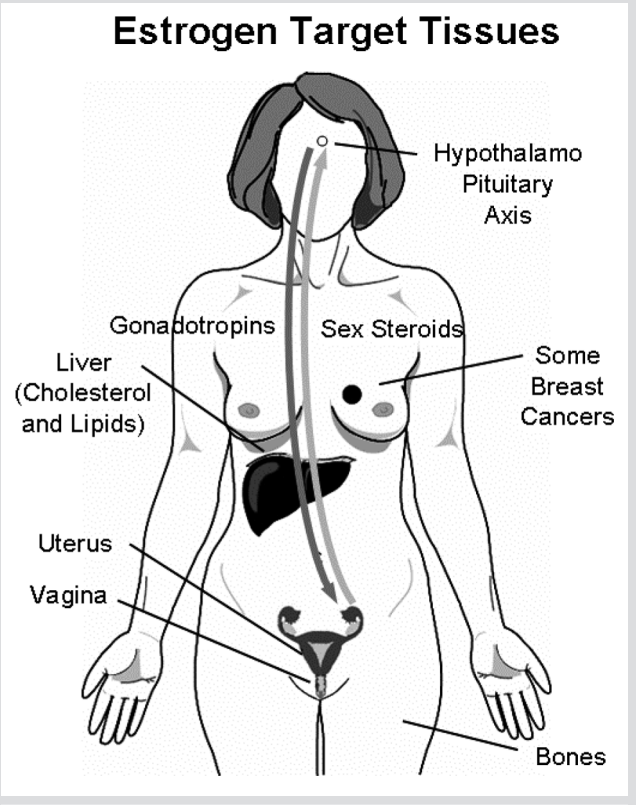

Fig. 1 The sites of action for estrogen.

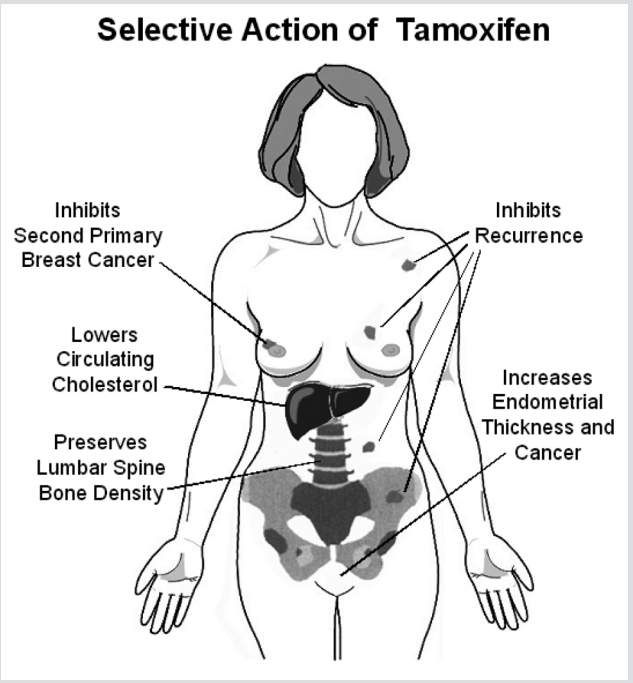

Fig. 2 The sites of action of tamoxifen.

Studies have shown that the partial agonist/antagonist properties depend on which associated coregulators are expressed when the receptor/ligand interaction occurs [15]. The details of the receptor/ligand interaction help us understand the mechanism of action of SERMs.

Mechanism of action

There are two aspects to the mechanism of action of SERMs: the pharmacokinetics or how the drug gets to the target site and the pharmacodynamics or what it does when it gets there. Tamoxifen (**Fig. 3**) is a lipophilic prodrug that is easily absorbed by the gut without modification and 98% is bound to albumin after entering the circulation. It undergoes extensive metabolism in the gastrointestinal (GI) tract and in the liver into its less active form *N*-desmethyltamoxifen and two most active forms, 4-hydroxytamoxifen and endoxifen [16], [17], [18], [19]. Each of the hydroxylated metabolites results from first pass metabolism in the liver. These compounds enter the bloodstream via the enterohepatic circulation to reach their target sites [18], [20], [21]. The metabolites of tamoxifen are excreted via the fecal route as has been shown by animal studies using ^{14}C radiolabeled tamoxifen [22]. These studies demonstrate that 67% of these metabolites enter the enterohepatic circulation and undergo further metabolism several times until excretion by the GI tract [23], [24]. 4-Hydroxytamoxifen, and endoxifen have the same affinity for the ER as estrogen. Other metabolites of tamoxifen do not have as much effect or affinity for the ER as they lack the 4-hydroxy group [18]. Recent studies demonstrate that the potent tamoxifen metabolite endoxifen is produced by the product of the CY2PD6 gene. In patients with mutations of the CYP2D6 gene or patients who take other medications that compete for the enzyme product, metabolism of tamoxifen to the potent metabolite endoxifen is affected and may therefore have less benefit [25], [26]. Raloxifene (**Fig. 3**), another SERM, is a polyphenol, which undergoes rapid conjugation in the GI tract and in the liv-

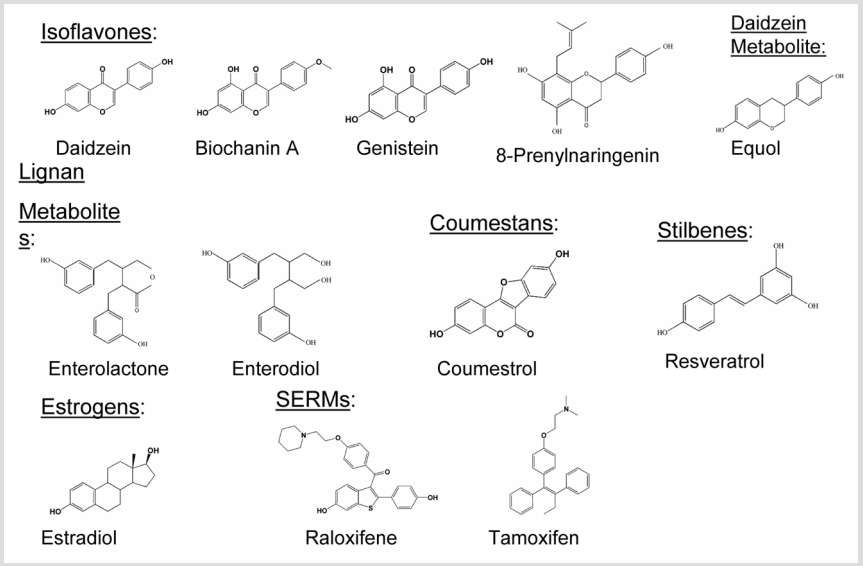

Fig. 3 A structural comparison of commonly studied phytoestrogens and phytoestrogen metabolites to SERMs.

er. In addition it also undergoes phase 3 metabolism by gut flora. The bacteria directly glucuronidate and sulfate this compound so that it is excreted [26], [27]. Since the drug does not reenter the enterohepatic circulation, it does not reach its targets as efficiently as tamoxifen. Also, a smaller percent enters the circulation as only 2% is bound to albumin and the half-life of raloxifene is 27 hours [28]. As a result of differences in metabolism and bioavailability, raloxifene is not as useful an agent in patients who already have breast cancer [29].

There are two isoforms of the ER, ERα and ERβ [6], [30] whose distribution and density varies depending on the target site. Both isoforms are found in the reproductive organs. Tamoxifen binds both receptors with equivalent affinity [31]. Endoxifen and 4-hydroxytamoxifen have similar affinities for both isoforms [32] and create similar gene expression profiles. Other ligands show preference for one isoform or the other, which may explain specific target tissue responses with various compounds. In many tissues, ERβ has anti-proliferative effects, whereas, ERα has proliferative effects [33]. Studies indicate that ER-β has an inhibitory effect on ER-α [34], [36], [35]. However, the biology is more complex than a simple agonist/antagonist interaction between the two receptors. The ratio of ERα to ERβ at a target site may be important in determining the overall action of a SERM on that tissue. A high ratio may correlate with high levels of cellular proliferation while a low ratio implies the opposite [36].

In the past, the interaction between SERMs and the ER was thought to be a simple case of a ligand switching its target receptor on or off. Through further research it is now known that this interaction is a more complex and dynamic process. Studies using phage display created a fingerprint of exposed surfaces when tamoxifen or estrogen was bound to the ER. Different conformational changes occur in the ER depending on the ligand that binds to the ER. In addition, the fingerprint was different in ER-α vs. ER-β when they were bound to identical ligands [37]. The discovery of the steroid receptor co-activator protein (SRC1) helped further to elucidate this complex interaction [40]. The binding of an SERM to the ER results in a conformational change in the ER [41], which results in the exposure of different amino acids on the receptor and the binding of different coactivators. Since the discovery of SRC1, dozens of other co-activator and co-repressor molecules have been discovered; all of which play some role in receptor modulation [15].

Finally, another dimension of signaling pathways can modulate the ER. Activation of the ER by other growth factor pathways can result in resistance to SERMs in a tumor.

This recruitment of specific co-regulators to the ligand receptor complex depends on the ligand that binds to the ER, the ER isoform, and "cross-talk" with other growth factor pathways [38]. SRC-3 is known to be important as a co-activator in breast cancer. In tumors and cancer cell lines that are HER2-positive and resistant to endocrine therapy with tamoxifen, studies demonstrate that SRC-3 is recruited to ER-α, but not ER-β in the presence of tamoxifen. In specimens from patients who were HER-2-negative and sensitive to endocrine therapy with tamoxifen, estrogen recruited SRC-3 to both ER isoforms, but tamoxifen did not [42]. Finally, when SRC-3 was knocked down, there was reduced expression of the estrogen target gene, pS2 in MCF7 cells. After the SRC-3 knockdown in cells derived from HER2-positive tumors, there was a decrease in cell proliferation and the cells regressed in the presence of tamoxifen [42].

To summarize the molecular process thus far: once an SERM binds to the ER it causes a change in the shape of the ER. This change of shape allows recruitment of co-activators, if it is destined to elicit an estrogenic response, or co-repressors if its response is anti-estrogenic. The binding of the coregulatory molecules leads to the activation of the promoter sequence of the estrogenic responsive gene [36]. This process is also controlled by the degradation and disassembly of complexes at the gene promoter site, which causes renewed activation of the signal to initiate RNA synthesis. In this way the SERM can specifically modulate the estrogen responsiveness of a target tissue (See review Jordan [36]).

Clinical relevance

The full details of the mechanism of action of SERMs have yet to be precisely described however, their clinical importance as an advance in medicine is proven. Tamoxifen was initially tested in humans in the early 1970 s, before extensive anti-tumor testing in animals [39], [40]. Animal testing [1], [9], [10] refocused efforts and targeted the ER [41], thereby opening the door for chemoprevention. Through animal studies tamoxifen was found to

have targeted anti-tumor activity and initially, anti-estrogenic activity correlated with anti-tumor activity. These findings led to extensive human trials that helped consolidate the actions of SERMs and refined their applications. In initial human studies tamoxifen, an "antiestrogen", was found to lower bone density in pre-menopausal women [42]. However, the "estrogen-like" actions of tamoxifen, maintained bone density in post-menopausal women [43], [44]. In the uterus tamoxifen acts as an agonist and increases the risk of endometrial cancer in post-menopausal women [45]. The next sections review the large-scale human chemoprevention trials of SERMs.

Chemoprevention

The first large human trial involving tamoxifen was the Royal Marsden study performed by Powles and colleagues [46], [47]. For this study approximately 3000 high-risk women were recruited and randomized to receive treatment with tamoxifen 20 mg/day for 8 years or placebo. High-risk status was determined by family history and a history of benign breast disease. The study found a decrease in LDL and loss of bone density in premenopausal women, but increased bone density in postmenopausal women and increased endometrial thickening on ultrasound study. Although this study initially showed no difference in the incidence of breast cancer, it was not powered to detect a difference in the development of breast cancer with either treatment group. Nevertheless, the twenty-year follow-up of this study does show a statistically significant reduction in the incidence of ER-positive breast cancer in the tamoxifen treatment arm after the 8 years of treatment [48].

The National Surgical Adjunctive Breast and Bowel Project (NSABP) P-1 trial by Bernard Fisher and colleagues was the first major chemoprevention trial in the Unites Stated with tamoxifen [49]. Over 13,000 women were recruited for this study in multiple centers around the US and Canada. Once again high-risk status was determined by family history, breast biopsy with pathological findings of lobular carcinoma *in situ* or atypical ductal hyperplasia, no children, menarche by 12 and age at birth of first child of over 30. The initial results of the NSABP trial showed a 49% reduction in the risk of invasive breast cancer and a 50% reduction in the risk of non-invasive breast cancer. Tamoxifen also reduced the incidence of osteoporotic fractures. No difference was seen in the risk of myocardial infarction but there was an increased risk of deep venous thrombosis, endometrial cancer and cataracts in the tamoxifen group.

Based on these clinical trials in 1998, tamoxifen was approved by the US FDA for reduction of the risk of breast cancer in high-risk women. Despite the positive results of the NSABP P-1 trial the side effects noted in the tamoxifen group resurrected the interest in other SERMs that had similar chemopreventive profiles to tamoxifen but with a more desirable side effect profile. This has led to human trials with raloxifene, an old compound, which had not been studied much since its discovery in the late 1970 s [50], [51].

Prevention of osteoporosis

In laboratory studies raloxifene was shown to inhibit DMBA-induced rat mammary carcinoma growth [52] and development [53], however, it was not as potent as tamoxifen. More importantly, raloxifene was as effective as tamoxifen in maintaining ovariectomized rat bone density but was less estrogen-like than tamoxifen in the rodent uterus [13] or in stimulating mouse endometrial tumor growth [54]. The short half-life of raloxifene makes it a difficult drug to dose, nonetheless; clinical trials with raloxifene have also helped define its pharmacology. The Multiple Outcomes for Raloxifene Evaluation (MORE) trial evaluated the effects of raloxifene in postmenopausal women [55], [60]. This study was extended to eight years as the Continuing Outcomes Relative to Evista (CORE) trial [61]. The results of the MORE/CORE trials demonstrated the effectiveness of raloxifene in preventing osteoporosis. In addition, raloxifene also inhibited the development of invasive breast cancer by 65% [61]. These clinical data justified the evaluation of raloxifene against tamoxifen to reduce the risk of breast cancer in high-risk postmenopausal women. The Study of Tamoxifen and Raloxifene (STAR) trial, was a phase III double-blinded study that randomized eligible postmenopausal women at a high risk for breast cancer, to receive tamoxifen 20 mg daily or raloxifene 60 mg daily [56]. The STAR trial demonstrated the equivalence of raloxifene and tamoxifen in reducing the incidence of invasive breast cancer. Furthermore, raloxifene had a better side effect profile with a lower incidence of endometrial cancer and hyperplasia, deep venous thromboses and cataracts. A drawback of raloxifene, however, was its decreased effectiveness in preventing the development of non-invasive breast cancer after two years, when compared to tamoxifen. Currently raloxifene is FDA-approved for the treatment and prevention of osteoporosis, and risk reduction for breast cancer in high-risk postmenopausal women.

Extending chemoprevention

The development of a chemopreventive agent such as tamoxifen but which has significant side effects had led to interest in whether naturally occurring compounds have similar chemopreventive effects. Epidemiologic observations have made this question even more seductive. While the etiology may be unclear, it has been well documented that Asian women have a lower incidence of breast and colorectal than Caucasian women [57]. Asian diets in particular are high in soy foods, which are felt to be responsible for this difference. When Asian women emigrate to western countries their incidence of breast cancer approaches that of the indigenous population [58]. This phenomenon has been observed in Japanese and Caucasian women who emigrate to the United States. It has also been observed that the risk of breast cancer in Asian Americans decreases in relation to increasing intake of soy derivatives [59]. Additionally, Chinese women who adopt a more westernized diet also appear to increase their incidence of breast cancer. All these findings have generated an interest in soy foods and its impact on hormone levels in the body. Phytoestrogens are the focus of current investigations. However, it should be stressed at the outset that despite beliefs of benefits from changes in diet and administration of supplements, there are dangers that breast cancer growth could be enhanced rather than prevented.

What are Phytoestrogens?

Phytoestrogens are plant derivatives that bear a structural similarity to 17-beta-estradiol and act in a similar manner. Structures of common phytoestrogens, SERMs and 17-beta-estradiol are shown in **Fig. 3**. The principal phytoestrogen groups are flavonoids, lignans, coumestans and stilbenes [60], [61], [62]. Phytoestrogens are present in common foods such as soybeans, grains, fruits and vegetables. An in-depth review of the various types of phytoestrogens is beyond the scope of this article, how-

ever, common properties of most phytoestrogens include their metabolism by gut flora to additional derivatives with varying estrogenic activity. Many studies have focused on isoflavones, which are a subgroup of the flavonoids, they include but are not limited to genistein, daidzein and biochanin A. These isoflavones have varying estrogenic activity [63] and isoflavones have been proposed as natural SERMs. Studies show that isoflavones act as antioxidants *in vitro* and exert antiproliferative activities [64], [65]. Equol (**Fig. 3**), an estrogenic metabolite of the isoflavonoids family [66], is produced from daidzein by the action of intestinal flora. This metabolic conversion however occurs in only 30% of the population [67].

Lignans, the most prevalent phytoestrogens in the diet are found in whole wheat, fruits and vegetables. Lignans are metabolized by the action of gut microflora into enterolactones and enterodiol [60] with very weak estrogenic properties [66]. While there are many studies on isoflavones, there are significantly fewer studies on coumestans and stilbenes. Coumestans are potent activators of the ER signaling pathway but are not as prevalent in the diet. Resveratrol is the most common stilbene and its use as a chemopreventive agent against breast cancer is actively being studied in rodent models [60]. In the next section we will consider the mechanism of action of phytoestrogens. The interaction of phytoestrogens with ERs is in some ways similar to the SERM/ER interaction, but there are significant differences that confound biological comparisons.

Mechanism of action of phytoestrogens

Hydroxylated SERMs in general have a higher binding affinity for both ERα and ERβ compared to phytoestrogens. As with SERMS, phytoestrogens can bind to either ERα or ERβ however, phytoestrogens appear to have a higher affinity for ERβ [68]. This affinity may be dose-dependent but overall phytoestrogens have a significantly lower affinity for the ER than estradiol [69], [70]. In addition the estrogenic potency of phytoestrogens varies within the particular phytoestrogen group. For example, within the flavonoid family genistein has greater potency than biochanin A, which has greater potency than daidzein [63]. Kuiper and colleagues [31] demonstrated that the stimulation of transcriptional activity by both subtypes of the ER vary depending on the estrogenic potency of the phytoestrogen and the further use of reporter gene assays demonstrate that synthetic estrogens and phytoestrogens have varying affinity for the ER and for each ER isoforms [68].

SERMs are non-steroidal estrogens that become antiestrogenic by virtue of their correctly positioned side chain. However, the antiestrogen side chain is not present in phytoestrogens and this structural deficit may therefore limit their classifications as SERMs. Nevertheless, the presence of a correctly positioned phenolic ring and also the distance between the two opposing phenolic oxygens in the isoflavone structure is similar to that of 17-beta-estradiol (**Fig. 3**). This similarity allows the isoflavones to bind to either subtype of ER, effectively displacing 17-beta-estradiol. Studies have found that isoflavones have both agonistic and antagonistic effects, although they are strong ERβ agonists and weak ERα agonists [71]. It is this pharmacological receptor interaction rather than competitive interaction at a single receptor site that may be responsible for some of the diverse biological actions of phytoestrogens. This action may explain how phytoestrogens protect against breast cancer, because ERβ inhibits mammary cell growth as well as the stimulatory effects of ERα [72]. However, there is yet another dimension of molecular action at the ER that might be important. It is not certain whether isoflavones displace the estradiol by binding to a primary site on the ER, causing competitive binding between the isoflavones and the estradiol, or whether the isoflavones bind to a secondary site on the ER [73]. In contrast, genistein has been found to bind to the active site of ERβ [74].

Recent studies have attempted to decipher the actual role of each receptor subtype in gene activation and physiological response. Part of the problem in determining the physiological actions of phytoestrogens is our ignorance of the actual role of the ERα and ERβ. For example, a study by Hertrampf and colleagues [75] shows that the osteoprotective effect of genistein is mediated through the ERα-dependent pathways and its effect is enhanced by physical activity. Also, the activation of ERβ may modulate ERα-mediated physiological effects *in vivo*.

Many factors such as the ligand, dose and interaction of the ligand and receptor all influence ER molecular biology at the target site [76].

As with the SERMs, studies have shown that the recruitment of coregulatory molecules may be important in determining the function of phytoestrogens. In particular, isoflavones appear to selectively trigger ERβ transcriptional pathways, especially transcriptional repression. This affinity for the ERβ results in the exposure of a weak activation function-2 (AF-2) on the surface of ERβ, which has greater affinity for certain coregulators compared to ERα [72]. Phytoestrogens also have differential activity on several ER associated signaling pathways. For example, Akt, which is normally phosphorylated secondary to activation of ERα, is up-regulated by genistein and daidzein in ER-positive breast cancer cell lines, while resveratrol has an inhibitory effect on the phosphorylation of Akt [77]. In contrast, in ER-negative cell lines, resveratrol and daidzein activate Akt and genistein inhibits activation of Akt [77]. This is clearly a non-ER event, but whether this is cancer-specific or a toxicity of studies conducted *in vitro* can only be resolved with studies *in vivo*.

Although the isoflavones have agonistic and antagonistic estrogenic effects, the phytoestrogens also induce differentiation as well as inhibit angiogenesis, cell proliferation, tyrosine kinase, and topoisomerase II; all of which will help prevent tumor growth. However, it is important to stress again that despite the fact that there have been numerous and extensive laboratory studies on the mechanisms of breast cancer chemoprevention with phytoestrogens, there is no definitive evidence that proves that phytoestrogens are chemopreventive but they may contribute to adverse outcomes in breast cancer [78].

Cell and animal studies on the effect of phytoestrogens

Phytoestrogens have been likened to natural SERMs, and a brief survey of cell and animal studies of phytoestrogens reveals some similarities to SERMs such as tamoxifen. The approach to these studies may be classified into three broad categories. The first are studies that focus primarily on the role of phytoestrogens as a chemopreventive agent. The second are those studies that focus on phytoestrogens as a treatment agent. The third are those studies that focus on the biological effects when phytoestrogens are used continuously from neonates to adults.

The first category focuses on the chemopreventive effects of phytoestrogens in animal models that are subsequently treated with a chemical carcinogen. Animal studies have shown that when rats are treated with phytoestrogens and then exposed to a carcinogen they are less likely to develop breast cancer if exposure to phytoestrogens occurs at an early age [79], [80]. Lamarti-

niere and colleagues [79] demonstrated that the timing of exposure to phytoestrogens whether pre- or post-puberty, may influence their action on preventing mammary carcinogenesis. Lamartiniere [79] found that neonatal injections of genistein reduced the incidence of DMBA-induced mammary tumors in rats. Further evaluation revealed that the overall effect of genistein on prepubertal rats appeared to be secondary to early differentiation in mammary tissues resulting in less active EGF signaling pathways in adulthood that may be protective against breast cancer. A recent meta-analyses by Warri et al. [81] revealed that pubertal exposure to phytoestrogens result in changes in the mammary gland morphology and signal pathways that mimic those induced by the estrogenic environment of early first pregnancy.

The second group of studies focus on the use of phytoestrogen treatments in both tumor-implanted athymic mice and breast cancer cell lines. Studies have shown that treating estrogen-sensitive MCF-7 cell lines with genistein has an inhibitory effect on their growth [82]. However, not all studies have had such conclusive findings such as that the action of phytoestrogens on breast cancer cells may be dose-dependent. At low concentrations phytoestrogens may stimulate growth, and at high concentrations inhibit growth [66], [82], [83], [84], [85]. The studies by Helferich help elucidate the dose-dependent actions of isoflavones [93], [86]. In animal studies, in which ovariectomized athymic mice were implanted with MCF-7 cells, genistein promotes the growth of ER+ MCF 7 cells and the effect of this isoflavone was dose-dependent. At concentrations as low as 10 nM genistein promoted growth of ER-dependent MCF-7 cells *in vitro* [86]. At higher concentration (> 20 microM) genistein inhibited the MCF-7 cell growth. In addition genistein can stimulate growth of MCF-7 cells *in vivo* in a dose-dependent manner [87]. Clearly, these data call for caution with the use of phytoestrogens in women with breast cancer.

Indeed, the early study by Welshons et al. [66] cautioned against the use of antihormonal therapies that did not block the ER for the treatment of breast cancer because high fiber or exclusively vegetarian diets with phytoestrogens-containing food supplements could enhance the probability of tumor recurrence and growth. Furthermore the combination of phytoestrogens and tamoxifen to treat breast cancer may result in decreased efficacy of tamoxifen. In a study evaluating the development of tumor and the tumor latency period, tamoxifen-treated mice fed a low dose isoflavone-enriched diet had a higher tumor incidence and a shorter tumor latency period than placebo-treated mice [95]. In addition tamoxifen-associated mammary tumor prevention was also significantly reduced. Nevertheless, certain phytoestrogens have also been noted to cause apoptosis of human breast cancer cells and this occurred at concentrations of 20 – 25 micromol/L [88], [89], [90]. While phytoestrogens have been observed to cause these various actions *in vitro*, it is unclear that *in vivo* the concentrations needed to achieve these actions are attainable. In animal studies a protective effect of phytoestrogens on the development of mammary cancer are conflicting [91], [92]. Santell and colleagues [92] have shown that while genistein may inhibit breast cancer cells *in vitro*, treatment of tumor-bearing athymic mice with genistein did not inhibit tumor growth, however in their study ER-negative human breast cancer cell lines were used. It would seem that the ability of phytoestrogens to be toxic *in vitro* at high concentrations does not extrapolate to models *in vivo* where the ability to maintain high local concentrations for long periods may be impaired.

A third approach is the study of the effects from early exposure to phytoestrogens from the perinatal periods and onwards. This approach was recently adopted by Mardon and colleagues [93]. Rats perinatally or lifelong exposed to a rich isoflavone diet exhibited higher body weight and fat mass at 24 months of age. Perinatal exposure to phytoestrogens led to higher bone mineral density in later life [93]. The translation of these data to human epidemiology and pharmacology is the challenge and has no immediate application to effects on mammary carcinogenesis. The observation is an estrogen-like action on bone rather than SERM related.

Human trials

Human trials on phytoestrogens differ from SERMs because unlike the SERMs, there are no major large-scale prospective studies of chemoprevention and pharmacology. Human studies on phytoestrogens can be divided into two broad categories. The first are studies that evaluate the effect of phytoestrogens on estrogen biosynthesis and excretion, the second are those studies that evaluate the overall impact of dietary phytoestrogens on specific clinical endpoints such as menopausal symptoms and bone mineral density presumably through a stimulatory action through the ER. Many studies have examined the use of phytoestrogens as chemopreventive agents; however, these studies are of limited value as they are retrospective.

Estrogen biosynthesis and excretion

Human studies on the effect of phytoestrogens on estrogen biosynthesis and excretion usually evaluate levels of circulating estrogen or steroid by-products and metabolites in the urine. In addition in many of these studies the levels of phytoestrogens are also measured and factors that affect these levels are explored. Human studies have shown conflicting results regarding the overall effect of phytoestrogens. Lu and colleagues [94] treated 10 pre-menopausal women with a soy-containing diet beginning on day two of the menstrual cycle to day two of the next cycle. Blood and urine samples were obtained before and during the initiation of the soy diet. Their results showed that the circulating levels of 17-beta-estradiol decreased by 25%, however, cycle length did not change [94]. A dietary intervention study by Kumar and co-workers showed similar findings [95]. This study randomized women to receive 40 mg of isoflavones day or placebo for a 12-week period. They found that serum free estradiol and estrone levels decreased. Serum hormone binding globulin increased and mean cycle length also increased. Conversely, a year-long dietary intervention study by Maskarinec and co-workers [96] in premenopausal women did not find any difference in cycle length or hormone levels. These studies raise the question that while dietary intake of phytoestrogens is important, intake alone may not be the determinant of a chemoprotective effect.

Since a Finnish case control study [97] suggests that high enterolactone concentrations are associated with decreased breast cancer risk., it is possible that lifestyle factors that affect enterolactone may be linked to breast cancer risk. Whether these lifestyle factors that control enterolactone levels are linked to breast cancer risk remains to be seen. Administration of antibiotics has been noted to decrease the serum concentration of enterolactone for a prolonged period [98]. Premenopausal women who are treated with long-term antibiotics for urinary tract infections seem to be at higher risk for breast cancer, presumably because it alters the gut metabolism of phytoestrogens [99]. Smoking and obesity have been noted to decrease plasma enterolac-

tone levels, however, tea, coffee, fiber and vegetables have the opposite effect [100]. In a study monitoring plasma enterolactone levels, women were noted to have a higher plasma concentration while on wheat bread 41.1 nmol/L compared to 15.4 nmol/L while on white bread [67]. Links to actual cancer risk do not exist but associations have been noted.

In human studies, it is often difficult to measure serum levels of phytoestrogens, because of a short half-life. Since most phytoestrogens are excreted in the urine, urine analysis of metabolites of phytoestrogens can be used to give an indication of exposure to phytoestrogens [101]. Urinary excretion of phytoestrogens varies in different regions of the world [102]. Women in areas with a low incidence of breast cancer have higher urinary isoflavonoids than women living in areas with a high incidence of breast cancer. Vegetarians also have a higher concentration of isoflavonoids in their urine than omnivores [103]. The excretion of equol in the urine has been proposed as a possible marker of the chemoprotective effect of phytoestrogens [112], [113]. Duncan and colleagues [104] studied the hormone profile of equol excretors versus equol non-excretors and found that regardless of the amount of phytoestrogens ingested in the diet, equol excretors had decreased levels of estrone, estrone sulfate, testosterone, DHEA and higher levels of steroid hormone binding globulin. This steroid hormone profile has been found to be a protective profile for breast cancer. The possible mechanisms to create a "change profile" may include the findings that phytoestrogens stimulate the production of sex steroid binding globulin by liver cells [103] and have inhibitory effects on the enzymes involved in the synthesis of estrogen. Phytoestrogens are known to decrease the conversion of androgens to estrogen by blocking the aromatase enzyme system. [105].

Phytoestrogens and cinical endpoints

The second group of human studies are those that focus on the effect of phytoestrogens on focal clinical endpoints. These endpoints vary and include alleviation of menopausal symptoms, maintenance of bone mineral density and development of breast cancer in some retrospective studies. Given recent concern regarding the possible adverse effects of hormone replacement therapy other alternatives for treatment of menopausal symptoms have been explored and phytoestrogens have played a significant role. A recent Cochrane review of the database revealed no clear evidence of the effectiveness of phytoestrogens in alleviating menopausal symptoms [106]. This notwithstanding, there are some small trials which show a benefit to using phytoestrogens for treating menopausal symptoms. In a double-blind prospective study sixty women were randomized to receive 60 mg of isoflavones daily for 3 months or placebo [107]. The menopausal symptoms before and after treatment were recorded. Women receiving the phytoestrogens treatment noted a 57% and 43% decrease in the incidence of hot flashes and night sweats, respectively. Similar results were seen in a small trial using a 6-week treatment of flaxseed for the treatment of menopausal symptoms [108]. Some investigators are evaluating the use of phytoestrogens as alternative agents to hormone replacement therapy (HRT) in the management of postmenopausal symptoms [107]. Recently, prenylated flavonoids derived from hops are being used to treat menopausal symptoms. One such compound is 8-prenylnaringenin (**Fig. 3**) that has strong estrogenic activity [109]. MenoHop an agent containing the phytoestrogen 8-prenylnaringenin, is currently being evaluated to treat menopausal complaints in Belgium [110].

The relationship between phytoestrogens and bone health remains unclear, with some studies showing a benefit associated with phytoestrogen treatment and others showing none [111]. Supplementation of diet with isoflavones has been shown to help maintain lumbar spine bone density [122], [112]. A randomized double-blind control trial was performed to compare with HRT, the effect of the phytoestrogen genistein on bone metabolism and bone mineral density [113]. Patients were randomized to receive either HRT daily (1 mg of 17-beta-estradiol and 0.5 mg norethisterone) or genistein 30 mg daily or placebo daily for a period of 1 year. On completion of this protocol women receiving the HRT and genistein had significantly increased bone mineral density in the femur compared to those in the placebo group. In another randomized control trial, Atkinson and colleagues [114] showed that women receiving an isoflavones extract had a decreased loss of lumbar spine bone mineral content and bone mineral density compared to placebo.

Direct studies on the efficacy of phytoestrogens in preventing breast cancer are difficult given the length of time required to perform such a study. Indeed, this obstacle with phytoestrogen research illustrates how powerful SERMS are to produce dramatic decreases in breast cancer incidence within 5 – 10 years [55], [115]. However, surrogate endpoints such as the effect of phytoestrogens on breast cell proliferation and mammographic density have been studied. Increased breast cell proliferation and increased mammographic density are risk factors for malignancy. Short-term dietary supplementation with phytoestrogens stimulates breast epithelial proliferation [116]. This finding has also been noted in premenopausal women treated with prolonged phytoestrogen intake [117]. This breast proliferation is evident on mammograms as increased mammographic densities and some of these parenchymal patterns are associated with a higher risk of breast cancer [118]. These histological findings are supported by the observation of increased high risk parenchymal sonographic patterns in women who report low dietary soy protein intake [119]. Other studies such as that by Maskarinec and colleagues [120] show a similar finding in mammographic density in women treated with prolonged phytoestrogen supplementation.

As noted in animal studies, [101] the age at which a woman is exposed to phytoestrogens and length of exposure to phytoestrogens may be important in determining whether a protective benefit is obtained. A prospective 12-ear study of diet and breast cancer by Key and colleagues [121] of over 30,000 women in Japan showed that there was no relationship found between soy food consumption and the development of breast cancer, however this study was comprised of mostly non-adolescent women. In contrast, Shu and colleagues [122] performed a retrospective case controlled study on Chinese women with breast cancer. Subjects completed a questionnaire regarding their dietary intake in adolescence. A high soy consumption as an adolescent was associated with a decreased incidence of breast cancer as an adult. This may also explain why when women emigrate to countries with a higher incidence of breast cancer than their native country, they are more likely to have a decreased incidence of breast cancer if they emigrated after puberty [123].

While there is increasing excitement at the possible role of phytoestrogens as chemopreventive agents or as complimentary alternative medicine for menopausal symptoms their safety profile remains largely unknown and concerns regarding this have been raised in two recent reviews [124], [125]. Isoflavones such as genistein have been found to stimulate the growth of MCF-7 cells [86], [93]. Some studies have shown that soy products in-

crease breast epithelial cell proliferation [125], [126], which may increase the risk of breast cancer. These findings suggest caution in the broad use of phytoestrogens. In addition the interaction of phytoestrogens and tamoxifen inbreast cancer patients may negate the protective effects of SERMs and caution has een advised against thebination of these two agents [126].

Conclusion

Since their discovery the use of SERMs in clinical practice continues to expand [127], [128], [129]. As our knowledge of phytoestrogens grows, so does our understanding of their interaction with the ER and ability to possibly act as a natural SERM or conversely to antagonize the actions of SERMs. However, based on their structure-function relationships, the molecular endocrinology of SERMs and phytoestrogens is very different and the phytoestrogens appear to act as ER agonists at low concentrations but may act as antagonists by biochemical mechanisms through the ER beta receptor complex. Despite the advances in the treatment of breast cancer, prevention if possible must be superior to treatment. Currently tamoxifen and raloxifene are the first important steps in the quest to develop a complete preventative agent. In the future, a role, if any for the phytoestrogens or their derivatives may emerge, but current research is too weak to provide any clinical guidelines beyond caution. Alternatively, clues from laboratory studies may prove to be important in future drug development. An example of this is the current interest in the pharmacology of resveratrol which may have valuable pharmacological actions not mediated via the ER [130], [131].

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References

1 *Jordan VC.* Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2003; 2: 205 – 13
2 *Beatson GT.* On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. Lancet 1896; 2: 104 – 7
3 *Boyd S.* On oophorectomy in cancer of the breast. B M J 1900; ii: 1161 – 7
4 *Allen E, Doisy EA.* An ovarian hormone: Preliminary reports on its localization, extraction and partial purification and action in test animals. JAMA 1923; 81: 810 – 21
5 *Lacassagne A.* Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer 1936; 27: 217 – 25
6 *Jensen EV, Jacobson HI.* Basic guides to the mechanism of estrogen action. Recent Prog Horm Res 1962; 18: 387 – 414
7 *Jensen EV, Jordan VC.* The estrogen receptor: a model for molecular medicine. Clin Cancer Res 2003; 9: 1980 – 9
8 *Lippman ME, Bolan G.* Oestrogen-responsive human breast cancer in long term tissue culture. Nature 1975; 256: 592 – 3
9 *Jordan VC.* Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 1974; 5: 354
10 *Jordan VC.* Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 1976; 12: 419 – 24
11 *Harper MJ, Walpole AL.* A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 1967; 13: 101 – 19
12 *Jordan VC, Robinson SP.* Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc 1987; 46: 1870 – 4
13 *Jordan VC, Phelps E, Lindgren JU.* Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987; 10: 31 – 5
14 *Jordan VC.* Selective estrogen receptor modulation: a personal perspective. Cancer Res 2001; 61: 5683 – 7
15 *Smith CL, O'Malley BW.* Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev 2004; 25: 45 – 71
16 *Jordan VC, Collins MM, Rowsby L, Prestwich G.* A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 1977; 75: 305 – 16
17 *Allen KE, Clark ER, Jordan VC.* Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 1980; 71: 83 – 91
18 *Borgna JL, Rochefort H.* Hydroxylated metabolites of tamoxifen are formed in vivo and bound to estrogen receptor in target tissues. J Biol Chem 1981; 256: 859 – 68
19 *Lien EA, Solheim E, Ueland PM.* Distribution of tamoxifen and its metabolites in rate and human tissues during steady-state treatment. Cancer Res 1991; 51: 4837 – 44
20 *Lien EA, Solheim E, Kvinnsland S, Ueland PM.* Identification of 4-hydroxy-*N*-desmethyltamoxifen as a metabolite of tamoxifen in human bile. Cancer Res 1988; 48: 2304 – 8
21 *Lien EA, Solheim E, Lea OA, Lundgren S, Kvinnsland S, Ueland PM.* Distribution of 4-hydroxy-*N*-desmethyltamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. Cancer Res 1989; 49: 2175 – 83
22 *Jordan VC.* New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer. Steroids 2007; 72: 829 – 42
23 *Fromson JM, Pearson S, Bramah S.* The metabolism of tamoxifen (I.C.I. 46,474). II. In female patients. Xenobiotica 1973; 3: 711 – 4
24 *Fromson JM, Pearson S, Bramah S.* The metabolism of tamoxifen (I.C.I. 46,474). I. In laboratory animals. Xenobiotica 1973; 3: 693 – 709
25 *Borges S, Desta Z, Li L, Skaar TC, Ward BA, Nguyen A et al.* Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. Clin Pharmacol Ther 2006; 80: 61 – 74
26 *Kemp DC, Fan PW, Stevens JC.* Characterization of raloxifene glucuronidation *in vitro*: contribution of intestinal metabolism to presystemic clearance. Drug Metab Dispos 2002; 30: 694 – 700
27 *Jeong EJ, Liu Y, Lin H, Hu M.* Species- and disposition model-dependent metabolism of raloxifene in gut and liver: role of UGT1A10. Drug Metab Dispos 2005; 33: 785 – 94
28 *Snyder KR, Sparano N, Malinowski JM.* Raloxifene hydrochloride. Am J Health Syst Pharm 2000; 76 – 8: 1669 – 75
29 *Buzdar AU, Marcus C, Holmes F, Hug V, Hortobagyi G.* Phase II evaluation of Ly156758 in metastatic breast cancer. Oncology 1988; 45: 344 – 5
30 *Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA.* Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 1996; 93: 5925 – 30
31 *Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S et al.* Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 1997; 138: 863 – 70
32 *Lim YC, Li L, Desta Z, Zhao Q, Rae JM, Flockhart DA et al.* Endoxifen, a secondary metabolite of tamoxifen, and 4-OH-tamoxifen induce similar changes in global gene expression patterns in MCF-7 breast cancer cells. J Pharmacol Exp Ther 2006; 318: 503 – 12
33 *Koehler KF, Helguero LA, Haldosen LA, Warner M, Gustafsson JA.* Reflections on the discovery and significance of estrogen receptor beta. Endocr Rev 2005; 26: 465 – 78
34 *Chang EC, Frasor J, Komm B, Katzenellenbogen BS.* Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. Endocrinology 2006; 147: 4831 – 42
35 *Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA.* Estrogen receptor beta inhibits 17-beta-estradiol-stimulated prolifera-

tion of the breast cancer cell line T47D. Proc Natl Acad Sci U S A 2004; 101: 1566 – 71
36 *Jordan VC.* Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nat Rev Cancer 2007; 7: 46 – 53
37 *Paige LA, Christensen DJ, Gron H, Norris JD, Gottlin EB, Padilla KM et al.* Estrogen receptor (ER) modulators each induce distinct conformational changes in ER alpha and ER beta. Proc Natl Acad Sci U S A 1999; 96: 3999 – 4004
38 *Mc Ilroy M, Fleming FJ, Buggy Y, Hill AD, Young LS.* Tamoxifen-induced ER-alpha-SRC-3 interaction in HER2 positive human breast cancer; a possible mechanism for ER isoform specific recurrence. Endocr Relat Cancer 2006; 13: 1135 – 45
39 *Cole MP, Jones CT, Todd ID.* A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer 1971; 25: 270 – 5
40 *Ward HW.* Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J 1973; 1: 13 – 4
41 *Jordan VC, Koerner S.* Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 1975; 11: 205 – 6
42 *Powles TJ, Hickish T, Kanis JA, Tidy A, Ashley S.* Effect of tamoxifen on bone mineral density measured by dual-energy x-ray absorptiometry in healthy premenopausal and postmenopausal women. J Clin Oncol 1996; 14: 78 – 84
43 *Kristensen B, Ejlertsen B, Dalgaard P, Larsen L, Holmegaard SN, Transbol I et al.* Tamoxifen and bone metabolism in postmenopausal low-risk breast cancer patients: a randomized study. J Clin Oncol 1994; 12: 992 – 7
44 *Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC et al.* Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 1992; 326: 852 – 6
45 *Jordan VC, Morrow M.* Should clinicians be concerned about the carcinogenic potential of tamoxifen? Eur J Cancer 1994; 30A: 1714 – 21
46 *Powles TJ, Hardy JR, Ashley SE, Farrington GM, Cosgrove D, Davey JB et al.* A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. Br J Cancer 1989; 60: 126 – 31
47 *Powles TJ, Jones AL, Ashley SE, O'Brien ME, Tidy VA, Treleavan J et al.* The Royal Marsden Hospital pilot tamoxifen chemoprevention trial. Breast Cancer Res Treat 1994; 31: 73 – 82
48 *Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M.* Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. J Natl Cancer Inst 2007; 99: 283 – 90
49 *Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM et al.* Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998; 90: 1371 – 88
50 *Lewis-Wambi JS, Jordan VC.* Case histories: raloxifene. In: Taylor J, Trigle D, editors. Comprehensive medicinal chemistry II, Vol. 8. Oxford, UK: Elsevier Limited; 2006: 103 – 21
51 *Black LJ, Jones CD, Falcone JF.* Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 1983; 32: 1031 – 6
52 *Clemens JA, Bennett DR, Black LJ, Jones CD.* Effects of a new antiestrogen, keoxifene (LY156758), on growth of carcinogen-induced mammary tumors and on LH and prolactin levels. Life Sci 1983; 32: 2869 – 75
53 *Gottardis MM, Jordan VC.* Antitumor actions of keoxifene and tamoxifen in the *N*-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987; 47: 4020 – 4
54 *Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC.* Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 1990; 50: 3189 – 92
55 *Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA et al.* The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple outcomes of raloxifene evaluation. JAMA 1999; 281: 2189 – 97
56 *Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN et al.* Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP study of tamoxifen and raloxifene (STAR) P-2 trial. JAMA 2006; 295: 2727 – 41
57 *Fund WCR.* Food, nutrition and the prevention of cancer: a global perspective. Washington: American Institute for Cancer Research; 1997
58 *Wu AH, Ziegler RG, Nomura AM, West DW, Kolonel LN, Horn-Ross PL et al.* Soy intake and risk of breast cancer in Asians and Asian Americans. Am J Clin Nutr 1998; 68: 1437S – 43S
59 *Wu AH, Ziegler RG, Horn-Ross PL, Nomura AM, West DW, Kolonel LN et al.* Tofu and risk of breast cancer in Asian-Americans. Cancer Epidemiol Biomarkers Prev 1996; 5: 901 – 6
60 *Limer JL, Speirs V.* Phyto-oestrogens and breast cancer chemoprevention. Breast Cancer Res 2004; 6: 119 – 27
61 *Moon YJ, Wang X, Morris ME.* Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. Toxicol In Vitro 2006; 20: 187 – 210
62 *Sirtori CR, Arnoldi A, Johnson SK.* Phytoestrogens: end of a tale? Ann Med 2005; 37: 423 – 38
63 *Zand RS, Jenkins DJ, Diamandis EP.* Steroid hormone activity of flavonoids and related compounds. Breast Cancer Res Treat 2000; 62: 35 – 49
64 *Peterson G.* Evaluation of the biochemical targets of genistein in tumor cells. J Nutr 1995; 125: 784S – 9S
65 *Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R et al.* Genistein, a dietary-derived inhibitor of in vitro angiogenesis. Proc Natl Acad Sci U S A 1993; 90: 2690 – 4
66 *Welshons WV, Murphy CS, Koch R, Calaf G, Jordan VC.* Stimulation of breast cancer cells *in vitro* by the environmental estrogen enterolactone and the phytoestrogen equol. Breast Cancer Res Treat 1987; 10: 169 – 75
67 *Adlercreutz H.* Phyto-oestrogens and cancer. Lancet Oncol 2002; 3: 364 – 73
68 *Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT et al.* Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 1998; 139: 4252 – 63
69 *McCarty MF.* Isoflavones made simple – genistein's agonist activity for the beta-type estrogen receptor mediates their health benefits. Med Hypotheses 2006; 66: 1093 – 114
70 *van der Woude H, Ter Veld MG, Jacobs N, van der Saag PT, Murk AJ, Rietjens IM.* The stimulation of cell proliferation by quercetin is mediated by the estrogen receptor. Mol Nutr Food Res 2005; 49: 763 – 71
71 *Fitzpatrick LA.* Phytoestrogens – mechanism of action and effect on bone markers and bone mineral density. Endocrinol Metab Clin North Am 2003; 32: 233 – 52, viii
72 *An J, Tzagarakis-Foster C, Scharschmidt TC, Lomri N, Leitman DC.* Estrogen receptor beta-selective transcriptional activity and recruitment of coregulators by phytoestrogens. J Biol Chem 2001; 276: 17 808 – 14
73 *Martin PM, Horwitz KB, Ryan DS, McGuire WL.* Phytoestrogen interaction with estrogen receptors in human breast cancer cells. Endocrinology 1978; 103: 1860 – 7
74 *Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engstrom O et al.* Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. EMBO J 1999; 18: 4608 – 18
75 *Hertrampf T, Gruca MJ, Seibel J, Laudenbach U, Fritzemeier KH, Diel P.* The bone-protective effect of the phytoestrogen genistein is mediated via ER alpha-dependent mechanisms and strongly enhanced by physical activity. Bone 2007; 40: 1529 – 35
76 *Chang EC, Charn TH, Park SH, Helferich WG, Komm B, Katzenellenbogen JA et al.* Estrogen receptors alpha and beta as determinants of gene expression: influence of ligand, dose, and chromatin binding. Mol Endocrinol 2008; 22: 1032 – 43
77 *Brownson DM, Azios NG, Fuqua BK, Dharmawardhane SF, Mabry TJ.* Flavonoid effects relevant to cancer. J Nutr 2002; 132: 3482S – 9S
78 *Mense SM, Hei TK, Ganju RK, Bhat HK.* Phytoestrogens and breast cancer prevention: possible mechanisms of action. Environ Health Perspect 2008; 116: 426 – 33
79 *Lamartiniere CA, Moore JB, Brown NM, Thompson R, Hardin MJ, Barnes S.* Genistein suppresses mammary cancer in rats. Carcinogenesis 1995; 16: 2833 – 40
80 *Murrill WB, Brown NM, Zhang JX, Manzolillo PA, Barnes S.* Molecular epidemiology and cancer prevention: Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. Carcinogenesis 1996; 17: 1451 – 7
81 *Warri A, Saarinen NM, Makela S, Hilakivi-Clarke L.* The role of early life genistein exposures in modifying breast cancer risk. Br J Cancer 2008; 98: 1485 – 93
82 *Fioravanti L, Cappelletti V, Miodini P, Ronchi E, Brivio M, Di Fronzo G.* Genistein in the control of breast cancer cell growth: insights into the mechanism of action *in vitro*. Cancer Lett 1998; 130: 143 – 52
83 *Constantinou AI, Krygier AE, Mehta RR.* Genistein induces maturation of cultured human breast cancer cells and prevents tumor growth in nude mice. Am J Clin Nutr 1998; 68: 1426S – 30S
84 *Hsu JT, Hung HC, Chen CJ, Hsu WL, Ying C.* Effects of the dietary phytoestrogen biochanin A on cell growth in the mammary carcinoma cell line MCF-7. J Nutr Biochem 1999; 10: 510 – 7

85 *Maggiolini M, Bonofiglio D, Marsico S, Panno ML, Cenni B, Picard D et al.* Estrogen receptor alpha mediates the proliferative but not the cytotoxic dose-dependent effects of two major phytoestrogens on human breast cancer cells. Mol Pharmacol 2001; 60: 595 – 602
86 *Hsieh CY, Santell RC, Haslam SZ, Helferich WG.* Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells *in vitro* and *in vivo.* Cancer Res 1998; 58: 3833 – 8
87 *Allred CD, Allred KF, Ju YH, Goeppinger TS, Doerge DR, Helferich WG.* Soy processing influences growth of estrogen-dependent breast cancer tumors. Carcinogenesis 2004; 25: 1649 – 57
88 *Katdare M, Osborne M, Telang NT.* Soy isoflavone genistein modulates cell cycle progression and induces apoptosis in HER-2/neu oncogene expressing human breast epithelial cells. Int J Oncol 2002; 21: 809 – 15
89 *Leung LK, Wang TT.* Bcl-2 is not reduced in the death of MCF-7 cells at low genistein concentration. J Nutr 2000; 130: 2922 – 6
90 *Vergote D, Cren-Olive C, Chopin V, Toillon RA, Rolando C, Hondermarck H et al.* (–)-Epigallocatechin (EGC) of green tea induces apoptosis of human breast cancer cells but not of their normal counterparts. Breast Cancer Res Treat 2002; 76: 195 – 201
91 *Lamartiniere CA.* Protection against breast cancer with genistein: a component of soy. Am J Clin Nutr 2000; 71: 1705S – 7
92 *Santell RC, Kieu N, Helferich WG.* Genistein inhibits growth ofindependent human breast cancer cells in culture but not in athymic mice. J Nutr 2000; 130: 1665 – 9
93 *Mardon J, Mathey J, Kati-Coulibaly S, Puel C, Davicco MJ, Lebecque P et al.* Influence of lifelong soy isoflavones consumption on bone mass in the rat. Exp Biol Med (Maywood) 2008; 233: 229 – 37
94 *Lu L-JW, Anderson KE, Grady JJ, Kohen F, Nagamani M.* Decreased ovarian hormones during a soya diet: implications for breast cancer prevention. Cancer Res 2000; 60: 4112 – 21
95 *Kumar NB, Cantor A, Allen K, Riccardi D, Cox CE.* The specific role of isoflavones on estrogen metabolism in premenopausal women. Cancer 2002; 94: 1166 – 74
96 *Maskarinec G, Williams AE, Inouye JS, Stanczyk FZ, Franke AA.* A randomized isoflavone intervention among premenopausal women. Cancer Epidemiol Biomarkers Prev 2002; 11: 195 – 201
97 *Pietinen P, Stumpf K, Mannisto S, Kataja V, Uusitupa M, Adlercreutz H.* Serum enterolactone and risk of breast cancer: a case-control study in eastern Finland. Cancer Epidemiol Biomarkers Prev 2001; 10: 339 – 44
98 *Kilkkinen A, Pietinen P, Klaukka T, Virtamo J, Korhonen P, Adlercreutz H.* Use of oral antimicrobials decreases serum enterolactone concentration. Am J Epidemiol 2002; 155: 472 – 7
99 *Knekt P, Adlercreutz H, Rissanen H, Aromaa A, Teppo L, Heliovaara M.* Does antibacterial treatment for urinary tract infection contribute to the risk of breast cancer? Br J Cancer 2000; 82: 1107 – 10
100 *Kilkkinen A, Stumpf K, Pietinen P, Valsta LM, Tapanainen H, Adlercreutz H.* Determinants of serum enterolactone concentration. Am J Clin Nutr 2001; 73: 1094 – 100
101 *Valentin-Blasini L, Blount BC, Caudill SP, Needham LL.* Urinary and serum concentrations of seven phytoestrogens in a human reference population subset. J Expo Anal Environ Epidemiol 2003; 13: 276 – 82
102 *Peeters PH, Keinan-Boker L, van der Schouw YT, Grobbee DE.* Phytoestrogens and breast cancer risk. Review of the epidemiological evidence. Breast Cancer Res Treat 2003; 77: 171 – 83
103 *Adlercreutz H.* Human health and phytoestrogens. In: Korach K, editor. Reproductive and developmental toxicology. New York: Marcel Dekker Inc; 1998: 299 – 371
104 *Duncan AM, Merz-Demlow BE, Xu X, Phipps WR, Kurzer MS.* Premenopausal equol excretors show plasma hormone profiles associated with lowered risk of breast cancer. Cancer Epidemiol Biomarkers Prev 2000; 9: 581 – 6
105 *Whitehead SA, Lacey M.* Phytoestrogens inhibit aromatase but not 17beta-hydroxysteroid dehydrogenase (HSD) type 1 in human granulosa-luteal cells: evidence for FSH induction of 17beta-HSD. Hum Reprod 2003; 18: 487 – 94
106 *Lethaby AE, Brown J, Marjoribanks J, Kronenberg F, Roberts H, Eden J.* Phytoestrogens for vasomotor menopausal symptoms. Cochrane Database Syst Rev; 2007: CD001395
107 *Cheng G, Wilczek B, Warner M, Gustafsson JA, Landgren BM.* Isoflavone treatment for acute menopausal symptoms. Menopause 2007; 14: 468 – 73
108 *Pruthi S, Thompson SL, Novotny PJ, Barton DL, Kottschade LA, Tan AD et al.* Pilot evaluation of flaxseed for the management of hot flashes. J Soc Integr Oncol 2007; 5: 106 – 12
109 *Gerhäuser C.* Beer constituents as potential cancer chemopreventive agents. Eur J Cancer 2005; 41: 1941 – 54
110 *Heyerick A, Vervarcke S, Depypere H, Bracke M, De Keukeleire D.* A first prospective, randomized, double-blind, placebo-controlled study on the use of a standardized hop extract to alleviate menopausal discomforts. Maturitas 2006; 54: 164 – 75
111 *Weaver CM, Cheong JM.* Soy isoflavones and bone health: the relationship is still unclear. J Nutr 2005; 135: 1243 – 7
112 *Alekel DL, Germain AS, Peterson CT, Hanson KB, Stewart JW, Toda T.* Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. Am J Clin Nutr 2000; 72: 844 – 52
113 *Morabito N, Crisafulli A, Vergara C, Gaudio A, Lasco A, Frisina N et al.* Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: a randomized double-blind placebo-controlled study. J Bone Miner Res 2002; 17: 1904 – 12
114 *Atkinson C, Compston JE, Day NE, Dowsett M, Bingham SA.* The effects of phytoestrogen isoflavones on bone density in women: a double-blind, randomized, placebo-controlled trial. Am J Clin Nutr 2004; 79: 326 – 33
115 *Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A et al.* Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 2005; 97: 1652 – 62
116 *McMichael-Phillips DF, Harding C, Morton M, Roberts SA, Howell A, Potten CS et al.* Effects of soy-protein supplementation on epithelial proliferation in the histologically normal human breast. Am J Clin Nutr 1998; 68: 1431S – 5S
117 *Petrakis NL, Barnes S, King EB, Lowenstein J, Wiencke J, Lee MM et al.* Stimulatory influence of soy protein isolate on breast secretion in pre- and postmenopausal women. Cancer Epidemiol Biomarkers Prev 1996; 5: 785 – 94
118 *Warner E, Lockwood G, Tritchler D, Boyd NF.* The risk of breast cancer associated with mammographic parenchymal patterns: a meta-analysis of the published literature to examine the effect of method of classification. Cancer Detect Prev 1992; 16: 67 – 72
119 *Jakes RW, Duffy SW, Ng FC, Gao F, Ng EH, Seow A et al.* Mammographic parenchymal patterns and self-reported soy intake in Singapore Chinese women. Cancer Epidemiol Biomarkers Prev 2002; 11: 608 – 13
120 *Maskarinec G, Williams AE, Carlin L.* Mammographic densities in a one-year isoflavone intervention. Eur J Cancer Prev 2003; 12: 165 – 9
121 *Key TJ, Sharp GB, Appleby PN, Beral V, Goodman MT, Soda M et al.* Soya foods and breast cancer risk: a prospective study in Hiroshima and Nagasaki, Japan. Br J Cancer 1999; 81: 1248 – 56
122 *Shu XO, Jin F, Dai Q, Wen W, Potter JD, Kushi LH et al.* Soyfood intake during adolescence and subsequent risk of breast Cancer among Chinese women. Cancer Epidemiol Biomarkers Prev 2001; 10: 483 – 8
123 *Ziegler RG, Hoover RN, Pike MC, Hildesheim A, Nomura AM, West DW et al.* Migration patterns and breast cancer risk in Asian-American women. J Natl Cancer Inst 1993; 85: 1819 – 27
124 *Duffy C, Perez K, Partridge A.* Implications of phytoestrogen intake for breast cancer. CA Cancer J Clin 2007; 57: 260 – 77
125 *Rice S, Whitehead SA.* Phytoestrogens and breast cancer – promoters or protectors? Endocr Relat Cancer 2006; 13: 995 – 1015
126 *This P, De La Rochefordiere A, Clough K, Fourquet A, Magdelenat H.* Phytoestrogens after breast cancer. Endocr Relat Cancer 2001; 8: 129 – 34
127 *Jordan VC, O'Malley BW.* Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 2007; 25: 5815 – 24
128 *Jordan VC.* Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. J Med Chem 2003; 46: 1081 – 111
129 *Jordan VC.* Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 1. Receptor interactions. J Med Chem 2003; 46: 883 – 908
130 *Khan N, Afaq F, Mukhtar H.* Cancer chemoprevention through dietary antioxidants: progress and promise. Antioxid Redox Signal 2008; 10: 475 – 510
131 *Whitsett T, Carpenter M, Lamartiniere CA.* Resveratrol, but not EGCG, in the diet suppresses DMBA-induced mammary cancer in rats. J Carcinogen 2006; 5: 15

Early breast cancer

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Adoption of urbanised lifestyles together with changes in reproductive behaviour might partly underlie the continued rise in worldwide incidence of breast cancer. Widespread mammographic screening and effective systemic therapies have led to a stage shift at presentation and mortality reductions in the past two decades. Loco-regional control of the disease seems to affect long-term survival, and attention to surgical margins together with improved radiotherapy techniques could further contribute to mortality gains. Developments in oncoplastic surgery and partial-breast reconstruction have improved cosmetic outcomes after breast-conservation surgery. Optimum approaches for delivering chest-wall radiotherapy in the context of immediate breast reconstruction present special challenges. Accurate methods for intraoperative assessment of sentinel lymph nodes remain a clinical priority. Clinical trials are investigating combinatorial therapies that use novel agents targeting growth factor receptors, signal transduction pathways, and tumour angiogenesis. Gene-expression profiling offers the potential to provide accurate prognostic and predictive information, with selection of best possible therapy for individuals and avoidance of overtreatment and undertreatment of patients with conventional chemotherapy. Short-term presurgical studies in the neoadjuvant setting allow monitoring of proliferative indices, and changes in gene-expression patterns can be predictive of response to therapies and long-term outcome.

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Introduction

Breast cancer remains the most common malignancy in women worldwide and is the leading cause of cancer-related mortality.[1] More than 1·2 million cases are diagnosed every year, affecting 10–12% of the female population and accounting for 500 000 deaths per year worldwide. Despite a higher prevalence of breast cancer in industrialised than in non-industrialised countries, incidence rates are steadily increasing in less affluent societies.[2] Breast cancer is mainly a postmenopausal disease, with more than three-quarters of tumours hormone responsive. This hormone dependency interacts with environmental and genetic factors to determine incidence and progression of the disease. Lifestyle and environmental effects are potentially modifiable risk factors and offer the prospect of interventions that might ultimately reduce the global burden of the disease.[3] The documented decrease in breast-cancer rates in the USA in 2003, after a gradual rise during the preceding 30 years, exemplifies the effect of risk modification. This reduction has been linked to decreased use of exogenous hormones after adverse reports of an association with increased breast-cancer risk[4] and a possible reduction in the uptake of screening mammography in view of its debatable modest benefit.

Mortality rates for breast cancer have fallen in many industrialised nations since around 1990, having previously been stable or increasing for several consecutive decades.[5–7] These falls in mortality have been attributed mainly to the introduction of mammographic screening programmes and the widespread use of adjuvant systemic therapies with tamoxifen.[8] A US population-based study showed that these mortality trends are accentuated in women with oestrogen-receptor-positive tumours compared with those with hormone-insensitive disease.[9] Moreover, this decrease in mortality was almost exclusively confined to women younger than 70 years (figure 1). This Seminar will focus on the epidemiology, diagnosis, and management of early breast cancer when there is no overt evidence of distant metastases and for which treatment intent is curative. Some patients can be cured with loco-regional treatment alone whereas many will have undetectable micrometastatic disease and require adjuvant systemic therapy.

Epidemiology

Age and female sex are major risk factors for breast cancer, with incidence rates rising rapidly between the ages of 35 and 39 years and subsequently levelling to a plateau after 80 years.[10] Nonetheless, the rate of increase slows around the age of 50 years, corresponding to the average age of menopause, which creates a point of inflection in the age-specific incidence curve known as Clemmesen's hook (figure 2).[11] This transition point is an indicator of the confluence of two separate rate curves for oestrogen-receptor-positive and negative tumours that have fairly favourable and poor prognoses, respectively.[10] The incidence of oestrogen-receptor-negative tumours increases rapidly until age 50 years and then flattens or decreases. By contrast, the incidence of oestrogen-receptor-positive tumours is similar up to the age of 50 years, but then continues to climb at a slower pace.

Search strategy and selection criteria

We searched the Cochrane Library and Medline between 1998, and 2008, with the term "breast cancer". In section of publications, we used discretion from our perception of the importance of the articles on the basis of citation within the medical published work and at major international conferences (San Antonio Breast Cancer Symposia; American Society of Clinical Oncology meetings). We restricted searches to the past 5 years, but some older referenced papers were collated from our personal collections. Some review articles have been cited to provide a more comprehensive list of references than is permissible in this Seminar.

Thus oestrogen-receptor-negative tumours tend to occur earlier in life and oestrogen-receptor-positive tumours are more common in older women. The peak ages of onset for these two tumour phenotypes are 50 and 70 years of age, respectively, and they seem to have different underlying causes and pathobiology. Reproductive and anthropomorphic factors have opposing effects, with nulliparity, obesity, and oral contraceptive use decreasing the risk of early-onset breast cancers while increasing the risk in older women.[12–14]

There are pronounced racial differences in the incidence and mortality of breast cancer.[15] Although age-standardised incidence rates are higher in white women than in those of African-American descent, these rates cross at about age 50 years. At younger ages, breast-cancer incidence rates are higher in African-American women, whereas rates are greater in their white counterparts at 50 years of age and older. Age-adjusted breast-cancer mortality rates were congruent between African-Americans and white Americans until the early 1980s, but thereafter a continued divergence was evident with higher mortality rates for African-American than for white people.[16] These differential mortality rates coincided with the introduction of mammographic screening in conjunction with adjuvant systemic therapy as an integral component of breast-cancer management.[16] Black women have a higher proportion of oestrogen-receptor-negative tumours than do white women and are therefore less likely to receive endocrine treatment. Furthermore, socioeconomic variation leads to inequalities in terms of health-insurance cover and educational attainment, which are likely to restrict access to new treatments and screening programmes for ethnic groups. A combination of intrinsic differences in response and health-care provision might account for the widening racial disparity in breast-cancer mortality rates within the USA.[16,17]

Familial forms of breast cancer incorporate both high and low genetic risk. When several first-degree relatives are affected, clustering is probably hereditary and attributable to high-risk susceptibility genes such as *BRCA1* and *BRCA2*.[18] These are tumour suppressor genes that display an autosomal dominant pattern of inheritance with variable penetrance. Mutations within these two genes account for around three-quarters of hereditary breast cancer cases (5–10% of all breast cancer) and confer a lifetime risk of between 80–85% by age 70 years.[19] Additionally, *BRCA1* mutations are associated with ovarian cancer risk of 20–40%. Within cells, the effects of *BRCA1* and *BRCA2* are recessive, and both copies of an allele must be lost or mutated for cancer

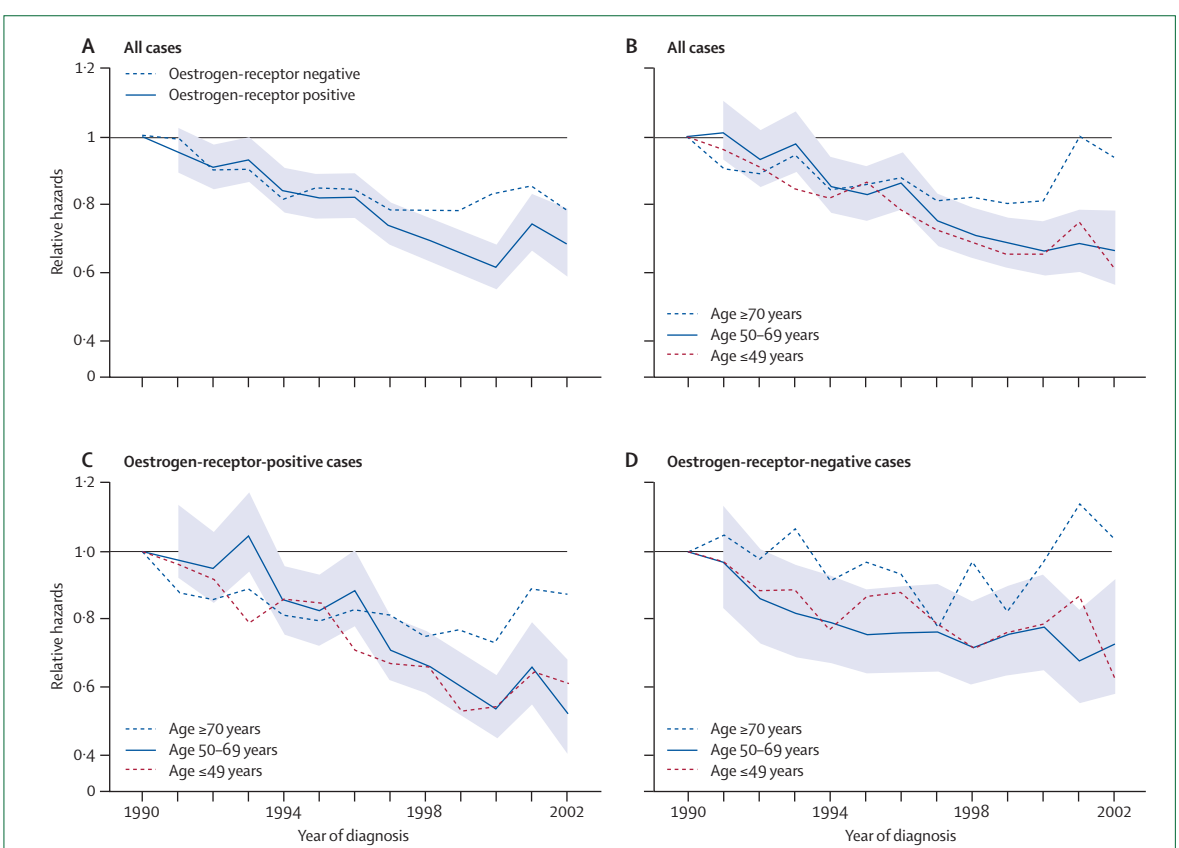

Figure 1: **Relative hazard rates of breast-cancer death according to oestrogen-receptor status and age at diagnosis**
Shading indicates 95% CIs. Reprinted with permission from the *Journal of Clinical Oncology*.[9]

progression. Individuals with a germline mutation in these genes have a dominantly inherited susceptibility, and the second so-called hit occurs in the somatic copy. Tumours from genetically predisposed patients show loss of heterozygosity in the wild-type *BRCA1* allele,[20] but mutations of *BRCA1* and *BRCA2* are uncommon in sporadic breast cancers. Other genes involved in genetic predisposition include *p53* (Li-Fraumeni syndrome), *AT* (ataxia telangiectasia), and *PTEN* (breast and thyroid cancer). Low penetrance genes such as *CHEK* mutations might collectively be responsible for up to 25% of familial cases; although they confer a reduced risk, they are more prevalent within the population.[21]

Breast-cancer risk is modulated by factors affecting the hormonal milieu; most mammary tumours are stimulated by oestrogens, and oestrogen-receptor-negative tumours can evolve from oestrogen-receptor-positive lesions rather than arising de novo. A woman's cumulative lifetime exposure to oestrogen determines the level of this environmental risk. Thus early menarche (<12 years *vs* 16 years) and late menopause (>55 years *vs* <45 years) are associated with relative risk increases of about 1·2.[3] Levels of oestrogen and rates of proliferation in breast epithelium are low after menopause, which when induced iatrogenically at younger than 40 years of age reduces the risk of breast cancer by almost two-thirds.[3] A first full-term pregnancy at younger than 20 years of age is protective for breast cancer, and high circulating concentrations of progesterone can cause terminal differentiation in pluripotential stem cells of immature breast tissue. Nulliparity is a well known risk factor for breast cancer since Ramizzini described *horrendis mammarium canceris* in Catholic nuns.[22] However, women who defer childbearing beyond 35 years of age have an increased relative risk compared with nulliparous women, which might have relevance to contemporary reproductive practices in which late pregnancies are associated with prolonged use of the oral contraceptive pill and a greater chance of pregnancy-related breast cancer.[23]

Hormone-replacement therapy increases the relative risk of breast cancer by roughly 35% after 10 years of use, although cancers developing in women who have ever used this therapy tend to be of more favourable prognosis.[24] Hormone-replacement therapy should be avoided in breast-cancer survivors, and more than doubles the risk of recurrence.[25] Moderately vigorous physical activity of up to 7 h per week can reduce the risk of breast cancer by almost 20%, and this effect is independent of menstrual function. Daily strenuous exercises reduce risk by up to 50% in women aged 14–22 years.[26] Alcohol consumption increases breast-cancer risk irrespective of the type of beverage consumed.[27] High mammographic breast density is a powerful independent predictor of breast-cancer risk and is associated with an increased ratio of glandular to fatty tissue.[28] Only a quarter of sporadic cases of breast cancer have any identifiable risk factor.

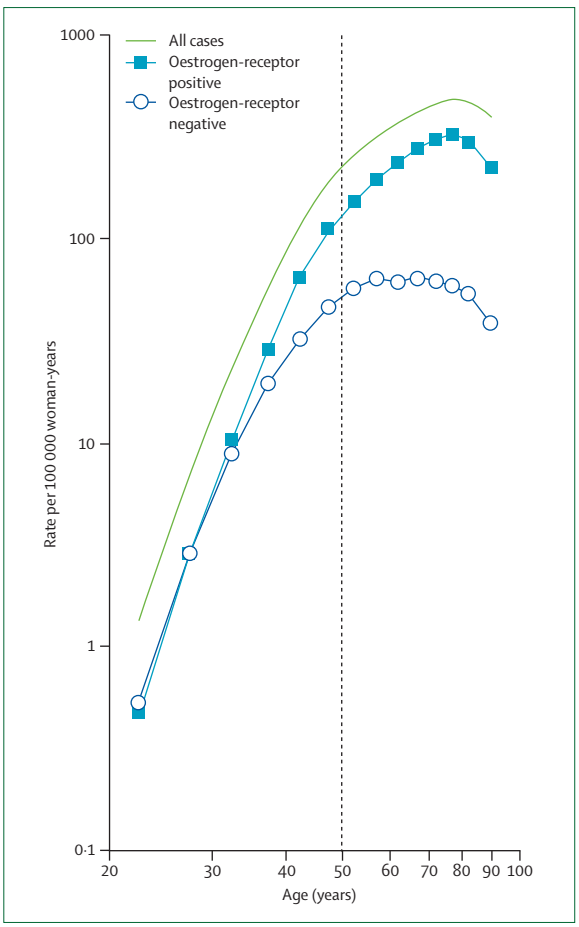

Figure 2: **Age-specific incidence rates (SEER Database) in female patients with breast cancer, showing point of inflexion (Clemmesen's Hook) at around 50 years of age**
SEER=Surveillance, Epidemiology and End Results program of the National Cancer Institute. Reproduced with permission from Springer-Verlag.

Diagnosis

About three-quarters of symptomatic breast cancers will present with a discrete breast lump. However, most patients referred to a breast clinic with a lump will have benign disease, and initial clinical examination will aim to establish whether a dominant mass or localised glandular nodularity is present. The physical characteristics of benign and malignant breast lumps overlap substantially. Thus complete clinical evaluation involves triple assessment, integrating information from clinical examination, radiological imaging, and percutaneous needle biopsy. A diagnostic mammogram can confirm a clinical suspicion of malignancy and typically shows a spiculate opacity or microcalcification. It can sometimes show the extent of malignancy (especially when associated with microcalcification) and identify occult (non-palpable) lesions in the ipsilateral or contralateral breast. Mammography does not show evidence of malignancy in 10% of patients with breast cancer.[29]

Breast ultrasound with 12–15 MHz transducers is complementary to mammography and increases diagnostic accuracy. It provides a measurement of tumour size, correlating well with pathological estimates.[30] Modern ultrasound devices incorporate a Doppler facility and are increasingly being used to image the axillary nodes and deselect patients for sentinel-lymph-node biopsy. Tissue diagnosis is essential and can be obtained with either fine-needle aspiration cytology or core biopsy. Before image-guided biopsy techniques, most tissue acquisition involved open excision or incision biopsy. Percutaneous needle-biopsy techniques can now provide a definitive diagnosis for most benign and malignant diseases.[31] Although fine-needle aspiration cytology simply, quickly, and cost-effectively establishes tissue diagnosis, it has lower sensitivity and specificity than does core biopsy.[31] Despite false-negative results with both, core biopsy with wide-bore needle is preferred to fine-needle aspiration cytology and yields solid cores of tissue that maintain tissue architecture and allow distinction between invasive and non-invasive carcinoma.[32] Biopsy samples of mass lesions can be taken with ultrasound or stereotactic guidance, whereas microcalcification usually mandates stereotactic methods.[33] The standard core-biopsy needle is either 14 or 16 gauge, but larger volumes of tissue can be obtained from vacuum-assisted core biopsy devices with a range of needle sizes, but most often an 11 gauge needle. Vacuum-assisted core-biopsy devices reduce the chance of underdiagnosis and increase the chance of obtaining a definitive preoperative diagnosis, allowing appropriate planning of breast-cancer surgery.[34]

When clinical examination, radiology, and core biopsy or fine-needle aspiration cytology show benign features only, the probability of malignancy is very low. A diagnostic (surgical) excision biopsy is warranted in the absence of concordance, although repeat core-needle biopsy can be attempted. MRI is used selectively in the diagnostic workup of breast-cancer patients to clarify the extent of a lesion and establish whether satellite foci are present in patients otherwise amenable to breast-conservation surgery.[35] However, evidence suggests that patients assessed with MRI are more likely to undergo (unnecessary) mastectomy instead of breast-conservation surgery.[36,37] Rates of ipsilateral breast tumour recurrence (IBTR) are fairly low—at 8 years rates are similar in patients receiving breast-conservation surgery with (3%) and without (4%) preoperative MRI imaging[38]—and additional lesions detected by MRI might not be clinically relevant or might be adequately treated with adjuvant therapies.[39]

A substantial proportion of breast cancers in the USA and western Europe are detected with screening mammography. Randomised controlled trials have confirmed that screening mammography with or without clinical breast examination in postmenopausal women reduces breast-cancer mortality by about 20%.[40] The use of screening mammography in premenopausal women remains controversial, and is probably not cost effective.[41] Some suggest that clinical breast examination should accompany mammographic screening, since some cancers are radiologically occult but clinically palpable.[42] Breast self-examination has not yet proven beneficial in clinical trials.[43] Breast MRI screening has been recommended for high-risk women with *BRCA1/2* mutation carriage.[44] The sensitivity of breast MRI and cancer-detection rates within this group are better than with mammography; however, data from prospective randomised controlled trials assessing the effect on breast-cancer mortality are scarce.

Biological hypotheses

Two biological notions of tumour pathogenesis have guided strategies for loco-regional and systemic treatment of breast cancer.[45] According to the Halstedian paradigm, breast cancer is a localised disease at inception with progressive and sequential spread from local tissues to lymph nodes and in turn haematogenous dissemination. IBTR is considered a cause of distant metastases, with the chance of cure related to the extent of primary loco-regional treatment. The Fisherian paradigm presupposes that breast cancer is predominantly a systemic disease at the outset, with cancer cells entering the bloodstream at an early stage of tumour development. Circulating tumour cells might be destroyed by the immune system, but some will establish viable micrometastatic foci at distant sites. Micrometastases at the time of diagnosis will determine a patient's clinical fate. IBTR is regarded as an indicator of distant-relapse risk and indicates a host-tumour relation that favours development of distant disease or activation of processes leading to a kick start of micrometastases. This notion of biological predeterminism has dominated approaches to breast-cancer management over the past three decades and emphasised the importance of systemic therapies targeting distant micrometastatic disease. Long-term follow-up of the largest breast conservation trial (NSABP B-06) at 20 years suggests that variations in extent of loco-regional treatments do not affect overall survival,[46] supporting the idea that local recurrence is an indicator of risk for development of distant disease that reflects intrinsic biology of the tumour.[47] Several studies have shown that IBTR is the strongest independent predictor of distant relapse, conferring an increased risk of up to three-fold to four-fold.[48] Although IBTR contributes roughly a third to the overall recurrence risk (Blamey R, University of Nottingham, personal communication), whether IBTR is causally related to distant relapse or merely associated with survival is unknown.

In a meta-analysis by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG), local radiation treatment to either the breast after breast-conservation surgery or the chest wall after mastectomy showed an overall survival benefit at 15 years.[49] For treatment comparisons in which the difference in local recurrence rates at 5 years was less than 10%, survival was unaffected. When

differences in local relapse were substantial (>10%), moderate reductions in breast-cancer-specific and overall mortality were recorded. Absolute reductions were 19% for local recurrence at 5 years and 5% for breast-cancer mortality at 15 years, representing one life saved for every four loco-regional recurrences prevented by radiotherapy at 5 years. This analysis showed conclusively that differences in loco-regional treatments that substantially improve rates of local control will affect long-term survival of patients with breast cancer. Local control does matter and rates of local recurrence should be kept to a minimum in the first 5 years. Up to a quarter of local recurrences will be a determinant and not simply an indicator of risk for distant relapse and death.

Molecular profiling can help to predict the biological behaviour and pattern of spread for individual tumours and avoid undertreatment and overtreatment with both loco-regional and systemic therapies.[50] Malignant stem cells are either quiescent or cycle fairly slowly, and are resistant to conventional chemotherapy. Their ability to self-renew provides the opportunity for regeneration and clinical recurrence of cancer. Identification of biochemical pathways that are unique to cancer stem cells will allow selective targeting of this important subpopulation of tumour cells. Cellular response to therapies should be anticipated and escape mechanisms co-targeted.

Surgery

The introduction of conservative surgery for breast cancer coincided with reduced tumour size at presentation and a shift in the underlying biological hypothesis. Breast-conservation surgery is now an established procedure and the preferred standard of care for management of women with early-stage breast cancer. Instigation of widespread mammographic screening has contributed to a stage shift for newly diagnosed disease, with an average tumour size at presentation of less than 2 cm. At least two-thirds of patients are eligible for breast-conservation surgery, but rates of mastectomy vary both geographically and institutionally.[51] Selection of patients for this surgery is crucial, with an inverse relation between competing oncological demands for surgical radicality and cosmesis. A balance exists between the risk of IBTR and cosmetic results, with oncoplastic surgery advancing the limits of surgical resection.

Two factors emerge as principal determinants of true local recurrence within the conserved breast: margin status and the presence or absence of an extensive in-situ component. Lymphatic invasion and young age (<35 years) are primary predictors for increased risk of IBTR. Consistent associations have been recorded for larger tumour size (>2 cm) and higher histological grade but not for tumour subtype or nodal status.[52] Results of the EBCTCG overview have reinforced the link between local control and mortality, leading to an emphasis on adequacy of surgical excision and other treatment-related variables such as radiotherapy.[49] Attainment of gross macroscopic clearance of the tumour at operation is no longer acceptable; all radial margins should be clear of tumour microscopically. A positive resection margin has not been uniformly defined, which has compounded issues relating to microscopically negative margins and degrees of surgical clearance—eg, how wide must a negative margin be to result in acceptable rates of local recurrence (1–2% per year)? Many surgeons regard a margin clearance of 2–3 mm to be appropriate, although up to 45% of American radiation oncologists consider a margin as negative provided that no tumour cells are at the inked edge.[53] Others strive for a radial margin clearance of 5 mm, which can lead to re-excision rates of up to 50% but is associated with very low rates of IBTR. Detection of further tumour is unusual when re-excision is done to achieve a wider margin rather than a negative margin. Singletary[54] has provided a useful analysis, showing median rates of IBTR of 3%, 6%, and 2% when margins of clearance were 1 mm, 2 mm, or just clear, respectively. Thus although rates of recurrence are determined by negative margin status, no direct relation exists between margin width and rates of local recurrence. When the first re-excision fails to achieve surgical clearance, mastectomy is often indicated and becomes necessary if margins remain positive after a reasonable number of surgical attempts (up to three).[55]

Practices are consistent with the notion that IBTR develops from re-growth of residual cancer cells in peritumoral tissue. Moreover, the invasive element confers the increased risk of distant failure; when local recurrence is exclusively ductal carcinoma in situ, features of the original primary tumour will determine systemic risk.

Most patients considered suitable for breast-conservation surgery will have a unifocal tumour measuring 3 cm or less and lying more than 2 cm from the nipple areolar complex. These patients usually have a favourable ratio of tumour to breast size and are amenable to conventional forms of wide local excision in which the tumour is excised with a roughly 2 cm margin of surrounding breast tissue without the need for any formal breast remodelling. Although a re-excision might be needed in up to a quarter of cases to achieve microscopically clear radial margins, an optimum cosmetic result should be attained after irradiation of the remaining breast tissue. Notwithstanding attempts at breast-conservation surgery, mastectomy is clearly indicated for some patients on the basis of tumour size or location, multifocality, an inflammatory component, or patient choice. Achievement of a good cosmetic outcome becomes progressively more difficult as the proportion of breast tissue removed increases. Although the absolute volume of tissue excised is surgeon dependent, a greater percentage is associated with larger tumours. When more than 10–20% of breast volume is removed, results might be unsatisfactory cosmetically.[56] Partial-breast reconstruction with oncoplastic procedures often allow wide resection of tissue, increasing the chance of tumour-free margins. Despite these techniques

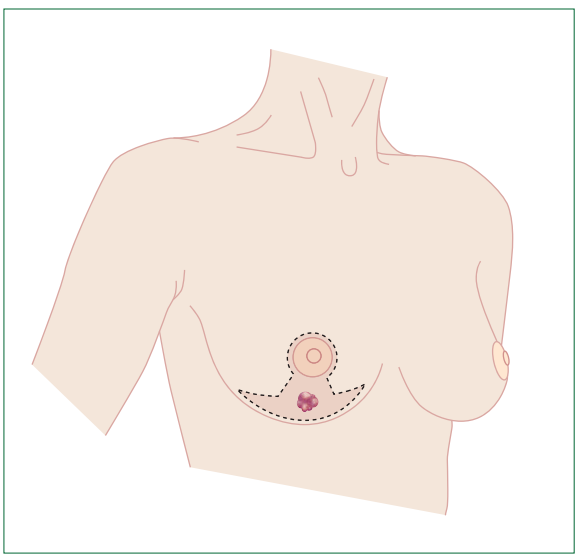

Figure 3: **A tumour in the inferior quadrant of the breast can be excised as part of a reduction mammoplasty specimen with wide surgical margins**

providing an opportunity for enhancing quality of life by improving cosmetic outcome and psychological well-being,[57] there are no long-term follow-up data to substantiate the claim for reduced rates of local relapse. Moreover, transposition of glandular tissue could jeopardise accurate targeting of any radiotherapy boost, unless it is given intraoperatively. Therapeutic mammoplasty can potentially improve cosmetic outcome when tumour size or location would otherwise lead to sub-optimum cosmesis after conventional breast-conservation surgery (figure 3).[58] Strict oncological selection criteria should still be applied;[59] when the estimated risk for IBTR is high, despite clear margins and a good cosmetic result, mastectomy with immediate breast reconstruction should be offered. The development of oncoplastic surgery and partial-breast reconstruction has improved the application of breast-conservation surgery to management of breast cancer, but careful patient selection is crucial. Often a contralateral procedure is required for symmetrisation, particularly when volume displacement rather than replacement techniques are used.[60]

Most patients who are healthy and younger than 70 years with a non-inflammatory or locally advanced tumour should be offered immediate breast reconstruction together with a skin-sparing mastectomy in which the nipple areolar complex is removed but much of the breast skin envelope remains. Skin-sparing mastectomy represents the latest phase in development of less mutilating forms of mastectomy and has revolutionised results of immediate breast reconstruction by preserving the inframammary fold and avoiding the need for resculpturing of any imported skin or tissue expansion of residual chest-wall skin. No evidence suggests increased rates of local recurrence with skin-sparing mastectomy, and the precise skin incision should be tailored to the individual patient with removal of any involved skin overlying a tumour.[61] Breast volume can be reconstituted with a variety of techniques including subpectoral tissue expander, extended autologous latissimus-dorsi flap, implant-assisted latissimus-dorsi flap, or a free/pedicled transverse rectus abdominus flap. Judicious patient selection and joint decision-making will help keep any disparity between patient expectation and clinical reality to a minimum and maximise satisfaction.

Many women now receive postmastectomy radiotherapy.[49,62,63] Anticipation of chest-wall irradiation will affect the choice of reconstructive technique; an implant-only reconstruction is generally avoided when postmastectomy radiotherapy is a possibility.[64] The potential problems of capsular contracture in this group of patients with implant-based reconstruction have led to a modified surgical approach with a delayed immediate reconstruction. A skin-sparing mastectomy can be undertaken initially with placement of a temporary tissue expander that acts as scaffolding for the skin flaps. Chest-wall irradiation can then be given and definitive reconstruction undertaken later. The viability of the native mastectomy flaps after radiation causes concern, and it might be preferable to proceed with immediate reconstruction with a latissimus-dorsi flap and implant for all patients and undertake implant exchange if and when required. An extended autologous latissimus-dorsi flap is not necessarily more tolerant of radiotherapy, and substantial donor-site morbidity occurs. However, delayed immediate reconstruction is a method that can potentially preserve the aesthetic benefits of immediate breast reconstruction with preservation of the three-dimensional skin envelope and more accurate targeting of tangential radiotherapy beams.

Methods for accurately staging the axilla continue to evolve, but remain dominated by sentinel-lymph-node biopsy, which is now widely practised and accepted as a standard of care. Dual labelling techniques with blue dye and isotope are associated with a shorter learning curve and optimum performance indicators such as rates of identification (>90%) and false negativity (5–10%).[65] Blue dye-assisted node sampling removes three to four blue and palpably suspicious nodes and can be a pragmatic and cost-effective method when radioisotope facilities are unavailable.[66] However, this method is associated with a higher false negative rate and lacks the reassurance provided by the absence of any residual radioactivity within the axilla. Results from the largest sentinel lymph node biopsy trial show an overall false negative rate of 9·8%, with higher rates when only one sentinel node is removed rather than two to three nodes.[67] Completion axillary-lymph-node dissection is recommended for all patients with either micrometastatic or macrometastatic deposits in the sentinel lymph node.[65] The chance of non-sentinel lymph-node involvement is related to the volume of disease in the sentinel node, but nomograms devised for estimation of this involvement are difficult to

reliably apply in practice and are less accurate when the predicted incidence of non-sentinel lymph-node positivity is low.[68] For some patients, the risk to benefit ratio for detection of positive cases of non-sentinel lymph nodes might not justify any delayed procedure. Low rates of axillary relapse are unlikely to translate into any meaningful reduction in long-term survival in an older group of patients with smaller non-high-grade tumours.[69] Methods for intraoperative assessment of sentinel lymph nodes obviate the need for a delayed axillary-lymph-node dissection, but detection of micrometastases with either touch imprint cytology or frozen section is problematic. New techniques based on reverse-transcriptase PCR can potentially overcome difficulties of limited node sampling and operating parameters set at a threshold for detection of metastases greater than 0·2 mm in size but not isolated tumour cells (≤0·2 mm).[70] Real-time PCR might allow quantitation and differentiation between macrometastases and micrometastases.

Rates of clinical regional recurrence in patients with negative sentinel-lymph-node biopsy who have not proceeded to axillary-lymph-node dissection range from 0 to 1·4%, with fairly short follow-up of 3 years or less.[71] Any residual disease within the axillary nodes will be low volume, and longer follow-up might be needed for any clinical manifestation of regional recurrence. Kujit and Roumen[72] report an actuarial rate of 5% at a median follow-up of 6·5 years, predicting that up to 10% of patients might eventually develop isolated axillary recurrence after a negative sentinel-lymph-node biopsy.

Radiotherapy

Long-term follow-up of breast-conservation trials confirm significantly increased rates of local relapse when radiotherapy is omitted.[46,73] However, rates of IBTR are acceptable when breast-conservation surgery is combined with whole-breast irradiation, usually delivered via conventional tangential breast fields at a total dose of 46–50 Gy in 25 fractions over 5 weeks with an optional booster dose (10–20 Gy). Within the NSABP B-06 trial, 39·2% of patients undergoing wide local excision only had developed local recurrence at 20 years' follow-up compared with 14·3% for those receiving radiotherapy after lumpectomy ($p<0·001$).[46] Moreover, cosmetic results are satisfactory when neither the volume of breast tissue excised or the radiation fraction size are excessive.[56] A group of patients for whom rates of IBTR are not further reduced by radiotherapy compared with observation or tamoxifen therapy alone has not been defined.[74–76] Omission of radiotherapy should be cautioned at present since it can lead to rates of IBTR approaching 30% for small tumours of favourable grade, and local control does affect overall survival.[49] Older women benefit in terms of breast-cancer-specific survival from radiotherapy after breast-conservation surgery, and tamoxifen alone cannot substitute for radiotherapy. Comorbidities can otherwise reduce life expectancy for some older women for whom any additional local control (3–6% risk absolute reduction) from radiotherapy might not be clinically significant.[77,78]

A group of techniques has been developed—accelerated partial-breast irradiation—that decrease the volume of breast tissue irradiated and the duration of treatment. More than three-quarters of true breast recurrences occur at the site of lumpectomy, and whole-breast irradiation might be unnecessary. These techniques are focused on the tumour bed and a zone of surrounding tissue of variable depth. The advent of CT-based treatment planning kept exposure of normal tissues to a minimum and helped radiotherapists cope with the challenges resulting from the peculiar shape of the breast and contiguity of important surrounding structures (eg, heart and lungs). Computer technology assisted with placement of multiplanar interstitial catheter implants for brachytherapy after lumpectomy. Treatment was aimed at the tumour bed and a margin of tissue to a depth of 1–2 cm. This technique allowed radiotherapy (34 Gy) to be completed within 1 week rather than 5 or 6 weeks. Single institution series with more than 5 years of follow-up show rates of local control to be similar to whole-breast irradiation for matched and appropriately selected subsets of patients.[79–81] Despite a US multicentre trial confirming reproducibility of these favourable results across institutions, the perceived complexity of brachytherapy detracted from its popularity and it remains available in only a few centres worldwide.[82]

Two further techniques of accelerated partial-breast irradiation have been pioneered: intraoperative radiotherapy and MammoSite. Intraoperative radiotherapy delivers a high dose of radiation as one fraction at the time of surgery, allowing precise application of radiation dose to the target area to eliminate tumour foci around the surgical bed. It potentially intensifies the tumour kill effect of surgery and radiotherapy, although some are concerned about the radiobiological equivalence of one dose of intraoperative radiotherapy (21 Gy) compared with conventional whole-breast irradiation. This concern applies particularly to the low-energy X-ray source (50 kV) used in the TARGIT trial[83] compared with electron beam therapy (electron intraoperative therapy)[84] for which depth of penetration is restricted. However, mathematical and laboratory models suggest that TARGIT might be better than conventional therapy,[85,86] and initial clinical results are encouraging.[83] Intraoperative radiotherapy facilitates an integrated approach to the multidisciplinary treatment of cancer, but requires specialised equipment and for electron intraoperative therapy a dedicated suite. An alternative technique of brachytherapy is given via a double lumen balloon catheter (MammoSite) placed within the surgical cavity.[87] This device delivers a total dose of 34 Gy in ten fractions (via a high-dose rate remote afterloader) and is now a common method of accelerated partial-breast irradiation, using equipment already present in many centres. Preliminary results with 5 years' follow-up show low rates of local recurrence (0–6%) and good to excellent cosmetic results in 80% of patients.[88,89]

Three-dimesional conformal radiotherapy is a form of accelerated partial-breast irradiation that uses an external beam to treat smaller volumes than whole-breast irradiation does. Despite being non-invasive, achieving high levels of conformality is difficult.[90,91] The related technique of intensity-modulated radiotherapy delivers conformal dose distributions and improves homogeneity.[92] The combined NSABP B-39/RTOG 0413 trial incorporates three techniques for accelerated partial-breast irradiation (three-dimensional conformal, interstitial brachytherapy, and MammoSite) and aims to assess these techniques in comparison with whole-breast irradiation with primary endpoints of local recurrence, disease-free survival, and overall survival.[93] The START trial has assessed accelerated hypofractionated whole-breast irradiation and showed that patients given a lower overall radiotherapy dose in fewer, larger fractions have similar local control and fewer adverse side-effects than does a dose of 50 Gy in a standard 5-week schedule. This finding supports hypofractionation as a safe and effective approach, but long-term follow-up is required to assess local control and late toxic effects.[94]

Radiotherapy after mastectomy encompasses irradiation of the chest wall and skin together with regional lymph nodes. The indications for this treatment continue to evolve, but all trials have shown that it reduces the proportional risk of local failure by two-thirds to three-quarters of patients, including those with tumours larger than 5 cm and four or more positive axillary lymph nodes. However, data for the benefit of postmastectomy radiotherapy in terms of overall survival are conflicting; an overview by the EBCTCG confirms that postmastectomy radiotherapy in node-positive women results in an absolute survival gain at 15 years.[49] Although postmastectomy radiotherapy reduced rates of local relapse in node-negative patients, mortality was not reduced. In the Danish and British Columbia trials of postmastectomy radiotherapy in premenopausal node-positive women receiving chemotherapy,[61,62] the proportional survival benefits were similar for patients with one to three and four or more positive nodes. However, some aspects of trial design were controversial and any survival advantage within the intermediate-risk groups (one–three nodes positive) could be masked by toxic effects from the radiotherapy. New radiation techniques using tangential fields minimise cardiotoxicity, as do methods such as intensity-modulated radiotherapy.[95] The SUPREMO trial is assessing whether modern chemotherapy regimens and postmastectomy radiotherapy can lead to overall-survival improvements in this intermediate-risk group.[96]

Adjuvant systemic therapies

Incorporation of adjuvant systemic therapies into the multidisciplinary management of breast cancer has led to improvements in rates of disease-free and overall survival.[97,98] The indication for adjuvant systemic therapy after definitive surgery is based on established prognostic factors, including age, comorbidities, axillary-lymph-node involvement, tumour size, and tumour grade.[99] In addition to these well established clinicopathological factors, molecular tests can assess the estimated risk of recurrence in patients with early-stage breast cancer and identify distinct biological classes of tumour. Three prognostic tests have been approved for clinical application in the USA:[100] Oncotype DX, MammaPrint, and H/I, which are based on a 21-gene profile, a 70-gene profile, and expression of the *HOXB13/IL17BR* genes, respectively. The Oncotype DX and *HOXB13-IL17BR* assays measure gene expression with reverse-transcriptase PCR and the MammaPrint assay uses complementary DNA microarray technology. One of the advantages of reverse-transcriptase PCR is that gene expression can be measured in formalin-fixed paraffin-embedded tumour tissue, whereas the microarrays need fresh frozen tissue. Although prognostic tests provide information about risk of recurrence and death, predictive markers are needed to select the optimum therapy for individual patients. The best characterised molecular predictive markers are the oestrogen receptor, the progesterone receptor, and the human epidermal growth factor receptor 2 (HER2).[101]

The responsiveness of breast tumours to hormonal manipulation provides a unique therapeutic opportunity in the form of targeted treatment. The antioestrogen tamoxifen confers a proportional reduction in mortality of 26% and up to 47% reduction in local recurrence at 10 years' follow up, with benefit confined to oestrogen-receptor-positive tumours.[98] Tamoxifen is effective in both premenopausal and postmenopausal women, although premenopausal women are eligible for ovarian suppression with either luteinising hormone-releasing hormone analogues or laparoscopic oophorectomy when disease is hormone responsive.[102] An advantage of luteinising hormone-releasing hormone agonists is their potentially reversible effects on cessation of treatment. Whether ovarian suppression can provide an alternative to chemotherapy in patients with oestrogen-receptor-positive disease, and whether luteinising hormone-releasing hormone agonists confer any additional benefit when combined with standard treatments, is being investigated.

The aromatase inhibitors represent an important advance in endocrine therapy of breast cancer. The oral agents anastrozole, letrozole, and exemestane are of comparable antitumour efficacy and are potentially interchangeable, although long-term data for side-effect profiles must be obtained before definitive pronouncements on clinical use. The American Society of Clinical Oncology Technology Assessment recommends that adjuvant hormonal therapy for postmenopausal women should include an aromatase inhibitor prescribed either as initial therapy or sequenced after tamoxifen for 2–3 years' (early switch) or 5 years' duration.[103] The largest study of adjuvant aromatase inhibitors showed a continuing divergence of the curves for disease-free survival at 68 months' follow-up, with evidence of a

carry-over effect and a reduction in time to distant recurrence favouring anastrozole.[104] The absolute benefit for time to recurrence has increased from 2·8% at 5 years to 4·8% at 100 months. This head-to-head comparison of tamoxifen versus anastrozole shows no difference in overall survival, although there is a non-significant trend for improved breast-cancer-specific survival in the latest analysis.[105] The fairly good prognostic parameters might ultimately obscure translation into a significant benefit for this endpoint.[104,106]

The IES trial is the only adjuvant study to show an overall-survival advantage for use of an aromatase inhibitor within the conventional 5-year treatment span.[107] These results, together with a meta-analysis of the Austrian (ABCSG 8/ARNO 95) and Italian (ITA) studies, support early sequencing with a switch to an aromatase inhibitor after 2–3 years of tamoxifen[108] as an efficacious approach, with improvement in overall survival for oestrogen-receptor-positive patients. The proportional risk reductions for disease-free survival are greater within the early switch than in head-to-head comparisons of tamoxifen and an aromatase inhibitor. Although interim results of the BIG 1-98 study[109] showed a disease-free survival benefit for 5 years of letrozole compared with 5 years of tamoxifen, definitive results of this study have not shown a clear advantage from an early switch policy in terms of recurrence rates compared with 5 years of an aromatase inhibitor.[110] At a median follow-up of 72 months, there was no significant difference in disease-free survival for 5 years of letrozole compared with either of the switch groups. However, pair-wise comparisons suggested a minor benefit for letrozole (5 years) compared with tamoxifen for 2 years followed by letrozole for 3 years. The inverse sequence of tamoxifen after 2 years of letrozole was equivalent to monotherapy, and patient cross-over from tamoxifen to letrozole precluded any updated comparison of the monotherapy groups.

On the basis of these data, some authorities recommend that patients at greatest risk of relapse might benefit most from an upfront aromatase inhibitor, whereas those with lower hazard rates for relapse might be best treated with an early-switch regimen involving tamoxifen for 2–3 years followed by an aromatase inhibitor for a total duration of 5 years. Although results of the BIG 1-98 study show greatest benefit from aromatase inhibitors for node-positive patients, the converse is true for the ATAC study.[105] Moreover, high-grade tumours derive no additional benefit from upfront aromatase inhibitors, and no conclusive evidence supports HER2 status as being predictive of response to aromatase inhibitors. Benefits in terms of disease-free and overall survival must be balanced against long-term adverse effects on bone health, cognitive function, and cost. Some patients at very low risk of relapse might derive minimal additional benefit from incorporation of an aromatase inhibitor into their treatment schedule and should receive tamoxifen only. Breast-cancer patients remain at chronic risk of relapse, and aromatase inhibitors offer the opportunity for extended adjuvant therapy beyond 5 years with use of an agent with a different mechanism of action. In the MA-17 trial of extended adjuvant treatment,[111] letrozole therapy significantly improved disease-free survival compared with placebo after completion of 5-years' standard tamoxifen treatment in node-positive patients.

Adjuvant chemotherapy improves rates of disease-free and overall survival for patients with early-stage breast cancer irrespective of nodal status.[112] US guidelines recommend adjuvant chemotherapy for healthy patients with axillary-node involvement and for node-negative disease when tumours are larger than 1 cm or in the presence of other adverse prognosticators (eg, age <35 years, negative oestrogen-receptor or progesterone-receptor status, high-grade tumour).[113] All patients within a subgroup are assumed to derive similar benefit from chemotherapy, but many are overtreated and do not have micrometastatic disease at presentation. Identification of patients with distant microscopic spread is particularly relevant in patients with node-negative, oestrogen-receptor

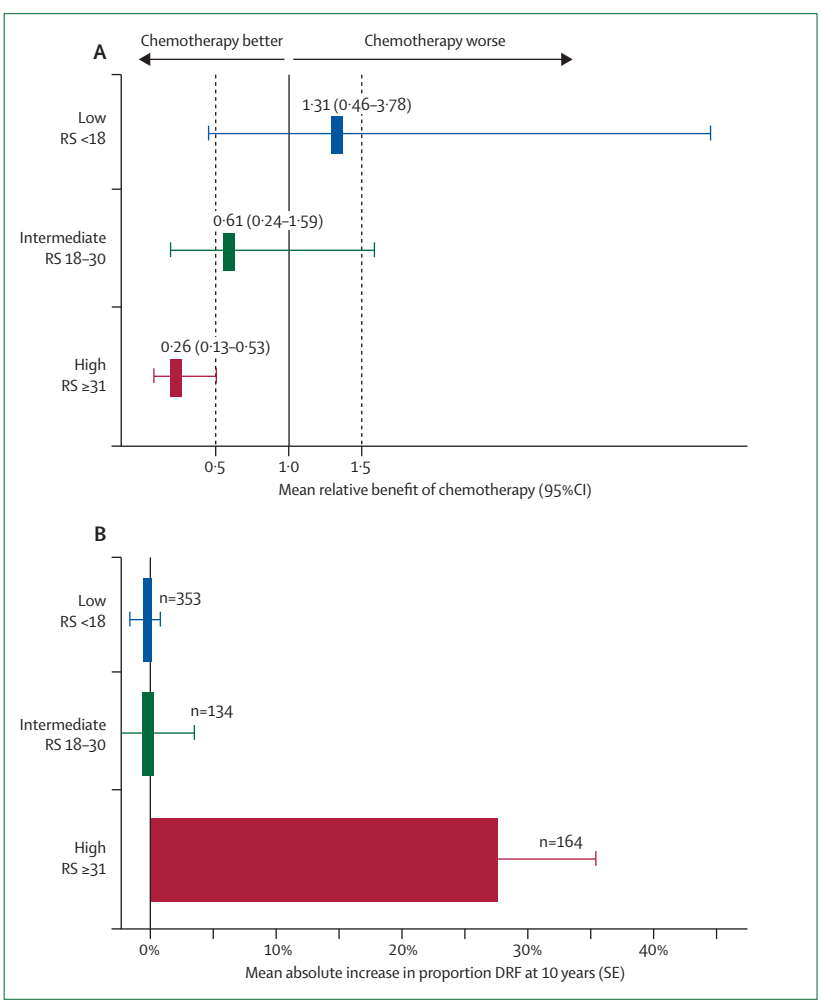

Figure 4: **Relative (A) and absolute (B) benefit of chemotherapy as a function of recurrence-score (RS) risk category in low, intermediate, and high RS groups**
DRF=distant recurrence free. Reprinted with permission from *Journal of Clinical Oncology*.[115]

or progesterone-receptor-positive breast cancer. Adjuvant endocrine therapy is highly effective in such cases and the contribution of chemotherapy not well defined. The prognostic and predictive roles of the Oncotype DX assay were assessed in archival tissue from treated node-negative, oestrogen-receptor-positive tumours. Tamoxifen was most effective when the recurrence score was low (≤18),[114] whereas patients whose primary tumours had a high (≥31) recurrence score derived more benefit from adjuvant chemotherapy (figure 4).

The 21-gene assay was also predictive of benefit from adjuvant chemotherapy in patients with node-positive breast cancer. A US trial randomly assigned premenopausal women with node-positive, oestrogen-receptor-positive breast cancer to tamoxifen or cyclophosphamide, doxorubicin, and fluorouracil (CAF) before or concurrently with tamoxifen. The sequential use of CAF followed by tamoxifen maximised rates of disease-free survival. However, when the Oncotype DX assay was applied to archival primary tissue, only patients with a high recurrence score benefited from CAF chemotherapy.[116] If prospective clinical trials confirm these data, we might be able to spare patients with low-risk breast cancer from undergoing chemotherapy, irrespective of the size of their primary tumours and degree of nodal involvement. Prognostic and predictive values of these molecular assays are based on retrospective subset analyses;[117] TAILORx is a prospective trial that randomises patients with oestrogen-receptor-positive node-negative breast cancer with an intermediate recurrence score (11–25) to chemotherapy and hormonal therapy, or hormonal therapy alone.[118]

The anthracyclines and taxanes are considered the most effective chemotherapies in the adjuvant setting. The taxanes (paclitaxel and docetaxel) have non-cross resistance with conventional agents, and their mechanism of action is to stabilise and prevent disaggregation of microtubules with disruption of the mitotic spindle. A randomised trial showed that four cycles of doxorubicin/cyclophosphamide (AC) followed by four cycles of paclitaxel improves survival compared with AC alone in patients with node-positive breast cancer.[119] Furthermore, giving AC and paclitaxel every 2 weeks (so-called dose-dense approach) improves disease-free survival compared with administration every 3 weeks.[120] Two trials investigating the efficacy of docetaxel in node-positive patients using more intensive anthracycline regimens noted significant improvements in overall survival for taxane-containing regimens.[115,121] A large trial randomised patients with node-positive breast cancer to four cycles of AC followed by either paclitaxel every 3 weeks, weekly paclitaxel, docetaxel every 3 weeks, or weekly docetaxel. Results showed that four cycles of AC followed by one dose of paclitaxel every week for 12 weeks improved overall survival.[122] Whether six or eight cycles are best, or four are sufficient, is unclear. Efforts are ongoing to identify gene-expression profiles that would help select patients for specific chemotherapies.[123] A popular combination is 5-fluorouracil, epirubicin, cyclophosphamide (FEC)-docetaxel for patients with involvement of four or more nodes. Provisional results from the TACT I trial suggest that adding four cycles of docetaxel to one of two standard regimens containing antracycline for unselected patients has little benefit.[124] Retrospective studies indicate that aberrant HER2 expression could correlate with benefit from paclitaxel in the adjuvant setting[125] and that modulation of topoisomerase II gene expression due to deletion or amplification might predict response to anthracycline-based chemotherapy.[126] Co-amplification of HER2 and the topoisomerase II amplicon is associated with increased response rates to anthracyclines.[126]

Trastuzumab (Herceptin) is a monoclonal antibody directed against the extracellular domain of HER2—a tyrosine kinase involved in cell growth and proliferation. Amplification of the *HER2* gene or otherwise overexpression of the cell-surface protein has been associated with a poor prognosis.[127] The HER2 status of the primary tumour or metastatic deposit should be assessed in all patients with breast cancer with either immunohistochemistry, fluorescence in-situ hybridisation, or chromogenic in-situ hybridisation.[128] If the tumour is HER2 positive, the patient is a good candidate for trastuzumab and for participation in clinical trials of novel HER2-directed treatments.

In patients with HER2-positive early-stage breast cancer, trastuzumab improves rates of disease-free and overall survival independent of age, axillary node metastases, and oestrogen-receptor or progesterone-receptor status.[129–131] Two US trials reported a significant reduction in risk of recurrence of about 50% and showed an early survival benefit favouring trastuzumab at 2 years (p=0·015).[129] The European trial (HERA) showed similar reductions in risk of recurrence but no overall-survival advantage.[130] Within the US trials, trastuzumab was given concurrently with an anthracycline-based chemotherapy (AC followed by paclitaxel/trastuzumab) and thereafter continued as single agent therapy for 52 weeks; however, Herceptin was prescribed only after completion of all chemotherapy (any regimen of ≥four cycles) in the European trial. A further international study (BCIRG 006) showed significant improvement in disease-free and overall survival for the non-anthracycline regimen TCH (docetaxel, carboplatin, and trastuzumab) compared with AC-T (doxorubicin/cyclophosphamide followed by docetaxel) and five-fold lower cardiotoxicity than with AC followed by docetaxel/trastuzumab (AC-TH).[131] The risk of cardiac toxicity in the adjuvant setting ranges from 0·5% to 4%.[132] Patients should undergo a baseline echocardiogram or cardiac scan to assess left ventricular ejection fraction before initiation of trastuzumab-based therapy. Serial assessments of left ventricular ejection fraction are recommended every 3 months while receiving trastuzumab with close follow-up in the first 2 years after

completion of treatment. In the event of cardiac toxicity, trastuzumab should be discontinued and left ventricular ejection fraction re-assessed in 4 weeks, although this decision should be made on an individual basis and consider recurrence risk and pre-existing cardiac morbidity.

Lapatinib (Tykerb) is a reversible small-molecule tyrosine-kinase inhibitor directed against epidermal growth factor receptor and HER2.[133] The combination of lapatinib and capecitabine improved rates of disease-free survival compared with capecitabine alone in heavily pretreated patients with metastatic breast cancer.[134] Inhibition of the HER2 kinase seems an important target for this type of molecule, because agents that target the epidermal growth factor receptor kinase selectively (eg, gefitinib and erlotinib) have shown insufficient efficacy in unselected patients with metastatic breast cancer.

Pertuzumab is a monoclonal antibody directed against HER2 that prevents formation of heterodimers between HER2 and other members of the HER family.[135] The binding sites of trastuzumab and pertuzumab localise to different domains of the HER2 protein. Preclinical studies showed a synergistic interaction between pertuzumab and trastuzumab, which is being explored.[135] Overexpression of insulin-like growth factor 1 receptor (IGF-IR) is associated with a poor prognosis and resistance to several drugs, including endocrine therapy and trastuzumab.[136] Approaches to inhibit IGF-IR include the use of monoclonal antibodies, small-molecule tyrosine-kinase inhibitors, and IGF binding proteins. Intracellular transduction pathways activated by growth factor receptors such as HER2 and IGF-IR are potential therapeutic targets, and the mitogen-activated protein kinase (MAPK) and the PI3K/Akt/mTOR pathways have been well characterised in breast-cancer cells.[137] Clinical trials are testing several inhibitors directed against different aspects of these signalling pathways. Postreceptor signalling pathways are not linear but form complex networks with much crosstalk. Multiple compensatory mechanisms exist with some functional redundancy, and blocking one protein (eg, mTOR) often leads to activation of more proximal steps (eg, Akt) and potentially increased proliferation.[137] An approach for overcoming the compensatory loops is to use a combination of inhibitors and to target central signalling nodes that are crucial for sustained growth-inhibitory effects. Novel approaches target not only the cancer cells but also the tumour microenvironment and new vessel formation. Preclinical and clinical studies have shown that blocking angiogenesis improves the efficacy of cytotoxic chemotherapy. Bevacizumab is a recombinant, humanised monoclonal antibody to vascular endothelial growth factor. A phase II trial of trastuzumab and bevacizumab showed that this combination was highly effective in patients with HER2 overexpressing metastatic breast cancer who had failed previous therapies.[138] Randomised clinical trials are in progress to establish the safety and efficacy of bevacizumab in combination with chemotherapy, endocrine therapy, and trastuzumab in all subtypes of breast cancer.

Primary systemic therapies

Primary systemic therapy, also known as neoadjuvant or preoperative therapy, was initially used for management of locally advanced breast cancers that could be rendered technically operable. Neoadjuvant approaches have increasingly been championed for treatment of operable tumours, with the expectation of improved outcomes and possible breast-conservation surgery.[139,140] Downstaging might reduce the requirement for mastectomy by up to half, and breast-conservation surgery is more likely for unifocal tumours located away from the nipple areolar complex.[141,142] Since the primary tumour remains in situ, primary systemic therapy allows serial core biopsies to be undertaken with monitoring of treatment effects. Primary systemic therapy constitutes a powerful in-vivo model providing potential information about pathological and molecular predictors of response and tumour biology, which in conjunction with imaging parameters, enables non-responders to be identified early and therapy changed accordingly.

Early trials of primary systemic therapy compared the same schedule of chemotherapy before or after standard surgical treatment. The NSABP B-18 trial randomised patients to four cycles of anthracycline-based chemotherapy before or after surgery. Overall survival was equivalent for both approaches, but patients receiving primary systemic therapy were more likely to undergo breast-conservation surgery.[141,142] However, rates of IBTR were higher when surgery followed rather than preceded chemotherapy, but this difference did not reach statistical significance (10·7% *vs* 7·6%, p=0·12).[142] Patients with a complete pathological response to primary systemic therapy have improved disease-free and overall survival, suggesting its use as a surrogate marker for trials comparing different schedules of primary systemic therapy.[141,143–145] The next generation of trials of primary systemic therapy aimed to establish whether different preoperative regimens could improve outcomes. In a small randomised trial, addition of docetaxel to a preoperative schedule compared with further anthracycline drugs doubled the complete pathological response with lengthening of disease-free and overall survival at 3 years.[146] By contrast, NSABP B-27 confirmed a doubling of the complete pathological response with addition of four cycles of docetaxel to four cycles of AC, but no improvement in overall survival.[147] Nonetheless, NSABP B-27 did show improved outcomes for patients who achieved a complete pathological response irrespective of schedule received.[147] Unlike adjuvant therapy trials, fewer numbers of patients and shorter follow-up are needed for assessment of primary systemic therapy. The activity of trastuzumab in the adjuvant setting has confirmed the benefits of this agent

when combined with chemotherapy in the neoadjuvant setting; complete pathological response rate more than doubled for combined therapy versus chemotherapy alone.[148] However, the potential for omission of surgical resection in patients with a complete pathological response remains limited in the absence of good clinico-radio-pathological correlation and prospective identification of this subset with imaging and percutaneous biopsy.

Oestrogen-receptor-negative tumours have higher rates of complete pathological response to primary chemotherapy than do hormone-sensitive tumours that exhibit lower rates.[147–150] Material from core biopsies or fine-needle aspirates can be processed for construction of DNA microarrays, allowing comparison of expression profiles between responders and non-responders.[151,152] A meta-analysis of neoadjuvant versus adjuvant systemic therapy for early-stage breast cancer shows that disease-free and overall survival are comparable for the two schedules.[153] Even if surgery causes a systemic perturbation that can be offset by induction chemotherapy, the assumption that a modest shift in the timing of chemotherapy relative to surgery would have any significant clinical effect is perhaps naive.[154] Moreover, increased rates of IBTR for neoadjuvant regimens could suggest inadequate surgery and cast doubt on the model of downstaging to allow breast-conservation surgery.

By analogy with neoadjuvant chemotherapy, hormonal treatment can be used preoperatively to downstage tumours. Hormonal therapies are less toxic and potential side-effects of chemotherapy can be avoided in elderly receptor-positive-patients and those with a poor performance status.[155] Moreover, oestrogen-receptor-positive patients are less likely to achieve a complete pathological response with primary chemotherapy than oestrogen-receptor-negative patients are.[147,149,150] Most studies relate to postmenopausal women for whom the aromatase inhibitors have consistently outperformed tamoxifen in the neoadjuvant setting, when endpoints include response rates and breast-conservation rates.[156,157] The amount of oestrogen-receptor expression is the main determinant of response, and the optimum duration of therapy might be longer than for chemotherapy.[158] In a study of patients with hormonally-sensitive locally-advanced and inoperable breast cancer, letrozole given as a preoperative schedule for 4 months yielded significantly better response rates than did tamoxifen for a similar period and allowed a significantly higher rate of breast-conservation surgery.[156] The IMPACT study randomised patients with operable hormone-responsive breast cancer to 3 months of anastrozole alone, tamoxifen alone, or a combination of the two. No significant difference in objective clinical response rates were recorded, but anastrozole was more effective than tamoxifen was in reducing Ki-67 expression and downstaging tumours to allow breast-conservation surgery according to surgeon assessment.[157]

Tumours do not necessarily shrink in a concentric manner in response to chemotherapy. Even if no viable cancer cells remain at the site of the original tumour periphery, this zone might contain unstable epithelium that is prone to malignant change.[159] Furthermore, tumour regression is difficult to assess radiologically even with MRI.[160] Functional imaging techniques have a potential role in assessment of disease extent before and after chemotherapy and can be especially useful in early assessment of tumour response as changes in metabolism, cell proliferation, and vascularity precede tumour regression.[161]

Primary systemic therapy can cause differential downstaging between sentinel and non-sentinel lymph nodes.[162] Biopsy of sentinel lymph nodes undertaken before chemotherapy will keep the risk of a false-negative result to a minimum and ensure that decisions for postmastectomy radiotherapy are based on accurate nodal staging.[163] However, there is no quantification of regional metastatic load, and some advocate biopsy of sentinel lymph nodes after primary systemic therapy to take advantage of nodal downstaging and avoidance of axillary dissection in up to 40% of patients.[162]

Chemoprevention

Tamoxifen is a pioneering non-steroidal antioestrogen whose primary action is to competitively antagonise oestrogen at the cellular-receptor level.[164] It has a proven efficacy in treatment of breast cancer over the past 30 years,[165] with substantial increases in survival in patients receiving long-term adjuvant therapy.[166] Furthermore, patients receiving adjuvant tamoxifen have a 47% reduction of contralateral tumours.[167] This accrual of a vast clinical database, underpinned by data from preclinical models and in-vitro studies,[168] catalysed the exploration of tamoxifen as a chemopreventive in high-risk women.[169] Several placebo-controlled chemoprevention trials of tamoxifen in high-risk premenopausal and postmenopausal women have shown up to a 50% reduction in the cumulative incidence of both invasive and non-invasive breast cancer, with primary effects confined to oestrogen-receptor-positive disease.[170,171] Moreover, recent data suggest that not only is tamoxifen effective during therapy, but also that chemoprevention is enhanced for many years after treatment ends.[171–173] This important observation shows the continuing antitumour action of tamoxifen, which occurs at a time when there are very few side-effects from the drug. The side-effect profile of tamoxifen and other potential agents are crucial considerations in the chemopreventive setting when the risk to benefit ratio is shifted and healthy women receive a pharmacological intervention for which the benefits are less tangible.

A modest increase in endometrial cancer in postmenopausal women has been well documented,[171] although neither this increase nor a raised risk of thromboembolism has been noted in premenopausal women.[171] Tamoxifen can result in hot flushes when used as adjuvant therapy in patients with breast cancer and is

a potential cause of non-compliance. This side-effect is especially pertinent in women who are considering use of tamoxifen for chemoprevention, and a selective serotonin reuptake inhibitor often needs to be co-prescribed. However, the latter can interfere with conversion of tamoxifen to its active metabolite endoxifen and should not be used;[174] the occurrence of hot flushes can indicate effective metabolism.[175] Additionally, mutations in *CYP2D6* can impede conversion of tamoxifen to endoxifen and might be relevant to patients considering use of tamoxifen for chemoprevention.

Concerns over increased incidence of endometrial cancer in women taking tamoxifen have led to re-assessment of other non-steroidal antioestrogens with attenuated uterotrophic activity in the rodent uterus.[176] The recognition that non-steroidal antioestrogens such as tamoxifen and raloxifene were selective oestrogen-receptor modulators with duality of action created a new dimension in therapeutics that is being exploited in chemoprevention strategies. If a selective oestrogen-receptor modulator is oestrogenic in bone but antioestrogenic in breast tissue, then perhaps it could be used to prevent osteoporosis with concomitant prophylaxis of breast cancer in post-menopausal women.[177] Raloxifene has been successfully tested for reduction of fractures in women at high risk for osteoporosis[178] and significantly reduces the incidence of oestrogen-receptor-positive breast cancer (77% risk reduction) in patients receiving long-term raloxifene for prevention of this disease.[179] These encouraging findings combined with the desire to minimise side-effects sporned the STAR trial:[180] a head-to-head comparison of tamoxifen and raloxifene as chemopreventive agents in high-risk postmenopausal women. Initial results have shown that raloxifene is equivalent to tamoxifen in reducing the incidence of oestrogen-receptor-positive breast cancer by 50%, but is less effective in prevention of non-invasive disease.[180] Raloxifene might therefore interfere with the progression of in-situ to invasive disease, but have no effect on premalignant to in-situ transition. Raloxifene had a more favourable side-effect profile than did tamoxifen, with marginally significant reductions of thromboembolic events, cataracts, lens replacement, and endometrial cancer.

The panel summarises US recommendations for use of selective oestrogen-receptor modulators on the basis of clinical-trial evidence and approvals from the Food and Drug Administration for risk reduction.[171,181] Recommendations for treatment duration are a single pulse of 5 years. However, the long-term use of raloxifene to prevent osteoporosis could mandate 10 or more years of therapy, but the apparent carry-over effect maintains the antitumour efficacy of raloxifene and tamoxifen beyond the actual treatment period.[180]

Future research should be directed at elucidating the mechanism of action of selective oestrogen-receptor modulators in different target tissues around the body. The configuration of the ligand/oestrogen receptor complex determines the recruitment of co-activators and co-repressors that bind to the external surface of the complex and activate oestrogen-response elements.[182] Individual selective oestrogen-receptor modulators have a clinical signature, with a range of structure-activity profiles that are site specific and confer differential and non-correlative mixed agonist or antagonist activity between species and tissues. Finally, clinical trials are assessing aromatase inhibitors in high-risk postmenopausal women as chemopreventive agents. IBIS II is a multicentre trial[183] that randomises healthy women at increased risk of breast cancer to either anastrozole or placebo. These inhibitors are associated with a greater reduction of contralateral breast cancer in adjuvant trials than is tamoxifen. They could potentially be combined with an luteinising hormone-releasing hormone agonist as a chemopreventive strategy in premenopausal women, but concerns exist about side-effects of profound oestrogen deprivation, and the optimum duration of therapy is unknown.

***Panel:* US recommendations for use of selective oestrogen-receptor modulators**

Tamoxifen

Recommended for high-risk premenopausal women for whom there is no significantly increased risk of endometrial cancer or blood clots

Raloxifene

Recommended for high-risk postmenopausal women for whom there is no significantly increased risk of endometrial cancer

Raloxifene

Recommended for treatment and prevention of osteoporosis. It reduces the risk of breast cancer with no increased risk of endometrial cancer

Conclusions

Despite an inexorable rise in the incidence of breast cancer, improvements in treatments together with screening have led to modest falls in mortality. Local control of disease does affect overall survival, and greater attention to surgical margins and improved radiotherapy techniques have reduced local recurrence after breast-conservation surgery. Oncoplastic surgical techniques are being used selectively to enhance cosmetic outcomes while satisfying oncological mandates. Long-term outcome is determined by the presence and behaviour of distant micrometastases, which have to be effectively managed to achieve disease control if not cure. Molecular profiling offers the potential to provide predictive information about individual tumour response, which will guide clinical application of targeted biological therapies and rationalise their integration with conventional systemic treatments.

Conflicts of interest

We declare that we have no conflicts of interest.

References

1 Hortobagyi GN, de la Garza Salazar J, Pritchard K, et al. The global breast cancer burden: variations in epidemiology and survival. *Clin Breast Cancer* 2005; **6:** 391–401.

2 Porter P. "Westernizing" women's risks? Breast cancer in lower-income countries. *N Engl J Med* 2008; **358:** 213–16.

3 Hankinson SE, Colditz GA, Willett WC. Towards an integrated model for breast cancer etiology: the lifelong interplay of genes, lifestyle, and hormones. *Breast Cancer Res Treat* 2004; **6:** 213–18.

4 Ravdin PM, Cronin KA, Howlader N, et al. The decrease in breast-cancer incidence in 2003 in the United States. *N Engl J Med* 2007; **356:** 1670–74.

5 Beral V, Hermon C, Reeves G, Peto R. Sudden fall in breast cancer death rates in England and Wales. *Lancet* 1995; **345:** 1642–43.

6 Peto R, Boreham J, Clarke M, Davies C, Beral V. UK and USA breast cancer deaths down 25% in year 2000 at ages 20–69 years. *Lancet* 2000; **355:** 1822.

7 Jatoi I, Miller AB. Why is breast-cancer mortality declining? *Lancet Oncol* 2003; **4:** 251–54.

8 Berry DA, Cronin KA, Plevritis SK, et al. Effect of screening and adjuvant therapy on mortality from breast cancer. *N Engl J Med* 2005; **353:** 1784–92.

9 Jatoi I, Chen BE, Anderson WF, Rosenberg PS. Breast cancer mortality trends in the United States according to estrogen receptor status and age at diagnosis. *J Clin Oncol* 2007; **25:** 1683–90.

10 Anderson WF, Jatoi I, Devesa SS. Distinct breast cancer incidence and prognostic patterns in the NCI's SEER program: suggesting a possible link between etiology and outcome. *Breast Cancer Res Treat* 2005; **90:** 127–37.

11 Clemmesen J. Carcinoma of the breast; results from statistical research. *Br J Rad* 1948; **21:** 583–90.

12 Adami HO, Hansen J, Jung B, Rimsten AJ. Age at first birth, parity and risk of breast cancer in a Swedish population. *Br J Cancer* 1980; **42:** 651–58.

13 Cleary MP, Maihle NJ. The role of body mass index in the relative risk of developing premenopausal versus postmenopausal breast cancer. *Proc Soc Exp Biol Med* (*New York*) 1997; **216:** 28–43.

14 Anderson WF, Matsuno RK, Sherman ME, et al. Estimating age-specific breast cancer risks: a descriptive tool to identify age interactions. *Cancer Causes Control* 2007; **18:** 439–47.

15 Byrne C, Schairer C, Wolfe J, et al. Mammographic features and breast cancer risk: effects with time, age and menopausal status. *J Natl Cancer Inst* 1995; **87:** 1622–29.

16 Jatoi I, Anderson WF, Rao SR, Devesa SS. Breast cancer trends among black and white women in the United States. *J Clin Oncol* 2005; **23:** 7836–41.

17 Jatoi I, Becher H, Leake CR. Widening disparity in survival between white and African-American patients with breast carcinoma treated in the US Department of Defense Healthcare system. *Cancer* 2003; **98:** 894–99.

18 Robson M, Offit K. Clinical practice. Management of an inherited predisposition to breast cancer. *N Engl J Med* 2007; **357:** 154–62.

19 Ford D, Easton DF. The genetics of breast and ovarian cancer. *Br J Cancer* 1995; **72:** 805–12.

20 Merajver SD, Pham TM, Caduff RF, et al. Somatic mutations in the BRCA1 gene in sporadic ovarian tumours. *Nat Genet* 1995; **9:** 439–43.

21 Baynes C, Healey CS, Pooley KA, et al. Common variants in the ATM, BRCA-1, BRCA-2, CHEK2 and TP53 cancer susceptibility genes are unlikely to increased breast cancer risk. *Breast Cancer Res Treat* 2007; **9:** R27.

22 Ramazzini B. De Morbis Artificum. Editio Secunda. Ultrajecti: Apud Guilielmum van de Water Academiae Typographum (courtesy, The Bodleian Library, Oxford): 156.

23 Petrek JA, Dukoff R, Rogatko A. Prognosis of pregnancy-associated breast cancer. *Cancer* 1991; **67:** 869–72.

24 Colditz GA. Estrogen, estrogen plus progestin therapy, and risk of breast cancer. *Clin Cancer Res* 2005; **11:** 909–17.

25 Holmberg L, Iversen O-E, Rudenstam CM, et al. Increased risk of recurrence after hormone replacement therapy in breast cancer survivors. *J Natl Cancer Inst* 2008; **100:** 475–82.

26 Monninkhof EM, Elias SG, Vlems FA, et al. Physical activity and breast cancer: a systematic review. *Epidemiology* 2007; **18:** 137–57.

27 Zhang SM, Lee IM, Manson JE, Cook NR, Willett WC, Buring JE. Alcohol consumption and breast cancer risk in the Women's Health Study. *Am J Epidemiol* 2007; **165:** 667–76.

28 Santen RJ, Boyd NF, Chlebowski RT, et al. Critical assessment of new risk factors for breast cancer: considerations for development of an improved risk prediction model. *Endocr Relat Cancer* 2007; **14:** 169–87.

29 Smith LF. Palpable cancer of the breast and negative mammography: the ongoing dilemma. *J Surg Oncol* 2007; **96:** 451–52.

30 Zonderland HM, Coerkamp EG, Hermans J, et al. Diagnosis of breast cancer: contributions of US as an adjunct to mammography. *Radiology* 1999; **213:** 413–22.

31 Britton PD, McCann J. Needle biopsy in the NHS Breast Screening Programme: how much and how accurate? *Breast* 1999; **8:** 5–11.

32 Stanley MW, Sidawy MK, Sanchez MA, Stahl RE, Goldfischer M. Current issues in breast cytopathology. *Am J Clin Path* 2000; **115:** S49–75.

33 Helbich TH, Matzek W, Fuchsjager MH. Stereotactic and ultrasound-guided breast biopsy. *Eur Radiol* 2004; **14:** 383–93.

34 Jackman RJ, Nowels KW, Rodriguez-Soto J, et al. Sterotactic, automated, large-core needle biopsy of nonpalpable breast lesions: false-negative and histologic underestimation rates after long term follow up. *Radiology* 1999; **210:** 799–805.

35 DeMartini W, Lehman C, Partridge S. Breast MRI for cancer detection and characterization: a review of evidence-based clinical applications. *Acad Radiol* 2008; **15:** 408–16.

36 Mameri CS, Kemp C, Goldman SM, et al. Impact of breast MRI on surgical treatment, axillary approach, and systemic therapy for breast cancer. *Breast J* 2008; **14:** 236–44.

37 Morrow M, Freedman G. A clinical oncology perspective on the use of breast MR. *Magn Reson Imaging Clin N Am* 2006; **14:** 363–78.

38 Solin LJ, Orel SG, Hwang WT, et al. Relationship of breast magnetic resonance imaging to outcome after breast conservation treatment with radiation for women with early stage invasive breast carcinoma or ductal carcinoma in situ. *J Clin Oncol* 2008; **26:** 386–91.

39 Morrow M. Magnetic resonance imaging in the breast cancer patient: curb your enthusiasm. *J Clin Oncol* 2008; **26:** 352–53.

40 Gotzsche PC, Nielsen M. Screening for breast cancer with mammography. *Cochrane Database Syst Rev* 2006; **3:** CD001877.

41 Moss SM, Cuckle H, Evans A, Johns L, Waller M, Bobrow L. Effect of mammographic screening from age 40 years on breast cancer mortality at 10 years' follow-up: a randomised controlled trial. *Lancet* 2006; **368:** 2053–60.

42 Barton MB, Harris R, Fletcher SW. The rational clinical examination. Does this patient have breast cancer? The screening clinical breast examination: should it be done? How? *JAMA* 1999; **282:** 1270–80.

43 Thomas DB, Gao DL, Ray RM, et al. Randomized trial of breast self-examination in Shanghai: final results. *J Natl Cancer Inst* 2002; **94:** 1445–57.

44 Saslow D, Boetes C, Burke W, et al. American Cancer Society guidelines for breast screening with MRI as an adjunct to mammography. *CA Cancer J Clin* 2007; **57:** 75–89.

45 Querci della Rovere G, Benson JR. Ipsilateral local recurrence of breast cancer: determinant or indicator of poor prognosis? *Lancet Oncol* 2002; **3:** 183–87.

46 Fisher B, Anderson S, Bryant J, et al. Twenty-year follow up of a randomized trial comparing total mastectomy, lumpectomy and lumpectomy plus irradiation for the treatment of invasive breast cancer. *N Engl J Med* 2002; **347:** 1233–41.

47 Fisher B. Laboratory and clinical research in breast cancer—a personal adventure. The David A Karnofsky Memorial Lecture. *Cancer Res* 1980; **40:** 3863–74.

48 Komoike Y, Akiyama F, Lino Y, et al. Ipsilateral breast tumour recurrence (IBTR) after breast conserving treatment for breast cancer. *Cancer* 2005; **106:** 35–39.

49 Early Breast Cancer Trialists Collaborative Group. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15 year survival: an overview of the randomised trials. *Lancet* 2005; **366:** 2087–106.

50 Huang E, Cheng SH, Dressman H, et al. Gene expression predictors of breast cancer outcomes. *Lancet* 2003; **361:** 1590–96.

51 Locker G, Sainsbury R, Cuzick J. Breast surgery in the ATAC trial: women in the United States are more likely to have mastectomy. *Breast Cancer Res Treat* 2002; **76:** S35.

52 IBTR. Breast cancer model. http://www.nemc.org/ibtr (accessed Nov 17, 2008).

53 Taghian A, Mohiuddin M, Jagsi R, et al. Current perceptions regarding surgical margin status after breast conserving therapy: results of a survey. *Ann Surg* 2005; **241:** 629–39.

54 Singletary SE. Surgical margins in patients with early stage breast cancer treated with breast conservation therapy. *Am J Surg* 2002; **184:** 383–93.

55 Morrow M, Harris JR. Practice guidelines for breast conserving therapy in the management of invasive breast cancer. *J Am Coll Surg* 2007; **205:** 362–76.

56 Cochrane R, Valasiadou P, Wilson A, et al. Cosmesis and satisfaction after breast conserving surgery correlates with percentage of breast volume excised. *Br J Surg* 2003; **90:** 1505–09.

57 Taylor ME, Perez CA, Halverson KJ, et al. Factors influencing cosmetic results after conservation therapy for breast cancer. *In J Rad Oncol Biol Phys* 1995; **31:** 753–64.

58 McCulley SJ, Macmillan RD. Planning and use of therapeutic mammoplasty–Nottingham approach. *Br J Plast Surg* 2005; **58:** 889–901.

59 Asgeirsson K, McCulley S, Pinder S, Macmillan R. Size of invasive breast cancer and risk of local recurrence after breast conservation therapy. *Eur J Cancer* 2003; **39:** 2462–69.

60 Benson JR, Absar MS. Volume replacement and displacement techniques in oncoplastic surgery. Advances in Breast Cancer 2008; **5:** 1–7.

61 Rivadeniera DE, Simmons RM, Fish SK, et al. Skin-sparing mastectomy with immediate breast reconstruction: a critical analysis of local recurrence. *Cancer J* 2000; **6:** 331–35.

62 Overgaard M, Hansen PS, Overgaard J, et al. Post-operative radiotherapy in high risk pre-menopausal women with breast cancer who receive adjuvant chemotherapy. *N Engl J Med* 1997; **337:** 949–55.

63 Ragaz J, Jackson SM, Le N, et al. Adjuvant radiotherapy and chemotherapy in node positive pre-menopausal women with breast cancer. *N Engl J Med* 1997; **337:** 956–62.

64 Behranwala KA, Dua RS, Ross GM, et al. The influence of radiotherapy on capsule formation and aesthetic outcome after immediate breast reconstruction using biodimensional anatomical expander implants. *J Plast Reconstr Aesthet Surg* 2006; **59:** 1043–51.

65 Lyman GH, Guiliano AE, Somerfield MR, et al. The Americal Society of Clinical Oncology Guideline Recommendations for Sentinel Lymph Node Biopsy in Early Stage Breast Cancer. *J Clin Oncol* 2005; **23:** 7703–20.

66 Benson JR, Querci della Rovere G. Management of the axilla in women with breast cancer. *Lancet Oncol* 2007; **8:** 331–48.

67 Krag DN, Anderson SJ, Julian TB, et al. Technical outcomes of sentinel-lymph-node resection and conventional axillary-lymph-node dissection in patients with clinically node-negative breast cancer: results from the NSABP B-32 randomised phase III trial. *Lancet Oncol* 2007; **8:** 881–88.

68 Pal A, Provenzano E, Duffy SW, et al. A model for predicting non-sentinel lymph node metastatic disease when the sentinel lymph node is positive. *Br J Surg* 2008; **95:** 302–09.

69 Park J, Fey JV, Naik AM, et al. A declining rate of completion axillary dissection in sentinel lymph node positive breast cancer patients is associated with the use of a multivariate nomogram. *Breast Cancer Res Treat* 2005; **100:** S80 (abstract 2001).

70 Julian TB, Blumencranz P, Deck K, et al. Novel intraoperative molecular test for sentinel lymph node metastasesin patients with early stage breast cancer. *J Clin Oncol* 2008; **26:** 3338–45.

71 Naik AM, Fey J, Gemignani M, et al. The risk of axillary relapse after sentinel lymph node biopsy for breast cancer is comparable with that of axillary lymph node dissection. *Ann Surg* 2004; **240:** 462–71.

72 Kujit GP, Roumen RMH. Second thoughts on sentinel lymph node biopsy in node negative breast cancer. *Br J Surg* 2008; **95:** 310–11.

73 Veronesi U, Cascinelli N, Mariani L, et al. Twenty-year follow-up of a randomized study comparing breast-conserving surgery with radical mastectomy for early breast cancer. *N Engl J Med* 2002; **347:** 1227–32.

74 Smith IE, Ross GM. Breast radiotherapy after lumpectomy—no longer always necessary. *N Engl J Med* 2004; **351:** 1021–23.

75 Smith BD, Gross CP, Smith GL, et al. Effectiveness of radiation therapy for older women with early breast cancer. *J Natl Cancer Inst* 2006; **98:** 681–90.

76 Clark RM, McCulloch PB, Levine MN, et al. Randomized clinical trial to assess the effectiveness of breast irradiation following lumpectomy and axillary dissection for node-negative breast cancer. *J Natl Cancer Inst* 1992; **84:** 683–89.

77 Hughes KS, Schnaper LA, Berry D, et al. Lumpectomy plus tamoxifen with or without irradiation in women 70 years of age or older with early breast cancer. *N Engl J Med* 2004; **351:** 971–77.

78 Fyles AW, McCready DR, Manchul LA, et al. Tamoxifen with or without breast irradiation in women 50 years of age or older with early breast cancer. *N Engl J Med* 2004; **351:** 963–70.

79 King TA, Bolton JS, Kuske RR, et al. Long-term results of wide-field brachytherapy as the sole method of radiation therapy after segmental mastectomy for T(is,1,2) breast cancer. *Am J Surg* 2000; **180:** 299–304.

80 Cuttino LW, Arthur DW, Todor DA, et al. CT- guided multi-catheter insertion technique for partial breast brachytherapy (PBB): Reliable target coverage and dose homogeneity. *Brachytherapy* 2003; **2:** 41–68.

81 Vicini FA, Kestin L, Chen P, et al. Limited-field radiation therapy in the management of early-stage breast cancer. *J Natl Cancer Inst* 2003; **95:** 1205–10.

82 Kuske RR, Winter K, Arthur DW, et al. Phase II trial of brachytherapy alone after lumpectomy for select breast cancer: toxicity analysis of RTOG 95-17. *Int J Radiat Oncol Biol Phys* 2006; **65:** 45–51.

83 Vaidya JS, Baum M, Tobias JS, et al. Targeted intra-operative radiotherapy (Targit): an innovative method of treatment for early breast cancer. *Ann Oncol* 2001; **12:** 1075–80.

84 Veronesi U, Orrechia R, Luini A, et al. A preliminary report of intraoperative radiotherapy (IORT) in limited-stage breast cancers that are conservatively treated. *Eur J Cancer* 2001; **37:** 2178–83.

85 Enderling H, Chaplain MA, Anderson AR, Vaidya JS. A mathematical model of breast cancer development, local treatment and recurrence. *J Theor Biol* 2007; **246:** 245–59.

86 Belletti B, Vaidya JS, D'Andrea S, et al. Targeted intraoperative radiotherapy impairs the stimulation of breast cancer cell proliferation and invasion caused by surgical wound healing. *Clin Cancer Res* 2008; **14:** 1325–32.

87 Keisch M, Vicini F, Kuske RR, et al. Initial clinical experience with the MammoSite breast brachytherapy applicator in women with early-stage breast cancer treated with breast-conserving therapy. *Int J Radiat Oncol Biol Phys* 2003; **55:** 289–93.

88 Benitez PR, Keisch ME, Vicini F, et al. Five-year results: the initial clinical trial of MammoSite balloon brachytherapy for partial breast irradiation in early-stage breast cancer. *Am J Surg* 2007; **194:** 456–62.

89 Vicini F, Beitch PD, Quiet CA, et al. Three-year analysis of treatment efficacy, cosmesis, and toxicity by the American Society of Breast Surgeons MammoSite Breast Brachytherapy Registry Trial in patients treated with accelerated partial breast irradiation (ABPI). *Cancer* 2008; **112:** 758–66.

90 Vicini FA, Remouchamps V, Wallace M, et al. Ongoing clinical experience utilizing 3D conformal external beam radiotherapy to deliver partial-breast irradiation in patients with early-stage breast cancer treated with breast-conserving therapy. *Int J Radiat Oncol Biol Phys* 2003; **57:** 1247–53.

91 Formenti SC, Truong MT, Goldberg JD, et al. Prone accelerated partial breast irradiation after breast-conserving surgery: preliminary clinical results and dose-volume histogram analysis. *Int J Radiat Oncol Biol Phys* 2004; **60:** 493–504.

92 Kestin LL, Sharpe MB, Frazier RC, et al. Intensity modulation to improve dose uniformity with tangential breast radiotherapy: initial clinical experience. *Int J Radiat Oncol Biol Phys* 2000; **48:** 1559–68.

93 Whelan T, MacKenzie R, Julian J, et al. Randomized trial of breast irradiation schedules after lumpectomy for women with lymph node-negative breast cancer. *J Natl Cancer Inst* 2002; **94:** 1143–50.

94 The START trialists' Group. The UK Standardisation of Breast Radiotherapy (START) Trail A of radiotherapy hypofractionation for treatment of early breast cancer: a randomised trial. *Lancet Oncol* 2008; **9:** 331–41.

95 Kruegrer EA, Fraass BA, McShan DL, et al. Potential gains for irradiation of chest wall and regional nodes with intensity modulated radiotherapy. *Int J Radiat Oncol Biol Phys* 2003; **56:** 1023–37.

96 MRC SUPREMO Trial. http://www.supremo-trial.com (accessed Nov 17, 2008).

97 Early Breast Cancer Trialists' Collaborative Group. Polychemotherapy for early breast cancer: an overview of the randomised trials. *Lancet* 1998; **352:** 930–42.

98 Early Breast Cancer Trialists Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998; **351:** 1451–61.

99 Ravdin PM, Siminoff LA, Davis GJ, et al. Computer program to assist in making decisions about adjuvant therapy for women with early breast cancer. *J Clin Oncol* 2001; **19:** 980–91.

100 Marchionni L, Wilson RF, Wolff AC, et al. Systematic review: gene expression profiling assays in early stage breast cancer. *Ann Intern Med* 2008; **148:** 358–69.

101 Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J Clin Oncol* 2007; **25:** 5287–12.

102 Scottish Cancer Trials Breast Group and IBU. Adjuvant ovarian ablation versus CMF chemotherapy in premenopausal women with pathological stage II breast carcinoma: the Scottish trial. *Lancet* 1993; **341:** 1293–98.

103 Lyman GH, Guiliano AE, Somerfield MR, et al. The American Society of Clinical Oncology Guideline Recommendations for Sentinel Lymph Node Biopsy in Early Stage Breast Cancer. *J Clin Oncol* 2005; **23:** 7703–20.

104 ATAC Trialist's Group. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years adjuvant treatment for breast cancer. *Lancet* 2005; **365:** 60–62.

105 The Arimidex, Tamoxifen, Alone or in Combination (ATAC) Trialists' Group. Effect of anastrozole and tamoxifen as adjuvant treatment for early stage breast cancer: 100 month analysis of the ATAC trial. *Lancet Oncol* 2008; **9:** 45–53.

106 Benson JR. The use or aromatase inhibitors in post-menopausal women with hormone receptor positive breast cancer. *J Clin Oncol* 2005; **23:** 6807–09.

107 Coombes RC, Kilburn LS, Snowdon CF, et al. Survival and safety of exemestane versus tamoxifen after 2–3 year's tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial. *Lancet* 2007; **369:** 559–70.

108 Jonat W, Gnant M, Boccado F, et al. Effectiveness of switching from adjuvant tamoxifen to anastrozole in postmenopausal women with hormone-sensitive early-stage breast cancer: a meta-analysis. *Lancet Oncol* 2006; **7:** 991–96.

109 Coates AS, Keshaviah A, Thurlimann B, et al. Five years of letrozole compared with tamoxifen as initial therapy for postmenopausal women with endocrine responsive early breast cancer: update of study BIG 1-98. *J Clin Oncol* 2007; **25:** 486–92.

110 Mouridsen H, Giobbie-Hurder A, Mauriac L, et al. BIG 1-98: a randomized double-blind phase III study evaluating letrozole and tamoxifen in sequence as adjuvant endocrine therapy for postmenopausal women with receptor-positive breast cancer. 31st San Antonio Breast Cancer Symposium, Texas, USA; December, 2008.

111 Goss PE, Ingle JN, Martino S, et al. Randomised trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17. *J Natl Cancer Inst* 2005; **97:** 1262–71.

112 Clarke M, Coates AS, Darby SC, et al. Adjuvant chemotherapy in oestrogen receptor poor breast cancer: patient level meta-analysis of randomised trials. *Lancet* 2008; **371:** 29–40.

113 Carlson RW, Brown E, Burstein HJ, et al. NCCN Task Force Report: adjuvant therapy for breast cancer. *J Natl Compr Cancer Netw* 2006; **4** (suppl)**:** S1–26.

114 Paik S, Shak S, Tang G, et al. A multigene assay to predict recurrence of tamoxifen-treated, node negative, estrogen receptor positive breast cancer. *J Clin Oncol* 2006; **24:** 3726–34.

115 Roche H, Fumoleau P, Spielman M, et al. Sequential adjuvant epirubicin based and docetaxel chemotherapy for node positive breast cancer patients: The FNCLCC PACS001 trial. *J Clin Oncol* 2006; **24:** 5664–71.

116 Albain KS, Barlow W, Shak S, et al. Prognostic and predictive value of the 21-gene recurrence score assay in postmenopausal, node positive, ER positive breast cancer. *Breast Cancer Res Treat* 2007; **96:** 10A.

117 Esteva FJ, Sahin AA, Cristofanilli M, et al. Prognostic role of a multigene reverse transcriptase-PCR assay in patients with node-negative breast cancer not receiving adjuvant systemic therapy. *Clin Cancer Res* 2005; **11:** 3315–19.

118 Sparano JA. TAILORx: trial assigning individualized options for treatment (Rx). *Clin Breast Cancer* 2006; **7:** 347–50.

119 Henderson IC, Berry DA, Demetri GD, et al. Improved outcomes from adding sequential Paclitaxel but not escalating Doxorubicin dose in an adjuvant chemotherapy regimen for patients with node positive primary breast cancer. *J Clin Oncol* 2003; **21:** 976–83.

120 Citron ML, Berry DA, Cirrincione C, et al. Randomised trial of dose-dense versus conventionally scheduled and sequential versus concurrent combination chemotherapy as postoperative adjuvant treatment of node positive primary breast cancer: first report of Intergroup Trial C. *J Clin Oncol* 2003; **21:** 1431–39.

121 Martin M, Pienkowski T, Mackay J, et al. Adjuvant docetaxol for node positive breast cancer. *N Engl J Med* 2005; **352:** 2302–13.

122 Sparano JA, Wang M, Martino S, et al. Weekly paclitaxel in the adjuvant treatment of breast cancer. *N Engl J Med* 2008; **358:** 1663–71.

123 Hess KR, Anderson K, Symmans WF, et al. Pharmacogenomic predictor of sensitivity to preoperative chemotherapy with Paclitaxel and Fluorouracil, Doxorubicin and Cyclophosphamide in breast cancer. *J Clin Oncol* 2006; **24:** 4236–44.

124 Ellis PA, Barrett-Lee PJ, Bloomfield D, et al. Preliminary results of the UK Taxotere as Adjuvant Chemotherapy (TACT) trial. *Breast Cancer Res Treat* 2007; **106:** S21 (abstract 78).

125 Hayes DF, Thor AD, Dressler LG, et al. HER2 and response to paclitaxel in node positive breast cancer. *N Engl J Med* 2008; **21:** 976–83.

126 Pritchard KI, Messersmith H, Elavathil L, et al. HER2 and topoisomerase II as predictors of response to chemotherapy. *J Clin Oncol* 2008; **26:** 736–44.

127 Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplication of the HER2/neu oncogene. *Science* 1987; **235:** 177–82.

128 Wolff AC, Hammond ME, Schartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol* 2007; **25:** 118–45.

129 Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2 positive breast cancer. *N Engl J Med* 2005; **353:** 1673–84.

130 Smith I, Procter M, Gelber RD, et al. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. *Lancet* 2007; **369:** 29–36.

131 Perez EA, Suman VJ, Davidson NE, et al. Cardiac safety analysis of doxorubicin and cyclophosphamide followed by paclitaxel with or without trastuzumab in the North Central Cancer Treatment Group N9831 adjuvant breast cancer trial. *J Clin Oncol* 2008; **26:** 1231–38.

132 Cancer International Research Group.Press Release BCIRG 006 FDA Approval. May 29, 2008. http://64.233.183.104/search?q=cache:L6vhvabBD1Q J:www.bcirg.org/NR/rdonl (accessed Aug 7, 2008).

133 Wood ER, Truesdale AT, McDonald OB, et al. A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate and receptor activity in tumour cells. *Cancer Res* 2004; **64:** 6652–59.

134 Geyer CE, Forster J, Lindquist D, et al. Lapatinib plus capecitibine for HER2 positive advanced breast cancer. *N Engl J Med* 2006; **355:** 2733–43.

135 Nahta R, Hung MC, Esteva FJ. The HER2 targetting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. *Cancer Res* 2004; **64:** 2343–46.

136 Nahta R, Yuan LX, Zhang B, et al. Insulin-like growth factor I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. *Cancer Res* 2005; **65:** 1118–28.

137 O'Reilly KE, Rojo F, She QB, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* 2006; **66:** 1500–08.

138 Pegram M, Chan D, Cichmann RA, et al. Phase II combined biological therapy targeting the HER2 proto-oncogene and the vascular endothelial growth factor (VEGF) using trastuzumab and bevacizumab as first-line treatment of HER2 amplified breast cancer. *Breast Cancer Res Treat* 2006; **100:** abstract 201.

139 Hortobagyii GN, Ames FC, Buzdar AU, et al. Management of stage III primary breast cancer with primary chemotherapy, surgery and radiation therapy. *Cancer* 1988; **62:** 2507–16.

140 Wolff AC, Davidson NE. Primary systemic therapy in operable breast cancer. *J Clin Oncol* 2000; **18:** 1558–69.

141 Fisher B, Brown A, Mamounas E, et al. Effect of preoperative chemotherapy on local-regional disease in women with operable breast cancer: findings from National Surgical Adjuvant Breast and Bowel Project B-18. *J Clin Oncol* 1997; **15:** 2483–93.

142 Wolmark N, Wang J, Mamounas E, et al. Preoperative chemotherapy in patients with operable breast cancer: nine-year results from National Surgical Adjuvant Breast and Bowel Project B-18. *J Natl Cancer Inst Monogr* 2001; **30:** 96–102.

143 Van der Hage JA, van de Velde CJH, Julien J-P, et al. Preoperative chemotherapy in primary operable breast cancer: results from the European Organization for Research and Treatment of Cancer trial 10902. *J Clin Oncol* 2001; **19:** 4224–37.

144 Makris A, Powles TJ, Ashley SE et al. A reduction in the requirements for mastectomy in a randomized trial of neoadjuvant chemoendocrine therapy in primary breast cancer. *Ann Oncol* 1998; **9:** 1179–84.

145 Scholl SM, Fourquet A, Asselain B et al. Neoadjuvant vs adjuvant chemotherapy in premenopausal women with tumors considered too large for breast conserving surgery: preliminary results of a randomised trial. *Eur J Cancer* 1994; **30A:** 645–52.

146 Smith IC, Heys SD, Hutcheon AW, et al. Neoadjuvant chemotherapy in breast cancer: significantly enhanced response with docetaxel. *J Clin Oncol* 2002; **20:** 1456–66.

147 Bear HD, Anderson S, Brown A, et al. The effect of tumor response of adding sequential preoperative docetaxel to preoperative doxorubicin and cyclophosphamide: Preliminary results from National Surgical Adjuvant Breast and Bowel Project Protocol B-27. *J Clin Oncol* 2003; **21:** 4165–74.

148 Buzdar AU, Ibrahim NK, Francis D, et al. Significant higher pathologic complete remission rate after neoadjuvant therapy with trastuzumab, paclitaxel, and epirubicin chemotherapy: results of a randomised trial in human epidermal growth factor receptor 2-positive operable breast cancer. *J Clin Oncol* 2005; **23:** 3676–85.

149 Guarneri V, Broglio K, Kau SW, et al. Prognostic value of pathologic complete response after primary chemotherapy in relation to hormone receptor status and other factors. *J Clin Oncol* 2006; **24:** 1037–44.

150 Gianni L, Baselga J, Eirmann W, et al. Feasibility and tolerability of sequential doxorubicin/paclitaxel followed by cyclophosphamide, methotrexate, and fluorouracil and its effects on tumor response as preoperative therapy. *Clin Cancer Res* 2005; **11:** 8715–21.

151 Chang JC, Wooten EC, Tsimelzon A, et al. Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. *Lancet* 2003; **362:** 362–69.

152 Ayers M, Symmans WF, Stec J, et al. Gene expression profiles predict complete pathological response to neoadjuvant paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide chemotherapy in breast cancer. *J Clin Oncol* 2004; **22:** 2284–93.

153 Mauri D, Pavlidis N, Ioannidis JP. Neoadjuvant versus adjuvant systemic treatment for breast cancer: a meta-analysis. *J Natl Cancer Inst* 2005; **97:** 188–194.

154 Davidson NE, Morrow M. Sometimes a great notion—an assessment of neoadjuvant systemic therapy for breast cancer. *J Natl Cancer Inst* 2005; **97:** 159–61.

155 Beresford MJ, Ravichandran D, Makris A. Neoadjuvant endocrine therapy in breast cancer. *Cancer Treat Rev* 2007; **33:** 48–57.

156 Eiermann W, Paepke S, Appfelastaedt J, et al. Pre-operative treatment of post-menopausal breast cancer with letrozole: a randomized double-blind multicenter study. *Ann Oncol* 2001; **12:** 1527–32.

157 Smith I, Dowsett M, Ebbs SR, et al. Neoadjuvant treatment of post-menopausal breast cancer with anastrozole, tamoxifen or both in combination: the Immediate Preoperative Anastrozole, Tamoxifen or Combined with Tamoxifen (IMPACT) Multicenter Double-Blind Randomized trial. *J Clin Oncol* 2005; **23:** 5108–16.

158 Dixon JM, Jackson J, Renshaw L, et al. Neoadjuvant tamoxifen and aromatase inhibitors: comparisons and clinical outcomes. *J Steroid Biochem Mol Biol* 2003; **86:** 295–99.

159 Veronesi U, Bonadonna G, Zurrida S, et al. Conservation surgery after primary chemotherapy in large carcinomas of the breast. *Ann Surg* 1995; **222:** 612–18.

160 Beresford M, Padhani AR, Goh V, et al. Imaging breast cancer response during neoadjuvant systemic therapy. *Expert Rev Anticancer Ther* 2005; **5:** 907–15.

161 Padhani AR, Ah-See M-L, Makris A. MRI in the detection and management of breast cancer. *Expert Rev Anticancer Ther* 2005; **5:** 239–52.

162 Mamounas EP, Brown A, Anderson S, et al. Sentinel node biopsy after neoadjuvant chemotherapy in breast cancer: results from National Surgical Adjuvant Breast and Bowel Project Protocol B-27. *J Clin Oncol* 2005; **23:** 2694–02.

163 Sabel MS, Schott AF, Kleer CG, et al. Sentinel node biopsy prior to neoadjuvant chemotherapy. *Am J Surg* 2003; **39:** 1089–96.

164 Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2003; **2:** 205–13.

165 Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. The Dorothy P Landon AACR Prize for Translational Research. *Clin Cancer Res* 2003; **9:** 1980–89.

166 Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005; **365:** 1687–717.

167 Cuzick J, Baum M. Tamoxifen and contralateral breast cancer. *Lancet* 1985; **2:** 282.

168 Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 2008; **44:** 30–38.

169 Powles TJ, Hardy JR, Ashley SE, et al. A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *Br J Cancer* 1989; **60:** 126–31.

170 Cuzick J, Powles T, Veronesi U, et al. Overview of the main outcomes in breast-cancer prevention trials. *Lancet* 2003; **361:** 296–300.

171 Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* 2005; **97:** 1652–62.

172 Cuzick J, Forbes JF, Sestak I, et al. Long term results of tamoxifen prophylaxis for breast cancer–96 month follow up of the randomized IBIS-I trial. *J Natl Camcer Inst* 2007; **99:** 272–82.

173 Powles TJ, Ashley S, Tidy A, et al. Twenty-year follow up of the Royal Marsden randomized, double blinded tamoxifen breast cancer prevention trial. *J Natl Cancer Inst* 2007; **99:** 283–90.

174 Stearns V, Johnson MD, Rae JM, et al. Active tamoxifen metabolite plasma concentrations after co-administration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. *J Natl Cancer Inst* 2003; **95:** 1758–64.

175 Goetz MP, Rae JM, Suman VJ, et al. Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. *J Clin Oncol* 2005; **23:** 9312–18.

176 Black LJ, Jones CD, Falcone JF, et al. Antagonism of estrogen action with a new benzothiaphene derived antiestrogen. *Life Sci* 1983; **32:** 1031–36.

177 Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Investl* 1988; **6:** 589–95.

178 Ettinger B, Black DM, Mitlak BH, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. *JAMA* 1999; **282:** 637–45.

179 Martino S, Cauley JA, Barrett-Connor E, et al, for the CORE Investigators Continuing Outcomes Relevant to Evista. Breast cancer incidence in postmenopausal osteoporotic women in a randomised trial of raloxifene. *J Natl Cancer Inst* 2004; **96:** 1751–61.

180 Vogel VG, Costantino JP, Wickerham DL, et al. The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA* 2006; **295:** 2727–41.

181 Barrett-Connor E, Mosca L, Collins P, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Engl J Med* 2006; **355:** 125–37.

182 Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. *Nature Rev* 2007; **7:** 46–53.

183 Cuzick J. Aromatase inhibitors in prevention–data from the ATAC trial and the design of IBIS-II (the second International Breast Cancer Intervention Study). *Recent Results Cancer Res* 2003; **163:** 96–103.

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The Paradox of Oestradiol-Induced Breast Cancer Cell Growth and Apoptosis

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Abstract

High dose oestrogen therapy was used as a treatment for postmenopausal patients with breast cancer from the 1950s until the introduction of the safer antioestrogen, tamoxifen in the 1970s. The anti-tumour mechanism of high dose oestrogen therapy remained unknown. There was no enthusiasm to study these signal transduction pathways as oestrogen therapy has almost completely been eliminated from the treatment paradigm. Current use of tamoxifen and the aromatase inhibitors seek to create oestrogen deprivation that prevents the growth of oestrogen stimulated oestrogen receptor (ER) positive breast cancer cells. However, acquired resistance to antihormonal therapy does occur, but it is through investigation of laboratory models that a vulnerability of the cancer cell has been discovered and is being investigated to provide new opportunities in therapy with the potential for discovering new cancer-specific apoptotic drugs. Laboratory models of resistance to raloxifene and tamoxifen, the selective oestrogen receptor modulators (SERMs) and aromatase inhibitors demonstrate an evolution of drug resistance so that after many years of oestrogen deprivation, the ER positive cancer cell reconfigures the survival signal transduction pathways so oestrogen now becomes an apoptotic trigger rather than a survival signal. Current efforts are evaluating the mechanisms of oestrogen-induced apoptosis and how this new biology of oestrogen action can be amplified and enhanced, thereby increasing the value of this therapeutic opportunity for the treatment of breast cancer. Several synergistic approaches to therapeutic enhancement are being advanced which involve drug combinations to impair survival signaling with the use of specific agents and to impair bcl-2 that protects the cancer cell from apoptosis. We highlight the historical understanding of oestrogen's role in cell survival and death and specifically illustrate the progress that has been made in the last five years to understand the mechanisms of oestrogen-induced apoptosis. There are opportunities to harness knowledge from this new signal transduction pathway to discover the precise mechanism of this oestrogen-induced apoptotic trigger. Indeed, the new biology of oestrogen action also has significance for understanding the physiology of bone remodeling. Thus, the pathway has a broad appeal in both physiology and cancer research.

Keywords

Breast cancer; oestrogen receptor; oestrogen action; apoptosis; tamoxifen; selective oestrogen receptor modulators

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HISTORICAL ORIGINS OF TARGETED HORMONAL THERAPY

In 1896 Beatson [1] published his findings of the beneficial effects of oophorectomy in premenopausal patients with advanced breast cancer. Beatson had based his approach on the role of the ovaries in mammalian lactation, and presumed that there would be a similar mechanism for breast cancer growth. The successful treatment of breast cancer by oophorectomy indicated that there was a principle in the ovary that travels around the body to control the growth of a cancer. The mechanism, however, was to remain unknown until 1923 when Allen and Doisy [2] showed that there is an ovarian hormone that they called oestrogen, which caused vaginal cornification and estrus in ovariectomized mice. Their method, employing ovariectomized mice, was subsequently used to test synthetic compounds for oestrogenic activity. Sir Charles Dodds [3] discovered the potent oestrogenic activity of the stilbenes and he was to discover and define the biological properties of diethylstilboestrol. The oestrogenic properties of triphenylethylene was also described around this time, but in contrast to diethylstilboestrol, triphenylethylene produces long term oestrogenic effects for many weeks [4].

Sir Alexander Haddow was particularly interested in the idea of chemical therapy so he initiated a program to explore the antitumour properties of polycyclic hydrocarbons in laboratory animals. Several compounds caused tumour regression but the fact that the chemicals were carcinogenic prohibited testing in humans. He reasoned that the triphenylethylene based oestrogens had a structural similarity to the polycyclic hydrocarbons and he found they also caused tumour regression in animals. He therefore chose to evaluate the application of oestrogens for the treatment of breast and prostate cancer. In 1944 Haddow [5] published the results of his clinical trial with the synthetic oestrogens triphenylchlorethylene, triphenylmethylethylene, and stilboestrol administered at high doses. He found that 10 out of 22 postmenopausal patients with advanced mammary carcinomas, who were treated with triphenylchlorethylene, had significant regression of tumour growth. Five patients out of 14 who were treated with high dose stilboestrol produced similar responses. Two patients with prostate cancer (out of 30 with diseases other than breast cancer) had a response. The finding that high doses of synthetic oestrogens induced regression of tumour growth in some, but not all patients with breast cancer was somewhat reminiscent of the apparent random responsiveness of oophorectomy in premenopausal patients with metastatic breast cancer [6]. However, Haddow [5] noted that the first successful use of a chemical therapy to treat cancer (breast and prostate) also had significant systemic side effects, such as nausea, areola pigmentation, uterine bleeding, and edema of the lower extremities.

By the 1960's, the standards for the endocrine treatment of breast cancer were established. Pre-menopausal women should be treated with ovarian irradiation therapy or bilateral oophorectomy. However, based on data from clinical trials, post-menopausal patients with advanced breast cancer should be treated with high dose of the most potent oestrogenic compound diethylstilboestrol [7]. Overall, one could anticipate that 36 % of patients would respond favorably to high dose oestrogen therapy [8].

The question that needed to be answered if endocrine therapy was to be further advanced was, who to treat? In other words, could one develop a test to predict responsiveness? Even as recently as 1970 Haddow [9] was not enthusiastic about the overall prospects of chemical therapy. He stated that there was unlikely to be a *chemotherapia specifica* in Ehrlich's sense because cancer cells are so similar to normal tissue. Also, unlike the antibiotics where one could pre-test responsiveness of the disease to a particular therapy, no such tests existed for cancer. Additionally, it was important that safer less toxic "oestrogens" were developed that might expand therapeutic use. There were clues that deciphering the mysteries of endocrine therapy could be of major benefit for patients. Haddow [10] noted that high dose oestrogen

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therapy was more successful as a treatment for breast cancer the farther the woman was from the menopause. Oestrogen-withdrawal somehow played a role in sensitizing tumours to the antitumour actions of oestrogen.

By the 1970's, interest in endocrine therapy waned with the advent of "successful" combinations of cytotoxic chemotherapies for the treatment of metastatic breast cancer. "Coopers Cocktail" of five different chemotherapeutic agents demonstrated dramatic response rates of up to 80% [11]. Cytotoxic chemotherapy was about to become the answer to cancer. But what happened to the triphenylethylene-based oestrogens? Was there another way to improve cancer therapy and to discover the cellular mechanisms of oestrogen action that control the life and death of breast cancer cells?

NON-STEROIDAL ANTIOESTROGENS: EVOLUTION TO TARGETED THERAPY

Leonard Lerner [12] reported the pharmacological properties of the first non-steroidal antioestrogen MER25 or ethamoxytriphetol. The compound was antioestrogenic in all species tested and exhibited no oestrogenic properties. However, the finding that MER25 was a postcoital contraceptive in laboratory animals [13] ignited an intense search of the structure activity relationships by medicinal chemists in the pharmaceutical industry. The goal was to find safer, more potent agents for clinical evaluation. The method chosen for drug discovery was Ehrlich's i.e.: study the structural organic chemistry using the clues provided by the lead compound MER25. The plan of the chemists was simple: place a strategically located alkylaminoethoxy side chain on numerous nonsteroidal oestrogens and then test them as postcoital contraceptives in rats and mice [14]. Although, the oestrogen receptor (ER) had been proposed as the conduit of oestrogen action in its target tissues [15], the actual ER protein was not isolated until 1966 [16]. As a result potential antioestrogens were not screened and identified using an ER assay but drug discovery followed Ehrlich's dictum of a laboratory model *thought* to represent human physiology. When the first clinically useful compound MRL41 or clomiphene was tested in women, it was not a contraceptive, but did the opposite; it induced ovulation. The basic reproductive endocrinology of the rat is completely different than that of a woman. Clomiphene is still used today as a profertility agent in subfertile women [17]. However, clinical trials of clomiphene in the early 1960's did move forward to evaluate activity in the treatment of breast cancer, but were terminated by the company because of concerns about the drug's potential to cause cataracts [17].

The story of the early development of tamoxifen (ICI 46,474) was similar to clomiphene's but the reason that clinical trials were terminated was not because of concerns about toxicity but rather the economic issue of insignificant projected profits that would accrue from marketing a drug for the induction of ovulation and a few thousand patients with metastatic breast cancer, treated for about a year [17].

Arthur Walpole was the leader of the antifertility program at Imperial Chemicals Industry (ICI) Pharmaceuticals (now Astra Zeneca), when ICI 46,474 the pure trans-isomer of a substituted triphenylethylene was discovered and described as a postcoital contraceptive in the rat [18] Earlier in his career [19], Walpole was interested in carcinogenesis and cancer chemotherapy. He also attempted to discover (unsuccessfully) why only some post-menopausal women with metastatic breast cancer respond to high dose oestrogen therapy [20]. It was Walpole who ensured that ICI 46,474 was tested in the clinic and placed on the market as an orphan drug while ICI invested in the scientific research by others in academia to conduct a systematic study of the anticancer actions of tamoxifen and its metabolites [21]. This investment reinvented tamoxifen as the first anticancer agent specifically targeted to the ER in the tumour and created the scientific principles to ultimately establish tamoxifen as the "gold standard" for the adjuvant

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therapy of breast cancer and as the first chemopreventative agent that reduces the incidence of breast cancer in women with elevated risk [22,23].

OESTROGEN RECEPTORS

The existence of the ER was predicted after Elwood Jensen [15] described the retention of subcutaneously administered tritiated 17β-oestradiol (E2) in target tissues such as uterus and vagina of the immature rats, but not in nontarget tissues such as kidney, liver, and muscle. The actual ER protein was extracted and identified from rat uterus in 1966 by Toft and Gorski [16]. Twenty years later in 1986 the ER gene was cloned [24], but afterwards was renamed as ERα because a second ER was cloned from a rat prostate cDNA based on the sequence similarity to ERα, and called ERβ [25]. Both ERs Fig. (1) are members of nuclear hormone receptor superfamily and bind oestrogens with high affinity and regulate transcription of oestrogen responsive genes [26].

The ERα gene is located on chromosome 6q25.1 [27] and encodes a 595 amino acid, 66 kDa protein composed of six domains [28] (Fig. (1). The first domain is called the amino-terminal A/B region and contains the ligand-independent and transcriptionally minor activating function-1 (AF-1). The second domain is called the C region that contains the DNA-binding domain (DBD), whose zinc fingers are responsible for ERα's binding to oestrogen response elements (EREs) found in the promoters of oestrogen responsive genes. The third domain is called the D region, which contains the nuclear localization signal. The fourth domain is called the E region and contains the ligand binding domain (LBD). The ligand-dependent and transcriptionally major activating function-2 (AF-2) is found in the LBD which mediates binding of the coactivators *via* nuclear receptor boxes composed of LXXLL-like motif [29]. The LBD is composed of 12 α helices, of which helix H3-H12 form a ligand-binding cavity with H12 acting as a "lid" for the cavity. The carboxy-terminal region of the receptor is called the F region. In the nucleus, unliganded monomeric ERα is bound with heat shock proteins (HSPs). Once in the nucleus, oestradiol binds to the LBD of the ERα-HSP complex, and leads to disassociation of HSPs. The LBD then undergoes a crucial conformational change in which H12 caps the ligand binding cavity, and the receptor homodimerizes with another ERα molecule and then binds with high affinity to oestrogen responsive elements (EREs) in the promoters, introns, or 3′ untranslated regions of target genes [30]. The ERE is composed of a 15 base pair palindromic inverted repeat consensus sequence: 5′-AGGTCAnnnTGACCT-3′ (n = any nucleotide). In addition to direct ERE binding, there is also evidence that ligand-bound ERs can interact with other transcription factor complexes like Fos/Jun (AP-1-responsive elements) [31,32] or SP-1 (GC-rich SP-1 motifs) [33] and influence transcription of genes whose promoters do not harbor EREs (i.e. "tethering mechanism). ERα and ERβ also interact with NF-κB to inhibit transcription [34]. Ligand-activated ERs mediate their genomic effects through interactions with coactivators (i.e. steroid receptor coactivator 1, 2, and 3) that recruit chromatin remodeling complexes, alter nucleosomal structure, enhance recruitment of general transcription factors, and increase recruitment of RNA polymerase II to transcribe target genes [35].

In addition to regulation by ligand binding and coregulator protein recruitment, ERs are regulated by phosphorylation in both a ligand-dependent [36] and ligand-independent manner [37]. Depending on which sites of ERα are phosphorylated, phosphorylation can either increase [38,39] or inhibit ERα-DNA binding [40] and alter nuclear localization [41], and thus alter gene transcription.

The ERβ gene is located on chromosome 14q23.2 and encodes a 530 amino-acid protein. As mentioned above ERα and ERβ share some sequence similarity, in particular they share the highest degree of amino acid sequence, 61 and 97% in LBD and DBD respectively, however

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A/B and D domains have only 27% and 26% amino acid homology respectively. ERβ is expressed in the testis, prostate, ovary, developing uterus, breast, vascular endothelium, smooth muscle, immune system, bone and some neurons. With lack of homology of A/B domain between ERα and ERβ, functional studies have indicated that ERβ lacks AF-1 activity [42].

However the real significance of ERβ in breast cancer remains unclear [43], and ERα is considered the molecular target for treating and preventing cancer [44], with the SERMs tamoxifen and raloxifene.

SELECTIVE OESTROGEN RECEPTOR MODULATION

Tamoxifen was originally referred to as a non-steroidal antioestrogen [18]. As more has become known about its molecular pharmacology it has become the pioneering Selective Oestrogen Receptor Modulator (SERM). Tamoxifen was first described as both a partial oestrogen agonist and antagonist in the rat uterus, and a full oestrogen in the mouse uterus and vagina [18]. These were the first important facts which helped to clarify the target-site-specific actions of SERMs. The concept of SERM action was defined by four main pieces of laboratory evidence: 1) ER-positive breast cancer cells inoculated into athymic mice grew into tumours in response to oestradiol, but not to tamoxifen (antioestrogenic action), however both oestradiol and tamoxifen induced uterine weight increase in mice (oestrogen action) [45]; 2) raloxifene (another nonsteroidal antioestrogen), which is less oestrogenic in rat uterus, maintained the bone density in ovariectomized rats (oestrogen action), as did tamoxifen [46], and prevented mammary carcinogenesis (antioestrogenic action) [47]; 3) tamoxifen blocked oestradiol-induced growth of ER-positive breast cancer cells in athymic mice (antioestrogenic action), but induced rapid growth of ER-positive endometrial carcinomas (oestrogenic action) [48]; 4) raloxifene was less effective in promoting endometrial cancer growth (less oestrogenic action) [49]. These laboratory results translated well into the clinic where it was shown that tamoxifen effectively can reduce the incidence of breast cancer in high-risk pre- and postmenopausal women, however increases the incidence of blood clots and endometrial cancer, which is linked to oestrogen-like actions of tamoxifen in these tissues in postmenopausal women, who have a low-oestrogen environment [23]. Furthermore, raloxifene maintains bone density in postmenopausal women and reduces fractures [50], but simultaneously reduces the incidence of breast cancer without increasing the incidence of endometrial cancer [51]. In the study of tamoxifen and raloxifene (STAR) both SERMs were equivalent at reducing the incidence of invasive breast cancer in high risk post-menopausal women, but raloxifene appeared to have a safer toxicity profile. There was, however, a nonstatistically significant higher risk of non-invasive breast cancer (DCIS) with raloxifene compared to tamoxifen [52]. The molecular reason for this difference is not known. With the recognition [53] and effective transition of SERMs to clinical practice, it is now important to understand their mechanism of action so new and novel applications can be developed [54].

MECHANISM OF SERM ACTION

The mechanism of SERM action is dependent upon a complex decision network in target tissues to program the cells to express oestrogenic or antioestrogenic actions. There are two ER's: ERα and ERβ and it is possible that a different ratio of both ER's could be important for chemoprevention with SERMs. A high ERα-ERβ ratio correlates with high cellular proliferation; in contrast the low ratio correlates with low cellular proliferation. In other words ERβ tends to suppress cell proliferation and may enhance apoptosis [55]. This is probably because ERα and ERβ have functional differences that can be traced back to differences in the AF1 domain, in particular that they share only 27% of homology in the amino-terminus of the protein. In contrast both ER's differ only by one amino acid in the C region (DNA binding region) and both ER's are able to regulate transcriptional activity of genes regulated by

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oestrogen response elements (EREs). In this regard ERβ does not have an active AF1 region which is the reason for its inhibitory properties within the cell.

Extensive structure-function relationship studies were initially used to develop a molecular model of oestrogen and antioestrogen action [56–58]. The hypothetical model required the envelopment of a planar oestrogen within the LBD of the ER complex. In contrast, the three-dimensional triphenylethylene binding in the LBD cavity prevents full ER's activation by keeping the LBD open. This structural perturbation of the ER complex is achieved by a correctly positioned bulky alkylaminoethoxy side chain on the SERM. This model was enhanced following studies to solve the X-ray crystallography of the LBD ER's bound with an oestrogen or an antioestrogen. The LBD of ERα is formed by H2-H11 helices and the hairpin β-sheet, while H12, in the agonist bound conformation closes over the LBD cavity filled with E2. The steroid is sealed within the hydrophobic pocket. Oestrogen is aligned in the cavity by hydrogen bonds at both ends of the ligand, particularly the 3-OH group at the A-ring end of E2 forms a hydrogen bond network with Glu353 and Arg394, while E2's 17β-OH group at the D-ring end of the ligand forms a hydrogen bond with ER's His524. This allows hydrophobic van der Waals contacts along the lipophilic rings of E2, in particular between Phe404 and E2's Aring, to promote a low energy conformation [59] (Fig. (2A). This results in sealing of the ligand-binding cavity by H12, and exposes the AF-2 surface for interaction with coactivators to promote transcriptional transactivation. In contrast, 4-hydroxytamoxifen binding to ER's LBD blocks the closure process by relocating H12 away from the binding pocket, thus preventing coactivator molecules from binding to the appropriate site on the external surface of the complex [60] (Fig. (2A). Therefore, it is the external shape of the ERs that is being modulated by the ligand which dictates the binding of coactivator molecules. In other words, the shape of the ligand actually causes the receptor to change shape and programs the ER complex to be able to bind coregulator molecules. However, the simple model of a coregulator controlling the biology of an ER complex is not that simple (Fig. (2B). The modulation of the oestrogen target gene is in fact, regulated by a dynamic process of assembly and destruction of transcription complexes at the promoter site of a target gene.

COREGULATORS AND OESTROGEN RECEPTOR ACTION

After ER is bound to an agonist ligand, its conformation changes allowing coregulator molecules to bind to the complex, for example, SRC-3. SRC-3 is a core coactivator that also attracts other coregulators that do not directly bind to ER, such as p300/CBP histone acetyltransferase, CARM1 methyltransferase, and ubiquitin ligases UbC and UbL. All of these coregulators perform specific subreactions within the protein complex of ER and DNA necessary for transcription of target genes, such as chromatin remodeling through methylation and acetylation modifications, and also direct their enzymatic activity towards adjacent factors, which promote dissociation of the coactivator complex and subsequent ubiquitinilation of select components for proteosomal degradation. As a result, this allows the next cycle of coactivator-receptor-DNA interactions to proceed and the binding and degradation of transcription complexes sustain gene transcription (Fig. (2B).

It is well established that ER is downregulated in the presence of E2 through ubiquitin proteasome pathway. Downregulation is crucial for ER's transcriptional activity. O'Malley [61] used ER positive MCF-7 cells to demonstrate that the 26S proteasome inhibitor MG132 ablated the transcriptional activity of ER, in luciferase activity assays as well as the endogenous transcription of oestrogen responsive genes, such as pS2 or progesterone receptor (PR) gene. Indeed this is a general principle as proteasome-mediated degradation is crucial for other nuclear receptors to function, such as PR and thyroid hormone receptor. The ubiquitin proteasome pathway is responsible for degradation and turnover of a number of transcriptional factors, such as NF-κB, and fos/jun. Through a number of enzymes (ubiquitin-activating

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[UBA], with which ubiquitin protein forms a high-energy thioester bond, and ubiquitin-conjugating/ubiquitin ligase enzymes), the ubiquitin protein covalently binds to proteins marked for degradation by the 26S proteasome, which subsequently degrades the targeted protein molecules. Lonard and coworkers [61] have demonstrated that blocking proteosomal degradation with MG132 attenuates the transcriptional activity through both the AF-1 and AF-2 domains, demonstrating that proteasome function is required for efficient transcription through either activation function. At the same time disruption of coactivator binding sites abrogates the ligand-mediated downregulation of the ER.

Armed with the knowledge that oestrogen agonists induce a conformation of the ER that stabilizes coactivator binding, it was logical to ask a question whether the binding of coactivators to ER cause a reciprocal stabilization of agonist ligand binding. This question was answered in studies with use of peptides with sequences derived from coactivator (SRC-1) binding sites on the ER (Nuclear-Receptor boxes), and tetrahydrochrysene-ketone (THC-ketone), DES and E2 as oestrogen antagonists [62]. Overall, coactivator peptides can stabilize the complex between the ER and agonist ligands (E2, DES, THC-ketone), with a marked reduction in ligand dissociation rate from the ligand-receptor complexes. Nevertheless, these coactivator peptides were much less effective in stabilizing ER-antagonist complexes, which was demonstrated in reporter-gene assays, where the elevation of SRC-1 levels increased the potency of E2, it decreased the potency of antioestrogens.

With this brief background of the molecular biology of oestrogenic and antioestrogenic modulation in target tissues we will survey the practical application of this knowledge for the treatment and prevention of breast cancer.

CLINICAL APPLICATIONS OF SERMS

The clinical application of the laboratory principle of targeting the ER with long-term antihormonal therapy [21] to treat breast cancer has become the standard of care. Two different approaches to adjuvant antihormonal therapy have been developed in the past 30 years: first, is the blockade of oestrogen-stimulated growth [44] at the tumour ERs, and the second one, is the use of aromatase inhibitors to block oestrogen biosynthesis in postmenopausal patients [63]. Aromatase inhibitors have an advantage in the therapy of post-menopausal patients over tamoxifen, firstly, because there are fewer side effects, such as blood clots or endometrial cancer, and aromatase inhibitors have a small, but still significant efficacy in increasing disease free survival [64]. However, most postmenopausal patients worldwide continue to undergo treatment with tamoxifen, either for economic reasons or because they were hysterectomized and also have a low risk of developing blood clots (low body mass index and are athletically active). In premenopausal women, long term tamoxifen is the antihormonal therapy of choice for the treatment of ductal carcinoma in situ (DCIS) [65], the treatment of ER-positive breast cancer [22] and the reduction of breast cancer incidence in those premenopausal women at elevated risk [23]. It is important to stress that premenopausal women treated with tamoxifen do not have elevations in endometrial cancer and blood clots, thus risk: benefit ratio is in favor of tamoxifen treatment [66].

The development of raloxifene from a laboratory concept [67] to an effective clinical strategy to prevent both osteoporosis and breast cancer [51], [52] has opened new opportunities for clinical applications of SERMs. However, the biggest advantage of raloxifene is that it does not increase the incidence of endometrial cancer [52], which was noted in post-menopausal women taking tamoxifen [23].

The current trend is to employ long-term treatment durations to treat disease with SERMs or aromatase inhibitors. Decades of raloxifene must be used to treat and prevent osteoporosis [68]. Additionally longer treatment trials for breast cancer with either aromatase inhibitors or

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tamoxifen are increasing the duration of therapy. Already aromatase inhibitors are used for a full 5 years after 5years of tamoxifen [69] and there is an ongoing trial ATLAS testing the effectiveness of long (10 years) against short (5 years) adjuvant tamoxifen treatment of breast cancer. The introduction of extended antihormonal therapy to treat and prevent breast cancer therefore has consequences with the development of antihormonal drug resistance.

Though, the clinical application of the SERM concept has proven itself to be successful, drug resistance remains an important issue arising from long-term SERM treatment. Studies have shown that after long-term SERM treatment, the pharmacology of the SERMs changes from an inhibitory antioestrogenic state to a stimulatory oestrogen-like response [70].

ANTIHORMONE DRUG RESISTANCE

Clinical and laboratory studies have identified three possible mechanism for the antihormone drug resistance to tamoxifen: the patient can influence the metabolism of tamoxifen, the ER-positive tumour can be intrinsically resistant or the ER positive tumour can initially respond and subsequently develop acquired tamoxifen resistance.

Activation of tamoxifen occurs when it is metabolized *via* demethylation to N-desmethyltamoxifen and subsequently gets transformed to the hydroxy metabolite endoxifen [71]. Endoxifen is formed by the CYP2D6 enzyme system [72], but there are genetic variants in the population that can influence drug metabolism. It is estimated that mutant alleles of the wild-type CYP2D6 enzyme variants are present in 10% of the population, thus meaning that these patients should be considered for an antioestrogenic therapy, other than tamoxifen i.e. aromatase inhibitors if they are postmenopausal. Side effects, that arise during treatment with tamoxifen, influence compliance and efficacy. An important side effect of tamoxifen is hot flashes and many patients become non compliant and stop therapy or use selective serotonin reuptake inhibitors (SSRIs) to reduce hot flashes. But it appears that hot flashes are good as tamoxifen must be metabolized into the potent antioestrogen endoxifen by the CYP2D6 enzyme. Unfortunately, SSRIs (fluoxetine and paroxetine) are also potent inhibitors of the CYP2D6 enzyme [73]. Therefore, symptom treatment can potentially undermine the efficacy of treatment with tamoxifen if the incorrect SSRI is employed. Venlafaxine is the recommended SSRI as there is a low affinity for the CYP2D6 enzyme system.

Forty percent of ER-positive metastatic breast cancers are intrinsically resistant to tamoxifen treatment. These tumours are identified as ER-positive and PR-negative tumours and only 40% [74] respond to antihormonal therapy. In contrast, ER/PR-positive tumours have an 80% response rate to endocrine therapy. In early studies it was noted that PR induction by oestrogen is impaired, through the epidermal growth factor receptor 1 (HER-1; EGFR) pathway [75], and that paracrine growth factor stimulation undermines the effectiveness of antioestrogen therapy [76]. These observations were expanded using breast cancer cells transfected with insulin-like growth factor receptor and by the examination of large tumour databases [77,78]. Insulin-like growth factor also reduces PR synthesis, so a general mechanism emerged that growth factor pathways impair ER signal transduction to initiate PR induction. Intrinsic tamoxifen resistance occurs in HER-2/neu-, PR negative, ER positive breast cancer cells that also have increased levels of SRC-3 coactivator [79]. Though, this patient category is only 10 to 15%, it provides a clue about who to test to avoid tamoxifen treatment. A retrospective analysis showed that patients with ER positive, PR-negative tumours would most likely respond better to aromatase inhibitor treatment than to tamoxifen [80], however, these data have subsequently not been confirmed [80,81].

Finally, and most intriguingly, long-term tamoxifen treatment can induce acquired resistance in breast cancers that are ER/PR-positive. Acquired resistance to tamoxifen is unique as the tumours are SERM stimulated for growth [82]. The first laboratory model [48,49,70] of

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transplantable tamoxifen resistant tumours demonstrated that 1) tamoxifen or oestrogen can cause tumours to grow, 2) tumours require a liganded receptor to grow, 3) an aromatase inhibitor (oestrogen deprivation) or a pure antioestrogen that causes ER destruction would be useful second line agents, 4) there was cross resistance with other SERMs [83].

However, it is the study of acquired antihormone resistance that has not only allowed the development of appropriate second line treatment strategies for patients (aromatase inhibitors or fulvestrant), but also has advanced our understanding of the apoptotic biology of high dose oestrogen as an effective therapy for breast cancer in the 1940's [5].

EVOLUTION OF ANTIHORMONE DRUG RESISTANCE

An obstacle to progress in therapeutics is a clear understanding of the changes that occur in the breast cancer cell, as a consequence of exhaustive antihormonal therapies. It is presumed that the cancer cell must create a sophisticated survival network and suppress the natural process of apoptosis to subvert the continuous inhibitory signal through the ER. Currently, numerous model systems exist to study antihormone resistance. Some are engineered to enhance the likelihood of resistance [79] and others are engineered by transfection of the aromatase gene to study resistance to aromatase inhibitors and compare them with tamoxifen [84]. In contrast, others have chosen to develop models naturally through selective pressure either *in vivo* or *in vitro*. The natural selection approach is either to continuously transplant the resulting SERM resistant breast cancer into SERM-treated athymic animals [85,86] or to employ strategies *in vitro* that use continuous SERM treatment [87–89] or long term oestrogen deprivation in culture [90,91].

In order to better understand the biological consequences of extended antioestrogen treatment on the survival of breast cancer, we have elucidated distinct phases of resistance with the use of unique models of tamoxifen-resistant breast cancer developed *in vivo* (Fig. 3. The model for the treatment phase of breast cancer was developed by injecting ERα-positive MCF-7 cells into athymic mice and supplementing them with post-menopausal doses of oestradiol (E2) (86–93 pg/ml) [92]. These MCF-7 tumours were oestradiol (E2)-stimulated and tamoxifen (TAM)-inhibited. Phase I TAM-resistant breast tumours developed with short term treatment (<2 years) with tamoxifen and were stimulated to grow by both E2 and tamoxifen [70,93]. The novel model of Phase II resistance to tamoxifen was observed when breast tumours were treated long-term with tamoxifen for more than 5 years (MCF-7TAMLT). These MCF-7TAMLT tumours were stimulated to grow with tamoxifen but paradoxically inhibited by E2 [85,94,95] (Fig. (3). Phase III resistance developed when all known therapies failed and only E2-inhibited growth [96]. However, during the progression from the treatment phase to Phase III resistance, a cyclic phenomenon was observed where initially E2-inhibited growth of Phase II TAM-resistant tumours followed by re-sensitization to E2 as a growth stimulant [94]. These E2 re-stimulated MCF-7 tumours from Phase II tamoxifen-resistant tumours were growth inhibited by no treatment, TAM, and fulvestrant demonstrating complete reversal of drug resistance to tamoxifen. In addition to tamoxifen-resistant tumours, oestradiol, at physiologic concentrations, has also been shown to induce apoptosis in long term oestrogen deprived (LTED) breast cancer cells *in vitro* and *in vivo*. It should be noted that in the past, pharmacologic oestrogen was a routinely employed therapy that resulted in durable responses with regression of disease [5]. Oestrogen therapy has yielded as high as 40% response rate as first-line treatment in patients with hormonally sensitive breast cancer with metastatic disease [97] and approximately 31% in patients heavily pre-treated with previous endocrine therapies [98] (Table 1A). What is still unclear, however, is the mechanism of oestradiol-induced apoptosis in breast cancer cells that are stimulated by tamoxifen or that grow spontaneously when deprived of oestradiol for a long time (> 1 year).

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MECHANISMS OF OESTROGEN INDUCED APOPTOSIS

Apoptosis is a form of programmed cell death that is executed by a family of proteases called caspases, which can be activated either by cell-surface death receptors (i.e., the extrinsic pathway) or by perturbation of the mitochondrial membrane (i.e., the intrinsic pathway) [99] (Fig. (4). Components of the extrinsic pathway include the death receptors FasR/FasL, DR4/DR5, and tumour necrosis factor (TNFR), whereas the intrinsic pathway centers on the mitochondria, which contain key apoptogenic factors such as cytochrome *c* and apoptosis inducing factor (AIF) [99] (Fig. (4). In the intrinsic pathway, the integrity of mitochondrial membranes is controlled primarily by a balance between the antagonistic actions of the pro-apoptotic and anti-apoptotic members of the Bcl-2 family. Bcl-2-family proteins comprise three principal subfamilies: (1) anti-apoptotic members, including Bcl-2/Bcl-x_L, which possess the Bcl-2 homology (BH) domains BH1, BH2, BH3, and BH4; (2) pro-apoptotic members, such as Bax, Bak, and Bok, which have the BH1, BH2, and BH3 domains; and (3) BH3-only proteins, such as Bid, Bim, Bad, Bik, and Puma, which generally possess only the BH3 domain [100]. The Bcl-2 family of proteins regulate apoptosis by altering mitochondrial membrane permeabilization and controlling the release of cytochrome *c*.

Mechanistic studies have used either SERM-stimulated models [88,95] or long-term oestrogen deprived MCF-7 breast cancer cell lines [90,91,101] to demonstrate a link between oestradiol-induced apoptosis and activation of the FasR/FasL death-signaling pathway. Osipo and coworkers [95,101] reported that physiologic levels of oestradiol induced regression of tamoxifen-stimulated breast cancer tumours by inducing the death receptor Fas and suppressing the antiapoptotic/prosurvival factors NF-κB and HER2/neu. A similar finding was reported by Liu and coworkers [88] for raloxifene (Ral)-resistant tumours. These investigators reported that the growth of Ral-resistant MCF-7/Ral cells *in vitro* and *in vivo* was repressed by oestradiol by a mechanism involving increased Fas expression and decreased NF-κB activity. Furthermore, Song and coworkers [90] showed that MCF-7 cells deprived of oestrogen for up to 24 months (MCF-7LTED) *in vitro* expressed high levels of Fas compared to the parental MCF-7 cells, which do not express Fas and treatment of the MCF-7/LTED cells with oestradiol resulted in a marked increase in Fas ligand (FasL) in these cells. Apart from the death receptor pathway, there is also evidence that the mitochondrial pathway is involved in oestradiol induced apoptosis. Oestradiol induced apoptosis occurs in a LTED breast cancer cell line named MCF-7:5C by activating the Bcl-2 family proteins (Fig. (4). In MCF-7:5C cells the expression of several pro-apoptotic proteins-including Bax, Bak, Bim, Noxa, Puma, and p53- are markedly increased with oestradiol treatment and blockade of Bax and Bim expression using siRNAs almost completely reversed the apoptotic effect of oestradiol. Oestradiol treatment also led to a loss of mitochondrial potential and a dramatic increase in the release of cytochrome c from the mitochondria, which resulted in activation of caspases and cleavage of PARP. Furthermore, overexpression of anti-apoptotic Bcl-x_L was able to protect MCF-7:5C cells from oestradiol-induced apoptosis. This particular study was one of the first to show a link between oestradiol-induced cell death and activation of the mitochondrial apoptotic pathway using a breast cancer cell model resistant to oestrogen withdrawal. It is worth noting that Song and coworkers [102] have also demonstrated the importance of Bcl-2 in mediating oestradiol-induced apoptosis in LTED cells. These investigators reported that basal bcl-2 level was markedly elevated in LTED cells and that knockdown of bcl-2 expression with siRNA dramatically sensitized these cells to the apoptotic action of oestradiol. At present, there is renewed interest in developing small molecule inhibitors of bcl-2 as anticancer cell and antiangiogenic agents [103] (Table 1B).

Apart from its action on the mitochondria, there is evidence that Bcl-2 also possesses antioxidant property. Bcl-2 overexpression increases cellular glutathione (GSH) level which is associated with increased resistance to chemotherapy-induced apoptosis [104]. GSH is a

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water-soluble tripeptide composed of glutamine, cysteine, and glycine. It is the most abundant intracellular small molecule thiol present in mammalian cells and it serves as a potent intracellular antioxidant protecting cells from toxins such free radicals [105,106]. Changes in GSH homeostasis have been implicated in the etiology and progression of a variety of human diseases, including breast cancer [107] and studies have shown that elevated levels of GSH prevent apoptotic cell death whereas depletion of GSH facilitates apoptosis [108]. Recently, our laboratory has found evidence which suggests that glutathione participates in retarding apoptosis in antihormone-resistant MCF-7:2A human breast cancer cells and that depletion of this molecule by L-buthionine sulfoximine (BSO), a potent inhibitor of glutathione biosynthesis, sensitizes these resistant cells to oestradiol-induced apoptosis [109]. GSH levels were elevated ~60% in antihormone-resistant MCF-7:2A cells compared to wild-type MCF-7 cells and unlike MCF-7:5C cells, the MCF-7:2A cells failed to undergo apoptosis following 1 week of treatment with physiological concentrations of oestradiol. In the presence of BSO (100 μM), however, 1 nM oestradiol caused a dramatic increase in apoptosis which was observed as early as 48 hours with maximum induction observed at day 7. It is worth noting that the concentration of BSO (100 μM) used in this study is clinically achievable [110]. Furthermore, early phase clinical trials of BSO at doses resulting in both peripheral and tumour GSH depletion show that BSO can be safely administered to patients with refractory disease [111]. Thus it is possible that future clinical studies of BSO infusions combined with low dose oestrogen hold the promise of improving disease control for patients with antihormone resistant ER positive metastatic breast cancer.

NONGENOMIC OESTROGEN ACTION

There is also evidence that E2 has “nongenomic or membrane-initiated” effects, that is, independent of ER-mediated transcription, that occur within minutes after E2 administration in a G-protein-coupled manner [112,113]. Since ERα lacks a transmembrane domain, how it gets to the plasma membrane is somewhat controversial but it appears to require palmitoylation [114]. ERα interacts with a number of proteins, including c-Src, the p85 subunit of PI3K, caveolin-1, and modulator of nongenomic activity of ER (MNAR) [115]. ERα has also been shown to interact with the epidermal growth factor receptor (EGFR), IGFR1, and HER2 in the plasma membrane of breast cancer cells [116]. In MCF-7 human breast cancer cells, E2 rapidly increased PIP2-phospholipase C activity [117], mobilized intracellular Ca^{2+}, and activated the MAPK [118] and PI3K/Akt pathways [119]. Since E2 activation of the “nongenomic” pathway occurs within minutes, it is unlikely that this pathway plays a major role in E2-induced apoptosis in our SERM-resistant or LTED-breast cancer cells which undergo apoptosis after hours of treatment.

CLINICAL EXPLOITATION OF OESTROGEN-INDUCED APOPTOSIS

Laboratory studies uniformly demonstrate that low concentrations of oestrogen can cause apoptotic tumour cell death following profound oestrogen deprivation with antihormones. This can be viewed as an enhanced vulnerability to oestrogen when Phase II antihormone resistance is developed consistent with the earlier use of high dose oestrogen to treat breast cancer in women 2–3 decades after menopause [5]. The question that needs now to be answered is how can this new laboratory knowledge be translated into patient care?

Several clinical trial groups are currently addressing this issue. In a recent study presented at the 31st annual San Antonio Breast Cancer Symposium, Ellis and coworkers [120] reported that a daily dose of 6 mg estradiol could stop the growth of tumors or even cause them to shrink in about 25% of women with metastatic breast cancer that had developed resistance to standard antihormonal therapy (Table 1A). In our own case, we are recruiting patients with metastatic breast cancer who have succeeded and experienced treatment failure with at least two

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successive endocrine therapies and we are determining the efficacy of a 12-week purge of high-dose oestradiol (30 mg daily) therapy (Fig. (5). The goal is to confirm and extend the previous study published by Lonning and colleagues [98] and then to determine the minimum dose of oestradiol necessary to induce the anticipated 30% response rate [98]. Based on our previous laboratory studies [94] we propose to re-treat responding patients with the aromatase inhibitor anastrozole to determine efficacy. Overall, our clinical program is part of a multi-institutional Center of Excellence grant BCO50277 entitled “A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Oestrogen-Induced Apoptosis” that will map the survival and death pathways of our models and integrate clinical material to determine the validity of the laboratory-derived molecular mechanisms and, ultimately, to address the issue of why the majority of tumours do not respond to oestrogen alone. We reason that knowledge of the new apoptotic biology of oestrogen could be enhanced in the future in much the same way as the modest responses of tamoxifen and raloxifene were enhanced to benefit patients. The philosophy is to deploy the right treatment at the right time, for the right patient.

PERSPECTIVES

Our proposed model clinical trial now provides opportunities to test compounds in associations with oestrogen as an apoptotic trigger. Pre-clinical data from our laboratory [109] clearly show that it is possible to enhance the apoptotic effect of low dose oestradiol by combining it with BSO. We propose that the combination of BSO and oestradiol could be used to improve the efficacy of oestradiol as an apoptotic agent if glutathione depletion is fundamental to tumour cell survival. Phase I clinical trials of BSO at doses resulting in both peripheral and tumour GSH depletion show that BSO can be safely administered to patients with refractory disease. BSO was administered intravenously twice daily either alone or together with chemotherapy to cancer patients whose disease had progressed despite multiple lines of previous chemotherapy [111,121].

We propose that inhibitors of survival pathways will enhance the apoptotic/growth inhibitory effects of oestrogen. Bcl-2 (B-cell lymphoma/leukemia-2) is a low molecular weight protein that is localized to the mitochondria and endoplasmic reticulum that acts as a key inhibitor of apoptosis. Expression of Bcl-2 is essential for growth of certain tumour cell lines *in vitro* and has been found to be upregulated in a variety of tumour types *in vivo* [122,123]. It is widely believed that some cancers evade apoptosis and obtain a survival advantage through aberrant expression of Bcl-2. To date, several independent groups have developed small-molecule inhibitors of Bcl-2 as antitumour agents [103]. These inhibitors encompass various drugs that bind the anti-apoptotic Bcl-2 family members with more or less efficacy. Oblimersen (Genasense; G3139; Genta Inc, Berkeley Heights, NJ) is an anti-Bcl-2 antisense oligonucleotide which has reached phase III clinical trials in combination therapy [124]. Peptide-based drugs have also been shown to attenuate Bcl-2 activity [125] and to activate Bax [126]. There are also natural inhibitors of Bcl-2 which include tea polyphenols such as catechins and theaflavins [127,128] and the natural polyphenol derivative gossypol [129]. Inspired by the potential of natural Bcl-2 inhibitors, several research groups have developed specific inhibitors of Bcl-2. HA14-1 was the first Bcl-2 binding ligand to be discovered using computer-based screening strategies using the predicted structure of Bcl-2 [130]. Other small-molecule inhibitors of Bcl-2 include TW-37 [130,131] and ABT-737 [132], both of which have better efficacy than HA14-1 [133] (Table 1B). Overall, the small-molecule inhibitors of Bcl-2, although they are not magic bullets, have great therapeutic potential and are proving to be an important investigative tool for understanding the function of Bcl-2.

There is strong clinical evidence that trastuzumab, a monoclonal antibody targeting the human epidermal growth factor receptor (HER) two tyrosine kinase receptor, is an important component of first-line treatment of patients with HER-2 positive metastatic breast cancer

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[134–136] (Table 1C). In particular, the combination with taxanes and vinorelbine has been established [137]. In the preoperative setting inclusion of trastuzumab has significantly increased the pathological complete response rate. Results from large phase III trials evaluating adjuvant therapy in HER-2 positive early breast cancer indicate that the addition of trastuzumab to chemotherapy improves disease-free and overall survival [138]. Based on our preclinical studies [88,95], we have found that HER-2 is an important target of oestradiol-induced apoptosis, hence, the possibility exist that the combination of oestradiol therapy with that of trastuzumab might have beneficial effects. In addition to trastuzumab, there is also pertuzumab (2C4, Omnitarg®) (Genentech Inc. San Francisco, CA, USA), a monoclonal antibody directed against HER-2 that sterically blocks dimerization of HER-2 with HER-1 and HER-3 [139, 140]. It is currently under early clinical evaluation, phase I data have shown that the drug is well tolerated and clinically active [141]. Ertumaxomab (Rexomun®) (Fresenius Biotech GmbH, Munich, Germany) is a novel trifunctional, bispecific antibody that targets HER-2 and CD3. A phase I study among 17 patients with HER-2 positive metastatic breast cancer has demonstrated strong immunologic responses with this antibody [142]. In addition, recent studies have reported that ertumaxomab induces cellular cytotoxicity against various tumour cell lines including cells with low expression of HER-2 [143]. Thus, this antibody may provide a new therapeutic option for breast cancer patients with low expression of HER-2.

Apart from monoclonal antibodies, the use of tyrosine kinase inhibitors to target HER-2 has also shown great promise. Lapatinib (Tyverb®, GW572016) (GlaxoSmithKline, Middlesex, UK) is a dual tyrosine kinase inhibitor of both HER-1 and HER-2, and of Akt and mitogen activated protein kinase (MAPK). Preclinical studies have demonstrated that this compound inhibits growth and induces apoptosis in breast cancer cell lines [144]. Results from phase I/II trials suggest that the compound has activity against several tumour types, in particularly breast cancer [144–147] (Table 1C). In addition, xenograph studies have shown that lapatinib may be able to restore tamoxifen sensitivity [148]. The compound has also been evaluated in combination with aromatase inhibitors in preclinical and clinical studies [149,150]. However, increased ER signaling has been demonstrated in biopsies from HER-2 positive breast tumours treated with lapatinib. This finding might indicate that ER signaling could be involved in lapatinib-resistance [151]. A phase I study of lapatinib in combination with the aromatase inhibitor letrozole in patients with solid tumours showed a positive response [149]. A phase III study comparing letrozole with letrozole plus lapatinib in patients with ER/PR-positive metastatic breast cancer has recently completed enrolment [152]. Data have not yet been published.

In recent years, compelling evidence suggests that increased growth factor signaling, in particular the EGFR/HER2 pathway, contributes to tamoxifen resistance [153]. Thus it is possible that exogenous inhibitors of the HER-signaling network and other mitogenic pathways can abrogate or improve the response rate of breast cancer with acquired phase II resistance during oestrogen therapy.

CONCLUSION

The discovery of a new biology of oestradiol-induced apoptosis provides a unique signal transduction pathway to exploit in the treatment of metastatic breast cancer that has become refractory to exhaustive antihormone therapy. The clinical clues with the use of high-dose oestrogen therapy [5,98,154] have now been supported by a wealth of laboratory data defining apoptotic mechanisms. It is plausible to consider that the methodical evaluation of monoclonal antibodies and small molecule tyrosine kinase inhibitors to prevent breast cancer survival could amplify the apoptotic actions of oestradiol in a select group of patients. Indeed, if a study of the molecular biology of oestrogen-induced apoptosis can precisely define the mechanism then the molecules involved will become the target for a new drug group. These new drugs may be

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able to precipitate apoptosis in ER-negative breast tumours or indeed be used universally to treat cancers other than breast cancer. All will depend on tissue selectivity.

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References

1. Beatson GT. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. Lancet 1886;2:162–165.
2. Allen E, Doisy EA. An ovarian hormone: Preliminary reports on its localization extraction and partial purification and action in test animals. JAMA 1923;81:810–821.
3. Dodds EC, Goldberg L, Lawson W, Robinson R. Estrogenic activity of certain synthetic compounds. Nature 1938;141:247–248.
4. Robson JM, Schonberg A. Estrous reactions, including mating, produced by triphenyl ethylene. Nature 1937;140:196.
5. Haddow A, Watkinson JM, Paterson E. Influence of synthetic oestrogens upon advanced malignant disease. Br Med J 1944:393–398.
6. Boyd S. On Oophorectomy in cancer of the breast. Br Med J 1900;2:134–141.
7. Kennedy BJ. Systemic effects of androgenic and estrogenic hormones in advanced breast cancer. J Am Geriatr Soc 1965;13:230–235. [PubMed: 14270629]
8. Kennedy BJ. Hormone therapy for advanced breast cancer. Cancer 1965;18:1551–1557. [PubMed: 5845796]
9. David A. Karnofsky memorial lecture. Thoughts on chemical therapy. Cancer 1970;26:737–754. [PubMed: 4918638]
10. Haddow A. The chemotherapy of cancer. Br Med J 1950;2:1271–1272. [PubMed: 14792004]
11. Cooper RG. Combination chemotherapy in hormone resistant breast cancer. Proc Am Assoc Cancer Res 1969;10:15.
12. Lerner LJ, Holthaus FJ Jr, Thompson CR. A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxy-phenylethanol. Endocrinology 1958;63:295–318. [PubMed: 13574085]
13. Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 1990;50:4177–4189. [PubMed: 2194650]
14. Jordan VC. Biochemical pharmacology of antiestrogen action. Pharmacol Rev 1984;36:245–276. [PubMed: 6395141]
15. Jensen EV, Jacobson HI. Basic guidelines to the mechanism of estrogen action. Recent Progr Horm Res 1962;18:387–414.
16. Toft D, Gorski J. A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc Natl Acad Sci USA 1966;55:1574–1581. [PubMed: 5227676]
17. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2003;2:205–213. [PubMed: 12612646]
18. Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 1967;13:101–119. [PubMed: 6066766]
19. Jordan VC. The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole Breast Cancer Res Treat 1988;11:197–209.
20. Walpole AL, Patterson E. Synthetic oestrogens in mammary cancer. Lancet 1948;2:783–786. [PubMed: 15391512]
21. Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 2008;44:30–38. [PubMed: 18068350]

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22. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687–1717. [PubMed: 15894097]
23. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998;90:1371–1388. [PubMed: 9747868]
24. Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J. Sequence and expression of human estrogen receptor complementary DNA. Science 1986;231:1150–1154. [PubMed: 3753802]
25. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 1996;93:5925–5930. [PubMed: 8650195]
26. Evans RM. The steroid and thyroid hormone receptor superfamily. Science 1988;240:889–895. [PubMed: 3283939]
27. Menasce LP, White GR, Harrison CJ, Boyle JM. Localization of the estrogen receptor locus (ESR) to chromosome 6q25.1 by FISH and a simple post-FISH banding technique. Genomics 1993;17:263–265. [PubMed: 8406468]
28. Kumar V, Green S, Stack G, Berry M, Jin JR, Chambon P. Functional domains of the human estrogen receptor. Cell 1987;51:941–951. [PubMed: 3690665]
29. Lees JA, Fawell SE, Parker MG. Identification of two transactivation domains in the mouse oestrogen receptor. Nucleic Acids Res 1989;17:5477–5488. [PubMed: 2762146]
30. Sanchez R, Nguyen D, Rocha W, White JH, Mader S. Diversity in the mechanisms of gene regulation by estrogen receptors. Bioessays 2002;24:244–254. [PubMed: 11891761]
31. Klinge CM. Estrogen receptor interaction with co-activators and co-repressors. Steroids 2000;65:227–251. [PubMed: 10751636]
32. Kushner PJ, Agard DA, Greene GL, et al. Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 2000;74:311–317. [PubMed: 11162939]
33. Saville B, Wormke M, Wang F, et al. Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. J Biol Chem 2000;275:5379–5387. [PubMed: 10681512]
34. Kalaitzidis D, Gilmore TD. Transcription factor cross-talk: the estrogen receptor and NF-kappaB. Trends Endocrinol Metab 2005;16:46–52. [PubMed: 15734144]
35. McKenna NJ, O'Malley BW. Minireview: nuclear receptor coactivators--an update. Endocrinology 2002;143:2461–2465. [PubMed: 12072374]
36. Lannigan DA. Estrogen receptor phosphorylation. Steroids 2003;68:1–9. [PubMed: 12475718]
37. Feng W, Webb P, Nguyen P, et al. Potentiation of estrogen receptor activation function 1 (AF-1) by Src/JNK through a serine 118-independent pathway. Mol Endocrinol 2001;15:32–45. [PubMed: 11145737]
38. Denton RR, Koszewski NJ, Notides AC. Estrogen receptor phosphorylation. Hormonal dependence and consequence on specific DNA binding. J Biol Chem 1992;267:7263–7268. [PubMed: 1559970]
39. Tzeng DZ, Klinge CM. Phosphorylation of purified estradiol-liganded estrogen receptor by casein kinase II increases estrogen response element binding but does not alter ligand stability. Biochem Biophys Res Commun 1996;223:554–560. [PubMed: 8687434]
40. Al-Dhaheri MH, Rowan BG. Protein kinase A exhibits selective modulation of estradiol-dependent transcription in breast cancer cells that is associated with decreased ligand binding, altered estrogen receptor alpha promoter interaction, and changes in receptor phosphorylation. Mol Endocrinol 2007;21:439–456. [PubMed: 17068199]
41. Picard N, Charbonneau C, Sanchez M, et al. Phosphorylation of activation function-1 regulates proteasome-dependent nuclear mobility and E6-associated protein ubiquitin ligase recruitment to the estrogen receptor beta. Mol Endocrinol 2008;22:317–330. [PubMed: 17962381]
42. Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ER-beta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 1999;140:5566–5578. [PubMed: 10579320]
43. Saji S, Hirose M, Toi M. Clinical significance of estrogen receptor beta in breast cancer. Cancer Chemother Pharmacol 2005;56 (Suppl 1):21–26. [PubMed: 16273360]

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44. Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. Clin Cancer Res 2003;9:1980–1989. [PubMed: 12796359]
45. Jordan VC, Robinson SP. Species-specific pharmacology of anti-estrogens: role of metabolism. Fed Proc 1987;46:1870–1874. [PubMed: 3556610]
46. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987;10:31–35. [PubMed: 3689979]
47. Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987;47:4020–4024. [PubMed: 3607747]
48. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 1988;48:812–815. [PubMed: 3338079]
49. Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 1990;50:3189–3192. [PubMed: 2334915]
50. Ettinger B, Black DM, Mitlak BH, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 1999;282:637–645. [PubMed: 10517716]
51. Cummings SR, Eckert S, Krueger KA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA 1999;281:2189–2197. [PubMed: 10376571]
52. Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen *vs* raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 2006;295:2727–2741. [PubMed: 16754727]
53. Jordan VC. Selective estrogen receptor modulation: a personal perspective. Cancer Res 2001;61:5683–5687. [PubMed: 11479197]
54. Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 2007;25:5815–5824. [PubMed: 17893378]
55. Paruthiyil S, Parmar H, Kerekatte V, Cunha GR, Firestone GL, Leitman DC. Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. Cancer Res 2004;64:423–428. [PubMed: 14729654]
56. Lieberman ME, Gorski J, Jordan VC. An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens *in vitro*. J Biol Chem 1983;258:4741–4745. [PubMed: 6833272]
57. Jordan VC, Lieberman ME, Cormier E, Koch R, Bagley JR, Ruenitz PC. Structural requirements for the pharmacological activity of nonsteroidal antiestrogens *in vitro*. Mol Pharmacol 1984;26:272–278. [PubMed: 6482874]
58. Jordan VC, Koch R, Mittal S, Schneider MR. Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis *in vitro*. Br J Pharmacol 1986;87:217–223. [PubMed: 3955300]
59. Brzozowski AM, Pike AC, Dauter Z, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997;389:753–758. [PubMed: 9338790]
60. Shiau AK, Barstad D, Loria PM, et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;95:927–937. [PubMed: 9875847]
61. Lonard DM, Nawaz Z, Smith CL, O'Malley BW. The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. Mol Cell 2000;5:939–948. [PubMed: 10911988]
62. Gee AC, Carlson KE, Martini PG, Katzenellenbogen BS, Katzenellenbogen JA. Coactivator peptides have a differential stabilizing effect on the binding of estrogens and antiestrogens with the estrogen receptor. Mol Endocrinol 1999;13:1912–1923. [PubMed: 10551784]
63. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007;72:7–25. [PubMed: 17169390]
64. Howell A, Cuzick J, Baum M, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 2005;365:60–62. [PubMed: 15639680]

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65. Fisher B, Dignam J, Wolmark N, et al. Tamoxifen in treatment of intraductal breast cancer: National Surgical Adjuvant Breast and Bowel Project B-24 randomised controlled trial. Lancet 1999;353:1993–2000. [PubMed: 10376613]
66. Gail MH, Costantino JP, Bryant J, et al. Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. J Natl Cancer Inst 1999;91:1829–1846. [PubMed: 10547390]
67. Jordan VC. SERMs: meeting the promise of multifunctional medicines. J Natl Cancer Inst 2007;99:350–356. [PubMed: 17341726]
68. Martino S, Cauley JA, Barrett-Connor E, et al. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 2004;96:1751–1761. [PubMed: 15572757]
69. Goss PE, Ingle JN, Martino S, et al. Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA. 17. J Natl Cancer Inst 2005;97:1262–1271. [PubMed: 16145047]
70. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term anti-estrogen administration. Cancer Res 1988;48:5183–5187. [PubMed: 3409244]
71. Stearns V, Johnson MD, Rae JM, et al. Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. J Natl Cancer Inst 2003;95:1758–1764. [PubMed: 14652237]
72. Desta Z, Ward BA, Soukhova NV, Flockhart DA. Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system *in vitro*: prominent roles for CYP3A and CYP2D6. J Pharmacol Exp Ther 2004;310:1062–1075. [PubMed: 15159443]
73. Borges S, Desta Z, Li L, et al. Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. Clin Pharmacol Ther 2006;80:61–74. [PubMed: 16815318]
74. Bloom ND, Tobin EH, Schreibman B, Degenshein GA. The role of progesterone receptors in the management of advanced breast cancer. Cancer 1980;45:2992–2997. [PubMed: 7388743]
75. Cormier EM, Wolf MF, Jordan VC. Decrease in estradiol-stimulated progesterone receptor production in MCF-7 cells by epidermal growth factor and possible clinical implication for paracrine-regulated breast cancer growth. Cancer Res 1989;49:576–580. [PubMed: 2783381]
76. Cormier EM, Jordan VC. Contrasting ability of antiestrogens to inhibit MCF-7 growth stimulated by estradiol or epidermal growth factor. Eur J Cancer Clin Oncol 1989;25:57–63. [PubMed: 2784101]
77. Cui X, Zhang P, Deng W, et al. Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells *via* the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: progesterone receptor as a potential indicator of growth factor activity in breast cancer. Mol Endocrinol 2003;17:575–588. [PubMed: 12554765]
78. Arpino G, Weiss H, Lee AV, et al. Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance. J Natl Cancer Inst 2005;97:1254–1261. [PubMed: 16145046]
79. Osborne CK, Bardou V, Hopp TA, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst 2003;95:353–361. [PubMed: 12618500]
80. Dowsett M, Cuzick J, Wale C, Howell T, Houghton J, Baum M. Retrospective analysis of time to recurrence in the ATAC trial according to hormone receptor status: An hypothesis-generating study. J Clin Oncol 2005;23:7512–7517. [PubMed: 16234518]
81. Jordan VC, Gelber RD. Problems with the progesterone receptor in practice? J Clin Oncol 2007;25:1957–1959. [PubMed: 17452674]
82. Howell A, Dodwell DJ, Anderson H, Redford J. Response after withdrawal of tamoxifen and progestogens in advanced breast cancer. Ann Oncol 1992;3:611–617. [PubMed: 1450042]
83. O'Regan RM, Gajdos C, Dardes RC, et al. Effects of raloxifene after tamoxifen on breast and endometrial tumor growth in athymic mice. J Natl Cancer Inst 2002;94:274–283. [PubMed: 11854389]
84. Brodie AH, Jelovac D, Long B. The intratumoral aromatase model: studies with aromatase inhibitors and antiestrogens. J Steroid Biochem Mol Biol 2003;86:283–288. [PubMed: 14623522]

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85. Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 1993;127:23–33. [PubMed: 8502820]
86. Lee ES, Schafer JM, Yao K, et al. Cross-resistance of triphenylethylene-type antiestrogens but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000;6:4893–4899. [PubMed: 11156249]
87. Herman ME, Katzenellenbogen BS. Response-specific antiestrogen resistance in a newly characterized MCF-7 human breast cancer cell line resulting from long-term exposure to trans-hydroxytamoxifen. J Steroid Biochem Mol Biol 1996;59:121–134. [PubMed: 9010327]
88. Liu H, Lee ES, Gajdos C, et al. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*. J Natl Cancer Inst 2003;95:1586–1597. [PubMed: 14600091]
89. Park WC, Liu H, Macgregor Schafer J, Jordan VC. Deregulation of estrogen induced telomerase activity in tamoxifen-resistant breast cancer cells. Int J Oncol 2005;27:1459–1466. [PubMed: 16211243]
90. Song RX, Mor G, Naftolin F, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 2001;93:1714–1723. [PubMed: 11717332]
91. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 2005;94:131–141. [PubMed: 15862958]
92. Robinson SP, Jordan VC. Antiestrogenic action of toremifene on hormone-dependent, -independent, and heterogeneous breast tumor growth in the athymic mouse. Cancer Res 1989;49:1758–1762. [PubMed: 2522347]
93. Osborne CK, Coronado E, Allred DC, Wiebe V, DeGregorio M. Acquired tamoxifen resistance: correlation with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxytamoxifen. J Natl Cancer Inst 1991;83:1477–1482. [PubMed: 1920493]
94. Yao K, Lee ES, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000;6:2028–2036. [PubMed: 10815929]
95. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 2003;95:1597–1608. [PubMed: 14600092]
96. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. Cancer Cell 2004;5:207–213. [PubMed: 15050912]
97. Ingle JN, Ahmann DL, Green SJ, et al. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. N Engl J Med 1981;304:16–21. [PubMed: 7001242]
98. Lonning PE, Taylor PD, Anker G, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat 2001;67:111–116. [PubMed: 11519859]
99. Peter ME, Krammer PH. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ 2003;10:26–35. [PubMed: 12655293]
100. Tsujimoto Y. Bcl-2 family of proteins: life-or-death switch in mitochondria. Biosci Rep 2002;22:47–58. [PubMed: 12418550]
101. Lewis JS, Meeke K, Osipo C, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 2005;97:1746–1759. [PubMed: 16333030]
102. Song RX, Zhang Z, Mor G, Santen RJ. Down-regulation of Bcl-2 enhances estrogen apoptotic action in long-term estradiol-depleted ER(+) breast cancer cells. Apoptosis 2005;10:667–678. [PubMed: 15909128]
103. Zeitlin BD, Zeitlin IJ, Nor JE. Expanding circle of inhibition: small-molecule inhibitors of Bcl-2 as anticancer cell and antiangiogenic agents. J Clin Oncol 2008;26:4180–4188. [PubMed: 18757333]
104. Voehringer DW. BCL-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity. Free Radic Biol Med 1999;27:945–950. [PubMed: 10569627]

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105. Schröder CP, Godwin AK, O'Dwyer PJ, Tew KD, Hamilton TC, Ozols RF. Glutathione and drug resistance. Cancer Invest 1996;14:158–168. [PubMed: 8597901]

106. Anderson ME. Glutathione: an overview of biosynthesis and modulation. Chem Biol Interact 1998;111–112:1–14.

107. Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. Biomed Pharmacother 2003;57:145–155. [PubMed: 12818476]

108. Anderson CP, Tsai JM, Meek WE, et al. Depletion of glutathione by buthionine sulfoxine is cytotoxic for human neuroblastoma cell lines *via* apoptosis. Exp Cell Res 1999;246:183–192. [PubMed: 9882527]

109. Lewis-Wambi JS, Kim H, Wambi C, et al. Buthionine Sulfoximide Sensitizes Antihormone-Resistant Human Breast Cancer Cells to Estrogen-Induced Apoptosis. Breast Cancer Research. 2008In Press

110. Bailey HH, Ripple G, Tutsch KD, et al. Phase I study of continuous-infusion L-S, R-buthionine sulfoximine with intravenous melphalan. J Natl Cancer Inst 1997;89:1789–1796. [PubMed: 9392620]

111. Bailey HH, Mulcahy RT, Tutsch KD, et al. Phase I clinical trial of intravenous L-buthionine sulfoximine and melphalan: an attempt at modulation of glutathione. J Clin Oncol 1994;12:194–205. [PubMed: 8270977]

112. Pedram A, Razandi M, Levin ER. Nature of functional estrogen receptors at the plasma membrane. Mol Endocrinol 2006;20:1996–2009. [PubMed: 16645038]

113. Arpino G, Wiechmann L, Osborne CK, Schiff R. Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. Endocr Rev 2008;29:217–233. [PubMed: 18216219]

114. Moriarty K, Kim KH, Bender JR. Minireview: estrogen receptor-mediated rapid signaling. Endocrinology 2006;147:5557–5563. [PubMed: 16946015]

115. Li L, Hisamoto K, Kim KH, et al. Variant estrogen receptor-c-Src molecular interdependence and c-Src structural requirements for endothelial NO synthase activation. Proc Natl Acad Sci USA 2007;104:16468–16473. [PubMed: 17921256]

116. Pietras RJ, Marquez-Garban DC. Membrane-associated estrogen receptor signaling pathways in human cancers. Clin Cancer Res 2007;13:4672–4676. [PubMed: 17699844]

117. Graber R, Sumida C, Vallette G, Nunez EA. Rapid and long-term effects of 17 beta-estradiol on PIP2-phospholipase C-specific activity of MCF-7 cells. Cell Signal 1993;5:181–186. [PubMed: 8388702]

118. Improta-Brears T, Whorton AR, Codazzi F, York JD, Meyer T, McDonnell DP. Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. Proc Natl Acad Sci USA 1999;96:4686–4691. [PubMed: 10200323]

119. Stoica GE, Franke TF, Moroni M, et al. Effect of estradiol on estrogen receptor-alpha gene expression and activity can be modulated by the ErbB2/PI 3-K/Akt pathway. Oncogene 2003;22:7998–8011. [PubMed: 12970748]

120. Ellis MJ, Dehdahti F, Kommareddy A, et al. A randomized phase 2 trial of low dose (6 mg daily) versus high dose (30 mg daily) estradiol for patients with estrogen receptor positive aromatase inhibitor resistant advanced breast cancer. Cancer Res 2009;69 (Suppl):67s–68s.

121. O'Dwyer PJ, Hamilton TC, LaCreta FP, et al. Phase I trial of buthionine sulfoximine in combination with melphalan in patients with cancer. J Clin Oncol 1996;14:249–256. [PubMed: 8558205]

122. Khor LY, Moughan J, Al-Saleem T, et al. Bcl-2 and Bax expression predict prostate cancer outcome in men treated with androgen deprivation and radiotherapy on radiation therapy oncology group protocol 92-02. Clin Cancer Res 2007;13:3585–3590. [PubMed: 17575222]

123. Lee KH, Im SA, Oh DY, et al. Prognostic significance of bcl-2 expression in stage III breast cancer patients who had received doxorubicin and cyclophosphamide followed by paclitaxel as adjuvant chemotherapy. BioMed Central Cancer 2007;7:63. [PubMed: 17430582]

124. Stein CA, Benimetskaya L, Mani S. Antisense strategies for oncogene inactivation. Semin Oncol 2005;32:563–572. [PubMed: 16338422]

125. Walensky LD, Kung AL, Escher I, et al. Activation of apoptosis *in vivo* by a hydrocarbon-stapled BH3 helix. Science 2004;305:1466–1470. [PubMed: 15353804]

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126. Oh KJ, Barbuto S, Pitter K, Morash J, Walensky LD, Korsmeyer SJ. A membrane-targeted BID BCL-2 homology 3 peptide is sufficient for high potency activation of BAX *in vitro*. J Biol Chem 2006;281:36999–37008. [PubMed: 16987815]

127. Leone M, Zhai D, Sareth S, et al. Cancer prevention by tea polyphenols is linked to their direct inhibition of antiapoptotic Bcl-2-family proteins. Cancer Res 2003;63:8118–8121. [PubMed: 14678963]

128. Zhai D, Jin C, Satterthwait AC, Reed JC. Comparison of chemical inhibitors of antiapoptotic Bcl-2-family proteins. Cell Death Differ 2006;13:1419–1421. [PubMed: 16645636]

129. Mohammad RM, Wang S, Aboukameel A, et al. Preclinical studies of a nonpeptidic small-molecule inhibitor of Bcl-2 and Bcl-X(L) [(−)-gossypol] against diffuse large cell lymphoma. Mol Cancer Ther 2005;4:13–21. [PubMed: 15657349]

130. Wang JL, Liu D, Zhang ZJ, et al. Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. Proc Natl Acad Sci USA 2000;97:7124–7129. [PubMed: 10860979]

131. Zeitlin BD, Joo E, Dong Z, et al. Antiangiogenic effect of TW37, a small-molecule inhibitor of Bcl-2. Cancer Res 2006;66:8698–8706. [PubMed: 16951185]

132. Oltersdorf T, Elmore SW, Shoemaker AR, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 2005;435:677–681. [PubMed: 15902208]

133. Mohammad RM, Goustin AS, Aboukameel A, et al. Preclinical studies of TW-37, a new nonpeptidic small-molecule inhibitor of Bcl-2, in diffuse large cell lymphoma xenograft model reveal drug action on both Bcl-2 and Mcl-1. Clin Cancer Res 2007;13:2226–2235. [PubMed: 17404107]

134. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 2001;344:783–792. [PubMed: 11248153]

135. Marty M, Cognetti F, Maraninchi D, et al. Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. J Clin Oncol 2005;23:4265–4274. [PubMed: 15911866]

136. Gasparini G, Gion M, Mariani L, et al. Randomized Phase II Trial of weekly paclitaxel alone versus trastuzumab plus weekly paclitaxel as first-line therapy of patients with Her-2 positive advanced breast cancer. Breast Cancer Res Treat 2007;101:355–365. [PubMed: 16850247]

137. Burstein HJ, Keshaviah A, Baron A, et al. Trastuzumab and vinorelbine or taxane chemotherapy for HER2+ metastatic breast cancer: the TRAVIOTA study. J Clin Oncol 2006;24ASCO Ann Meet Proc Part I; abstract 650

138. Robert N, Leyland-Jones B, Asmar L, et al. Randomized phase III study of trastuzumab, paclitaxel, and carboplatin compared with trastuzumab and paclitaxel in women with HER-2-overexpressing metastatic breast cancer. J Clin Oncol 2006;24:2786–2792. [PubMed: 16782917]

139. Bernard-Marty C, Lebrun F, Awada A, Piccart MJ. Monoclonal antibody-based targeted therapy in breast cancer: current status and future directions. Drugs 2006;66:1577–1591. [PubMed: 16956305]

140. Johnston JB, Navaratnam S, Pitz MW, et al. Targeting the EGFR pathway for cancer therapy. Curr Med Chem 2006;13:3483–3492. [PubMed: 17168718]

141. Agus DB, Gordon MS, Taylor C, et al. Phase I clinical study of pertuzumab, a novel HER dimerization inhibitor, in patients with advanced cancer. J Clin Oncol 2005;23:2534–2543. [PubMed: 15699478]

142. Kiewe P, Hasmüller S, Kahlert S, et al. Phase I trial of the trifunctional anti-HER2 x anti-CD3 antibody ertumaxomab in metastatic breast cancer. Clin Cancer Res 2006;12:3085–3091. [PubMed: 16707606]

143. Jager M, Ruf P, Schoberth A, Hess J, Lindhofer H. Effect of low HER2(+1) expressing tumor cells of the trifunctional antibody ertumaxomab. J Clin Oncol 2008;26abstract 3071

144. Konecny GE, Pegram MD, Venkatesan N, et al. Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. Cancer Res 2006;66:1630–1639. [PubMed: 16452222]

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145. Gomez HL, Chavez MA, Doval DC, et al. Results from a phase II randomized study of lapatinib as the first-line treatment for patients with HER2-amplified locally advanced or metastatic breast cancer. Breast Cancer Res Treat 2006;100(Suppl 1)abstract 1090

146. Nelson MH, Dolder CR. Lapatinib: a novel dual tyrosine kinase inhibitor with activity in solid tumors. Ann Pharmacother 2006;40:261–269. [PubMed: 16418322]

147. Cameron D, Casey M, Press M, et al. A phase III randomized comparison of lapatinib plus capecitabine versus capecitabine alone in women with advanced breast cancer that has progressed on trastuzumab: updated efficacy and biomarker analyses. Breast Cancer Res Treat 2008;112:533–543. [PubMed: 18188694]

148. Xia W, Bacus S, Hegde P, et al. A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. Proc Natl Acad Sci USA 2006;103:7795–7800. [PubMed: 16682622]

149. Chu QS, Cianfrocca ME, Goldstein LJ, et al. A phase I and pharmacokinetic study of lapatinib in combination with letrozole in patients with advanced cancer. Clin Cancer Res 2008;14:4484–4490. [PubMed: 18628463]

150. Frassoldati A, Guarneri V, Piacentini F, et al. Letrozole versus letrozole plus Lapatinib (GW572016) in hormone-sensitive, HER2-negative operable breast cancer: a double-blind, randomized, phase II study with biomarker evaluation (EGF109077-LAP107692/LETLOB). Clin Breast Cancer 2008;8:97–100. [PubMed: 18501066]

151. Konecny G, Pauletti G, Pegram M, et al. Quantitative association between HER-2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. J Natl Cancer Inst 2003;95:142–153. [PubMed: 12529347]

152. Whenham N, D'Hondt V, Piccart MJ. HER2-positive breast cancer: from trastuzumab to innovatory anti-HER2 strategies. Clin Breast Cancer 2008;8:38–49. [PubMed: 18501058]

153. Shou J, Massarweh S, Osborne CK, et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu crosstalk in ER/HER2-positive breast cancer. J Natl Cancer Inst 2004;96:926–935. [PubMed: 15199112]

154. Kennedy BJ, Nathanson IT. Effects of intensive sex steroid hormone therapy in advanced breast cancer. J Am Med Assoc 1953;152:1135–1141. [PubMed: 13052446]

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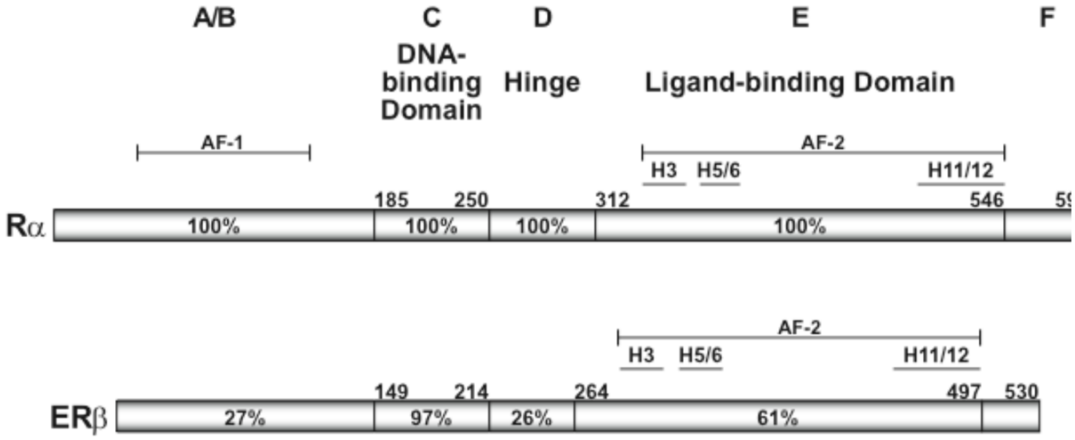

Fig. 1.

ERα and ERβ functional domains. ERα and ERβ proteins both are members of nuclear hormone receptor superfamily and bind oestrogens and regulate transcription of oestrogen responsive genes. They share the highest degree of amino acid sequence, 61 and 97% in LBD and DBD respectively, however A/B and D domains have only 27% and 26% amino acid homology respectively, however with lack of homology of A/B domain between ERα and ERβ, functional studies have indicated that ERβ lacks AF-1 activity.

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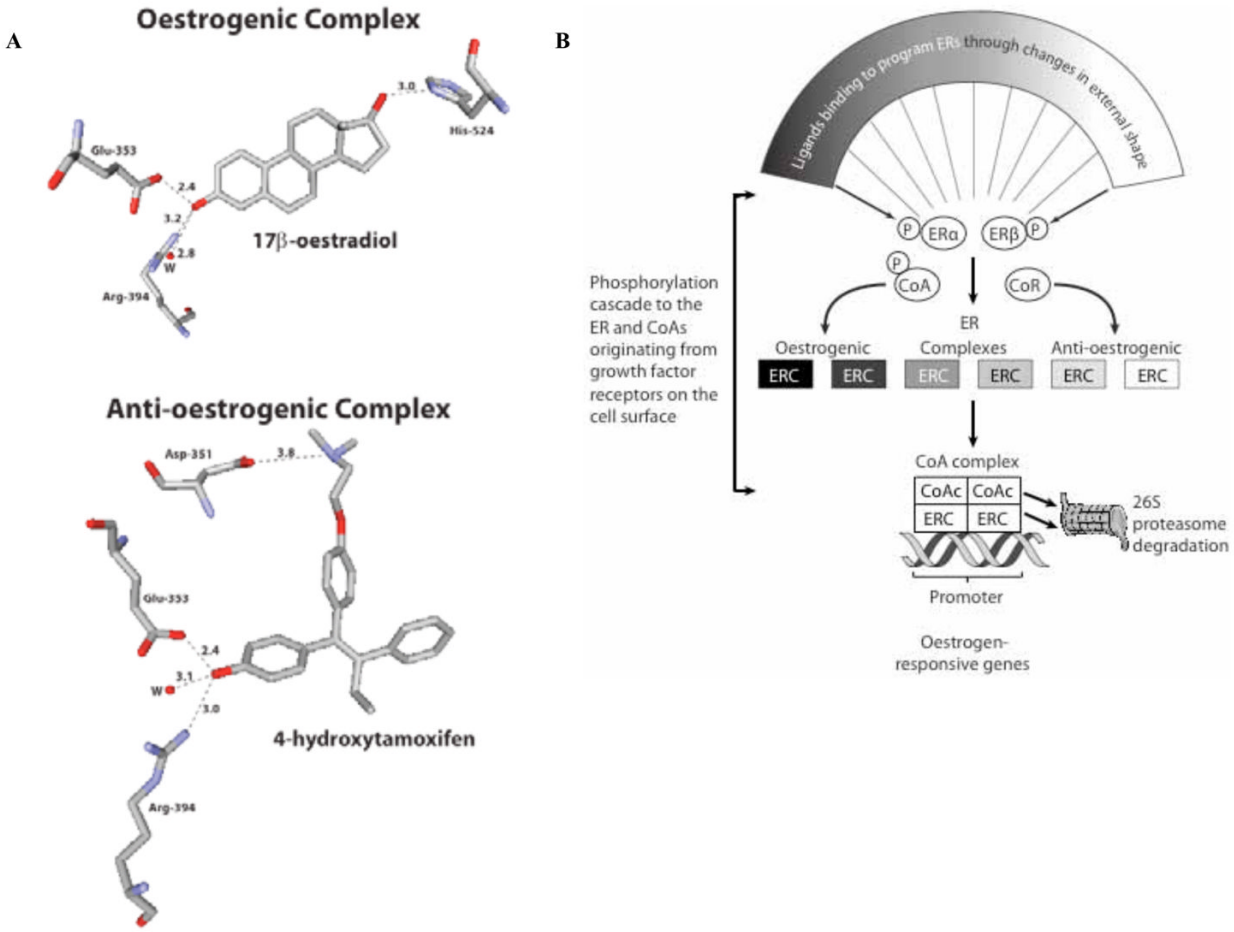

Fig. 2.

Coregulators and Oestrogen receptor action.

A: Molecular interactions between oestradiol, 4-hydroxytamoxifen and ERα. using the X-ray crystallographic structures. Oestrogen is aligned in the cavity by hydrogen bonds at both ends of the ligand, particularly the 3-OH group at the A-ring end of E2 forms a hydrogen bond network with Glu353 and Arg394, while E2's 17β-OH group at the D-ring end of the ligand forms a hydrogen bond with ER's His524. This results in sealing of the ligand-binding cavity by H12, and exposes the AF-2 surface for interaction with coactivators to promote transcriptional transactivation. In contrast, 4-hydroxytamoxifen binding to ER's LBD blocks the closure process by relocating H12 away from the binding pocket, thus preventing coactivator molecules from binding to the appropriate site on the external surface of the complex.

B: Structurally different ligands change the conformation of the oestrogen receptor and thus program them to bind either to coactivators with different enzymatic activities or co-repressors. Coactivators are necessary for the receptor to bind to the promotor regions of oestrogen responsive genes and then subsequently be degraded by the 26S proteasome complex, which is necessary for sustaining gene transcription. In the case of antioestrogen binding, co-repressors prevent activation of oestrogen responsive gene transcription.

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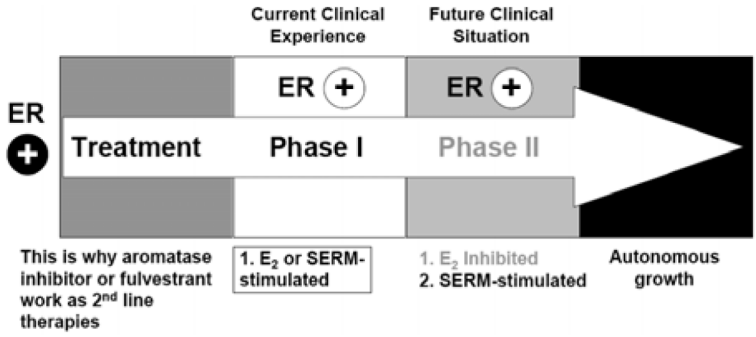

Fig. 3.

Evolution of antihormone resistance. Acquired resistance of breast cancer cells occurs during long-term treatment with SERMs and is evidenced by SERM-stimulated growth of these cells. Tumours still exploit oestrogen for growth when the SERM treatment is stopped, meaning that a promiscuous transduction pathway has developed. At this point aromatase inhibitors are effective as they destroy the ER. This phase of drug resistance is called phase I. Continued exposure to SERMs eventually leads to oestrogen-independent autonomous growth of breast cancer cells, continuing to be SERM-stimulated. However, these cells now respond to oestradiol by triggering aopoptosis rather than growth. This phase of drug resistance is called phase II.

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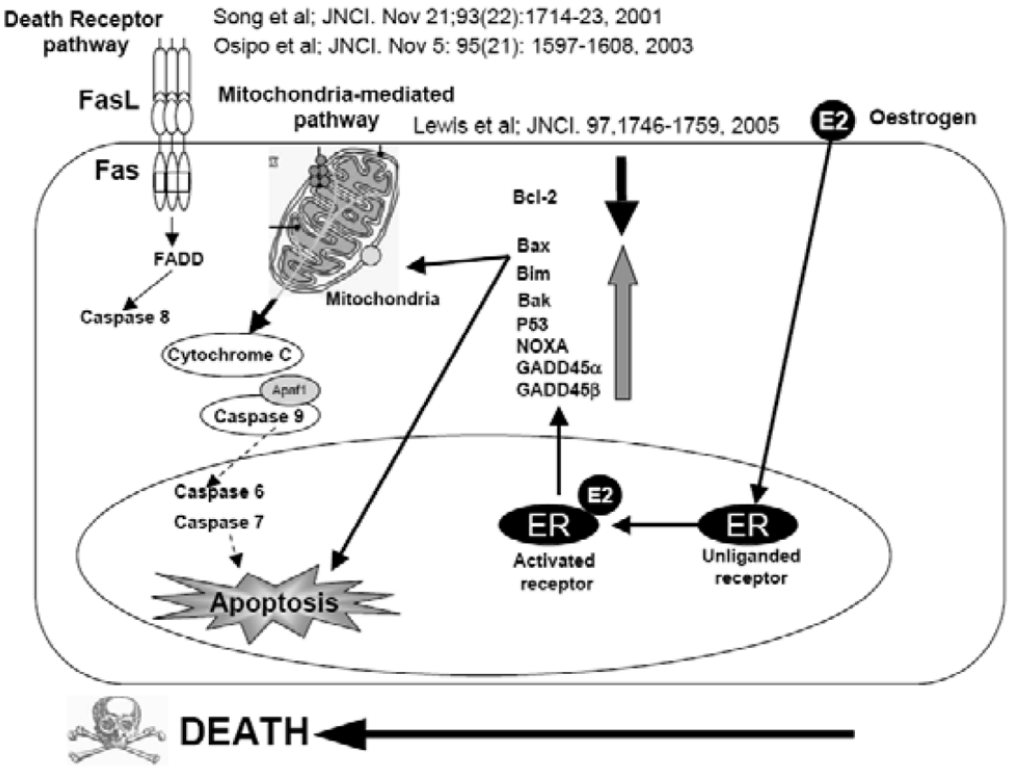

Fig. 4.

The proposed subcellular mechanism for oestrogen induced apoptosis of SERM resistant (Phase II) and long term oestrogen deprived (aromatase inhibitor-resistant) breast cancer. In SERM-resistant breast cancer, there is an induction of the Fas receptor/Fas ligand resulting in activation of caspase 8 and induction of apoptosis (programmed cell death). In long term oestrogen deprived breast cancer, the oestradiol ER complex alters Bcl-2 family protein expression which then activates the mitochondria causing cytochrome *c* release, caspase 9 activation, and PARP cleavage, ultimately resulting in cell death.

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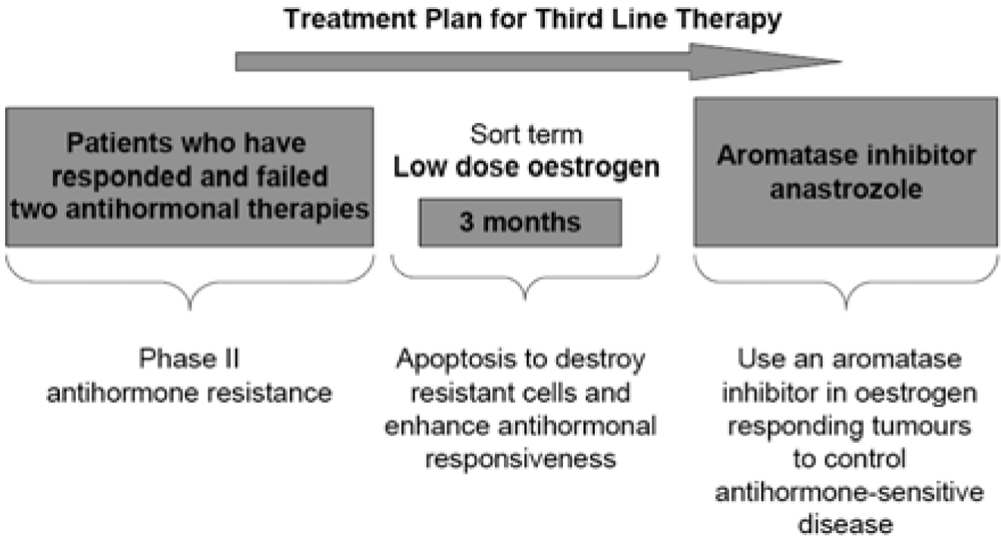

Fig. 5.

Clinical protocol to investigate the efficacy of oestradiol treatment to induce apoptosis in long-term endocrine refractory breast cancer. An anticipated treatment plan for third-line endocrine therapy. Patients must have responded and experience treatment failure with two successive antihormone therapies to be eligible for a course of low-dose oestradiol therapy for 3 months. The anticipated response rate is 30% and responding patients will be treated with anastrozole until relapse. The overall goal is to increase response rates and maintain patients for longer on antihormone strategies before chemotherapy is required.

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Table 1

Results from Previous Clinical Trials and Ongoing Trials with Oestrogen and Other Targeted Therapies

A: Results of clinical trails of low dose oestrogen (DES and oestradiol) treatment in patients with advanced breast cancer. CR- complete response, PR- partial response, CI- confidence interval, SD- stable disease. B: Results of preclinical trials of Bcl-2 inhibitors [-]-glossypol and TW-37 in diffuse large cell lymphoma xenograft model. T/C- reduction of tumour weight in treated group of mice relative to control in percents, T-C- time in days to weight equivalence of tumours in treated group of mice with tumour weight in control group of mice. C: Results of clinical trials of HER2 inhibitor antibodies in patients with advanced breast cancer. ORR- overall response rate.

A.

Oestrogen/mg	CR	PR	CI, %	SD	Reference
DES/15mg	4/32	6/32	N/A	2/32	Lonning[a]
Oestradiol/6mg	N/A	1/32	15–37	7/32	Ellis[b]
Oestradiol/30mg	N/A	3/34	19–42	7/34	Ellis[b]

B.

Bcl-2 Inhibitor	T/C, %	T-C, Days	No. of Animals	Reference
[-]-Glossypol	56	3	5	Mohammad[c]
TW-37	57	4	5	Mohammad[d]

C.

HER2 Inhibitors	ORR	Phase	Reference
Trastuzumab+paclitaxel or doxorubicin+cyclophosphamide	50% vs. 32%	Phase III	Slamon[e]
Trastuzumab+docetaxel	61% vs. 34%	Phase II	Marty[f]
Lapatinib	24%	Phase III	Gomez[g]
Lapatinib+capecitabine	24% vs. 36%	Phase III	Cameron[h]

[a] reference [98]

[b] reference [120]

[c] reference [129]

[d] reference [133]

[e] reference [134]

[f] reference [135]

[g] reference [145]

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$h_{\text{reference [147]}}$

Review

Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit?

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Abstract

The link between estrogen and the development and proliferation of breast cancer is well documented. Estrogen stimulates growth and inhibits apoptosis through estrogen receptor-mediated mechanisms in many cell types. Interestingly, there is strong evidence that estrogen induces apoptosis in breast cancer and other cell types. Forty years ago, before the development of tamoxifen, high-dose estrogen was used to induce tumor regression of hormone-dependent breast cancer in post-menopausal women. While the mechanisms by which estrogen induces apoptosis were not completely known, recent evidence from our laboratory and others demonstrates the involvement of the extrinsic (Fas/FasL) and the intrinsic (mitochondria) pathways in this process. We discuss the different apoptotic signaling pathways involved in E2 (17β-estradiol)-induced apoptosis, including the intrinsic and extrinsic apoptosis pathways, the NF-κB (nuclear factor-kappa-B)-mediated survival pathway as well as the PI3K (phosphoinositide 3-kinase)/Akt signaling pathway. Breast cancer cells can also be sensitized to estrogen-induced apoptosis through suppression of glutathione by BSO (L-buthionine sulfoximine). This finding has implications for the control of breast cancer with low-dose estrogen and other targeted therapeutic drugs.

Introduction

Breast cancer is one of the most frequently diagnosed cancers among women, with an estimated 184,450 new cases of invasive disease and 40,930 deaths in 2008. There is strong evidence that estrogen plays a role in its development and progression [1]. Breast cancer was first recognized to be estrogen-dependent when the British surgeon George Beatson [2] published his findings of the beneficial effects of oophorectomy in a pre-menopausal patient with advanced breast cancer. Beatson had based his approach on the role of the ovaries in mammalian lactation and presumed that there would be a similar mechanism for breast cancer growth. Since that time, there has been an expanding clinical database that implicates estrogen in the development and progression of breast cancer. Evidence to support this conclusion comes from clinical studies of hormone replacement therapy, which were initially designed to determine the benefits of replacement approaches on post-menopausal women's health [3,4], and the successful clinical strategy of treating breast cancer by blocking estrogen action using the anti-estrogen tamoxifen [5] or preventing estrogen synthesis using aromatase inhibitors (AIs) [6].

Estrogens are a class of sex steroid hormones that are synthesized from cholesterol and are secreted primarily by the ovaries, with secondary contributions from placenta, adipose tissue, testes, and adrenal glands. After menopause, ovarian estrogen biosynthesis is minimal, with circulating estrogens being derived principally from peripheral aromatization of adrenal androgens. Estrogens are essential to the function of the female reproductive system and are required for the proliferation and differentiation of healthy breast epithelium. Estrogens occur naturally in several structurally related forms; however, the predominant intracellular estrogen is 17β-estradiol (E2). In mammary glands, E2 promotes cell proliferation in both normal and transformed epithelial cells by modifying the expression of hormone-responsive genes involved in the cell cycle and/or programmed cell

AI = aromatase inhibitor; AP-1 = activator protein 1; Bad = Bcl-2/Bcl-X_L-associated death domain protein; Bak = Bcl-2 homologous antagonist-killer protein; Bax = Bcl-2-associated X protein; Bcl-2 = B-cell lymphoma-2; Bcl-X_L = Bcl-2-related gene, long form; BH = Bcl-2 (B-cell lymphoma-2) homology; Bid = Bcl-2-interacting domain; Bim = Bcl-2-interacting mediator of cell death; BSO = L-buthionine sulfoximine; CDK = cyclin-dependent kinase; CR = complete response; DES = diethylstilbestrol; E2 = 17β-estradiol; ER = estrogen receptor; FasL = Fas ligand; GSH = glutathione; IL = interleukin; JNK = c-jun N-terminal kinase; LTED = long-term estrogen-deprived; MAPK = mitogen-activated protein kinase; NF-κB = nuclear factor-kappa-B; OPG = osteoprotegerin; p53 = 53 kDa protein; PARP = poly(ADP-ribose)polymerase; PI3K = phosphoinositide 3-kinase; PKB/Akt = protein kinase B; PKC-α = protein kinase C-alpha; Puma = p53-upregulated modulator of apoptosis; RANK-L = ligand of the receptor activator of nuclear factor-kappa-B; SERM = selective estrogen receptor modulator; siRNA = short interfering RNA; Sp-1 = specificity protein 1; TNF = tumor necrosis factor.

death. In estrogen receptor (ER)-positive MCF-7 human breast cancer cells, one of the principal mechanism by which E2 stimulates growth is through the induction of G_1- to S-phase transition. This induction is associated with the rapid and direct upregulation of c-myc, which controls cyclin D1 expression along with activation of cyclin-dependent kinase (CDK) and phosphorylation of retinoblastoma protein [7]. E2 also rapidly activates cyclin E-CDK2 complexes, accelerating the G_1-to-S transition [8]. Additionally, E2 has 'non-genomic or membrane-initiated' effects (that is, independent of ER-mediated transcription) that occur within minutes after E2 administration [9-11]. Specifically, ER-α interacts with a number of proteins, including c-Src, the p85 subunit of phosphoinositide 3-kinase (PI3K), caveolin 1, and modulator of non-genomic activity of ER (MNAR) [10,12], epidermal growth factor receptor (EGFR), insulin-like growth factor receptor 1 (IGFR1), and HER2 [13], and it rapidly increases PIP2-phospholipase C activity and activates the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways [9,12,13]. More importantly, E2 is a potent inhibitor of apoptosis and it regulates the expression of several apoptotic proteins, including Bcl-2 in MCF-7, T47-D, and ZR-75-1 breast cancer cells [14].

Remarkably, there is another dimension to estrogen action which contrasts with its ability to stimulate growth and inhibit apoptosis. Physiologic E2 is also capable of inducing apoptosis in breast cancer cells that have been long-term estrogen-deprived (LTED) or have been treated exhaustively with anti-estrogens [15-23], prostate cancer cells [24], neuronal cells [25], bone-derived cells [26], thymocytes [27], and ER-transfected cells [28,29]. These data are particularly interesting because high-dose estrogen therapy was used as a treatment for post-menopausal patients with metastatic breast cancer from the 1940s until the introduction of the safer anti-estrogen tamoxifen in the 1970s [30]. At that time, however, the mechanism of estrogen-induced tumor regression was not known. In this review, we will discuss the current understanding of estrogen-induced apoptosis in breast cancer and will summarize the possible mechanisms involved in this estrogen-mediated process.

Estrogen-induced apoptosis: laboratory observations

Recent *in vitro* studies from our laboratory [18,31] and other investigators [19,20,32] have shown that long-term estrogen deprivation of hormone-dependent MCF-7 breast cancer cells causes them to undergo adaptive changes in which estradiol switches from being a proliferative agent to paradoxically inhibiting growth and inducing apoptosis. Interestingly, LTED cells also exhibit enhanced sensitivity to estradiol in that an estradiol concentration that is three logs lower can stimulate proliferation of these cells compared with wild-type MCF-7 cells [19]. The development of hypersensitivity to estradiol as a result of LTED is associated with the upregulation of ER-α and the MAPK, PI3K, and mTOR (mammalian target of rapamycin) growth factor pathways [33]. In contrast, the apoptotic mechanisms of estradiol in LTED cells are thought to involve the death receptors as well as the mitochondrial pathways. Specific molecular events include the activation of the Fas death receptor/Fas ligand (FasL) complex [20], the release of cytochrome *c* from the mitochondria and alterations in Bcl-2 [18,32], and the downregulation of the anti-apoptotic factor nuclear factor-κ [31,34]. It is important to note that estradiol also induces apoptosis in *in vivo* models of anti-hormone drug resistance [23,35,36]; however, the mechanisms by which this occurs are not completely known.

Estrogen therapy and breast cancer: clinical observations

Clinical data support the use of high-dose estrogen to treat hormonally sensitive breast tumors [37-41]. In 1944, Sir Alexander Haddow and colleagues [37] published the results of their clinical trial with the synthetic estrogens triphenylchlorethylene, triphenylmethylethylene, and stilbestrol administered at high doses. They found that 10 out of 22 post-menopausal patients with advanced mammary carcinomas, who were treated with triphenylchlorethylene, had significant regression of tumor growth. Five patients out of 14 who were treated with high-dose stilbestrol produced similar responses. Interestingly, the duration of the post-menopausal period was found to be a critical factor affecting the success of this therapy. For example, when the synthetic estrogen diethylstilbestrol (DES) was administered at 15 mg per day, women who had experienced the onset of menopause less than 1 year prior to therapy did not respond to DES; women who had experienced the onset of menopause within 5 years of menopause experienced a 7.9% objective response rate; and women who reached menopause more than 10 years earlier experienced a 22% response rate [41]. Despite the benefits, however, there were significant systemic side effects associated with high-dose estrogen therapy [37].

Cole and colleagues [39] reported the first clinical trial of the anti-estrogen tamoxifen in women with late or recurrent breast cancer and compared their findings with historical data from women receiving DES. They concluded that the levels of response were similar for DES and tamoxifen; however, tamoxifen had a lower incidence of side effects. Ingle and colleagues [30] compared tamoxifen with DES directly and noted that response rates were similar but tamoxifen had fewer side effects. Based on these data, the use of high-dose estrogen for treatment of advanced breast cancer fell out of favor, and tamoxifen became the standard first-line endocrine therapy. The Ingle study [30] that compared DES-treated and tamoxifen-treated patients was followed up but surprisingly showed a survival advantage for DES-treated patients [41]. Another small trial was conducted by Lonning and colleagues [40] in post-menopausal patients with advanced breast cancer exposed to multiple endocrine therapies and revealed a 31% objective response rate with DES therapy. More recently, Ellis and colleagues [42]

reported that a daily dose of 6 mg of E2 could stop the growth of tumors or even cause them to shrink in about 25% of women with metastatic breast cancer that had developed resistance to standard anti-hormonal therapy. These clinical observations that estrogen can induce tumor regression after several years of anti-hormonal therapy provide a clue that the adaptation of cancer cells to low levels of estrogen might sensitize cells to the apoptotic effect of estrogen. While the mechanisms by which estrogen exerts its pro-apoptotic/anti-tumor effect are not known, a growing body of evidence suggests the involvement of the extrinsic (death receptor) and intrinsic (mitochondrial) pathways in this process.

Two main pathways involved in apoptosis regulation

Apoptosis is a form of programmed cell death that plays a critical role in the maintenance of tissue homeostasis [43]. It is a highly regulated physiologic mechanism that removes excess or damaged cells [43]. The dysregulation of apoptosis is a hallmark of cancer, with both the loss of pro-apoptotic signals and the gain of anti-apoptotic mechanisms contributing to tumorigenesis [44]. The induction of apoptosis in many cell types is achieved through the activation of the extrinsic and the intrinsic pathways [45]. The extrinsic pathway (Figure 1) is initiated by the interaction between specific ligands and surface receptors, such as CD95/Fas/Apo1, tumor necrosis factor (TNF) receptor 1 (TNFR1), TNF receptor 2 (TNFR2), and death receptors 3-6 (DR3-6) [46], which are able to deliver a death signal from the extracellular microenvironment to the cytoplasm. Binding of the ligand to the receptor induces receptor multimerization, binding of Fas-associated death domain (FADD) adapter protein, formation of the death-induced signaling complex (DISC) which recruits the initiator caspases 8 and 10, and subsequently activation of the effector caspases 3 and 7 [46]. In the intrinsic pathway (Figure 1), the integrity of the mitochondrial membrane is controlled primarily by a balance between the antagonistic actions of the proapoptotic and antiapoptotic members of the Bcl-2 family [47] (please see Table 1 for a detail description of common abbreviations used in apoptosis). Bcl-2 family proteins comprise three principal subfamilies: (a) anti-apoptotic members, including Bcl-2/Bcl-x_L, which possess the Bcl-2 homology (BH) domains BH1, BH2, BH3, and BH4; (b) pro-apoptotic members, such as Bax, Bak, and Bok, which have the BH1, BH2, and BH3 domains; and (c) BH3-only proteins, such as Bid, Bim, Bad, Bik, and Puma, which generally possess only the BH3 domain [47]. The Bcl-2 family of proteins regulates apoptosis by altering mitochondrial membrane permeabilization and controlling the release of cytochrome *c*. Several lines of evidence demonstrate that the Bcl-2 family functions are controlled by growth factor signaling pathways, including the PI3K/Akt, the JAK (Janus kinase)/Stat (signal transducer and activator of transcription), and the Ras/MAPK pathways [48]. Phosphorylation and dephosphorylation of the members of the Bcl-2 family of proteins by the above pathways regulate the stabilization of mitochondrial homeostasis [48].

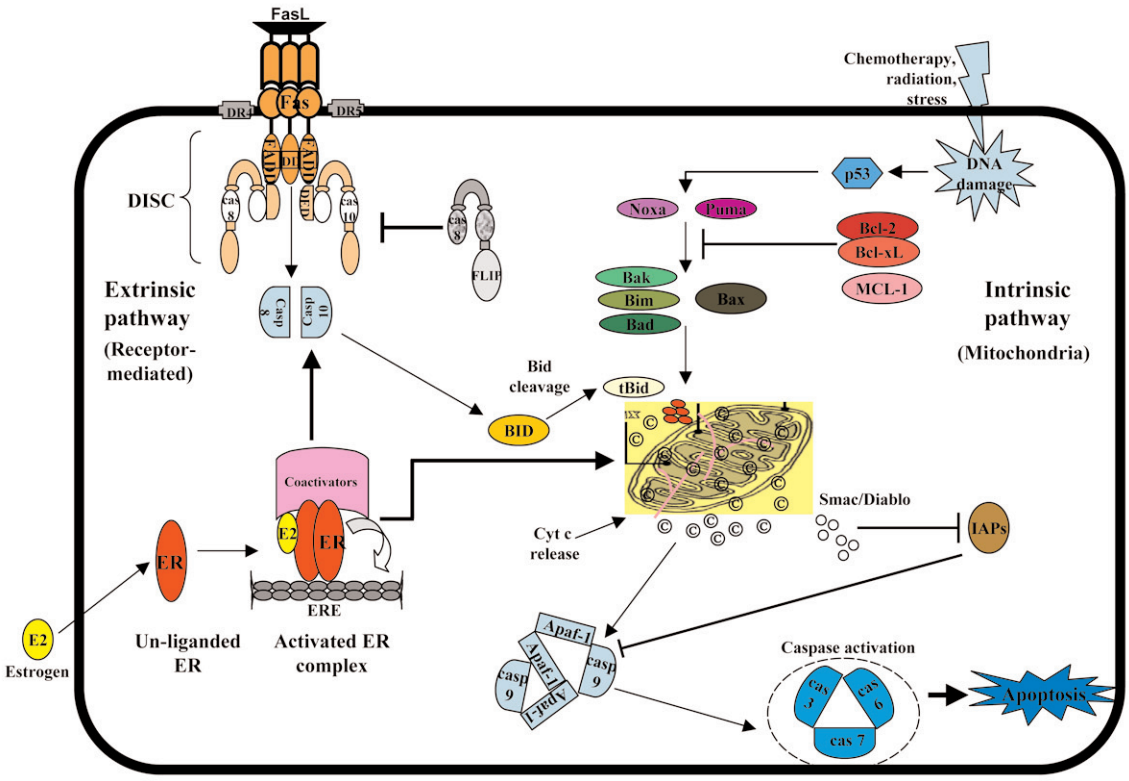
Signaling pathways implicated in estrogen-induced apoptosis

The extrinsic (receptor-mediated) pathway

Mechanistic studies have used either LTED MCF-7 breast cancer cells [18,20,31,34] or selective ER modulator (SERM) (tamoxifen or raloxifene)-stimulated tumor models [23,35,36,49-51] to demonstrate the involvement of the Fas/FasL death signaling pathway in the paradoxical apoptotic/anti-tumor effects of E2. Song and colleagues [20] were the first to demonstrate that E2 caused apoptosis in breast cancer cells that were adapted to grow in an E2-free environment for prolonged periods. They reported that their LTED cells, which were derived by growing wild-type MCF-7 breast cancer cells under long-term (6 to 24 months) estrogen-deprived conditions, expressed high levels of Fas compared with the parental MCF-7 cells and that treatment of these cells with E2 resulted in a marked increase in FasL. This finding was confirmed by Osipo and colleagues [35], who reported that physiologic levels of E2 induced regression of tamoxifen-resistant breast cancer tumors by inducing Fas expression and suppressing the anti-apoptotic/pro-survival factors nuclear factor-kappa-B (NF-κB) and HER2/neu. A similar finding was reported by Liu and colleagues [49] in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*. In addition, Tonetti and colleagues [50,51] previously reported that stable overexpression of protein kinase C-alpha (PKC-α) in hormone-responsive T47D:A18 breast cancer cells (T47D:A18/PKC-α) produced a hormone-independent/tamoxifen-resistant and E2-inhibitory phenotype *in vivo* [50,51]. Using the T47D:A18/PKC-α-overexpressing tumor model, they further demonstrated that E2-induced regression and apoptosis were due to increased expression of Fas/FasL proteins and downregulation of the pro-survival Akt pathway [36]. In all of these model systems, the ER-α was shown to be critical for E2-induced tumor regression and apoptosis. Blockade of the ER-α signaling pathway using the pure anti-estrogen fulvestrant completely inhibited the apoptotic effect of E2 [20,35,36,49].

It is worth noting that a putative estrogen-responsive element (ERE) has been identified in the promoter region of the FasL gene [52], suggesting direct estrogen effects on FasL expression. In addition, a number of transactivating factors have been identified as regulators of FasL gene expression, including activator protein 1 (AP-1) [53] and specificity protein 1 (Sp-1) [54]. Sp-1 is involved in the transcriptional regulation of many genes and has also been identified to be important in the regulation of FasL gene expression and apoptosis. Indeed, this transcription factor is able to activate FasL promoter via a distinct recognition element, and inducible FasL promoter activation is abrogated by the expression of the dominant-negative mutant form of Sp-1 [54]. Functional studies have demonstrated that Sp-1 is a crucial effector of E2 signal in enhancing FasL gene expression. For instance, it is well known that ERs can transactivate gene promoters without directly binding to DNA

Figure 1



The two main pathways involved in apoptosis regulation. **(a)** The extrinsic pathway begins outside the cell through the activation of receptors on the cell surface by specific molecules known as pro-apoptotic ligands, including CD95L/FasL (receptor CD95/Fas). Once activated, the death domains of these receptors bind to the adapter protein Fas-associated death domain (FADD), resulting in the assembly of death-induced signaling complex (DISC) and recruitment and assembly of initiator caspases 8 and 10. The two caspases are stimulated and processed, releasing active enzyme molecules into the cytosol, where they activate caspases 3, 6, and 7, thereby converging on the intrinsic pathway. **(b)** The intrinsic (mitochondrial) pathway is initiated in response to cellular signals resulting from DNA damage, a defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of cell survival factors, or other types of severe cell stress. This triggers activation of specific members of the pro-apoptotic Bcl-2 protein family involved in the promotion of apoptosis, Puma and Noxa, which in turn activate the pro-apoptotic proteins Bax or Bak. These two proteins move to the mitochondrial membrane and disrupt the anti-apoptotic function of the Bcl-2 family proteins, allowing for permeabilization of the mitochondrial membrane. Apaf-1, apoptotic protease activating factor 1; Bad, Bcl-2/Bcl-X_L-associated death domain protein; Bak, Bcl-2 homologous antagonist-killer protein; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; Bcl-X_L, Bcl-2-related gene, long form; Bid, Bcl-2-interacting domain; Bim, Bcl-2-interacting mediator of cell death; Casp, caspase; Cyt c, cytochrome *c*; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen-responsive element; FasL, Fas ligand; FLIP, FLICE-inhibitory protein; IAP, inhibitor of apoptosis; Noxa, phorbol-12-myristate-13-acetate-induced protein 1; Puma, p53-upregulated modulator of apoptosis.

but instead through interaction with other DNA-bound factors in promoter regions lacking TATA box. This has been investigated extensively in relation to protein complexes involving Sp-1 and ER-α at GC boxes, which are classic binding sites for members of the Sp-1 family of transcription factors. Sp-1 protein plays an important role in the regulation of mammalian and viral genes, and recent results have shown that E2 responsiveness of c-fos, cathepsin D, retinoic acid, and insulin-like grow factor-binding protein 4 gene expression in breast cancer cells is linked to specific GC-rich promoter sequences that bind ER/Sp-1 complex in which only Sp-1 protein binds DNA [55-59]. Thus, it is possible that, when E2 upregulates FasL production in these different model systems, an apoptotic signal is initiated by FasL binding on Fas receptor.

The intrinsic (mitochondrial) pathway

Over the last several years, there has been accumulating evidence that, apart from the extrinsic/receptor-mediated pathway, the mitochondrial (intrinsic) pathway plays a role in E2-induced apoptosis. Indeed, we [18] have previously reported that, in our LTED breast cancer cell line, MCF-7:5C, E2 treatment markedly increased the expression of several pro-apoptotic proteins, including, Bax, Bak, Bim, Noxa, Puma, and p53, and that blockade of Bax and Bim expression using short interfering RNAs (siRNAs) almost completely reversed

Table 1

Description of common abbreviations used in apoptosis and signal transduction

Abbreviation	Meaning	Synonyms
Bad	Bcl-2/Bcl-X_L-associated death domain protein	BH3-only member of the Bcl-2 family
Bak	Bcl-2 homologous antagonist-killer protein	Multi-BH domain pro-apoptosis protein
Bax	Bcl-2-associated X protein	Multi-BH domain pro-apoptosis protein
Bcl-2	B-cell lymphoma-2	Defining member of the family; originally characterized as an oncogene
Bcl-X_L	Bcl-2-related gene, long form	Bcl-X_S is a shorter splice variant that is pro-apoptotic
Bim	Bcl-2-interacting mediator of cell death	BH3-only member of the Bcl-2 family
IκB	Inhibitor of NF-κB	Interacts with NF-κB
IKK	IκB kinase	Phosphorylates IκB to promote its degradation
MDM2	Murine double minute	Negative regulator of the p53 tumor suppressor
NF-κB	Nuclear factor-kappa type B	Originally linked with enhancement of immunoglobulin kappa light-chain gene
p53	53 kDa protein	Tumor-suppressor protein
PDK-1	3-phosphoinositide-dependent protein kinase 1	Master kinase that is crucial for the activation of Akt/PKB
PI3K	Phosphoinositide 3-kinase	Phosphatidylinositol 3-kinase; PI 3-kinase; PtdIns3K
PKB	Protein kinase B	Akt; RACK (related to A and C kinase); has PH domain
PMAIP-1/Noxa	Phorbol-12-myristate-13-acetate-induced protein 1	BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis
PUMA	p53-upregulated modulator of apoptosis	BH3-only member of the Bcl-2 family

BH, Bcl-2 (B-cell lymphoma-2) homology.

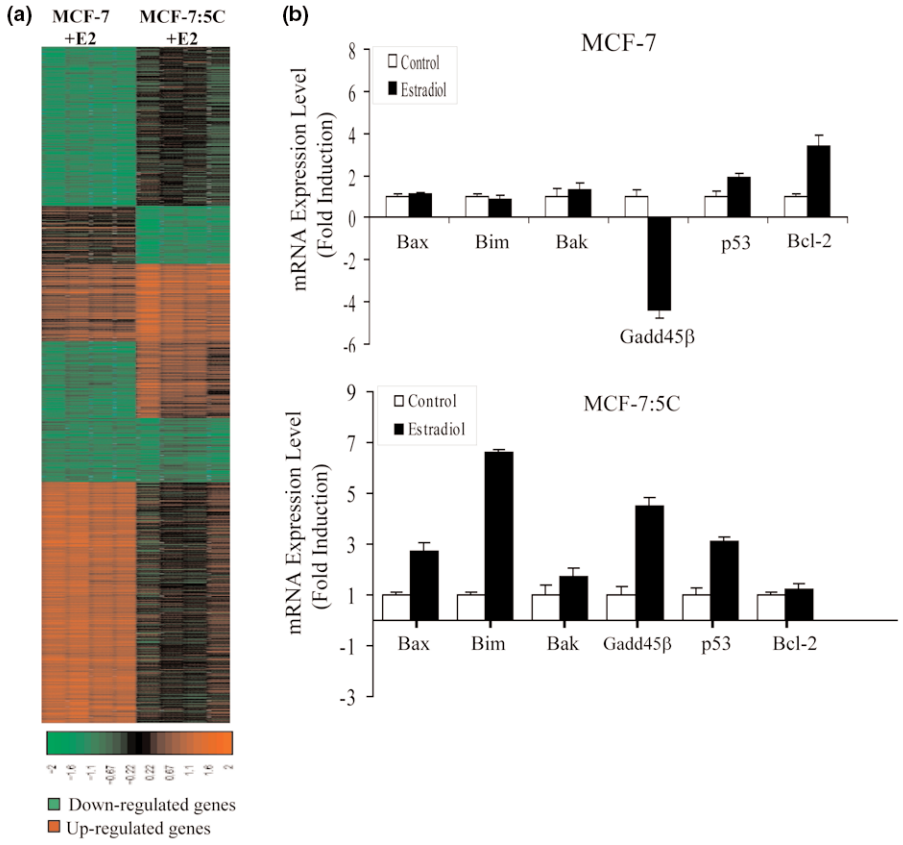
the apoptotic effect of E2 in these cells. E2 treatment also led to a loss of mitochondrial potential and a dramatic increase in the release of cytochrome *c* from the mitochondria, which resulted in activation of caspases 7 and 9 and cleavage of poly(ADP-ribose)polymerase (PARP). Furthermore, over-expression of anti-apoptotic Bcl-x_L completely blocked E2-induced apoptosis in MCF-7:5C cells. Interestingly, microarray analysis of wild-type MCF-7 cells and LTED MCF-7:5C cells revealed significant differences in the gene expression profile between the two cell lines following E2 treatment (Figure 2a). In particular, E2 treatment caused a marked increase in several pro-apoptotic genes in MCF-7:5C cells compared with wild-type MCF-7 cells (Figure 2b).

In two other estrogen-deprived breast cancer lines, LTED and E8CASS, basal Bcl-2 level was greatly elevated and knock-down of Bcl-2 expression with siRNA markedly sensitized these cells to the apoptotic action of E2 [32]. A similar finding was reported for another LTED breast cancer cell line, MCF-7:2A, which expressed elevated basal levels of Bcl-2 and was initially resistant to E2-induced apoptosis [34]. We found that suppression of Bcl-2 expression in these cells enhanced the apoptotic effect of E2 by almost fivefold [34], thus suggesting an important role for this protein in the apoptotic action of E2. Currently, there is renewed interest in developing small-molecule inhibitors of Bcl-2 [60] as anti-cancer cell and anti-angiogenic agents. The Bcl-2 antisense oligonucleotide, Oblimersen (Genasense; Genta Incorporated, Berkeley Heights, NJ, USA), which works by blocking Bcl-2 protein production, is now in a phase III clinical trial [61].

Inhibition of the survival pathways Akt and nuclear factor-kappa-B

The existence of various checkpoints in apoptosis reveals a complex balance between cell survival and cell death in cells. Two of the main signaling pathways involved in cell survival are the Akt and NF-κB signaling pathways (Figure 3). The PI3K/Akt/protein kinase B (PKB) pathway plays a central role in a variety of cellular processes, including cell growth, proliferation, motility, and survival in both normal and tumor cells. It impinges upon a remarkable array of intracellular events that influence either directly or indirectly whether a cell will undergo apoptosis. Many of the transforming events in breast cancer are a result of enhanced signaling of the PI3K/Akt pathway. Akt, also called PKB, is the human homologue of the viral oncogene v-akt [62], which regulates multiple targets, including several apoptotic genes. In a series of publications [63,64], Akt was found to mediate phosphorylation and hence inactivation of pro-apoptotic factors like Bad, which controls the release of cytochrome *c*,

Figure 2



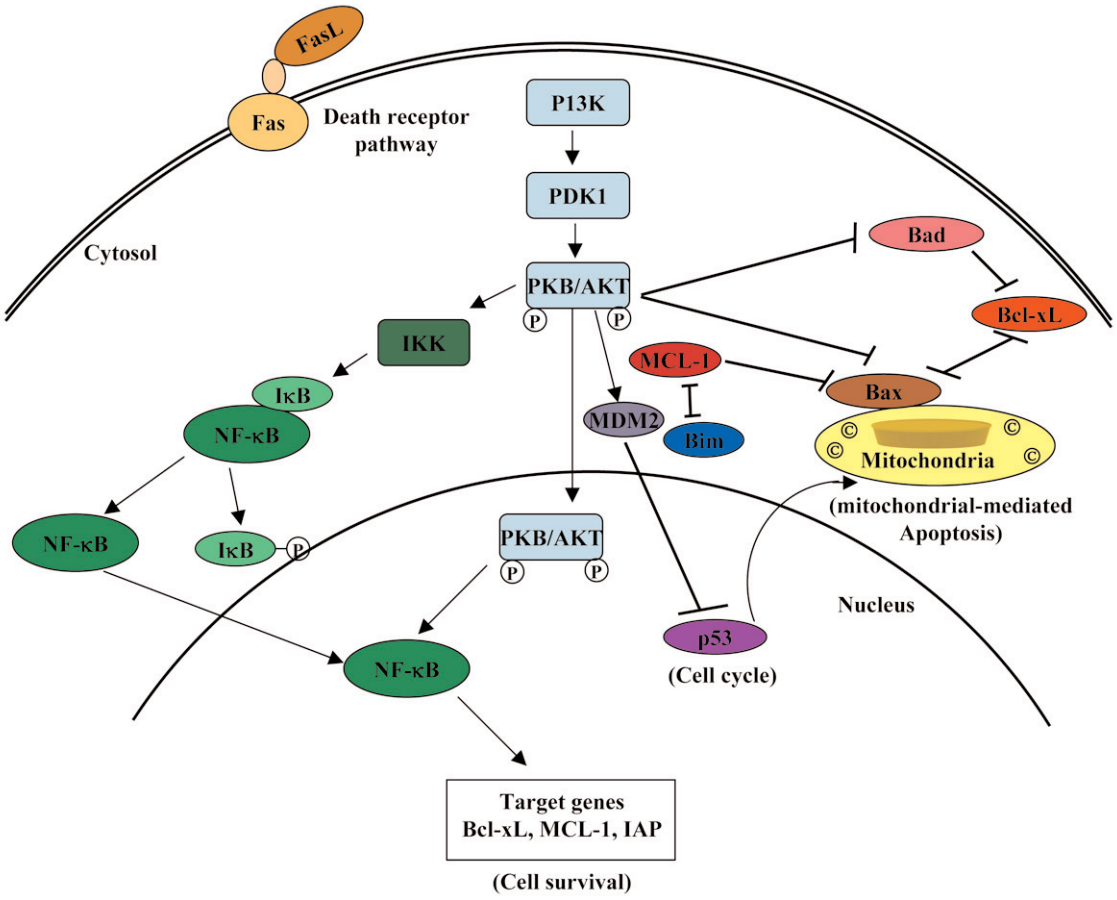
Gene expression profile of wild-type MCF-7 cells and long-term estrogen-deprived (LTED) MCF-7:5C breast cancer cells following 17β-estradiol (E2) treatment. Cells were treated with 1 nM E2 for 48 hours, and RNA was hybridized to the Affymetrix Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA). **(a)** Hierarchical clustering dendogram of E2-regulated genes in MCF-7 and MCF-7:5C cells. Microarray expression data for each cell line were first filtered for minimal intra-replicate standard deviation (<0.25) and a standard deviation between all samples of at least 0.25. This generated a total of 2,743 genes. In addition, genes displaying a minimum of twofold upregulation or downregulation by E2 in either MCF-7 or MCF-7:5C cells were extracted, revealing a set of 539 differentially expressed, E2-regulated genes. **(b)** E2 regulation of pro-apoptotic and anti-apoptotic genes in MCF-7 cells (top panel) and MCF-7:5C cells (bottom panel). Bak, Bcl-2 homologous antagonist-killer protein; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; Bim, Bcl-2-interacting mediator of cell death; GADD45β, growth arrest and DNA damage; p53, 53 kDa protein.

procaspase 9, and Forkhead transcription factors. Akt also activates anti-apoptotic genes, including cyclic-AMP response element-binding protein (CREB) and IκB (inhibitor of NF-κB) kinase (IKK), the primary regulator of NF-κB activity.

Several groups have demonstrated that E2 can also inhibit the P13K/Akt signaling pathway and consequently induce apoptosis of cancer cells. In tamoxifen-resistant PKC-α-overexpressing cells, E2-induced tumor regression is associated with the downregulation of phosphorylated Akt [36]. In addition, in LTED MCF-7:5C and MCF-7:2A breast cancer cells, the basal level of phosphorylated Akt is markedly upregulated and E2 treatment significantly reduces its expression (Figure 4). There is also evidence that, in MCF-7.beclin-overexpressing cells, E2 treatment significantly reduces Akt phosphorylation, which is associated with a decrease in cell proliferation [65]. Akt, therefore, is considered a rational target for cancer therapies and inhibitors of the PI3K/Akt pathway have been identified [66].

NF-κB is one of the most studied transcription factors in mammalian cells. Its family is composed of five members: RELA (p65), RELB, REL (cRel), NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) [67]. These proteins form homodimeric and heterodimeric complexes, and the activity of these proteins is regulated by two major pathways: the classical or canonical NF-κB activation pathway [67] and the non-canonical NF-κB activation pathway [67]. One of the most documented functions of NF-κB is its ability to promote cell survival through the induction of target genes (Figure 3), the products of which inhibit the apoptotic machinery in normal and malignant cells

Figure 3



Summary of some of the key processes regulated in the cytoplasm, at the mitochondria, in the nucleus, or in the cytosol by the PI3K/Akt pathway in controlling apoptosis. The positive events controlled either directly or indirectly by PI3K/Akt are indicated by arrows, whereas blocked lines represent events that have inhibitory effects. Bad, Bcl-2/Bcl-X_L-associated death domain protein; Bax, Bcl-2-associated X protein; Bcl-X_L, Bcl-2-related gene, long form; Bim, Bcl-2-interacting mediator of cell death; FasL, Fas ligand; IAP, inhibitor of apoptosis; IκB, inhibitor of nuclear factor-kappa-B; IKK, IκB (inhibitor of nuclear factor-kappa-B) kinase; Mcl-1, myeloid cell leukemia **1**; Mdm2, murine double minute; NF-κB, nuclear factor-kappa-B; p53, 53 kDa protein; PDK-1, phosphoinositide-dependent protein kinase 1; PI3K, phosphoinositide 3-kinase; PKB/Akt, protein kinase B.

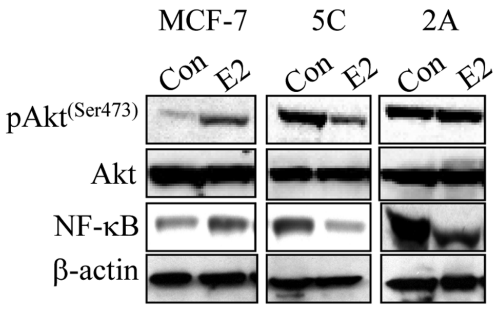
[68]. Indeed, overall reduction in NF-κB activity is associated with an increased apoptotic index in many cell types [68]. Furthermore, NF-κB activation has been shown to inhibit p53-dependent apoptosis following expression of the oncogene AP12/MALT1 [69]. Thus, blocking this signaling pathway might be a promising option to improve the efficacy of conventional anti-cancer therapies.

Several studies have shown that E2 can inhibit the activity of NF-κB and thereby increase apoptosis. For example, Osipo and colleagues [35] reported that, in tamoxifen-resistant MCF-7 tumors, E2 treatment almost completely down-regulated the level of the NF-κB p65 subunit protein, which correlated with the anti-proliferative and pro-apoptotic effects of E2 in this model system. These investigators also reported that cyclooxygenase 2 (COX-2), an NF-κB-responsive gene, was markedly reduced in E2-treated tamoxifen-stimulated MCF-7 tumors [35]. They concluded from this finding that E2-induced apoptosis and tumor regression in tamoxifen-resistant MCF-7 tumors occurred, in part, through suppression of the pro-survival/anti-apoptotic factor NF-κB. It should be noted that NF-κB expression is also markedly elevated in raloxifene-resistant MCF-7 breast cancer cells [49] and LTED breast cancer cells (Figure 4) and its downregulation by E2 is associated with the suppression of proliferation and the induction of apoptosis [31,32,70].

Glutathione suppression and estrogen-induced apoptosis

Previous studies have reported that, apart from its action on the mitochondria, Bcl-2 functions as an anti-oxidant to block apoptosis and that Bcl-2 protein levels and glutathione (GSH) intracellular concentration are coordinately regulated, with a decrease in either favoring cell death [71]. It is

Figure 4



E2 (17β-estradiol) regulation of survival pathways in wild-type MCF-7 cells and long-term estrogen-deprived MCF-7:5C and MCF-7:2A breast cancer cells. Cells were treated with 1 nM E2 for 72 hours, and protein lysates were analyzed by Western blot for p-Akt, Akt, and nuclear factor-kappa-B (NF-κB). β-actin was used as a loading control.

believed that one mechanism by which Bcl-2 may function as an anti-oxidant is through upregulation of GSH, leading to rapid detoxification of reactive oxygen species and inhibition of free radical-mediated mitochondrial damage. Bcl-2 also has the ability to shift the entire cellular redox potential to a more reduced state which is independent of its effect on GSH levels [72].

GSH is a water-soluble tripeptide composed of glutamine, cysteine, and glycine. It is the most abundant intracellular small-molecule thiol present in mammalian cells, and it serves as a potent intracellular anti-oxidant, protecting cells from toxins such as free radicals [73]. Changes in GSH homeostasis have been implicated in the etiology and progression of a variety of human diseases, including breast cancer [74], and studies have shown that elevated levels of GSH prevent apoptotic cell death whereas depletion of GSH facilitates apoptosis [75]. L-buthionine sulfoximine (BSO) is a specific γ-glutamylcysteine synthetase inhibitor that blocks the rate-limiting step of GSH biosynthesis and, in doing so, depletes the intracellular GSH pool in both cultured cells and whole animals [73].

Recently, we reported that GSH participates in retarding apoptosis in anti-hormone-resistant LTED MCF-7:2A human breast cancer cells and that depletion of this molecule by BSO, a potent inhibitor of GSH biosynthesis, sensitized these resistant cells to E2-induced apoptosis [34]. GSH levels were elevated approximately 60% in MCF-7:2A cells compared with wild-type MCF-7 cells and these cells failed to undergo apoptosis following 1 week of E2 treatment. In the presence of BSO (100 μM), however, 1 nM E2 caused a dramatic increase in apoptosis which was observed as early as 48 hours, with maximum induction observed at day 7. The apoptotic effect of E2 plus BSO in MCF-7:2A cells was associated with a marked decreased in Bcl-2 and phosphorylated Bcl-2 protein levels, mitochondrial membrane disruption and cytochrome *c* release, caspase 7 activation, and PARP cleavage [34]. It is important to note that the concentration of BSO (100 μM) used in this study is clinically achievable without significant side effects [76]. Furthermore, early-phase clinical trials of BSO at doses resulting in both peripheral and tumor GSH depletion show that BSO can be safely administered with melphalan (L-PAM) to patients with refractory disease [77,78]. Thus, it is possible that future clinical studies of BSO infusions combined with low-dose estrogen hold the promise of improving disease control for patients with anti-hormone-resistant ER-positive metastatic breast cancer.

c-Jun N-terminal kinase signaling pathway

There is also evidence that E2 induces apoptosis by regulating the c-Jun N-terminal kinase (JNK) pathway. JNKs are a group of MAPKs that bind the NH2-terminal activation domain of the transcription factor c-jun and phosphorylate c-jun on amino acid residues Ser-63 and Ser-73 [79]. JNKs are stimulated by multiple factors, including cytokines, DNA-damaging agents, and environmental stresses, and are important in controlling programmed cell death or apoptosis. The inhibition of JNKs has been shown to enhance chemotherapy-induced inhibition of tumor cell growth, suggesting that JNKs may provide a molecular target for the treatment of cancer [79]. Recently, Altiok and colleagues [80] reported that, under low growth-stimulated conditions, high concentrations (1 μM) of E2 induced apoptosis and concomitantly increased phosphorylation of c-jun in ER-positive MCF-7 breast cancer cells but not in ER-negative MDA-MB 231 cells, thus suggesting an ER-mediated event. Interestingly, when the JNK signaling pathway was disrupted by the JNK inhibitor SP600125, the ability of E2 to inhibit the growth of MCF-7 cells and to induce apoptosis was completely blocked. More recently, we reported that JNK activation (as measured by the increased levels of phospho-JNK1/2 and the JNK substrate phospho-c-Jun) was increased by low-dose E2 in the presence of BSO in anti-hormone-resistant MCF-7:2A cells [34]. While the exact mechanism by which JNK promotes apoptosis is not currently known, the phosphorylation of transcription factors such as c-jun and p53, as well as pro- and anti-apoptotic Bcl-2 family members, has been suggested to be of importance [81]. The treatment with BSO plus E2 markedly increased phosphorylated c-jun in MCF-7:2A cells and decreased phosphorylated Bcl-2 in these cells. These findings thus suggest that BSO plus E2 might mediate their apoptotic effect, in part, through activation of JNK.

Clinical exploitation of estrogen-induced apoptosis

Laboratory studies uniformly demonstrate that low concentrations of estrogen can cause apoptotic tumor cell death following profound estrogen deprivation with anti-hormones. The question that now needs to be answered is how can this new laboratory knowledge be translated into patient care?

Recently, Ellis and colleagues [42] reported that low-dose E2 (6 mg daily: 2 mg three times a day) produced a 25% response rate for patients with ER-positive AI-resistant advanced breast cancer. This number is slightly lower than the 31% objective response rate reported by Lonning and colleagues [40] with DES (5 mg three times a day) in post-menopausal women heavily pre-treated with endocrine therapy. The Lonning study [40] recruited patients with advanced breast cancer who were previously treated with exhaustive endocrine therapy. Of the 32 patients enrolled, four patients obtained complete response (CR) and six patients obtained partial response. In contrast, the Ellis study [42] recruited patients who were treated with an AI with 24 or more weeks of progression-free survival or who had a relapse after 2 or more years of adjuvant AI. Interestingly, there were no CRs in the Ellis study [42]. Clinical observations suggest that the duration of the post-menopausal period is one of the crucial factors affecting the success of low-dose estrogen therapy. In other words, the longer the estrogen deprivation period, the higher the likelihood of a response to low-dose estrogen. The fact that there were four CRs in the Lonning study [40] but none in the Ellis study [42] suggests the need for extensive estrogen blockade or withdrawal to get the best effects from low-dose estrogen.

Estrogen and bone remodeling

In addition to its role in female sexual development and reproductive physiology, estrogen plays a key role in bone cell metabolism. Estrogen contributes to the strength of a woman's skeleton by maintaining bone density. Bone is a dynamic tissue that is constantly being reshaped by osteoblasts, which build bone, and osteoclasts, which resorb bone [82]. This dynamic process is called remodeling. Osteoblasts are derived from pleiotropic mesenchymal stem cells in the bone marrow, whereas osteoclasts are multinuclear macrophage-like cells derived from hematopoietic stem cells also in the bone marrow. Bone resorption and deposition are tightly coupled, and their balance defines both bone mass as well as quality. The regulation of bone remodeling is complex; however, estrogen is thought to play a key role in this process [82]. Estrogen inhibits bone remodeling and bone resorption and enhances bone formation. Conversely, loss of estrogen, due to menopause or surgical oophorectomy, leads to an increased rate of remodeling and tilts the balance between bone resorption and formation in favor of the former [83]. Estrogen deficiency in post-menopausal women frequently leads to osteoporosis, the most common skeletal disorder. The imbalance in bone turnover that is induced by estrogen deficiency in women and female rodents can be ameliorated with estrogen/progestin hormone therapy or SERMs [84].

The main effect of estrogen on bone remodeling is to decrease activation frequency and subsequently decrease the numbers of osteoclasts and osteoblasts. Its effects on osteoclasts are mainly indirect and mediated by products secreted by the osteoblast. These products include RANK-L (the ligand of the receptor activator of NF-κB), colony-stimulating factor 1 (CSF-1), and osteoprotegerin (OPG). They regulate the differentiation of osteoclast precursors to osteoclasts and then modulate the activity of the mature osteoclasts and regulate its rate of apoptosis. Estrogen also decreases the secretion of the pro-inflammatory cytokines interleukin (IL) 1, IL-6, and TNF-α by marrow monocytes, resulting in decreased production of OPG and RANK-L by the osteoblasts, thereby decreasing the rate of production of osteoclasts, their activity, and their survival [82]. There is also evidence that estrogen has direct effects on osteoclast lineage cells. It induces apoptosis of these cells and it suppresses RANK-L-induced osteoclast differentiation by blocking RANK-L/macrophage colony-stimulating factor (M-CSF)-induced AP-1-dependent transcription through a reduction of c-jun activity [85]. Moreover, estrogen has been shown to inhibit the activity of mature osteoclasts through direct, receptor-mediated mechanisms. Indeed, a recent study by Nakamura and colleagues [86] reported that estrogen, acting via the ER-α, induced apoptosis in osteoclasts through activation of the Fas/FasL system and that this leads to suppression of bone resorption through truncating the short life span of differentiated osteoclasts.

Future perspective

Estrogen is a potent mitogen that stimulates cell proliferation and prevents cell death in breast cancer cells through activation of the ER. Paradoxically, estrogen is also capable of inducing tumor regression of hormone-dependent breast cancer in post-menopausal women who have been treated exhaustively with anti-hormones. Pre-clinical studies suggest that the evolution of anti-hormone resistance over years of therapy reconfigures the survival mechanism of the breast cancer cell so that estrogen no longer functions as a survival factor but as a death signal. It is this reconfiguration that helps to explain the 'two faces' of estrogen: the ability to stimulate growth and to induce death. Interestingly, estrogen also induces tumor regression in raloxifene-resistant endometrial tumors (G Balaburski and VC Jordan, personal communication) and it prevents bone loss by inducing apoptosis in osteoclasts, suggesting a universal principle.

Pre-clinical data [34] clearly show that it is possible to enhance the apoptotic effect of low-dose E2 by combining it with BSO. Hence, the combination of BSO and E2 could be used to improve the efficacy of E2 as an apoptotic agent if GSH depletion is fundamental to tumor cell survival. Phase I clinical trials of BSO at doses resulting in both peripheral and tumor GSH depletion show that BSO can be safely administered to patients with refractory disease. BSO was administered intravenously twice daily either alone or together with chemotherapy to cancer patients whose disease had progressed despite multiple lines of previous chemotherapy [78].

Inhibitors of survival pathways also have the ability to enhance the apoptotic/growth-inhibitory effects of E2. Several groups

have developed small-molecule inhibitors of Bcl-2 as anti-tumor agents [87]. These inhibitors encompass various drugs that bind the anti-apoptotic Bcl-2 family members with more or less efficacy. Oblimersen (Genasense; G3139) is an anti-Bcl-2 antisense oligonucleotide that has reached phase III clinical trials in combination therapy [88]. There are also natural inhibitors of Bcl-2, which include tea polyphenols such as catechins and theaflavins [89].

Conclusions

The discovery of a new biology of E2-induced apoptosis provides a unique signal transduction pathway to exploit in the treatment of metastatic breast cancer that has become refractory to exhaustive anti-hormone therapy. The clinical clues with the use of high-dose estrogen therapy have now been supported by a wealth of laboratory data defining apoptotic mechanisms. It is plausible to consider that the methodical evaluation of monoclonal antibodies and small-molecule tyrosine kinase inhibitors to prevent breast cancer survival could amplify the apoptotic actions of estradiol in a select group of patients. Indeed, if a study of the molecular biology of estrogen-induced apoptosis can define the mechanism precisely, then the molecules involved will become the target for a new drug group. These new drugs may be able to precipitate apoptosis in ER-negative breast tumors or indeed be used universally to treat cancer types other than breast cancer.

Competing interests

The authors declare that they have no competing interests.

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References

1. Russo IH, Russo J: **Role of hormones in mammary cancer initiation and progression.** *J Mammary Gland Biol Neoplasia* 1998, **3:**49-61.
2. Beatson CT: **On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases.** *Lancet* 1896, **2:**101-111.
3. Beral V: **Breast cancer and hormone-replacement therapy in the Million Women Study.** *Lancet* 2003, **362:**419-427.
4. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J; Writing Group for the Women's Health Initiative Investigators: **Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial.** *JAMA* 2002, **288:**321-333.
5. **Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group**. *Lancet* 1998, **351:**1451-1467.
6. Iwase H: **Current topics and perspectives on the use of aromatase inhibitors in the treatment of breast cancer.** *Breast Cancer* 2008, **15:**278-290.
7. Altucci L, Addeo R, Cicatiello L, Dauvois S, Parker MG, Truss M, Beato M, Sica V, Bresciani F, Weisz A: **17beta-Estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells.** *Oncogene* 1996, **12:**2315-2324.
8. Foster JS, Wimalasena J: **Estrogen regulates activity of cyclin-dependent kinases and retinoblastoma protein phosphorylation in breast cancer cells.** *Mol Endocrinol* 1996, **10:**488-498.
9. Pietras RJ, Marquez-Garban DC: **Membrane-associated estrogen receptor signaling pathways in human cancers.** *Clin Cancer Res* 2007, **13:**4672-4676.
10. Moriarty K, Kim KH, Bender JR: **Minireview: estrogen receptor-mediated rapid signaling.** *Endocrinology* 2006, **147:**5557-5563.
11. Pedram A, Razandi M, Levin ER: **Nature of functional estrogen receptors at the plasma membrane.** *Mol Endocrinol* 2006, **20:**1996-2009.
12. Stoica GE, Franke TF, Wellstein A, Czubayko F, List HJ, Reiter R, Morgan E, Martin MB, Stoica A: **Estradiol rapidly activates Akt via the ErbB2 signaling pathway.** *Mol Endocrinol* 2003, **17:**818-830.
13. Arpino G, Wiechmann L, Osborne CK, Schiff R: **Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance.** *Endocr Rev* 2008, **29:**217-233.
14. Gompel A, Somai S, Chaouat M, Kazem A, Kloosterboer HJ, Beusman I, Forgez P, Mimoun M, Rostene W: **Hormonal regulation of apoptosis in breast cells and tissues.** *Steroids* 2000, **65:**593-598.
15. Jordan VC: **The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer—survival or death?** *J Clin Oncol* 2008, **26:**3073-3082.
16. Jordan VC, Lewis JS, Osipo C, Cheng D: **The apoptotic action of estrogen following exhaustive antihormonal therapy: a new clinical treatment strategy.** *Breast* 2005, **14:**624-630.
17. Lewis JS, Cheng D, Jordan VC: **Targeting oestrogen to kill the cancer but not the patient.** *Br J Cancer* 2004, **90:**944-949.
18. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, Jordan VC: **Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation.** *J Natl Cancer Inst* 2005, **97:**1746-1759.
19. Santen R, Jeng MH, Wang JP, Song R, Masamura S, McPherson R, Santner S, Yue W, Shim WS: **Adaptive hypersensitivity to estradiol: potential mechanism for secondary hormonal responses in breast cancer patients.** *J Steroid Biochem Mol Biol* 2001, **79:**115-125.
20. Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, Yue W, Wang J, Santen RJ: **Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol.** *J Natl Cancer Inst* 2001, **93:**1714-1723.
21. Swaby RF, Jordan VC: **Low-dose estrogen therapy to reverse acquired antihormonal resistance in the treatment of breast cancer.** *Clin Breast Cancer* 2008, **8:**124-133.
22. Wolf DM, Jordan VC: **A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy.** *Recent Results Cancer Res* 1993, **127:**23-33.
23. Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, Jordan VC: **Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice.** *Clin Cancer Res* 2000, **6:**2028-2036.
24. Robertson CN, Roberson KM, Padilla GM, O'Brien ET, Cook JM, Kim CS, Fine RL: **Induction of apoptosis by diethylstilbestrol in hormone-insensitive prostate cancer cells.** *J Natl Cancer Inst* 1996, **88:**908-917.
25. Nilsen J, Mor G, Naftolin F: **Estrogen-regulated developmental neuronal apoptosis is determined by estrogen receptor subtype and the Fas/Fas ligand system.** *J Neurobiol* 2000, **43:**64-78.
26. Saintier D, Khanine V, Uzan B, Ea HK, de Vernejoul MC, Cohen-Solal ME: **Estradiol inhibits adhesion and promotes apoptosis in murine osteoclasts *in vitro*.** *J Steroid Biochem Mol Biol* 2006, **99:**165-173.
27. Okasha SA, Ryu S, Do Y, McKallip RJ, Nagarkatti M, Nagarkatti PS: **Evidence for estradiol-induced apoptosis and dysregulated T cell maturation in the thymus.** *Toxicology* 2001, **163:**49-62.

28. Jiang SY, Jordan VC: **Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor.** *J Natl Cancer Inst* 1992, **84:**580-591.
29. Levenson AS, Jordan VC: **Transfection of human estrogen receptor (ER) cDNA into ER-negative mammalian cell lines.** *J Steroid Biochem Mol Biol* 1994, **51:**229-239.
30. Ingle JN, Ahmann DL, Green SJ, Edmonson JH, Bisel HF, Kvols LK, Nichols WC, Creagan ET, Hahn RG, Rubin J, Frytak S: **Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer.** *N Engl J Med* 1981, **304:**16-21.
31. Lewis JS, Osipo C, Meeke K, Jordan VC: **Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal.** *J Steroid Biochem Mol Biol* 2005, **94:**131-141.
32. Song RX, Zhang Z, Mor G, Santen RJ: **Down-regulation of Bcl-2 enhances estrogen apoptotic action in long-term estradiol-depleted ER(+) breast cancer cells.** *Apoptosis* 2005, **10:**667-678.
33. Santen RJ, Song RX, Masamura S, Yue W, Fan P, Sogon T, Hayashi S, Nakachi K, Eguchi H: **Adaptation to estradiol deprivation causes up-regulation of growth factor pathways and hypersensitivity to estradiol in breast cancer cells.** *Adv Exp Med Biol* 2008, **630:**19-34.
34. Lewis-Wambi JS, Kim HR, Wambi C, Patel R, Pyle JR, Klein-Szanto AJ, Jordan VC: **Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis.** *Breast Cancer Res* 2008, **10:**R104.
35. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC: **Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer.** *J Natl Cancer Inst* 2003, **95:**1597-1608.
36. Zhang Y, Zhao H, Asztalos S, Chisamore M, Sitabkhan Y, Tonetti DA: **Estradiol-induced regression in T47D:A18/PKCalpha tumors requires the estrogen receptor and interaction with the extracellular matrix.** *Mol Cancer Res* 2009, **7:**498-510.
37. Haddow A, Watkinson JM, Paterson E: **Influence of synthetic oestrogens upon advanced malignant disease.** *BMJ* 1944, **2:**393-398.
38. Carter AC, Sedransk N, Kelley RM, Ansfield FJ, Ravdin RG, Talley RW, Potter NR: **Diethylstilbestrol: recommended dosages for different categories of breast cancer patients. Report of the Cooperative Breast Cancer Group.** *JAMA* 1977, **237:**2079-2078.
39. Cole MP, Jones CT, Todd ID: **A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474.** *Br J Cancer* 1971, **25:**270-275.
40. Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jorgensen LM, Mella O, Howell A: **High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy.** *Breast Cancer Res Treat* 2001, **67:**111-116.
41. Peethambaram PP, Ingle JN, Suman VJ, Hartmann LC, Loprinzi CL: **Randomized trial of diethylstilbestrol vs. tamoxifen in postmenopausal women with metastatic breast cancer. An updated analysis.** *Breast Cancer Res Treat* 1999, **54:**117-122.
42. Ellis MJ, Dehdahti F, Kommareddy A, Jamalabadi-Majidi S, Crowder R, Jeffe DB, Gao F, Fleming G, Silverman P, Dickler M, L Carey, Marcom PK: **A randomized phase 2 trial of low dose (6 mg daily) versus high dose (30 mg daily) estradiol for patients with estrogen receptor positive aromatase inhibitor resistant advanced breast cancer [abstract].** *Cancer Res* 2009, **69:**s67.
43. Degterev A, Boyce M, Yuan J: **A decade of caspases.** *Oncogene* 2003, **22:**8543-8567.
44. Hanahan D, Weinberg RA: **The hallmarks of cancer.** *Cell* 2000, **100:**57-70.
45. Ziegler DS, Kung AL: **Therapeutic targeting of apoptosis pathways in cancer.** *Curr Opin Oncol* 2008, **20:**97-103.
46. Peter ME, Krammer PH: **The CD95(APO-1/Fas) DISC and beyond.** *Cell Death Differ* 2003, **10:**26-35.
47. Tsujimoto Y: **Bcl-2 family of proteins: life-or-death switch in mitochondria.** *Biosci Rep* 2002, **22:**47-58.
48. Talapatra S, Thompson CB: **Growth factor signaling in cell survival: implications for cancer treatment.** *J Pharmacol Exp Ther* 2001, **298:**873-878.
49. Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, Loweth J, McKian K, De Los Reyes A, Wing L, Jordan VC: **Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*.** *J Natl Cancer Inst* 2003, **95:**1586-1597.
50. Chisamore MJ, Ahmed Y, Bentrem DJ, Jordan VC, Tonetti DA: **Novel antitumor effect of estradiol in athymic mice injected with a T47D breast cancer cell line overexpressing protein kinase Calpha.** *Clin Cancer Res* 2001, **7:**3156-3165.
51. Tonetti DA, Chisamore MJ, Grdina W, Schurz H, Jordan VC: **Stable transfection of protein kinase C alpha cDNA in hormone-dependent breast cancer cell lines.** *Br J Cancer* 2000, **83:**782-791.
52. Mor G, Kohen F, Garcia-Velasco J, Nilsen J, Brown W, Song J, Naftolin F: **Regulation of fas ligand expression in breast cancer cells by estrogen: functional differences between estradiol and tamoxifen.** *J Steroid Biochem Mol Biol* 2000, **73:**185-194.
53. Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A, Green DR: **DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1.** *Mol Cell* 1998, **1:**543-551.
54. Kavurma MM, Santiago FS, Bonfoco E, Khachigian LM: **Sp1 phosphorylation regulates apoptosis via extracellular FasL-Fas engagement.** *J Biol Chem* 2001, **276:**4964-4971.
55. Krishnan V, Wang X, Safe S: **Estrogen receptor-Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells.** *J Biol Chem* 1994, **269:**15912-15917.
56. Porter W, Saville B, Hoivik D, Safe S: **Functional synergy between the transcription factor Sp1 and the estrogen receptor.** *Mol Endocrinol* 1997, **11:**1569-1580.
57. Duan R, Porter W, Safe S: **Estrogen-induced c-fos protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation.** *Endocrinology* 1998, **139:**1981-1990.
58. Sun G, Porter W, Safe S: **Estrogen-induced retinoic acid receptor alpha 1 gene expression: role of estrogen receptor-Sp1 complex.** *Mol Endocrinol* 1998, **12:**882-890.
59. Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson JA, Safe S: **Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements.** *J Biol Chem* 2000, **275:**5379-5387.
60. Kang MH, Reynolds CP: **Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy.** *Clin Cancer Res* 2009, **15:**1126-1132.
61. O'Brien S, Moore JO, Boyd TE, Larratt LM, Skotnicki A, Koziner B, Chanan-Khan AA, Seymour JF, Bociek RG, Pavletic S, Rai KR: **Randomized phase III trial of fludarabine plus cyclophosphamide with or without oblimersen sodium (Bcl-2 antisense) in patients with relapsed or refractory chronic lymphocytic leukemia.** *J Clin Oncol* 2007, **25:**1114-1120.
62. Bellacosa A, Testa JR, Staal SP, Tsichlis PN: **A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region.** *Science* 1991, **254:**274-277.
63. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME: **Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery.** *Cell* 1997, **91:**231-241.
64. del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G: **Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt.** *Science* 1997, **278:**687-689.
65. John S, Nayvelt I, Hsu HC, Yang P, Liu W, Das GM, Thomas T, Thomas TJ: **Regulation of estrogenic effects by beclin 1 in breast cancer cells.** *Cancer Res* 2008, **68:**7855-7863.
66. Garcia-Echeverria C, Sellers WR: **Drug discovery approaches targeting the PI3K/Akt pathway in cancer.** *Oncogene* 2008, **27:**5511-5526.
67. Ghosh S, May MJ, Kopp EB: **NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses.** *Annu Rev Immunol* 1998, **16:**225-260.
68. Dutta J, Fan Y, Gupta N, Fan G, Gelinas C: **Current insights into the regulation of programmed cell death by NF-kappaB.** *Oncogene* 2006, **25:**6800-6816.
69. Stoffel A, Levine AJ: **Activation of NF-kappaB by the API2/MALT1 fusions inhibits p53 dependant but not FAS induced apoptosis: a directional link between NF-kappaB and p53.** *Cell Cycle* 2004, **3:**1017-1020.
70. Lobanova YS, Scherbakov AM, Shatskaya VA, Evteev VA,

Krasil'nikov MA: **NF-kappaB suppression provokes the sensitization of hormone-resistant breast cancer cells to estrogen apoptosis.** *Mol Cell Biochem* 2009, **324:**65-71.
71. Voehringer DW: **BCL-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity.** *Free Radic Biol Med* 1999, **27:**945-950.
72. Ellerby LM, Ellerby HM, Park SM, Holleran AL, Murphy AN, Fiskum G, Kane DJ, Testa MP, Kayalar C, Bredesen DE: **Shift of the cellular oxidation-reduction potential in neural cells expressing Bcl-2.** *J Neurochem* 1996, **67:**1259-1267.
73. Anderson CP, Tsai JM, Meek WE, Liu RM, Tang Y, Forman HJ, Reynolds CP: **Depletion of glutathione by buthionine sulfoxine is cytotoxic for human neuroblastoma cell lines via apoptosis.** *Exp Cell Res* 1999, **246:**183-192.
74. Schroder CP, Godwin AK, O'Dwyer PJ, Tew KD, Hamilton TC, Ozols RF: **Glutathione and drug resistance.** *Cancer Invest* 1996, **14:**158-168.
75. Schnelldorfer T, Gansauge S, Gansauge F, Schlosser S, Beger HG, Nussler AK: **Glutathione depletion causes cell growth inhibition and enhanced apoptosis in pancreatic cancer cells.** *Cancer* 2000, **89:**1440-1447.
76. Bailey HH: **L-S,R-buthionine sulfoximine: historical development and clinical issues.** *Chem Biol Interact* 1998, **111-112:** 239-254.
77. Bailey HH, Mulcahy RT, Tutsch KD, Arzoomanian RZ, Alberti D, Tombes MB, Wilding G, Pomplun M, Spriggs DR: **Phase I clinical trial of intravenous L-buthionine sulfoximine and melphalan: an attempt at modulation of glutathione.** *J Clin Oncol* 1994, **12:**194-205.
78. Bailey HH, Ripple G, Tutsch KD, Arzoomanian RZ, Alberti D, Feierabend C, Mahvi D, Schink J, Pomplun M, Mulcahy RT, Wilding G: **Phase I study of continuous-infusion L-S,R-buthionine sulfoximine with intravenous melphalan.** *J Natl Cancer Inst* 1997, **89:**1789-1796.
79. Vasilevskaya I, O'Dwyer PJ: **Role of Jun and Jun kinase in resistance of cancer cells to therapy.** *Drug Resist Updat* 2003, **6:** 147-156.
80. Altiok N, Koyuturk M, Altiok S: **JNK pathway regulates estradiol-induced apoptosis in hormone-dependent human breast cancer cells.** *Breast Cancer Res Treat* 2007, **105:**247-254.
81. Park J, Kim I, Oh YJ, Lee K, Han PL, Choi EJ: **Activation of c-Jun N-terminal kinase antagonizes an anti-apoptotic action of Bcl-2.** *J Biol Chem* 1997, **272:**16725-16728.
82. Riggs BL, Khosla S, Melton LJ 3rd: **Sex steroids and the construction and conservation of the adult skeleton.** *Endocr Rev* 2002, **23:**279-302.
83. Teitelbaum SL: **Osteoclasts: what do they do and how do they do it?** *Am J Pathol* 2007, **170:**427-435.
84. Delmas PD, Ensrud KE, Adachi JD, Harper KD, Sarkar S, Gennari C, Reginster JY, Pols HA, Recker RR, Harris ST, Wu W, Genant HK, Black DM, Eastell R; Mulitple Outcomes of Raloxifene Evaluation Investigators: **Efficacy of raloxifene on vertebral fracture risk reduction in postmenopausal women with osteoporosis: four-year results from a randomized clinical trial.** *J Clin Endocrinol Metab* 2002, **87:**3609-3617.
85. Srivastava S, Toraldo G, Weitzmann MN, Cenci S, Ross FP, Pacifici R: **Estrogen decreases osteoclast formation by down-regulating receptor activator of NF-kappa B ligand (RANKL)-induced JNK activation.** *J Biol Chem* 2001, **276:**8836-8840.
86. Nakamura T, Imai Y, Matsumoto T, Sato S, Takeuchi K, Igarashi K, Harada Y, Azuma Y, Krust A, Yamamoto Y, Nishina H, Takeda S, Takayanagi H, Metzger D, Kanno J, Takaoka K, Martin TJ, Chambon P, Kato S: **Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts.** *Cell* 2007, **130:**811-823.
87. Zeitlin BD, Zeitlin IJ, Nor JE: **Expanding circle of inhibition: small-molecule inhibitors of Bcl-2 as anticancer cell and antiangiogenic agents.** *J Clin Oncol* 2008, **26:**4180-4188.
88. Stein CA, Benimetskaya L, Mani S: **Antisense strategies for oncogene inactivation.** *Semin Oncol* 2005, **32:**563-572.
89. Leone M, Zhai D, Sareth S, Kitada S, Reed JC, Pellecchia M: **Cancer prevention by tea polyphenols is linked to their direct inhibition of antiapoptotic Bcl-2-family proteins.** *Cancer Res* 2003, **63:**8118-8121.

Potential of Selective Estrogen Receptor Modulators as Treatments and Preventives of Breast Cancer

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Abstract: Estrogen plays vital roles in human health and diseases. Estrogen mediates its actions almost entirely by binding to estrogen receptors (ER), alpha and beta which further function as transcription factors. Selective estrogen receptor modulators (SERMs) are synthetic molecules which bind to ER and can modulate its transcriptional capabilities in different ways in diverse estrogen target tissues. Tamoxifen, the prototypical SERM, is extensively used for targeted therapy of ER positive breast cancers and is also approved as the first chemo-preventive agent for lowering breast cancer incidence in high risk women. The therapeutic and preventive efficacy of tamoxifen was initially proven by series of experiments in the laboratory which laid the foundation of its clinical use. Unfortunately, use of tamoxifen is associated with de-novo and acquired resistance and some undesirable side effects. The molecular study of the resistance provides an opportunity to precisely understand the mechanism of SERM action which may further help in designing new and improved SERMs. Recent clinical studies reveal that another SERM, raloxifene, which is primarily used to treat post-menopausal osteoporosis, is as efficient as tamoxifen in preventing breast cancers with fewer side effects. Overall, these findings open a new horizon for SERMs as a class of drug which not only can be used for therapeutic and preventive purposes of breast cancers but also for various other diseases and disorders. Major efforts are therefore directed to make new SERMs with a better therapeutic profile and fewer side effects.

Key Words: Breast cancer, osteoporosis, estrogen receptor, tamoxifen, raloxifene, SERMs, endocrine therapy, drug resistance.

1. INTRODUCTION

Breast cancer incidences and death rates have dropped significantly during recent years, which is associated strongly with improvement in early detection methods and decrease of menopausal hormone replacement therapy (HRT) [1, 2]. HRT, in the form of estrogen alone or estrogen plus progesterone, had been widely used since the 1960s until recent years, to treat conditions associated with aging as well as unpleasant menopausal symptoms. HRT was also known to protect post-menopausal women from osteoporosis and also thought to protect women from heart disease and Alzheimer's disease. However, the Women's Health Initiative (WHI) study indicated that taking estrogen with or without progesterone for 5 or more years placed the women at higher risk of breast cancer, Alzheimer's disease, heart disease, blood clot and stroke, although HRT is effective to reduce the risks of osteoporosis and colon cancer [3, 4]. The Million Women Study (MWS) conducted in the UK also showed that women taking HRT were more likely to develop breast cancer [5], endometrial cancer [6] and ovarian caner [7]. In the US, the use of estrogen-plus-progestin HRT has dropped almost 50% when the WHI announced their findings in 2002, and this was followed by a sharp 7% decrease of new breast cancers in 2003 [2]. Although the decrease of HRT uses is not the sole reason leading to less breast cancer incidences, much effort has been focused on finding more effective and safer compounds to replace HRT which not only relieve menopausal symptoms but also prevent and treat hormone-responsive cancers. One most promising approach is to use selective estrogen receptor modulators (SERMs).

SERMs are synthetic compounds that bind to estrogen receptors alpha and/or beta (ERα and/or ERβ) and exert estrogenic or anti-estrogenic activities in a tissue/cell-specific manner. The first SERM that has been used successfully in the clinic to prevent and treat breast cancer is tamoxifen, a failed postcoital contraceptive that evolved into the "gold standard" for breast cancer treatment [8, 9]. Tamoxifen is estimated to have saved the lives of over 400,000 women with breast cancer [8]. The second generation SERM, raloxifene (formally called keoxifene), failed as a treatment for breast cancer but is effective against osteoporosis and prevents breast cancer at the same time. Raloxifene is as effective as tamoxifen to reduce invasive breast cancer risks without an increase in the risk of endometrial cancer observed with tamoxifen [10]. Indeed a recent study suggests that raloxifene might even be effective in preventing endometrial cancer [11]. These findings have acted as a catalyst for the search of new SERMs which are estrogen-like in bones and circulating lipids but antiestrogenic in women's reproductive organs and therefore are anti-cancer agents. However, there are problems associated with the current SERMs such as drug resistance and side effects. For example, both tamoxifen and raloxifene increase both hot flashes and blood clots [12].

Besides SERMs, other endocrine therapies target the ER indirectly to prevent and treat breast cancer. Aromatase inhibitors (AIs) that block the synthesis of estrogen from androgen in peripheral tissues have been extensively studied and show efficacy equivalent or superior to tamoxifen to treat postmenopausal breast cancer [13]. Since the mechanism for AIs to treat ER-positive breast cancer is to deplete estrogen in postmenopausal patients, they do not increase risks of endometrium cancer or blood clot and may be a better choice for postmenopausal breast cancer patients than tamoxifen. However, AIs are not effective in premenopausal women with actively functioning ovaries because AIs do not inhibit ovarian estrogen production. In addition, AIs lack the estrogenic protective function for cardiovascular diseases or osteoporosis. As a result, the side effects of AIs are mostly consistent with estrogen deprivation. AIs are associated with a greater incidence of bone loss and musculoskeletal symptoms, and probably higher risk of cardiovascular disease suggested by adjuvant trials comparing AIs and tamoxifen [14]. However, AIs are associated with a lower incidence of gynecological symptoms, thromboembolic diseases and hot flashes compared to tamoxifen in adjuvant setting [14]. Another strategy is to use selective estrogen receptor down-regulators (SERDs), such as fulvestrant, that cause degradation of ERs. Fulvestrant has been approved to treat advanced breast cancer after tamoxifen failure, and a recent phase III trial indicated that fulvestrant and AI exemestane were equally effective with a similar safety profile [15].

Resistance is a common problem associated with endocrine therapy therefore alternative treatment strategies without cross-resistance are necessary. Compared to the pure anti-estrogenic actions

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like AIs or fulvestrant, an ideal SERM with beneficial estrogenic effects has great potential for breast cancer prevention and treatment, especially in postmenopausal women as they often suffer from unpleasant symptoms resulting from lower estrogen. A perfect SERM would reduce the risk of breast cancer, ovarian cancer and uterine cancer, as well as strengthen the bone, prevent coronary heart disease, strokes and Alzheimer's disease, and relieve menopausal discomfort like hot flashes and vaginal atrophy [12].

The complicated outcome of SERMs action cannot just be explained by turning on or off the ERs and their downstream genes. Although much new knowledge is being developed, we are still evolving in our understanding of the detailed mechanism of SERMs and their interaction with the ERs. In the past decade, another group of protein factors, nuclear receptor coregulators, have been identified that are essential for modulating the functions of SERMs and ERs. In this article, we will review the evolving understanding of the molecular mechanisms of SERMs action in the context of other signal transduction pathways and nuclear receptor coregulators, as well as the problems associated with the application of SERMs as a treatment or preventative for breast cancer. Finally, the new SERMs with potential as new agents to treat or prevent breast cancer will be described.

2. MECHANISM OF ESTROGEN ACTION

2.1. Structure and Function of ER

The existence of estrogen binding protein was first predicted by Elwood Jensen and colleagues in early 1960's [16]. The first ER cDNA, now known as ERα, was later cloned in the mid-1980's [17, 18]. In 1996, an additional ER was cloned from rat prostate [19] and designated as ERβ. The action of estrogen in cells is therefore almost entirely mediated by these two related but distinct subtype of estrogen receptors, ERα and ERβ. Both receptors function as ligand activated transcription factors which can bind the cognate DNA sequences known as estrogen responsive elements (ERE), and activate transcription. The ER proteins can be structurally subdivided into six domains on the basis of the functions controlled by the region, as shown in Fig. (**1**). The A/B domain contains one of the two transcriptional activation functions (AFs), designated as AF1 which is involved in estrogen-independent activation of transcription. Another activation function domain, AF2, is located in the E domain which also harbors the ligand binding domain (LBD), and is involved in estrogen/ ligand dependent activation [20, 21]. The ERβ has 97% homology in the DBD and 61% homology in the LBD with ERα suggesting differential ligand binding capability of ERs [21].

SERMs, the molecules which can bind to ERα and/or β and can either stimulate estrogen-like actions (agonist) or oppose estrogen actions (antagonist) in various estrogen target tissues and cells. This pharmacologic knowledge advanced studies to decipher the details of the molecular mechanism of estrogen action in different cell and tissue types.

The structural studies of a SERM complexed with the LBD of ERα and ERβ reveal that re-orientation of the AF2 helix (helix 12) after the binding of the SERM to the hydrophobic pocket of the LBD [22, 23]. The interaction of amino acid Asp351 of ERα with the alkylaminoethoxyphenyl side chain of tamoxifen or raloxifene is crucial to prevent the recruitment of coactivators to the SERM-receptor complex surface [22, 23]. Using different mutants of ERα for the amino acid Asp351, it was shown that shielding and neutralization of Asp351 by the side chain of raloxifene is critical in defining the antiestrogenicity of this SERM. Furthermore, it has been shown that changing the Asp351 from aspartate to glycine (D351G) abolishes the estrogen-agonist activity of the tamoxifen-ER complex, while retaining its antagonistic property. The AF2 region of the agonist-bound receptor is particularly important for the interactions of steroid receptor coactivators (SRCs 1-3) *via* the interacting amino acid motif LxxLL, known as nuclear receptor interacting domain (NRID). It is important to note that the affinity of ERs for these NRIDs of SRCs is highly dependent upon the ER subtype, α and β, and ligand bound to the ER [24-26]. Recruitment

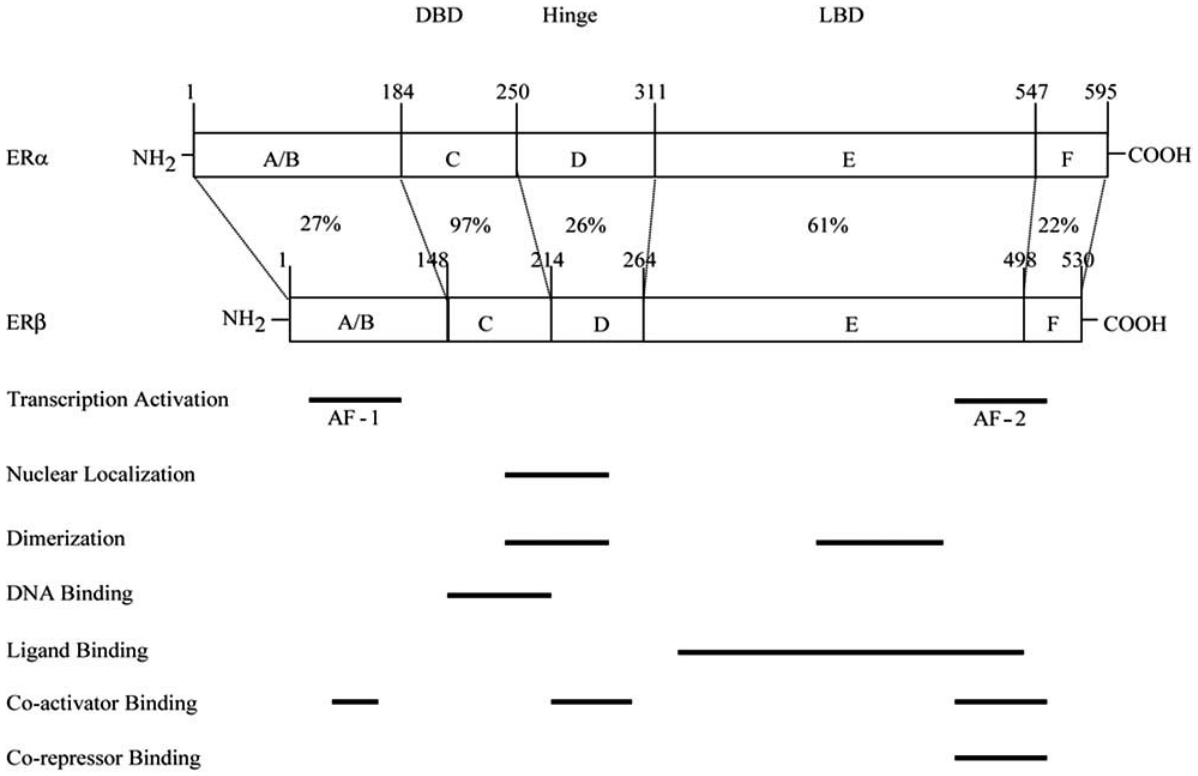


Fig. (1). Schematic comparison of human ER-α and ER-β structure. The structural domains are shown, and the percentage of amino acid identity shared by the two ERs is indicated for each domain. The horizontal bars highlight areas of different functions.

of these co-activator(s) is also responsible for facilitating the activation of estrogen responsive genes by modifying the chromatin structure and activating the transcriptional machinery. Additionally, SERMs may also show differential AF1 activity mediated by co-repressor binding. Using ERE-reporter constructs, it has been shown that the AF1 domain of ERα is actively involved in agonist-induced gene expression whereas the AF1 domain of ERβ is involved very weakly [27].

Estrogen can also modulate the expression of genes by another mechanism in which the receptor complex can interact with other transcription factors such as activating protein 1 (AP1) or stimulating protein 1 (Sp1) through a process known as a tethering mechanism. Intriguing differences are observed in the mechanism of action between ERα and ERβ through an AP1 site. In the presence of estrogen, ERα induces AP1 driven reporter activity but ERβ has no effect [28]. The raloxifene bound ERβ complex can induce transcriptional activity through the AP1 site but the activity through ERα bound to raloxifene is negligible.

ERs also act in a non-genomic manner initiated from the cell membrane. These actions are very fast (seconds to minutes) and occur without RNA or protein synthesis. They often mobilize second messenger molecules such as Ca^{2+} and cAMP, and are associated with protein kinase cascades such as PI3K/Akt and MAPK [29, 30]. Several explanations have been offered to explain these effects. There could be a subpopulation of nuclear ERs associate with the plasma membrane, either through posttranslational modification such as palmitoylation or mediated by scaffold proteins such as caveolin-1 and MNAR, since ERs do not have a transmembrane domain [29, 30]. Another membrane bound protein, G protein coupled receptor GPR30, was identified in recent years that mediates non-genomic actions of estrogen [31, 32]. The cellular localization of GPR30 is still controversial. Some evidence suggests it is at plasma membrane [33, 34] and other evidence suggests it is in the endoplasmic reticulum [32]. GPR30 binds to 17β-estradiol, tamoxifen and fulvestrant with high affinity [33] and is associated with breast cancer metastasis and transactivation of the epidermal growth factor receptor (EGFR) [35].

2.2. Co-Regulators

The co-regulators are protein molecules which can physically interact with the liganded or un-liganded ERs and modulate the transcription of the genes. The transcriptional activation or repression of the responsive genes is a combinatorial function of ligand-receptor interaction, recognition of cognate DNA sequence and recruitment of specific co-regulators onto the promoter of the gene. The assembly of the whole transcriptional complex is also dependent upon the affinity of the above mentioned individual components among themselves and their relative concentrations in the cell. Co-regulators play defining roles in the final tissue outcome in terms of transcriptional activation or repression mediated by estrogen or SERMs. The co-regulators can be broadly classified on the basis of their function, as co-activators which promote the activation of the transcriptional process, or co-repressors which are associated with repression of transcription of genes (Fig. (**2**)).

2.2.1. Co-Activators

Presently, around 200 co-activators are known, which are associated with 48 nuclear receptors [36]. The family of p160 proteins known as steroid receptor co-activators (SRCs) have been studied extensively. The relative abundance of SRC1 in uterine cells is responsible for the agonistic activity of tamoxifen, whereas in breast cancer cells, with low SRC1 levels, tamoxifen acts as an estrogen antagonist [37]. However, raloxifene, another related SERM, does not recruit SRC-1 even in the uterine cells [37], suggesting that the interaction with specific ligand which elicits a unique conformation of the receptor is critical for the interaction of co-regulators. These observations further provide an explanation for the earlier studies, where tamoxifen have been reported to induce growth of endometrial cancer cells but not of breast cancer cells in athymic mice [38] and also that estrogen agonistic properties of raloxifene is less in endometrial cancer cells [39]. These finding also translate very well to clinical experience [40]. In addition, the SERMs can enhance the stability of the co-activators (SRC1 and SRC3) and thereby influence the transcriptional capability of other nuclear receptors [41]. Post-translational modifications of the co-activators, including but not limited to phosphorylation, methylation, ubiquitylation, sumoylation and acetylation, can also regulate the gene activation by influencing the ability of the co-activators to interact with ER and other components of the transcriptional complex [34-36]. The understanding of structure-function relationship of ligands at the ER has formed the basis of designing effective new SERMs with fewer side effects.

2.2.2. Co-Repressors

Co-repressors are functional counterparts of co-activators, which are associated with transcriptionally inactive promoters and help repress the expression of genes [42]. Fewer co-repressors have been reported compared to the co-activators. In the case of ER, the co-repressors are known to interact with the un-liganded and/or antagonist bound receptor. The two most extensively studied co-repressors in connection with ER are Nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). The ER bound to raloxifene or 4-hydroxytamoxifen (a potent antagonist metabolite of tamoxifen) is known to recruit NCoR and SMRT to the promoters of estrogen responsive genes and repress transcription [43-45]. It has been shown that inhibition of NCoR or SMRT with monoclonal antibodies can enhance the agonistic property of 4-hydroxytamoxifen [46]. Moreover, using fibroblasts from NCoR null mice, 4-hydroxytamoxifen was shown to be a relatively potent ERα agonist [47]. The critical role of NCoR and SMRT in 4-hydroxytamoxifen-induced arrest of cell proliferation of ERα positive breast cancer cells is confirmed because 4-hydroxytamoxifen-stimulated cell cycle progression now occurs in NCoR-and-SMRT-deficient breast cancer cells [48]. However not all estrogen responsive genes are activated by 4-hydroxytamoxifen in NCoR and SMRT deficient cells, clearly indicating that additional molecules are important in SERM-induced repression of estrogen responsive genes. Indeed, there are several other co-repressor proteins known for ER. Metastasis associated protein 1 (MTA1) is a corepressor found to mediate the ER transcriptional repression [49]. Another corepressor, known as repressor of estrogen action (REA) potentiates the inhibitory effects of anti-estrogens including 4-hydroxytamoxifen. Additionally, REA interacts with ER and competes with the co-activator SRC1 for binding to the estrogen bound ER [50, 51]. This again emphasizes the fact that the relative levels of co-regulators may be important in deciding the outcome of the SERM action. The proteasomal regulation of NCoR is another factor which may influence the SERM action. Degradation of NCoR occurs through the 26S proteasome, which is mediated by seven in absentia homologue 2 (Siah2) [52]. Interestingly, estrogen mediated upregulation of Siah2 in ER positive breast cancer cells has been implicated in the proteasomal degradation of NCoR, and subsequent de-repression of NCoR regulated genes [53].

In addition to acting as a "transcriptional adapter" between the receptors and the transcriptional machinery, the coregulator itself or its complex possess various enzymatic activities such as acetylation, phosphorylation, methylation or de-acetylation by which they are able to modify the local chromatin structure thereby making the local environment conducive for gene expression or repression. Intrinsic histone acetyl transferase activity was found to be associated with co-activator SRC1 which helps in the activation of transcriptional expression [54]. In contrast, the 4-hydroxytamoxifen bound ER complex which recruits the co-repressors NCoR and SMRT is associated with histone de-acetylases and other chromatin modifying enzymes [37, 55]. The deacetylase activity promotes

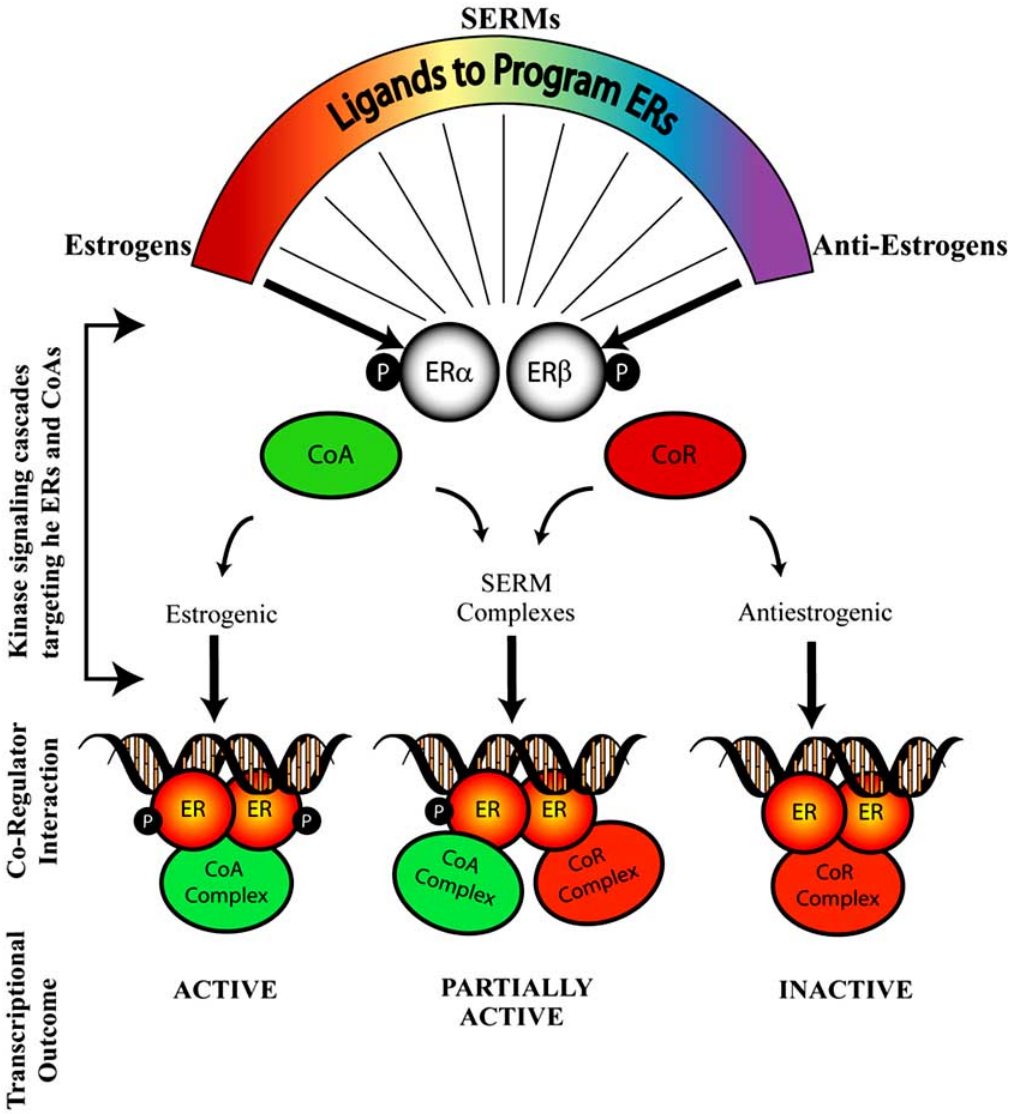


Fig. (2). Schematic representation of different liganded-ER complexes interacting with co-regulators and consequent transcriptional activities. ERs that bind to estrogenic ligands interact with co-activators (CoA) and activate transcription. Anti-estrogen liganded-ER complexes interact with co-repressors (CoR) and inactivate transcription of responsive genes. Selective estrogen receptor modulators (SERMs) bind to ERs and interact with either co-activator or co-repressor complexes eliciting partial transcriptional activity depending upon the cellular context.

transcriptional repression [37, 55]. Interestingly, another enzyme in the co-activator complex, CARM1 (coactivator associated arginine methyltransferase 1) has recently been implicated in modifying the coactivator itself and inducing the degradation of the complex [56]. This suggests the ability of the enzymes in the complex to modify other proteins in its own complex apart from a role in the modification of chromatin.

With this background of the molecular biology of SERM action, it is now appropriate to describe our evolving understanding about drug resistance. This is important not only because tumor drug resistance is the consequence of long term SERM administration, but also because new knowledge will aid patients with the development of novel treatment strategies for SERM-resistant breast cancer.

3. DRUG RESISTANCE TO SERMS

There are three types of resistance to SERMs based on the mechanism: metabolic resistance, intrinsic resistance and acquired resistance [57].

3.1. Metabolic Resistance

Metabolic resistance to tamoxifen is mostly related to CYP2D6, an enzyme product that metabolizes tamoxifen into its active forms 4-hydroxytamoxifen and endoxifen [58]. This has been extensively reviewed recently and will only be briefly mentioned here [13, 59]. CYP2D6 is genetically polymorphic and 5-8% of Caucasian subjects are CYP2D6 “poor metabolizers” thus are less likely to benefit from tamoxifen treatment, although it has been shown that these women tolerate tamoxifen better and tend to remain on the drug for longer [59]. The genotype of CYP2D6 has been shown in multiple clinical trials to be directly related to the outcome of tamoxifen use, however, the results are not always consistent. Eight studies indicated that CYP2D6 “poor metabolizer” genotypes have worse outcome of breast cancer patients who received tamoxifen but two studies contradicted this conclusion [60]. In addition to the genotype of CYP2D6, it is important to consider that other drugs may interact with the enzyme system and block the metabolic activation of tamoxifen. Unfortunately, selective serotonin reuptake inhibitors (SSRIs) that are used to relieve the menopausal side effects of ta-

moxifen are also metabolized by CYP2D6 and block the metabolic activation of tamoxifen. The proper choice of SSRI is therefore important so as not to impair tamoxifen metabolism. The SSRI of choice is venlafaxine that has only a low affinity for the CYP2D6 enzyme [61]. Although these emerging data about CYP2D6 genotypes and the drug interaction between tamoxifen and SSRIs are important, it is perhaps too early to use CYP2D6 status to routinely choose between tamoxifen and aromatase inhibitors to treat postmenopausal women with breast cancer. At present, an international consortium is evaluating the overall CYP2D6 status of completed clinical trials with tamoxifen to assemble a large scale retrospective analysis of the worth of genotyping. The aim is to answer the question of whether "poor metabolizers" should avoid tamoxifen use.

3.2. Intrinsic Resistance

Approximately 30% ER-positive breast cancer patients do not respond to tamoxifen [62]. This type of resistance is referred to as "de novo" resistance or intrinsic resistance. Clinical studies showed that only 40% patients with ER-positive, progesterone receptor (PR)-negative breast cancers are responsive to anti-estrogen treatment (tamoxifen or endocrine ablation) compared to 80% responsive rate in ER-and-PR-positive patients [58, 59]. Historically, the status of PR has been regarded as an indicator of a functional ER pathway, since expression of PR is regulated by estrogen. On the other hand, recent evidence suggested that the absence of PR is associated with excessive growth factor signaling such as overexpression of HER2 [63, 64], which has been known to impair estrogen induction of PR and reduce the effectiveness of tamoxifen treatment for breast cancer [65]. However, the negative association between PR and HER2 seems more evident in older women (> 45 yrs) [66] and it remains controversial that PR-status could be used for clinical decision on choosing between tamoxifen or AIs [67].

Growth factor signaling, especially through epidermal growth factor (EGF) pathway, has been studied extensively in the past two decades and linked to SERM resistance. This has been recently reviewed [68] and will only be briefly summarized here. EGF binds to ErbB family of cell surface receptors that include four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4). Overexpression of HER2 has been clinically linked to less response to endocrine therapies and worse prognosis [69-71], so has the overexpression of EGFR [72]. Different ErbB family members can form heterodimers and activate multiple signalling pathways including PI3K/Akt and MAPK. The major molecular mechanisms leading to SERM resistance can be summarized as follows: 1. Activation of downstream kinase cascade results in the phosphorylation of ER at key residues (Ser106/107, 118, 167, 305 and Thr 311) which activates transcription in a ligand-independent manner. Phosphorylation may change the binding of ER with ligands, DNA and coregulators, which may ultimately alter the activity of SERMs [73]. For example, phosphorylation of ER at Ser167 by Akt and Ser118 by the MAPK pathway both cause ligand-independent activation [74-76]. A recent study showed that phosphorylation of ER at Ser305 altered the orientation between the C-terminus of ER and SRC-1 that led to the recruitment of ER transcription coactivators and RNA polymerase II even in the presence of tamoxifen [77]. 2. Phosphorylation of ER co-regulators is equally important as the phosphorylation of ER itself, since phosphorylated co-activators have increased activity in the presence of SERMs [78-80]. Phosphorylation of co-repressors such as SMRT is associated with the co-repressor's nuclear export and impaired transcriptional suppressing function [81]. 3. Other than enhancing the transcriptional activity of the ER by phosphorylation, overexpression of EGFR or HER2 increases the non-genomic actions of ER, and SERMs may now act as estrogen agonists *via* the membrane effects of ER [82, 83]. In addition to the EGF signal pathway, the insulin-like growth factor (IGF) signal pathway is also involved in tamoxifen resistance [84]. It can activate PI3K/Akt pathway [71] and turn on genes that are otherwise activated by estrogen [85, 86].

Dysregulation of ER co-regulators is another major contributor to intrinsic SERM resistance. Overexpression of both AIB1 (SRC-3, ACTR, p/CIP, RAC3, TRAM-1) and HER2 have been shown to convert tamoxifen into an estrogen agonist in breast cancer cells [79]. Elevated AIB1 was found to associate with tamoxifen resistance, DNA-nondiploidy, high S-phase fraction and HER2 amplification in samples from clinical study [87]. Although a study indicated that high expression of AIB1 was not associated with relapse during tamoxifen treatment [88], AIB1 was shown to associate with tamoxifen resistance in breast cancers that overexpressed ErbB family proteins [88, 89]. AIB1 might be a predictor marker for tamoxifen ineffectiveness in ER-positive, HER2-positive and PR-negative breast cancer. On the other hand, low expression of ER co-repressor NcoR is associated with shorter relapse-free survival in breast cancer patients who only received tamoxifen after surgery [90]. Based on the emerging importance of co-regulators and tamoxifen resistance, one novel approach to overcome tamoxifen resistance is by the use of disulfide benzamide (DIBA) to disrupt the zinc finger in the ERα DNA binding domain. The approach facilitates ERα dissociation from coactivator AIB1 and concomitant association of corepressor NcoR without changing the phosphorylation of HER2, MAPK, Akt or AIB1 [91].

Another group of regulators associated with tamoxifen resistance are microRNAs (miRNA). These are naturally occurring single-stranded RNAs with the length of 21-23 nucleotides that do not code for proteins. They regulate gene expression mainly by inducing target mRNA degradation or inhibiting translation (protein synthesis). Dysregulation of miRNAs is associated with many cancers including breast cancer [92, 93]. Two recent studies show that miRNA-221/222 are upregulated in tamoxifen-resistant breast cancer cells and primary tumors, and they may contribute to tamoxifen resistance by down-regulating p27Kip1 or ERα [94, 95].

3.3. Acquired Resistance

Breast cancer patients who initially respond to tamoxifen later develop "acquired resistance" that is characterized by tamoxifen stimulated growth. This can be replicated in the laboratory with MCF-7 xenograft tumors implanted in ovariectomized athymic mice. Tamoxifen initially inhibits estrogen stimulated tumor growth but eventually some tumors start to grow during tamoxifen therapy [96]. These tumors now grow in response to either estrogen or tamoxifen and stop growing with no treatment or during treatment with fulvestrant [96]. The laboratory model is consistent with the clinic observation that aromatase inhibitor or fulvestrant are equally effective after the failure of tamoxifen treatment [97, 98]. It therefore appears that ER remains fully functional in the laboratory model of acquired tamoxifen resistance. In clinical studies, only 17-28% patients with acquired tamoxifen resistance have a loss of ER function [99, 100], and it is more likely that acquired resistance is associated with the stimulation of other growth/survival pathways [101]. For example, activated mammalian target of rapamycin (mTOR, downstream of PI3K/Akt and MAPK pathway) and c-Src (downstream of EGFR/HER2) were observed in breast cancer cells and mTOR and c-Src inhibitors can restore tamoxifen sensitivity in these cells, respectively [102, 103]. Several genes involved in cell proliferation and survival have altered expression level in breast cancer cells with acquired tamoxifen resistance. Examples of genes which down regulation is associated with acquired tamoxifen resistance include cyclin-dependent kinase inhibitors p21Cip [104] and p27Kip [105]. Examples of genes which upregulation is associated with tamoxifen acquired resistance include cyclin-dependent kinase 10 (CDK10) [106] and anti-apoptotic protein survivin [107].

Laboratory observation showed that acquired tamoxifen-resistant breast cancer cells/tumors respond differently to estrogen, and three phases of tamoxifen-resistance have been described,

which seems to depend on the length of tamoxifen exposure [12]. Tumors with phase I resistance are stimulated by estrogen and tamoxifen but inhibited by AIs and fulvestrant; tumors with phase II resistance are stimulated by tamoxifen but are inhibited by estrogen due to apoptosis; tumors with phase III resistance (automatous growth) grow in a hormone-independent manner that is not responsive to either AIs or fulvestrant or SERMs, but estrogen still exerts apoptotic actions on those tumors [12]. The laboratory models suggest a new treatment strategy, in which limited duration, low-dose estrogen can be used to purge phase II- or phase III-resistant breast cancer cells so that the tumors will be responsive to antiestrogen therapy again. Phase II clinical study is ongoing to test this treatment plan [108].

Most studies on SERM-resistance are related to tamoxifen and little is known about raloxifene resistance. Based on a few studies on raloxifene resistance using cell culture and animal models, raloxifene-resistant tumors are likely to have similar properties as tamoxifen-resistant ones [109]. Raloxifene-resistant MCF7 cells generated by long-term exposure to raloxifene *in vitro* are also resistant to tamoxifen *in vitro and in vivo*. They exhibit phase II SERM-resistance as estradiol treatment causes tumor regression by inducing G2/M cell cycle arrest and apoptosis [110]. Another raloxifene-resistant breast tumor model generated by exposing MCF7 breast tumors to raloxifene *in vivo* exhibits phase I SERM-resistance whose growth is stimulated by tamoxifen, raloxifene and estrogen [109]. Interestingly, protein levels of EGFR and HER2 are also increased in this phase I raloxifene-resistant tumor model, which suggests raloxifene-resistant tumors share similar molecular mechanisms as tamoxifen-resistant ones [109].

Overall, the classifications of different forms of antihormonal drug resistance can be used as a basis to evaluate the pharmacology of new SERMs. The goal is to improve on tamoxifen, the pioneer that over the past 30 years found ubiquitous long term applications in the treatment and prevention of breast cancer.

4. NEW SERMs

The discovery of the first antibiotic penicillin initiated a search for further antibiotics to delay drug resistance and to target specific diseases. Similarly, the successful clinical application of tamoxifen in medicine has resulted in the investigation of numerous related molecules to develop the "ideal SERM". However, it has been challenging to find a SERM that is superior to tamoxifen, which retains or extends its benefit to treat and prevent breast cancer but with fewer side effects. Tamoxifen maintains bone density in animals [111] and humans [112] so SERMs are being developed to treat osteoporosis, but the potential to prevent breast cancer and uterine cancer will also increase their clinical value and commercial success. The core structures of SERMs are diverse, including triphenylethylene, benzothiophene, chromene (benzopyran), naphthalene, indole and steroid, but each of the newer SERM is really a mimic of tamoxifen, raloxifene or estradiol. The development of dozens of SERMs have been discontinued due to ineffectiveness for human disease or severe side effects, but several new SERMs are under active investigations with great potential in breast cancer treatment and/or prevention, alone or in combination with other type of drugs. In addition, since the identification of ERβ in 1996 [19], ER subtype selective SERMs have been developed which could potentially be used as breast cancer preventives. Thus this area of medicinal chemistry remains an important topic of interest as new ER regulated targets emerge. We will review the current status of several agents that are either approved or in the process of drug development (summarized in Table **1**).

4.1. Tamaoxifen-Like SERMs

4.1.1. Toremifene (Fareston)

Toremifene (**2**) is a chlorinated tamoxifen analogue which has been approved in the US and several other countries for the treatment of metastatic breast cancer. Its structure is shown in Fig. (**3**). Toremifene is as effective as tamoxifen in the treatment of ER-positive breast cancer but with the potential of fewer genotoxic effects, since it does not produce DNA adducts in rat liver and human endometrium [113]. The mechanism for the reduced genotoxicity of toremifene can be explained as follows: Tamoxifen-DNA adducts are primarily formed *via* sulfonation of the α-hydroxylated tamoxifen metabolites, but the α-hydroxy metabolites of toremifene is poorly esterified or sulfonated, and even sulfonated α-hydroxy toremifene, α-sulfoxytoremifene, reacts poorly with DNA [114, 115]. However, there are some reports to show toremifene induces DNA damages and hepatocarcinogenesis in rats [116, 117].

The effects of toremifene and tamoxifen on bones are similar [118], as are the endometrial effects. However, a recent safety evaluation demonstrates that secondary endometrial cancer incidence is lower with toremifene than with tamoxifen and is similar to that with raloxifene [119]. Nevertheless, toremifene stimulates the growth of human endometrial cancer implanted in athymic mice in the same way as tamoxifen [120]. The positive effects of toremifene on lipid profiles are superior to tamoxifen's. Toremifene lowers the low-density lipoprotein (LDL) cholesterol to a level similar to that seen with tamoxifen, but unlike tamoxifen, toremifene slightly increases high-density lipoprotein (HDL) cholesterol and lowers triglycerides in the serum [121, 122].

Cross-resistance with tamoxifen is an important issue to consider when using toremifene for recurrent breast cancer because the majority of patients have received or failed adjuvant tamoxifen. Toremifene is completely cross-resistant with tamoxifen in human breast tumors implanted in athymic mice [123], as well as in breast cancer patients [124, 125]. Therefore, toremifene would not be effective as a second-line endocrine therapy after tamoxifen failure and may offer no therapeutic advantages over tamoxifen as an adjuvant therapy.

In recent years, toremifene has been developed to treat other estrogen-related diseases. Toremifene is effective to treat mastalgia in some small phase II trials [126, 127], and is also effective at decreasing prostate cancer incidences in a high-risk population [128]. In addition, a recent multicenter randomized phase III trial showed that toremifene increased bone density and improved lipid profile in men receiving androgen deprivation therapy for prostate cancer [129, 130].

4.1.2. Ospemifene (Deaminohydroxytoremifene, FC-1271a)

Ospemifene (**3**), or deaminohydroxytoremifene, is a metabolite of toremifene (Fig. (**3**)). Like toremifene, ospemifene is generally well tolerated and has a favorable safety profile. It does not induce DNA adducts in mice [131], rats [132] and monkey [133]. Ospemifene exerts a very weak estrogenic effect on endometrial histology, like raloxifene and decreases cholesterol [134]. However, unlike tamoxifen or raloxifene, ospemifene has significant estrogenic effects on vaginal epithelium [134-136] and is being developed for postmenopausal vaginal atrophy, a chronic condition experienced by about 40% postmenopausal women. Ospemifene is being evaluated in a phase III trial that has already recruited 826 women. Early results suggested that a 12-week course of ospemifene treatment significantly relieves symptoms of dryness in the vagina.

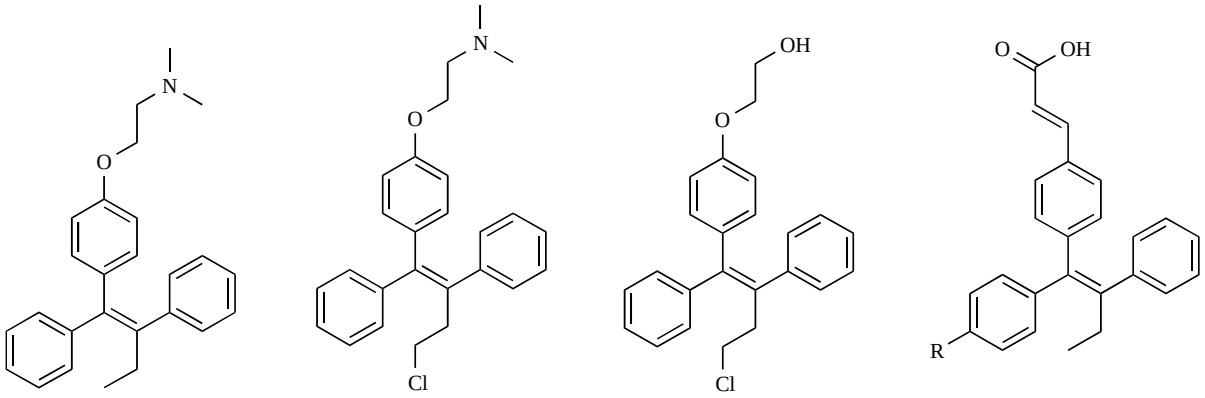
Ospemifene has showed promise in the prevention and treatment of osteoporosis. Cell culture studies indicated that ospemifene inhibits osteoclast formation and bone resorption and protects osteoblast-derived cells from apoptosis [137, 138]. In a recent phase II trial to compare effects of ospemifene and raloxifene on biochemical markers of bone turnover in postmenopausal women, ospemifene showed similar effects as raloxifene in regulating most of the bone markers examined, and at the 90-mg dose, ospemifene increased procollagen type I N propeptide (PINP) more than raloxifene [139]. Ospemifene is currently in phase III development for the treatment of postmenopausal osteoporosis.

Table 1. Current Status of New SERMs

Drug Name	Category (Structure)	Effects	Preclinical Results	Clinical Status
Toremifene	Tamoxifen-like	Breast cancer treatment	Fewer genotoxic effects than tamoxifen [113], bone effects similar to tamoxifen [119]	FDA approved for metastatic breast cancer
		Heart protection		Phase II trial (65 women) better than tamoxifen regulating lipid metabolism [121, 122]
		Mastalgia treatment		Phase II trials (62 and 195 women) effective [126, 127]
		Prostate cancer prevention		Phase II trial (514 men) decreases prostate cancer incidence [128]
		Relieve side effects of androgen deprivation therapy		Phase III trial (1,389 men) improves lipid profiles [130] Phase III trial (1,392 men) increases bone mineral density [129]
Ospemifene	Tamoxifen-like	Vaginal atrophy treatment	Estrogenic effects on vaginal epithelium that is not observed with tamoxifen or raloxifene [134-136]	Phase III trial (826 women) relieves vaginal dryness
		Osteoporosis treatment		Phase II trial (118 women): Comparable to or slightly better than raloxifene [139]
				Phase III trial planned (detail not available)
		Breast cancer prevention	Inhibits tumor growth in animal models as effective as tamoxifen [140, 141]	Not available
GW5638 (DPC974) & GW7604	Tamoxifen-like	Breast cancer treatment (2^{nd} line therapy)	Works as a SERM and as a SERD [148], effective in tamoxifen-resistant tumors [144, 145]; functions as an ER agonist in bone and cardiovascular system but an antagonist in breast and endometrium [142]	Phase I trial (9 patients who failed first-line hormone therapy) low toxicity [ASCO meeting 2002, abstract 452]
Arzoxifene (LY353381)	Raloxifene-like	Breast cancer treatment	Antiestrogenic in breast and endometrium, estrogenic in bone and lipids [150]	Phase III trial (200 patients) inferior to tamoxifen [154]
		Breast cancer prevention	Effective to prevent ER-positive and ER-negative mammary tumors especially in combination with LG100268 [140, 155]	Phase I trials (50 and 76 women) low toxicity and favorable biomarker profile [156]
Lasofoxifene (CP-336156, Fablyn)	Raloxifene-like	Osteoporosis treatment and prevention	Higher potency than tamoxifen and raloxifene [158]; higher oral bioavailability than raloxifene [160]	Phase III trial (1,907 women) significantly increases bone mineral density compared to placebo, no endometrial effects, no association with thromboembolic disorder [159]
				Phase III trial to compare with raloxifene (CORAL trial, details not available)
		Vaginal atrophy treatment		Phase III trial (445 patients) improves vaginal atrophy compared to placebo
		Breast cancer treatment and prevention	Effects similar to tamoxifen to prevent and treat NMU-induced mammary tumor in rats [163]	Phase III trial (PEARL trial with 8,556 women), reduces ER-positive breast cancer incidence compared to placebo; slightly decreases major coronary disease risk; reduces vertebral and non-vertebral fractures; increases risks of venous thromboembolic events but not stroke; no endometrial effects [SABCS 2008, abstract 11]
		Heart disease prevention		
Pipendoxifene (ERA-923)	Raloxifene-like	Breast cancer treatment	Inhibits tamoxifen-sensitive and -resistant tumors in mice and rats no uterotrophic activities compared to raloxifene [167]	Phase II trial to treat tamoxifen-refractory breast cancer in postmenopausal women (details not available)
Bazedoxifene (TSE-424 WAY-140424)	Raloxifene-like	Osteoporosis treatment and prevention	Increases bone density with little uterine or vasomotor effects	Phase III trial (7,492 women) reduces vertebral and non-vertebral fracture incidences, while raloxifene is not effective against non-vertebral fracture [171]
				Phase III trial (497 women) reduces endometrial thickness, unique property among known SERMs [170]
		Breast cancer prevention	Inhibits estrogen-stimulated breast cancer cells growth [169]	Not available
Acolbifene (EM-652, SCH57068) & EM-800 (SCH57050)	Raloxifene-like	Breast cancer treatment (2^{nd} line therapy)	Highest affinity for ER, inhibit growth of multiple breast cancer cells *in vitro* and *in vivo* [180]	Phase III trial, less effective than anastrozole to treat tamoxifen-resistance breast cancer, study halted [182]
		Breast cancer treatment (1st line therapy)		Phase III trial planned [182]
		Breast cancer prevention		Phase II trial (started in February, 2009) for premenopausal women

(Table 1) contd….

Drug Name	Category (Structure)	Effects	Preclinical Results	Clinical Status
CHF4227	Raloxifene-like	Breast cancer and osteoporosis prevention	Prevents DMBA-induced mammary tumors and preserves bone mass in rats [184, 185]	Phase I trials (24 and 56 women) beneficial on bone markers and lipid metabolism; no effects on endometrium; not causing hot flashes
SP500263	Raloxifene-like	Breast cancer and osteoporosis treatment	Inhibits breast cancer cell growth *in vitro* and *in vivo* without stimulating uterine weight gain [188, 189], protects bone *in vitro* [190]	Not available
HMR3339	Steroidal	Osteoporosis and cardiovascular disease prevention	Better than raloxifene to protect cancellous bones [191]	Phase II trials (96 and 118 and 94 women) better than raloxifene at improving some beneficial cardiovascular markers [192-194]
PSK3471	Steroidal	Osteoporosis and breast cancer prevention and treatment	Prevents bone loss *in vivo*, inhibits growth of breast cancer cells *in vivo* [197]	Not available
Trilostane (Modrenal)	ER subtype-selective	Breast cancer treatment Prostate cancer treatment	Increases estradiol binding to ERβ, increases ERβ expression, partially inhibits estrogen production [214, 215]	Approved in UK to treat advanced postmenopausal breast cancer after relapse to initial hormone therapy Phase III trial (714 women with advanced breast cancer) effective for both ER-positive and ER-negative breast cancer, effective for endocrine therapy-resistant cancer Phase II trial for use in premenopausal breast cancer (details not available) Phase II trial with hormone-refractory prostate cancer (details not available)
TAS-108 (SR16234)	Steroidal, ER subtype-selective	Breast cancer treatment and prevention	Inhibits growth of tamoxifen- and AI-sensitive and resistant cancer cells *in vitro* and *in vivo*; inhibits DMBA-induced tumor growth in rats [216]	Phase I trials (16 and 15 women) effective and well tolerated [218, 219] Phase II trails (145 and 97 postmenopausal women with advanced breast cancer) beneficial effects, well tolerated [SABCS, 2008, abstract 2131and 2132]



(**1**) Tamoxifen (**2**) Toremifene (**3**) Ospemifene (**4**) GW5638 R=H (**5**) GW7604 R=OH

Fig. (3). SERMs with a structure mimicking tamoxifen containing a triphenylethylene core.

Studies based on animal models suggest ospemifene might be effective in breast cancer prevention. Ospemifene prevented dimethylbenzanthracene (DMBA)-induced mammary tumors in female Sencar mice as effectively as tamoxifen, while raloxifene was not effective [140]. In a transplantable mouse model of ductal carcinoma *in situ* (DCIS), ospemifene had inhibitory effects equivalent to tamoxifen in terms of tumor growth and progression [141]. Nevertheless, the chemoprevention effects of ospemifene in breast cancer need to be further studied and substantiated by clinical trials.

4.1.3. GW5638 (DPC974) and GW7604

GW5638 (**4**) is a derivative of tamoxifen with an acrylate side chain in place of the dimethylaminoethoxy side chain in tamoxifen. GW7604 (**5**) is the 4-hydroxy version of GW5638, analogous to the major metabolite of tamoxifen, 4-hydroxytamoxifen. Their structures are shown in Fig. (**3**). GW5638 functions as a full ER agonist in bone and the cardiovascular system but as an antagonist in breast and endometrial system in rodent models [142].

Although the structures of tamoxifen and GW5638/GW7604 are similar, GW5638/GW7604 acts with a mechanism different from tamoxifen/4-hydroxy tamoxifen as suggested by the following evidence: 1. GW7604 acts as an antagonist in MDA-MB-231 cells transfected with wild-type ERα, but 4-hydroxytamoxifen acts as an agonist [143]; 2. Phage display experiments indicate that GW7604 bound ERα or ERβ is associated with different peptides from 4-hydroxytamoxifen, raloxifene or fulvestrant bound ERs [144]; 3. GW5638 inhibits the growth of tamoxifen-resistant breast tumor xenograft [144, 145]; 4. The crystal structure of the ERα LBD

bound by GW5638 shows that GW5638 induces a distinct conformation of H12 in the ERα AF2 region, which increased exposure of hydrophobic residues and results in ERα destabilization in MCF7 cells [146].

GW5638 and GW7604 are also classified as selective estrogen receptor down-regulators (SERDs) because they induce ERα degradation, a property observed with the pure antiestrogen fulvestrant which was approved for the treatment of metastatic breast cancer [147]. However, a recent report [148] indicates that GW5638 induces ERα degradation through a different mechanism from fulvestrant and another SERD RU58,668, as the protein/protein interaction surface on ER required for fulvestrant-induced degradation is not necessary for GW5638-induced degradation. The fact that GW5638 has a unique mechanism to antagonize estrogen function and induces ER degradation in breast cancer cells makes it a possible second line therapy after tamoxifen failure and as an alternative to fulvestrant. Currently, GW5638 is under clinical development under the name DPC974 [148].

4.2. Raloxifene-Like SERMs

4.2.1. Arzoxifene (LY353381)

Arzoxifene (**7**) is a derivative of raloxifene with the ketone group replaced by an ether group and the hydroxy group is replaced by a methoxy group (Fig. (**4**)). These modifications have improved the pharmokinetic properties [149]. Arzoxifene has antiestrogenic effects on breast and endometrium but pro-estrogenic effects on bone and lipids [150]. Arzoxifene is cross-resistant in some but not all tamoxifen-stimulated breast tumor xenografts [151]. Phase II clinical trials indicate that arzoxifene is effective to treat tamoxifen-sensitive or tamoxifen-refractory patients with advanced or metastatic breast cancer [152] and patients with recurrent or advanced endometrial cancer [153] with minimus toxicity. However, a phase III trial showed arzoxifene was inferior to tamoxifen to treat patients with locally advanced and metastatic breast cancer [154]. The main role of arzoxifene may reside in its chemoprevention potential since it is more potent than raloxifene in pre-clinical studies [149].

The breast cancer chemoprevention property of arzoxifene has been studied with animal models and small short-term clinical trials. Arzoxifene effectively prevented nitrosomethylurea (NMU)-induced mammary tumor in rats [140] and induced apoptosis of breast cancer cells in rodent models especially when used in combination with rexinoid LG100268, a selective ligand for the retinoid X receptors (RXR) [155]. In two phase I clinical trails of women with newly diagnosed ductal carcinoma *in situ* or T1/T2 invasive cancer, arzoxifene did not demonstrate a significant reduction of tumor cell proliferation compared to placebo in 2-6 weeks treatment [156]. However, there were some favorable findings, such as a decrease of serum insulin like growth factor I (IGF-1) vs IGF binding protein 3 (IGFBP3) ratio and an increase of sex hormone binding globulin [156]. Another interesting aspect of the pharmacology of arzoxifene is that it might have chemopreventive properties for ER-negative breast cancer when used in combination with LG100268. A recent study showed that both SERMs arzoxifene and acolbifene alone prevent ER-negative mammary tumor in a mouse model and the effect is synergized with LG100268 [155]. Although the SERMs by themselves are not functional in the treatment of established tumors, together with LG100268 they inhibit proliferation and induce apoptosis in the ER-negative mammary tumors [155]. The mechanism how SERMs prevent tumorigenesis of ER-negative breast tissue is unknown, but the results suggest that arzoxifene has the potential for further clinical development as a chemoprevention drug of both ER-positive and negative breast cancer, especially in combination with rexinoids.

4.2.2. Lasofoxifene (CP-336156, Fablyn)

Lasofoxifene (**8**) has a naphthalene core structure, which is different from all the other SERMs discussed in this article (Fig. (**4**)). However, the crystal structure shows that lasofoxifene fits into the ERα LBD pocket in a similar manner as other ligands [157]. In addition, lasofoxifene-bound ERα LBD has similar conformational features as other SERM-bound ERα LBDs, such as tamoxifen or raloxifene, in which H12 in the "antagonist-bound" conformation and occludes the coactivator binding surface [157]. Lasofoxifene has a high affinity for ER with an IC_{50} of 1.5 nM, which is comparable to 17β-estradiol and higher than tamoxifen and raloxifene [158]. It preserves bone density and lowers serum cholesterol, and also has chemopreventive and chemotherapeutic effects in rat mammary tumor models without any uterine hypertrophic effects [159]. Lasofoxifene is currently undergoing an extensive clinical evaluation for the prevention and treatment of osteoporosis [159]. One advantage of lasofoxifene over raloxifene is its increased oral bioavailability due to the nonpolar naphthalene structure that makes it a poor substrate for intestinal wall glucuronidation [160]. In addition to its effects on bone, lasofoxifene significantly improves symptoms of vaginal atrophy [161] and a recently completed phase III trial indicated that lasofoxifene decreased vaginal pH and improved the vaginal-cell maturation index in osteoporotic postmenopausal women. These effects may be due to the increased vaginal ERβ and androgen receptor protein levels [162]. Lasofoxifene acts as a chemopreventive and treatment in the NMU-induced rat mammary tumor model. The results are similar to the comparator drug tamoxifen [163]. Phase III trials are currently ongoing to evaluate its ability to prevent breast cancer and cardiovascular diseases in postmenopausal women [164].

4.2.3. Pipendoxifene (ERA-923)

Pipendoxifene (**9**) has an indole core structure (Fig. (**4**)). It was designed by adding an alkylaminoethoxyphenyl side chain to zindoxifene (D-16726), a 2-phenylindol based SERM which failed as a treatment for breast cancer [165]. Pipendoxifene, also named ERA-923, mimics the structure of raloxifene and is devoid of uterotrophic activities in immature rats and ovariectomized mice compared to raloxifene [166]. It inhibits the growth of tamoxifen-sensitive and -resistant tumors in rats and mice [167] and is under phase II clinical development for the treatment of tamoxifen-resistant metastatic breast cancer. In a recent study, a combination of pipendoxifene and temsirolimus, which is a mammalian target of rapamycin (mTOR) inhibitor, synergistically inhibited growth of MCF-7 cells and xenograft models even at suboptimal doses, primarily by causing G1 cell cycle arrest [168]. This suggested that combination of a SERM and an mTOR inhibitor might be of clinic value as breast cancer treatments.

4.2.4. Bazedoxifene (TSE-424, WAY-140424)

Bazedoxifene (**10**) is another indole SERM, designed and synthesized at the same time as pipendoxifene with a slight structural difference, as shown in Fig. (**4**) [166]. This SERM is being actively developed to treat osteoporosis with the potential to prevent breast cancer. Bazodoxifene binds to ERα and ERβ with an affinity lower than raloxifene but is less selective for ERα [169]. It inhibits estrogen-mediated proliferation of breast cancer MCF7 cells and increases bone density with little uterine or vasomotor effects in rat models [169]. A Phase III trial with 497 healthy postmenopausal women showed that 6-month bazedoxifene treatment decreases endometrium thickness and uterine bleeding, suggesting antagonistic effects of bazedoxifene in endometrium [170]. Bazedoxifene is currently under review by the Food and Drug Administration (FDA) for the prevention and treatment of postmenopausal osteoporosis. The completed 3-year phase III trial which enrolled 7,492 postmenopausal women with moderate to severe osteoporosis showed bazedoxifene significantly reduced the incidences of vertebral and non-vertebral fracture compared to placebo, while raloxifene was not effective against non-vertebral fracture [171]. No safety concerns related to breast or endometrium were observed, however, a statistical insignificant increase of venous thromboembolic events was observed with groups treated with either baze-

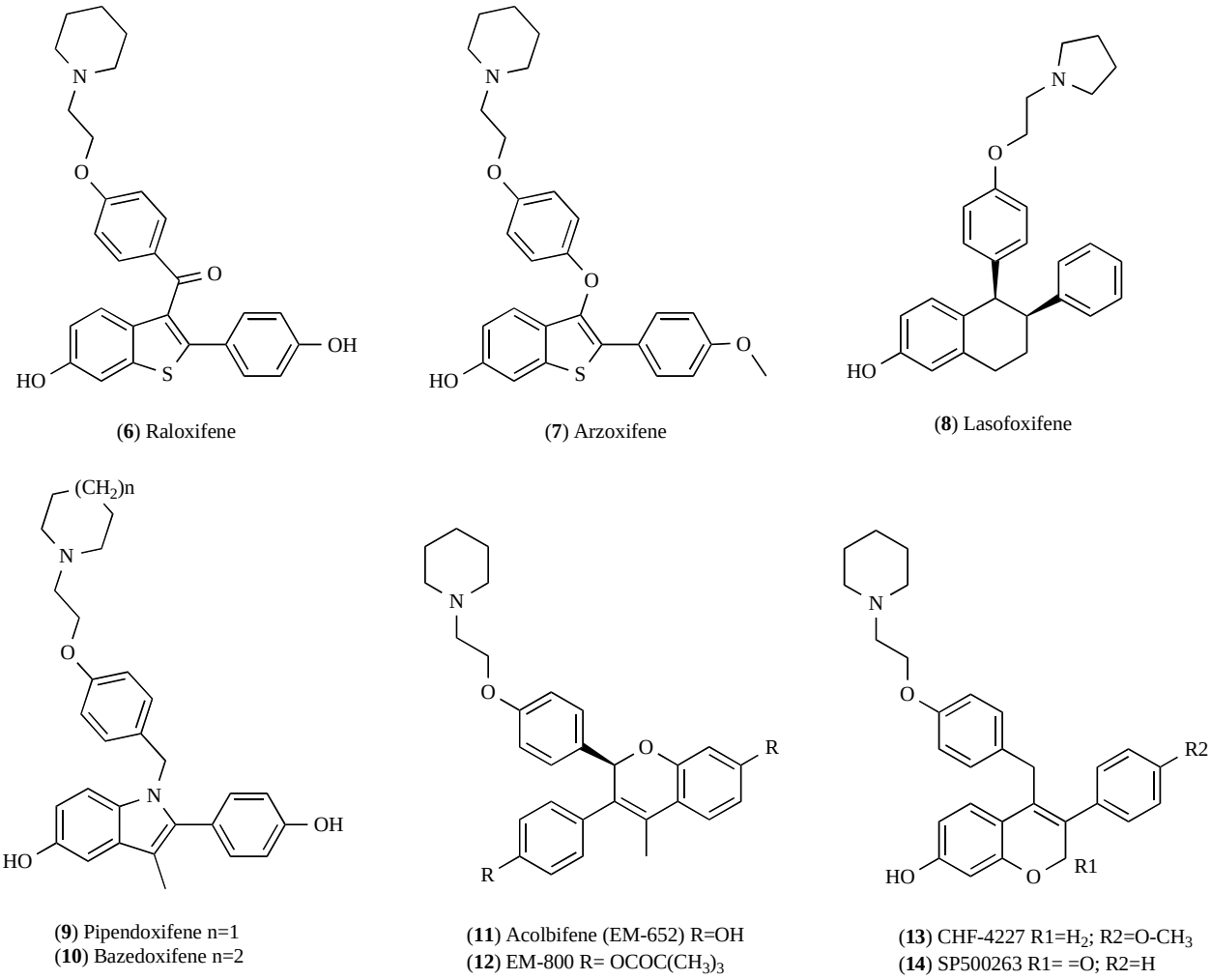


Fig. (4). SERMs with a structure mimicking raloxifene.

doxifene or raloxifene in the same study [172]. Based on studies using rodent models, combination of bazedoxifene and conjugated estrogens exerted positive vasomotor, lipid, and skeletal responses with minimal uterine stimulation [173]. This suggested that pairing SERMs and estrogen might be effective in the treatment of menopausal symptoms and prevention of osteoporosis. However, further studies are needed to examine the effectiveness of bazedoxifene in breast cancer prevention.

4.2.5. Acolbifene (EM-652, SCH57068) and EM-800 (SCH57050)

Acolbifene (EM-652) (**11**) and its orally active prodrug EM-800 (**12**) have a chromene core structure (Fig. (**4**)). They were initially misclassified as pure antiestrogens and their side chain was depicted by analogy with the pure antiestrogen fulvestrant [174]. However, the structure of acolbifene is actually similar to that of raloxifene, and unlike fulvestrant, the antiestrogenic side chain of acolbifene does not mask the mutant ER amino acid D351Y to produce an estrogenic action [175]. In addition, acolbifene and EM-800 act as antiestrogens in mammary and uterine tissues, but have estrogenic effects to prevent bone loss and have a favored function in the regulation of lipid metabolism by lowering plasma cholesterol and triglyceride in rodent models [176, 177]. Therefore, acolbifene and EM-800 should be classified as SERMs.

Acolbifene has the highest ER-binding affinity among all known compounds [178]. Preclinical studies indicated that acolbifene and EM-800 were more potent than tamoxifen, idoxifene, raloxifene, GW-5638, toremifene and droloxifene to inhibit the growth of breast cancer cell lines MCF-7, ZR-75-1, MCF-7 and T47D as well as ZR-75-1 xenograft in mice models [179, 180]. Interestingly, acolbifene caused disappearance of 65% ZR-75-1 xenograft in ovariectomized nude mice, while other SERMs tested (tamoxifen, toremifene, raloxifene, droloxifene, idoxifene and GW 5638) only decreased the tumor growth rate stimulated by estrone [180]. Acolbifene was evaluated as a second line therapy for tamoxifen-refractory breast cancers, since it was regarded as a pure anti-estrogen. In a small clinical trail involved 43 postmenopausal or ovariectomized women with breast cancer who had received tamoxifen for over a year but relapsed, the objective response to EM-800 was 12% with 1 complete response and 4 partial responses [181]. In a phase III trial to compare acolbifene with the aromatase inhibitor anastrozole in breast cancer patients who did not respond to tamoxifen, acolbifene did not show superior antitumor activity to anastrozole and the study was halted [182]. However, acolbifene and EM-800 may be more suitable as first line therapy and a phase III trial for untreated metastatic breast cancer patients is planned [182].

Recent studies indicate that acolbifene might be used in combination with other drugs. Acolbifene synergizes with rexinoid LG-100268 in the prevention and treatment of mice with ER-negative mammary tumor [155]. It also synergizes with dehydroepiandrosterone (DHEA), which is a naturally produced prohormone for androgen and estrogen, in the prevention of dimethylbenzanthracene (DMBA)-induced mammary tumors in the rats [183]. A phase

III trials of acolbifene plus DHEA for vaginal atrophy and uterine safety has been planned.

4.2.6. CHF4227

CHF4227 (**13**) is a SERM with a chromene (benzopyran) core structure, as shown in Fig. (**4**). Compared with raloxifene, CHF4227 binds to ERα and ERβ with higher affinity and inhibits the uterotropic action of 17alpha-ethynyl estradiol with more potency [184]. CHF4227 significantly prevents the development of DMBA-induced mammary tumors in rats [184]. It preserves bone mass without affecting uterine weight and decreases serum cholesterol and fat mass in ovariectomized rats [185]. A recent phase I study showed CHF4227 is well-tolerated, as 28 days of treatment has a positive effect on the serum lipid profile and bone markers without any negative effects on the endometrium or the fibrinolytic system. Additionally, CHF4227 does not cause vaginal bleeding or hot flashes [186]. These results suggest that CHF4227 is safe and worthy of further clinical development for osteoporosis and the chemoprevention of breast cancer.

4.2.7. SP500263

SP500263 (**14**) was discovered in a screen to identify ER agonist in bone cells [187]. It has a chromene core structure and binds to both ERα and ERβ with high affinity similar to raloxifene's (Fig. (**4**)) [187]. SP500263 inhibits the growth of breast cancer MCF7 cells and xenografts in nude mice, and does not stimulate uterine weight gain in immature rats or ovariectomized adult rats [188, 189]. SP500263 also blocks osteoclastogenesis in human bone cell model [190]. These preclinical results suggest that SP500263 has potential for the treatment of both breast cancer and osteoporosis. However, clinical value of this drug has yet to be determined.

4.3. Steroidal SERMs

4.3.1. HMR3339

All of the SERMs described to this point are non-steroidal. Recently, steroidal SERMs have been described (Fig. (**5**)). In rats, HMP3339 (**15**) not only increases bone mineral density but also restores the mechanical strength at multiple sites even after ovariectomy, and it affects both cortical and cancellous bones, while raloxifene was effective only at cancellous sites [191]. HMR3339 has entered clinical investigation for the prevention of osteoporosis and cardiovascular diseases. In a series of small phase II trials with healthy postmenopausal women, HMR3339 was found to reduce total cholesterol, LDL cholesterol, C-reactive protein (CRP, a pro-inflammatory cytokine and a cardiovascular disease risk factor), asymmetric dimethylarginine (AMDA, a nitric oxide synthase inhibitor) and homocysteine [192-194]. Elevation of AMDA or homocysteine is linked to a high incidence of cardiovascular disease but raloxifene treatment does not reduce the level of either AMDA or homocysteine [192]. HMR3339 reduces concentrations of pro-carboxypeptidase U (pro-CpU, an inhibitor of fibrinolysis), anti-thrombin and fibrinogen to a degree similar to raloxifene and shows beneficial effects on some markers of fibrinolysis [195, 196]. Therefore, HMR3339 has potential to prevent cardiovascular diseases and possibly also osteoporosis. However, whether or not there is potential as a cancer preventive has not been determined.

4.3.2 PSK3471

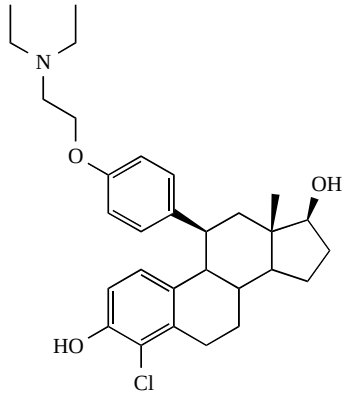
PSK3471 (**16**) is a newly developed SERM with a structure similar to HMR3339 (Fig. (**5**)). It was reported to prevent gonadectomy-induced bone loss in male and female mice, and antagonize estradiol-stimulated MCF-7 cell proliferation [197].

4.4. ER Subtype Selective SERMs

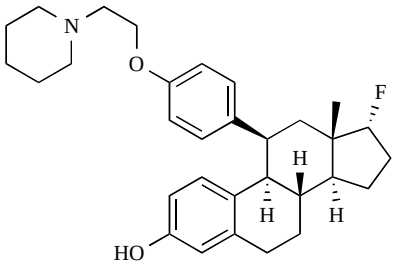
ERα and ERβ have a different tissue distribution and have overlapping but distinct biological functions [198]. Unlike ERα, ERβ expression is not routinely examined in the clinic and its function in breast cancer remains unclear. ERβ expression is found in both normal and breast cancer specimens but does not correlate with ERα expression [199]. It seems that ERβ functions differently if it is expressed alone or co-expressed with ERα in breast cancers. In ERα-positive breast tumors, ERβ often antagonizes the pro-proliferation actions of ERα [200, 201] and its expression is associated with better response to endocrine therapy and a favorable clinical outcome in most cases [202]. Thus ERβ seems to function as a tumor suppressor. However in ERα-negative breast tumors, several studies indicated that the expression of ERβ correlates with proliferation markers such as Ki67 and cyclin A [202, 203], which suggested that ERβ might stimulate cancer growth. In the latter situation, ERβ could serve as an endocrine therapy target in those patients who would otherwise be regarded as ER-negative and have limited choice but chemotherapy. The presence of ERβ in ERα-negative breast cancers may partly explain why some "ER-negative" patients respond to SERMs. The reason that ERβ functions differently in the absence or presence of ERα might be due to the different activities between the ERα/β heterodimer and ERα or ERβ homodimers.

A new direction to consider is the estrogen related receptor (ERR) [204, 205]. There is emerging evidence that ERRα is critical for the growth of ER-negative breast-cancer MDA-MD-231 xenografts in mice [206], as ERRα appears to be involved in angiogenesis by inducing the expression of vascular endothelial growth factor (VEGF) [207, 208]. Novel therapeutic agents targeted to ERRα would be valuable to treat breast cancer.

Several ER-subtype selective SERMs have been reported, although it is difficult to design subtype selective ligands given the fact that only two amino acids are different in the ligand binding pocket between ERα and ERβ (despite that they have 61% amino



(**15**) HMR3339



(**16**) PSK3471

Fig. (5). Steroidal SERMs

acid identity in LBD). All the SERMs discussed previously were designed against ERα and have low subtype selection in terms of binding affinity. In contrast to the focus on ERα and breast cancer, most of ER subtype selective SERMs are developed for diseases other than breast cancer. In animal models, ERβ-selective agonists ERB041 and diaryl-propionitrile (DPN) have been shown to have anti-inflammatory properties and antidepressant-like effects, respectively [209, 210]. An ERβ agonist, 8-vinylestra-1,3,5 (10)-triene-3,17β-diol, stimulates ovarian follicular development in hypophysectomized rats and gonadotropin-releasing hormone a tagonist-treated mice [211], thus this drug could be used to enhance fertility [198]. A few ERβ agonists are being developed for clinical applications in Alzheimer's disease and rheumatoid arthritis [212]. For breast cancer prevention and treatment, it is conceivable that ERβ agonist might have potential for ERα-and-β-positive tumors, especially in combination of an ERα selective antagonist, since the preclinical studies indicate a protective role of ERβ. However, this strategy poses a difficult pharmacologic issue of tissue pharmacodynamics. Nevertheless, a couple of ERβ modulators have been shown with positive effects to treat advanced postmenopausal breast cancer, which will be discussed below.

4.4.1. Trilostane (Modrenal)

Trilostane (**17**) (Fig. (**6**)) is an inhibitor of 3β-hydroxysteroid dehydrogenase, a critical enzyme in the conversion of DHEA to estradiol in breast tumors [213]. Trilostane increases the maximum binding of estradiol to ERβ but not ERα in MCF-7 breast cancer cells [214], and it increased the expression of ERβ in MCF-7 cells and rat uterine [215]. Trilostane is approved in UK to treat advanced postmenopausal breast cancer after relapse to initial hormone therapy and is currently under investigation to be used in prostate cancer and premenopausal breast cancer [213].

4.4.2. TAS-108 (SR16234)

Another type of subtype selective SERM that might be relevant to breast cancer is a combined ERα antagonist but ERβ agonist. TAS-108 (**18**) (Fig. (**5**)) is a steroidal antiestrogen for ERα and a partial agonist on ERβ [216]. TAS-108 has pure antiestrogenic effects for ERα in the presence or absence of estrogen but exhibits partial agonist activity on ERβ using *in vitro* reporter assay. TAS-108 inhibits the growth of tamoxifen-resistant breast cancer cells, DMBA-induced mammary tumor in rats and estrogen-stimulated growth of MCF7 xenografts with little uterotrophic effect [216, 217]. Phase I trial indicate that TAS-108 has anti-tumor activity, is well tolerated, and does not have effects on an endometrial thickness based on an evaluation using trans-vaginal ultrasound [218, 219]. Similar results were obtained in Phase II trials that recruited postmenopausal women with advanced breast cancer, according to presentations at San Antonio Breast Cancer Symposium (SABCS) in December, 2008. A phase III trial is planned [217]. TAS-108 did not increase bone loss like fulvestrant, which could be due to its agonistic property on ERβ. Another advantage over fulvestrant is that TAS-108 is orally administered [220]. TAS-108 is therefore a promising breast cancer drug, even for patients who have relapsed after tamoxifen.

5. CONCLUDING REMARKS

Endocrine therapy targeting to ERα has been very successful in the treatment and prevention of breast cancer [221, 222]. It is very effective and less toxic compared to combinational cytotoxic chemotherapy that was the only option 30 years ago. In the ensuing period, multiple strategies have been developed to antagonize estrogen action. Most experience has accumulated with the competitive inhibitor of estrogen action tamoxifen, but targeting aromatase to deplete estrogen with AIs in postmenopausal patients or to induce ER degradation with SERDs have been valuable innovations in therapies. The goal for treatment is to create a "no-estrogen environment". However, SERMs that maintain the beneficial effects of estrogen but antagonize the harmful effects of estrogen have great potential in the prevention of multiple diseases in common. It is clear that many new SERMs are being developed that could provide better choices for patients in the future.

To overcome the unwanted side effects and problems with drug resistance, combination therapy might be another important direction in addition to the development of new SERMs. For example, combination of SERM acolbifene and DHEA could be protective against breast cancer and osteoporosis with beneficial effects to stimulate vaginal maturation and decrease skin dryness [182]. As traditional HRT is less acceptable to regulatory authorities because of the increased risk of breast cancer, a combination of HRT and a SERM may be a reasonable idea to relieve unpleasant menopausal effects while decrease breast cancer risks. With regards to avoiding drug resistance, combining a SERM and an inhibitor targeting significant survival signal transduction pathway is under active evaluation. By way of example, a combination of tamoxifen and inhibitors of the HER2 signal transduction pathway may prevent acquired tamoxifen resistance [223]. Similarly, SERM pipendoxifene and mTOR inhibitor temsirolimus synergistically inhibits the proliferation of MCF7 breast cancer cells and xenograft at suboptimal concentrations [168]. Additionally, combinations of a SERM (arzoxifene or acolbifene) and a rexinoid LG100268 are effective to prevent and treat ER-negative mammary tumors in animal models [155]. The potential combination seems endless but the marriage of molecular biology and medicine holds great promise for advances in targeted therapeutics based on the SERM model.

In summary, it is clear that the original idea of targeting specific hormone receptor with selective medicine has proven its worth by advancing medicine with the SERMs tamoxifen and raloxifene. Now there are a whole range of new SERMs poised for clinical applications. But this is not the end of the story. Novel selective modulators of all members of the nuclear receptor superfamily are under investigation addressing the treatment or prevention of diseases never before considered possible [57, 222].

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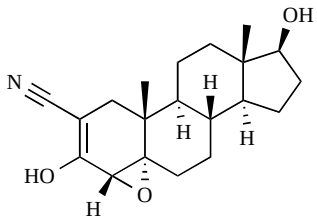
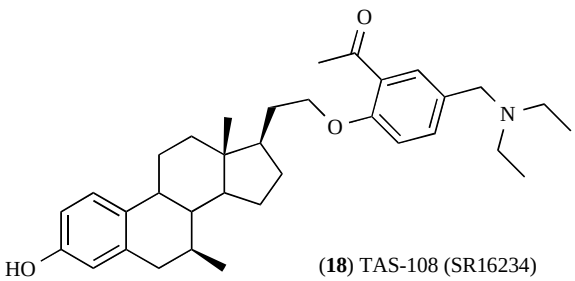


(**17**) Trilostane



(**18**) TAS-108 (SR16234)

Fig. (6). Structure of ERβ-selective agonists.

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REFERENCES

[1] Berry, D.A.; Cronin, K.A.; Plevritis, S.K.; Fryback, D.G.; Clarke, L.; Zelen, M.; Mandelblatt, J.S.; Yakovlev, A.Y.; Habbema, J.D.; Feuer, E.J. Effect of screening and adjuvant therapy on mortality from breast cancer. *N. Engl. J. Med.*, **2005**, *353*(17), 1784-1792.

[2] Ravdin, P.M.; Cronin, K.A.; Howlader, N.; Berg, C.D.; Chlebowski, R.T.; Feuer, E.J.; Edwards, B.K.; Berry, D.A. The Decrease in Breast-Cancer Incidence in 2003 in the United States. *N. Engl. J. Med.*, **2007**, *356*(16), 1670-1674.

[3] Writing Group for the Women's Health Initiative, I. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the women's health initiative randomized controlled trial. *JAMA*, **2002**, *288*(3), 321-333.

[4] The Women's Health Initiative Steering, C. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the women's health initiative randomized controlled trial. *JAMA*, **2004**, *291*(14), 1701-1712.

[5] Beral, V. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet*, **2003**, *362*(9382), 419-427.

[6] Beral, V.; Bull, D.; Reeves, G. Endometrial cancer and hormone-replacement therapy in the Million Women Study. *Lancet*, **2005**, *365*(9470), 1543-1551.

[7] Beral, V.; Bull, D.; Green, J.; Reeves, G. Ovarian cancer and hormone replacement therapy in the Million Women Study. *Lancet*, **2007**, *369*(9574), 1703-1710.

[8] Jordan, V.C. Tamoxifen: a most unlikely pioneering medicine. *Nat. Rev. Drug Discov*, **2003**, *2*(3), 205-213.

[9] Veronesi, U.; Boyle, P.; Goldhirsch, A.; Orecchia, R.; Viale, G. Breast cancer. *Lancet*, **2005**, *365* (9472), 1727-1741.

[10] Cummings, S.R.; Eckert, S.; Krueger, K.A.; Grady, D.; Powles, T.J.; Cauley, J.A.; Norton, L.; Nickelsen, T.; Bjarnason, N.H.; Morrow, M.; Lippman, M.E.; Black, D.; Glusman, J.E.; Costa, A.; Jordan, V.C. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the more randomized trial. *JAMA*, **1999**, *281*(23), 2189-2197.

[11] DeMichele, A.; Troxel, A.B.; Berlin, J.A.; Weber, A.L.; Bunin, G.R.; Turzo, E.; Schinnar, R.; Burgh, D.; Berlin, M.; Rubin, S.C.; Rebbeck, T.R.; Strom, B.L. Impact of raloxifene or tamoxifen use on endometrial cancer risk: a population-based case-control study. *J. Clin. Oncol.*, **2008**, *26*(25), 4151-4159.

[12] Jordan, V.C. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell*, **2004**, *5*(3), 207-213.

[13] Jordan, V.C.; Brodie, A.M. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids*, **2007**, *72*(1), 7-25.

[14] Perez, E.A. Safety profiles of tamoxifen and the aromatase inhibitors in adjuvant therapy of hormone-responsive early breast cancer. *Ann. Oncol.*, **2007**, *18*(Suppl. 8), viii26-35.

[15] Chia, S.; Gradishar, W.; Mauriac, L.; Bines, J.; Amant, F.; Federico, M.; Fein, L.; Romieu, G.; Buzdar, A.; Robertson, J.F.; Brufsky, A.; Possinger, K.; Rennie, P.; Sapunar, F.; Lowe, E.; Piccart, M. Double-blind, randomized placebo controlled trial of fulvestrant compared with exemestane after prior nonsteroidal aromatase inhibitor therapy in postmenopausal women with hormone receptor-positive, advanced breast cancer: results from EFECT. *J. Clin. Oncol.*, **2008**, *26*(10), 1664-1670.

[16] Jensen, E.V.; Jacobson, H.I. Basic guide to the mechanism of estrogen action. *Recent Prog. Hormone Res.*, **1962**, *18*, 387-414.

[17] Green, S.; Walter, P.; Kumar, V.; Krust, A.; Bornert, J.M.; Argos, P.; Chambon, P. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature*, **1986**, *320*(6058), 134-139.

[18] Greene, G.L.; Gilna, P.; Waterfield, M.; Baker, A.; Hort, Y.; Shine, J. Sequence and expression of human estrogen receptor complementary DNA. *Science*, **1986**, *231*(4742), 1150-1154.

[19] Kuiper, G.G.; Enmark, E.; Pelto-Huikko, M.; Nilsson, S.; Gustafsson, J.A. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. USA*, **1996**, *93*(12), 5925-5930.

[20] Tsai, M.J.; O'Malley, B.W. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.*, **1994**, *63*, 451-486.

[21] MacGregor, J.I.; Jordan, V.C. Basic guide to the mechanisms of antiestrogen action. *Pharmacol. Rev.*, **1998**, *50*(2), 151-196.

[22] Brzozowski, A.M.; Pike, A.C.; Dauter, Z.; Hubbard, R.E.; Bonn, T.; Engstrom, O.; Ohman, L.; Greene, G.L.; Gustafsson, J.A.; Carlquist, M. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*, **1997**, *389*(6652), 753-758.

[23] Pike, A.C.; Brzozowski, A.M.; Hubbard, R.E.; Bonn, T.; Thorsell, A.G.; Engstrom, O.; Ljunggren, J.; Gustafsson, J.A.; Carlquist, M. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO J.*, **1999**, *18*(17), 4608-4618.

[24] Bramlett, K.S.; Wu, Y.; Burris, T.P. Ligands specify coactivator nuclear receptor (NR) box affinity for estrogen receptor subtypes. *Mol. Endocrinol.*, **2001**, *15*(6), 909-922.

[25] Wong, C.W.; Komm, B.; Cheskis, B.J. Structure-function evaluation of ER alpha and beta interplay with SRC family coactivators. ER selective ligands. *Biochemistry*, **2001**, *40*(23), 6756-6765.

[26] Warnmark, A.; Almlof, T.; Leers, J.; Gustafsson, J.A.; Treuter, E. Differential recruitment of the mammalian mediator subunit TRAP220 by estrogen receptors ERalpha and ERbeta. *J. Biol. Chem.*, **2001**, *276*(26), 23397-23404.

[27] Hall, J.M.; McDonnell, D.P. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology*, **1999**, *140*(12), 5566-5578.

[28] Paech, K.; Webb, P.; Kuiper, G.G.; Nilsson, S.; Gustafsson, J.; Kushner, P.J.; Scanlan, T.S. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science*, **1997**, *277*(5331), 1508-1510.

[29] Bjornstrom, L.; Sjoberg, M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol. Endocrinol.*, **2005**, *19*(4), 833-842.

[30] Manavathi, B.; Kumar, R. Steering estrogen signals from the plasma membrane to the nucleus: two sides of the coin. *J. Cell. Physiol.*, **2006**, *207*(3), 594-604.

[31] Filardo, E.J.; Quinn, J.A.; Frackelton, A.R., Jr.; Bland, K.I. Estrogen action *via*. the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol. Endocrinol.*, **2002**, *16*(1), 70-84.

[32] Revankar, C.M.; Cimino, D.F.; Sklar, L.A.; Arterburn, J.B.; Prossnitz, E.R. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science*, **2005**, *307*(5715), 1625-1630.

[33] Thomas, P.; Pang, Y.; Filardo, E.J.; Dong, J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology*, **2005**, *146*(2), 624-632.

[34] Funakoshi, T.; Yanai, A.; Shinoda, K.; Kawano, M.M.; Mizukami, Y. G protein-coupled receptor 30 is an estrogen receptor in the plasma membrane. *Biochem. Biophys. Res. Commun.*, **2006**, *346*(3), 904-910.

[35] Filardo, E.J.; Quinn, J.A.; Sabo, E. Association of the membrane estrogen receptor, GPR30, with breast tumor metastasis and transactivation of the epidermal growth factor receptor. *Steroids*, **2008**, *73*(9-10), 870-873.

[36] Lonard, D.M.; O'Malley, B.W. The expanding cosmos of nuclear receptor coactivators. *Cell*, **2006**, *125*(3), 411-414.

[37] Shang, Y.; Brown, M. Molecular Determinants for the Tissue Specificity of SERMs. *Science*, **2002**, *295*(5564), 2465-2468.

[38] Gottardis, M.M.; Robinson, S.P.; Satyaswaroop, P.G.; Jordan, V.C. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res.*, **1988**, *48*(4), 812-815.

[39] Gottardis, M.M.; Ricchio, M.E.; Satyaswaroop, P.G.; Jordan, V.C. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. *Cancer Res.*, **1990**, *50*(11), 3189-3192.

[40] Vogel, V.G.; Costantino, J.P.; Wickerham, D.L.; Cronin, W.M.; Cecchini, R.S.; Atkins, J.N.; Bevers, T.B.; Fehrenbacher, L.; Pajon, E.R., Jr.; Wade, J.L., 3rd; Robidoux, A.; Margolese, R.G.; James, J.; Lippman, S.M.; Runowicz, C.D.; Ganz, P.A.; Reis, S.E.; McCaskill-Stevens, W.; Ford, L.G.; Jordan, V.C.; Wolmark, N. Ef-

fects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA*, **2006**, *295*(23), 2727-2741.

[41] Lonard, D.M.; Tsai, S.Y.; O'Malley, B.W. Selective estrogen receptor modulators 4-hydroxytamoxifen and raloxifene impact the stability and function of SRC-1 and SRC-3 coactivator proteins. *Mol. Cell. Biol.*, **2004**, *24*(1), 14-24.

[42] McKenna, N.J.; Lanz, R.B.; O'Malley, B.W. Nuclear receptor coregulators: cellular and molecular biology. *Endocr. Rev.*, **1999**, *20*(3), 321-344.

[43] Shang, Y.; Hu, X.; DiRenzo, J.; Lazar, M.A.; Brown, M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell*, **2000**, *103*(6), 843-852.

[44] Shang, Y.; Brown, M. Molecular determinants for the tissue specificity of SERMs. *Science*, **2002**, *295*(5564), 2465-2468.

[45] Smith, C.L.; Nawaz, Z.; O'Malley, B.W. Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. *Mol. Endocrinol.*, **1997**, *11*(6), 657-666.

[46] Lavinsky, R.M.; Jepsen, K.; Heinzel, T.; Torchia, J.; Mullen, T.M.; Schiff, R.; Del-Rio, A.L.; Ricote, M.; Ngo, S.; Gemsch, J.; Hilsenbeck, S.G.; Osborne, C.K.; Glass, C.K.; Rosenfeld, M.G.; Rose, D.W. Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc. Natl. Acad. Sci. USA*, **1998**, *95*(6), 2920-2925.

[47] Jepsen, K.; Hermanson, O.; Onami, T.M.; Gleiberman, A.S.; Lunyak, V.; McEvilly, R.J.; Kurokawa, R.; Kumar, V.; Liu, F.; Seto, E.; Hedrick, S.M.; Mandel, G.; Glass, C.K.; Rose, D.W.; Rosenfeld, M.G. Combinatorial roles of the nuclear receptor corepressor in transcription and development. *Cell*, **2000**, *102*(6), 753-763.

[48] Keeton, E.K.; Brown, M. Cell cycle progression stimulated by tamoxifen-bound estrogen receptor-alpha and promoter-specific effects in breast cancer cells deficient in N-CoR and SMRT. *Mol. Endocrinol.*, **2005**, *19*(6), 1543-1554.

[49] Mazumdar, A.; Wang, R.A.; Mishra, S.K.; Adam, L.; Bagheri-Yarmand, R.; Mandal, M.; Vadlamudi, R.K.; Kumar, R. Transcriptional repression of oestrogen receptor by metastasis-associated protein 1 corepressor. *Nat. Cell. Biol.*, **2001**, *3*(1), 30-37.

[50] Montano, M.M.; Ekena, K.; Delage-Mourroux, R.; Chang, W.; Martini, P.; Katzenellenbogen, B.S. An estrogen receptor-selective coregulator that potentiates the effectiveness of antiestrogens and represses the activity of estrogens. *Proc. Natl. Acad. Sci. USA*, **1999**, *96*(12), 6947-6952.

[51] Delage-Mourroux, R.; Martini, P.G.; Choi, I.; Kraichely, D.M.; Hoeksema, J.; Katzenellenbogen, B.S. Analysis of estrogen receptor interaction with a repressor of estrogen receptor activity (REA) and the regulation of estrogen receptor transcriptional activity by REA. *J. Biol. Chem.*, **2000**, *275*(46), 35848-35856.

[52] Zhang, J.; Guenther, M.G.; Carthew, R.W.; Lazar, M.A. Proteasomal regulation of nuclear receptor corepressor-mediated repression. *Genes Dev.*, **1998**, *12*(12), 1775-1780.

[53] Frasor, J.; Danes, J.M.; Funk, C.C.; Katzenellenbogen, B.S. Estrogen down-regulation of the corepressor N-CoR: mechanism and implications for estrogen derepression of N-CoR-regulated genes. *Proc. Natl. Acad. Sci. USA*, **2005**, *102*(37), 13153-13157.

[54] Spencer, T.E.; Jenster, G.; Burcin, M.M.; Allis, C.D.; Zhou, J.; Mizzen, C.A.; McKenna, N.J.; Onate, S.A.; Tsai, S.Y.; Tsai, M.J.; O'Malley, B.W. Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature*, **1997**, *389*(6647), 194-198.

[55] Liu, X.F.; Bagchi, M.K. Recruitment of distinct chromatin-modifying complexes by tamoxifen-complexed estrogen receptor at natural target gene promoters *in vivo*. *J. Biol. Chem.*, **2004**, *279*(15), 15050-15058.

[56] Feng, Q.; Yi, P.; Wong, J.; O'Malley, B.W. Signaling within a coactivator complex: methylation of SRC-3/AIB1 is a molecular switch for complex disassembly. *Mol. Cell. Biol.*, **2006**, *26*(21), 7846-7857.

[57] Jordan, V.C.; O'Malley, B.W. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. *J. Clin. Oncol.*, **2007**, *25*(36), 5815-5824.

[58] Jin, Y.; Desta, Z.; Stearns, V.; Ward, B.; Ho, H.; Lee, K.H.; Skaar, T.; Storniolo, A.M.; Li, L.; Araba, A.; Blanchard, R.; Nguyen, A.; Ullmer, L.; Hayden, J.; Lemler, S.; Weinshilboum, R.M.; Rae, J.M.; Hayes, D.F.; Flockhart, D.A. CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. *J. Natl. Cancer Inst.*, **2005**, *97*(1), 30-39.

[59] Weinshilboum, R. Pharmacogenomics of endocrine therapy in breast cancer. *Adv. Exp. Med. Biol.*, **2008**, *630*, 220-231.

[60] Flockhart, D. CYP2D6 genotyping and the pharmacogenetics of tamoxifen. *Clin. Adv. Hematol. Oncol.*, **2008**, *6*(7), 493-494.

[61] Borges, S.; Desta, Z.; Li, L.; Skaar, T.C.; Ward, B.A.; Nguyen, A.; Jin, Y.; Storniolo, A.M.; Nikoloff, D.M.; Wu, L.; Hillman, G.; Hayes, D.F.; Stearns, V.; Flockhart, D.A. Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. *Clin. Pharmacol. Ther.*, **2006**, *80*(1), 61-74.

[62] Harvell, D.M.; Richer, J.K.; Singh, M.; Spoelstra, N.; Finlayson, C.; Borges, V.F.; Elias, A.D.; Horwitz, K.B. Estrogen regulated gene expression in response to neoadjuvant endocrine therapy of breast cancers: tamoxifen agonist effects dominate in the presence of an aromatase inhibitor. *Breast Cancer Res. Treat.*, **2008**, *112*(3), 489-501.

[63] Kim, H.-J.; Cui, X.; Hilsenbeck, S.G.; Lee, A.V. Progesterone Receptor Loss Correlates with Human Epidermal Growth Factor Receptor 2 Overexpression in Estrogen Receptor-Positive Breast Cancer. *Clin. Cancer Res.*, **2006**, *12*(3), 1013s-1018.

[64] Arpino, G.; Weiss, H.; Lee, A.V.; Schiff, R.; De Placido, S.; Osborne, C.K.; Elledge, R.M. Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance. *J. Natl. Cancer Inst.*, **2005**, *97*(17), 1254-1261.

[65] Osborne, C.K.; Schiff, R.; Arpino, G.; Lee, A.S.; Hilsenbeck, V.G. Endocrine responsiveness: understanding how progesterone receptor can be used to select endocrine therapy. *Breast*, **2005**, *14*(6), 458-465.

[66] Huang, H.J.; Neven, P.; Drijkoningen, M.; Paridaens, R.; Wildiers, H.; Van Limbergen, E.; Berteloot, P.; Amant, F.; Christiaens, M.R.; Vergote, I. Association between HER-2/neu and the progesterone receptor in oestrogen-dependent breast cancer is age-related. *Breast Cancer Res. Treat.*, **2005**, *91*(1), 81-87.

[67] Jordan, V.C.; Gelber, R.D. Problems with the progesterone receptor in practice? *J. Clin. Oncol.*, **2007**, *25*(15), 1957-1959.

[68] Arpino, G.; Wiechmann, L.; Osborne, C.K.; Schiff, R. Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. *Endocr. Rev.*, **2008**, *29*(2), 217-233.

[69] Slamon, D.J.; Clark, G.M.; Wong, S.G.; Levin, W.J.; Ullrich, A.; McGuire, W.L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*, **1987**, *235*(4785), 177-182.

[70] Konecny, G.; Pauletti, G.; Pegram, M.; Untch, M.; Dandekar, S.; Aguilar, Z.; Wilson, C.; Rong, H.M.; Bauerfeind, I.; Felber, M.; Wang, H.J.; Beryt, M.; Seshadri, R.; Hepp, H.; Slamon, D.J. Quantitative association between HER-2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. *J. Natl. Cancer Inst.*, **2003**, *95*(2), 142-153.

[71] De Laurentiis, M.; Arpino, G.; Massarelli, E.; Ruggiero, A.; Carlomagno, C.; Ciardiello, F.; Tortora, G.; D'Agostino, D.; Caputo, F.; Cancello, G.; Montagna, E.; Malorni, L.; Zinno, L.; Lauria, R.; Bianco, A.R.; De Placido, S. A meta-analysis on the interaction between HER-2 expression and response to endocrine treatment in advanced breast cancer. *Clin. Cancer Res.*, **2005**, *11*(13), 4741-4748.

[72] Tsutsui, S.; Ohno, S.; Murakami, S.; Hachitanda, Y.; Oda, S. Prognostic value of epidermal growth factor receptor (EGFR) and its relationship to the estrogen receptor status in 1029 patients with breast cancer. *Breast Cancer Res. Treat.*, **2002**, *71*(1), 67-75.

[73] Likhite, V.S.; Stossi, F.; Kim, K.; Katzenellenbogen, B.S.; Katzenellenbogen, J.A. Kinase-specific phosphorylation of the estrogen receptor changes receptor interactions with ligand, deoxyribonucleic acid, and coregulators associated with alterations in estrogen and tamoxifen activity. *Mol. Endocrinol.*, **2006**, *20*(12), 3120-3132.

[74] Campbell, R.A.; Bhat-Nakshatri, P.; Patel, N.M.; Constantinidou, D.; Ali, S.; Nakshatri, H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. *J. Biol. Chem.*, **2001**, *276*(13), 9817-9824.

[75] Bunone, G.; Briand, P.A.; Miksicek, R.J.; Picard, D. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J.*, **1996**, *15*(9), 2174-2183.

[76] Kato, S.; Endoh, H.; Masuhiro, Y.; Kitamoto, T.; Uchiyama, S.; Sasaki, H.; Masushige, S.; Gotoh, Y.; Nishida, E.; Kawashima, H.; *et. al*. Activation of the estrogen receptor through phosphorylation by mitogen- activated protein kinase. *Science*, **1995**, *270*(5241), 1491-1494.

[77] Zwart, W.; Griekspoor, A.; Berno, V.; Lakeman, K.; Jalink, K.; Mancini, M.; Neefjes, J.; Michalides, R. PKA-induced resistance to tamoxifen is associated with an altered orientation of ERalpha towards co-activator SRC-1. *EMBO J.*, **2007**, *26*(15), 3534-3544.

[78] Wu, R.C.; Qin, J.; Hashimoto, Y.; Wong, J.; Xu, J.; Tsai, S.Y.; Tsai, M.J.; O'Malley, B.W. Regulation of SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) Coactivator activity by I kappa B kinase. *Mol. Cell. Biol.*, **2002**, *22*(10), 3549-3561.

[79] Font de Mora, J.; Brown, M. AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Mol. Cell. Biol.*, **2000**, *20*(14), 5041-5047.

[80] Lopez, G.N.; Turck, C.W.; Schaufele, F.; Stallcup, M.R.; Kushner, P.J. Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. *J. Biol. Chem.*, **2001**, *276*(25), 22177-22182.

[81] Hong, S.H.; Privalsky, M.L. The SMRT corepressor is regulated by a MEK-1 kinase pathway: inhibition of corepressor function is associated with SMRT phosphorylation and nuclear export. *Mol. Cell. Biol.*, **2000**, *20*(17), 6612-6625.

[82] Shou, J.; Massarweh, S.; Osborne, C.K.; Wakeling, A.E.; Ali, S.; Weiss, H.; Schiff, R. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J. Natl. Cancer Inst.*, **2004**, *96*(12), 926-935.

[83] Schiff, R.; Massarweh, S.A.; Shou, J.; Bharwani, L.; Mohsin, S.K.; Osborne, C.K. Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. *Clin. Cancer Res.*, **2004**, *10*(1 Pt 2), 331S-336S.

[84] Nicholson, R.I.; Hutcheson, I.R.; Knowlden, J.M.; Jones, H.E.; Harper, M.E.; Jordan, N.; Hiscox, S.E.; Barrow, D.; Gee, J.M.W. Nonendocrine Pathways and Endocrine Resistance: Observations with Antiestrogens and Signal Transduction Inhibitors in Combination. *Clin. Cancer Res.*, **2004**, *10*(1), 346S-354.

[85] Lee, A.V.; Weng, C.N.; Jackson, J.G.; Yee, D. Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. *J. Endocrinol.*, **1997**, *152*(1), 39-47.

[86] Creighton, C.J.; Casa, A.; Lazard, Z.; Huang, S.; Tsimelzon, A.; Hilsenbeck, S.G.; Osborne, C.K.; Lee, A.V. Insulin-like growth factor-I activates gene transcription programs strongly associated with poor breast cancer prognosis. *J. Clin. Oncol.*, **2008**, *26*(25), 4078-4085.

[87] Dihge, L.; Bendahl, P.O.; Grabau, D.; Isola, J.; Lovgren, K.; Ryden, L.; Ferno, M. Epidermal growth factor receptor (EGFR) and the estrogen receptor modulator amplified in breast cancer (AIB1) for predicting clinical outcome after adjuvant tamoxifen in breast cancer. *Breast Cancer Res. Treat.*, **2008**, *109*(2), 255-262.

[88] Kirkegaard, T.; McGlynn, L.M.; Campbell, F.M.; Muller, S.; Tovey, S.M.; Dunne, B.; Nielsen, K.V.; Cooke, T.G.; Bartlett, J.M. Amplified in breast cancer 1 in human epidermal growth factor receptor - positive tumors of tamoxifen-treated breast cancer patients. *Clin. Cancer Res.*, **2007**, *13*(5), 1405-1411.

[89] Osborne, C.K.; Bardou, V.; Hopp, T.A.; Chamness, G.C.; Hilsenbeck, S.G.; Fuqua, S.A.; Wong, J.; Allred, D.C.; Clark, G.M.; Schiff, R. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J. Natl. Cancer Inst.*, **2003**, *95*(5), 353-361.

[90] Girault, I.; Lerebours, F.; Amarir, S.; Tozlu, S.; Tubiana-Hulin, M.; Lidereau, R.; Bieche, I. Expression analysis of estrogen receptor {alpha} coregulators in breast carcinoma: evidence that NCOR1 expression is predictive of the response to tamoxifen. *Clin. Cancer Res.*, **2003**, *9*(4), 1259-1266.

[91] Wang, L.H.; Yang, X.Y.; Zhang, X.; An, P.; Kim, H.J.; Huang, J.; Clarke, R.; Osborne, C.K.; Inman, J.K.; Appella, E.; Farrar, W.L. Disruption of estrogen receptor DNA-binding domain and related intramolecular communication restores tamoxifen sensitivity in resistant breast cancer. *Cancer Cell*, **2006**, *10*(6), 487-499.

[92] Lu, J.; Getz, G.; Miska, E.A.; Alvarez-Saavedra, E.; Lamb, J.; Peck, D.; Sweet-Cordero, A.; Ebert, B.L.; Mak, R.H.; Ferrando, A.A.; Downing, J.R.; Jacks, T.; Horvitz, H.R.; Golub, T.R. MicroRNA expression profiles classify human cancers. *Nature*, **2005**, *435*(7043), 834-838.

[93] Iorio, M.V.; Ferracin, M.; Liu, C.G.; Veronese, A.; Spizzo, R.; Sabbioni, S.; Magri, E.; Pedriali, M.; Fabbri, M.; Campiglio, M.; Menard, S.; Palazzo, J.P.; Rosenberg, A.; Musiani, P.; Volinia, S.; Nenci, I.; Calin, G.A.; Querzoli, P.; Negrini, M.; Croce, C.M. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res.*, **2005**, *65*(16), 7065-7070.

[94] Miller, T.E.; Ghoshal, K.; Ramaswamy, B.; Roy, S.; Datta, J.; Shapiro, C.L.; Jacob, S.; Majumder, S. MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27(Kip1). *J. Biol. Chem.*, **2008**, *283*(44), 29897-29903.

[95] Zhao, J.-J.; Lin, J.; Yang, H.; Kong, W.; He, L.; Ma, X.; Coppola, D.; Cheng, J.Q. MicroRNA-221/222 negatively regulates ERalpha and associates with tamoxifen resistance in breast cancer. *J. Biol. Chem.*, **2008**, *283*(45), 31079-31086.

[96] Gottardis, M.M.; Jordan, V.C. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res.*, **1988**, *48*(18), 5183-5187.

[97] Osborne, C.K.; Pippen, J.; Jones, S.E.; Parker, L.M.; Ellis, M.; Come, S.; Gertler, S.Z.; May, J.T.; Burton, G.; Dimery, I.; Webster, A.; Morris, C.; Elledge, R.; Buzdar, A. Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. *J. Clin. Oncol.*, **2002**, *20*(16), 3386-3395.

[98] Howell, A.; Robertson, J.F.; Quaresma Albano, J.; Aschermannova, A.; Mauriac, L.; Kleeberg, U.R.; Vergote, I.; Erikstein, B.; Webster, A.; Morris, C. Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. *J. Clin. Oncol.*, **2002**, *20*(16), 3396-3403.

[99] Johnston, S.R.; Saccani-Jotti, G.; Smith, I.E.; Salter, J.; Newby, J.; Coppen, M.; Ebbs, S.R.; Dowsett, M. Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. *Cancer Res.*, **1995**, *55*(15), 3331-3338.

[100] Gutierrez, M.C.; Detre, S.; Johnston, S.; Mohsin, S.K.; Shou, J.; Allred, D.C.; Schiff, R.; Osborne, C.K.; Dowsett, M. Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. *J. Clin. Oncol.*, **2005**, *23*(11), 2469-2476.

[101] Normanno, N.; Di Maio, M.; De Maio, E.; De Luca, A.; de Matteis, A.; Giordano, A.; Perrone, F. Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. *Endocr. Relat. Cancer*, **2005**, *12*(4), 721-747.

[102] deGraffenried, L.A.; Friedrichs, W.E.; Russell, D.H.; Donzis, E.J.; Middleton, A.K.; Silva, J.M.; Roth, R.A.; Hidalgo, M. Inhibition of mTOR activity restores tamoxifen response in breast cancer cells with aberrant Akt activity. *Clin. Cancer Res.*, **2004**, *10*(23), 8059-8067.

[103] Chu, I.; Sun, J.; Arnaout, A.; Kahn, H.; Hanna, W.; Narod, S.; Sun, P.; Tan, C.K.; Hengst, L.; Slingerland, J. p27 phosphorylation by Src regulates inhibition of cyclin E-Cdk2. *Cell*, **2007**, *128*(2), 281-294.

[104] Abukhdeir, A.M.; Vitolo, M.I.; Argani, P.; De Marzo, A.M.; Karakas, B.; Konishi, H.; Gustin, J.P.; Lauring, J.; Garay, J.P.; Pendleton, C.; Konishi, Y.; Blair, B.G.; Brenner, K.; Garrett-Mayer, E.; Carraway, H.; Bachman, K.E.; Park, B.H. Tamoxifen-stimulated growth of breast cancer due to p21 loss. *Proc. Natl. Acad. Sci. USA*, **2008**, *105*(1), 288-293.

[105] Yuan, Y.; Qin, L.; Liu, D.; Wu, R.-C.; Mussi, P.; Zhou, S.; Songyang, Z.; Xu, J. Genetic screening reveals an essential role of p27kip1 in restriction of breast cancer progression. *Cancer Res.*, **2007**, *67*(17), 8032-8042.

[106] Iorns, E.; Turner, N.C.; Elliott, R.; Syed, N.; Garrone, O.; Gasco, M.; Tutt, A.N.J.; Crook, T.; Lord, C.J.; Ashworth, A. Identification of CDK10 as an important determinant of resistance to endocrine therapy for breast cancer. *Cancer Cell*, **2008**, *13*(2), 91-104.

[107] Moriai, R.; Tsuji, N.; Moriai, M.; Kobayashi, D.; Watanabe, N. Survivin plays as a resistant factor against tamoxifen-induced apoptosis in human breast cancer cells. *Breast Cancer Res. Treat.*, **2008**.

[108] Jordan, V.C.; Lewis, J.S.; Osipo, C.; Cheng, D. The apoptotic action of estrogen following exhaustive antihormonal therapy: a new clinical treatment strategy. *Breast*, **2005**, *14*(6), 624-630.

[109] O'Regan, R.M.; Osipo, C.; Ariazi, E.; Lee, E.S.; Meeke, K.; Morris, C.; Bertucci, A.; Sarker, M.A.; Grigg, R.; Jordan, V.C. Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. *Clin. Cancer Res.*, **2006**, *12*(7 Pt 1), 2255-2263.

[110] Liu, H.; Lee, E.S.; Gajdos, C.; Pearce, S.T.; Chen, B.; Osipo, C.; Loweth, J.; McKian, K.; De Los Reyes, A.; Wing, L.; Jordan, V.C. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*. *J. Natl. Cancer Inst.*, **2003**, *95*(21), 1586-1597.

[111] Jordan, V.C.; Phelps, E.; Lindgren, J.U. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res. Treat.*, **1987**, *10*(1), 31-35.

[112] Love, R.R.; Mazess, R.B.; Barden, H.S.; Epstein, S.; Newcomb, P.A.; Jordan, V.C.; Carbone, P.P.; DeMets, D.L. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N. Engl. J. Med.*, **1992**, *326*(13), 852-856.

[113] Umemoto, A.; Lin, C.X.; Ueyama, Y.; Komaki, K.; Santosh Laxmi, Y.R.; Shibutani, S. Absence of DNA adduct in the leukocytes from breast cancer patients treated with toremifene. *Chem. Res. Toxicol.*, **2006**, *19*(3), 421-425.

[114] Shibutani, S.; Ravindernath, A.; Terashima, I.; Suzuki, N.; Laxmi, Y.R.; Kanno, Y.; Suzuki, M.; Apak, T.I.; Sheng, J.J.; Duffel, M.W. Mechanism of lower genotoxicity of toremifene compared with tamoxifen. *Cancer Res.*, **2001**, *61*(10), 3925-3931.

[115] da Costa, G.G.; Pereira, P.C.; Churchwell, M.I.; Beland, F.A.; Marques, M.M. DNA adduct formation in the livers of female Sprague-Dawley rats treated with toremifene or alpha-hydroxytoremifene. *Chem. Res. Toxicol.*, **2007**, *20*(2), 300-310.

[116] Dragan, V.P.; Vaughan, J.; Jordan, V.C.; Pitot, H.C. Comparison of the effects of tamoxifen and toremifene on liver and kidney tumor promotion in female rats. *Carcinogenesis*, **1995**, *16*(11), 2733-2741.

[117] Sargent, L.M.; Dragan, Y.P.; Sattler, C.; Bahnub, N.; Sattler, G.; Martin, P.; Cisneros, A.; Mann, J.; Thorgeirsson, S.; Jordan, V.C.; Pitot, H.C. Induction of hepatic aneuploidy *in vivo* by tamoxifen, toremifene and idoxifene in female Sprague-Dawley rats. *Carcinogenesis*, **1996**, *17*(5), 1051-1056.

[118] Erkkola, R.; Mattila, L.; Powles, T.; Heikkinen, J.; Toivola, B.; Korhonen, P.; Mustonen, M. Bone mineral density and lipid changes during 5 years of follow-up in a study of prevention of breast cancer with toremifene in healthy, high-risk pre- and postmenopausal women. *Breast Cancer Res. Treat.*, **2005**, *93*(3), 277-287.

[119] Harvey, H.A.; Kimura, M.; Hajba, A. Toremifene: an evaluation of its safety profile. *Breast*, **2006**, *15*(2), 142-157.

[120] O'Regan, R.M.; Cisneros, A.; England, G.M.; MacGregor, J.I.; Muenzner, H.D.; Assikis, V.J.; Bilimoria, M.M.; Piette, M.; Dragan, Y.P.; Pitot, H.C.; Chatterton, R.; Jordan, V.C. Effects of the antiestrogens tamoxifen, toremifene, and ICI 182,780 on endometrial cancer growth. *J. Natl. Cancer Inst.*, **1998**, *90*(20), 1552-1558.

[121] Kusama, M.; Kaise, H.; Nakayama, S.; Ota, D.; Misaka, T.; Aoki, T. Crossover trial for lipid abnormality in postmenopausal breast cancer patients during selective estrogen receptor modulators (SERMs) administrations. *Breast Cancer Res. Treat.*, **2004**, *88*(1), 9-16.

[122] Kusama, M.; Miyauchi, K.; Aoyama, H.; Sano, M.; Kimura, M.; Mitsuyama, S.; Komaki, K.; Doihara, H. Effects of toremifene (TOR) and tamoxifen (TAM) on serum lipids in postmenopausal patients with breast cancer. *Breast Cancer Res. Treat.*, **2004**, *88*(1), 1-8.

[123] Lee, E.S.; Schafer, J.M.; Yao, K.; England, G.; O'Regan, R.M.; De Los Reyes, A.; Jordan, V.C. Cross-resistance of triphenylethylene-type antiestrogens but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice. *Clin. Cancer Res.*, **2000**, *6*(12), 4893-4899.

[124] Stenbygaard, L.E.; Herrstedt, J.; Thomsen, J.F.; Svendsen, K.R.; Engelholm, S.A.; Dombernowsky, P. Toremifene and tamoxifen in advanced breast cancer--a double-blind cross-over trial. *Breast Cancer Res. Treat.*, **1993**, *25*(1), 57-63.

[125] Vogel, C.L.; Shemano, I.; Schoenfelder, J.; Gams, R.A.; Green, M.R. Multicenter phase II efficacy trial of toremifene in tamoxifen-refractory patients with advanced breast cancer. *J. Clin. Oncol.*, **1993**, *11*(2), 345-350.

[126] Gong, C.; Song, E.; Jia, W.; Qin, L.; Guo, J.; Jia, H.; Hu, X.; Su, F. A double-blind randomized controlled trial of toremifen therapy for mastalgia. *Arch. Surg.*, **2006**, *141*(1), 43-47.

[127] Oksa, S.; Luukkaala, T.; Maenpaa, J. Toremifene for premenstrual mastalgia: a randomised, placebo-controlled crossover study. *BJOG*, **2006**, *113*(6), 713-718.

[128] Price, D.; Stein, B.; Sieber, P.; Tutrone, R.; Bailen, J.; Goluboff, E.; Burzon, D.; Bostwick, D.; Steiner, M. Toremifene for the prevention of prostate cancer in men with high grade prostatic intraepithelial neoplasia: results of a double-blind, placebo controlled, phase IIB clinical trial. *J. Urol.*, **2006**, *176*(3), 965-970; discussion 970-961.

[129] Smith, M.R.; Malkowicz, S.B.; Chu, F.; Forrest, J.; Price, D.; Sieber, P.; Barnette, K.G.; Rodriguez, D.; Steiner, M.S. Toremifene increases bone mineral density in men receiving androgen deprivation therapy for prostate cancer: interim analysis of a multicenter phase 3 clinical study. *J. Urol.*, **2008**, *179*(1), 152-155.

[130] Smith, M.R.; Malkowicz, S.B.; Chu, F.; Forrest, J.; Sieber, P.; Barnette, K.G.; Rodriquez, D.; Steiner, M.S. Toremifene improves lipid profiles in men receiving androgen-deprivation therapy for prostate cancer: interim analysis of a multicenter phase III study. *J. Clin. Oncol.*, **2008**, *26*(11), 1824-1829.

[131] Hellmann-Blumberg, U.; Taras, T.L.; Wurz, G.T.; DeGregorio, M.W. Genotoxic effects of the novel mixed antiestrogen FC-1271a in comparison to tamoxifen and toremifene. *Breast Cancer Res. Treat.*, **2000**, *60*(1), 63-70.

[132] Qu, Q.; Zheng, H.; Dahllund, J.; Laine, A.; Cockcroft, N.; Peng, Z.; Koskinen, M.; Hemminki, K.; Kangas, L.; Vaananen, K.; Harkonen, P. Selective estrogenic effects of a novel triphenylethylene compound, FC1271a, on bone, cholesterol level, and reproductive tissues in intact and ovariectomized rats. *Endocrinology*, **2000**, *141*(2), 809-820.

[133] Wurz, G.T.; Hellmann-Blumberg, U.; DeGregorio, M.W. Pharmacologic effects of ospemifene in rhesus macaques: a pilot study. *Basic Clin. Pharmacol. Toxicol.*, **2008**, *102*(6), 552-558.

[134] Komi, J.; Lankinen, K.S.; Harkonen, P.; DeGregorio, M.W.; Voipio, S.; Kivinen, S.; Tuimala, R.; Vihtamaki, T.; Vihko, K.; Ylikorkala, O.; Erkkola, R. Effects of ospemifene and raloxifene on hormonal status, lipids, genital tract, and tolerability in postmenopausal women. *Menopause*, **2005**, *12*(2), 202-209.

[135] Voipio, S.K.; Komi, J.; Kangas, L.; Halonen, K.; DeGregorio, M.W.; Erkkola, R.U. Effects of ospemifene (FC-1271a) on uterine endometrium, vaginal maturation index, and hormonal status in healthy postmenopausal women. *Maturitas*, **2002**, *43*(3), 207-214.

[136] Rutanen, E.M.; Heikkinen, J.; Halonen, K.; Komi, J.; Lammintausta, R.; Ylikorkala, O. Effects of ospemifene, a novel SERM, on hormones, genital tract, climacteric symptoms, and quality of life in postmenopausal women: a double-blind, randomized trial. *Menopause*, **2003**, *10*(5), 433-439.

[137] Kallio, A.; Guo, T.; Lamminen, E.; Seppanen, J.; Kangas, L.; Vaananen, H.K.; Harkonen, P. Estrogen and the selective estrogen receptor modulator (SERM) protection against cell death in estrogen receptor alpha and beta expressing U2OS cells. *Mol. Cell. Endocrinol.*, **2008**, *289*(1-2), 38-48.

[138] Michael, H.; Harkonen, P.L.; Kangas, L.; Vaananen, H.K.; Hentunen, T.A. Differential effects of selective oestrogen receptor modulators (SERMs) tamoxifen, ospemifene and raloxifene on human osteoclasts *in vitro*. *Br. J. Pharmacol.*, **2007**, *151*(3), 384-395.

[139] Komi, J.; Lankinen, K.S.; DeGregorio, M.; Heikkinen, J.; Saarikoski, S.; Tuppurainen, M.; Halonen, K.; Lammintausta, R.; Vaananen, K.; Ylikorkala, O.; Erkkola, R. Effects of ospemifene and raloxifene on biochemical markers of bone turnover in postmenopausal women. *J. Bone. Miner. Metab.*, **2006**, *24*(4), 314-318.

[140] Wurz, G.T.; Read, K.C.; Marchisano-Karpman, C.; Gregg, J.P.; Beckett, L.A.; Yu, Q.; Degregorio, M.W. Ospemifene inhibits the

growth of dimethylbenzanthracene-induced mammary tumors in Sencar mice. *J. Steroid. Biochem. Mol. Biol.*, **2005**, *97*(3), 230-240.

[141] Namba, R.; Young, L.J.; Maglione, J.E.; McGoldrick, E.T.; Liu, S.; Wurz, G.T.; DeGregorio, M.W.; Borowsky, A.D.; MacLeod, C.L.; Cardiff, R.D.; Gregg, J.P. Selective estrogen receptor modulators inhibit growth and progression of premalignant lesions in a mouse model of ductal carcinoma *in situ*. *Breast Cancer Res.*, **2005**, *7*(6), R881-889.

[142] Willson, T.M.; Norris, J.D.; Wagner, B.L.; Asplin, I.; Baer, P.; Brown, H.R.; Jones, S.A.; Henke, B.; Sauls, H.; Wolfe, S.; Morris, D.C.; McDonnell, D.P. Dissection of the molecular mechanism of action of GW5638, a novel estrogen receptor ligand, provides insights into the role of estrogen receptor in bone. *Endocrinology*, **1997**, *138*(9), 3901-3911.

[143] Bentrem, D.; Dardes, R.; Liu, H.; MacGregor-Schafer, J.; Zapf, J.; Jordan, V. Molecular mechanism of action at estrogen receptor alpha of a new clinically relevant antiestrogen (GW7604) related to tamoxifen. *Endocrinology*, **2001**, *142*(2), 838-846.

[144] Connor, C.E.; Norris, J.D.; Broadwater, G.; Willson, T.M.; Gottardis, M.M.; Dewhirst, M.W.; McDonnell, D.P. Circumventing tamoxifen resistance in breast cancers using antiestrogens that induce unique conformational changes in the estrogen receptor. *Cancer Res.*, **2001**, *61*(7), 2917-2922.

[145] Dardes, R.C.; O'Regan, R.M.; Gajdos, C.; Robinson, S.P.; Bentrem, D.; De Los Reyes, A.; Jordan, V.C. Effects of a new clinically relevant antiestrogen (GW5638) related to tamoxifen on breast and endometrial cancer growth *in vivo*. *Clin. Cancer Res.*, **2002**, *8*(6), 1995-2001.

[146] Wu, Y.L.; Yang, X.; Ren, Z.; McDonnell, D.P.; Norris, J.D.; Willson, T.M.; Greene, G.L. Structural basis for an unexpected mode of SERM-mediated ER antagonism. *Mol. Cell*, **2005**, *18*(4), 413-424.

[147] Fan, M.; Rickert, E.L.; Chen, L.; Aftab, S.A.; Nephew, K.P.; Weatherman, R.V. Characterization of molecular and structural determinants of selective estrogen receptor downregulators. *Breast Cancer Res. Treat.*, **2007**, *103*(1), 37-44.

[148] Wittmann, B.M.; Sherk, A.; McDonnell, D.P. Definition of functionally important mechanistic differences among selective estrogen receptor down-regulators. *Cancer Res.*, **2007**, *67*(19), 9549-9560.

[149] Sporn, M.B. Arzoxifene: a promising new selective estrogen receptor modulator for clinical chemoprevention of breast cancer. *Clin. Cancer Res.*, **2004**, *10*(16), 5313-5315.

[150] Munster, P.N. Arzoxifene: the development and clinical outcome of an ideal SERM. *Expert. Opin. Invest. Drugs*, **2006**, *15*(3), 317-326.

[151] Schafer, J.M.; Lee, E.S.; Dardes, R.C.; Bentrem, D.; O'Regan, R.M.; De Los Reyes, A.; Jordan, V.C. Analysis of cross-resistance of the selective estrogen receptor modulators arzoxifene (LY353381) and LY117018 in tamoxifen-stimulated breast cancer xenografts. *Clin. Cancer Res.*, **2001**, *7*(8), 2505-2512.

[152] Buzdar, A.; O'Shaughnessy, J.A.; Booser, D.J.; Pippen, J.E., Jr.; Jones, S.E.; Munster, P.N.; Peterson, P.; MeleMed., A.S.; Winer, E.; Hudis, C. Phase II, randomized, double-blind study of two dose levels of arzoxifene in patients with locally advanced or metastatic breast cancer. *J. Clin. Oncol.*, **2003**, *21*(6), 1007-1014.

[153] McMeekin, D.S.; Gordon, A.; Fowler, J.; MeleMed., A.; Buller, R.; Burke, T.; Bloss, J.; Sabbatini, P. A phase II trial of arzoxifene, a selective estrogen response modulator, in patients with recurrent or advanced endometrial cancer. *Gynecol. Oncol.*, **2003**, *90*(1), 64-69.

[154] Deshmane, V.; Krishnamurthy, S.; MeleMed., A.S.; Peterson, P.; Buzdar, A.U. Phase III double-blind trial of arzoxifene compared with tamoxifen for locally advanced or metastatic breast cancer. *J. Clin. Oncol.*, **2007**, *25*(31), 4967-4973.

[155] Liby, K.; Rendi, M.; Suh, N.; Royce, D.B.; Risingsong, R.; Williams, C.R.; Lamph, W.; Labrie, F.; Krajewski, S.; Xu, X.; Kim, H.; Brown, P.; Sporn, M.B. The combination of the rexinoid, LG100268, and a selective estrogen receptor modulator, either arzoxifene or acolbifene, synergizes in the prevention and treatment of mammary tumors in an estrogen receptor-negative model of breast cancer. *Clin. Cancer Res.*, **2006**, *12*(19), 5902-5909.

[156] Fabian, C.J.; Kimler, B.F.; Anderson, J.; Tawfik, O.W.; Mayo, M.S.; Burak, W.E., Jr.; O'Shaughnessy, J.A.; Albain, K.S.; Hyams, D.M.; Budd, G.T.; Ganz, P.A.; Sauter, E.R.; Beenken, S.W.; Grizzle, W.E.; Fruehauf, J.P.; Arneson, D.W.; Bacus, J.W.; Lagios, M.D.; Johnson, K.A.; Browne, D. Breast cancer chemoprevention phase I evaluation of biomarker modulation by arzoxifene, a third generation selective estrogen receptor modulator. *Clin. Cancer Res.*, **2004**, *10*(16), 5403-5417.

[157] Vajdos, F.F.; Hoth, L.R.; Geoghegan, K.F.; Simons, S.P.; LeMotte, P.K.; Danley, D.E.; Ammirati, M.J.; Pandit, J. The 2.0 A crystal structure of the ERalpha ligand-binding domain complexed with lasofoxifene. *Protein Sci.*, **2007**, *16*(5), 897-905.

[158] Ke, H.Z.; Paralkar, V.M.; Grasser, W.A.; Crawford, D.T.; Qi, H.; Simmons, H.A.; Pirie, C.M.; Chidsey-Frink, K.L.; Owen, T.A.; Smock, S.L.; Chen, H.K.; Jee, W.S.; Cameron, K.O.; Rosati, R.L.; Brown, T.A.; Dasilva-Jardine, P.; Thompson, D.D. Effects of CP-336,156, a new, nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. *Endocrinology*, **1998**, *139*(4), 2068-2076.

[159] Gennari, L. Lasofoxifene: a new type of selective estrogen receptor modulator for the treatment of osteoporosis. *Drugs Today (Barc)*, **2006**, *42*(6), 355-367.

[160] Rosati, R.L.; Da Silva Jardine, P.; Cameron, K.O.; Thompson, D.D.; Ke, H.Z.; Toler, S.M.; Brown, T.A.; Pan, L.C.; Ebbinghaus, C.F.; Reinhold, A.R.; Elliott, N.C.; Newhouse, B.N.; Tjoa, C.M.; Sweetnam, P.M.; Cole, M.J.; Arriola, M.W.; Gauthier, J.W.; Crawford, D.T.; Nickerson, D.F.; Pirie, C.M.; Qi, H.; Simmons, H.A.; Tkalcevic, G.T. Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryltetrahydronaphthalene. *J. Med. Chem.*, **1998**, *41*(16), 2928-2931.

[161] Gass, M.; Kagan, R.; Simon, J.; Spino, C.; Barcomb, L. Objective Measures of vaginal atrophy are improved with lasofoxifene, a next-generation selective estrogen receptor modulator. *Obstet. Gynecol.*, **2005**, *105*(4), 71S.

[162] Wang, X.N.; Simmons, H.A.; Salatto, C.T.; Cosgrove, P.G.; Thompson, D.D. Lasofoxifene enhances vaginal mucus formation without causing hypertrophy and increases estrogen receptor beta and androgen receptor in rats. *Menopause*, **2006**, *13*(4), 609-620.

[163] Cohen, L.A.; Pittman, B.; Wang, C.X.; Aliaga, C.; Yu, L.; Moyer, J.D. LAS, a novel selective estrogen receptor modulator with chemopreventive and therapeutic activity in the N-nitroso-N-methylurea-induced rat mammary tumor model. *Cancer Res.*, **2001**, *61*(24), 8683-8688.

[164] Vogelvang, T.E.; van der Mooren, M.J.; Mijatovic, V.; Kenemans, P. Emerging selective estrogen receptor modulators: special focus on effects on coronary heart disease in postmenopausal women. *Drugs*, **2006**, *66*(2), 191-221.

[165] Stein, R.C.; Dowsett, M.; Cunningham, D.C.; Davenport, J.; Ford, H.T.; Gazet, J.C.; von Angerer, E.; Coombes, R.C. Phase I/II study of the anti-oestrogen zindoxifene (D16726) in the treatment of advanced breast cancer. A Cancer Research Campaign Phase I/II Clinical Trials Committee study. *Br. J. Cancer*, **1990**, *61*(3), 451-453.

[166] Miller, C.P.; Collini, M.D.; Tran, B.D.; Harris, H.A.; Kharode, Y.P.; Marzolf, J.T.; Moran, R.A.; Henderson, R.A.; Bender, R.H.; Unwalla, R.J.; Greenberger, L.M.; Yardley, J.P.; Abou-Gharbia, M.A.; Lyttle, C.R.; Komm, B.S. Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. *J. Med. Chem.*, **2001**, *44*(11), 1654-1657.

[167] Greenberger, L.M.; Annable, T.; Collins, K.I.; Komm, B.S.; Lyttle, C.R.; Miller, C.P.; Satyaswaroop, P.G.; Zhang, Y.; Frost, P. A new antiestrogen, 2-(4-hydroxy-phenyl)-3-methyl-1-[4-(2-piperidin-1-yl-ethoxy)-benzyl]-1H-in dol-5-ol hydrochloride (ERA-923), inhibits the growth of tamoxifen-sensitive and -resistant tumors and is devoid of uterotropic effects in mice and rats. *Clin. Cancer Res.*, **2001**, *7*(10), 3166-3177.

[168] Sadler, T.M.; Gavriil, M.; Annable, T.; Frost, P.; Greenberger, L.M.; Zhang, Y. Combination therapy for treating breast cancer using antiestrogen, ERA-923, and the mammalian target of rapamycin inhibitor, temsirolimus. *Endocr. Relat. Cancer*, **2006**, *13*(3), 863-873.

[169] Komm, B.S.; Kharode, Y.P.; Bodine, P.V.; Harris, H.A.; Miller, C.P.; Lyttle, C.R. Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity. *Endocrinology*, **2005**, *146*(9), 3999-4008.

[170] Ronkin, S.; Northington, R.; Baracat, E.; Nunes, M.G.; Archer, D.F.; Constantine, G.; Pickar, J.H. Endometrial effects of baze-

doxifene acetate, a novel selective estrogen receptor modulator, in postmenopausal women. *Obstet. Gynecol.*, **2005**, *105*(6), 1397-1404.

[171] Silverman, S.L.; Christiansen, C.; Genant, H.K.; Vukicevic, S.; Zanchetta, J.R.; de Villiers, T.J.; Constantine, G.D.; Chines, A.A. Efficacy of bazedoxifene in reducing new vertebral fracture risk in postmenopausal women with osteoporosis: results from a 3-year, randomized, placebo- and active-controlled clinical trial*. *J. Bone Miner. Res.*, **2008**, *23*(12) 1923-1934.

[172] No authors listed. Bazedoxifene: Bazedoxifene Acetate, TSE 424, TSE-424, WAY 140424. *Drugs R D*, **2008**, *9*(3), 191-196.

[173] Kharode, Y.; Bodine, P.V.N.; Miller, C.P.; Lyttle, C.R.; Komm, B.S. The pairing of a selective estrogen receptor modulator, bazedoxifene, with conjugated estrogens as a new paradigm for the treatment of menopausal symptoms and osteoporosis prevention. *Endocrinology*, **2008**, en.2008-0817.

[174] Tremblay, A.; Tremblay, G.B.; Labrie, C.; Labrie, F.; Giguere, V. EM-800, a novel antiestrogen, acts as a pure antagonist of the transcriptional functions of estrogen receptors alpha and beta. *Endocrinology*, **1998**, *139*(1), 111-118.

[175] Schafer, J.I.; Liu, H.; Tonetti, D.A.; Jordan, V.C. The interaction of raloxifene and the active metabolite of the antiestrogen EM-800 (SC 5705) with the human estrogen receptor. *Cancer Res.*, **1999**, *59*(17), 4308-4313.

[176] Martel, C.; Picard, S.; Richard, V.; Belanger, A.; Labrie, C.; Labrie, F. Prevention of bone loss by EM-800 and raloxifene in the ovariectomized rat. *J. Steroid. Biochem. Mol. Biol.*, **2000**, *74*(1-2), 45-56.

[177] Lemieux, C.; Gelinas, Y.; Lalonde, J.; Labrie, F.; Richard, D.; Deshaies, Y. Hypocholesterolemic action of the selective estrogen receptor modulator acolbifene in intact and ovariectomized rats with diet-induced hypercholesterolemia. *Metabolism*, **2006**, *55*(5), 605-613.

[178] Martel, C.; Provencher, L.; Li, X.; St Pierre, A.; Leblanc, G.; Gauthier, S.; Merand, Y.; Labrie, F. Binding characteristics of novel nonsteroidal antiestrogens to the rat uterine estrogen receptors. *J. Steroid. Biochem. Mol. Biol.*, **1998**, *64*(3-4), 199-205.

[179] Simard, J.; Labrie, C.; Belanger, A.; Gauthier, S.; Singh, S.M.; Merand, Y.; Labrie, F. Characterization of the effects of the novel non-steroidal antiestrogen EM-800 on basal and estrogen-induced proliferation of T-47D, ZR-75-1 and MCF-7 human breast cancer cells *in vitro*. *Int. J. Cancer*, **1997**, *73*(1), 104-112.

[180] Gutman, M.; Couillard, S.; Roy, J.; Labrie, F.; Candas, B.; Labrie, C. Comparison of the effects of EM-652 (SCH57068), tamoxifen, toremifene, droloxifene, idoxifene, GW-5638 and raloxifene on the growth of human ZR-75-1 breast tumors in nude mice. *Int. J. Cancer*, **2002**, *99*(2), 273-278.

[181] Labrie, F.; Champagne, P.; Labrie, C.; Roy, J.; Laverdiere, J.; Provencher, L.; Potvin, M.; Drolet, Y.; Pollak, M.; Panasci, L.; L'Esperance, B.; Dufresne, J.; Latreille, J.; Robert, J.; Samson, B.; Jolivet, J.; Yelle, L.; Cusan, L.; Diamond, P.; Candas, B. Activity and safety of the antiestrogen EM-800, the orally active precursor of acolbifene, in tamoxifen-resistant breast cancer. *J. Clin. Oncol.*, **2004**, *22*(5), 864-871.

[182] Labrie, F. Future perspectives of selective estrogen receptor modulators used alone and in combination with DHEA. *Endocr. Relat. Cancer*, **2006**, *13*(2), 335-355.

[183] Luo, S.; Sourla, A.; Labrie, C.; Belanger, A.; Labrie, F. Combined effects of dehydroepiandrosterone and EM-800 on bone mass, serum lipids, and the development of dimethylbenz(A)anthracene-induced mammary carcinoma in the rat. *Endocrinology*, **1997**, *138*(10), 4435-4444.

[184] Galbiati, E.; Caruso, P.L.; Amari, G.; Armani, E.; Ghirardi, S.; Delcanale, M.; Civelli, M. Pharmacological actions of a novel, potent, tissue-selective benzopyran estrogen. *J. Pharmacol. Exp. Ther.*, **2002**, *303*(1), 196-203.

[185] Armamento-Villareal, R.; Sheikh, S.; Nawaz, A.; Napoli, N.; Mueller, C.; Halstead, L.R.; Brodt, M.D.; Silva, M.J.; Galbiati, E.; Caruso, P.L.; Civelli, M.; Civitelli, R. A new selective estrogen receptor modulator, CHF 4227.01, preserves bone mass and microarchitecture in ovariectomized rats. *J. Bone. Miner. Res.*, **2005**, *20*(12), 2178-2188.

[186] Civelli, M.; Preti, A.P.; Cenacchi, V.; Rondelli, I.; Guastalla, D.; Tarral, A.; Dostert, P.; Guillevic, Y.; Homery, M.C. Single and multiple ascending dose studies of a novel tissue-selective oestrogen receptor modulator, CHF 4227, in healthy postmenopausal women. *Br. J. Clin. Pharmacol.*, **2007**, *64*(3), 304-316.

[187] Brady, H.; Doubleday, M.; Gayo-Fung, L.M.; Hickman, M.; Khammungkhune, S.; Kois, A.; Lipps, S.; Pierce, S.; Richard, N.; Shevlin, G.; Sutherland, M.K.; Anderson, D.W.; Bhagwat, S.S.; Stein, B. Differential response of estrogen receptors alpha and beta to SP500263, a novel potent selective estrogen receptor modulator. *Mol. Pharmacol.*, **2002**, *61*(3), 562-568.

[188] Brady, H.; Desai, S.; Gayo-Fung, L.M.; Khammungkhune, S.; McKie, J.A.; O'Leary, E.; Pascasio, L.; Sutherland, M.K.; Anderson, D.W.; Bhagwat, S.S.; Stein, B. Effects of SP500263, a novel, potent antiestrogen, on breast cancer cells and in xenograft models. *Cancer Res.*, **2002**, *62*(5), 1439-1442.

[189] Sutherland, M.K.; Brady, H.; Gayo-Fung, L.M.; Leisten, J.; Lipps, S.G.; McKie, J.A.; O'Leary, E.; Patnaik, N.; Anderson, D.W.; Bhagwat, S.S.; Stein, B. Effects of SP500263, a novel selective estrogen receptor modulator, on bone, uterus, and serum cholesterol in the ovariectomized rat. *Calcif. Tissue Int.*, **2003**, *72*(6), 710-716.

[190] Kung Sutherland, M.S.; Lipps, S.G.; Patnaik, N.; Gayo-Fung, L.M.; Khammungkune, S.; Xie, W.; Brady, H.A.; Barbosa, M.S.; Anderson, D.W.; Stein, B. SP500263, a novel SERM, blocks osteoclastogenesis in a human bone cell model: role of IL-6 and GM-CSF. *Cytokine*, **2003**, *23*(1-2), 1-14.

[191] Ammann, P.; Bourrin, S.; Brunner, F.; Meyer, J.M.; Clement-Lacroix, P.; Baron, R.; Gaillard, M.; Rizzoli, R. A new selective estrogen receptor modulator HMR-3339 fully corrects bone alterations induced by ovariectomy in adult rats. *Bone*, **2004**, *35*(1), 153-161.

[192] Vogelvang, T.E.; Mijatovic, V.; Kenemans, P.; Teerlink, T.; van der Mooren, M.J. HMR 3339, a novel selective estrogen receptor modulator, reduces total cholesterol, low-density lipoprotein cholesterol, and homocysteine in healthy postmenopausal women. *Fertil. Steril.*, **2004**, *82*(6), 1540-1549.

[193] Vogelvang, T.E.; Mijatovic, V.; Kenemans, P.; Schalkwijk, C.G.; van der Mooren, M.J. Effect of HMR 3339, a novel selective estrogen receptor modulator, on C-reactive protein levels in healthy postmenopausal women. *Am. J. Cardiol.*, **2004**, *94*(9), 1205-1208.

[194] Verhoeven, M.O.; Teerlink, T.; Kenemans, P.; Vogelvang, T.E.; van der Mooren, M.J. Effects on asymmetric dimethylarginine of HMR 3339, a novel selective estrogen receptor modulator: a 12-week, randomized, placebo-controlled, double-blind, dose-ranging study in healthy postmenopausal women. *Menopause*, **2007**, *14*(2), 235-242.

[195] Vogelvang, T.E.; Leurs, J.R.; Mijatovic, V.; Willemse, J.; van der Mooren, M.J. HMR 3339, a novel selective estrogen receptor modulator, reduces concentrations of procarboxypeptidase U, an inhibitor of fibrinolysis. A randomized, placebo-controlled study in postmenopausal women. *J. Thromb. Haemost.*, **2005**, *3*(5), 1090-1092.

[196] Vogelvang, T.E.; Mijatovic, V.; Kenemans, P.; Emeis, J.J.; Heijst, J.A.; van der Mooren, M.J. The effects of 12 weeks of HMR 3339, a novel selective estrogen receptor modulator, on markers of coagulation and fibrinolysis: a randomized, placebo-controlled, double-blind, dose-ranging study in healthy postmenopausal women. *Am. J. Obstet. Gynecol.*, **2005**, *193*(4), 1384-1394.

[197] Windahl, S.H.; Galien, R.; Chiusaroli, R.; Clement-Lacroix, P.; Morvan, F.; Lepescheux, L.; Nique, F.; Horne, W.C.; Resche-Rigon, M.; Baron, R. Bone protection by estrens occurs through non-tissue-selective activation of the androgen receptor. *J. Clin. Invest.*, **2006**, *116*(9), 2500-2509.

[198] Zhao, C.; Dahlman-Wright, K.; Gustafsson, J.A. Estrogen receptor beta: an overview and update. *Nucl. Recept. Signal*, **2008**, *6*, e003.

[199] Skliris, G.P.; Leygue, E.; Watson, P.H.; Murphy, L.C. Estrogen receptor alpha negative breast cancer patients: estrogen receptor beta as a therapeutic target. *J. Steroid. Biochem. Mol. Biol.*, **2008**, *109*(1-2), 1-10.

[200] Chang, E.C.; Frasor, J.; Komm, B.; Katzenellenbogen, B.S. Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. *Endocrinology*, **2006**, *147*(10), 4831-4842.

[201] Lin, C.Y.; Strom, A.; Li Kong, S.; Kietz, S.; Thomsen, J.S.; Tee, J.B.; Vega, V.B.; Miller, L.D.; Smeds, J.; Bergh, J.; Gustafsson, J.A.; Liu, E.T. Inhibitory effects of estrogen receptor beta on spe-

cific hormone-responsive gene expression and association with disease outcome in primary breast cancer. *Breast Cancer Res.*, **2007**, *9*(2), R25.

[202] Murphy, L.C.; Watson, P.H. Is oestrogen receptor-beta a predictor of endocrine therapy responsiveness in human breast cancer? *Endocr. Relat. Cancer*, **2006**, *13*(2), 327-334.

[203] Rosa, F.E.; Caldeira, J.R.; Felipes, J.; Bertonha, F.B.; Quevedo, F.C.; Domingues, M.A.; Moraes Neto, F.A.; Rogatto, S.R. Evaluation of estrogen receptor alpha and beta and progesterone receptor expression and correlation with clinicopathologic factors and proliferative marker Ki-67 in breast cancers. *Hum. Pathol.*, **2008**, *39*(5), 720-730.

[204] Ariazi, E.A.; Jordan, V.C. Estrogen-related receptors as emerging targets in cancer and metabolic disorders. *Curr. Top. Med. Chem.*, **2006**, *6*(3), 203-215.

[205] Stein, R.A.; McDonnell, D.P. Estrogen-related receptor {alpha} as a therapeutic target in cancer. *Endocr. Relat. Cancer*, **2006**, *13*(Suppl. 1), S25-32.

[206] Stein, R.A.; Chang, C.-y.; Kazmin, D.A.; Way, J.; Schroeder, T.; Wergin, M.; Dewhirst, M.W.; McDonnell, D.P. Estrogen-related receptor {alpha} is critical for the growth of estrogen receptor-negative breast cancer. *Cancer Res.*, **2008**, *68*(21), 8805-8812.

[207] Arany, Z.; Foo, S.Y.; Ma, Y.; Ruas, J.L.; Bommi-Reddy, A.; Girnun, G.; Cooper, M.; Laznik, D.; Chinsomboon, J.; Rangwala, S.M.; Baek, K.H.; Rosenzweig, A.; Spiegelman, B.M. HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. *Nature*, **2008**, *451*(7181), 1008-1012.

[208] Ao, A.; Wang, H.; Kamarajugadda, S.; Lu, J. Involvement of estrogen-related receptors in transcriptional response to hypoxia and growth of solid tumors. *Proc. Natl. Acad. Sci. USA*, **2008**, *105*(22), 7821-7826.

[209] Leventhal, L.; Brandt, M.R.; Cummons, T.A.; Piesla, M.J.; Rogers, K.E.; Harris, H.A. An estrogen receptor-beta agonist is active in models of inflammatory and chemical-induced pain. *Eur. J. Pharmacol.*, **2006**, *553*(1-3), 146-148.

[210] Meyers, M.J.; Sun, J.; Carlson, K.E.; Marriner, G.A.; Katzenellenbogen, B.S.; Katzenellenbogen, J.A. Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *J. Med. Chem.*, **2001**, *44*(24), 4230-4251.

[211] Hegele-Hartung, C.; Siebel, P.; Peters, O.; Kosemund, D.; Muller, G.; Hillisch, A.; Walter, A.; Kraetzschmar, J.; Fritzemeier, K.H. Impact of isotype-selective estrogen receptor agonists on ovarian function. *Proc. Natl. Acad. Sci. USA*, **2004**, *101*(14), 5129-5134.

[212] Barker, S. Non-steroidal anti-estrogens in the treatment of breast cancer. *Curr. Opin. Invest. Drugs*, **2006**, *7*(12), 1085-1091.

[213] Puddefoot, J.R.; Barker, S.; Vinson, G.P. Trilostane in advanced breast cancer. *Expert. Opin. Pharmacother.*, **2006**, *7*(17), 2413-2419.

[214] Puddefoot, J.R.; Barker, S.; Glover, H.R.; Malouitre, S.D.; Vinson, G.P. Non-competitive steroid inhibition of oestrogen receptor functions. *Int. J. Cancer*, **2002**, *101*(1), 17-22.

[215] Barker, S.; Malouitre, S.D.; Glover, H.R.; Puddefoot, J.R.; Vinson, G.P. Comparison of effects of 4-hydroxy tamoxifen and trilostane on oestrogen-regulated gene expression in MCF-7 cells: up-regulation of oestrogen receptor beta. *J. Steroid. Biochem. Mol. Biol.*, **2006**, *100*(4-5), 141-151.

[216] Yamamoto, Y.; Shibata, J.; Yonekura, K.; Sato, K.; Hashimoto, A.; Aoyagi, Y.; Wierzba, K.; Yano, S.; Asao, T.; Buzdar, A.U.; Terada, T. TAS-108, a novel oral steroidal antiestrogenic agent, is a pure antagonist on estrogen receptor alpha and a partial agonist on estrogen receptor beta with low uterotrophic effect. *Clin. Cancer Res.*, **2005**, *11*(1), 315-322.

[217] Buzdar, A.U. TAS-108: a novel steroidal antiestrogen. *Clin. Cancer Res.*, **2005**, *11*(2 Pt 2), 906s-908s.

[218] Blakely, L.J.; Buzdar, A.; Chang, H.Y.; Frye, D.; Theriault, R.; Valero, V.; Rivera, E.; Booser, D.; Kuritani, J.; Tsuda, M. A phase I and pharmacokinetic study of TAS-108 in postmenopausal female patients with locally advanced, locally recurrent inoperable, or progressive metastatic breast cancer. *Clin. Cancer Res.*, **2004**, *10*(16), 5425-5431.

[219] Saeki, T.; Noguchi, S.; Aogi, K.; Inaji, H.; Tabei, T.; Ikeda, T. Evaluation of the safety and tolerability of oral TAS-108 in postmenopausal patients with metastatic breast cancer. *Ann. Oncol.*, **2009**.

[220] Ariazi, E.A.; Ariazi, J.L.; Cordera, F.; Jordan, V.C. Estrogen receptors as therapeutic targets in breast cancer. *Curr. Top. Med. Chem.*, **2006**, *6*(3), 181-202.

[221] Jensen, E.V.; Jordan, V.C. The estrogen receptor: a model for molecular medicine. *Clin. Cancer Res.*, **2003**, *9*(6), 1980-1989.

[222] Jordan, V.C. Tamoxifen: catalyst for the change to targeted therapy. *Eur. J. Cancer*, **2008**, *44*(1), 30-38.

[223] Massarweh, S.; Osborne, C.K.; Creighton, C.J.; Qin, L.; Tsimelzon, A.; Huang, S.; Weiss, H.; Rimawi, M.; Schiff, R. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. *Cancer Res.*, **2008**, *68*(3), 826-833.

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Review

Targeting of tamoxifen to enhance antitumour action for the treatment and prevention of breast cancer: The 'personalised' approach?

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ABSTRACT

Tamoxifen is a standard endocrine therapy for the treatment of steroid receptor positive breast cancer. Tamoxifen efficacy depends on the formation of clinically active metabolites 4-hydroxytamoxifen and endoxifen which have a greater affinity to the oestrogen receptor and ability to control cell proliferation as compared to the parent drug. The cytochrome P450 2D6 enzyme plays a key role in this biotransformation and lack of tamoxifen efficacy has been linked to low activity. There is now considerable mechanistic, pharmacologic and clinical pharmacogenetic evidence in support of the notion that CYP2D6 genetic variants and phenocopying effects through drug interaction by CYP2D6 inhibitors influence plasma concentrations of active tamoxifen metabolites and negatively impact tamoxifen outcome. These interrelations are particularly critical for patients with non-functional (poor metaboliser) and severely impaired (intermediate metaboliser) CYP2D6 variants, and, moreover, for patients in need of co-medication such as serotonin re-uptake inhibitors to control adverse effects such as hot flashes and other menopausal symptoms. Therefore, in the future, a personalised approach for an optimal tamoxifen benefit should consider a CYP2D6 genotype guided adjuvant endocrine treatment strategy and avoid non-adherence as well as strong CYP2D6 inhibitors such as co-medications.

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Review criteria

Apart from select historically relevant papers and review articles, literature was identified by searching the PubMed database for relevant publications written in English between December 2003 and April 2009. Search terms included 'tamoxifen, CYP2D6 metabolism', 'tamoxifen outcome, CYP2D6', and 'tamoxifen adherence' matched by 'pharmacogenetics' and/or 'hot flashes'.

1. Introduction

Tamoxifen, a non-steroidal antioestrogen[1] (Fig. 1), is used for the treatment of all stages of breast cancer[2–4] and in the US is available to reduce the incidence of breast cancer in both pre- and postmenopausal women at elevated risk.[5–7] It is important to remember that during early clinical studies tamoxifen did not show any improvement in efficacy over standard hormonal treatments (high dose oestrogen or androgen) for

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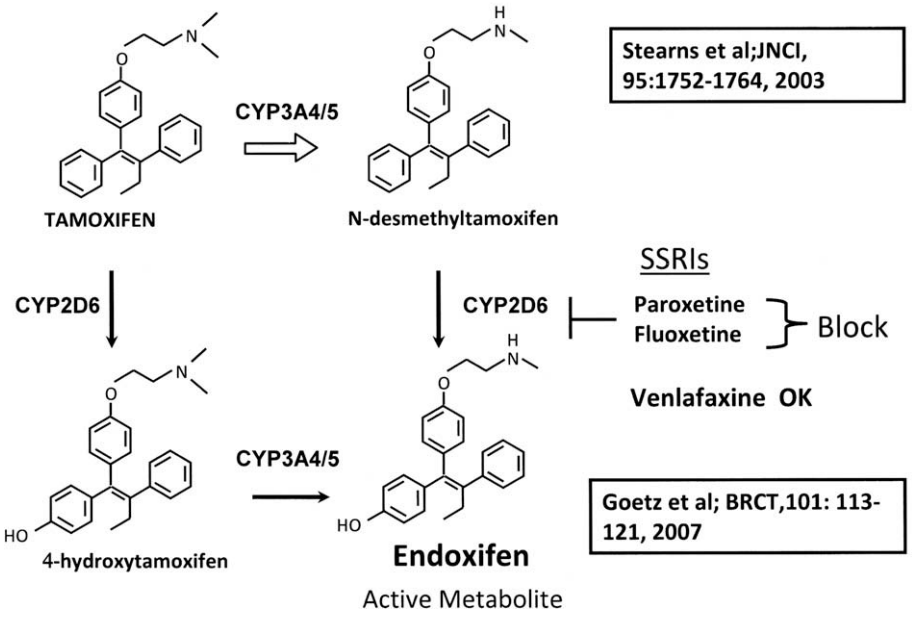

Fig. 1 – The principal metabolites of tamoxifen illustrating the route of metabolism for endoxifen via the CYP2D6 enzyme. An increase in the intensity of hot flashes and menopausal symptoms during tamoxifen therapy has prompted the widespread use of selective serotonin re-uptake inhibitors (SSRIs) to improve the quality of life. However, the SSRIs such as paroxetine and fluoxetine are also metabolised by the CYP2D6 enzyme as it can block the production of endoxifen. Venlafaxine has a low affinity for the CYP2D6 enzyme and is therefore recommended as an alternative to ameliorate menopausal symptoms.

metastatic breast cancer.[2,8] The only advantage of tamoxifen was a reduced incidence of side effects for those 30% of patients who responded for about 1 year. However, laboratory studies to target the tumour oestrogen receptor (ER)[9] employed long term adjuvant therapy[10] and considered the chemoprevention of breast cancer.[11,12] Tamoxifen was thus reinvented from an orphan drug to the 'gold standard' for the endocrine treatment of breast cancer between 1984 and 2004. The targeting of tamoxifen to block oestrogen stimulated breast tumour growth with long term (5 years) adjuvant tamoxifen therapy[13] resulted in a major improvement in patient survivorship and has contributed significantly to the reduction in national death rates from breast cancer.[14,15] The recent development of aromatase inhibitors as an effective treatment for breast cancer in postmenopausal patients[16] has improved disease-free survival and reduced the side effects of endometrial cancer and blood clots noted with tamoxifen.[17–20] However, aromatase inhibitors are not universally available in national health care systems worldwide because of significant financial constraints. Tamoxifen remains a cheap, life-saving, targeted therapy for both pre- and postmenopausal patients with breast cancer.

The application of the ER as a tumour target to treat breast cancer patients appropriately provided a valuable, but admittedly not perfect, test to increase the probability of tumour growth control during long term adjuvant treatment. Tamoxifen does not enhance either disease-free or overall survival in patients with ER negative tumours.[14,15]

At present, there are no universally accepted tumour markers to improve response rates for patients with ER positive tumours. However, emerging data on the pharmacogenomics of tamoxifen metabolism through the CYP2D6 enzyme and new knowledge of potential drug interactions with selective serotonin re-uptake inhibitors (SSRIs), to control hot flashes, provide valuable new information to aid in the selection of the appropriate long term endocrine treatment for breast cancer patients with ER positive disease.

The goal of this concise review is to describe the new understanding of the metabolic activation of tamoxifen to its putative active agent endoxifen[21–23] and consider the clinical significance of CYP2D6 polymorphisms together with phenocopying effects through drug interaction. We will summarise the actions necessary to improve the value of tamoxifen as a 'personalised targeted treatment for breast cancer'.

2. Clinical pharmacology of tamoxifen

2.1. *Tamoxifen efficacy*

Our evolving understanding of the relevance of tamoxifen metabolism for its pharmacology has recently been reviewed.[24] Nevertheless, the important pharmacological issues and conclusions will be restated to provide a scientific background for evaluating the role of the CYP2D6 enzyme and underlying genetics for the antitumour actions of tamoxifen.

Tamoxifen is a pro-drug that requires metabolic activation to 4-hydroxytamoxifen[25,26] and 4-hydroxy-N-desmethyltamoxifen (endoxifen) (Fig. 1) in order to exert its therapeutic effect.[3,4,22,23] 4-Hydroxylation of tamoxifen and its major metabolite N-desmethyltamoxifen increases the affinity for the ER,[26–28] and although both metabolites are equipotent with respect to ER binding and inhibition of 17beta-oestradiol induced cell proliferation, it is proposed that endoxifen is the

principal antioestrogenic metabolite for the antitumour activity observed in breast cancer patients treated at the 20 mg daily dose of tamoxifen.[29] Endoxifen was found at more than six-fold higher concentrations in the plasma of tamoxifen treated patients as compared to 4-hydroxytamoxifen. The metabolism of interest is illustrated in Fig. 1 and the principal metabolites of interest are 4-hydroxytamoxifen and endoxifen. Both metabolites induce similar changes on global gene expression patterns, i.e. the gene array analysis of the spectrum of genes activated or suppressed by 4-hydroxytamoxifen and endoxifen in MCF-7 breast cancer cells is almost the same.[30] There are 4062 total genes either up or down regulated by oestradiol but in the presence of oestradiol and 4-hydroxytamoxifen or endoxifen, 2444 and 2390 genes were affected, respectively. Both tamoxifen metabolites showed overlapping effects on 1365 oestradiol sensitive genes and there was reasonable confirmation with selected genes by RT-PCR. The overall conclusion was that 4-hydroxytamoxifen and endoxifen are almost identical.[30] Together with the ER binding profile and the antiproliferative action of 4-hydroxytamoxifen and endoxifen in MCF-7 cells being identical,[28] but the circulating levels of endoxifen in patients being higher than that of 4-hydroxytamoxifen,[23,29] based on the Law of Mass Action, endoxifen would be anticipated to be the principal metabolite blocking the binding of oestradiol at the tumour ER.

An intriguing aspect of the investigations of the molecular pharmacology of endoxifen is the recent report that the antioestrogen targets ERα for rapid destruction in breast cancer cells.[31] The implication is that the shape of the endoxifen ERα complex is perturbed significantly for rapid proteasomal degradation. Profound structural perturbations of the ER are noted with the pure antioestrogen ICI164384[32] and the SERM GW5638[33] with both compounds causing rapid destruction of ER. In contrast, the structure of endoxifen is almost identical to the related metabolite 4-hydroxytamoxifen (Fig. 1) that causes accumulation of the ER. The structure of the 4 hydroxyl tamoxifen ER complex has been resolved.[34] Perhaps crystallisation of the endoxifen ER complex would provide insight into the actions of endoxifen at the ER.

2.2. *Tamoxifen pharmacogenomics*

2.2.1. *The role of cytochrome P450 2D6*

Numerous drug metabolising enzymes, particularly of the cytrochrome P450 (CYP) iso-enzyme family, are involved in the metabolism of tamoxifen. Among these, CYP2D6 plays the dominant role in the conversion from the major, but clinically inactive, metabolite N-desmethyltamoxifen into the clinically active endoxifen (Fig. 1).[35] Together with CYP2B6, CYP2C9, CYP2C19 and CYP3A4, it is also involved in the formation of 4-hydroxytamoxifen. With CYP2D6 being at the heart of tamoxifen action, host factors, by virtue of the patients genetic makeup, must be taken into account, in addition to tumour characteristics such as ER status, in order to understand drug efficacy. This is owing to the fact that the CYP2D6 phenotype is variable and that this variability differs with respect to degree, underlying genetic variation and frequencies across ethnic groups. By way of clinical observation, the first CYP2D6 phenotypic variation (sparteine/debrisoquine polymorphism) distinct from an extensive metaboliser (EM) phenotype was identified more than 30 years ago and termed poor metaboliser (PM).[36,37] Since then, based on drug oxidation capacity, four different CYP2D6 phenotypes, namely EM, intermediate metaboliser (IM), PM, and ultrarapid metaboliser (UM), have been identified.[38–40] Their frequencies and global distributions have been investigated and extensively reviewed.[41] Although not all CYP2D6 phenotypic variations can be attributed to genetic variations, as of today, there are more than 100 known different alleles of the CYP2D6 (http://www.cypalleles.ki.se).

The PM phenotype is present in 7 to 10% of the European population with PM individuals carrying two non-functional (null) alleles leading to a loss of enzyme function. Of the numerous known null alleles the CYP2D6 *3, *4, and *5 alleles are prevalent in populations of European descent with *4 being present in 70–90% of all PMs. PM status, i.e. lack of catalytic function, can be deduced with greater than 99% certainty from the presence of two non-functional alleles and, therefore, can be accurately predicted from the patients genotype without the need to phenotype.[38,40,42,43] Ten to 15% of Europeans are IM, characterised by severely impaired CYP2D6 expression and function due to the presence of *9, *10, and *41 alleles.[39,44–46] IMs are genetically either homozygous for IM mutations or compound heterozygous for an IM allele in combination with one null allele.[45,47] The EM phenotype results from the presence of one or two alleles with normal expression level and catalytic function such as *1 and *2 and represents the most frequent CYP2D6 phenotype within the European population accounting for 60–70%. EMs can be separated into homozygous or heterozygous EMs depending on whether they carry two or one functional allele. Heterozygous EMs carrying one *1 or *2 allele in combination with an IM or PM allele have a somewhat impaired enzyme expression and function, a reason why they have been classified as 'intermediate metabolisers' assuming a gene dose effect. However, due to the substantial overlap both in enzyme content and activity between homozygous and heterozygous EMs, this is not correct and, therefore, the predictive value of the heterozygous EM genotype is rather poor. Importantly, the IM is a phenotype and genotype distinct from the heterozygous EM based on the presence of *9, *10, and *41 and/or non-functional alleles.[39,46] The UM phenotype is present in 10–15% of the European population and a gene duplication with up to 13 gene copies involving *1, *2, and *4 alleles has been identified as an underlying molecular mechanism.[48,49] Such an increase in enzyme activity can have profound consequences on the plasma concentrations of drug metabolites[50,51]; however, only 20–30% of the UM phenotype in the Caucasian population are accessible through genotyping and, therefore, the predictive value is rather low.[38,40,52]

While CYP2D6 tamoxifen pharmacogenomics for patients of European descent must primarily focus on PM and IM, but also include UM, the PMs play a minor role in individuals of non-European descent. Rather, within Asian populations, IMs are prevalent based on a much higher frequency of the *10 allele, i.e. 57% in Han Chinese[41] and, therefore, tamoxifen pharmacogenomics in Asia requires a focus on IM. Furthermore, North Eastern African populations would require a focus on gene duplication due to a much higher frequency

e.g. 29% in Ethiopia[53] and 21% in Saudi Arabia[54] as compared to 1–5% in populations of European descent.[41,43,55]

2.2.2. CYP2D6 genotype – tamoxifen outcome relationship

Within recent years an increasing awareness of the CYP2D6 phenotypes and underlying genotypes[29,56] sparked a number of international clinical studies to assess retrospectively the potential value of tamoxifen pharmacogenomics for the prediction of treatment outcome of (mainly) early breast cancer. The first evidence linking CYP2D6 variants with treatment response was obtained by Goetz et al.[57] from a US prospective randomised phase III trial of postmenopausal women with primary ER positive breast cancer (North Central Cancer Treatment Group Adjuvant Breast Cancer Trial 89-30-52) investigating the effect of adding the androgen fluoxymestrone, for 1 year, to the standard 5-year adjuvant tamoxifen (20 mg/day). Patients who had received 20 mg/daily adjuvant tamoxifen ($n = 223$ of 256 eligible; mainly of European descent) were genotyped for CYP2D6 variants *4 and *6. Their genomic DNA was obtained from paraffin-embedded tissue specimens. Of the 190 patients for whom CYP2D6 (*4) genotyping was possible, 137 (72.1%) had wt/wt, 40 (21.1%) wt/*4, and 13 (6.8%) *4/*4 genotype. The concordance rate between the genotype obtained from additional buccal cells in 17 patients and the corresponding tumour tissue was 100%. After a median follow-up of 11.4 years, the CYP2D6 *4/*4 genotype was associated with poor patient outcome. CYP2D6 *4/*4 was associated with worse relapse-free ($P = 0.023$) and disease-free survival ($P = 0.012$). The genotype did not have an impact on overall survival ($P = 0.169$). The authors confirmed their findings in an extended study of 256 patients.[58]

A robust association between CYP2D6 genotypes and treatment outcome has been obtained by Schroth et al.[59] from a non-randomised retrospective cohort of ER-positive postmenopausal breast cancer cases from Germany. The study included 206 breast cancer patients treated with adjuvant tamoxifen monotherapy (standard dose) and 280 patients without tamoxifen. The comprehensive genotyping approach using constitutional DNA derived from formalin-fixed paraffin-embedded normal breast tissues included the CYP2D6 variants *4, *5, *10, and *41 to cover the vast majority of PM and IM genotypes (e.g. 95% and 90%, respectively). The analyses were aimed at the investigation of approximately 15–25% of patients expected to be carriers of PM or IM alleles and genotypes. At a median follow-up of 71 months, carriers of CYP2D6 *4, *5 *10 and *41 alleles had significantly more breast cancer recurrences, shorter relapse-free time (hazard ratio (HR) = 2.24; 95% confidence interval (CI), 1.16–4.33; $P = 0.02$), and worse event-free survival (HR = 1.89; 95% CI, 1.10–3.25; $P = 0.02$) compared to carriers of functional alleles (Fig. 2). These associations were not observed in postmenopausal ER positive patients not treated with tamoxifen. This study also included other tamoxifen metabolising genes (i.e. CYP2C19, CYP2B6, CYP2C9, and CYP3A5) and variants. Interestingly, the CYP2C19*17 (UM) allele also had a favourable effect on tamoxifen treatment outcome in that patients with a homozygous *17 genotype had significantly less breast cancer recurrences, longer relapse-free time and better event-free survival (HR = 0.45; 95% CI, 0.21–0.92; $P = 0.03$) compared to non *17 carriers.[59] Altogether, this study suggested that genotyping for CYP2D6 *4, *5, *10 and *41 can identify patients who will derive little benefit from adjuvant tamoxifen. However, EM patients, accounting for approximately 50% of all patients, are likely to benefit from tamoxifen and this benefit will be at a maximum for those with a combination of CYP2D6 functional and CYP2C19 UM alleles. The latter applies to approximately one third of all patients pointing to the relevance of tamoxifen pharmacogenomics for a large fraction of patients receiving endocrine treatment.

Supportive evidence has been provided by studies from Korea,[60] China[61] and Japan.[62] As expected for Asian populations, the CYP2D6 *10 allele significantly contributed to the overall fraction of IM genotypes and observed effects in these patient cohorts. The Korean study by Lim et al.[60] included 202 patients with either primary or metastatic breast cancer treated with 20 mg/daily tamoxifen for more than 8 weeks. Genotype frequencies were 31.6% for wt/wt, 44% for wt/*10, and 24.2% for *10/*10. Patients with *10/*10 genotype ($n = 49$) had significantly lower steady-state plasma concentrations of endoxifen and 4-OH-tamoxifen than those with other

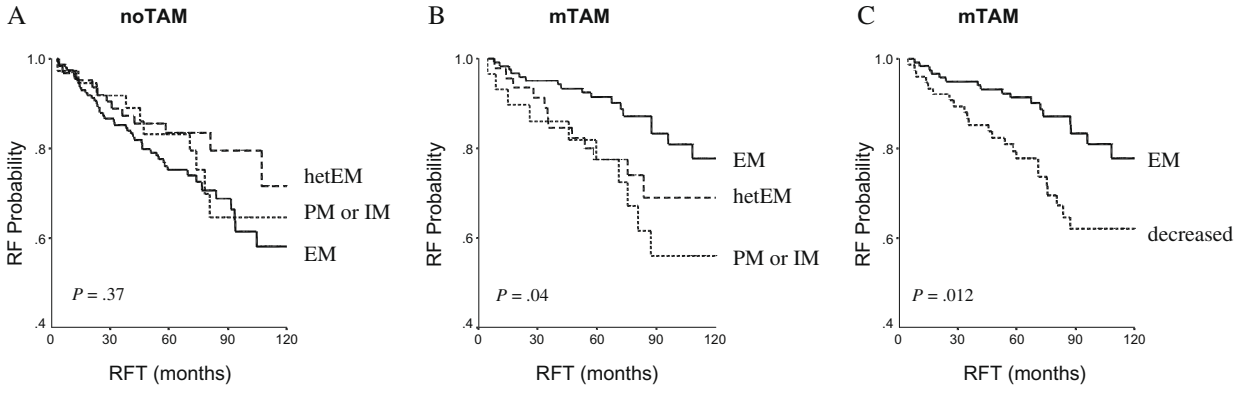

Fig. 2 – Kaplan–Meier estimates of relapse-free time (RFT) for CYP2D6 metaboliser phenotypes predicted from genotypes. (A) Patients not treated with tamoxifen (noTAM); (B) patients treated with adjuvant tamoxifen monotherapy (mTAM); (C) carriers of one or two impaired CYP2D6 alleles predictive for decreased enzyme activity were combined; EM, IM, PM, extensive, intermediate, poor metaboliser, hetEM, heterozygous extensive.[59]

genotypes ($n = 153$). In a small cohort of 21 patients with metastatic breast cancer and treated with tamoxifen, all six patients with progressive or stable disease lasting less than 24 weeks carried the *10/*10 genotype ($P = 0.0186$). The median time to progression for CYP2D6*10/*10 patients was significantly shorter than that for all other genotypes (5.0 versus 21.8 months, $P = 0.0032$). The Chinese study by Xu et al.[61] investigated 152 patients with 20 mg/daily adjuvant tamoxifen for 5 years and a cohort of 141 patients not treated with tamoxifen. Overall genotype frequencies were 24% for *10 wt/wt (C/C), 28% for wt/*10 (C/T), and 48% for *10/*10 (T/T). At a median follow-up time of 63 months, carriers of the CYP2D6 *10/*10 genotype had a significantly worse disease-free survival (89% versus 96%, $P = 0.005$), an association that was not observed in the patient cohort not treated with tamoxifen. Moreover, among 37 patients taking tamoxifen for at least 4 weeks, 4-hydroxytamoxifen levels were significantly lower in CYP2D6*10 homozygous genotype carriers than in patients with homozygous CYP2D6 wt/wt genotype ($P = 0.04$). The Japanese study by Kiyotani et al.[62] investigated 67 patients treated with 20 mg/daily tamoxifen for 5 years with a median follow-up of 10 years. Frequencies were 29.9% for CYP2D6 *1/*1 (wt/wt), 34.3% for *1/*10 and 22.4% for *10/*10. Patients with a CYP2D6 *10/*10 genotype showed a significantly higher incidence of recurrence than those with a CYP2D6 *1/*1 genotype ($P = 0.0057$) or a combined patient group carrying at least one *1 allele ($P = 0.0031$ for trend). Although some of the sample sizes in the Asian studies were low, their findings of an implication of CYP2D6 genotypes predictive for tamoxifen outcome are in line with the studies from Europe[59] and the US.[57,58]

No favourable association of CYP2D6 genetics and tamoxifen outcome was reported in studies from the US, by Nowell et al. (162 patients with tamoxifen, 175 patients without tamoxifen), and Sweden, by Wegmann et al. (112 patients with 40 mg/daily tamoxifen, and mean follow-up of 10.7 years), respectively.[63,64] While Nowell et al. reported no association between CYP2D6 *4 and tamoxifen response or breast cancer prognosis,[63] Wegman et al. reported a decrease in the number of recurrences in tamoxifen treated patients who carried the CYP2D6 *4 variant (odds ratio (OR) = 0.28; 95% CI, 0.11–0.74; $P = 0.0089$).[64] Wegman et al. in addition investigated a cohort of 677 tamoxifen-treated postmenopausal patients, 238 of whom were randomised to 2 versus 5 years of treatment. Patients homozygous for CYP2D6 *4 had a significantly better disease-free survival compared to patients homozygous or heterozygous for the *1 allele ($P = 0.05$ and $P = 0.04$, respectively); however, this effect was not significant in multivariate Cox analysis ($P = 0.055$).[65]

So far, most available evidence in favour of a relationship between CYP2D6 variation and tamoxifen treatment outcome is derived from patients with mainly adjuvant tamoxifen treatment. However, preliminary evidence suggests that this relationship may also play a role in breast cancer chemoprevention as reported from the Italian tamoxifen trial including 5408 healthy hysterectomised women aged 35–70 years who were randomly assigned to receive 20 mg daily tamoxifen or placebo. In a nested case-control study including 46 women who developed breast cancer and 136 controls, the frequency of CYP2D6 *4/*4 genotype was significantly higher in women who developed breast cancer than in those who did not: all women (tamoxifen and placebo): 9% versus 1% $P = 0.015$); tamoxifen treated women: 15% versus 2% ($P = 0.04$).[66] Unexpectedly, hot flashes were reported for all three CYP2D6 *4/*4 allele carriers who developed breast cancer during tamoxifen treatment.

Finally, a small study of hereditary breast cancer patients being tumour suppressor mutation carriers of BRCA1 (47 patients) or BRCA2 (68 patients) and treated with tamoxifen suggested a relationship between CYP2D6 PM status and worse survival.[67] This relationship was observed for BRCA2 but not for BRCA1 carriers. Due to small numbers, as well as ER positive and ER negative patients being included in the analysis, further investigation will be needed to distinguish a pharmacogenetic effect from a poor prognosis effect.

2.3. *Effects of metabolite levels and drug interaction on tamoxifen efficacy and outcome*

It is clear that patients must complete a 5-year course of tamoxifen because 5 years of tamoxifen is superior to 1 or 2 years of adjuvant treatment. This principle is elegantly demonstrated in the overview analysis of clinical trials for premenopausal patients with ER positive breast cancer (Fig. 3).[15] Although, in general, rates of tamoxifen adherence are higher than those observed for other medications, discontinuation of adjuvant tamoxifen in older women with ER positive breast cancer has been evaluated. Randomised clinical trials of adjuvant therapy reported 5-year discontinuance rates of 23% and 40%[4,68], and the primary prevention trial reported a 5-year dis-

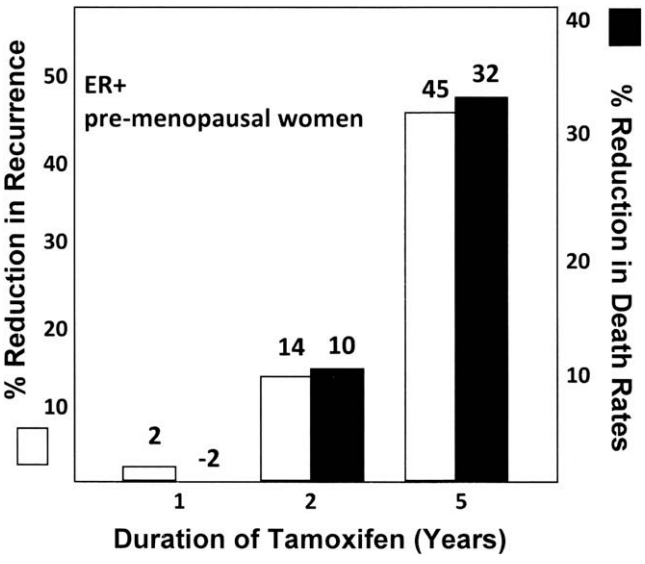

Fig. 3 – The influence of the durations of adjuvant tamoxifen therapy administered to premenopausal patients with oestrogen receptor (ER) positive (+) breast cancer.[15] The enhancement of a reduction of recurrences and a reduction of death rates between women taking only 1 year of adjuvant tamoxifen compared to 5 years serves to illustrate the benefits of the drug, the need for compliance, and the need to ensure that patients are neither poor metabolisers by virtue of aberrations of CYP2D6 or phenocopying by taking SSRIs to reduce menopausal symptoms.

continuance rate of 24%.[69] In clinical practice, discontinuation rates range from 15% to 50%.[70–74] Health-care data-based analyses revealed that as many as half of the patients stop their medication in the course of the 5-year adjuvant treatment with tamoxifen and as many as 15% and 22% of patients stop taking tamoxifen during the first year.[75–77]

The main obstacle to compliance is unacceptable side effects such as severe hot flashes and related menopausal symptoms.[70] However, there is accumulating evidence that hot flashes are an indicator of tamoxifen efficacy and, therefore, the patient's lack of compliance imposes an obstacle to successful treatment. This has recently been suggested by data from the Women's Healthy Eating and Living trial (WHEL)[78] which enrolled primary breast cancer patients ($n = 3088$ between 18 and 70 years of age) between 2 to 48 month after initial diagnosis to either dietary intervention ($n = 1537$) or observation ($n = 1551$) alone. At study entry, among the 864 women taking tamoxifen 78% reported hot flashes, and among those 69% also reported night sweats; only 4% reported night sweats without hot flashes, and 18% did not report either hot flashes or night sweats. Patients reporting hot flashes at baseline were less likely to develop recurrent breast cancer than those who did not report hot flashes (12.9% versus 21%, $P = 0.01$; 127 women had a confirmed breast cancer recurrence after 7.3 years follow-up). Moreover, hot flashes were more predictive of outcome than age, grade, hormone receptor status, or stage II cancer.[78] Goetz et al. showed that the incidence of hot flashes during adjuvant tamoxifen improved therapeutic outcomes and correlated with the CYP2D6 genotype.[57] In their study none of the patients with CYP2D6 *4/*4 genotype (0/13) reported moderate or severe hot flashes compared to 20% (36/177) in the groups of *4/wt and wt/wt patients ($P = 0.064$). Accordingly, hot flashes can be attributed to higher tamoxifen metabolite levels in patients with functional CYP2D6 and drug efficacy. These data which link the occurrence of hot flashes with CYP2D6 genotype and adjuvant tamoxifen outcome, clearly extend previous prospective cohort studies of adjuvant tamoxifen treatment that have already demonstrated that there is a wide inter-individual variability in the formation of tamoxifen metabolites and that steady-state endoxifen plasma concentrations during tamoxifen treatment are substantially reduced in women carrying CYP2D6 genetic variants.[23,29,56] Similar relationships have been reported in studies from Asia[60,61] and Europe.[79] Moreover, at the level of chemoprevention, higher levels of N-desmethyltamoxifen (i.e. endoxifen precursor, Fig. 1) have been reported for carriers of CYP2D6 variants after 1 year of tamoxifen, suggesting that the conversion into the clinically active endoxifen may be impaired.[80] In the light of these genotype–metabolite relationships it is of utmost importance that patients experiencing hot flashes sustain adjuvant tamoxifen despite the discomfort of adverse reactions.

To aid compliance, patients are routinely prescribed selective serotonin re-uptake inhibitors (SSRIs, Fig. 4) that reduce menopausal symptoms.[81–83] This, however, imposes a new challenge because it is known, that some SSRIs have a high affinity for the CYP2D6 enzyme[84,85] and, therefore, SSRIs can inhibit CYP2D6 activity and interfere with tamoxifen efficacy by blocking the conversion of tamoxifen to endoxifen. The relative inhibitory concentrations of SSRIs for the CYP2D6 enzyme product are noted in the legend of Fig. 4. While the effect of SSRIs on the plasma levels of endoxifen had been previously reported by Stearns et al.,[23] this endoxifen lowering effect has been subsequently linked to the patients' CYP2D6 genotype by Jin et al.[29] Plasma concentrations after 4 months of tamoxifen therapy were significantly lower in patients with a CYP2D6 homozygous variant (20 nM; 95% CI: 11.1–28.9 nM) or heterozygous genotype (43.1 nM, 95% CI: 33.3–52.9 nM) than those with homozygous wild type (78.0 nM; 95% CI: 65.1–90.1 nM) (both $P = 0.003$). In this study, 24 of the 78 patients took CYP2D6 inhibitors including paroxetine, fluoxetine, sertraline, citalopram, amiodarone and metoclopramide. Among patients who carried a homozygous wild type genotype, the mean plasma endoxifen concentration for patients using CYP2D6 inhibitors was 58% lower than that of patients not using SSRI co-medication (38.6 nM versus 91.4 nM, $P = 0.0025$), and in patients who were heterozygous

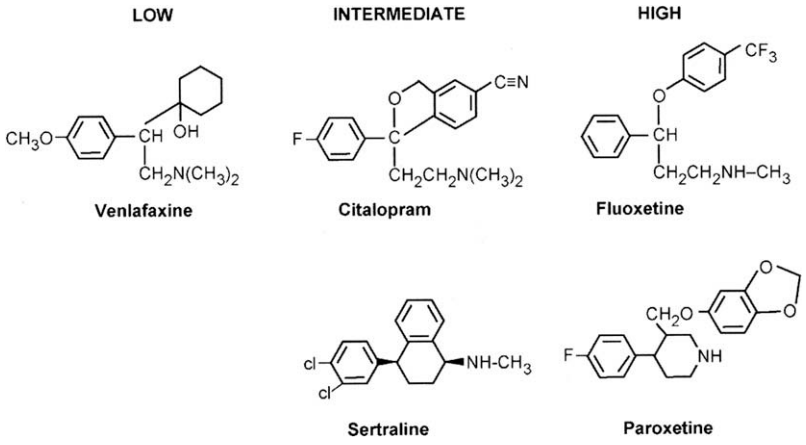

Fig. 4 – The selective serotonin re-uptake inhibitors (SSRIs) used to ameliorate hot flashes and menopausal symptoms during tamoxifen therapy. The SSRIs are CYP2D6 substrates and compete with N-desmethyltamoxifen for the CYP2D6 enzyme. Thy can be classified in high, intermediate, and low binding substrates for the CYP2D6 enzyme. The inhibitor constants for venlafaxine (low), citalopram (intermediate), and sertraline (intermediate), fluoxetine and paroxetine (high) are 33, 7, 1.5, 0.17 and 0.05, respectively.

for a non-functional CYP2D6 allele (wt/vt) this difference was 38% (31.0 nM versus 51.7, $P = 0.08$). Moreover, women taking the weak CYP2D6 inhibitor venlafaxine (a serotonine noradrenaline re-uptake inhibitor (SNRI)) had slightly reduced plasma endoxifen concentrations compared to women taking the potent CYP2D6 inhibitor paroxetine.[29] These findings suggest that both pharmacogenomic effects and pharmacological interactions of drugs at CYP2D6 have an influence on the metabolism of tamoxifen and, therefore, ultimately affect drug efficacy.

The extended investigations of Borges et al.[56] scrutinised the quantitative relationship between CYP2D6 variants, i.e. PM, IM and UM genotype, on endoxifen plasma concentrations in 158 patients at 4 months of 20 mg daily tamoxifen. They found that CYP2D6 genoptypes are highly associated with endoxifen plasma concentrations and account for their variability. While there were no significant differences in mean plasma concentrations of tamoxifen, N-desmethyltamoxifen and 4-hydroxytamoxifen between users and non-users of concomitant CYP2D6 inhibitors, the mean endoxifen plasma concentration was significantly lower in patients taking CYP2D6 inhibitors compared to that in patients who did not (39.6 ± 28.4 nmol/L versus 71.5 ± 41.2 nmol/L; $P < 0.01$).[56] When the authors divided the CYP2D6 inhibitors into potent (paroxetine, fluoxetine, $n = 19$) and weak (SSRI: sertraline and citalopram [$n = 14$] as well as celecoxib, diphenydramine and chlorpheniramine [$n = 13$]), they found a more pronounced decrease in mean endoxifen plasma concentrations with potent inhibitors than with weak inhibitors. Concomitant use of venlafaxine, which is considered the least potent inhibitor, did not show any significant effect. Taking into account CYP2D6 genotypes, the authors observed that the mean plasma endoxifen concentration was significantly lower in CYP2D6 EM patients who were taking potent CYP2D6 inhibitors compared to that in patients who were not (23.5 ± 9.5 nmol/L versus 84.1 ± 39.4 nmol/L, $P < 0.001$).[56] Thus, CYP2D6 genotype and concomitant potent CYP2D6 inhibitors are highly associated with plasma endoxifen concentrations and may substantially impact outcome during tamoxifen treatment by phenocopying effects i.e. converting an EM into a PM phenotype.

The phenocopying effect of SSRI with respect to their interplay with CYP2D6 genotype and effect on clinical outcome was explored by Goetz et al. in their recent follow-up of the NCCTG trial.[58] They investigated the role of CYP2D6 inhibitors in 256 patients that had been randomised to the tamoxifen alone arm. Patients with CYP2D6 wt/wt genotype who did not take CYP2D6 inhibitors were classified as EM ($n = 115$), whereas patients with either one or two *4 alleles or those taking a CYP2D6 inhibitor were classified as IM or PM ($n = 65$), depending on the strength of the inhibitor. Following these assignments, patients with decreased metabolism had shorter time to breast recurrence ($P = 0.015$), relapse-free ($P = 0.007$), disease-free ($P = 0.009$), and overall survival ($P = 0.082$) compared to those with extensive CYP2D6 metabolism.[58] The authors concluded that CYP2D6 metabolism, as measured by genetic variation and enzyme inhibition, is an independent predictor of breast cancer outcome in postmenopausal primary breast cancer patients receiving adjuvant tamoxifen. Accordingly, outcome during tamoxifen treatment may be influenced by its pharmacogenetics as well as co-prescription of drugs interfering with the CYP2D6 mediated tamoxifen metabolism.

3. Conclusion

In summary, we can conclude that endoxifen is formed by the CYP2D6 enzyme[21–23,28,35] and it is therefore anticipated that aberrant genotypes and other medicines that are metabolised by the same enzyme impair the actions of tamoxifen in patients.[29] We addressed the veracity of the hypothesis from the current literature to explore the possibility of targeting tamoxifen to improve women's health. There is now strong evidence that hot flashes are indicators of tamoxifen efficacy and that tamoxifen efficacy and outcome depend on the drug's metabolism which is subject to CYP2D6 genotype and pharmaco-interations. Data from numerous international studies[29,56–62] yielded consistent results in linking active tamoxifen metabolite plasma concentrations with genetically determined CYP2D6 metaboliser status, interference with strong CYP2D6 inhibitors, as well as clinical outcome. Few conflicting data[63–65] may be explained by variations in patient inclusion criteria into respective studies (e.g. variations in tamoxifen doses, length of treatment, additional chemotherapy regimens, lack of consistent ER testing). Importantly, most authors agree that genetic CYP2D6 variants, as well as CYP2D6 inhibition by prescribed co-medications such as antidepressants, may decrease tamoxifen metabolism, and thus negatively impact tamoxifen efficacy and treatment outcome.

There are a number of potential clinical consequences from these emerging data. First of all, strict compliance with tamoxifen treatment is critical for efficacy and outcome and, therefore, deviations from the prescribed course of adjuvant tamoxifen must be avoided even when side effects occur. Second, potent SSRIs such as paroxetine or fluoxetine should not be used for the relief of hot flashes in breast cancer patients receiving tamoxifen. Even though SSRIs are one of the few evidence-based therapy options for menopausal vasomotor symptoms,[86] available data indicate that they may compromise tamoxifen efficacy due to their interference with CYP2D6 dependent tamoxifen metabolism. Yet, this interference depends on the strength of the CYP2D6 inhibitor.[84,85] If treatment of hot flashes is indicated, a SSRI such as citalopram or escitalopram or a SNRI such as venlafaxine should be used because these substances showed no significant inhibition of CYP2D6.[29] Third, the CYP2D6 genotype/phenotype-treatment outcome relationship points to the possible benefit of upfront CYP2D6 genotyping prior to the initiation of endocrine treatment. A comprehensive robust, standardised, and quality controlled CYP2D6 genotyping test will need to analyse all relevant genetic variants that may affect tamoxifen metabolism which should include common PM alleles (*3, *4 and *5) and IM alleles depending on the individual's ethnic origin.[57–62] Of note, *41 is the most frequent IM allele in Europeans, *17 is the principal IM allele in Africans, and *10 dominates in Asians (in addition *9 should also be considered).[41] Other areas of interest with respect to clinical application are the measurement of endoxifen plasma levels as a surrogate of CYP2D6 phenotype and a possible dose increase of

tamoxifen to overcome impaired CYP2D6 metabolism; however, the latter option requires further investigation before definite conclusions can be made.

Given alternative treatment options, i.e. tamoxifen versus aromatase inhibitors (AI), and considering the available scientific and clinical evidence, an individualised approach for endocrine treatment of postmenopausal breast cancer patients is desirable. One might speculate that tamoxifen alone may be adequate for CYP2D6 EM/EM (wt/wt) carriers whereas postmenopausal patients with variant CYP2D6 alleles may fare better with upfront AI therapy. However, currently, a formal recommendation on the integration of CYP2D6 genotypes in treatment decisions must await their validation in statistically powered and/or prospective clinical trials. While these may be under way it will be interesting to see whether the small difference in the outcome benefit of AI as compared to tamoxifen recently reported from landmark trials BIG 1–98[87] and ATAC[17,88] can be attributed to the lack of CYP2D6 genotype stratification. This possibility should be considered particularly in the light of insights from a biomathematical modelling exercise of the estimated benefit of adjuvant tamoxifen according to CYP2D6 gene status. Using the BIG 1–98 information on recurrence probabilities and assuming that AI metabolism was CYP2D6 independent, it has been suggested that the benefit of 5 years of adjuvant tamoxifen may even exceed that of upfront AI treatment in postmenopausal CYP2D6 wt/wt patients.[89] In the meantime, the International Tamoxifen Pharmacogenetics Consortium (http://www.pharmgkb.org/views/project.jsp?pId=63) is making an effort towards pooled analysis of available data to further strengthen our understanding of the relationship between CYP2D6 metabolism status and tamoxifen efficacy.

Finally, the personalised approach in targeting tamoxifen seems feasible and should await timely translation into clinical practise. Indeed, the CYP2D6 genotype might be one of the first predictors of therapeutic response in cancer care. Because this approach is genome-based by utilising CYP2D6 genotyping for the prediction of a patient's metaboliser phenotype, ethical issues need to be sufficiently addressed. In the light of acceptable alternatives, an informed choice about adjuvant endocrine treatment and, most importantly, avoidance of a therapy that might potentially lack efficacy must be prime interests. It will therefore be important to make patients and their care takers aware of these issues and also to initiate discussions with regulatory authorities.

Conflict of interest statement

None declared.

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REFERENCES

1. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2003;**2**(3):205–13.
2. Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. *Br J Cancer* 1971;**25**(2):270–5.
3. NATO. Controlled trial of tamoxifen as adjuvant agent in management of early breast cancer. Interim analysis at four years by Nolvadex Adjuvant Trial Organisation. *Lancet* 1983;**1**(8319):257–61.
4. Fisher B, Costantino J, Redmond C, et al. A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. *N Engl J Med* 1989;**320**(8):479–84.
5. Cuzick J, Powles T, Veronesi U, et al. Overview of the main outcomes in breast-cancer prevention trials. *Lancet* 2003;**361**(9354):296–300.
6. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* 2005;**97**(22):1652–62.
7. Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. *J Natl Cancer Inst* 2007;**99**(4):283–90.
8. Ingle JN, Ahmann DL, Green SJ, et al. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *N Engl J Med* 1981;**304**(1):16–21.
9. Jordan VC, Koerner S. Tamoxifen (ICI 46, 474) and the human carcinoma 8S oestrogen receptor. *Eur J Cancer* 1975;**11**(3):205–6.
10. Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 1980;**16**(2):239–51.
11. Jordan VC. Effect of tamoxifen (ICI 46, 474) on initiation and growth of DMBA-induced rat mammary carcinomata. *Eur J Cancer* 1976;**12**(6):419–24.
12. Powles TJ, Hardy JR, Ashley SE, et al. A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *Br J Cancer* 1989;**60**(1):126–31.
13. Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 2008;**44**(1):30–8.
14. EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. *Lancet* 1998;**351**(9114):1451–67.
15. EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005;**365**(9472):1687–717.
16. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* 2007;**72**(1):7–25.
17. Howell A, Cuzick J, Baum M, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* 2005;**365**(9453):60–2.
18. Goss PE, Ingle JN, Martino S, et al. A randomized trial of letrozole in postmenopausal women after five years of

tamoxifen therapy for early-stage breast cancer. *N Engl J Med* 2003;**349**(19):1793–802.
19. Coombes RC, Hall E, Gibson LJ, et al. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. *N Engl J Med* 2004;**350**(11):1081–92.
20. Thurlimann B, Keshaviah A, Coates AS, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med* 2005;**353**(26):2747–57.
21. Lien EA, Solheim E, Kvinnsland S, Ueland PM. Identification of 4-hydroxy-N-desmethyltamoxifen as a metabolite of tamoxifen in human bile. *Cancer Res* 1988;**48**(8):2304–8.
22. Lien EA, Solheim E, Lea OA, Lundgren S, Kvinnsland S, Ueland PM. Distribution of 4-hydroxy-N-desmethyltamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. *Cancer Res* 1989;**49**(8):2175–83.
23. Stearns V, Johnson MD, Rae JM, et al. Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. *J Natl Cancer Inst* 2003;**95**(23):1758–64.
24. Jordan VC. New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer. *Steroids* 2007;**72**(13):829–42.
25. Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* 1977;**75**(2):305–16.
26. Allen KE, Clark ER, Jordan VC. Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure–activity relationships. *Br J Pharmacol* 1980;**71**(1):83–91.
27. Lim YC, Desta Z, Flockhart DA, Skaar TC. Endoxifen (4-hydroxy-N-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen. *Cancer Chemother Pharmacol* 2005;**55**(5):471–8.
28. Johnson MD, Zuo H, Lee KH, et al. Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. *Breast Cancer Res Treat* 2004;**85**(2):151–9.
29. Jin Y, Desta Z, Stearns V, et al. CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. *J Natl Cancer Inst* 2005;**97**(1):30–9.
30. Lim YC, Li L, Desta Z, et al. Endoxifen, a secondary metabolite of tamoxifen, and 4-OH-tamoxifen induce similar changes in global gene expression patterns in MCF-7 breast cancer cells. *J Pharmacol Exp Ther* 2006;**318**(2):503–12.
31. Wu X, Hawse JR, Subramaniam M, Goetz MP, Ingle JN, Spelsberg TC. The tamoxifen metabolite, endoxifen, is a potent antiestrogen that targets estrogen receptor alpha for degradation in breast cancer cells. *Cancer Res* 2009;**69**(5):1722–7.
32. Pike AC, Brzozowski AM, Walton J, et al. Structural insights into the mode of action of a pure antiestrogen. *Structure* 2001;**9**(2):145–53.
33. Wu YL, Yang X, Ren Z, et al. Structural basis for an unexpected mode of SERM-mediated ER antagonism. *Mol Cell* 2005;**18**(4):413–24.
34. Shiau AK, Barstad D, Loria PM, et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 1998;**95**(7):927–37.
35. Desta Z, Ward BA, Soukhova NV, Flockhart DA. Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: prominent roles for CYP3A and CYP2D6. *J Pharmacol Exp Ther* 2004;**310**(3):1062–75.
36. Eichelbaum M, Spannbrucker N, Dengler HJ. Proceedings: N-oxidation of sparteine in man and its interindividual differences. *Naunyn Schmiedebergs Arch Pharmacol* 1975;**287**(Suppl.):R94.
37. Mahgoub A, Idle JR, Dring LG, Lancaster R, Smith RL. Polymorphic hydroxylation of Debrisoquine in man. *Lancet* 1977;**2**(8038):584–6.
38. Griese EU, Zanger UM, Brudermanns U, et al. Assessment of the predictive power of genotypes for the in-vivo catalytic function of CYP2D6 in a German population. *Pharmacogenetics* 1998;**8**(1):15–26.
39. Raimundo S, Toscano C, Klein K, et al. A novel intronic mutation, 2988G>A, with high predictivity for impaired function of cytochrome P450 2D6 in white subjects. *Clin Pharmacol Ther* 2004;**76**(2):128–38.
40. Sachse C, Brockmoller J, Bauer S, Roots I. Cytochrome P450 2D6 variants in a Caucasian population: allele frequencies and phenotypic consequences. *Am J Hum Genet* 1997;**60**(2):284–95.
41. Sistonen J, Sajantila A, Lao O, Corander J, Barbujani G, Fuselli S. CYP2D6 worldwide genetic variation shows high frequency of altered activity variants and no continental structure. *Pharmacogenet Genomics* 2007;**17**(2):93–101.
42. Marez D, Legrand M, Sabbagh N, et al. Polymorphism of the cytochrome P450 CYP2D6 gene in a European population: characterization of 48 mutations and 53 alleles, their frequencies and evolution. *Pharmacogenetics* 1997;**7**(3): 193–202.
43. Zanger UM, Turpeinen M, Klein K, Schwab M. Functional pharmacogenetics/genomics of human cytochromes P450 involved in drug biotransformation. *Anal Bioanal Chem* 2008;**392**(6):1093–108.
44. Raimundo S, Fischer J, Eichelbaum M, Griese EU, Schwab M, Zanger UM. Elucidation of the genetic basis of the common 'intermediate metabolizer' phenotype for drug oxidation by CYP2D6. *Pharmacogenetics* 2000;**10**(7):577–81.
45. Toscano C, Klein K, Blievernicht J, et al. Impaired expression of CYP2D6 in intermediate metabolizers carrying the *41 allele caused by the intronic SNP 2988G>A: evidence for modulation of splicing events. *Pharmacogenet Genomics* 2006;**16**(10):755–66.
46. Zanger UM, Fischer J, Raimundo S, et al. Comprehensive analysis of the genetic factors determining expression and function of hepatic CYP2D6. *Pharmacogenetics* 2001;**11**(7):573–85.
47. Zanger UM, Raimundo S, Eichelbaum M. Cytochrome P450 2D6: overview and update on pharmacology, genetics, biochemistry. *Naunyn Schmiedebergs Arch Pharmacol* 2004;**369**(1):23–37.
48. Dahl ML, Johansson I, Bertilsson L, Ingelman-Sundberg M, Sjoqvist F. Ultrarapid hydroxylation of debrisoquine in a Swedish population. Analysis of the molecular genetic basis. *J Pharmacol Exp Ther* 1995;**274**(1):516–20.
49. Johansson I, Lundqvist E, Bertilsson L, Dahl ML, Sjoqvist F, Ingelman-Sundberg M. Inherited amplification of an active gene in the cytochrome P450 CYP2D locus as a cause of ultrarapid metabolism of debrisoquine. *Proc Natl Acad Sci USA* 1993;**90**(24):11825–9.
50. Bertilsson L, Dahl ML, Sjoqvist F, et al. Molecular basis for rational megaprescribing in ultrarapid hydroxylators of debrisoquine. *Lancet* 1993;**341**(8836):63.
51. Dalen P, Dahl ML, Bernal Ruiz ML, Nordin J, Bertilsson L. 10-Hydroxylation of nortriptyline in white persons with 0, 1, 2, 3, and 13 functional CYP2D6 genes. *Clin Pharmacol Ther* 1998;**63**(4):444–52.
52. Lovlie R, Daly AK, Matre GE, Molven A, Steen VM. Polymorphisms in CYP2D6 duplication-negative individuals with the ultrarapid metabolizer phenotype: a role for the CYP2D6*35 allele in ultrarapid metabolism? *Pharmacogenetics* 2001;**11**(1):45–55.

53. Aklillu E, Persson I, Bertilsson L, Johansson I, Rodrigues F, Ingelman-Sundberg M. Frequent distribution of ultrarapid metabolizers of debrisoquine in an ethiopian population carrying duplicated and multiduplicated functional CYP2D6 alleles. *J Pharmacol Exp Ther* 1996;**278**(1):441–6.
54. McLellan RA, Oscarson M, Seidegard J, Evans DA, Ingelman-Sundberg M. Frequent occurrence of CYP2D6 gene duplication in Saudi Arabians. *Pharmacogenetics* 1997;**7**(3):187–91.
55. Ingelman-Sundberg M. Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity. *Pharmacogenomics J* 2005;**5**(1):6–13.
56. Borges S, Desta Z, Li L, et al. Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. *Clin Pharmacol Ther* 2006;**80**(1):61–74.
57. Goetz MP, Rae JM, Suman VJ, et al. Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. *J Clin Oncol* 2005;**23**(36):9312–8.
58. Goetz MP, Knox SK, Suman VJ, et al. The impact of cytochrome P450 2D6 metabolism in women receiving adjuvant tamoxifen. *Breast Cancer Res Treat* 2007;**101**(1):113–21.
59. Schroth W, Antoniadou L, Fritz P, et al. Breast cancer treatment outcome with adjuvant tamoxifen relative to patient CYP2D6 and CYP2C19 genotypes. *J Clin Oncol* 2007;**25**(33):5187–93.
60. Lim HS, Ju LH, Seok LK, Sook LE, Jang IJ, Ro J. Clinical implications of CYP2D6 genotypes predictive of tamoxifen pharmacokinetics in metastatic breast cancer. *J Clin Oncol* 2007;**25**(25):3837–45.
61. Xu Y, Sun Y, Yao L, et al. Association between CYP2D6 *10 genotype and survival of breast cancer patients receiving tamoxifen treatment. *Ann Oncol* 2008;**19**(8):1423–9.
62. Kiyotani K, Mushiroda T, Sasa M, et al. Impact of CYP2D6*10 on recurrence-free survival in breast cancer patients receiving adjuvant tamoxifen therapy. *Cancer Sci* 2008;**99**(5):995–9.
63. Nowell SA, Ahn J, Rae JM, et al. Association of genetic variation in tamoxifen-metabolizing enzymes with overall survival and recurrence of disease in breast cancer patients. *Breast Cancer Res Treat* 2005;**91**(3):249–58.
64. Wegman P, Vainikka L, Stal O, et al. Genotype of metabolic enzymes and the benefit of tamoxifen in postmenopausal breast cancer patients. *Breast Cancer Res* 2005;**7**(3):R284–90.
65. Wegman P, Elingarami S, Carstensen J, Stal O, Nordenskjold B, Wingren S. Genetic variants of CYP3A5, CYP2D6, SULT1A1, UGT2B15 and tamoxifen response in postmenopausal patients with breast cancer. *Breast Cancer Res* 2007;**9**(1):R7.
66. Bonanni B, Macis D, Maisonneuve P, et al. Polymorphism in the CYP2D6 tamoxifen-metabolizing gene influences clinical effect but not hot flashes: data from the Italian Tamoxifen Trial. *J Clin Oncol* 2006;**24**(22):3708–9.
67. Newman WG, Hadfield KD, Latif A, et al. Impaired tamoxifen metabolism reduces survival in familial breast cancer patients. *Clin Cancer Res* 2008;**14**(18):5913–8.
68. Powles T, Eeles R, Ashley S, et al. Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomised chemoprevention trial. *Lancet* 1998;**352**(9122):98–101.
69. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 1998;**90**(18):1371–88.
70. Demissie S, Silliman RA, Lash TL. Adjuvant tamoxifen: predictors of use, side effects, and discontinuation in older women. *J Clin Oncol* 2001;**19**(2):322–8.
71. Fink AK, Gurwitz J, Rakowski W, Guadagnoli E, Silliman RA. Patient beliefs and tamoxifen discontinuance in older women with estrogen receptor-positive breast cancer. *J Clin Oncol* 2004;**22**(16):3309–15.
72. Lash TL, Fox MP, Westrup JL, Fink AK, Silliman RA. Adherence to tamoxifen over the five-year course. *Breast Cancer Res Treat* 2006;**99**(2):215–20.
73. McCowan C, Shearer J, Donnan PT, et al. Cohort study examining tamoxifen adherence and its relationship to mortality in women with breast cancer. *Br J Cancer* 2008;**99**(11):1763–8.
74. Partridge AH, Wang PS, Winer EP, Avorn J. Nonadherence to adjuvant tamoxifen therapy in women with primary breast cancer. *J Clin Oncol* 2003;**21**(4):602–6.
75. Barron TI, Connolly R, Bennett K, Feely J, Kennedy MJ. Early discontinuation of tamoxifen: a lesson for oncologists. *Cancer* 2007;**109**(5):832–9.
76. Fallowfield L. Acceptance of adjuvant therapy and quality of life issues. *Breast* 2005;**14**(6):612–6.
77. Owusu C, Buist DS, Field TS, et al. Predictors of tamoxifen discontinuation among older women with estrogen receptor-positive breast cancer. *J Clin Oncol* 2008;**26**(4):549–55.
78. Mortimer JE, Flatt SW, Parker BA, et al. Tamoxifen, hot flashes and recurrence in breast cancer. *Breast Cancer Res Treat* 2008;**108**(3):421–6.
79. Gjerde J, Hauglid M, Breilid H, et al. Effects of CYP2D6 and SULT1A1 genotypes including SULT1A1 gene copy number on tamoxifen metabolism. *Ann Oncol* 2008;**19**(1):56–61.
80. Decensi A, Gandini S, Serrano D, et al. Randomized dose-ranging trial of tamoxifen at low doses in hormone replacement therapy users. *J Clin Oncol* 2007;**25**(27): 4201–9.
81. Loprinzi CL, Kugler JW, Sloan JA, et al. Venlafaxine in management of hot flashes in survivors of breast cancer: a randomised controlled trial. *Lancet* 2000;**356**(9247):2059–63.
82. Loprinzi CL, Sloan JA, Perez EA, et al. Phase III evaluation of fluoxetine for treatment of hot flashes. *J Clin Oncol* 2002;**20**(6):1578–83.
83. Stearns V, Beebe KL, Iyengar M, Dube E. Paroxetine controlled release in the treatment of menopausal hot flashes: a randomized controlled trial. *JAMA* 2003;**289**(21):2827–34.
84. Crewe HK, Lennard MS, Tucker GT, Woods FR, Haddock RE. The effect of selective serotonin re-uptake inhibitors on cytochrome P4502D6 (CYP2D6) activity in human liver microsomes. *Br J Clin Pharmacol* 1992;**34**(3):262–5.
85. Jeppesen U, Gram LF, Vistisen K, Loft S, Poulsen HE, Brosen K. Dose-dependent inhibition of CYP1A2, CYP2C19 and CYP2D6 by citalopram, fluoxetine, fluvoxamine and paroxetine. *Eur J Clin Pharmacol* 1996;**51**(1):73–8.
86. Hickey M, Davis SR, Sturdee DW. Treatment of menopausal symptoms: what shall we do now? *Lancet* 2005;**366**(9483):409–21.
87. Coates AS, Keshaviah A, Thurlimann B, et al. Five years of letrozole compared with tamoxifen as initial adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer: update of study BIG 1–98. *J Clin Oncol* 2007;**25**(5):486–92.
88. Forbes JF, Cuzick J, Buzdar A, Howell A, Tobias JS, Baum M. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 100-month analysis of the ATAC trial. *Lancet Oncol* 2008;**9**(1):45–53.
89. Punglia RS, Burstein HJ, Winer EP, Weeks JC. Pharmacogenomic variation of CYP2D6 and the choice of optimal adjuvant endocrine therapy for postmenopausal breast cancer: a modeling analysis. *J Natl Cancer Inst* 2008;**100**(9):642–8.

Leading Article

Chemoprevention of Breast Cancer with Selective Estrogen Receptor Modulators

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Thirty years ago, there were no practical measures available in clinical practice to reduce the incidence of breast cancer in women. Today, through effective translational research, scientific principles from the laboratory have enhanced patient options to reduce their risk of developing breast cancer. Tamoxifen and raloxifene are selective estrogen receptor (ER) modulators (SERMs) that are estrogen-like and enhance bone density in postmenopausal women, but are also antiestrogenic and prevent breast cancer growth and development. Clinical studies have demonstrated that tamoxifen is safe and effective in reducing the incidence of breast cancer in premenopausal high-risk women. However, tamoxifen increases the risk of endometrial cancer in postmenopausal high-risk women. Raloxifene represents a useful and safe alternative chemopreventive therapy that does not elevate the incidence of endometrial cancer in postmenopausal women. Most importantly, the use of raloxifene to prevent osteoporosis in osteopenic postmenopausal women has the added advantage of preventing breast cancer and endometrial cancer as a beneficial side effect. This article will review the history of chemoprevention in breast cancer from concept to clinical practice.

The lost origins of the idea of chemoprevention

The early clinical observation that there is a link between the removal of the ovaries and the regression of approximately one-third of metastatic breast cancers in pre-menopausal women acted as a catalyst for investigations to discover the cause of this observation [1,2]. In 1916, Lathrop and Loeb demonstrated that early oophorectomy would prevent the development of spontaneous mammary tumors in high-incidence strains of mice [3]. In the 1930s, Lacassagne followed up this observation with the demonstration that various estrogens increase mouse mammary tumorigenesis [4]. He proposed that "if one accepts the consideration of adenocarcinoma of the breast as a consequence of a special hereditary sensibility to the proliferative action of estrone, one is led to imagine a therapeutic preventative for subjects predisposed by their heredity to this cancer" [5].

The challenge was, where to start? The mechanism of estrogen-mediated mammary tumorigenesis was unknown, there was no target or model system for screening drugs, and there were no known synthetic antiestrogenic compounds with which to start a systematic search for clinically useful medicines. The discovery of the ER and the first non-steroidal antiestrogen in the late 1950s would open the door to innovation in the field of targeted therapy for breast cancer and accelerate progress in the practical application of chemoprevention [6,7].

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The discovery of selective ER modulation

The development of tamoxifen in the late 1970s as the first targeted therapy for breast cancer created a new dimension for the long-term adjuvant treatment of ER-positive disease (**Figure 1**) [8]. In comparison to chemotherapy, the side effects were minimal. For 20 years, tamoxifen became the "gold standard" for the treatment of all stages of ER-positive breast cancer worldwide, and is credited with not only enhancing survivorship [9] but also reducing the incidence of contralateral breast cancer by 50% [10]. The latter clinical observation, the extensive clinical experience with patients receiving extended tamoxifen treatment, and the prior laboratory studies propelled tamoxifen forward as the agent of choice for evaluation as a chemopreventive therapy for women at high-risk of breast cancer [11–16]. By the 1980s, an enormous clinical database on tamoxifen use had been accrued, and its side effects were apparently well documented; that is, except for the most important one – an elevation in the risk of endometrial cancer in postmenopausal women.

Laboratory studies in the mid-1980s documented that the so-called non-steroidal antiestrogens tamoxifen and subsequently raloxifene (**Figure 1**) could either stimulate or block the effects of estrogen in its target tissues around an animal's body [17]. Most importantly, human breast and endometrial cancers bitransplanted into the same athymic (immunodeficient) ovariectomized mouse were found to respond differently to tamoxifen [18]. Tamoxifen inhibited estrogen-stimulated breast tumor growth but stimulated the growth of human endometrial cancer. The investigators who conducted that laboratory study suggested that patients treated with adjuvant tamoxifen should be evaluated for the growth of occult endometrial cancer. Examination of data from clinical trials evaluating different durations of adjuvant tamoxifen for the treatment of breast cancer against placebo, demonstrated a small, but significant, rise in the incidence of endometrial cancer in postmenopausal patients [19,20]. Clearly, this was an important side effect to evaluate in subsequent chemoprevention studies.

An additional concern about the evaluation of an "antiestrogen" for the chemoprevention of breast cancer was the potential for interference with the normal physiological requirements of estrogen in a woman's body. If estrogen was essential to maintain bone density in women, perhaps an antiestrogen would precipitate severe osteoporosis in postmenopausal women. However, the finding that both tamoxifen and raloxifene would, in fact, maintain bone density in ovariectomized rats suggested that the estrogen-like properties of these compounds in bone could benefit women [21]. Thus, the use of tamoxifen as a chemopreventive agent could potentially preserve bone density in postmenopausal women; this was subsequently found to be correct [22]. Nonetheless, the concerns about the risk of endometrial cancer in postmenopausal women meant that either clinical trials of tamoxifen would have to recruit hysterectomized women, or a safer SERM was needed to prevent osteoporosis and breast cancer at the same time [7]. The subsequent development of raloxifene to prevent both osteoporosis and breast cancer in postmenopausal women, but without an increase in endometrial cancer, provided physicians with two valuable approved medicines that have now been evaluated in international clinical trials [23].

Although efforts continue to improve breast cancer prevention by improving the side-effect profile of tamoxifen with low-dose therapy [24–26], improving the efficiency and reducing the side effects of tamoxifen with aromatase inhibitors in postmenopausal women [27], and reducing the incidence of ER-negative breast cancer [28], we have chosen to focus on the use of the currently available SERMs in clinical practice in this review.

Clinical trial evidence

Cuzick et al. conducted an overview of the results of the randomized chemoprevention trials of tamoxifen [29], also including the early evaluation of the reduction in breast cancer

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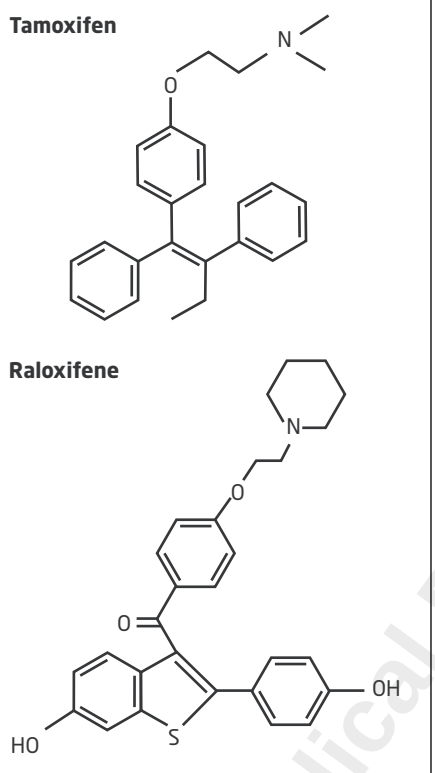
Figure 1. A structural comparison of tamoxifen and raloxifene.



incidence when raloxifene was used to prevent osteoporosis [30]. Overall, tamoxifen was found to reduce the incidence of breast cancer in high-risk women, but the main effect observed in the largest randomized trial, the National Surgical Adjuvant Breast and Bowel Project (NSABP) P-1 study [11,16], was diluted by the results of the smaller trials – the Royal Marsden study [12], the Italian Tamoxifen Prevention Study [14], and IBIS-I (the International Breast Cancer Intervention Study) [15].

The NSABP P-1 study recruited 13 338 pre- and postmenopausal women at high risk as determined by the Gail model [31]. The study design is summarized in **Figure 2**. Volunteers were randomized to tamoxifen 20 mg daily or placebo with an intended treatment duration of 5 years. The study was unblinded early because the trial achieved its objective; tamoxifen reduced the incidence of ER-positive breast cancer in all categories of women at risk. The incidence of ER-negative breast cancer was unaffected; however, a recent re-analysis demonstrated that ER-negative tumors were detected earlier during tamoxifen treatment [32]. Blood clots, stroke, and endometrial cancer were all significantly more frequent in tamoxifen-treated women, but it must be stressed that these side effects were confined to postmenopausal women [11,16].

Overall, in the randomized trials of tamoxifen chemoprevention, although tamoxifen produced a pronounced early and prolonged benefit in reducing the risk of breast cancer, no survival advantage was noted. This is not surprising, as all of the studies were underpowered to detect such an advantage and survival was never a stated endpoint. Be that as it may, the fact that the application of a cheap medicine (by modern standards) leads to fewer breast cancers to treat reduces the burden for the healthcare system. Most importantly, there was no excess of deaths as a result of tamoxifen treatment in the NSABP P-1 study [11], the Italian study [14], or the Royal Marsden study [12]. In contrast, in the IBIS-1 study tamoxifen treatment was associated with an increased death rate from thrombotic events [15]. However, the investigators attributed their findings to the observation that the fatal clotting occurred only in women who had elective surgery scheduled and did not know to stop their tamoxifen (or did not even know they were taking tamoxifen).

Two other important scientific observations that deserve comment emerged from the Italian study [14] and the Royal Marsden study [12]. The Italian study recruited women without the requirement for them to be at high risk, and women were permitted to remain on hormone-replacement therapy (HRT) irrespective of whether they were randomized to placebo or tamoxifen. The reason for this was that many women in the trial had previously undergone an elective ovariohysterectomy. Subsequent analysis demonstrated that tamoxifen treatment blocked the rise in breast cancer incidence observed with HRT [33]. This is an important result, as current trials of novel SERMs are evaluating the co-administration of HRT to

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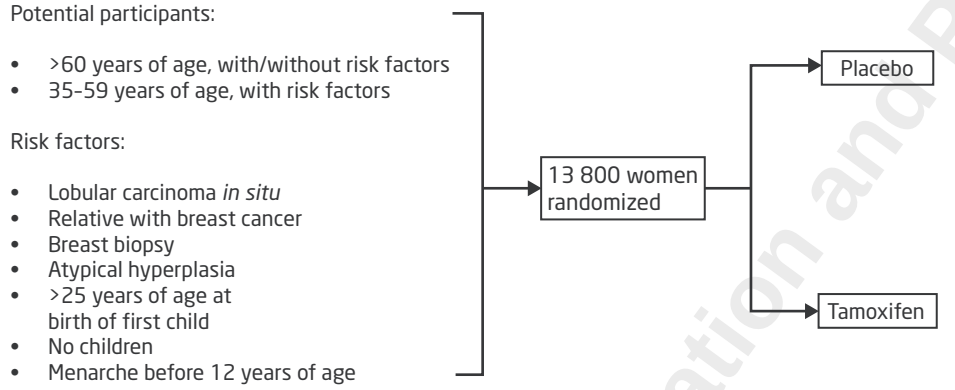
Figure 2. A summary of the design of the NSABP P-1 trial [11]. Over 13 000 women determined to be high risk by the Gail model were randomized to receive placebo or tamoxifen (20 mg/day) for 5 years.



NSABP: National Surgical Adjuvant Breast and Bowel Project.

reduce hot flashes and block estrogen-induced carcinogenesis in the uterus and breast at the same time. These data are consistent with the recent report that a decrease in the prescription of HRT correlates with a decrease in breast cancer incidence [34].

The interim analysis of the Royal Marsden study did not show a reduction in the incidence of breast cancer among patients taking tamoxifen [12]; however, 20 years after the study started a significant reduction in the incidence of breast cancer was observed [35]. Thus, the greatest impact of tamoxifen treatment is noted long after therapy is stopped and at a time when there are no significant side effects. Indeed, this is a general characteristic of tamoxifen that has been observed in all of the trials [16,36] and was first noted in adjuvant treatment trials with the dual endpoint of contralateral breast cancer and recurrence of disease following surgery [9,37]. Tamoxifen in some way creates a cellular state that is ultimately lethal for occult or nascent breast cancer during the 5-year treatment period. This could be described as the antiestrogenic effect of tamoxifen on proliferation. However, tamoxifen is not necessarily apoptotic or lethal to cells. It is as if tamoxifen somehow sensitizes the tumor to vulnerability by enhancing survival mechanisms that collapse once tamoxifen is stopped. Although the exact mechanism is unknown, it may be related to the known evolution of drug resistance during tamoxifen therapy [38] to phase II resistance to tamoxifen that results in breast cancer cells perceiving physiological estrogen as an apoptotic trigger [39,40].

SERMs to prevent multiple diseases

Raloxifene was reinvented from the failed breast cancer drug keoxifene to become the first multifunctional medicine for women's health [17,23]. The evidence-based hypothesis underlying its development proposed that selective ER modulation could be applied to prevent multiple diseases in women [17]. Nearly 2 decades later, the veracity of this hypothesis can be assessed through the results of prospective, randomized, clinical trials. The ongoing evaluation of raloxifene as a preventive agent for osteoporosis now extends for more than a decade [41]. The overall conclusion of the major breast cancer study was that raloxifene significantly reduces the rate of breast cancer development in low-risk women who are treated to prevent osteoporosis [30]. There is no increase in endometrial cancer with raloxifene, and the 40–50% reduction in breast cancer incidence is consistent across multiple trials of normal and high-risk women

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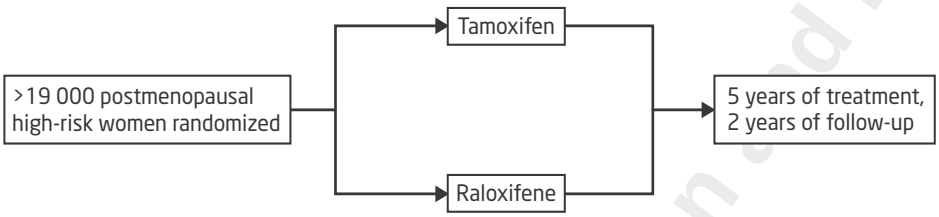
Figure 3. A summary of the design of STAR [43]. Over 19 000 high-risk postmenopausal women were randomized to receive raloxifene (60 mg/day) or tamoxifen (20 mg/day) for 5 years and then followed for 2 additional years after completion of treatment. A self-reporting subproject was also undertaken to compare endometrial and uterine characteristics.



STAR: the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene/P-2 trial.

[42,43]. STAR (the NSABP Study of Tamoxifen and Raloxifene/P-2 Trial; **Figure 3**), which recruited >19 000 high-risk postmenopausal women, demonstrated that raloxifene is equally as effective as tamoxifen at reducing breast cancer incidence but with an improved safety profile [43]. There are fewer hysterectomies because of uterine bleeding and concerns about endometrial cancer, fewer cataract operations, and fewer blood clots in patients receiving raloxifene compared with tamoxifen.

Overall, the last decade has seen significant advances in the application of SERMs to address the need to reduce breast cancer incidence. Several new SERMs are currently entering clinical evaluation, so it is likely that in the future there will be a whole menu of medicines available to address individual patient needs [44]. With this in mind, it is now perhaps important to summarize the issues that need to be considered by physicians with regard to the risks and benefits of the current SERMs for chemoprevention.

Risks of chemoprevention

Evaluation of the pharmacology of tamoxifen is ongoing. The medicine is perhaps the most investigated yet cost-effective cancer therapeutic agent. Hot flashes are a concern during long-term therapy with tamoxifen, as they can encourage the patient to either stop treatment prematurely, thereby reducing the effectiveness of chemoprevention, or seek medical help in the form of selective serotonin reuptake inhibitors (SSRIs). In fact, it appears that hot flashes are a predictive sign that therapy is optimal [45,46]. Tamoxifen needs to be metabolically activated to its more active metabolites, one of which – endoxifen – is a major factor in the antitumor actions of tamoxifen. In other words, the more metabolism via cytochrome P450 2D6 (CYP2D6), the more endoxifen, and the more effective the therapy; hot flashes are a surrogate for metabolic activation to a more potent antiestrogenic product (**Figure 4**). The ability of SSRIs to attenuate hot flashes may involve blocking tamoxifen's metabolic activation at the CYP2D6 enzyme, and may therefore reduce the effectiveness of tamoxifen [47]. Thus, the agent of choice to reduce hot flashes is venlafaxine, as it has a low affinity for the CYP2D6 enzyme system.

An additional concern with the use of tamoxifen as a chemopreventive agent is that it may not be activated at all in patients with polymorphisms in CYP2D6. Should the US Food and Drug Administration choose to relabel tamoxifen in the future based on evidence from the application of the antiestrogen as a treatment, it would be wise to consider genotyping patients to ensure maximal efficacy during long-term therapy.

The most notable risks of tamoxifen as a chemopreventive are the aforementioned increase in endometrial cancer in post-menopausal women and an increase in blood clots. Raloxifene is an appropriate substitute without these risks.

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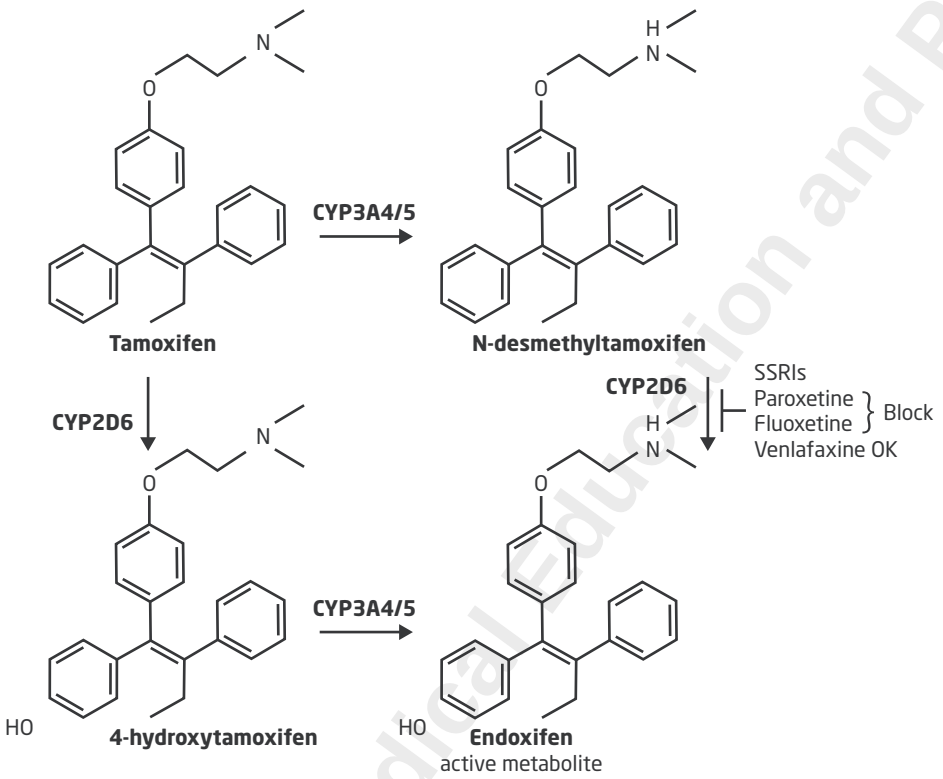
Figure 4. Undermining the actions of tamoxifen. The figure depicts the metabolism of tamoxifen to its active metabolite, endoxifen, via CYP2D6. SSRIs block this step and reduce tamoxifen's effectiveness. Venlafaxine does not block the conversion of tamoxifen to endoxifen but does reduce hot flashes [51,52].



CYP2D6: cytochrome P450 2D6; SSRI: selective serotonin reuptake inhibitors.

Benefits of chemoprevention

Approximately 1 million women worldwide are diagnosed with breast cancer each year [48]. Employing tamoxifen as a cheap and effective chemopreventive agent has an enormous public health benefit. In premenopausal patients, tamoxifen does not increase blood clots or endometrial cancer, and reduces breast cancer incidence by 50%. In fact, the benefits extend far beyond the time of therapy, and continuing and enhanced protection can be anticipated for a decade after treatment. The worldwide availability of raloxifene for the prevention of osteoporosis in postmenopausal women provides the opportunity to address the prevention of two diseases at the same time. Raloxifene is a superior medicine to tamoxifen for public health, as it is approved for both the prevention of osteoporosis and the reduction of breast cancer risk in the US. Recent evidence suggests that for those postmenopausal women who choose to take tamoxifen, there is a significant reduction in fracture rates. However, this is only true during treatment [49]. As mentioned above, raloxifene, in contrast to tamoxifen, does not increase the risk of endometrial cancer [50] and has an overall lower incidence of side effects [43].

Chemoprevention in clinical practice

It is now possible to summarize the appropriate use of the current SERMs in clinical practice (**Table 1**). Tamoxifen is only approved for reducing the risk of breast cancer in high-risk populations in the US, but raloxifene is approved for the treatment and prevention of osteoporosis in dozens of countries worldwide;

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Table 1. Breast cancer prevention and practice in 2008: a summary of the appropriate use of SERMs in current clinical practice [53–55].

Population	SERM	Outcome
High-risk premenopausal women	Tamoxifen	Reduction in the risk of breast cancer and no increase in blood clots or endometrial cancer
High-risk postmenopausal women	Raloxifene	Reduction in the risk of breast cancer and no increase in endometrial cancer
Patients with or at risk of osteoporosis	Raloxifene	Treatment of and reduction in the risk of osteoporosis, reduction in the risk of breast cancer, and no increase in endometrial cancer

SERM: selective estrogen receptor modulator.

thus, breast cancer incidence is being reduced indirectly. The past 3 decades have seen enhanced optimism about the early detection and first effective targeted treatment of breast cancer with adjuvant tamoxifen, as this practice has led to a trend towards increased patient survivorship [37]. However, early detection is a "wait and see" philosophy, while chemoprevention seeks to intervene to block the natural history of carcinogens. Unlike with lung cancer, in which one can choose not to smoke, no lifestyle change will prevent breast cancer (except not taking HRT). The SERMs are the first practical medicines that can reduce the incidence of breast cancer by 50%. Although the approach is not perfect, and breast cancer is not completely eradicated, the present progress is an essential first step in the ongoing process to prevent breast cancer completely.

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References

1. Beatson GT. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. *Lancet* 1896;2:104–7.
2. Boyd S. On oophorectomy in cancer of the breast. *BMJ* 1900;2:1161–7.
3. Lathrop AE, Loeb L. Further investigations on the origins of tumors in mice. III. On the part played by internal secretion in the spontaneous development of tumors. *J Cancer Res* 1916;1:1–19.
4. Lacassagne A. A comparative study of the carcinogenic action of certain oestrogenic hormones. *Am J Cancer* 1936;28:735–40.
5. Lacassagne A. Hormonal pathogenesis of adenocarcinoma of the breast. *Am J Cancer* 1936;27:217–25.
6. Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. *Clin Cancer Res* 2003;9:1980–9.
7. Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. *Cancer Res* 1990;50:4177–89.
8. Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 2008;44:30–8.
9. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005;365:1687–717.
10. Cuzick J, Baum M. Tamoxifen and contralateral breast cancer. *Lancet* 1985;2:282.
11. Fisher B, Costantino JP, Wickerham DL et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 1998;90:1371–88.
12. Powles T, Eeles R, Ashley S et al. Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomised chemoprevention trial. *Lancet* 1998;352:98–101.
13. Powles TJ, Hardy JR, Ashley SE et al. A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *Br J Cancer* 1989;60:126–31.
14. Veronesi U, Maisonneuve P, Costa A et al. Prevention of breast cancer with tamoxifen: preliminary findings from the Italian randomised trial among hysterectomised women. Italian Tamoxifen Prevention Study. *Lancet* 1998;352:93–7.
15. Cuzick J, Forbes J, Edwards R et al. First results from the International Breast Cancer Intervention Study (IBIS-I): a randomised prevention trial. *Lancet* 2002;360:817–24.
16. Fisher B, Costantino JP, Wickerham DL et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* 2005;97:1652–62.
17. Jordan VC. Selective estrogen receptor modulation: a personal perspective. *Cancer Res* 2001;61:5683–7.
18. Gottardis MM, Robinson SP, Satyaswaroop PG et al. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 1988;48:812–5.
19. Fornander T, Rutqvist LE, Cedermark B et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* 1989;1:117–20.
20. Fisher B, Costantino, JP, Redmond CK et al. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. *J Natl Cancer Inst* 1994;86:527–37.

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21. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 1987;10:31–5.
22. Love RR, Mazess RB, Barden HS et al. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med* 1992;326:852–6.
23. Jordan VC. The rise of raloxifene and the fall of invasive breast cancer. *J Natl Cancer Inst* 2008;100:831–3.
24. Guerrieri-Gonzaga A, Robertson C, Bonanni B et al. Preliminary results on safety and activity of a randomized, double-blind, 2 × 2 trial of low-dose tamoxifen and fenretinide for breast cancer prevention in premenopausal women. *J Clin Oncol* 2006;24:129–35.
25. Decensi A, Gandini S, Serrano D et al. Randomized dose-ranging trial of tamoxifen at low doses in hormone replacement therapy users. *J Clin Oncol* 2007;25:4201–9.
26. Decensi A, Robertson C, Guerrieri-Gonzaga A et al. Randomized double-blind 2 × 2 trial of low-dose tamoxifen and fenretinide for breast cancer prevention in high-risk premenopausal women. *J Clin Oncol* 2009;27:3749–56.
27. Cuzick J. Aromatase inhibitors for breast cancer prevention. *J Clin Oncol* 2005;23:1636–43.
28. Li Y, Brown PH. Prevention of ER-negative breast cancer. *Recent Results Cancer Res* 2009;181:121–34.
29. Cuzick J, Powles T, Veronesi U et al. Overview of the main outcomes in breast-cancer prevention trials. *Lancet* 2003;361:296–300.
30. Cummings SR, Eckert S, Krueger et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* 1999;281:2189–97.
31. Gail MH, Brinton LA, Byar DP et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst* 1989;81:1879–86.
32. Shen Y, Costantino JP, Qin J. Tamoxifen chemoprevention treatment and time to first diagnosis of estrogen receptor-negative breast cancer. *J Natl Cancer Inst* 2008;100:1448–53.
33. Veronesi U, Maisonneuve P, Sacchini V et al. Tamoxifen for breast cancer among hysterectomised women. *Lancet* 2002;359:1122–4.
34. Ravdin PM, Cronin KA, Howlader N et al. The decrease in breast-cancer incidence in 2003 in the United States. *N Engl J Med* 2007;356:1670–4.
35. Powles TJ, Ashley S, Tidy A et al. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. *J Natl Cancer Inst* 2007;99:283–90.
36. Cuzick J, Forbes JF, Sestak I et al. Long-term results of tamoxifen prophylaxis for breast cancer – 96-month follow-up of the randomized IBIS-I trial. *J Natl Cancer Inst* 2007;99:272–82.
37. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;351:1451–67.
38. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;5:207–13.
39. Yao K, Lee ES, Bentrem DJ et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;6:2028–36.
40. Swaby RF, Jordan VC. Low-dose estrogen therapy to reverse acquired antihormonal resistance in the treatment of breast cancer. *Clin Breast Cancer* 2008;8:124–33.
41. Martino S, Cauley JA, Barrett-Connor E et al. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J Natl Cancer Inst* 2004;96:1751–61.
42. Barrett-Connor E, Mosca L, Collins P et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Engl J Med* 2006;355:125–37.
43. Vogel VG, Costantino JP, Wickerham DL et al.; National Surgical Adjuvant Breast and Bowel Project (NSABP). Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 Trial. *JAMA* 2006;295:2727–41.
44. Ariazi EA, Ariazi JL, Cordera F et al. Estrogen receptors as therapeutic targets in breast cancer. *Curr Top Med Chem* 2006;6:181–202.
45. Jin Y, Desta Z, Stearns V et al. CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. *J Natl Cancer Inst* 2005;97:30–9.
46. Goetz MP, Rae JM, Suman VJ et al. Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. *J Clin Oncol* 2005;23:9312–8.
47. Stearns V, Beebe KL, Iyengar M et al. Paroxetine controlled release in the treatment of menopausal hot flashes: a randomized controlled trial. *JAMA* 2003;289:2827–34.
48. Garcia M, Jemal A, Ward EM et al. *Global Cancer Facts & Figures 2007*. Atlanta, GA: American Cancer Society, 2007:10–2.
49. Cooke AL, Metge C, Lix L et al. Tamoxifen use and osteoporotic fracture risk: a population-based analysis. *J Clin Oncol* 2008;26:5227–32.
50. DeMichele A, Troxel AB, Berlin JA et al. Impact of raloxifene or tamoxifen use on endometrial cancer risk: a population-based case-control study. *J Clin Oncol* 2008;26:4151–9.
51. Stearns V, Johnson MD, Rae JM et al. Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. *J Natl Cancer Inst* 2003;95:1758–64.
52. Goetz MP, Knox SK, Suman VJ et al. The impact of cytochrome P450 2D6 metabolism in women receiving adjuvant tamoxifen. *Breast Cancer Res Treat* 2007;101:113–21.
53. Jordan VC. Optimising endocrine approaches for the chemoprevention of breast cancer. Beyond the Study of Tamoxifen and Raloxifene (STAR) Trial. *Eur J Cancer* 2006;42:2909–13.
54. Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. *Nat Rev Cancer* 2007;7:46–53.
55. Jordan VC. SERMs: meeting the promise of multifunctional medicines. *J Natl Cancer Inst* 2007;99:350–6.

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Review

Progress in endocrine approaches to the treatment and prevention of breast cancer

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ABSTRACT

Tamoxifen had been the only available hormonal option for the systemic treatment for breast cancer from 1973 to 2000. Enormous efforts have led to the development of potent and selective third generation aromatase inhibitors including anastrozole, letrozole and exemestane. Due to their superior efficacy to tamoxifen, aromatase inhibitors are presently approved as first line agents for the treatment of advanced estrogen receptor (ER) positive breast cancer and adjuvant therapy in early ER positive early breast cancer in postmenopausal women. Selective ER Modulators (SERMS), tamoxifen and raloxifene are the only agents presently used in breast cancer prevention in high risk women and their use has increased substantially over the last decade. Third generations SERMS, lasofoxifene and bazedoxifene have shown significant reduction in bone loss compared to placebo in postmenopausal women and are currently approved in the European Union for the treatment of postmenopausal osteoporosis. This review outlines the current strategies employed in the use of endocrine therapy in the management and prevention of breast cancer.

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1. Introduction

The journey to determine the mechanism that lies behind the growth of breast cancer started more than 100 years ago. The first medical evidence was the suppression of estrogen levels through oophorectomy to cause regression of metastatic breast cancer [1].

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Similar antitumor effects were observed following adrenelectomy and hypophysectomy in postmenopausal women with breast cancer [2]. This led to the evolution of endocrine therapies, with the principal goal of depriving tumor cells of estrogen to induce tumor regression. The story of the reinvention of tamoxifen to become the gold standard for the adjuvant treatment of breast cancer and the pioneering medicine for the reduction of breast cancer incidence in high risk women, has been told in detail elsewhere [3,4]. The translational laboratory research work in the 1970s [5] catalyzed the move from orphan drug for the adjuvant treatment and prevention of breast cancer resulting in tamoxifen becoming the standard of care for the long term adjuvant therapy of ER positive breast cancer and the extension of the lives of millions of women worldwide. Despite the clinical success of tamoxifen, development of drug resistance and endometrial cancer led to the requirement of alternative hormonal therapy to avoid these issues. The clinical efficacy of third generation non steroidal aromatase inhibitors (AIs), anastrozole and letrozole and steroidal AI, exemestane has been extensively studied in comparison to tamoxifen. Although AIs have shown some superiority to tamoxifen as first-line agents in the treatment of postmenopausal women with breast cancer [6–8] selective estrogen receptor modulators (SERMs) remain the mainstay of treatment in breast cancer prevention. In this review, we focus on current published data on the treatment strategies using hormonal therapy in the treatment and prevention of breast cancer.

2. Tamoxifen versus aromatase inhibitors

2.1. Advanced breast cancer

A meta-analysis [9] of comparative studies of AIs with tamoxifen, in postmenopausal women with advanced breast cancer demonstrated a significant difference favoring AIs over tamoxifen as first line agents in overall response rate (ORR; OR, 1.56; 95% CI, 1.17–2.07; $p = 0.002$) and clinical benefit (CB; OR, 1.70; 95% CI, 1.24–2.33; $p = 0.0009$). Although the overall survival (OS) was increased for the AIs arm compared to the tamoxifen arm, the differences observed were not statistically significant (OR, 1.95; 95% CI, 0.88–4.30; $p = 0.10$).

2.2. Adjuvant monotherapy

In estrogen receptor (ER) positive early breast cancer, 5 years of adjuvant tamoxifen significantly reduces breast cancer recurrence and mortality throughout the first 10 years and 15 years respectively [10]. Incorporation of AIs as adjuvant therapy in breast cancer has been extensively studied. Several randomized trials [11–13] have compared AIs to 5 years of tamoxifen as primary adjuvant treatment of postmenopausal women with early breast cancer. The results are summarized in Table 1. Although anastrozole and letrozole showed significant improvements for disease free survival (DFS) and time to distant recurrence (TTDR) and exemestane only improved TTDR, none of the AIs showed significant overall survival (OS). A meta-analysis of the ATAC and BIG trials [14] revealed that the AIs achieved a 2.9% absolute decrease in recurrence (9.6% for AI vs. 12.6% for tamoxifen; $p < 0.00001$) and a nonsignificant reduction in breast cancer mortality. In both studies, the incidence of bone fractures was observed more frequently in the AI arm but gynecological problems were more frequent with tamoxifen therapy. At 10 year follow up of the ATAC trial, the incidence of most cancers was similar between groups and continue to be increased with anastrozole for colorectal (66 vs. 44; OR 1.51, 1.01–2.27)) and lung cancer (51 vs. 34; OR 1.51, 95% CI 0.96–2.41)), and lower for endometrial cancer (6 vs. 24; OR 0.25, 95% CI 0.08–0.63), melanoma (8 vs. 19; 0.42, 0.16–1.00), and ovarian cancer (17 vs. 28). Although long term effects of AIs are not yet established, it is suggested that bisphosphonates be added to AIs regimens to prevent AI associated bone loss. Furthermore, concerns have been raised about the potential increase of myocardial infarction with AIs. This has been addressed in clinical trials, which revealed no significant difference between AIs and tamoxifen [15]. However combined analysis [16] of multiple randomized controlled trials comparing AIs to tamoxifen, demonstrated that AIs were associated with a higher incidence of grade 3 and 4 cardiovascular events ($p = 0.038$) while thromboembolic events were more frequent in the tamoxifen arm ($p < 0.0001$).

2.3. Sequential therapy

It is well known that despite an initial response to tamoxifen, disease progression can occur due to acquired resistance. Prevention of breast cancer recurrences and improvement of survival have been explored with the use of sequential therapy with AIs after 2–3 years of tamoxifen to a total of 5 years of endocrine therapy. Pooled analysis [14] of 4 trials [15,17,18] in which 2–3 years of tamoxifen is switched to either 2–3 years of AIs or tamoxifen revealed that AI therapy was associated with an absolute 3.1% (SE = 0.6%) reduction in recurrence (5% for AI vs. 8.1% for tamoxifen; $2p < 0.00001$) and an absolute 0.7% (SE = 0.3%) decrease in breast cancer mortality (1.7% for AI vs. 2.4% for tamoxifen; $2p = 0.02$) after approximately 5 years of hormonal therapy. Whereas breast cancer mortality was significantly reduced, none of the individual trials reported a significant overall survival. However, updated data from the Anastrozole-Nolvadex (ARNO)-95trial, showed significant reduction in the risk of recurrences ($p = 0.049$) and improved overall survival ($p = 0.045$) with sequential treatment with anastrozole compared to tamoxifen monotherapy [19].

Two studies compared primary AI monotherapy with sequential therapy including tamoxifen followed by an AI. In addition to assessment of letrozole monotherapy compared to tamoxifen, the BIG 1-98 trial [12] also evaluated sequential therapy of 2 years of letrozole followed by 3 years of tamoxifen or 2 years of tamoxifen followed by 3 years of letrozole. A median follow up of 71 months revealed that there was no significant difference in terms of DFS with either sequential therapy when compared with letrozole alone. The TEAM trial was initially designed to evaluate the clinical efficacy of exemestane compared to 5 years of tamoxifen as initial adjuvant endocrine therapy. The study design was changed, based on the results of the Intergroup Exemestane Study (IES) trial, to include the sequential use of exemestane after 2.5–3 years of tamoxifen treatment. Updated analysis from the TEAM trial [20] at 5.1 years follow up showed that there was no significant difference in DFS between exemestane alone and tamoxifen followed by exemestane (Fig. 1).

Therefore current recommendation in adjuvant endocrine treatment of ER positive breast cancer (Fig. 2.) is that postmenopausal women take AIs as a primary agent for 5 years or for 2–3 years after tamoxifen, while tamoxifen is recommended as a first line treatment for pre or peri-menopausal women [21]. However which AI to use as either initial or sequential adjuvant therapy is yet to be determined. Studies [22] have shown that letrozole was more potent than anastrozole in the inhibition of aromatization and estrogen suppression in postmenopausal women with locally advanced and invasive ER positive breast cancer. But the superiority of letrozole was not observed in the head to head comparison of letrozole and anastrozole as second line agents in metastatic breast cancer [23]. The ACSOG trial [24] compared the clinical efficacy of all three AIs in the neoadjuvant treatment of locally advanced breast cancer. Preliminary results showed no significant difference in the clinical

Table 1
Third generation aromatase inhibitors versus tamoxifen as first line adjuvant therapy.

TRIAL	ARM	Median follow-up (months)	*n*	DFS	TTDR
ATAC [11]	ANA vs.TAM	120	6241	HR 0.91, 95% CI 0.83–0.99 $p = 0.04$	HR 0.87, 95% CI 0.77–0.99 $p = 0.03$
BIG [12]	LET vs. TAM	76	4922	HR 0.88, 95%CI 0.78–0.99 $p = 0.03$	HR 0.85, 95% CI 0.72–1.00 $p = 0.05$
TEAM [13]	EXE vs. TAM	33	9766	HR 0.91, 95% CI 0.83–0.99 $p = 0.12$	HR 0.81, 95% CI 0.67–0.98 $p < 0.03$

ANA, anastrozole; ATAC, Arimidex, Tamoxifen, Alone or in combination; BIG, Breast International Group; DFS, disease free survival; EXE, exemestane; LET, letrozole; LET, letrozole TAM, tamoxifen; TEAM, Tamoxifen, Exemestane Adjuvant Multicenter; TTDR, time to distant recurrence.

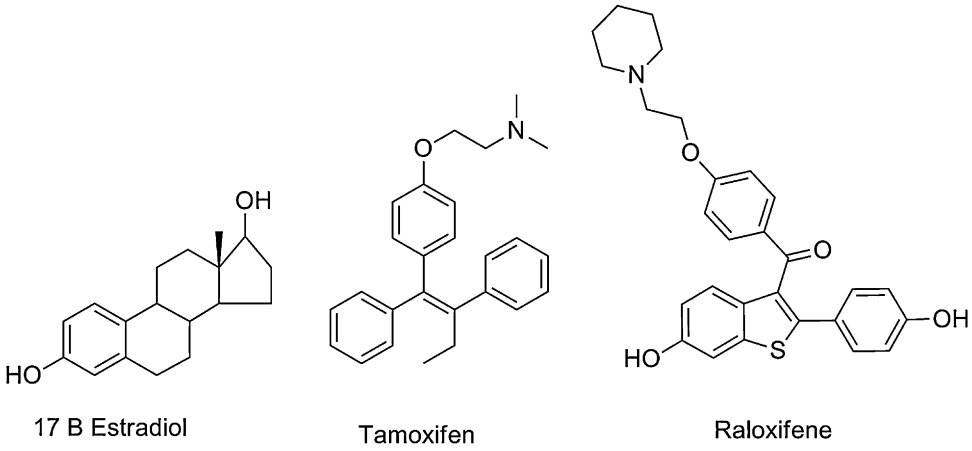

Fig. 1. Chemical Structures of SERMS currently used in breast cancer prevention. The structure of estradiol is included for comparison.

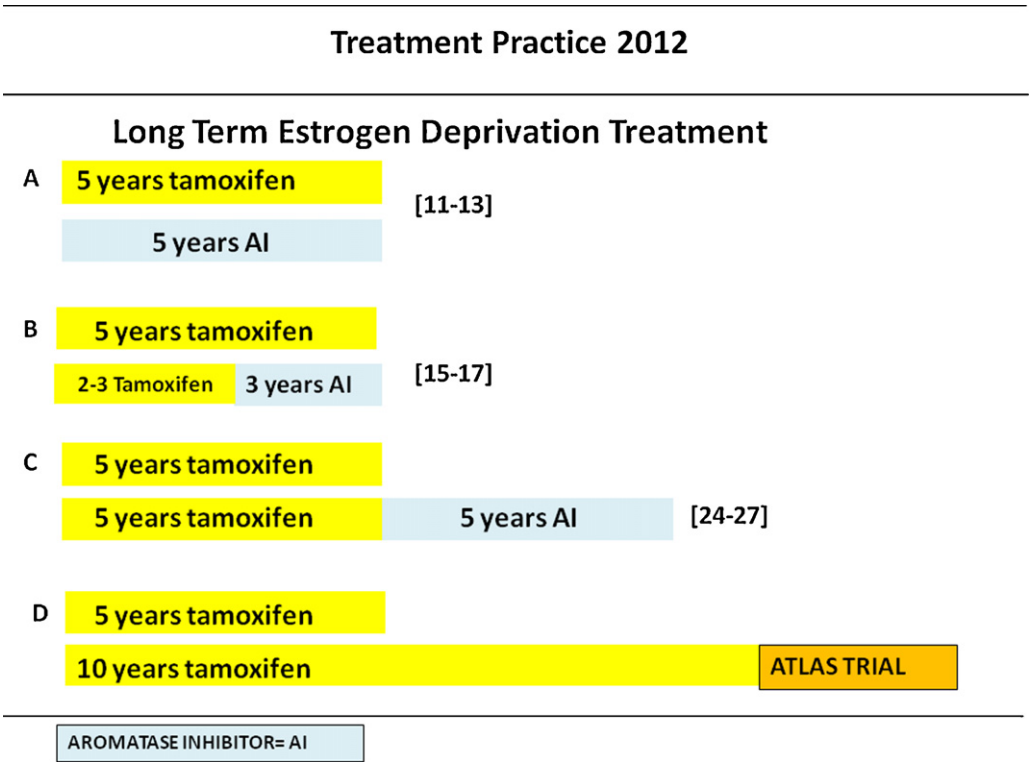

Fig. 2. Clinical guidelines in the adjuvant treatment of estrogen positive breast cancer in postmenopausal patients (A–C) or pre/postmenopausal patients (D). A. Five years of Tamoxifen or aromatase inhibitors can be used as first line adjuvant hormonal therapy in pre or perimenopausal or postmenopausal women respectively. B. In postmenopausal women, sequential therapy with aromatase inhibitor after 2–3 years of tamoxifen is comparable alternative to AI monotherapy. C. Additional 5 years with AIs after 5 years of tamoxifen, have shown significant disease free survival. D. Investigation of extension of tamoxifen beyond 5 years is presently ongoing.

response rate. To date no meaningful clinical differences have been demonstrated between third generation AIs.

2.4. Extended therapy

The MA-17 [25] randomly assigned 5187 patients who have completed 5 years of tamoxifen to 5 years of letrozole or placebo to determine the risk of recurrence. The study was stopped early when the first interim analysis showed that letrozole significantly lowered recurrence rate at a median follow up of 2.4 years. As a result the study was unblinded and 66% of patients on placebo crossed over to the letrozole group. An updated intent to treat analysis [26] revealed that letrozole treatment achieved a 2.9% improvement in DFS at 4 years (HR 0.68 $p = 0.0001$). Similarly, ABCSG-6a [27] evaluated anastrozole for 3 years in comparison with placebo. Favorable results were obtained with anastrozole which resulted in a significant reduction in risk of recurrence ($p = 0.031$). Exemestane was also compared with placebo after tamoxifen adjuvant therapy by the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-33. Similar to the MA-17, the study was stopped prematurely and unblinded due to significant improvement in DFS [28]. However all 3 extended adjuvant trials showed no significant improvement in overall survival. Although 10 year follow up of patients who received 5 years of tamoxifen yielded beneficial effects compared with 2 years of tamoxifen [29] extension of adjuvant therapy with tamoxifen beyond 5 years is not yet recommended. Results are currently awaited from the Adjuvant Tamoxifen-Longer Against Shorter (ATLAS) and adjuvant Tamoxifen Treatment offer more (aTTOM) which should give more insight to extending tamoxifen beyond 5 years. Furthermore, no data is available for the use of AIs beyond 5 years, therefore the recommended limit on AIs is 5 years total across strategies [21].

3. The SERM concept and breast cancer prevention

As a result of a focused effort to decipher the pharmacology and toxicology of tamoxifen, conclusions were built one upon the other, in the same laboratory, to define the properties of a new drug group called the SERMs and to articulation a roadmap to apply that drug group to prevent multiple diseases in women health. The mention of "modulation" at an ER target site occurred with the examination of the structure–function relationships of estrogenic triphenylethylene derivatives of tamoxifen at a prolactin gene target *in vitro* [30]. The estrogenic compounds could activate or suppress prolactin synthesis by altering the shape of the ER complex between the extremes of an "antiestrogenic" or an "estrogenic" conformation [31]. This idea of the molecular modulation of the receptor at a single target site was then expanded to consider the physiologic responses that occurred with nonsteriodal antiestrogen at multiple target sites in the body simultaneously. A simultaneous series of translational studies focused on the uterus, breast (mammary gland) and bone together created the laboratory rationale for further clinical trials by the pharmaceutical industry [32–35]. It was clear in 1990 that the toxicological issues with tamoxifen e.g. endometrial cancer [35,36] needed another approach. A roadmap was stated because few women would have a prevention of breast cancer even in high risk populations; all would be exposed to side effects: "We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may as a side effect, significantly retard the development of breast cancer" [37].

Although tamoxifen is the pioneering SERM, raloxifene is the medicine that first exploited the 'roadmap" successfully starting in 1992 [38]. Scientists [39] confirmed the concept in animal models measuring bone density, uterine weights and circulating cholesterol and initiated the Multiple Outcomes of Raloxifene Evaluation (MORE) trial. Raloxifene would be the first SERM to be approved for two of the three properties of the "ideal SERM": reduction in the incidence of fractures in osteoporosis and the reduction in the incidence of breast cancer [40–42]. Raloxifene does not reduce the risk of coronary heart disease [43]. It is however, perhaps pertinent to note that the original work on the prevention of rat mammary carcinogenesis [34] concluded that because the pharmacokinetics of tamoxifen were superior to raloxifene then raloxifene was unlikely to be superior clinically in breast chemoprevention. Initially, data demonstrated that raloxifene was extremely effective at preventing ER positive breast cancer in 90% of osteoporotic women [41] but in the Study of Tamoxifen and Raloxifene or STAR trial in healthy postmenopausal women tamoxifen and raloxifene were equivalent in producing a 50% decrease in breast cancer incidence [42]. However, the latter evaluations were during treatment with the SERMs. If an evaluation of breast cancer incidence occurs after the end of a 5 year treatment regimen tamoxifen is superior to raloxifene that is only 78% as effective as tamoxifen 3 years following stopping treatment [44]. The laboratory study was accurate. As a result, continuous treatment with raloxifene can be considered and is efficacious at maintaining an antitumor environment [45]. Most importantly, there is no increased risk of endometrial cancer with raloxifene this again demonstrating the veracity of the translational research. Due to its breast cancer and osteoporosis preventive effects, raloxifene is recommended to be the ideal treatment of choice in high risk postmenopausal women.

4. New generation SERMS

The development of third generation SERMs was based on preclinical studies which showed beneficial estrogenic effects on the bone without the detrimental stimulation on the endometrium or breast tissue [46,47]. Lasofoxifene, Bazedoxifene, Arzoxifene and Ospemifene (Fig. 3) have been assessed in the treatment and prevention of osteoporosis as well as prevention of breast cancer. The Osteoporosis Prevention and Lipid Lowering (OPAL) and PEARL studies evaluated lasofoxifene, a third generation SERM in the treatment of osteoporosis. The OPAL study consists of two identical double-blind placebo-controlled studies assessing the vaginal and bone effects of lasofoxifene in nonosteoporotic women. Bone mineral density (BMD) was significantly reduced with an improvement in vaginal pH after 2 years of therapy [48–50]. The PEARL trial [51] is a randomized placebo controlled study involving 8556 postmenopausal women with low bone density. Five years treatment with 0.5 mg of lasofoxifene induced a significant 79% reduction of all breast cancers as well as a statistically significant reduction of vertebral (42%) and non vertebral fractures (24%), major coronary events (32%) and stroke (36%) when compared to placebo [52]. The CORAL trial [53] compared the effects of lasofoxifene, raloxifene and placebo on BMD of postmenopausal women. Although lasofoxifene and raloxifene had a similar adverse effect profile, lasofoxifene significantly improved lumbar spine BMD ($P \leq 0.05$), and significantly reduced low-density lipoprotein cholesterol levels ($P \leq 0.05$) at 2 years of therapy compared to raloxifene and placebo. Lasofoxifene was approved for the treatment of osteoporosis in the European Union in March 2009; however it is still under review by the FDA in the United States. The medicine has not been marketed.

A 2 year randomized double-blind study [54] assessed the clinical efficacy of bazedoxifene compared with placebo. Raloxifene was

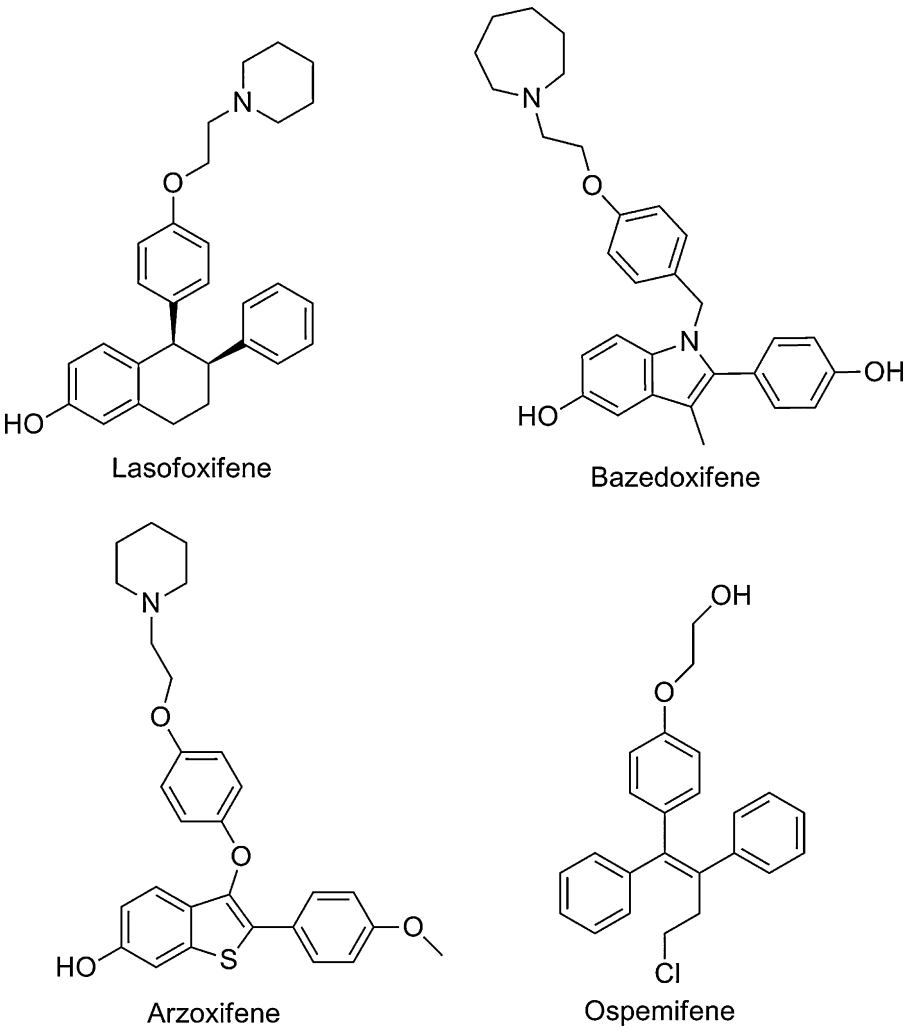

Fig. 3. Chemical structures of third generation SERMS.

added as a positive control. 10 mg, 20 mg and 40 mg of bazedoxifene had superior advantage over placebo in the improvement of BMD at all skeletal sites ($p < 0.001$). These effects were comparable to that obtained with 60 mg raloxifene. Incidence of cardiovascular disease, thromboembolic events, endometrial abnormalities and breast cancer did not significantly differ between treatment groups. Silverman and Colleagues [55] reported that the incidence of new vertebral fractures was significantly lower in the bazedoxifene group compared to placebo ($p < 0.05$), while incidence of non vertebral fractures was not statistically different from the placebo group at 3 years. This trend was maintained on extension of the study for an additional 2 years [56]. A post hoc analysis of a subgroup of women at higher fracture risk showed that bazedoxifene induced a 50% and 44% reduction in nonvertebral fracture risk relative to placebo ($p = 0.02$) and raloxifene ($p = 0.05$). This effect by bazedoxifene in comparison to placebo was supported by a re-analysis using the fracture probability tool, FRAX [57]. Although incidence of breast cancer was lower in the bazedoxifene group, there were no significant differences noted in the incidence of breast or endometrial carcinoma as well as endometrial hyperplasia among treatment groups. Because of the favorable outcomes seen with bazedoxifene on the endometrium and bone, the (Selective Estrogen Menopause and Response to Therapy) SMART-1 [58,59] trial investigated the combination of bazedoxifene(BZA) and conjugated estrogens(CE) compared to placebo using endometrial hyperplasia and BMD as the primary endpoints. Although treatment with BZA/CE did not significantly reduce the incidence of endometrial hyperplasia over placebo at 2 years, BMD was increased significantly with BZA/CE at the lumbar spine and total hip. Perhaps the endometrial protective effects of BZA/CE may be seen with longer follow-up. This may alleviate the need for progestins in postmenopausal women with intact uterus on hormone replacement therapy. Bazedoxifene is currently approved for the treatment of osteoporosis in the European Union. Arzoxifene showed potential in the reduction of vertebral fractures but it was withdrawn from future clinical development based on nonvertebral efficacy [60]. Presently, FDA approval is being sought for the use of ospemifene in the treatment of vulvovaginal atrophy [61].

5. Conclusion

Tamoxifen continues to play a major role in the treatment and prevention of breast cancer. Parallel studies have shown that AIs are superior to tamoxifen in the management of metastatic breast cancer as well as an adjuvant agent in early breast cancer. Although most differences were statistically significant, however differences in overall survival was either non significant or was somewhat marginal. Clinical trials involving head to head comparison of AI are needed to determine the superiority (if any) in efficacy in tumor suppression. This will clarify the initial or sequential order in which these agents are used in clinical management. Tamoxifen and raloxifene are the only endocrine agents approved in the prevention

of breast cancer in high risk women. Newer SERMs, lasofoxifene and bazedoxifene are well tolerated agents and could possibly act as an alternative in the prevention of postmenopausal osteoporosis. These SERMs have shown comparable efficacy to raloxifene. However clinical validation is needed to confirm beneficial effects in the reduction of the incidence of breast cancer, cardiovascular and thromboembolic events. Progress with the new SERMS is currently dependent upon the financial advantages of new agents over old SERMS now as generics (tamoxifen) or ending their patent life (raloxifene). So what about no estrogen at all for chemoprevention? Two trials were established to evaluate the efficacy of anastrozole (IBISII trial) and exemestane (MAP.3) with placebo in the prevention of breast cancer in high risk postmenopausal women. Recently MAP.3 has demonstrated the value of reducing breast cancer incidence by a reported low incidence of side effects [62]. Nevertheless, AIs are not currently recommended for breast cancer risk reduction outside of a clinical trial. No other drugs have shown greater efficacy than those currently approved for breast cancer treatment and prevention.

In summary, it is reasonable to note that much progress has been made in women's health and a menu of medicines is now available and validated approaches are proven compared to none when all this started nearly 40 years ago [5].

Contributors

Ifeyinwa Obiorah MBBS, MSc: contribution: researched and wrote the article. V. Craig Jordan OBE, PhD, DSc, FMedSci: contribution: advised on referencing, diagrams and content.

Competing interests

The authors do not have any competing interests.

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References

[1] Beatson G. On the treatment of inoperable cases of carcinoma of the mamma. Suggestions for a new method of treatment with illustrative cases. Lancet 1896;2:104–7.
[2] Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 2009;69:1243–54.
[3] Jordan VC. Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 2006;147:S269–76.
[4] Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2003;2:205–13.
[5] Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 2008;44:30–8.
[6] Bonneterre J, Buzdar A, Nabholtz JM, et al. Anastrozole is superior to tamoxifen as first-line therapy in hormone receptor positive advanced breast carcinoma. Cancer 2001;92:2247–58.
[7] Mouridsen H, Gershanovich M, Sun Y, et al. Phase III study of letrozole versus tamoxifen as first-line therapy of advanced breast cancer in postmenopausal women: analysis of survival and update of efficacy from the International Letrozole Breast Cancer Group. J Clin Oncol 2003;21:2101–9.
[8] Paridaens RJ, Dirix LY, Beex LV, et al. Phase III study comparing exemestane with tamoxifen as first-line hormonal treatment of metastatic breast cancer in postmenopausal women: the European Organisation for Research and Treatment of Cancer Breast Cancer Cooperative Group. J Clin Oncol 2008;26:4883–90.
[9] Xu HB, Liu YJ, Li L. Aromatase inhibitor versus tamoxifen in postmenopausal woman with advanced breast cancer: a literature-based meta-analysis. Clin Breast Cancer 2011;11:246–51.
[10] Early Breast Cancer Trialists' Collaborative Group E. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 2011;378:771–84.
[11] Cuzick J, Sestak I, Baum M, et al. Effect of anastrozole and tamoxifen as adjuvant treatment for early stage breast cancer: 10-year analysis of the ATAC trial. Lancet Oncol 2010;11:1135–41.
[12] Mouridsen H, Giobbie-Hurder A, Goldhirsch A, et al. Letrozole therapy alone or in sequence with tamoxifen in women with breast cancer. N Engl J Med 2009;361:766–76.
[13] Jones S, Seynaeve C, Hasenburg A, et al. Results of the first planned analysis of the TEAM (tamoxifen exemestane adjuvant multinational) prospective randomized phase III trial in hormone sensitive postmenopausal early breast cancer. Cancer Res 2009;69(Suppl.):15.
[14] Dowsett M, Cuzick J, Ingle J, et al. Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 2010;28:509–18.
[15] Jakesz R, Jonat W, Gnant M, et al. Switching of postmenopausal women with endocrine-responsive early breast cancer to anastrozole after 2 years' adjuvant tamoxifen: combined results of ABCSG trial 8 and ARNO 95 trial. Lancet 2005;366:455–62.
[16] Cuppone F, Bria E, Verma S, et al. Do adjuvant aromatase inhibitors increase the cardiovascular risk in postmenopausal women with early breast cancer. Cancer 2008;112:260–7.
[17] Boccardo F, Rubagotti A, Guglielmini P, et al. Switching to anastrozole versus continued tamoxifen treatment of early breast cancer. Updated results of the Italian tamoxifen anastrozole (ITA) trial. Ann Oncol 2006;17:vii10–4.
[18] Coombes RC, Kilburn LS, Snowdon CF, et al. Survival and safety of exemestane versus tamoxifen after 2–3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial. Lancet 2007;369:559–70.
[19] Kaufmann M, Jonat W, Hilfrich J, et al. Improved overall survival in postmenopausal women with early breast cancer after anastrozole initiated after treatment with tamoxifen compared with continued tamoxifen: the ARNO 95 Study. J Clin Oncol 2007;25:2664–70.
[20] van de Velde CJ, Rea D, Seynaeve C, et al. Adjuvant tamoxifen and exemestane in early breast cancer (TEAM): a randomised phase 3 trial. Lancet 2011;377:321–31.
[21] Burstein HJ, Prestrud AA, Seidenfeld J, et al. American Society of Clinical Oncology clinical practice guideline: update on adjuvant endocrine therapy for women with hormone receptor-positive breast cancer. J Clin Oncol 2010;28:3784–96.
[22] Geisler J, Helle H, Ekse D, et al. Letrozole is superior to anastrozole in suppressing breast cancer tissue and plasma estrogen levels. Clin Cancer Res 2008;14:6330–5.
[23] Rose C, Vtoraya O, Pluzanska A, et al. An open randomised trial of second-line endocrine therapy in advanced breast cancer: comparison of the aromatase inhibitors letrozole and anastrozole. Eur J Cancer 2003;39:2318–27.
[24] Ellis MJ, Suman VJ, Hoog J, et al. Randomized phase II neoadjuvant comparison between letrozole, anastrozole, and exemestane for postmenopausal women with estrogen receptor-rich stage 2–3 breast cancer: clinical and biomarker outcomes and predictive value of the baseline PAM50-based intrinsic subtype – ACOSOG Z1031. J Clin Oncol 2011;29:2342–9.
[25] Goss PE, Ingle JN, Martino S, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early stage breast cancer. N Engl J Med 2003;349:1793–802.
[26] Ingle JN, Tu D, Pater JL, et al. Intent-to-treat analysis of the placebo-controlled trial of letrozole for extended adjuvant therapy in early breast cancer: NCIC CTG MA.17. Ann Oncol 2008;19:877–82.

[27] Jakesz R, Greil R, Gnant M, et al. Extended adjuvant therapy with anastrozole among postmenopausal breast cancer patients: results from the randomized Austrian Breast and Colorectal Cancer Study Group Trial 6a. J Natl Cancer Inst 2007;99:1845–53.
[28] Mamounas EP, Jeong JH, Wickerham DL, et al. Benefit from exemestane as extended adjuvant therapy after 5 years of adjuvant tamoxifen: intention-to-treat analysis of the National Surgical Adjuvant Breast And Bowel Project B-33 trial. J Clin Oncol 2008;26:1965–71.
[29] Hackshaw A, Roughton M, Forsyth S, et al. Long-term benefits of 5 years of tamoxifen: 10-year follow-up of a large randomized trial in women at least 50 years of age with early breast cancer. J Clin Oncol 2011;29:1657–63.
[30] Jordan VC, Koch R, Mittal S, Schneider MR, Oestrogenic. antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. Br J Pharmacol 1986;87:217–23.
[31] Lieberman ME, Gorski J, Jordan VC. An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 1983;258:4741–5.
[32] Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc 1987;46:1870–4.
[33] Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987;10:31–5.
[34] Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987;47:4020–4.
[35] Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 1988;48:812–5.
[36] Fornander T, Rutqvist LE, Cedermark B, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1989;1:117–20.
[37] Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 1990;50:4177–89.
[38] Lewis J, Jordan V. Comprehensive medicinal chemistry. In: Taylor J, Triggle D, editors. Case Histories: Raloxifene. Oxford Edition UK: Elsevier Limited; 2006, 103–121.
[39] Black LJ, Sato M, Rowley ER, et al. Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 1994;93:63–9.
[40] Ettinger B, Black DM, Mitlak BH, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 1999;282:637–45.
[41] Cummings SR, Eckert S, Krueger KA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple outcomes of Raloxifene evaluation. JAMA 1999;281:2189–97.
[42] Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 2006;295:2727–41.
[43] Barrett-Connor E, Mosca L, Collins P, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 2006;355:125–37.
[44] Vogel VG, Costantino JP, Wickerham DL, et al. Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 trial: preventing breast cancer. Cancer Prev Res 2010;3:696–706.
[45] Martino S, Cauley JA, Barrett-Connor E, et al. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 2004;96:1751–61.
[46] Komm BS, Lyttle CR. Developing a SERM: stringent preclinical selection criteria leading to an acceptable candidate (WAY-140424) for clinical evaluation. Ann N Y Acad Sci 2001;949:317–26.
[47] Ke HZ, Paralkar VM, Grasser WA, et al. Effects of CP-336,156, a new, nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. Endocrinology 1998;139:2068–76.
[48] McClung M, Siris E, Cummings S. Lasofoxifene increased BMD of the spine and hip and decreased bone turnover markers in postmenopausal women with low or normal BMD. J Bone Miner Res 2005;20:F429.
[49] Davidson M, Moffett A, Welty F, et al. Extraskeletal effects of lasofoxifene on postmenopausal women. J Bone Miner Res 2005;20:S173.
[50] Peterson GM, Naunton M, Tichelaar LK, Gennari L. Lasofoxifene: selective estrogen receptor modulator for the prevention and treatment of postmenopausal osteoporosis. Ann Pharmacother 2011;45:499–509.
[51] Cummings SR, Ensrud K, Delmas PD, et al. Lasofoxifene in postmenopausal women with osteoporosis. N Engl J Med 2010;362:686–96.
[52] LaCroix AZ, Powles T, Osborne CK, et al. Breast cancer incidence in the randomized PEARL trial of lasofoxifene in postmenopausal osteoporotic women. J Natl Cancer Inst 2010;102:1706–15.
[53] McClung MR, Siris E, Cummings S, et al. Prevention of bone loss in postmenopausal women treated with lasofoxifene compared with raloxifene. Menopause 2006;13:377–86.
[54] Miller PD, Chines AA, Christiansen C, et al. Effects of bazedoxifene on BMD and bone turnover in postmenopausal women: 2-yr results of a randomized, double-blind, placebo-, and active-controlled study. J Bone Miner Res 2008;23:525–35.
[55] Silverman SL, Christiansen C, Genant HK, et al. Efficacy of bazedoxifene in reducing new vertebral fracture risk in postmenopausal women with osteoporosis: results from a 3-year, randomized, placebo-, and active-controlled clinical trial. J Bone Miner Res 2008;23:1923–34.
[56] Silverman SL, Chines AA, Kendler DL, et al. Sustained efficacy and safety of bazedoxifene in preventing fractures in postmenopausal women with osteoporosis: results of a 5-year, randomized, placebo-controlled study. Osteoporos Int 2011. Epub. July 21. doi:10.1007/s00198-011-1691-1.
[57] Kanis JA, Johansson H, Oden A, McCloskey EV. Bazedoxifene reduces vertebral and clinical fractures in postmenopausal women at high risk assessed with FRAX. Bone 2009;44:1049–54.
[58] Pickar JH, Yeh IT, Bachmann G, Speroff L. Endometrial effects of a tissue selective estrogen complex containing bazedoxifene/conjugated estrogens as a menopausal therapy. Fertil Steril 2009;92:1018–24.
[59] Lindsay R, Gallagher JC, Kagan R, et al. Efficacy of tissue-selective estrogen complex of bazedoxifene/conjugated estrogens for osteoporosis prevention in at-risk postmenopausal women. Fertil Steril 2009;92:1045–52.
[60] Silverman SL. New selective estrogen receptor modulators (SERMs) in development. Curr Osteoporos Rep 2010;8:151–3.
[61] Bachmann GA, Komi JO. Group aTOS. Ospemifene effectively treats vulvovaginal atrophy in postmenopausal women: results from a pivotal phase 3 study. Menopause 2010;17:480–6.
[62] Goss PE, Ingle JN, Alés-Martínez JE, et al. Exemestane for breast-cancer prevention in postmenopausal women. N Engl J Med 2011;364:2381–91.

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Models and Mechanisms of Acquired Antihormone Resistance in Breast Cancer: Significant Clinical Progress Despite Limitations

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Abstract

Translational research for the treatment and prevention of breast cancer depends upon the four Ms: models, molecules, and mechanisms in order to create medicines. The process, to target the estrogen receptor (ER) in estrogen-dependent breast cancer, has yielded significant advances in patient survivorship and the first approved medicines (tamoxifen and raloxifene) to reduce the incidence of any cancer in high- or low-risk women. This review focuses on the critical role of the few ER-positive cell lines (MCF-7, T47D, BT474, ZR-75) that continue to advance our understanding of the estrogen-regulated biology of breast cancer. More importantly, the model cell lines have provided an opportunity to document the development and evolution of acquired antihormone resistance. The description of this evolutionary process that occurs in micrometastatic disease during up to a decade of adjuvant therapy would not be possible in the patient. The use of the MCF-7 breast cancer cell line in particular has been instrumental in discovering a vulnerability of ER-positive breast cancer exhaustively treated with antihormone therapy. Physiologic estradiol acts as an apoptotic trigger to cause tumor regression. These unanticipated findings in the laboratory have translated to clinical advances in our knowledge of the paradoxical role of estrogen in the life and death of breast cancer.

Never in the field of breast cancer research [human conflict] was so much owed by so many to so few.

(With apologies to the late Winston Spencer Churchill, Prime Minister, August 20, 1940 reporting on the successful winning of the Battle of Britain).

Introduction

The past four decades have witnessed the successful evolution of effective breast cancer therapies as scientific research has translated into clinical practice. Breast cancer therapy began its story with combination cytotoxic chemotherapy. Chemotherapy, though able to create complete responses in some cases of breast cancer, works non-specifically, causing harmful and sometimes intolerable, life-threatening side effects. Antiestrogen therapies, by contrast, provide significant therapeutic improvement by focusing on a target, the tumor estrogen receptor (ER) [1]. It is important to point out that the ER was initially used not as a therapeutic target, but as a predictor of response to endocrine ablation, such as oophorectomy [2]. The innovation of targeting the tumor ER specifically using the non-steroidal antiestrogen tamoxifen (Figure 1) [3] ultimately changed the prognosis of women

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with breast cancer by proposing two new treatment strategies: a new approach to therapy with long-term early adjuvant tamoxifen treatment following surgery and subsequently the possibility of using tamoxifen for chemoprevention [1,2]. In both cases the target would be the ER, to be blocked by tamoxifen.

Tamoxifen is approved by the Food and Drug Administration (FDA) to treat node-positive and node-negative breast cancer patients with long-term adjuvant therapy, and is approved to lower the incidence of breast cancer in high-risk pre- and postmenopausal women. In both applications, clinical trials established and confirmed that patients with ER-positive breast cancer are the ones who benefit. Tumors that are ER-negative do not respond to tamoxifen. In addition to blocking estrogen's binding to its receptor, another means of limiting estrogenic activity in breast tissue is by blocking the synthesis of estrogen. Aromatase inhibitors block estrogen's conversion from its androgen precursor thereby limiting the production of estrogen [4,5]. This approach has proven beneficial clinically with fewer side effects than tamoxifen and improvements in recurrence rates and survival for postmenopausal patients [6–9].

The benefit of antihormone (data primarily from tamoxifen trials) therapy targeted to the ER is impressive in terms of both recurrence-free survival and decreases in mortality [7,10]. Millions of women now live longer, healthier lives based on the application of translational research [1]. Women of any age with ER-positive tumors experience an approximately 30% mortality reduction when treated with long-term (5 year) adjuvant tamoxifen [7,10]. Postmenopausal women, however, receive greater clinical benefit with aromatase inhibitors rather than tamoxifen, in terms of lower breast cancer recurrence rates and fewer side effects [6]. Aromatase inhibitors can be used instead of tamoxifen for five years, after tamoxifen for five years, or by switching to an aromatase inhibitor after a year or two of tamoxifen. The important principle is to ensure compliance so that at least five years of antihormone treatment is used.

Breast cancer prevention trials built on the previous clinical experience with tamoxifen to demonstrate tamoxifen's efficacy in preventing ER-positive invasive breast cancer in women at high-risk [11]. However, few high-risk women benefit from population-based chemoprevention with tamoxifen while many are exposed to side effects such as endometrial cancer and thromboembolic events [12]. As a result, a paradigm shift occurred with the finding that non-steroidal antiestrogens are, in fact, selective ER modulators (SERMs). The laboratory discovery that SERMs can maintain bone density but prevent mammary carcinogenesis led to the idea of treating osteoporosis while preventing breast cancer at the same time [13–15]. It is fair to say that the laboratory finding [16] that tamoxifen increases the growth of human endometrial cancer but stops the growth of breast cancer, and its subsequent clinical confirmation [16,17], really stressed the need to find a new chemopreventive medicine. Raloxifene is a drug similar in structure to tamoxifen (Figure 1) which is now prescribed indefinitely as a medicine to prevent osteoporosis, offering a beneficial side effect of breast cancer prevention in postmenopausal women [18,19]. Additionally, raloxifene is FDA-approved as a prevention strategy to reduce the incidence of ER-positive breast cancer in at-risk postmenopausal women without increasing the incidence of endometrial cancer as occurs with tamoxifen [20,21]. Figure 1 illustrates the structures of estradiol, raloxifene, tamoxifen, and related metabolites.

With this brief clinical background of progress in the quality of life and survivorship for women with breast cancer, and the practical progress in reducing the incidence of breast cancer, several principles emerge to focus laboratory efforts to enhance further advances. Long-term therapy is the key to successful increases in survivorship and only ER-positive tumors are responsive to antihormone therapy. However, because of the finding that five or

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more years of therapy can control recurrences of the growth of micrometastatic primary breast cancer, it is acquired resistance to antihormone therapy that must be addressed. Models must replicate clinical experience with the ER-positive tumor. The surviving cells whose growth is not blocked by antihormones have the plasticity to respond to treatment in a Darwinian model of continued growth and replication.

We will first describe the limited types of ER-positive breast cancer cells available to the scientific community and the strategies used in the laboratory to create models to mimic clinical experience i.e. years of antihormone therapy. Through the creation of reproducible models, mechanisms can be deciphered to apply to new clinical treatment strategies.

Cell lines as platforms for modeling acquired antihormone resistance

The Early Breast Cancer Trialists' Collaborative Group recently showed that after about 5 years of tamoxifen therapy for women with ER-positive breast tumors (10,645 women), yearly breast cancer mortality rate was reduced by 30% for 15 years after treatment initiation [7]. If we estimate that ER-positive breast cancer, the most prevalent type, accounts for 75% of all breast cancer, it follows that about half of the breast cancers may have or acquire resistance to antihormone therapy. This, combined with the fact that over 200,000 new cases of breast cancer [22] are expected to occur each year, makes acquired resistance a critical issue in breast cancer research and women's health. Prevention of primary breast cancer or the maintenance of patients to prevent recurrence of the disease is an important advance in translational research that continues to reduce healthcare costs and improve survivorship for millions of patients worldwide. Although it is fair to say that few women at high risk for breast cancer elect the chemoprevention option, there are more than half a million women using raloxifene to prevent osteoporosis and prevent breast cancer at the same time [18]. However, tumors that form during long-term raloxifene treatment [19] have acquired resistance to this SERM.

It is currently impossible to analyze the cell biology of every patient's individual breast tumor and predict outcomes, both practically and financially. The actual relationship of the cancer cell with supporting stroma of an individual tumor cannot yet be reconstructed under laboratory conditions, but what can be achieved at this stage is the interrogation of available cell lines to focus on a specific group of ER-positive tumors and obtain general principles with which to plan treatments. In other words, laboratory models *in vitro* and *in vivo* represent the medium for a conversation between the laboratory and the clinic. These models represent important subgroups of breast tumors in patients.

Breast cancer cell lines that are ER-positive are of specific value to conduct translational research to understand the mechanisms by which hormone-responsive breast tumors may develop acquired antihormone resistance. The ER-positive models to be discussed here are: ZR-75, BT-474, T47D, and MCF-7. Each cell line is available from the American Type Culture Collection (ATCC) but there are individual variants maintained in specific laboratories. The current ER statuses (Figure 2), ER protein regulation (Figure 2), hormone responsiveness to the principal steroidal estrogens estradiol and estrone (Figure 3), and the relative ability of tamoxifen and its metabolites to block combined circulating levels of estrone and estradiol (Figure 4) are illustrated. All cells tested have been confirmed by DNA fingerprinting.

The ZR-75 breast cancer cell line

The ZR-75 human breast cancer cell line was derived in the late 1970s from a 63-year-old postmenopausal female patient with metastatic ductal carcinoma of the breast. The cells were taken from the ascites three months after initiation of tamoxifen treatment and exhibit

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estrogen and insulin responsiveness [23]. As ZR-75 cells are passaged they retain their epithelial morphology, remaining similar in appearance to their original source biopsy, though their chromosome count decreases from approximately 75 to 72 after 38 passages [23]. ZR-75 cells are ER-positive, glucocorticoid receptor (GR)-positive, androgen receptor (AR)-positive, and progesterone receptor (PR)-positive [23]. Tamoxifen (10^{-6} M) causes growth inhibition and the cells die [24]. Also, the cells are specifically growth-stimulated by insulin, and inhibited by androgens and glucocorticoids [23].

The BT-474 breast cancer cell line

The BT-474 cell line comprises ER-positive, PR-positive epithelial cancer cells derived from invasive ductal breast carcinoma of a 60-year-old female patient [25]. Notably, these cells also express the nuclear receptor human epidermal growth factor receptor 2 (HER2) [26]. With 55 chromosomes, they grow in adherent patches in tissue culture, and are tumorigenic [25]. BT-474 cells grow in response to estradiol, via their ER (see Figure 3).

The T47D breast cancer cell line

The T47D cell line originates from a pleural effusion of a 54-year-old female patient with infiltrating ductal breast carcinoma. The cells have approximately 60 to 70 chromosomes, multiple mitochondria, and irregular nuclei and nucleoli [27]. They maintain their epithelial morphology after several years of passage, can produce casein, and can be grown in a monolayer *in vitro* [27]. First described as an ER-positive, PR-positive, AR-positive, GR-positive, epithelial cell carcinoma model, it has since been established that the nuclear receptor levels and hormone responsiveness depend on the culture conditions [28]. T47D cells express ER and PR in estrogen-rich media, but lose most PR and ER expression when grown in the absence of estrogen [28].

Classically, estradiol stimulates proliferation of the T47D cell line through the ER, and stimulates estrogen-regulated proteins such as PR, while tamoxifen inhibits this growth [29]. The stimulatory action of physiologic estrogens and the inhibition caused by tamoxifen and its principal metabolites are shown in Figures 3 and 4, respectively. Without the nuclear receptors, however, neither estradiol nor tamoxifen can influence growth since their mechanism of action through ER is eliminated [28].

The MCF-7 breast cancer cell line

The majority of investigations into acquired antiestrogen drug resistance have utilized the MCF-7 cell line so prevalent in breast cancer laboratories. The MCF-7 cell line has been the topic of an earlier review [30]. MCF-7 cells are used ubiquitously in research for ER-positive breast cancer cell experiments and many subclones have been established, representing different classes of ER-positive tumors with varying nuclear receptor expression levels.

The MCF-7 cell line was derived from the pleural effusion of a 69-year-old female patient with a diagnosis of adenocarcinoma of the breast [31]. This particular patient had undergone three years of radiotherapy and hormone therapy, most likely high-dose diethylstilbestrol (DES), a synthetic estrogen (the cell line was created before tamoxifen was available for clinical use). The cells were noted to be ER-positive [32]. In the mid-1970s Lippman [33,34] demonstrated that nonsteroidal antiestrogens in general and tamoxifen in particular could stop the growth of MCF-7 cells in culture, and this could be reversed with the administration of exogenous estradiol.

In the early 1980s, MCF-7 cells were shown to form tumors *in vivo* [35] with estrogen administration, but estrogen did not significantly stimulate growth of the same cells *in vitro*

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[36]. At the time, it was proposed that a factor existing in the animal but not in culture, be it a second messenger system or peptide growth factor, was required for the profound growth influence of estrogen on MCF-7 cells [36]. However, a landmark discovery occurred in 1986 identifying a contaminant of phenol red (phenolsulfonphthalein) (Figure 5), the pH indicator in media, as estrogenic [37,38]. The media was therefore causing cells to grow [37]. All previous studies measuring estrogen's impact on the cells were undermined since the effects were confounded by additional estrogen in the media. The discovery allowed complete withdrawal of estrogen from the cells and the subsequent ability to document the real impact of estrogen on various cell functions including proliferation and apoptosis of MCF-7 cells [39–43].

Being ER-positive, the MCF-7 cell line grows and proliferates with estrogens, in concentrations as low as 10^{-11} M estradiol (Figure 3) [30]. Tamoxifen competitively inhibits DNA synthesis in MCF-7 cells, binding to the same ER as do estrogens, though with a 1000-fold lower affinity than estradiol [30]. When added to the cells simultaneously, estradiol can reverse this inhibition at a concentration 100-fold lower than tamoxifen (10^{-7} M vs. 10^{-8} M) causing cell growth (Figure 4) [30]. The actions of tamoxifen and its metabolites on estrogen-stimulated proliferation are shown in Figure 4. Pure antiestrogens, such as fulvestrant, that destroy ER, also inhibit growth of MCF-7 cells [44].

ER regulation in ER-positive breast cancer cell lines

Figure 2 illustrates ER expression in the four described ER-positive breast cancer cell lines in different media conditions. ZR-75, BT-474, and MCF-7 cells increase expression of ER in the absence of estrogens, represented here by phenol red-free media supplemented with charcoal-stripped fetal bovine serum (SFS). Estrogen exposure to these cells causes decreased ER mRNA and protein levels [45]. T47D cells, by contrast, express more ER in an estrogenic environment, shown here as red media with fetal bovine serum (FBS) [45]. As previously stated, T47D ER expression is lost in an estrogen-free environment. Tamoxifen causes increased ER protein levels in MCF-7 and T47D cells, while fulvestrant causes decreased protein levels in both cell lines [45]. The alternate models of ER regulation in the cell lines has previously been summarized [45] and is now updated and illustrated in Figure 6 for convenience. The consistent model (Model I) of ER regulation is an upregulation of ER in the absence of estrogen. However, T47D does not conform and requires estrogen for ER synthesis (Model II).

Models of acquired antihormone resistance *in vitro*

ER-negative breast cancer cells, such as the MDA-MB-231 and SKBr3 cell lines, do not respond to antihormone treatment. There are some ER-positive cell lines that also exhibit intrinsic resistance; that is, antihormones do not create a subpopulation of these cells that are resistant over time. They simply do not respond initially, perhaps via growth factor receptor overexpression allowing other mechanisms of growth stimulation. Osborne's group showed in 1992 [46] that when ER-positive MCF-7 cells are transfected with HER2, the cells are intrinsically resistant to antihormones such as tamoxifen, presenting HER2 as a potentially important factor for tamoxifen sensitivity and drug resistance.

To investigate the properties of acquired antihormone-resistant breast cancer cells, populations of MCF-7 cells have been created that are adapted to various antihormone environments. MCF-7 cells, more than the other three ER-positive cell lines T47D, BT-474, and ZR-75, are well-suited for antihormone resistance studies since they are easily cultured and retain ER expression when treated with antihormones; they are routinely used in the laboratory and have produced more data of practical knowledge for patient care than any

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other breast cancer cell line (see final section). Figure 7 illustrates the lineages of different subtypes of MCF-7 cells maintained in the laboratory.

One such *in vitro* model illustrating the varied attributes of tamoxifen-resistant cells are the MCF-7 LCC subclones (see Figure 7). The MCF-7:LCC1 variant represents an estrogen-independent breast cancer cell line obtained from *in vivo* selection in oophorectomized nude mice and re-cultured *in vitro* to become a stable cell line [47,48]. Though estrogen-independent, the cells are still tamoxifen-sensitive [47]. When this cell line was selected for tamoxifen resistance *in vitro*, the MCF-7:LCC2 clone was created. MCF-7:LCC2 cells are stable, ER-positive, and respond to the pure antiestrogen, fulvestrant [49]. Along the same lineage, MCF-7:LCC9 cells were derived by selecting *in vitro* MCF-7:LCC1 cells for fulvestrant resistance, and subsequently, these cells exhibit cross-resistance to tamoxifen [50].

Another early antiestrogen-resistant variant of MCF-7 cells is the LY2 line. MCF-7:LY2 cells are resistant to LY117018, a potent antiestrogen related to raloxifene [51]. The LY2 cells also exhibit cross-resistance to tamoxifen and continue to be responsive to estrogen but with lower ER levels than MCF-7. The cell line was created by selection with increasing the concentration of LY117018 up to 1 μM as MCF-7 cells became resistant [51]. A related MCF-7 raloxifene-resistant line MCF-7/RAL was created by growing MCF-7 cells in estrogen-free culture with 1 μM raloxifene for over a year [52]. These cells grow in response to estradiol and raloxifene, and are growth-inhibited by fulvestrant [53]. Most importantly the cells exhibit an unusual apoptotic response to estradiol *in vivo* (see next section). The MCF-7/F cell line was established by culturing the parental MCF-7 cells in fulvestrant-containing estrogen-free media for 18 months. ER expression was lost, and the cells became resistant to all antihormone therapies [54].

Short-term estrogen deprivation causes distinct responses of MCF-7 cells in comparison to long-term (over six months) estrogen deprivation. These studies are important to mimic the early response of ER-positive breast cancer to aromatase inhibition. Culture of MCF-7 cells in media that is phenol red-free with charcoal-stripped serum (estrogen-free) causes immediate proliferation inhibition [39,43]. Slowed proliferation continues for about a month after estrogen removal, indicating the cells have not yet found adaptive or compensatory growth mechanisms. When stimulated with estradiol, the proliferation rate of these short-term estrogen-deprived cells increases, and antiestrogens again inhibit growth [39,40]. Over time, MCF-7 cells deprived of estrogen eventually adapt their growth in estrogen-free media, losing their estrogen sensitivity, but antiestrogens continue to inhibit growth [40]. The ER is retained and expanded.

In 1995, Santen's group hypothesized [55] MCF-7 cells develop hypersensitivity to minute concentrations of estradiol (or indeed any available estrogen) after estradiol deprivation as a means of adapting to estrogen withdrawal and spontaneous growth. They noted that when MCF-7 cells are deprived of estrogen for 1–6 months, a 10^4-fold lower concentration of estradiol is needed for maximal growth, when compared to normally cultured MCF-7 cells. This model suggests an explanation for spontaneous growth that occurs after estrogen withdrawal; that is, the breast cancer cells are hypersensitive to minute environmental concentrations of estrogen [55]. Indeed this is a valid hypothesis as the estrogen-deprived cell population adapts by selecting any available cell to grow in the environment: a Darwinian model.

Long-term estrogen deprived (LTED) MCF-7 cells form a stable cell line that has been used to investigate estrogen's effect on breast cancer cells over varied exposures and lengths of time. MCF-7:LTED cells, in contrast to their short-term estrogen-deprived counterparts, are

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able to grow despite lack of estrogen in the media, and are growth-inhibited by estradiol [40].

MCF-7:5C cells were developed by long-term estrogen withdrawal from the parental wild-type MCF-7 breast cancer cells [56,57]. The ER in MCF-7:5C cells is wild-type, and expression levels are similar to MCF-7 [56] (see Figure 2). This hormone-independent, ER-positive, PR-negative clonal population proved useful in representing the behavior of long-term estrogen-deprived breast cancer cells; that is, those of postmenopausal women decades after menopause, or patients who have undergone long-term antihormone therapy, e.g. 5-year aromatase inhibitor treatment [57]. MCF-7:5C cells are unresponsive to 4-hydroxytamoxifen, and estradiol does not enhance growth [56,57] but triggers estradiol-induced apoptosis [41].

The MCF-7:2A cell line is similar to the MCF-7:5C cell line and was generated from long-term estrogen withdrawal from MCF-7 cells. Uniquely, MCF-7:2A cells express two forms of the ER, a 66 kDa wild-type and a 77 kDa mutant (see Figure 2) [45,58]. The wild-type ER, expressed 4- to 10-fold higher than the mutant, is still functional, whereas the mutant ER, containing a repeat of exons 6 and 7 in the ER gene [59], can no longer bind estrogens nor antiestrogens. MCF-7:2A cells grow in estrogen-free media since they are estrogen-independent. In contrast to its parental cell line, the 2A cells show no response to estradiol during the first seven days of treatment, then begin to die via apoptosis during week two. Both tamoxifen and pure antiestrogens block growth in these cells [45,58].

In search of other *in vitro* models illustrating antihormone-resistant breast cancer cells, the T47D cell line can offer additional information. T47D cells differ from MCF-7 cells in that their tumor suppressor protein p53 is mutated on one allele of the gene (194 Leu-->Phe) [60]. Also, MCF-7 cells continually express ER whereas T47D lose ER expression when estrogen is withdrawn for extended periods of time [61]. The T47D:A18 variant is ER-positive and PR-positive, derived from culturing the T47D cell line in estrogen-rich media [61]. They grow in response to estrogen and are inhibited by 4-hydroxytamoxifen [61]. T47D:C4 cells, in contrast, were established by culturing T47D cells in estrogen-free media [28,61]. The parental cells are transformed into ER-negative, PR-negative cells which are unresponsive to antihormone therapy [62].

To address mechanistic issues of antihormone resistance, T47D-r cells, also derived from the parental T47D line, were created to be resistant to fulvestrant [63]. Proteomic analysis was used to compare T47D versus T47D-r cells to identify 38 proteins with significantly (2-fold up- or down-regulation) different expression [63]. Furthermore, mRNA expression differed for 11 of the proteins. These data are evidence supporting the molecular and mechanistic changes that occur to T47D breast cancer cells as they become increasingly resistant to antiestrogens [63].

The T47Dco subclone is estrogen- and antiestrogen-resistant, and expresses PR regardless of estrogen stimulation. Progestins inhibit proliferation of T47Dco cells [64]. Initially described as ER-negative [64], it was subsequently shown that the cells express three mutant ERs that have no ability to bind ligand [65]. This cell line allows for extensive study on progestins' effect on breast cancer independently of estrogen, as well as on ER mutations as a mechanism of hormone resistance.

When ZR-75 cells are treated with tamoxifen for six months, both ER and PR levels decrease, but the antihormone is still able to impede the cancer growth. Tamoxifen resistance occurs after a year of tamoxifen treatment, as evidenced by the tamoxifen-resistant subclone ZR-75-9a1, a distinct ER-negative, PR-negative cell line [66]. Table 1

summarizes the discussed cell lines' subclones used for modeling ER-positive breast cancer cells *in vitro*.

Models of acquired antihormone resistance *in vivo*

Laboratory studies of endometrial cancer *in vivo* aided in the understanding of acquired resistance to tamoxifen. Estradiol significantly increases the growth rate of human ER-positive endometrial cancer transplanted into ovariectomized nude mice, while the growth rate of ER-negative endometrial cancer in this model is unaffected by estradiol treatment [67]. However, ER-positive endometrial tumors implanted in nude mice also grew more quickly in response to tamoxifen or estradiol treatment than the control-treated mice [68]. When medroxyprogesterone acetate (MPA) (a standard therapy for endometrial cancer) was administered to the tamoxifen-treated animals implanted with endometrial tumors, inhibition of growth was increased in comparison to the tamoxifen-treated tumors alone. In contrast, the growth of ER-negative endometrial cancer injected into athymic mice was unaffected by all treatments [68].

Subsequently, the human endometrial tumor EnCa101 was pivotal in enhancing knowledge of the target site specificity of tamoxifen, as well as by other similar triphenylethylene antiestrogens (e.g. clomiphene, trioxifene, nafoxidine) [69]. Athymic mice transplanted with both MCF-7 breast and EnCa101endometrial tumors, and treated with either estradiol, tamoxifen or the combination, demonstrated that estradiol increases the growth in both tumors. Tamoxifen, however, blocks breast cancer growth while enhancing the growth of endometrial cancer [16]. These data were rapidly translated to patient care [17], with breast cancer patients being given routine gynecological examinations to detect endometrial cancer that was slightly but significantly increased during adjuvant tamoxifen therapy. The target site specific action of tamoxifen in breast and endometrium was hypothesized to be dependent on differential modulation of the estrogenic actions of tamoxifen in different target tissues [70]. The concept was supported by studies of antiestrogens with reduced estrogenic action. Keoxifene (subsequently called raloxifene) and LY117018 are less estrogenic in the rodent uterus and have less of an effect on EnCa101 growth stimulation [69,71]. Further, ICI 164,384, since it is a pure antiestrogen with no intrinsic estrogenicity, did not stimulate EnCa101 tumor growth, and was able to block tamoxifen-induced growth [15]. Clinical studies demonstrate that unlike tamoxifen, raloxifene [18] and fulvestrant [72] have no estrogen-like action in the human uterus.

MCF-7 models *in vitro* eventually evolved one step further toward clinical practice when they were adapted into models *in vivo* which mirror more closely clinical care. Models *in vivo* create a new dimension to assess the importance of a functioning physiologic interaction between cancer cells, the interaction of angiogenesis, cellular metabolism, and respiration that are not created in cell culture. The first studies of MCF-7 cells implanted into nude mice were published in the 1980s. MCF-7 cells implanted into mice with intact ovaries, or simultaneously with estrogen into ovariectomized mice, grew in an estrogen-dependent manner [35].

In the 1980s, transplanted models of MCF-7 human breast cancer into athymic mice were used to investigate the unique aspects of acquired resistance to SERMs. Tamoxifen acts as a competitive inhibitor of estradiol-stimulated growth, i.e. the action of tamoxifen as an antitumor agent is reversed by increasing the dose of estradiol [73]. Similarly, months of tamoxifen therapy do not destroy implanted MCF-7 tumors [74,75], as estrogen can reactivate tumor growth. Eventually acquired resistance to tamoxifen occurred after four months of treatment, wherein neither tamoxifen nor estrogen deprivation could produce

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significant tumor regression [76]. Breast tumors then grew despite tamoxifen treatment demonstrating that acquired resistance to antihormone therapy had developed.

However, a similar study came to a different conclusion; MCF-7 tumors grew in the athymic mouse not despite tamoxifen therapy but because of tamoxifen therapy [77]. When the MCF-7 tumors resistant to tamoxifen were transplanted into new athymic animals, these ER-positive, PR-positive tumors were found to grow in response to either estradiol or tamoxifen treatment. It is also noteworthy that the tamoxifen-stimulated tumors expressed twice the level of ER when compared to their estradiol-stimulated counterparts [77]. A survey of other steroidal and non-steroidal antiestrogens demonstrated that tamoxifen-stimulated growth is dependent on the estrogen-like actions of tamoxifen. Less estrogenic agents do not increase the growth of acquired tamoxifen resistance in MCF-7 tumors [78]. There is cross-resistance with other antiestrogens e.g. toremifene or raloxifene [79,80] but not fulvestrant. Overall, this model mimics the development of acquired resistance to tamoxifen during the treatment of metastatic breast cancer. The tumors become resistant to therapy in about two years.

Many of the previously discussed MCF-7 subclones have been examined in animal models. When the MCF-7/RAL cells are transplanted into athymic ovariectomized mice, they are able to form tumors when treated with either estradiol or raloxifene. Eventually, after about eight months of re-transplantation, the tumors grow only in response to raloxifene, and are inhibited by estradiol [53].

MCF-7/LCC1 cells are estrogen-responsive and tamoxifen-sensitive *in vivo*. MCF-7/LCC2 cells, on the other hand, behave estrogen-independently *in vivo*. They continue to exhibit tamoxifen resistance *in vivo* as they do *in vitro* [49]. The MCF-7/LCC9 cell line, consistent with its *in vitro* action, can form tumors in the athymic ovariectomized mouse, and are unresponsive to fulvestrant [50].

Similarly, MCF-7 cells with acquired resistance to tamoxifen (MCF-7:Tam) *in vivo* implanted in athymic ovariectomized mice grow in response to tamoxifen or estradiol but the steroidal antiestrogen RU 39,411 or ICI 164,384 inhibit growth [78]. However, long-term transplantation of MCF-7:Tam tumors into athymic mice eventually results in a change in response to physiologic estradiol with rapid tumor regression [81,82]. Similarly, MCF-7:5C cells injected into athymic ovariectomized mice undergo apoptosis when treated with estradiol, causing complete tumor regression [41]. This unusual change in the biology of the tumors will be revisited in the next section.

T47D cells have also been examined *in vivo* to evaluate the role of SERMs to create acquired antihormone resistance. T47D cells transplanted into athymic ovariectomized mice can generate tumors in response to estradiol, and tamoxifen can inhibit this estrogen-stimulated growth. However, after high-dose (1.5 mg daily) tamoxifen treatment, the tumor cells become tamoxifen-resistant after about eight weeks, wherein tamoxifen begins to stimulate tumor growth [83]. The T47D cells giving rise to tamoxifen-stimulated tumors produce a subtype of T47D cell named T47D:Tam. Other SERMs, Arzoxifene and LY117018, did not increase growth of T47D:Tam tumors *in vivo*; likewise, Arzoxifene and LY117018 did not increase the growth of estradiol-stimulated T47D tumors either. This indicates a lack of cross-resistance between tamoxifen and the other antiestrogens in T47D cells *in vivo* [84].

In addition to SERM studies, models *in vivo* also examined the effect of aromatase inhibition on ER-positive cell lines. In 1994, nude mice were injected with MCF-7 cells transfected with the human aromatase gene to study the action of aromatase inhibitors *in vivo* for the treatment of breast cancer [85]. In the normal nude mouse, tumors grew in response to ovarian estrogen and were inhibited by aromatase inhibitors and tamoxifen. The

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aromatase substrate, androstenedione, was administered to the ovariectomized mice in order to model human disease since mice express no androgen precursor. Ovariectomized nude mice injected with aromatase-transfected MCF-7 cells grew tumors utilizing estrogen produced through the aromatization of androstenedione via the aromatase pathway. Aromatase inhibitors (4-hydroxyadrostenedione and CGS 16949A) and tamoxifen were able to block the tumor growth. This latter model represents postmenopausal women whose tumors grow not in response to ovarian estrogen, but estrogen generated through the aromatization of androgens found primarily in the adipose tissue. MCF-7 cells transfected with the aromatase gene and injected into ovariectomized mice were inhibited better with the combination treatment of fulvestrant and anastrozole than either agent alone. This suggests the targeting of both aromatase and the ER for better treatment of postmenopausal breast cancer patients [86]. These studies provide a rationale behind aromatase inhibitors' efficacy in the clinical setting [85].

Laboratory models set the stage for intense evaluation of antihormone-resistant breast cancer cells. By continuing investigation of mechanisms of resistance, many unique and sometimes paradoxical effects of hormones and antihormones on ER-positive breast tumors have been discovered. The finding that an estrogen and an antiestrogen could eventually stimulate breast cancer growth demonstrated the unique qualities of acquired resistance to SERMs [77]. The aforementioned individual findings now began to form models for the evolution of acquired resistance that can not only be interrogated in the laboratory but applied to clinical care.

Evolution of acquired antihormone resistance

Based on laboratory evidence from both individual reports and studies of up to a decade, the evolution of acquired resistance to SERMs can now be described in distinct phases following long-term SERM treatment and long-term experiments *in vitro* and *in vivo* (Figure 8) [87,88]. The evolution (Figure 8) of acquired resistance occurs after an initial period of therapeutic success where antiestrogenic activity predominates and the SERMs are competitive inhibitors of estrogen-stimulated tumor growth in athymic mice [73,74]. The therapeutic phase of SERM action can be maintained for a year or two (at most) but eventually tumors start to grow despite continued tamoxifen [76]. However, these tumors can be re-transplanted into other tamoxifen-treated ovariectomized athymic mice [77]. Paradoxically, both physiologic estradiol and tamoxifen (there is cross-resistance with raloxifene and toremifene) [79] can then cause growth, indicating Phase I resistance. The pure antiestrogens ICI 164,382 and fulvestrant block Phase I growth with either tamoxifen or estradiol. A similar form of acquired resistance to tamoxifen occurs with the T47D breast cancer cell line [83,84]. This type of acquired resistance is characteristic of resistance to tamoxifen during the treatment of metastatic ER-positive breast cancer and is why either fulvestrant or an aromatase inhibitor are effective second-line therapeutic agents in the clinic [89,90]. The laboratory principles are illustrated in Figure 8.

However, these laboratory data are inconsistent with the successful adjuvant treatment of node-positive and node-negative ER-positive breast cancer with five years of tamoxifen [7]. In fact, not only is tamoxifen effective during adjuvant therapy but it is also effective at maintaining recurrence-free survival and reducing mortality by 30% from the 10 years following tamoxifen being stopped. Laboratory studies have now provided an insight into this clinical advance.

Repeated transplantation of tamoxifen-resistant tumors into subsequent generations of tamoxifen-treated athymic mice results in a change in the clonal selection of tumor cells. Not only do the tumors remain tamoxifen-dependent for growth over a five-year period but

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the constant exposure to tamoxifen changes the tumor response to estradiol from being a survival signal to an apoptotic trigger. Tumor regression occurs in response to physiologic estrogen and this has been proposed as a mechanism to explain the decreasing mortality of tamoxifen-treated patients following adjuvant tamoxifen [81,82]. In other words, short-term adjuvant tamoxifen only pushes acquired resistance into Phase I resistance where estradiol is still a growth stimulator once tamoxifen is stopped. In contrast, longer tamoxifen forces clonal selection into Phase II resistance where apoptosis occurs upon exposure to a woman's own estrogen. This is illustrated when a comparison between Figures 9A and 9B is made. Indeed it was proposed that since tumors that regress and subsequently regrow in response to physiological estrogen can again respond to subsequent antihormone treatments, then this could be applied in the clinic [82]. This experiment has recently been reported in a clinical study by Ellis [91].

The evolution of cell populations to long-term antihormone therapies has been replicated with raloxifene in a 10-year study *in vivo* [53]. The reason for doing this is because raloxifene will be used indefinitely to prevent osteoporosis [19] and breast cancer [21]. The same evolution of acquired resistance occurs with the development of Phase I and Phase II raloxifene resistance characterized by Phase I resistance with estradiol- or raloxifene-stimulated tumor growth and Phase II resistance characterized with estradiol-induced tumor regression. It is perhaps relevant to point out that MCF-7 cells exposed to both raloxifene and estrogen deprivation *in vitro* rapidly advance to Phase II resistance with estradiol-induced apoptosis *in vivo* [52].

Additionally, there are a couple of other clinically relevant points that can be made about acquired SERM resistance in the laboratory. The T47D cell line advances to Phase I tamoxifen resistance but does not progress to Phase II. The fact that T47D cells have mutant p53 may be relevant as estrogen-induced apoptosis does not develop.

The pure antiestrogen fulvestrant is an excellent antiestrogen/antitumor agent in the laboratory but results have been disappointing clinically until the recent successful use of twice the recommended dose [92]. Laboratory studies with Phase II tamoxifen-resistant tumors grown in athymic mice suggest that the second-line use of fulvestrant in an environment of physiologic estrogen is destined to fail and, in fact, cause enhanced tumor growth [93]. The reason for this is unknown.

The fact that aromatase inhibitors are now the adjuvant treatment of choice for postmenopausal patients with ER-positive breast cancer makes an examination of acquired resistance mandatory. Suffice to say that the principles first described for SERMs are true for aromatase inhibitors and the development of acquired resistance to estrogen deprivation *in vivo* [94–96] and *in vitro* [30,39–41,97].

Mechanisms of acquired antihormone resistance

Breast cancer can be resistant to antihormones in varied ways. As previously noted, intrinsic resistance can occur *de novo* wherein antihormone therapy generates no disease regression. This occurs in ER-negative tumors, as well as in some subgroups of ER-positive tumors. However, we will focus on the mechanisms involved in the evolution of acquired antihormone resistance. Acquired resistance to antihormone therapy can be caused by three main mechanisms to be discussed here: loss of ER function, aberrant growth factor signaling, and estrogen-induced apoptosis.

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Loss of ER function as a mechanism of acquired antihormone resistance

Experiments *in vitro* provide an initial platform for studying the mechanisms of acquired antihormone resistance. Firstly, if the ER in breast cancer cells is altered, the effects of antihormones will be altered accordingly. If ER expression is lost, the whole mechanism of endocrine therapy will be undermined; ER-mediated actions will no longer contribute to proliferation or apoptosis. Similarly, if ER is mutated in such a way that no longer binds its ligands, resistance will occur. Nonetheless, ER mutation is not a major factor in drug resistance but one example that has provided insight into ER modulation of antiestrogen action [98–101].

If the promoter regions of ER target genes are hypermethylated during acquired resistance, transcription of ER target genes is again blocked, abrogating antihormone efficacy *in vitro* [102]. Coupling of ubiquitin conjugation to ER degradation (CUE) domains are approximately 50 amino acids long and bind monoubiquitin molecules used in trafficking and ubiquitylation [103]. CUE domain-containing protein-2 (CUEDC2) is shown to have an inverse correlation with ER protein expression in breast cancer cells *in vitro*. High levels of CUEDC2 protein expression correlate with tamoxifen resistance, probably due to loss of ER via the ubiquitin/proteosome pathway [104].

If the ER is inactivated because of histone methylation or deacetylation, treating breast cancer cells that have acquired resistance to antihormones with a histone deacetylase (HDAC) inhibitor can re-activate the ER. This concept has been illustrated using ER-negative MDA-MB-231 wherein an HDAC inhibitor generates both ER and aromatase expression. Letrozole can then be used as effective treatment [105], suggesting a potential treatment mechanism for ER-positive cells that have lost ER expression during acquired resistance. Loss or reduction of ER as a primary cell survival pathway can also be replaced by an increase in the mosaic of growth factor signaling pathways. These pathways can modulate and subvert steroid hormone receptor synthesis and action [106,107]

Growth factor signaling as a compensatory mechanism of survival

Growth factor signaling and ER crosstalk are consistent mechanisms by which acquired resistance to antihormones develops. It provides the breast cancer cells a means of escape from suppressive signaling and a way to continue proliferation. Growth factors may be able to contribute enough proliferative signal to drive ER-target gene transcription even without normal ER ligand [108]. Growth factor signaling contributes indirectly to ER function, both genomically and non-genomically [108].

An important mechanism for bypassing antihormone-induced apoptosis is through increased expression of membrane receptor tyrosine kinases, including epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), fibroblast growth factor receptor (FGFR) and HER2. These membrane receptors can activate not only the ER signaling pathway [109], but also the MAPK and AKT signal transduction pathways through increased phosphorylation of p42/44. This is demonstrated *in vitro* using MCF-7:LTED cell growth inhibition by IGFR knockdown [110]. OSI-906, an IGFR tyrosine kinase inhibitor, prevents MCF-7:LTED growth both *in vitro* and *in vivo* [110].

When EGFR is transfected into ZR-75 cells, the cells become estrogen-independent. These cells become ER-negative when tamoxifen is introduced and continued to grow using EGF and its receptor, indicating a possible growth mechanism for antihormone resistant breast cancer cells [111]. Further, ZR-75 cells treated with a 5-azacytidine (a DNA methylation inhibitor used to study influence of epigenetic changes on acquired estrogen independence) develop estrogen independence when grown in estrogen-free media, increasing their HER2 and EGFR expression. Growth of these antihormone-resistant cells can be slowed by an

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anti-EGFR antibody, indicating a crucial role of EGFR and growth factor signaling in the progression of antihormone resistance in ZR-75 cells [112]. When EGF-stimulated growth was measured in MCF-7 cells, it was not able to be blocked by tamoxifen, 4-hydroxytamoxifen, nor ICI 164384, suggesting an important growth factor influence on their proliferation [113]. Further, breast cancer cells with amplified FGFR show increased resistance to 4-hydroxytamoxifen *in vitro*, reversible with FGFR-targeted siRNA, indicating a mechanism driving endocrine resistance [114].

If cancer cells are using downstream signaling pathways to continue their growth independent of ER, then blocking key signaling molecules could reveal additional mechanisms of escape. Antagonists of downstream ER signaling pathway proteins, such as mammalian target of rapamycin (mTOR) and phosphoinositide 3-kinase (PI3K), provide potential targets to prevent breast cancer growth after antihormone resistance occurs. The combination of tamoxifen and the mTOR inhibitor RAD001 have an additive effect on MCF-7 cells, together blocking tumor growth *in vitro* better than either agent alone [115], identifying mTOR as an important target to delay the development of antihormone resistance.

Breast cancer cells that have acquired letrozole resistance highly overexpress the growth factor progranulin when compared to their letrozole-sensitive counterparts *in vitro* [116]. Progranulin is shown in the laboratory to cause breast cancer cells to acquire letrozole resistance, and knocking down this growth factor can confer letrozole-sensitivity to cells that had acquired letrozole resistance, thereby blocking their proliferation [116]. This example again demonstrates the complexity and flexibility of breast cancer cells to utilize growth factor signaling for survival after long-term antihormone therapy [116].

Long-term estrogen-deprived ER-positive breast cancer cells transfected with the human aromatase gene were studied in ovariectomized athymic nude mice to elucidate mechanisms of acquired resistance to aromatase inhibitors *in vivo*. Similar concepts emerge *in vivo* as have been described *in vitro*. Letrozole-resistant tumors express decreased levels of ER compared with letrozole-sensitive tumors *in vivo*, and an increase in HER2 (6-fold) and IGFR tyrosine kinase receptors and their downstream signaling proteins (e.g. MAPK), suggesting a shift in signaling pathways away from ER [96,117–120]. Inhibiting these tumors with the anti-HER2 trastuzumab restores letrozole sensitivity [120,121] by downregulating HER2 and restoring ER expression [105]. This indicates that letrozole-resistant ER-positive tumors utilize HER2 signaling to survive despite therapy. HER2 and ER expression were shown *in vivo* to correlate inversely with one another; that is, when HER2 is inactivated by trastuzumab or herceptin, ER expression increases and the cells become re-sensitized to antihormones and aromatase inhibition [96,118]. EGFR inhibitors are also able to restore letrozole sensitivity [119].

Proteins involved in the MAPK signaling pathway, p-Raf, p-Mek1/2, and p-MAPK, are increased in tumors *in vivo* that have acquired resistance to letrozole [119,120,122], suggesting the activation of aberrant signaling for compensatory proliferation after long-term aromatase inhibition. Blocking ER with fulvestrant simultaneously with the PI3K inhibitor wortmannin is more effective than antihormone alone, suggesting that the pathway involving PI3K provides a means of growth escape to long-term antihormone-treated breast cancers [123].

Growth factors, e.g. the nuclear coactivator Amplified in Breast Cancer-1 (AIB1, also called SRC-3 and NCoA-3) can activate the ER pathway during antihormone treatment. In the clinical setting, high levels of AIB1 expression in tamoxifen-treated tumors is associated with worse disease-free survival for breast cancer patients, illustrating the importance of

AIB1 in the resistance pathway [124]. AIB1 exerts control over many of the growth factor signaling pathways relevant to acquired antihormone resistance, such as EGFR, HER2, PI3K, and mTOR, and interacts with many proteins associated with transcription, cell cycle regulation, and protein degradation [125,126].

Estrogen-induced apoptosis mechanisms during acquired Phase II resistance

The most significant aspect of the evolution of antihormone resistance is the drift toward reconfiguring signaling networks to make the cell survive with no estrogen, but this creates a vulnerability to estrogen-induced apoptosis. After five years of treatment with antihormones, the sophisticated growth pathways become sensitive and paradoxically collapsed by estrogen, once a growth and survival signal. Clinically in the past, women with breast cancer have been successfully treated with high-dose estrogen therapy [127,128]. This was the first effective chemical therapy for any cancer and was the standard-of-care before tamoxifen [129]. Investigation has sought to uncover mechanisms by which apoptosis occurs in Phase II acquired resistance, and how estrogen makes this switch in signaling.

B-cell lymphoma 2 (Bcl-2) is a signaling molecule expressed in 40–80% of primary breast cancers that functions to prevent apoptosis [130], thereby contributing to malignancy and resistance. It acts as an anti-apoptotic signal in long-term estrogen-deprived ER-positive breast cancer cells [131] to subvert estrogen-induced apoptosis. Inhibition of Bcl-2 via siRNA *in vitro* confers caspase-7 and caspase-9 activation and causes the cells to be synergistically sensitive to estrogen-induced apoptosis [131], making Bcl-2 an interesting therapeutic target. Bcl-2-interacting killer (BIK) regulates calcium release from the endoplasmic reticulum that triggers downstream mitochondria-mediated apoptosis, also inhibiting Bcl-2. High levels of BIK's inhibitory chaperone, GRP78, in ER-positive breast cancer cells, prevents apoptosis and causes endocrine resistance, [132], thereby asserting itself as another potential therapeutic target.

Studies of varied ER-positive breast cancer cells began to investigate the unique properties of physiologic estrogen that causes tumor regression in postmenopausal women [40]. Santen's group showed in 2001 [40] estrogen-independent growth of MCF-7:LTED cells, and significant reduction of tumor growth when treated with estradiol. Using annexin V staining and Western blot analysis, the experiments demonstrated induction of FasL, a death receptor ligand associated with the apoptosis cascade, when cells were treated with estradiol [40]. This finding established the notion of estrogen-inducing Fas-mediated apoptosis in LTED breast cancer cells. Apoptosis via the Fas/FasL pathway was increased seven-fold in the estradiol-treated LTED breast cancer cells when compared to the vehicle-treated LTED cells [40]. Fas mRNA and protein were also increased in MCF-7:Tam tumors *in vivo*, correlated with decreases in NF-κB expression. The laboratory experiment showed that increased Fas signaling and simultaneous suppression of NF-κB's anti-apoptotic signaling may be characteristic of estradiol-induced apoptosis [93].

Estrogen-induced apoptosis can also originate through the intrinsic mitochondrial apoptosis pathway, when cytochrome C is released from the mitochondria [41]. This is shown in the laboratory using MCF-7:5C cells *in vivo* [41]. MCF-7:5C cells injected into ovariectomized athymic mice exhibited increased apoptotic protein (e.g. Bax, Bim, p53) expression and tumor regression when treated with estradiol [41].

In tamoxifen-stimulated (Phase II resistant) MCF-7 xenografts, fulvestrant can reverse estrogen-induced apoptosis, stimulating growth and expression of phosphorylated HER2, HER3, p-ERK1/2, and p-GSK3α and β proteins [133]. Pertuzumab blocks the interaction of p-HER2 and HER3 and is able to decrease tumor growth in this model *in vivo*, suggesting

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that fulvestrant stimulation of antihormone-resistant ER-positive breast cancers depend not on ER or ER target genes, but on the HER2/HER3 signaling pathway [133].

Additionally, AIB1 is required for estrogen-induced apoptosis in MCF-7:5C cells *in vitro*. The Wellstein group found that AIB1 is involved in signaling pathways that encourage apoptosis in this context, most prominently through associations with G-protein-coupled receptors, PI3K, Wnt, and Notch signaling pathways [126]. MCF-7 gene expression was examined for the WS8 (wild-type), 5C, and 2A derived cell lines to examine differences in gene regulation during Phase II estrogen-induced apoptosis [97]. For the cell line most sensitive to estrogen-induced apoptosis (MCF-7:5C), genes associated with estrogen signaling, endoplasmic reticulum stress, and inflammation were upregulated, along with apoptotic genes such as BIM and caspase-4, in comparison to WS8 and 2A cells. Analysis of the gene regulation and protein expression indicates that estrogen-induced apoptosis is induced through an inflammatory response in the breast cancer cells, inducing proinflammatory genes (e.g. IL, IFM, arachidonic acid) [97]. The aforementioned examples allow translational research to apply laboratory-revealed mechanisms of acquired resistance to antihormones toward treatment strategies for overcoming or preventing such resistance in ER-positive breast cancer.

Clinical translation via cell models of ER-positive breast cancer

Laboratory models *in vitro* and *in vivo* are the invaluable link to clinical translation and enhanced patient survivorship. During the past three decades, the ER-positive breast cancer cell line MCF-7 has been indispensable in this process not only to test therapeutic strategies but also to advance our understanding of hormone-dependent cancer growth [30]. The MCF-7 cell line was the first hormone-responsive breast cancer cell line used effectively to decipher hormone action in breast cancer [30]. Additionally, the ER from MCF-7 cells was prepared on an “industrial scale” to prepare the first monoclonal antibodies [134,135]. These antibodies are now used ubiquitously to determine the ER status of a patient’s tumor by immunohistochemistry [136–139] or flow cytometry [140–142]. However, it was the acquisition of monoclonal antibodies that permitted the cloning and sequencing of the human ER [143–145]. This advance has had a major impact on our understanding of the structure-function relationships of ER-mediated cell regulation.

The availability of ER-positive breast cancer cells and the development of models to test therapeutic strategies continues to play an essential part in the development of clinical trials. By way of example, we will close by considering the role of the MCF-7 cell line in patient care. To set the scene we will place the comments in the context of current clinical practice. There are two therapeutic scenarios to consider: disease in the premenopausal patient and disease in the postmenopausal patient.

Premenopausal women who present with ER-positive breast tumors are generally prescribed combination cytotoxic chemotherapy with five years of adjuvant tamoxifen treatment, while postmenopausal women with ER-positive breast cancer are likely to receive an aromatase inhibitor. If these antiestrogenic approaches fail to prevent recurrence, fulvestrant is used as a second-line antihormone treatment [146].

The strategy of targeting the ER in the tumor micrometastases with long-term adjuvant tamoxifen was created using the 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat mammary carcinoma model [1,3,147]. The first specific aromatase inhibitor, 4-hydroxyandrostenedione (formestane) was compared and contrasted to tamoxifen in the DMBA-induced rat mammary carcinoma model [148–150], but with the development of the model of estrogen-simulated MCF-7 tumors grown in athymic mice in the early 1980s [35,36], the DMBA model was discarded. Initial studies in the athymic mouse model [74]

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only served to confirm the previous results in the DMBA model but the breakthrough with the MCF-7 model really occurred with the discovery of the evolution of drug resistance to either tamoxifen (or indeed any SERMs) or aromatase inhibitors. We will consider several examples of progress using models of resistance in available breast cancer cell lines that are changing patient care.

The discovery that *in vivo* acquired tamoxifen resistance is unique, as the tumors grow with either tamoxifen or physiologic estrogen [77], recreated a new dimension to consider in therapeutics: the tumor was amplifying the weak estrogen-like properties of tamoxifen by cell selection. An antiestrogenic strategy of no estrogen (an aromatase inhibitor) or an antiestrogen with no estrogen-like properties was required. The genesis and development of fulvestrant, the injectable long-acting pure steroidal antiestrogen is long, dating back to the mid-1970s, but only now is the clinical community able to apply the drug optimally for appropriate patient care [92].

The idea for studying the therapeutic value of 6,7-substituted estradiol analogs was started through a joint research scheme between ICI pharmaceutical division and Leeds University. The idea was to develop a cytotoxic carrier molecule based on the binding of estradiol to ER that would invariably target and destroy ER-positive metastases [151]. The last compound tested in the series was a 7-substituted $(-CH_2-)_{10}$ chain with the alkylating function on the end. This was based on the knowledge from Roussel Uclaf chemists who had made resin columns to extract and purify the ER [152]. The 7-substitution was an appropriate substitution to retain ER binding. The project to discover ER-targeted cytotoxic agents was abandoned but subsequently, and independently, scientists at ICI pharmaceuticals discovered the merits of this class of molecules to create a “pure” antiestrogen [153]. The lead compound, ICI 164,384, first tested successfully in the tamoxifen-stimulated MCF-7 tumor athymic mouse model [78], provided the reassurance necessary for the clinical development of fulvestrant [44] or an aromatase inhibitor as a second-line agent following the failure of tamoxifen [89,90]. The clinical results mimicked the animal data.

Osborne’s group made the important discovery that transfection of the HER2/neu gene would enhance and accelerate the development of resistance in MCF-7 cells to tamoxifen [46]. This has had important implications for the selection of breast cancer patients for tamoxifen treatment. Indeed, it is the important interplay and interaction of the ER and growth factor receptor pathways that is currently a major focus of translational research. The question has become, “what are the mechanisms and changes that occur in breast cancer cell populations that cause acquired resistance?” Once this question is answered, it will be followed by a different question of, “how do we use the knowledge to delay the process and improve survivorship?” A clinical trial was launched in 2009 comparing lapatinib, a HER2 tyrosine kinase inhibitor, with letrozole versus letrozole alone in postmenopausal hormone receptor-positive patients who have acquired tamoxifen resistance [154]. Lapatinib increases progression-free survival in these patients better than the aromatase inhibitor alone, illustrating a compensatory mechanism of antihormone-resistant cells via HER2 after tamoxifen failure [154]. There are ongoing preclinical and clinical trials investigating the EGFR pathway as a growth mechanism after acquired resistance, comparing antihormone treatments, such as tamoxifen and aromatase inhibitors, with and without EGFR inhibitors, such as gefitinib and erlotinib [155,156].

Breast cancer cells that have acquired resistance to antiestrogen therapy are shown to remain sensitive to therapies targeted against the PI3K pathway [157]. Signaling molecules in the PI3K pathway are frequently mutated in antihormone-resistant ER-positive breast cancer, and comprise a targetable pathway to inhibit for effective therapy [157]. Multiple Phase I and Phase II prospective randomized trials focused on combinations of PI3K pathway

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inhibitors (e.g. everolimus, trastuzumab, lapatinib, gefitinib, enzastaurin, tipifarnib, BMS-754807, IMCA12, AMG479) and antihormone treatments (e.g. letrozole, exemestane, tamoxifen, anastrozole, fulvestrant) are underway [157] and predicted to provide valuable information.

The encouraging study of mTOR inhibitors in antihormone resistance has advanced to a successful Phase II trial comparing the effectiveness of letrozole, an aromatase inhibitor, treatment alone versus letrozole plus the mTOR inhibitor, everolimus, in patients with ER-positive breast cancer. The results [158] demonstrate increased response rates for the combination arm, which has prompted the initiation of a Phase III clinical trial comparing everolimus in combination with exemestane, a different aromatase inhibitor, for postmenopausal women with ER-positive breast cancer resistant to other aromatase inhibitors [159,160].

Brodie's group has advanced knowledge of the development of acquired resistance to aromatase inhibitors. Fulvestrant (to destroy the ER) plus an aromatase inhibitor is superior to either strategy alone [86] and trastuzumab reverses letrozole resistance and amplifies the sensitivity of breast cancer cells to estrogen [161]. Each of these strategies have been addressed in clinical trials [162–164] recruiting patients with ER-positive tumors in late-stage breast cancer, but it will be in the adjuvant setting that most gains may occur for patient survivorship. Osborne's group [155,165] has independently pioneered the strategy of using multiple inhibitors of the growth factor receptor family in combination with either estrogen deprivation or tamoxifen therapy and these strategies are moving into clinical trial.

However, it is the laboratory knowledge derived from the evolution of acquired resistance to long-term antihormone therapy that is providing an insight into past clinical research and future opportunities. All MCF-7 or T47D laboratory models for SERM resistance *in vivo* develop acquired resistance within a year or two. This is consistent with the endocrine treatment of metastatic breast cancer but does not explain the remarkable success of five years adjuvant tamoxifen to create a 30% decrease in mortality, not only during therapy but sustained for ten years after therapy stops [7]. The treatment of micrometastatic disease with tamoxifen is clearly different than treatment of established tumors. A breakthrough occurred in the early 1990s with the finding that three repeated transplantations of small MCF-7 tumor pieces into subsequent generations of tamoxifen-treated athymic mice for more than five years exposes a vulnerability to the tumor cells that rapidly die during physiologic estrogen treatment [81,82]. This phenomenon was originally advanced [81] to explain the sustained anti-tumor action of tamoxifen when adjuvant treatment is stopped. It was suggested that women's own estrogen causes apoptosis in micrometastases during Phase II of acquired resistance. Subsequent studies *in vitro* with estrogen-deprived MCF-7 breast cancer cells demonstrated estradiol-induced apoptosis [40,41].

Based on these studies with MCF-7 cells alone, clinical trials have demonstrated the effectiveness of both high- and low-dose estrogen therapy to treat breast cancer following the development of acquired resistance to antihormone therapy in metastatic disease [91,166]. The approach [81,167] is now being applied indirectly to adjuvant clinical trials of long-term adjuvant therapy (Study of Letrozole Extension), where it is anticipated that a three-month drug holiday per year for five years may reduce recurrence rates during letrozole adjuvant therapy. This is the same principle that is now applied to explain [168] the efficacy of low-dose estrogen replacement alone to reduce the incidence of breast cancer in women with a median of 20 years past their menopause (i.e. long-term estrogen deprivation) [169].

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For the future of research in cellular models of breast cancer and acquired resistance to antihormone therapy there are four new developments. Firstly, new primary breast cancer cell lines are being developed and tested both *in vivo* and *in vitro* for drug sensitivity. Secondly, a huge pool of human breast cancer cell lines has been interrogated for drug sensitivity and pathway analysis completed to procure new clinical strategies for treatment [170,171]. Thirdly, signatures have been created to define acquired drug resistance to tamoxifen in existing breast cancer cell lines [114,172] that can be applied to clinical trial. Finally, new methodologies are now available to enrich for breast cancer stem cells and expanding this populations for drug sensitivity testing [173]. Should the future of the "many" new cell systems from primary tumors deliver the promise achieved by the "few" cell lines in the past then there is every reason to believe that enormous progress will occur in the successful treatment and prevention of breast cancer in the coming decades.

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References

1. Jordan VC. Tamoxifen: Catalyst for the change to targeted therapy. Eur J Cancer. 2008; 44:30–38. [PubMed: 18068350]
2. Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. Clinical Cancer Res. 2003; 9:1980–1989. [PubMed: 12796359]
3. Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer. 1975; 11:205–206. [PubMed: 165942]
4. Brodie A. Aromatase and its inhibitors--an overview. J Steroid Biochem Mol Biol. 1991; 40:255–261. [PubMed: 1958529]
5. Chumsri S, Howes T, Bao T, Sabnis G, Brodie A. Aromatase, aromatase inhibitors, and breast cancer. J Steroid Biochem Mol Biol. 2011; 125:13–22. [PubMed: 21335088]
6. Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, Bliss J, Buyse M, Baum M, Buzdar A, Colleoni M, Coombes C, Snowdon C, Gnant M, Jakesz R, Kaufmann M, Boccardo F, Godwin J, Davies C, Peto R. Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol. 2010; 28:509–518. [PubMed: 19949017]
7. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet. 2011; 378:771–784. [PubMed: 21802721]
8. van de Velde CJ, Rea D, Seynaeve C, Putter H, Hasenburg A, Vannetzel JM, Paridaens R, Markopoulos C, Hozumi Y, Hille ET, Kieback DG, Asmar L, Smeets J, Nortier JW, Hadji P, Bartlett JM, Jones SE. Adjuvant tamoxifen and exemestane in early breast cancer (TEAM): a randomised phase 3 trial. Lancet. 2011; 377:321–331. [PubMed: 21247627]
9. Regan MM, Neven P, Giobbie-Hurder A, Goldhirsch A, Ejlertsen B, Mauriac L, Forbes JF, Smith I, Lang I, Wardley A, Rabaglio M, Price KN, Gelber RD, Coates AS, Thurlimann B. for the members of the BIG 1–98 Collaborative Group and the International Breast Cancer Study Group (IBCSG). Assessment of letrozole and tamoxifen alone and in sequence for postmenopausal women with steroid hormone receptor-positive breast cancer: the BIG 1–98 randomised clinical trial at 8·1 years median follow-up. Lancet Oncol. 2011; 12:1101–1108. [PubMed: 22018631]
10. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005; 365:1687–1717. [PubMed: 15894097]

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11. Fisher B, Constantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N. other National Surgical Adjuvant Breast and Bowel Project Investigators. Tamoxifen for prevention of breast cancer: Report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst. 1998; 90:1371–1388. [PubMed: 9747868]
12. Fisher B, Constantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, Atkins JN, Margolese RG, Runowicz CD, James JM, Ford LG, Wolmark N. Tamoxifen for the prevention of breast cancer: Current status of the national surgical adjuvant breast and bowel project P-1 study. J Natl Cancer Inst. 2005; 97:1652–1662. [PubMed: 16288118]
13. Jordan VC, Phelps E, Lindgren JU. Effects of antiestrogens on bone in castrated and intact female rats. Breast Cancer Res Treat. 1987; 10:31–35. [PubMed: 3689979]
14. Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res. 1987; 47:4020–4024. [PubMed: 3607747]
15. Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res. 1990; 50:4177–4189. [PubMed: 2194650]
16. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res. 1988; 48:812–815. [PubMed: 3338079]
17. Fornander T, Cedermark B, Mattsson A, Skloog L, Theve T, Askergren J, Rutqvist L, Glas U, Silfversward C, Somell A, Wilking N, Hjalmar ML. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet. 1989; 1(8630):117–120. [PubMed: 2563046]
18. Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, Norton L, Nickelsen T, Bjarnason NH, Morrow M, Lippman ME, Black D, Glusman JE, Costa A, Jordan VC. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA. 1999; 281:2189–2197. [PubMed: 10376571]
19. Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, Secrest RJ, Cummings SR. CORE Investigators. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst. 2004; 96:1751–1761. [PubMed: 15572757]
20. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER Jr, Wade JL 3rd, Robidoux A, Margolese RG, James J, Lippman SM, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N. National Surgical Adjuvant Breast and Bowel Project (NSABP). Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA. 2006; 295:2727–2741. [PubMed: 16754727]
21. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER, Wade JL 3rd, Robidoux A, Margolese RG, James J, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N. National Surgical Adjuvant Breast and Bowel Project. Update of the national surgical adjuvant breast and bowel project study of tamoxifen and raloxifene (STAR) P-2 trial: Preventing breast cancer. Cancer Prev Res. 2010; 3:696–706.
22. Jemal A, Siegel R, Xu J, Ward E. Cancer Statistics, 2010. CA: A Cancer Journal for Clinicians. 2010; 60:277–300. [PubMed: 20610543]
23. Engel LW, Young NA, Tralka TS, Lippman ME, O'Brien SJ, Joyce MJ. Establishment and characterization of three new continuous cell lines derived from human breast carcinomas. Cancer Res. 1978; 38:3352–3364. [PubMed: 688225]
24. Allegra JC, Lippman ME. The effects of 17β estradiol and tamoxifen on the ZR-75-1 human breast cancer cell line in defined medium. Eur J Cancer. 1980; 16(8):1007–1015. [PubMed: 7439217]
25. Lasfargues EY, Coutinho WG, Redfield ES. Isolation of two human tumor epithelial cell lines from solid breast carcinomas. J Natl Cancer Inst. 1978; 61:967–978. [PubMed: 212572]

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26. Kraus MH, Popescu NC, Amsburgh SC, King CR. Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J. 1987; 6:605–610. [PubMed: 3034598]
27. Keydar I, Chen L, Karby S, Weiss FR, Delarea J, Radu M, Chaitcik S, Brenner HJ. Establishment and characterization of a cell line of human breast carcinoma origin. Eur J Cancer. 1979; 15:659–670. [PubMed: 228940]
28. Murphy CS, Meisner LF, Wu SQ, Jordan VC. Short- and long-term estrogen deprivation of T47D human breast cancer cells in culture. Eur J Cancer Clin Oncol. 1989; 25:1777–1788. [PubMed: 2632259]
29. Chalbos D, Vignon F, Keydar I, Rochefort H. Estrogens stimulate cell proliferation and induce secretory proteins in a human breast cancer cell line (T47D). J Clin Endocrinol Metab. 1982; 55:276–283. [PubMed: 7045150]
30. Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res. 1997; 57:3071–3078. [PubMed: 9242427]
31. Soule MD, Vazquez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst. 1973; 51:1409–1413. [PubMed: 4357757]
32. Brooks SC, Locke ER, Soule HD. Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J Biol Chem. 1973; 248:6251–6253. [PubMed: 4353636]
33. Lippman ME, Bolan G. Oestrogen-responsive human breast cancer in long term tissue culture. Nature. 1975; 258:339–341. [PubMed: 172794]
34. Lippman M, Bolan G, Huff K. The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture. Cancer Res. 1976; 36:4595–4601. [PubMed: 1000504]
35. Soule HD, McGrath CM. Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice. Cancer Lett. 1980; 10:177–189. [PubMed: 7459836]
36. Shafie SM. Estrogen and the growth of breast cancer: new evidence suggests indirect action. Science. 1980; 209:701–702. [PubMed: 6994231]
37. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A. 1986; 83:2496–2500. [PubMed: 3458212]
38. Bindal RD, Katzenellenbogen JA. Bis(4-hydroxyphenyl)[2 from commercial preparations of phenol red (phenolsulfonphtalein). J Med Chem. 1988; 31(10):1978–1983. [PubMed: 3172132]
39. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. Cancer Res. 1987; 47(16):4355–4360. [PubMed: 3607768]
40. Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, Yue W, Wang J. Santen - (phenoxysulfonyl)phenyl]methane: isolation and structure elucidation of a novel estrogen RJ. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst. 2001; 93:1714–1723. [PubMed: 11717332]
41. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, Jordan VC. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst. 2005; 97:1746–1759. [PubMed: 16333030]
42. Cho HS, NG PA, Katzenellenbogen BS. Differential regulation of gene expression by estrogen in estrogen growth-independent and -dependent MCF-7 human breast cancer cell sublines. Mol Endocrinol. 1991; 5:1323–1330. [PubMed: 1722871]
43. Welshons WV, Jordan VC. Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. Eur J Cancer Clin Oncol. 1987; 23:1935–1939. [PubMed: 3436356]
44. Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. Cancer Res. 1991; 51:3867–3873. [PubMed: 1855205]
45. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. Cancer Res. 1996; 56:2321–2330. [PubMed: 8625307]

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46. Benz CC, Scott GK, Sarup JC, Johnson RM, Tripathy D, Coronado E, Shepard HM, Osborne CK. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res Treat. 1992; 24(2):85–95. [PubMed: 8095168]
47. Clarke R, Brunner N, Katzenellenbogen BS, Thompson EW, Norman MJ, Koppi C, Paik S, Lippman ME, Dickson RB. Progression of human breast cancer cells from hormone-dependent to hormone-independent growth both in vitro and in vivo. Proc. Natl. Acad. Sci. 1989; 86:3649–3653. [PubMed: 2726742]
48. Brunner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R. Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. Cancer Res. 1993; 53:283–290. [PubMed: 8380254]
49. Brunner N, Frandsen TL, Holst-Hansen C, Bei M, Thompson EW, Wakeling AE, Lippman ME, Clarke R. MCF7/LCC2: a 4-hydroxytamoxifen resistant human breast cancer variant that retains sensitivity to the steroidal antiestrogen ICI 182,780. Cancer Res. 1993; 53:3229–3232. [PubMed: 8324732]
50. Brunner N, Boysen B, Jirus S, Skaar TC, Holst-Hansen C, Lippman J, Frandsen T, Spang-Thomsen M, Fuqua SA, Clarke R. MCF7/LCC9: an antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen. Cancer Res. 1997; 57:3486–3493. [PubMed: 9270017]
51. Bronzert DA, Greene GL, Lippman ME. Selection and characterization of a breast cancer cell line resistant to the antiestrogen LY117018. Endocrinol. 1985; 117:1409–1417.
52. Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, Loweth J, McKian K, De Los Reyes A, Wing L, Jordan VC. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst. 2003; 95:1586–1597. [PubMed: 14600091]
53. Balaburski GM, Dardes RC, Johnson M, Haddad B, Zhu F, Ross EA, Sengupta S, Klein-Szanto A, Liu H, Lee ES, Kim H, Jordan VC. Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. Int J Oncol. 2010; 37:387–398. [PubMed: 20596666]
54. Liu H, Cheng D, Weichel AK, Osipo C, Wing LK, Chen B, Louis TE, Jordan VC. Cooperative effect of gefitinib and fumitremorgin c on cell growth and chemosensitivity in estrogen receptor alpha negative fulvestrant-resistant MCF-7 cells. Int J Oncol. 2006; 29:1237–1246. [PubMed: 17016657]
55. Masamura S, Santner SJ, Heitjan DF, Santen RJ. Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. J Clin Endocrinol Metab. 1995; 80(10):2918–2925. [PubMed: 7559875]
56. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol. 1992; 90:77–86. [PubMed: 1301400]
57. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol. 2005; 94:131–141. [PubMed: 15862958]
58. Pink JJ, Jiang SY, Fritsch M, Jordan VC. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. Cancer Res. 1995; 55:2583–2590. [PubMed: 7780972]
59. Pink JJ, Wu SQ, Wolf DM, Bilimoria MM, Jordan VC. A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. Nucleic Acids Res. 1996; 24(5):962–969. [PubMed: 8600466]
60. Vojtesek B, Lane DP. Regulation of p53 protein expression in human breast cancer cell lines. J Cell Sci. 1993; 105:607–612. [PubMed: 8408290]
61. Murphy CS, Pink JJ, Jordan VC. Characterization of a receptor-negative, hormone-nonresponsive clone derived from a T47D human breast cancer cell line kept under estrogen-free conditions. Cancer Res. 1990; 50:7285–7292. [PubMed: 2224859]

$watermark-text

$watermark-text

$watermark-text

62. Pink JJ, Bilimoria MM, Assikis J, Jordan VC. Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. Br J Cancer. 1996; 74:1227–1236. [PubMed: 8883409]
63. Huber M, Bahr I, Kratzschmar JR, Becker A, Muller EC, Donner P, Pohlenz HD, Schneider MR, Sommer A. Comparison of proteomic and genomic analyses of the human breast cancer cell line T47D and the antiestrogen-resistant derivative T47D-r. Mol Cell Proteomics. 2004; 3:43–55. [PubMed: 14557597]
64. Horwitz KB, Freidenberg GR. Growth inhibition and increase of insulin receptors in antiestrogen-resistant T47DCO human breast cancer cells by progestins: implications for endocrine therapies. Cancer Res. 1985; 45:167–173. [PubMed: 3838085]
65. Graham ML 2nd, Krett NL, Miller LA, Leslie KK, Gordon DF, Wood WM, Wei LL, Horwitz KB. T47DCO cells, genetically unstable and containing estrogen receptor mutations, are a model for the progression of breast cancers to hormone resistance. Cancer Res. 1990; 50:6208–6217. [PubMed: 2400987]
66. van ben Berg HW, Lynch M, Martin J, Nelson J, Dickson GR, Crockard AD. Characterisation of a tamoxifen-resistant variant of the ZR-75-1 human breast cancer cell line (ZR-75-9a1) and the ability of the resistant phenotype. Br J Cancer. 1989; 59:522–526. [PubMed: 2713239]
67. Satyaswaroop PG, Zaino RJ, Mortel R. Human endometrial adenocarcinoma transplanted into nude mice: growth regulation by estradiol. Science. 1983; 219:58–60. [PubMed: 6849115]
68. Zaino RJ, Satyaswaroop PG, Mortel R. Hormonal therapy of human endometrial adenocarcinoma in a nude mouse model. Cancer Res. 1985; 45:539–541. [PubMed: 3967228]
69. Jordan VC, Gottardis MM, Satyaswaroop PG. Tamoxifen-stimulated growth of human endometrial carcinoma. Ann N Y Acad Sci. 1991; 622:439–446. [PubMed: 1905895]
70. Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc. 1987; 46:1870–1874. [PubMed: 3556610]
71. Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res. 1990; 50:3189–3192. [PubMed: 2334915]
72. Addo S, Yates RA, Laight A. A phase I trial to assess the pharmacology of the new oestrogen receptor antagonist fulvestrant on the endometrium in healthy postmenopausal volunteers. Br J Cancer. 2002; 87:1354–1359. [PubMed: 12454761]
73. Ilino Y, Wolf DM, Langan-Fahey SM, Johnson DA, Ricchio M, Thompson ME, Jordan VC. Reversible control of oestradiol-stimulated growth of MCF-7 tumours by tamoxifen in the athymic mouse. Br J Cancer. 1991; 64:1019–1024. [PubMed: 1764361]
74. Osborne CK, Hobbs K, Clark GM. Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res. 1985; 45:584–590. [PubMed: 3967234]
75. Gottardis MM, Robinson SP, Jordan VC. Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumoristatic action of tamoxifen. J Steroid Biochem. 1988; 30:311–314. [PubMed: 3386259]
76. Osborne CK, Coronado EB, Robinson JP. Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. Eur J Cancer Clin Oncol. 1987; 23:1189–1196. [PubMed: 3653212]
77. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res. 1988; 48:5183–5187. [PubMed: 3409244]
78. Gottardis MM, Jiang SY, Jeng MH, Jordan VC. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res. 1989; 49:4090–4093. [PubMed: 2743303]
79. Lee ES, Schafer JM, Yao K, England G, O'Regan RM, De Los Reyes A, Jordan VC. Cross-resistance of triphenylethylene-type antiestrogens but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res. 2000; 6:4893–4899. [PubMed: 11156249]
80. O'Regan RM, Cisneros A, England GM, MacGregor JI, Muenzner HD, Assikis VJ, Bilimoria MM, Piette M, Dragan YP, Pitot HC, Chatterton R, Jordan VC. Effects of the antiestrogens tamoxifen,

$watermark-text

toremifene, and ICI 182,780 on endometrial cancer growth. J Natl Cancer Inst. 1998; 90:1552–1558. [PubMed: 9790548]

81. Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res. 1993; 127:23–33. [PubMed: 8502820]
82. Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, Jordan VC. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res. 2000; 6:2028–2036. [PubMed: 10815929]
83. Schafer JM, Lee ES, O'Regan RM, Yao K, Jordan VC. Rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice. Clin Cancer Res. 2000; 6:4373–4380. [PubMed: 11106256]
84. Schafer JM, Lee ES, Dardes RC, Bentrem D, O'Regan RM, De Los Reyes A, Jordan VC. Analysis of cross-resistance of the selective estrogen receptor modulators arzoxifene (LY353381) and LY117018 in tamoxifen-stimulated breast cancer xenografts. Clin Cancer Res. 2001; 7:2505–2512. [PubMed: 11489833]
85. Yue W, Zhou D, Chen S, Brodie A. A new nude mouse model for postmenopausal breast cancer using MCF-7 cells transfected with the human aromatase gene. Cancer Res. 1994; 54:5092–5095. [PubMed: 7923123]
86. Macedo LF, Sabnis GJ, Goloubeva OG, Brodie A. Combination of anastrozole with fulvestrant in the intratumoral aromatase xenograft model. Cancer Res. 2008; 68:3516–3522. [PubMed: 18451180]
87. Jordan VC. Selective estrogen receptor modulation: Concept and consequences in cancer. Cancer Cell. 2004; 5:207–213. [PubMed: 15050912]
88. Jordan VC. The 38th David A. Karnofsky Lecture: The Paradoxical Actions of Estrogen in Breast Cancer—Survival or Death? J Clin Oncol. 2008; 26:3078–3082.
89. Osborne CK, Pippen J, Jones SE, Parker LM, Ellis M, Come S, Gertler SZ, May JT, Burton G, Dimery I, Webster A, Morris C, Elledge R, Buzdar A. Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. J Clin Oncol. 2002; 20:3386–3395. [PubMed: 12177098]
90. Howell A, Robertson JF, Quaresma Albano J, Aschermannova A, Mauriac L, Kleeberg UR, Vergote I, Erikstein B, Webster A, Morris C. Fulvestrant, formerly ICI 182,780 is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. J Clin Oncol. 2002; 20:3396–3403. [PubMed: 12177099]
91. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A, Jamalabadi-Majidi S, Crowder R, Siegel BA. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA. 2009; 302:774–780. [PubMed: 19690310]
92. Di Leo A, Jerusalem G, Petruzelka L, Torres R, Bondarenko IN, Khasanov R, Verhoeven D, Pedrini JL, Smirnova I, Lichinitser MR, Pendergrass K, Garnett S, Lindemann JP, Sapunar F, Martin M. Results of the CONFIRM phase III trial comparing fulvestrant 250 mg with fulvestrant 500 mg in postmenopausal women with estrogen receptor-positive advanced breast cancer. J Clin Oncol. 2010; 28:4594–4600. [PubMed: 20855825]
93. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst. 2003; 95:1597–1608. [PubMed: 14600092]
94. Long BJ, Jelovac D, Thiantanawat A, Brodie AM. The effect of second-line antiestrogen therapy on breast tumor growth after first-line treatment with aromatase inhibitor letrozole: long-term studies using the intratumoral aromatase postmenopausal breast cancer model. Clin Cancer Res. 2002; 8(7):2378–2388. [PubMed: 12114443]
95. Brodie A, Jelovac D, Long BJ. Predictions from a preclinical model: studies of aromatase inhibitors and antiestrogens. Clin Cancer Res. 2003; 9:455S–459S. [PubMed: 12538500]
96. Brodie A, Macedo L, Sabnis G. Aromatase resistance mechanisms in model systems in vivo. J Steroid Biochem Mol Biol. 2010; 118:283–287. [PubMed: 19778610]

$watermark-text

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97. Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, Tapia C, Kim HR, Yerrum S, Sharma CG, Nicolas E, Balagurunathan Y, Ross EA, Jordan VC. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci U S A. 2011; 108:18879–18886. [PubMed: 22011582]

98. Wolf DM, Jordan VC. The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat. 1994; 31:129–138. [PubMed: 7981453]

99. Levenson AS, Jordan VC. The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. Cancer Res. 1998; 58:1872–1875. [PubMed: 9581827]

100. MacGregor Schafer J, Liu H, Bentrem DJ, Zapf JW, Jordan VC. Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. Cancer Res. 2000; 60:5097–5105. [PubMed: 11016635]

101. Liu H, Lee ES, Deb Los Reyes A, Zapf JW, Jordan VC. Silencing and reactivation of the selective estrogen receptor-estrogen receptor alpha complex. Cancer Res. 2001; 61:3632–3639. [PubMed: 11325832]

102. Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB, Davidson NE. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res. 1994; 54:2552–2555. [PubMed: 8168078]

103. Prag G, Misra S, Jones EA, Ghirlando R, Davies BA, Horazdovsky BF, Hurley JH. Mechanism of ubiquitin recognition by the CUE domain of Vps9p. Cell. 2003; 113:609–620. [PubMed: 12787502]

104. Pan X, Zhou T, Tai YH, Wang C, Zhao J, Cao Y, Chen Y, Zhang PJ, Yu M, Zhen C, Mu R, Bai ZF, Li HY, Li AL, Liang B, Jian Z, Zhang WN, Man JH, Gao YF, Gong WL, Wei LX, Zhang XM. Elevated expression of CUEDC2 protein confers endocrine resistance in breast cancer. Nat Med. 2011; 17:708–714. [PubMed: 21572428]

105. Chumsri S, Sabnis GJ, Howes T, Brodie AM. Aromatase inhibitors and xenograft studies. Steroids. 2011; 76:730–735. [PubMed: 21420993]

106. Cormier EM, Wolf MF, Jordan VC. Decrease in estradiol-stimulated progesterone receptor production in MCF-7 cells by epidermal growth factor and possible clinical implication for paracrine-regulated breast cancer growth. Cancer Res. 1989; 49:576–580. [PubMed: 2783381]

107. Robinson SP, Jordan VC. The paracrine stimulation of MCF-7 cells by MDA-MB-231 cells: possible role in antiestrogen failure. Eur J Cancer Clin Oncol. 1989; 25:493–497. [PubMed: 2703004]

108. Arpino G, Wiechmann L, Osborne CK, Schiff R. Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: Molecular mechanism and clinical implications for endocrine therapy resistance. Endocr. Rev. 2008; 29:217–233. [PubMed: 18216219]

109. Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. Annu Rev Med. 2011; 62:233–247. [PubMed: 20887199]

110. Fox EM, Miller TW, Balko JM, Kuba MG, Sanchez V, Smith RA, Liu S, Gonzalez-Angulo AM, Mills GB, Ye F, Shyr Y, Manning HC, Buck E, Arteaga CL. A Kinome-Wide Screen Identifies the Insulin/IGF-I Receptor Pathway as a Mechanism of Escape from Hormone Dependence in Breast Cancer. Cancer Res. 2011; 71:6773–6784. [PubMed: 21908557]

111. van Agthoven T, van Agthoven TL, Portengen H, Foekens JA, Dorssers LC. Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. Cancer Res. 1992; 52:5082–5088. [PubMed: 1516065]

112. van Agthoven T, van Agthoven TL, Dekker A, Foekens JA, Dorssers LC. Induction of estrogen independence of ZR-75-1 human breast cancer cells by epigenetic alterations. Mol Endocrinol. 1994; 8:1474–1483. [PubMed: 7533260]

113. Cormier EM, Jordan VC. Contrasting ability of antiestrogens to inhibit MCF-7 growth stimulated by estradiol or epidermal growth factor. Eur J Cancer Clin Oncol. 1989; 25:57–63. [PubMed: 2784101]

114. Turner N, Pearson A, Sharpe R, Lambros M, Geyer F, Lopez-Garcia MA, Natrajan R, Marchio C, Iorns E, Mackay A, Gillett C, Grigoriadis A, Tutt A, Reis-Filho JS, Ashworth A. FGFR1

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$watermark-text

amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer. Cancer Res. 2010; 70:2085–2094. [PubMed: 20179196]

115. Treeck O, Wackwitz B, Haus U, Ortmann O. Effects of a combined treatment with mTOR inhibitor RAD001 and tamoxifen in vitro on growth and apoptosis of human cancer cells. Gynecol Oncol. 2006; 102:292–299. [PubMed: 16443261]
116. Abrhale T, Brodie A, Sabnis G, Macedo L, Tian C, Yue B, Serrero G. GP88 (PC-Cell Derived Growth Factor, progranulin) stimulates proliferation and confers letrozole resistance to aromatase overexpressing breast cancer cells. BMC Cancer. 2011; 11:231. [PubMed: 21658239]
117. Brodie A, Sabnis G. Adaptive changes result in activation of alternate signaling pathways and acquisition of resistance to aromatase inhibitors. Clin Cancer Res. 2011; 17:4208–4213. [PubMed: 21415222]
118. Sabnis G, Brodie A. Adaptive changes results in activation of alternate signaling pathways and resistance to aromatase inhibitor resistance. Mol Cell Endocrinol. 2011; 340:142–147. [PubMed: 20849912]
119. Brodie A, Sabnis G, Macedo L. Xenograft models for aromatase inhibitor studies. J Steroid Biochem Mol Biol. 2007; 106:119–124. [PubMed: 17611101]
120. Macedo LF, Sabnis G, Brodie A. Preclinical modeling of endocrine response and resistance: focus on aromatase inhibitors. Cancer. 2008; 112:679–688. [PubMed: 18072255]
121. Sabnis G, Brodie A. Understanding resistance to endocrine agents: molecular mechanisms and potential for intervention. Clin Breast Cancer. 2010; 10:E6–E15. [PubMed: 20133251]
122. Brodie A, Jelovac D, Sabnis G, Long B, Macedo L, Goloubeva O. Model systems: mechanisms involved in the loss of sensitivity to letrozole. J Steroid Biochem Mol Biol. 2005; 95:41–48. [PubMed: 15967659]
123. Sabnis G, Goloubeva O, Jelovac D, Schayowitz A, Brodie A. Inhibition of the phosphatidylinositol 3-kinase/Akt pathway improves response of long-term estrogen-deprived breast cancer xenografts to antiestrogens. Clin Cancer Res. 2007; 13:2751–2757. [PubMed: 17473209]
124. Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, Fuqua SA, Wong J, Allred DC, Clark GM, Schiff R. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst. 2003; 95:353–361. [PubMed: 12618500]
125. Lahusen T, Henke RT, Kagan BL, Wellstein A, Riegel AT. The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer. Breast Cancer Res Treat. 2009; 116:225–237. [PubMed: 19418218]
126. Hu ZZ, Kagan BL, Ariazi EA, Rosenthal DS, Zhang L, Li JV, Huang H, Wu C, Jordan VC, Riegel AT, Wellstein A. Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. PLoS One. 2011; 6:e20410. [PubMed: 21738574]
127. Haddow A, Watkinson JM, Paterson E. Influence of synthetic oestrogens upon advanced malignant disease. Br Med J. 1944; 2:393–398. [PubMed: 20785660]
128. Ingle JN, Ahmann DL, Green SJ, Edmonson JH, Bisel HF, Kvols LK, Nichols WC, Creagan ET, Hahn RG, Rubin J, Frytak S. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. N Engl J Med. 1981; 304:16–21. [PubMed: 7001242]
129. Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, Bonds D, Brunner R, Brzyski R, Caan B, Chlebowski R, Curb D, Gass M, Hays J, Heiss G, Hendrix S, Howard BV, Hsia J, Hubbell A, Jackson R, Johnson KC, Judd H, Kotchen JM, Kuller L, LaCroix AZ, Lane D, Langer RD, Lasser N, Lewis CE, Manson J, Margolis K, Ockene J, O'Sullivan MJ, Phillips L, Prentice RL, Ritenbaugh C, Robbins J, Rossouw JE, Sarto G, Stefanick ML, Van Horn L, Wactawski-Wende J, Wallace R, Wassertheil-Smoller S. Women's Health Initiative Steering Committee. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. JAMA. 2004; 291:1701–1712. [PubMed: 15082697]
130. Nahta R, Esteva FJ. Bcl-2 antisense oligonucleotides: A potential novel strategy for the treatment of breast cancer. Semin Oncol. 2003; 30:143–149. [PubMed: 14613035]

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131. Song RX, Zhang Z, Mor G, Santen RJ. Down-regulation of Bcl-2 enhances estrogen apoptotic action in long-term estradiol-depleted ER(+) breast cancer cells. Apoptosis. 2005; 10:667–678. [PubMed: 15909128]
132. Zhou H, Zhang Y, Fu Y, Chan L, Lee AS. Novel mechanism of anti-apoptotic function of 78-kDa glucose-regulated protein (GRP78): endocrine resistance factor in breast cancer, through release of B-cell lymphoma 2 (BCL-2) from BCL-2-interacting killer (BIK). J Biol Chem. 2011; 286:25687–25696. [PubMed: 21622563]
133. Osipo C, Meeke K, Cheng D, Weichel A, Bertucci A, Liu H, Jordan VC. Role for HER2/neu and HER3 in fulvestrant-resistant breast cancer. Int J Oncol. 2007; 30:509–520. [PubMed: 17203234]
134. Greene GL, Nolan C, Engler JP, Jensen EV. Monoclonal antibodies to human estrogen receptor. Proc Natl Acad Sci U S A. 1980; 77(9):5115–5119. [PubMed: 7001472]
135. Greene GL, Fitch FW, Jensen EV. Monoclonal antibodies to estrophilin: probes for the study of estrogen receptors. Proc Natl Acad Sci U S A. 1980; 77:157–161. [PubMed: 6928610]
136. Poulsen HS, Ozzello L, King WJ, Greene GL. The use of monoclonal antibodies to estrogen receptors (ER) for immunoperoxidase detection of ER in paraffin sections of human breast cancer tissue. J Histochem Cytochem. 1985; 33:87–92. [PubMed: 2578501]
137. DeSombre ER, Thorpe SM, Rose C, Blough RR, Andersen KW, Rasmussen BB, King WJ. Prognostic usefullness of estrogen receptor immunocytochemical assays for human breast cancer. Cancer Res. 1986; 46:4256s–4264s. [PubMed: 2425944]
138. Bevitt DJ, Milton ID, Piggot N, Henry L, Carter MJ, Toms GL, Lennard TW, Westley B, Angus B, Horne CH. New monoclonal antibodies to oestrogen and progesterone receptors effective for paraffin section immunohistochemistry. J Pathol. 1997; 183:228–232. [PubMed: 9390038]
139. King WJ, Greene GL. Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature. 1984; 307:745–747. [PubMed: 6700704]
140. Ford CH, Al-Bader M, Al-Ayadhi B, Francis I. Reassessment of estrogen receptor expression in human breast cancer cell lines. Anticancer Res. 2011; 31:521–527. [PubMed: 21378333]
141. Cao S, Hudnall SD, Kohen F, Lu LJ. Measurement of estrogen receptors in intact cells by flow cytometry. Cytometry. 2000; 41:109–114. [PubMed: 11002266]
142. Girdler F, Browell DA, Cunliffe WJ, Shenton BK, Hemming JD, Scorer P, Young JR, Brotherick I. Use of the monoclonal antibody DAKO-ERbeta (8D5-1) to measure oestrogen receptor beta in breast cancer cells. Cytometry. 2001; 45:65–72. [PubMed: 11598948]
143. Walter P, Green S, Greene G, Krust A, Bornert JM, Jeltsch JM, Staub A, Jensen E, Scrace G, Waterfield M. Cloning of the human estrogen receptor cDNA. Proc Natl Acad Sci U S A. 1985; 82:7889–7893. [PubMed: 3865204]
144. Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J. Sequence and expression of human estrogen receptor complementary DNA. Science. 1986; 231(4742):1150–1154. [PubMed: 3753802]
145. Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P, Chambon P. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature. 1986; 320:134–139. [PubMed: 3754034]
146. Catania C, Ascione G, Adamoli L, De Pas T, Medici M, Franceschelli L, Verri E, Magni E, Sanna G, Torrisi R, Goldhirsch A, Nolè F. Fulvestrant in heavily pre-treated patients with advanced breast cancer: results from a single compassionate use programme centre. Breast Cancer Res Treat. 2007; 106:97–103. [PubMed: 17260095]
147. Jordan VC, Allen KE. Evaluation of the antitumor activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer. 1980; 16:239–251. [PubMed: 6768559]
148. Brodie AM, Schwarzel WC, Shaikh AA, Brodie HJ. The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. Endocrinol. 1977; 100:1684–1695.
149. Brodie AM, Marsh DA, Wu JT, Brodie HJ. Aromatase inhibitors and their use in controlling oestrogen-dependent processes. J Steroid Biochem. 1979; 11:107–112. [PubMed: 114710]

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150. Brodie AM, Garrett WM, Hendrickson JR, Tsai-Morris CH. Effects of aromatase inhibitor 4-hydroxyandrostenedione and other compounds in the 7,12-dimethylbenz(a)anthracene-induced breast carcinoma model. Cancer Res. 1982; 42:3360s–3364s. [PubMed: 6805949]

151. Jordan VC, Fenuik L, Allen KE, Cotton RC, Richardson D, Walpole AL, Bowler J. Structural derivatives of tamoxifen and oestradiol 3-methyl ether as potential alkylating antioestrogens. Eur J Cancer. 1981; 17:193–200. [PubMed: 7262157]

152. Bucourt R, Vignau M, Torelli V. New biospecific adsorbents for the purification of estradiol receptor. J Biol Chem. 1978; 253:8221–8228. [PubMed: 556354]

153. Wakeling AE, Bowler J. Steroidal pure antioestrogens. J Endocrinol. 1987; 112(3):R7–R10. [PubMed: 3559447]

154. Johnston S, Pippen J Jr, Pivot X, Lichinitser M, Sadeghi S, Dieras V, Gomez HL, Romieu G, Manikhas A, Kennedy MJ, Press MF, Maltzman J, Florance A, O'Rourke L, Oliva C, Stein S, Pegram M. Lapatinib combined with letrozole versus letrozole and placebo as first-line therapy for postmenopausal hormone receptor-positive metastatic breast cancer. J Clin Oncol. 2009; 27:5538–5546. [PubMed: 19786658]

155. Osborne CK, Neven P, Dirix LY, Mackey JR, Robert J, Underhill C, Schiff R, Gutierrez C, Migliaccio I, Anagnostou VK, Rimm DL, Magill P, Sellers M. Gefitinib or placebo in combination with tamoxifen in patients with hormone receptor-positive metastatic breast cancer: a randomized phase II study. Clin Cancer Res. 2011; 17:1147–1159. [PubMed: 21220480]

156. Cristofanilli M, Valero V, Mangalik A, Royce M, Rabinowitz I, Arena FP, Kroener JF, Curcio E, Watkins C, Bacus S, Cora EM, Anderson E, Magill PJ. Phase II, randomized trial to compare anastrozole combined with gefitinib or placebo in postmenopausal women with hormone receptor-positive metastatic breast cancer. Clin Cancer Res. 2010; 16:1904–1914. [PubMed: 20215537]

157. Miller TW, Balko JM, Arteaga CL. Phosphatidylinositol 3-kinase and antiestrogen resistance in breast cancer. J Clin Oncol. 2011; 29:4452–4461. [PubMed: 22010023]

158. Baselga J, Semiglazov V, van Dam P, Manikhas A, Bellet M, Mayordomo J, Campone M, Kubista E, Greil R, Bianchi G, Steinseifer J, Molloy B, Tokaji E, Gardner H, Phillips P, Stumm M, Lane HA, Dixon JM, Jonat W, Rugo HS. Phase II Randomized study of neoadjuvant everolimus plus letrozole compared with placebo plus letrozole in patients with estrogen receptor-positive breast cancer. J Clin Oncol. 2009; 27:2630–2637. [PubMed: 19380449]

159. ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US); Novartis Pharmaceuticals. Everolimus in Combination With Exemestane in the Treatment of Postmenopausal Women With Estrogen Receptor Positive Locally Advanced or Metastatic Breast Cancer Who Are Refractory to Letrozole or Anastrozole (BOLERO-2). 2000- [cited 2011 Nov 16]. Available from: http://clinicaltrials.gov/show/NCT00863655 NLM Identifier: NCT00863655.

160. Baselga J, Campone M, Piccart M, Burris HA, Rugo HS, Sahmoud T, Noguchi S, Gnant M, Pritchard KI, Lebrun F, Beck JT, Ito Y, Yardley D, Deleu I, Perez A, Bachelot T, Vittori L, Xu Z, Mukhopadhyay P, Lebwohl D, Hortobagyi GN. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med. 2011 Dec 7. [Epub ahead of print].

161. Sabnis G, Schayowitz A, Goloubeva O, Macedo L, Brodie A. Trastuzumab reverses letrozole resistance and amplifies the sensitivity of breast cancer cells to estrogen. Cancer Res. 2009; 69:1416–1428. [PubMed: 19190349]

162. Dodwell D, Coombes G, Bliss JM, Kilburn LS, Johnston S. SoFEA Trial Management Group. Combining fulvestrant (Faslodex) with continued oestrogen suppression in endocrine-sensitive advanced breast cancer: the SoFEA trial. Clin Oncol (R Coll Radiol). 2008; 20:321–324. [PubMed: 18387790]

163. Swiss Group for Clinical Cancer Research. ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US); Trastuzumab and Letrozole in Treating Postmenopausal Women With Progressive Advanced Breast Cancer. 2000- [cited 2011 Nov 15]. Available from: http://clinicaltrials.gov/show/NCT00238290 NLM Identifier: NCT00238290.

164. Koeberle D, Ruhstaller T, Jost L, Pagani O, Zaman K, von Moos R, Oehlschlegel C, Crowe S, Pilop C, Thuerlimann B. Swiss Group for Clinical Cancer Research (SAKK). Combination of trastuzumab and letrozole after resistance to sequential trastuzumab and aromatase inhibitor

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monotherapies in patients with estrogen receptor-positive, HER-2-positive advanced breast cancer: a proof-of-concept trial (SAKK 23/03). Endocr Relat Cancer. 2011; 18:257–264. [PubMed: 21317203]

165. Massarweh S, Osborne CK, Creighton CJ, Qin L, Tsimelzon A, Huang S, Weiss H, Rimawi M, Schiff R. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. Cancer Res. 2008; 68:826–833. [PubMed: 18245484]
166. Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jorgensen LM, Mella O, Howell A. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat. 2001; 67:111–116. [PubMed: 11519859]
167. Sabnis GJ, Macedo LF, Goloubeva O, Schayowitz A, Brodie AM. Stopping treatment can reverse acquired resistance to letrozole. Cancer Res. 2008; 68:4518–4524. [PubMed: 18559495]
168. Jordan VC, Ford LG. Paradoxical clinical effect of estrogen on breast cancer risk: a "new" biology of estrogen-induced apoptosis. Cancer Prev Res (Phila). 2011; 4:633–637. [PubMed: 21478501]
169. LaCroix AZ, Chlebowski RT, Manson JE, Aragaki AK, Johnson KC, Martin L, Margolis KL, Stefanick ML, Brzyski R, Curb JD, Howard BV, Lewis CE, Wactawski-Wende J. WHI Investigators. Health outcomes afters stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. JAMA. 2011; 305:1305–1314. [PubMed: 21467283]
170. Heiser LM, Wang NJ, Talcott CL, Laderoute KR, Knapp M, Guan Y, Hu Z, Ziyad S, Weber BL, Laquerre S, Jackson JR, Wooster RF, Kuo WL, Gray JW, Spellman PT. Integrated analysis of breast cancer cell lines reveals unique signaling pathways. Genome Biol. 2009; 10:R31. [PubMed: 19317917]
171. Heiser LM, Sadanandam A, Kuo WL, Benz SC, Goldstein TC, Ng S, Gibb WJ, Wang NJ, Ziyad S, Tong F, Bayani N, Hu Z, Billig JI, Dueregger A, Lewis S, Jakkula L, Korkola JE, Durinck S, Pepin F, Guan Y, Purdom E, Neuvial P, Bengtsson H, Wood KW, Smith PG, Vassilev LT, Hennessy BT, Greshock J, Bachman KE, Hardwicke MA, Park JW, Marton LJ, Wolf DM, Collisson EA, Neve RM, Mills GB, Speed TP, Feiler HS, Wooster RF, Haussler D, Stuart JM, Gray JW, Spellman PT. Subtype and pathway specific responses to anticancer compounds in breast cancer. Proc Natl Acad Sci U S A. 2011 [Epub ahead of print].
172. Mendes-Pereira AM, Sims D, Dexter T, Fenwick K, Assiotis I, Kozarewa I, Mitsopoulos C, Hakas J, Zvelebil M, Lord CJ, Ashworth A. Genome-wide functional screen identifies a compendium of genes affecting sensitivity to tamoxifen. Proc Natl Acad Sci U S A. 2011 [Epub ahead of print].
173. Liu X, Ory V, Chapman S, Yuan H, Timofeeva OA, Albanese C, Haddad B, Rhim JS, Dritschilo A, Riegel A, McBride A, Schlegel R. ROCK inhibitor and feeder cells induce the conditional immortalization of epithelial cells. Am J Path. in review.
174. Jordan VC, Fritz NF, Lagan-Fahey S, Thompson M, Tormey DC. Alteration of endocrine parameters in premenopausal women with breast cancer during long-term adjuvant therapy with tamoxifen as the single agent. J Natl Cancer Inst. 1991; 83:1488–1491. [PubMed: 1920495]
175. Murdter TE, Schroth W, Bacchus-Gerybadze L, Winter S, Heinkele G, Simon W, Fasching PA, Fehm T, Eichelbaum M, Schwab M, Brauch H. German Tamoxifen and AI Clinicians Group. Activity levels of tamoxifen metabolites at the estrogen receptor and the impact of genetic polymorphisms of phase I and II enzymes on their concentration levels in plasma. Clin Pharmacol Ther. 2011; 89:708–717. [PubMed: 21451508]
176. Welshons WV, Wolf MF, Murphy CS, Jordan VC. Estrogenic activity of phenol red. Mol Cell Endocrinol. 1988; 57:169–178. [PubMed: 3402660]

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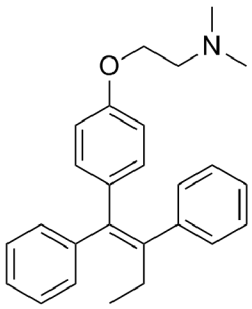
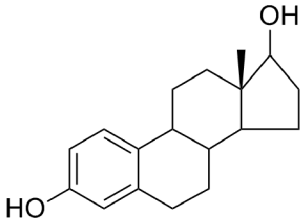
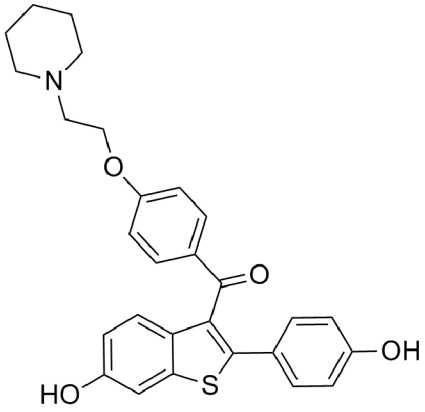


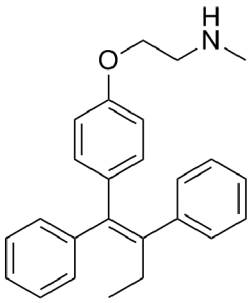
Tamoxifen
Originally ICI 46,474
a failed "morning after" pill



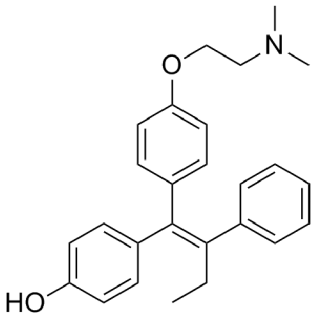
17 β-Estradiol



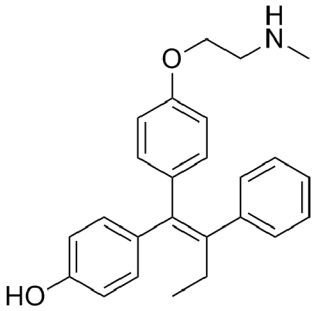
Raloxifene
originally Keoxifene
a failed breast cancer drug



N desmethyl tamoxifen
Major metabolite of tamoxifen



4 Hydroxytamoxifen



Endoxifen
Major hydroxylated
metabolite of tamoxifen

Figure 1.
Chemical structures of 17β-Estradiol, raloxifene, tamoxifen, and tamoxifen's metabolies n-desmethyl tamoxifen, 4-hydroxytamoxifen, and endoxifene.

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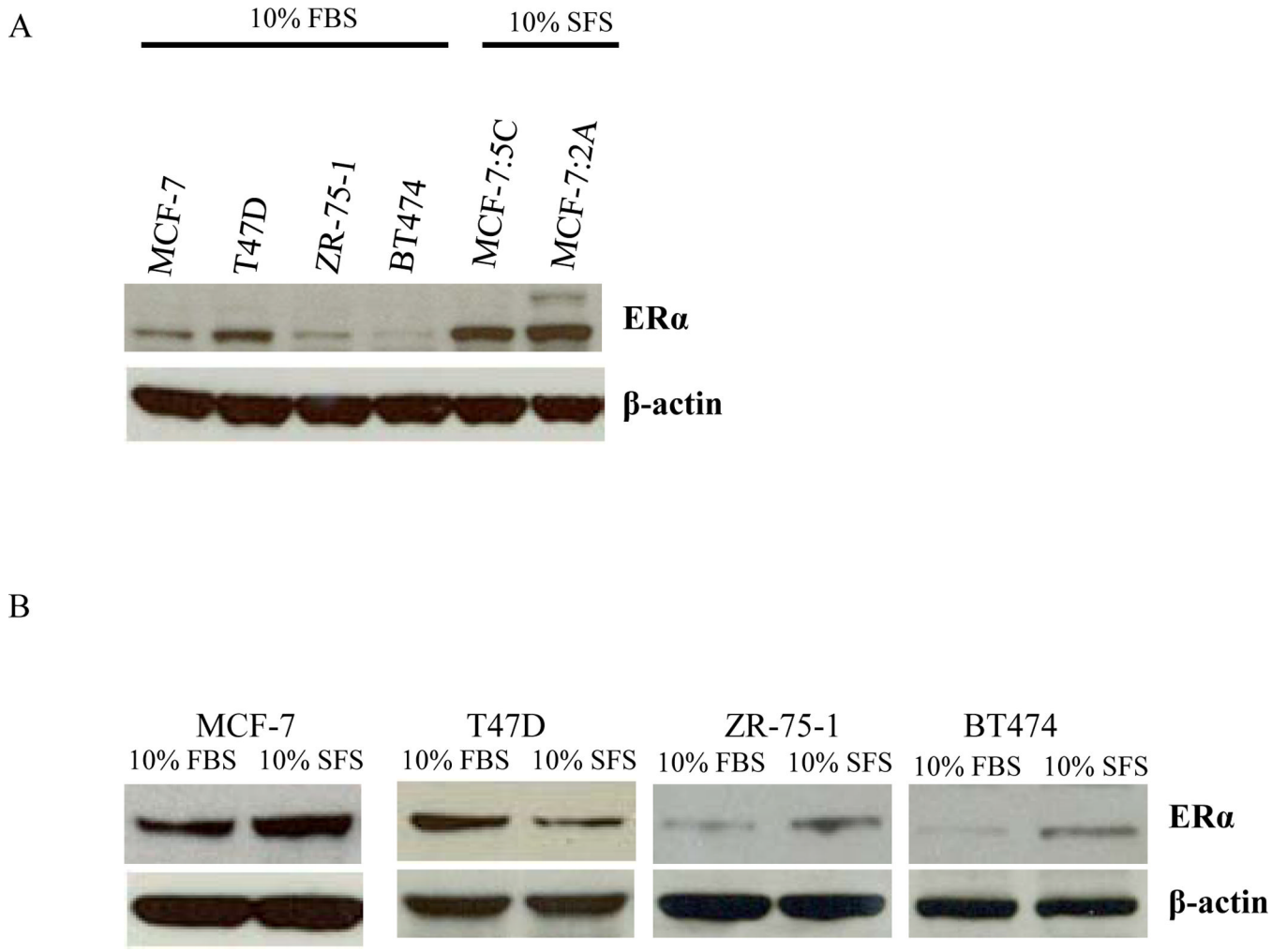

Figure 2.

A. ERα expression levels in different ER positive cells. Cell lysates of MCF-7, T47D, ZR-75-1, BT474, MCF-7:5C, and MCF-7:2A were harvested. MCF-7, T47D, ZR-75-1 and BT474 cells were cultured under conditions with estrogen (10% FBS), while MCF-7:5C and MCF-7:2A cells were cultured under estrogen-free conditions (10%SFS). ERα expression levels were examined by immunoblotting with primary antibody. Immunoblotting for β-actin was determined for loading control. B. Modulation of ERα expression in the absence of estrogen. Wild-type ER positive MCF-7, T47D, ZR-75-1, and BT474 cells were cultured under conditions with estrogen (10% FBS) or without estrogen (10% SFS) for 3 days, respectively. Cell lysates were harvested. ERα expression levels were examined by immunoblotting with primary antibody. Immunoblotting for β-actin was determined for loading control.

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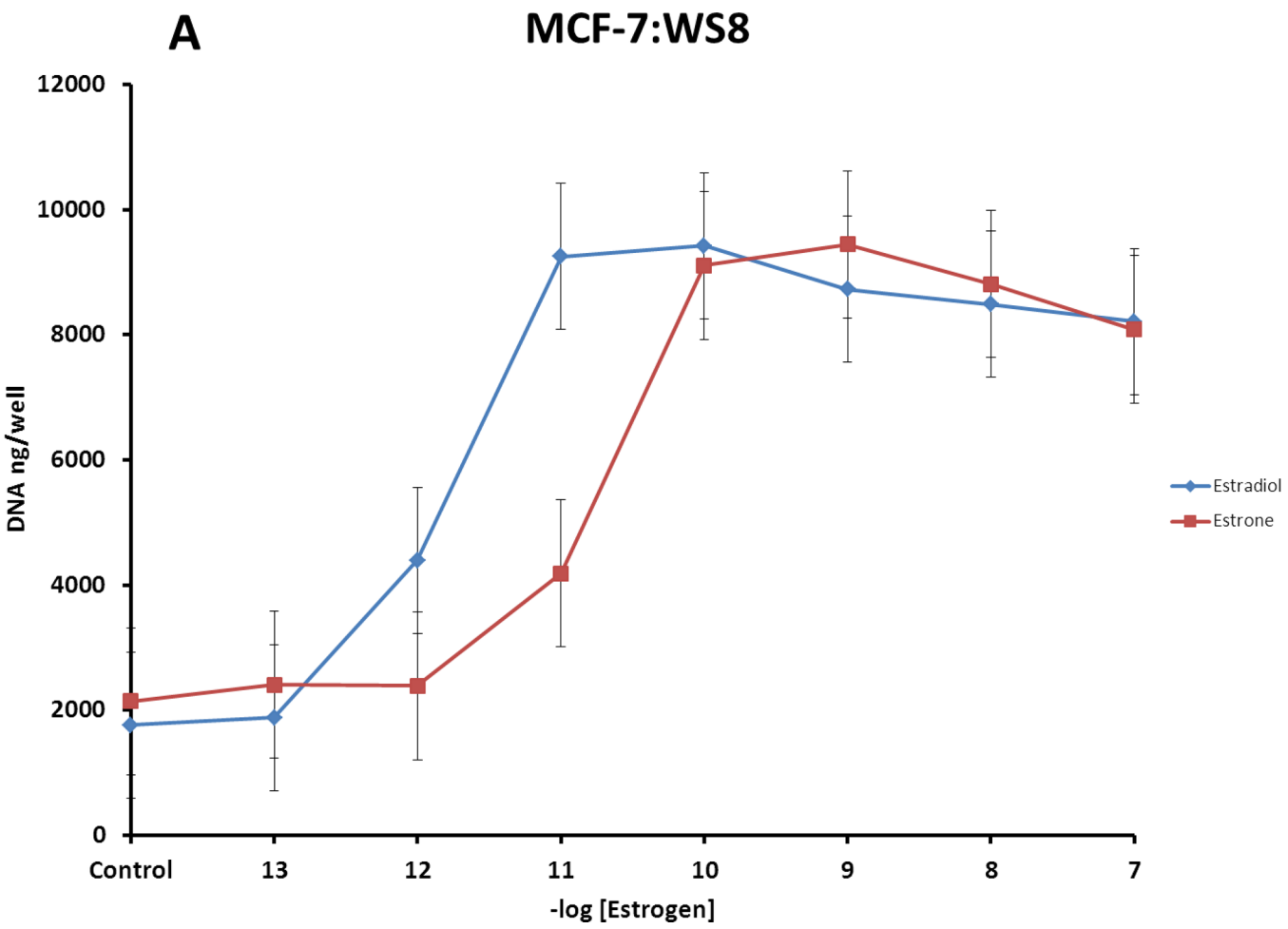

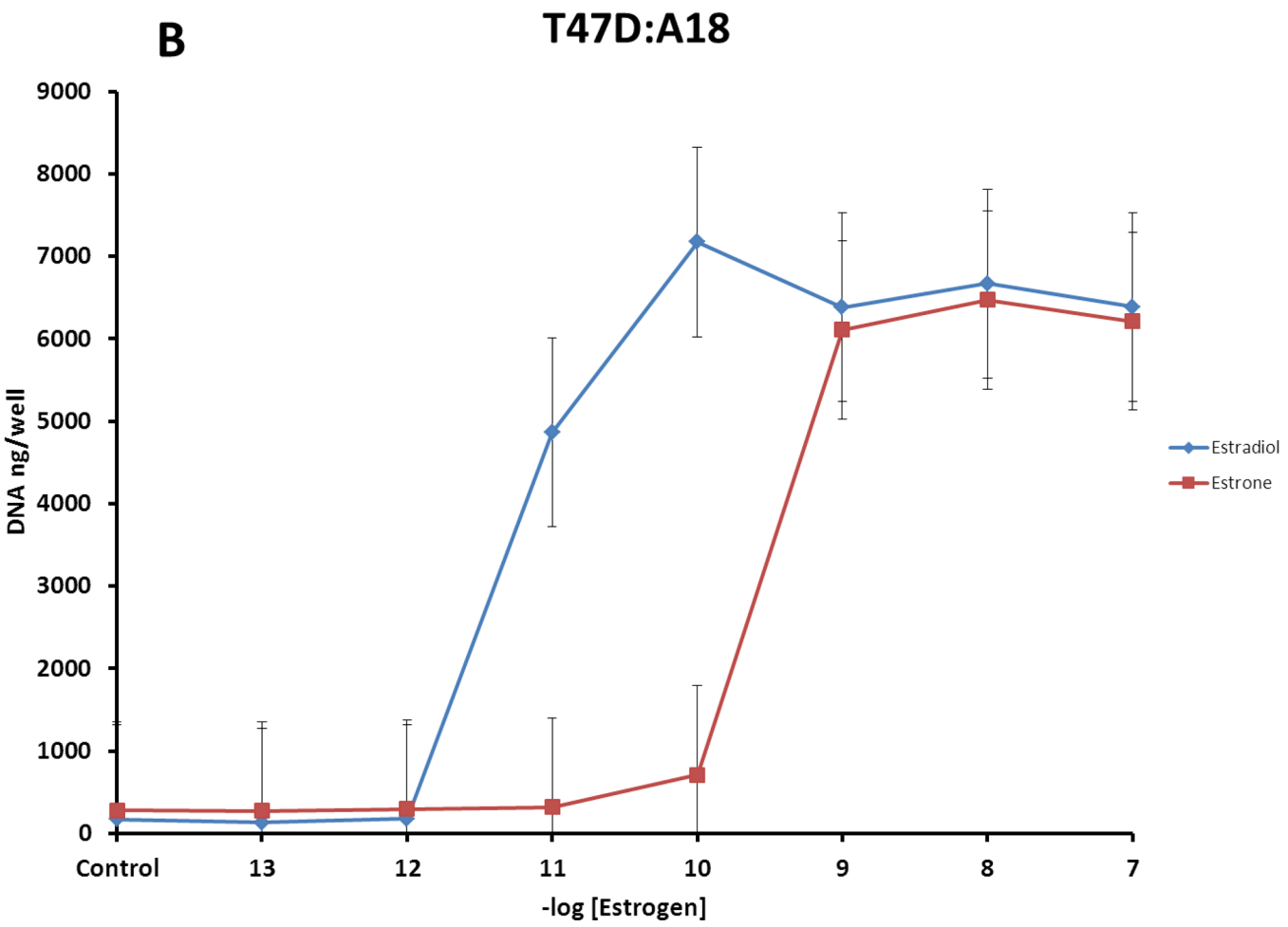

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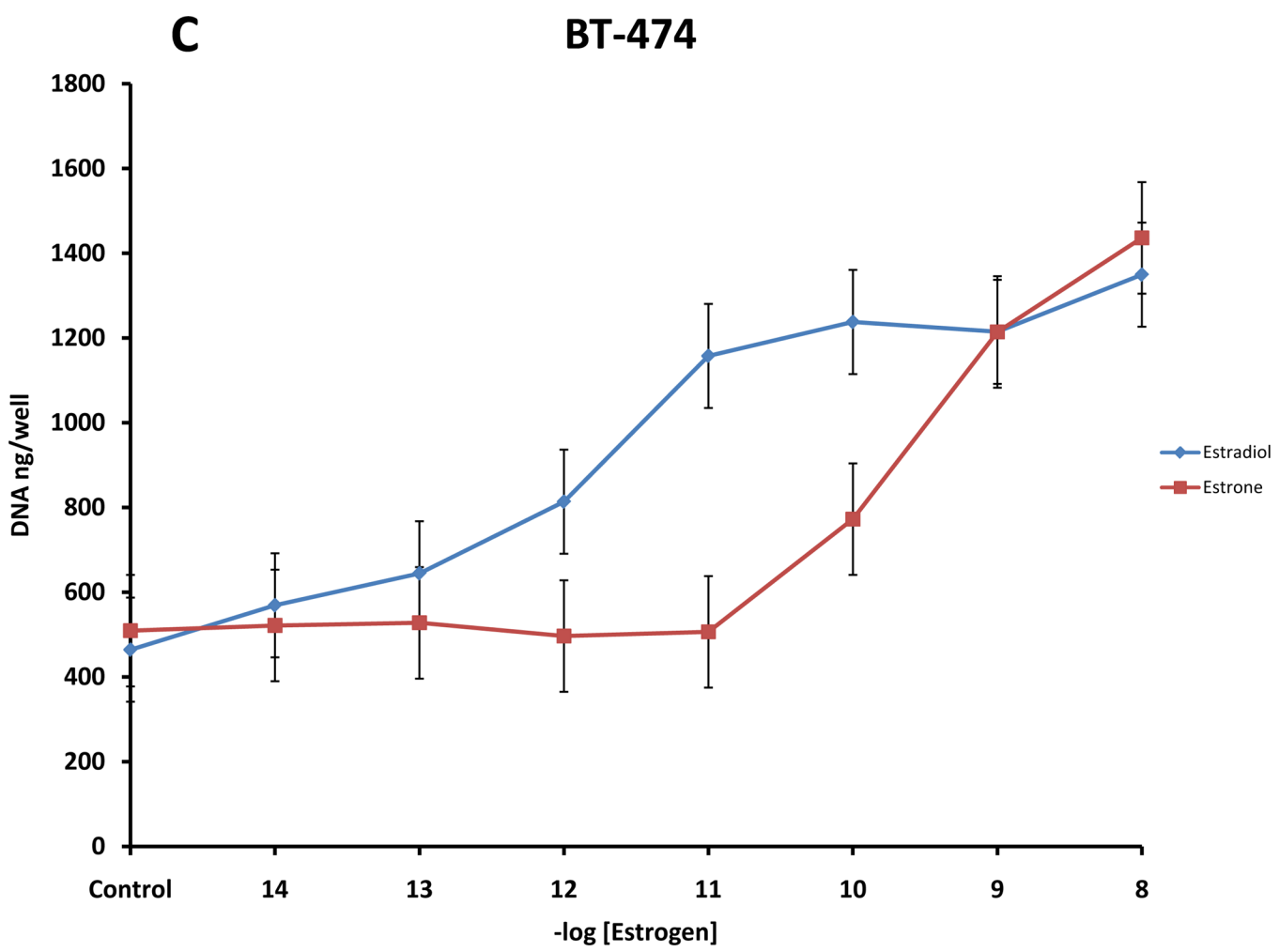

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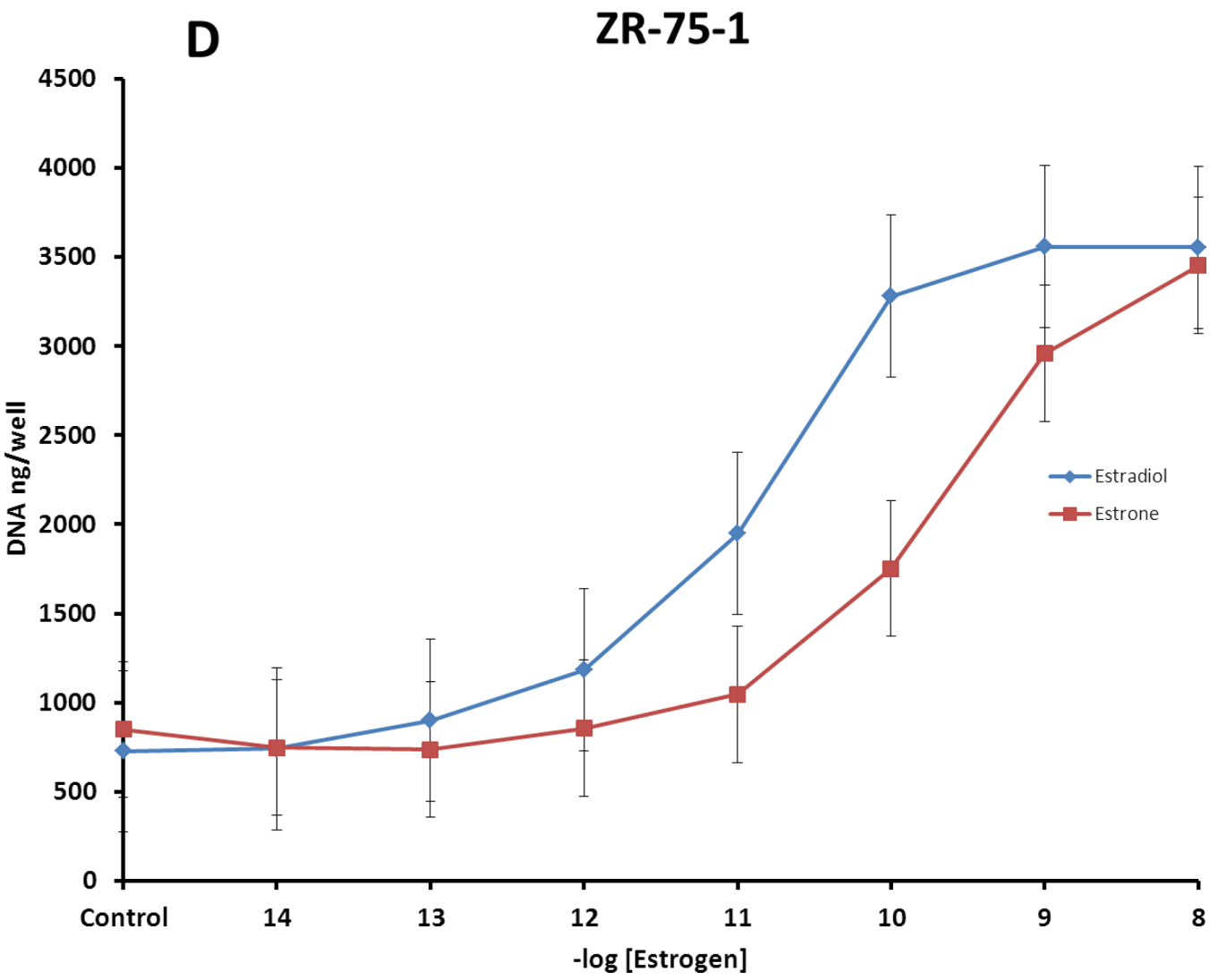

Figure 3.

Proliferative responses of different ER-positive breast cancer cell lines to treatments with estradiol (E_2) and estrone (E_1). Growth of cells was determined by measuring DNA per well after 7 day treatments. A. MCF-7:WS8 cells, hypersensitive clones of MCF-7 cell line; B. T47D:A18 cells, hypersensitive clone of T47D cell line; C. BT474 ER-positive breast cancer cells (ATCC); D. ZR-75-1 ER-positive breast cancer cells (ATCC). Estradiol is the most potent of the natural estrogens in a woman's body, and estrone, with the 17β hydroxyl oxidized to a ketone, is less potent. It does, however, significantly contrinue to breast cancer cell growth.

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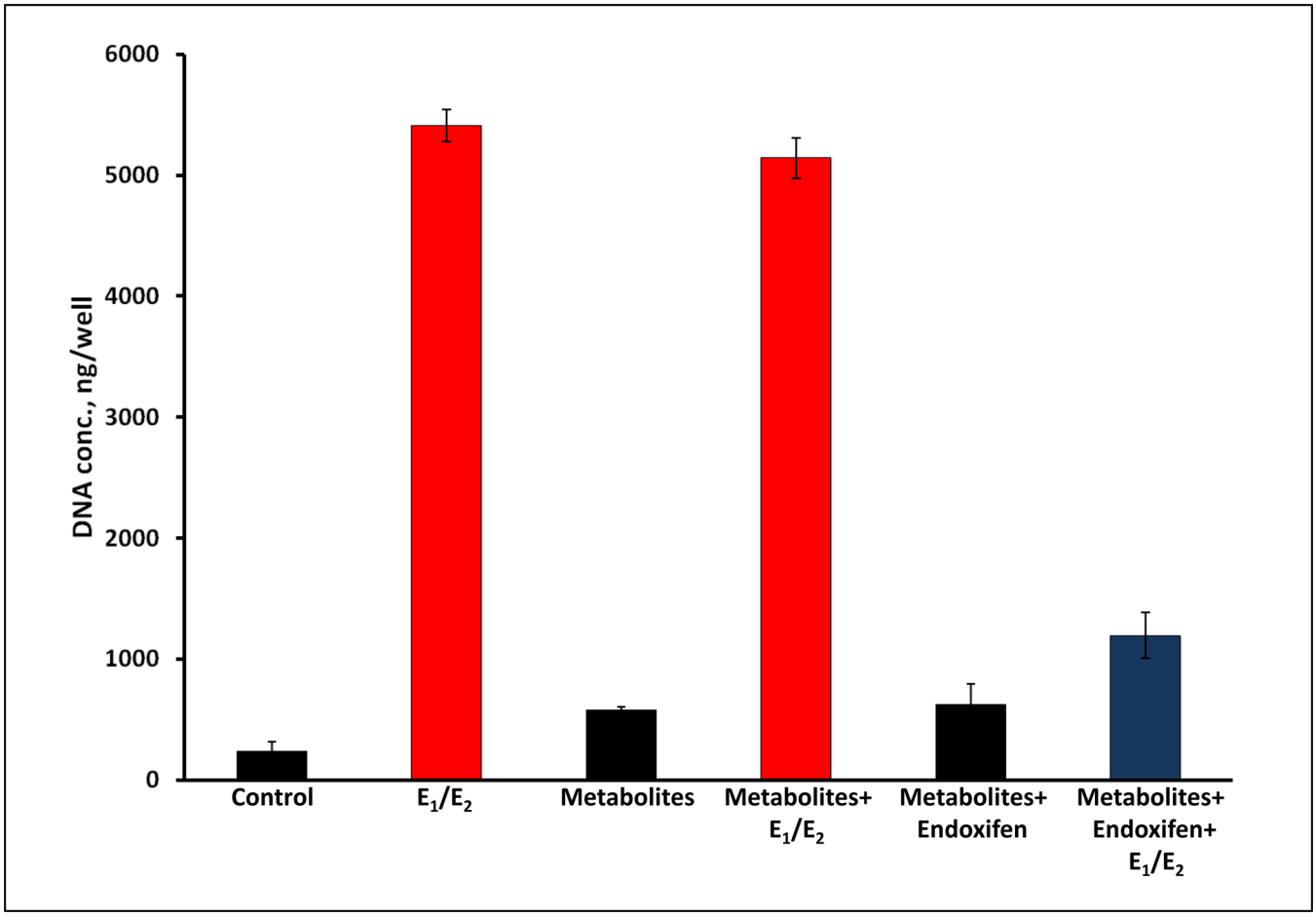
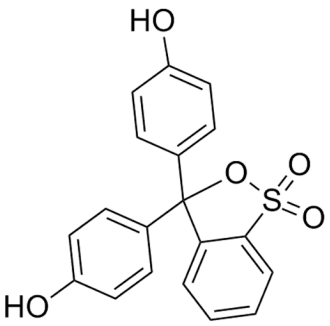
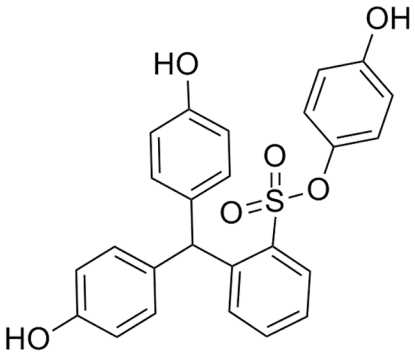


Figure 4.

Biological response of MCF-7 cells after 7 day treatment with premenopausal levels of estrone (E_1, 8nM) and estradiol (E_2, 4nM) found in plasma of premenopausal women during follicular phase of menstrual cycle [174] and tamoxifen metabolites 4OHT (6.3 nM), N-desmethyl-Tam (558 nM), tamoxifen (386 nM) and endoxifene (35.6 nM) at concentrations found in plasma of extensive metabolizers of tamoxifen [175]. As shown in the figure, combination of E_1/E_2 induce cell growth and treatment with combination of tamoxifen and its metabolites has minor effect on cells. Combination treatment of E_1/E_2 and tamoxifen metabolites does not ablate the proliferation of the cells. However, addition of another tamoxifen metabolite endoxifen at concentrations found in plasma of extensive metabolizers of tamoxifen (35.6 nM) produces almost complete inhibitory effect on cell growth. Treatment with combination of all tamoxifen metabolites (including endoxifen) does not have any major biological effect.



Phenol Red



bis(4-hydroxyphenyl)[2-(phenoxysulfonyl)phenyl]methane

Figure 5.

Phenolsulfonphthalein (phenol red), the pH indicator in cell culture media, is structurally similar to the natural estrogen estradiol (Figure 1) and synthetic estrogens. Unlike normal chemical titration analyses that use a pH indicator at very low concentrations, phenol red is incorporated at μM levels in culture media. The estrogenicity was found to vary from batch to batch [176]. However, a potent estrogenic contaminant (right) exerts growth stimulatory effects on breast cancer cells [38].

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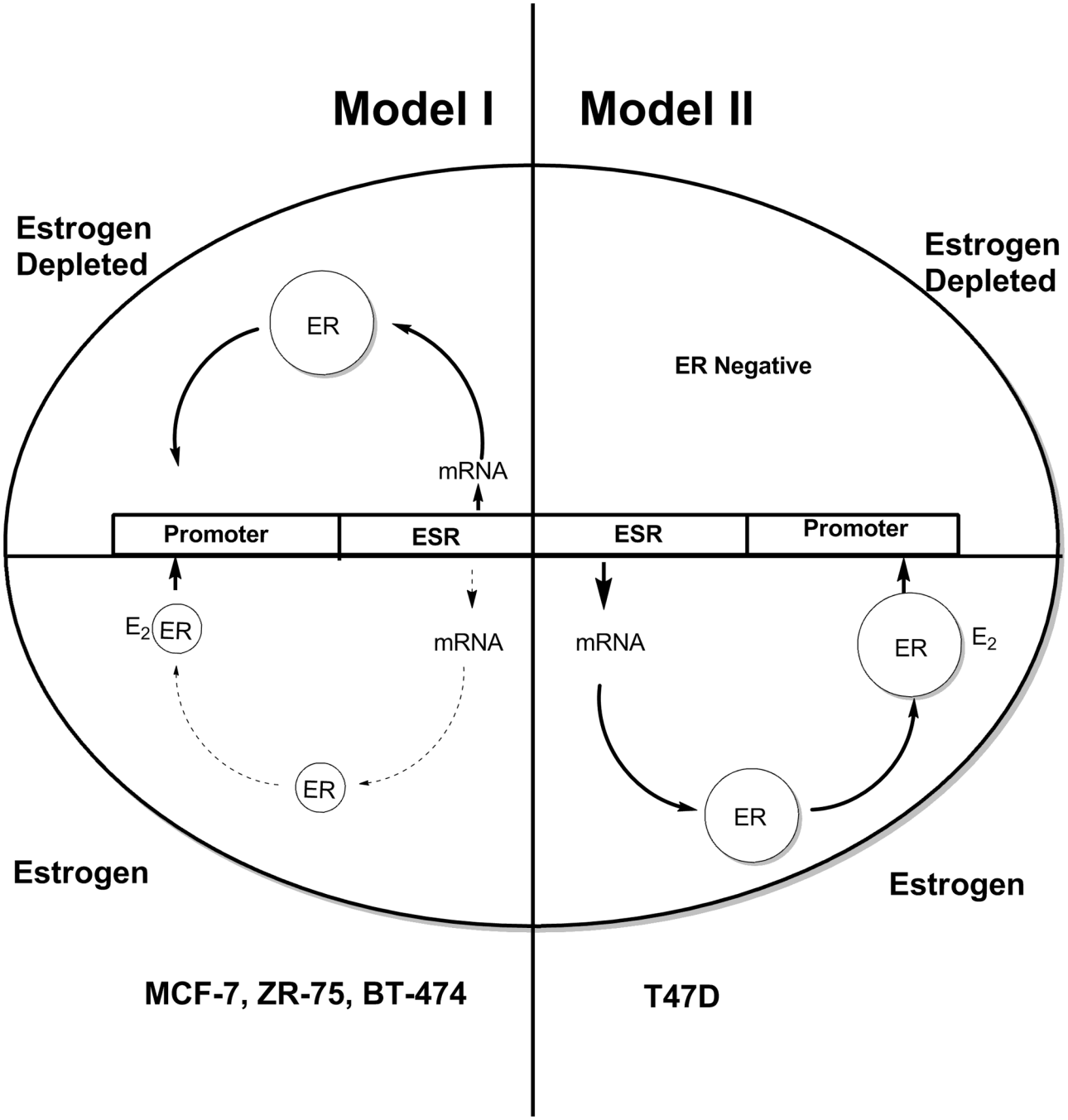

Figure 6.

The diagrammatic representation of cellular estrogen receptor (ER) regulation in media with or without estradiol (E_2). This diagram is based on the general responses to estrogen illustration by Western blotting in Figure 2 and presented in detail in [45]. Model I ER regulation (MCF-7, ZR-75, BT-474) has an upregulation of ER message and protein in an estrogen-depleted environment, but ER is downregulated at the mRNA and protein level in the presence of estrogen. Model II ER regulation (T47D) has upregulation of ER message and protein in an estrogen-containing environment but ER is not produced in an estrogen-depleted environment. Cells lose ER to become ER-negative.

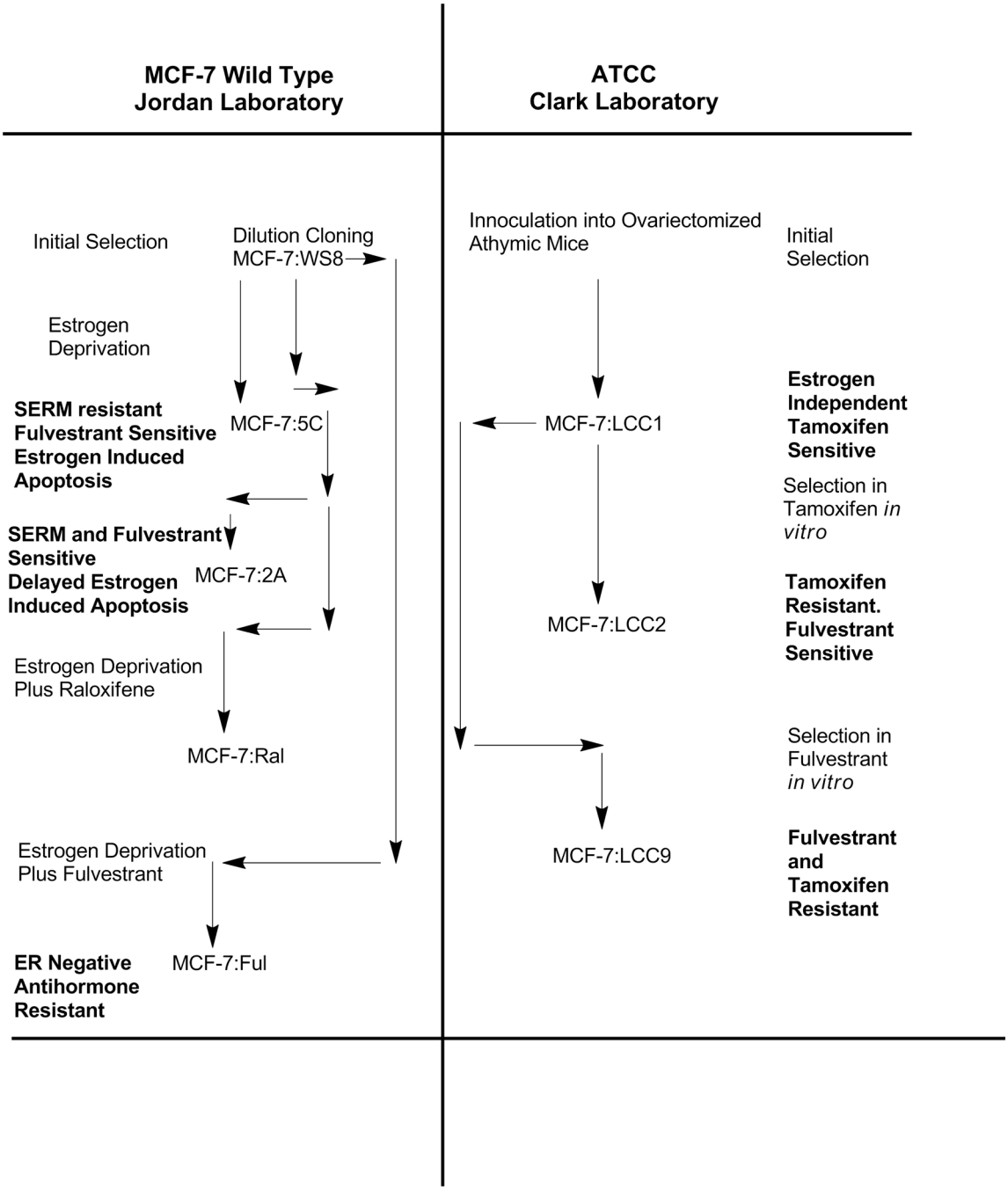

Figure 7.

A flow diagram representation of the defined antihormone-resistant cell lines derived from MCF-7 cells. The Jordan laboratory obtained original “Soule” MCF-7 cells from the Michigan Cancer Foundation as a gift from Dr. Dean Edwards who was then at the University of Texas. The Clark laboratory obtained MCF-7 cells from the ATCC cell collection. All cells are genotyped by DNA fingerprinting.

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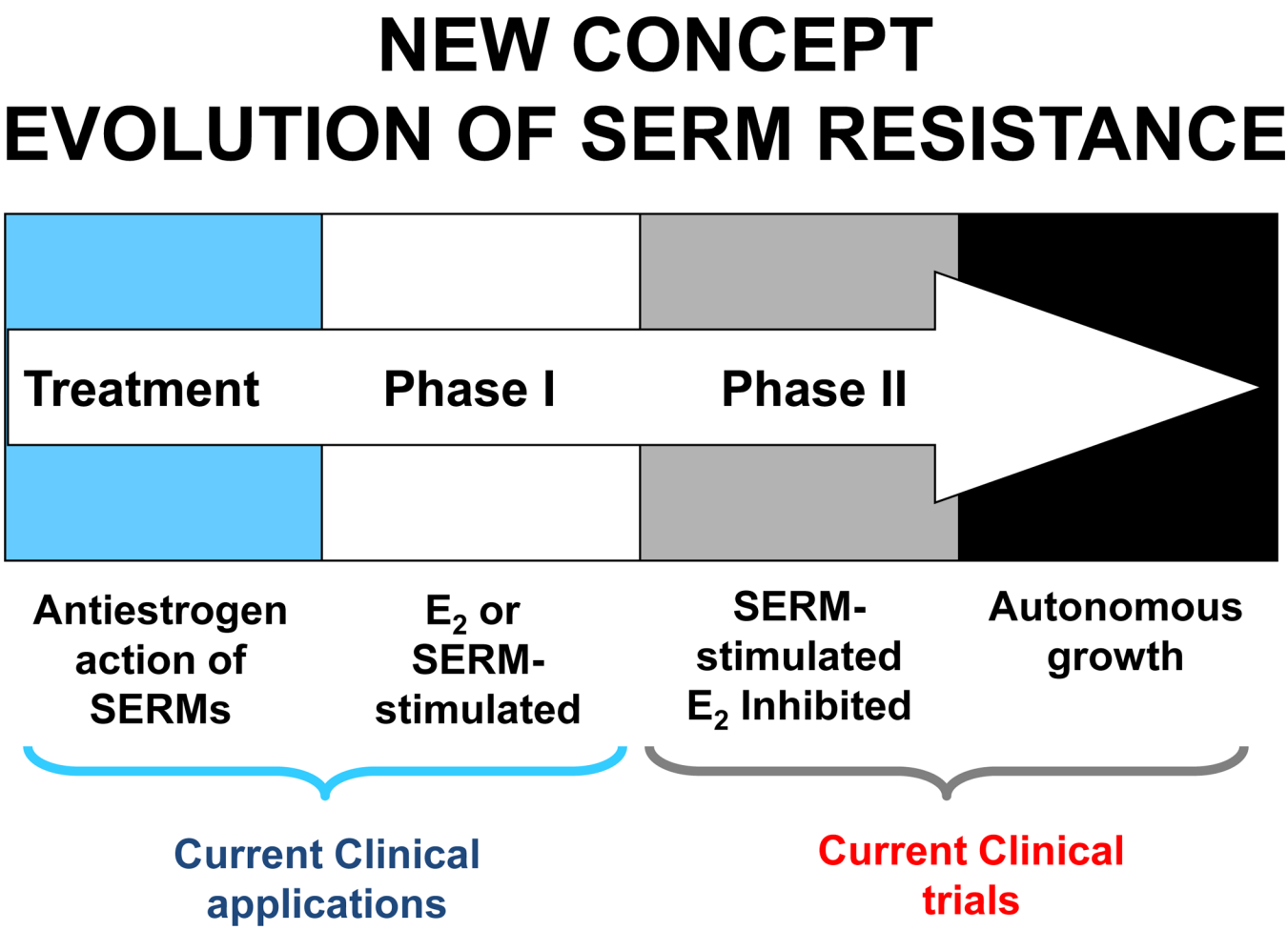
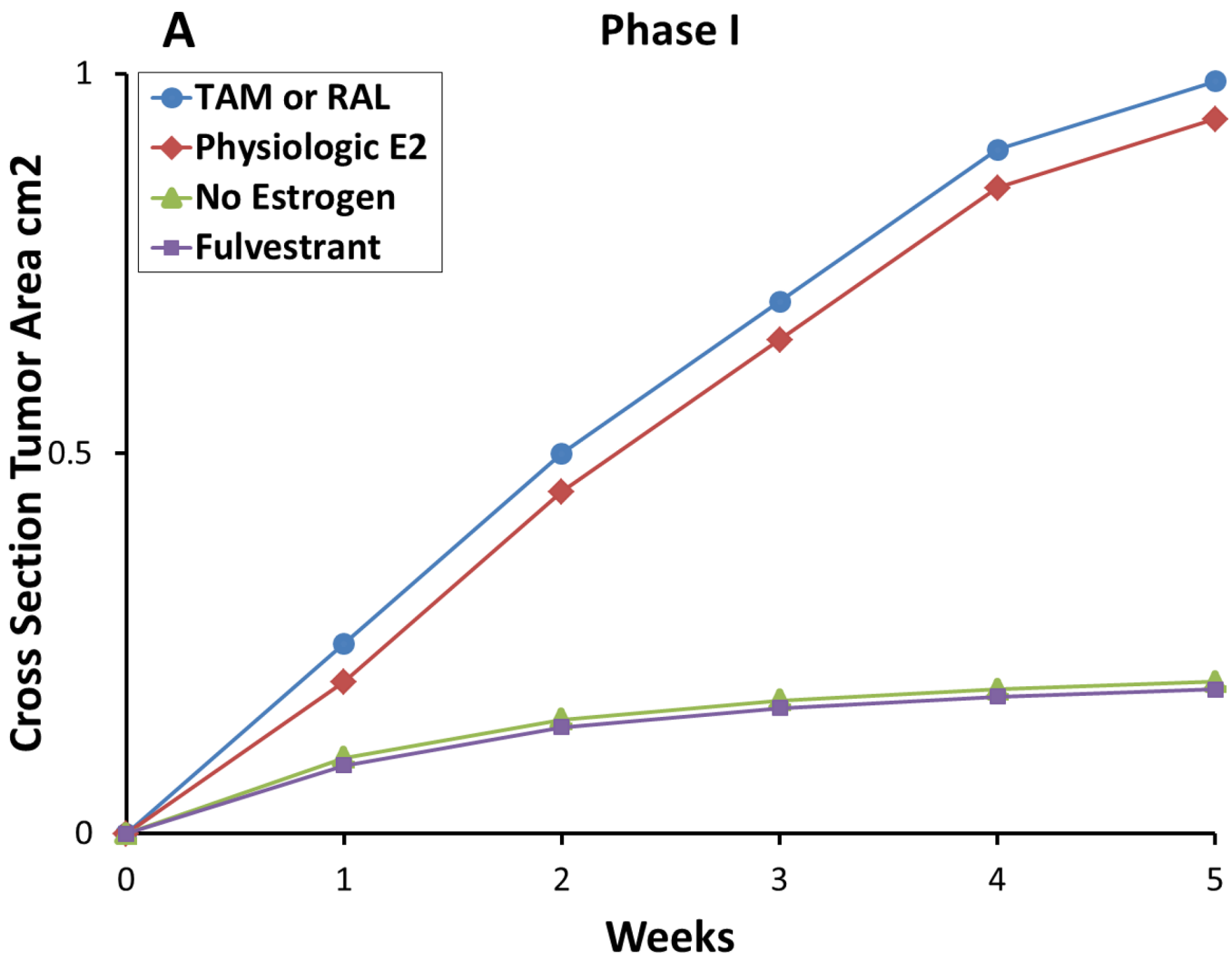


Figure 8.

Evolution of acquired SERM resistance. After long-term treatment with SERMs (1–2 years *in vivo*), initially responsive ER-positive tumors become resistant to treatment and are stimulated by SERMs (Phase I of resistance) as well as by E_2. After long-term transplantation into SERM-treated animal (5+ years), breast tumor growth is inhibited by E_2, though still stimulated by SERMs (Phase II of resistance). A stylized representation of MCF-7 tumor growth is illustrated in Figure 9. This process with SERMs *in vivo* is replicated with estrogen deprivation with MCF-7 breast cancer cells *in vitro;* cells initially start to grow spontaneously but estrogen still induces growth (hypersensitivity). This is Phase I. Long-term estrogen deprivation causes spontaneous growth in culture but apoptosis with physiologic estrogens both *in vitro* and *in vivo* (Phase II).



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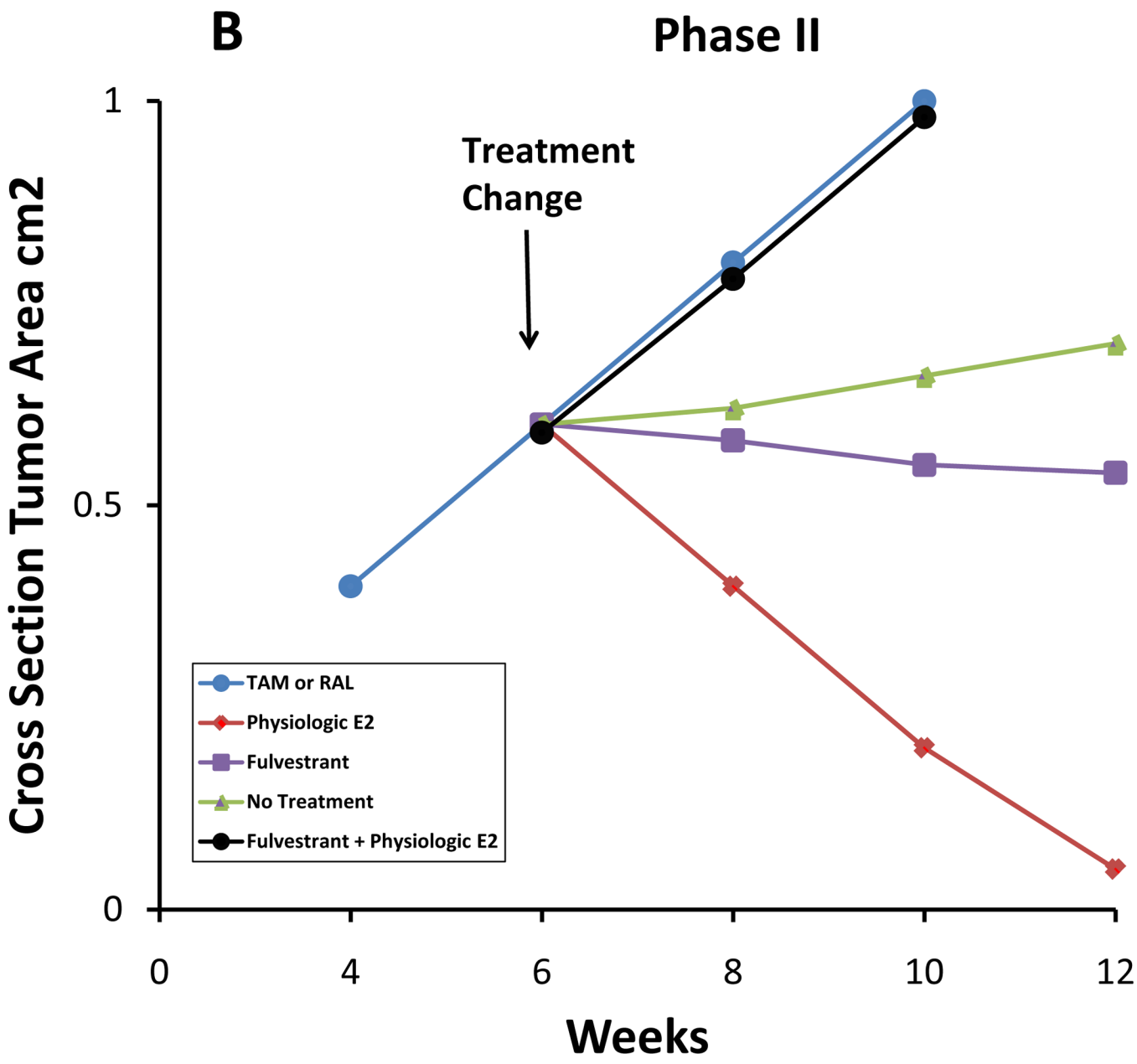

Figure 9.

Diagram of the growth rates of MCF-7 tumors during the evolution of drug resistance to selective estrogen receptor modulators (SERMs). A. During Phase I SERM resistance, tumors transplanted into athymic mice grow in response to either a SERM, tamoxifen (Tam) or raloxifene (Ral), or estrogen, but no estrogen (equivalent to the use of an aromatase inhibitor used clinically after Tam resistance occurs) or fulvestrant does not support growth (fulvestrant in used in this indication as a second-line therapy). B. During Phase II SERM resistance, tumors transplanted into athymic mice treated with SERMs now grow with a SERM (Tam or Ral). No treatment (equivalent to an aromatase inhibitor clinically) causes growth to slow, as does administering fulvestrant, but physiologic estradiol (E_2) causes dramatic apoptosis and tumor regression. Paradoxically, physiologic E_2 plus fulvestrant actually causes tumor growth. The low concentration of fulvestrant cancels out the apoptotic

effect of E_2 thereby redirecting E_2 as a growth signal, but higher concentrations of fulvestrant now have effective antitumor effects. This is now noted clinically [92].

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Table 1

Various subclones generated from different ER-positive breast cancer cell lines. To simulate different scenarios of therapy and development of resistance to SERMs, cells were cultured in different environments to create stable cell lines. Fulv: fulvestrant, Tam: tamoxifen, Ral: raloxifene, Ref: reference number

Parental Line	Subclone	How subclone was generated	Subclone's resistance	Ref
ZR-75	9a1	long-term tam treatment	tam	66
T47D	ER-negative	estrogen withdrawal	antihormones	28
T47D	−r	long-term fulv treatment	fulv	63
T47D	A18	estrogen-rich culture	-	61
T47D	C4	estrogen withdrawal	antihormones	61
T47D	co	PR expression selection without estrogen	estrogen, antiestrogen	65
MCF-7	Ral	long-term ral treatment	ral	52
MCF-7	F	long-term fulv treatment without estrogen	fulv	54
MCF-7	5C	estrogen withdrawal	tam	56, 57
MCF-7	2A	estrogen withdrawal	-	58
MCF-7	LY2	LY117018 selection	tam, LY117018	51
MCF-7	LCC1	estrogen withdrawal	-	47, 48
MCF-7	LCC2	estrogen withdrawal, tam selection	tam	49

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The Discovery and Development of Selective Estrogen Receptor Modulators (SERMs) for Clinical Practice

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Abstract: Selective estrogen receptor modulators (SERMs) are structurally different compounds that interact with intracellular estrogen receptors in target organs as estrogen receptor agonists or antagonists. These drugs have been intensively studied over the past decade and have proven to be a highly versatile group for the treatment of different conditions associated with postmenopausal women's health, including hormone responsive cancer and osteoporosis. Tamoxifen, a failed contraceptive is currently used to treat all stages of breast cancer, chemoprevention in women at high risk for breast cancer and also has beneficial effects on bone mineral density and serum lipids in postmenopausal women. Raloxifene, a failed breast cancer drug, is the only SERM approved internationally for the prevention and treatment of postmenopausal osteoporosis and vertebral fractures. However, although these SERMs have many benefits, they also have some potentially serious adverse effects, such as thromboembolic disorders and, in the case of tamoxifen, uterine cancer. These adverse effects represent a major concern given that long-term therapy is required to prevent osteoporosis or prevent and treat breast cancer.

The search for the 'ideal' SERM, which would have estrogenic effects on bone and serum lipids, neutral effects on the uterus, and antiestrogenic effects on breast tissue, but none of the adverse effects associated with current therapies, is currently under way. Ospemifene, lasofoxifene, bazedoxifene and arzoxifene, which are new SERM molecules with potentially greater efficacy and potency than previous SERMs, have been investigated for use in the treatment and prevention of osteoporosis. These drugs have been shown to be comparably effective to conventional hormone replacement therapy in animal models, with potential indications for an improved safety profile. Clinical efficacy data from ongoing phase III trials are available or are awaited for each SERM so that a true understanding of the therapeutic potential of these compounds can be obtained.

In this article, we describe the discovery and development of the group of medicines called SERMs. The newer SERMs in late development: ospemifene, lasofoxifene, bazedoxifene, are arzoxifene are described in detail.

Keywords: Arzoxifene, bazedoxifene, lasofoxifene, ospemifene, raloxifene, selective estrogen receptor modulator, tamoxifen.

THE QUEST TO PREVENT BREAST CANCER

The idea of using a chemical to prevent (chemo-prevention) breast cancer is a noble goal that has achieved significant successes in the past three decades. This is however not a new concept as Professor Antoine Lacassagne [1] had the vision which he stated at the Annual Meeting of the American Association for Cancer Research in 1936:

> *"If one accepts the consideration of adenocarcinoma of the breast as a consequence of a special hereditary sensibility to the proliferative action of oestrone, one is led to imagine a therapeutic preventive for subjects predisposed by their heredity to this cancer, to stop the congestion of oestrone in the breast."*

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However, his vision was based on his laboratory experiments with oophorectomy to prevent or estrogen replacement to enhance, tumorigenesis in strains of mice with a high incidence of mammary cancer. Most importantly, chemoprevention could not advance in humans because therapeutic knowledge was not available in the 1930's. The first antiestrogens would not be reported until the late 1950's more than 20 years later [2].

The non-steroidal antiestrogens initially had no major clinical impact during the first decade since the discovery of the first non-steroidal antiestrogen MER25 [3] in 1958. The early compounds were studied as antifertility agents in the laboratory, but clomiphene did the opposite in humans, so it was used successfully to induce ovulation in subfertile women. Clomiphene, a mixture of estrogenic (zuclomiphene) and antiestrogenic (enclomiphene) geometric isomer has been used for over 50 years for the induction of ovulation [4, 5]. This therapeutic advance set the scene for the subsequent breakthroughs in molecular pharmacology and medicines seen in the latter half of the 20th century (Fig. **1**). The

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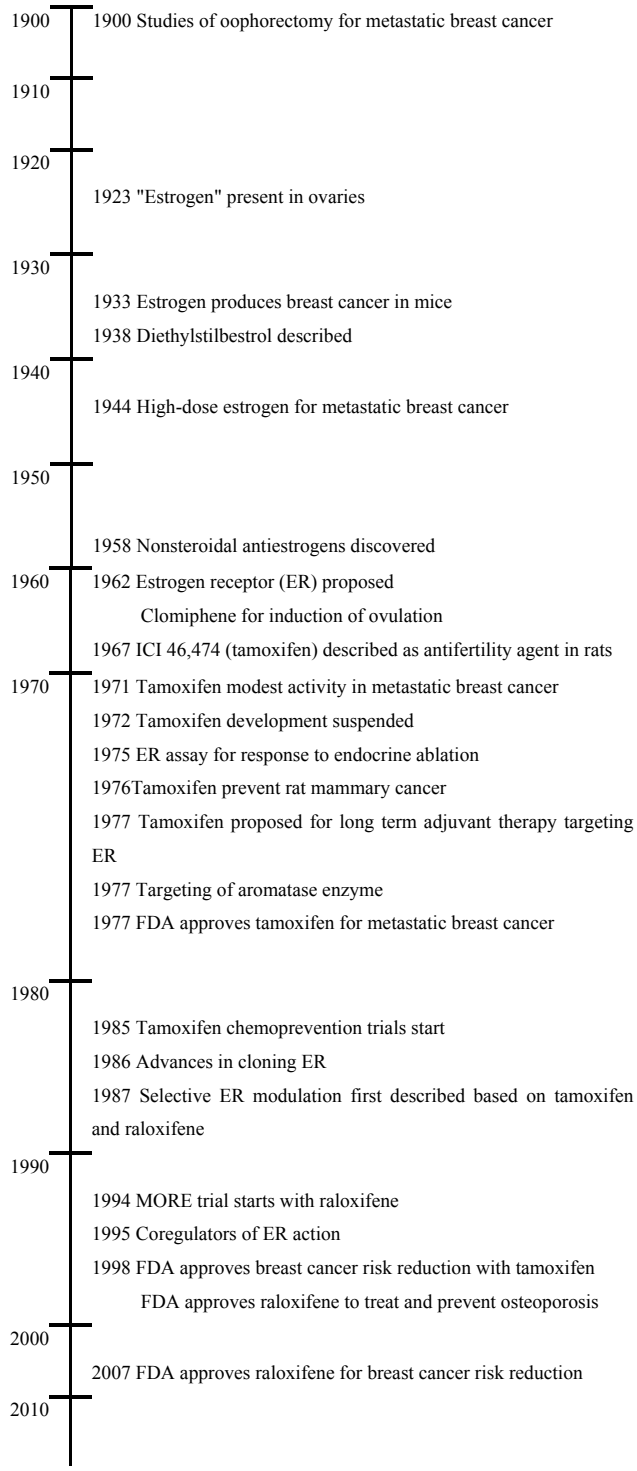

Fig. (1). Timeline of the major landmarks in estrogen action, anti-estrogens and SERMs for the treatment and prevention of breast cancer, and osteoporosis.

endocrinology of clomiphene was studied in some detail [6], for the obvious reason that the medicine was used to induce ovulation in healthy women, but toxicological issues prevented further drug development for other potential applications in women's health eg. breast cancer treatment and prevention. Then came tamoxifen, ICI 46,474, the failed contraceptive [7, 8] and orphan drug looking for a therapeutic application. Initial clinical studies demonstrated that it was safe and effective for the induction of ovulation in subfertile women [9, 10] and for the treatment of metastatic breast cancer in postmenopausal women [11, 12].

The story of the reinvention of tamoxifen to become the gold standard for the adjuvant treatment of breast cancer and the pioneering medicine for the reduction of breast cancer incidence in high risk women, has been told in detain elsewhere [13, 14]. Suffice to say the translational laboratory research work in the 1970's [15] that catalyzed tamoxifen's move from orphan drug resulted in tamoxifen becoming the standard of care for the long term adjuvant therapy of estrogen receptor (ER) positive breast cancer and, as a result, extended the lives of millions of women worldwide. The approvals for the use of tamoxifen are unique amongst anticancer agents and include the treatment of metastatic breast cancer, adjuvant therapy with chemotherapy, adjuvant therapy alone, the treatment of ductal carcinoma in situ, risk reduction in high risk pre- and postmenopausal women and breast cancer treatment in men. The advance was achieved based on the premise that tamoxifen, the pure *trans* isomer of a triphenylethylene was the lead member of the group of drugs known as nonsteroidal antiestrogens [16]. If estrogen was indicated in the growth of some breast cancer then an antiestrogenic drug would be effective as a treatment. But fashions in science and medicine change and this was about to happen in the 1980's with a new approach to the management of breast cancer: chemoprevention

Professor Trevor Powles was the first to initiate a pilot study for the chemoprevention of breast cancer in a small group of high risk women using tamoxifen. He selected women with a first degree relative that had already had breast cancer. His pilot toxicology study was initiated in 1985 and published in 1989 [17]. However, there were significant toxicological issues that had to be addressed in the laboratory and translated to clinical trial before an "antiestrogen" could be considered to be tested in large populations of healthy women for the chemoprevention of breast cancer. Tamoxifen was noted in the laboratory [18] and clinic [19] to increase the growth and incidence of endometrial cancer. Also at that time in the 1980's it was believed, that estrogen was useful to protect women from coronary heart disease and osteoporosis. Clearly there would be no advantage of using a drug classified as a "non-steroidal antiestrogen" to block estrogen mediated breast carcino-genesis in the few, but expose the whole experimental population to crushing osteoporosis or an elevation of the incidence of coronary heart disease. Studies conducted at the University of Wisconsin Comprehensive Cancer Center [2, 18, 20-26] were instrumental in providing clarity to these questions and created the new drug group – Selective ER Modulators or SERMs.

The mention of "modulation" at an ER target site first occurred with the examination of the structure function relationships of estrogenic triphenylethylene derivatives of tamoxifen at a prolactin gene target *in vitro* [27]. The

estrogenic compounds could activate or suppress prolactin synthesis by altering the shape of the ER complex between the extremes of an "antiestrogenic" or an "estrogenic" conformation [28]. This idea of the molecular modulation of the receptor at a single target site was then expanded to consider the physiologic responses that occurred with nonsteroidal antiestrogen at multiple target sites in the body – simultaneously.

A cluster of translational studies focused on the uterus, breast (mammary gland) and bone together created the data base for further confirmatory studies and the clinical trials by the pharmaceutical industry that resulted in the reinvention of the failed breast cancer drug keoxifene to become raloxifene the first clinically available SERM to prevent both osteoporosis and breast cancer [29-32]. Each of the laboratory studies provided an interlocking network of knowledge relevant to the practical application of a new drug group in medical practice. The fundamental concept of SERMs action described first in the late 1980s [2, 23] and later refined and defined as a balance of receptors and coregulators (Fig. **2**) is similar to the subsequent description of Protean agonists of the G-protein-coupled receptors [33].

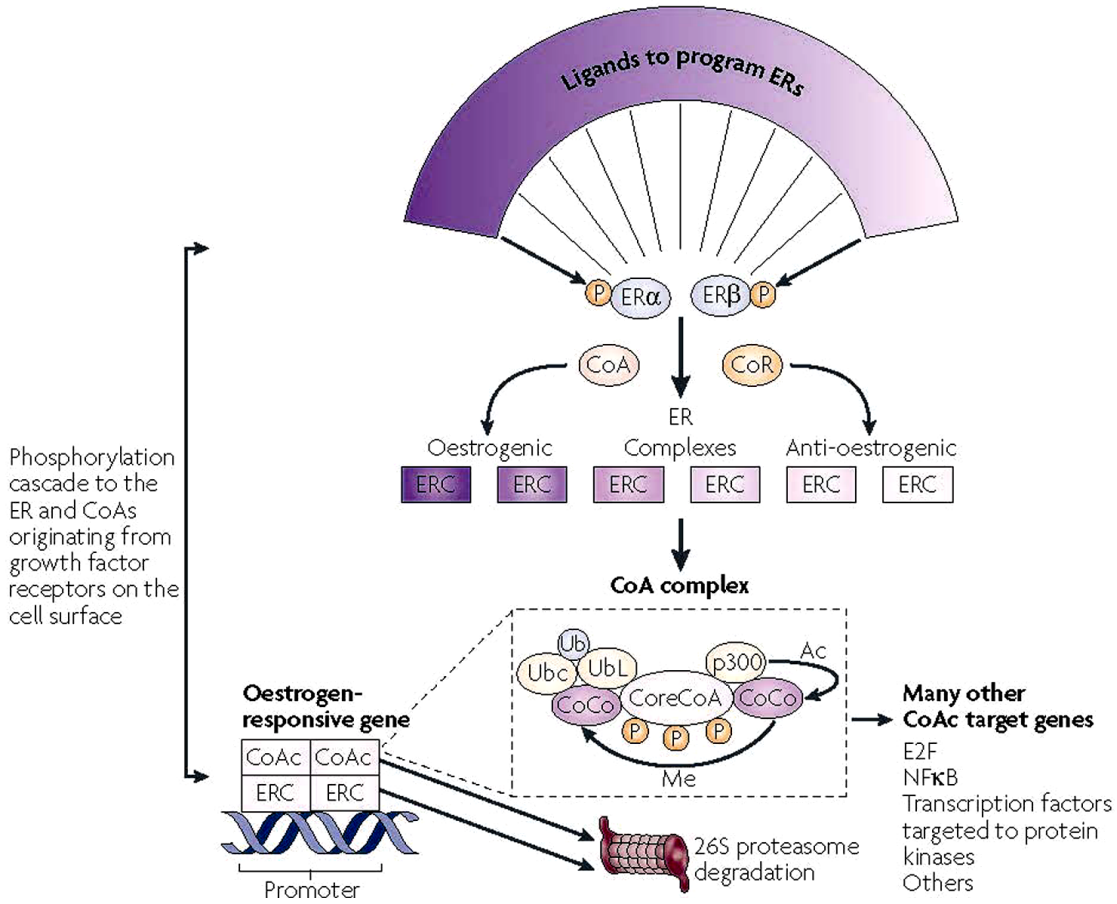

Fig. (2). Molecular networks potentially influence the expression of SERM action in a target tissue. The shape of the ligands that bind to the estrogen receptors (ERs)α and β programmes the complex to become an estrogenic or anti-estrogenic signal. The context of the ER complex (ERC) can influence the expression of the response through the numbers of co-repressors (CoR) or coactivators (CoA). In simple terms, a site with few CoAs or high levels of CoRs might be a dominant anti-estrogenic site. However, the expression of estrogenic action is not simply the binding of the receptor complex to the promoter of the estrogen-responsive gene, but a dynamic process of CoA complex assembly and destruction [101]. A core CoA, for example, steroid receptor coactivator protein 3 (SRC3), and the ERC are influenced by phosphorylation cascades that phosphorylate target sites on both complexes. The core CoA then assembles an activated multiprotein complex containing specific co-co-activators (CoCo) that might include p300, each of which has a specific enzymatic activity to be activated later. The CoA complex (CoAc) binds to the ERC at the estrogen-responsive gene promoter to switch on transcription. The CoCo proteins then perform methylation (Me) or acetylation (Ac) to activate dissociation of the complex. Simultaneously, ubiquitiylation by the bound ubiquitin-conjugating enzyme (Ubc) targets ubiquitin ligase (UbL) destruction of protein members of the complex through the 26S proteasome. The ERs are also ubiquitylated and destroyed in the 26S proteasome. Therefore, a regimented cycle of assembly, activation and destruction occurs on the basis of the preprogrammed ER complex [101]. However, the co-activator, specifically SRC3, has ubiquitous action and can further modulate or amplify the ligand-activated trigger through many modulating genes [215] that can consolidate and increase the stimulatory response of the ERC in a tissue. Therefore, the target tissue is programmed to express a spectrum of responses between full estrogen action and anti-estrogen action on the basis of the shape of the ligand and the sophistication of the tissue-modulating network. NFκB, nuclear factor κB. This figure is published with permission from Nature Publishing group. Jordan, V.C. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nature Reviews Cancer, 2007 Jan; 7(1): 46-53.

The first public description of the clinical concept of SERMs as useful medicines for women's health was at the First International Chemoprevention meeting in New York in 1987. The vision was stated as follows: "The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused on the identification of a population of high-risk women to test 'chemopreventive' agents. But, are resources being used less than optimally? An alternative would be to seize on the developing clues provided by an extensive clinical investigation of available antiestrogens. Could analogues be developed to treat osteoporosis or even retard the development of atherosclerosis? If this proved to be true, then a majority of women in general would be treated for these conditions as soon as menopause occurred. Should the agent also retain antibreast tumor actions, then it might be expected to act as a chemosuppressive on all developing breast cancers if these have an evolution from hormone-dependent disease to hormone independent disease. A bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century [23]".

Subsequently the "roadmap" for the pharmaceutical industry was refined and defined more precisely in the Cain Memorial Award lecture presented before the American Association for Cancer Research in 1989 for advances in laboratory research leading to the discovery and development of new therapeutic agents for the treatment of cancer. "We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids, so apparently, derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high-risk group to prevent breast cancer [2]".

Indeed, the discovery that tamoxifen and raloxifene had target site selective estrogenic and antiestrogenic actions around the body would stimulate all subsequent research on SERMs [34].

PHARMACOKINETICS OF TAMOXIFEN AND RALOXIFENE

Tamoxifen a long acting drug with a long biological half-life that is metabolically activated, whereas raloxifene is a very short acting drug that is rapidly conjugated and then excreted through the biliary tract. The metabolism, pharmacogenomics and pharmacokinetics of SERMs continue to present challenges. Just when everything appears to be straightforward, old drugs create unanticipated surprises and in contrast ideas to alter the pharmacokinetics of raloxifene from a short to a long acting drug do not result in success. Initially, there was little pharmacologic information or interest in the metabolism of tamoxifen in animals and man; this was not a major requirement to register a drug to treat advanced breast cancer in the 1970's [14]. The situation remained the same during the 1980's when tamoxifen was about to become the standard of care as the adjuvant antihormonal treatment of ER positive breast cancer and studies were planned to evaluate the worth of tamoxifen to prevent the breast cancer in high risk women [14]. At that time, it was accepted that tamoxifen was either metabolically activated to 4-hydroxytamoxifen [35, 36], a minor metabolite with high binding affinity to the ER but with a short biological half-life [37] or was demethylated to N-desmethyltamoxifen, a compound with low binding affinity for the ER but a long biological half-life. N-Desmethyltamoxifen was further demethylated to desdimethyltamoxifen and subsequently deaminated to the weakly antiestrogenic glycol derivative of tamoxifen referred to as metabolite Y [38]. These antiestrogenic metabolites deactivate the ER but based on concentrations of metabolites and their affinity, all were considered to play a role in blocking estrogen action.

The ubiquitous application of tamoxifen as a long-term, well tolerated treatment for breast cancer during the past two decades and its use as a preventive in high risk women, resulted in the close examination of symptom management, especially hot flashes, to enhance compliance. Selective serotonin reuptake inhibitors (SSRIs) are effective in controlling hot flashes experienced by up to 45% of treated patients. However, the identification and characterization [39-41] of the high affinity metabolite of tamoxifen 4-hydroxy-N-desmethyltamoxifen (endoxifen) and the finding that endoxifen levels are reduced by the co-administration of SSRIs [42-44] is an important observation that has potential therapeutic implications. It follows that since SSRIs block CYP2D6, thereby inhibiting the metabolism of tamoxifen to endoxifen, then the efficacy of tamoxifen as an anticancer agent (treatment or chemoprevention) could be impaired by either the ubiquitous use of SSRIs to prevent hot flashes or the administration of tamoxifen to women with a defect in the CYP2D6 enzyme that no longer converts tamoxifen to endoxifen. Preliminary evidence suggests that this might be the case [44, 45]. However, the proposition that patients should be genotyped to identify poor metabolizers who will be less likely to respond to tamoxifen remains controversial. Be as it may, it is probably unwise to use SSRI to reduce hot flashes in patients taking tamoxifen. Venlafaxine, a drug with low potential to interact with the CYP2D6 enzyme, is the agent of choice for symptom control.

The knowledge that tamoxifen was metabolically activated to hydroxylated metabolites with high affinity for the ER [35] created the opportunity for chemists in the pharmaceutical industry to design the high affinity SERMs, raloxifene, basedoxifene and lasofoxifene. However, the pharmacokinetics and pharmacodynamics of these polyphenolic compounds now creates a complex new set of problems to get an orally active drug constantly to the breast tissues to prevent estrogen-stimulated growth Raloxifene and other SERM members that are benzothiophene derivatives, are short acting [46-48]. However, raloxifene has a plasma elimination half-life of approximately 27 hours which apparently results from reversible Phase II metabolism which conjugates the polyphenolic drugs prior to excretion as sulphates and glucuronides. There appear to be two

aspects for consideration for a polyphenolic SERM to be an effective chemopreventive for breast cancer. Firstly, raloxifene is conjugated by the human intestinal enzymes UGTIA8 and UGTIA10 [49] but it is the dynamic relationship between absorption, Phase II metabolism and excretion in the intestine [50] that controls the 2% bioavailability of raloxifene [48]. The second aspect for consideration is the retention of raloxifene in the target tissue. This depends on local sulphation which inactivates the SERM prior to diffusion out of the tissue. Here again, there are disparities in the efficacy of multiple sulphation enzymes (sulphotransferases, SULTs) to terminate bioactivity of raloxifene in a target site. By way of example: 4-hydroxytamoxifen [35] is only sulphated by three of seven SULT isoforms whereas raloxifene is sulphated by all seven [51]. Additionally, SULTIEI, which sulphates raloxifene in endometrial tissue, is only expressed in the secretory phase [51] of the menstrual cycle following ovulation [52]. All these issues promted chemists in industry to improve the breast cancer treatment potential of SERMs by improving the pharmacokinetics by designing the long acting "raloxifene" named arzoxifene (see later section). Similarly lasofoxifene creates a very interesting innovation in enhanced pharmacokinetics. Lasofoxifene is extensively metabolized in rats and monkeys with tissues achieving maximal concentrations within one hour of oral administration of ^{14}C labeled lasofoxifene [53]. There was greater than 95% of lasofoxifene and metabolites excreted in feces through the biliary route with only a small amount of glucoronide. It is reasoned that increased oral bioavailability results from the fact that the non-planar lasofoxifene is a poor substrate for glucuronidation. Lasofoxifene exists in two enantiomer; the l-enantiomer has high ER binding and increased bioavailability, compared to the d-enantiomer [54]. This property of the molecule improves pharmacokinetics so that a clinical dose of 0.5mg daily is proven effective in clinical trial to prevent bone loss and prevent breast cancer [55]. This is $1/100^{th}$ the daily dose of raloxifene!

With this background of the challenges that the medicinal chemist faces and must solve to create a successful SERM, we now turn to the story that evolved during the 1980's that formed the basis for all future drug discoveries by the pharmaceutical industry. Simply stated; what were the circumstances that created the SERMs, what were the challenges for the clinical community and where did the new SERMs we study today have their origins?

THE BIOLOGICAL BASIS OF SERM ACTION: TARGET TISSUE SPECIFIC ACTIONS

In this section we will present the translational data, obtained primarily during the 1980's that proved to be the database that created the concept to move forward to clinical testing and advance novel SERMs for clinical applications. We will cluster each estrogen target tissue group studied in the 1980's that advanced the new SERM concept [2, 23] into clinical testing and validation during the 1990's.

Uterus, Breast and Endometrial Cancer

The development of the athymic (immune deficient) mouse models provided an invaluable opportunity to study human tumor cell lines *in vivo*. The ER positive breast cancer cell line MCF-7 [56] can be inoculated into ovariectomized athymic mice and will grow into tumors in response to the administration of sustained release physiologic estradiol. However, the pharmacology of tamoxifen is species specific; the compound is classified as an anti-estrogen in the rat but an estrogen in the mouse [7]. Administration of tamoxifen to athymic mice implanted with MCF-7 tumors demonstrated that only estradiol would cause the human breast tumor to grow, tamoxifen did not [22]. Nevertheless, the ovariectomized mouse uterus grew in response to either tamoxifen or estradiol. There was target site specificity and the conclusions in a pivotal paper [22] clearly stated the idea "The species differences observed with tamoxifen are the result of differences in the interpretation of the drug-ER complex by the cell. The drug-ER complex is perceived as either a stimulatory or an inhibitory signal in the different target tissues from different species". Nevertheless, the results could have been the result of species differences in pharmacology and not tissue specific pharmacology. To address this question two approaches were taken 1) the target site specificity of two human tumors were compared and contrasted implanted in the same athymic mouse and 2) inbred strains of mice with a high incidence of mammary tumors were used to determine whether there was target site specificity to prevent mammary cancer in the same species of rodent.

Bitransplantation of ovariectomized mice with a MCF-7 breast tumor in one axillary fat pad and an EnCa101 human endometrial tumor in the other provides an ideal translational model to evaluate the responsiveness of two human tumors in the same therapeutic environment. The analogy would be the responsiveness of the breast cancer patient to adjuvant tamoxifen but with an occult endometrial tumor. At the time of the experiments in 1987 there were no reports of an increase in endometrial cancer incidence in any adjuvant clinical trials. The laboratory study demonstrated that tamoxifen blocked breast tumor growth but tamoxifen enhanced estrogen-stimulated endometrial cancer growth [18].

Even before the start of the tamoxifen chemoprevention trials in the early 1990's it was clear that a new approach to the chemoprevention of breast cancer was necessary. Firstly the targeted population for preventing breast cancer was only a small percent of the potential population at risk ie: only about 8-10 women will develop breast cancer per 1000 high risk women per year. However, all women will be exposed to the side effects of tamoxifen. An increased risk of developing endometrial cancer was obviously significant to women so a solution needed to be addressed. Another medicine was necessary but clues were already in the refereed literature to formulate a strategy for the new drug class – the SERMs. An important clue was to be found using the 'nonsteroidal antiestrogen' keoxifene abandoned by Eli Lilly following its failure in testing as a breast cancer drug competitor to tamoxifen in 1987. Keoxifene was not as estrogen-like as tamoxifen in the rodent uterus [57] but was used as a comparator compound to illustrate that different antiestrogens would modulate the growth of human endometrial carcinoma implanted in to athymic mice [58]. Keoxifene did not have the same efficacy as tamoxifen to

enhance the growth of human endometrial carcinoma under laboratory conditions. Indeed keoxifene could block full tamoxifen stimulated endometrial carcinoma growth [58]. This was important pharmacological evidence published in the refereed literature years before raloxifene (a.k.a. keoxifene) advanced the path for progress in women's health after 1992.

The additional important target site specific evidence to support the clinical development of SERMs for women's health was the use of inbred strains of mice with a high incidence of spontaneous mammary cancer. The question to be addressed was whether tamoxifen could prevent mouse mammary carcinogenesis if the drug was classified as an estrogen in the mouse. Professor Antoine Lacassagne had used this model to support his hypothesis stating earlier that "Therapeutic compounds could be found to stop the congestion of oestrone in the breast" [1]. However, tamoxifen was classified as an estrogen in the mouse [7]. Studies comparing and contrasting tamoxifen and oophorectomy in the C3H/OUJ mouse strain demonstrated that long term tamoxifen treatment was effective in preventing mouse mammary tumorigenesis, was superior to oophorectomy, and that tamoxifen's action as an estrogen in the uterus was target site specific in the same species [59, 60]. Overall these mouse studies (athymic and high incidence mammary cancer strains) demonstrated "targeted estrogenic and antiestrogenic actions".

Summary and Conclusion

As a result of the finding in the laboratory [18], Fornander and colleagues [19] reported a significant increase in the risk of developing endometrial cancer during tamoxifen therapy. Practice changes occurred immediately and regular gynecologic examinations were recommended for women taking tamoxifen. It is important to note, however, that the risk of developing endometrial cancer is only elevated in postmenopausal women. The laboratory testing and reinvention of raloxifene as an antiestrogen with no uterine effects was to be critical to exploit the discovery of the estrogen-like effects of tamoxifen and raloxifene in bone.

Bone and Mammary Tumorigenesis

The fact that estrogens build bone and estrogen deprivation during the postmenopausal period enhances the risk of osteoporosis was a major concern for implementing a safe strategy of breast chemoprevention with the nonsteroidal antiestrogen tamoxifen. An antiestrogenic drug may prevent breast cancer in a few but enhance the risk of osteoporosis in the majority. Laboratory research and clinical translation would change that perspective and deliver the SERMs as a new drug group.

An early report using clomiphene (the mixture of estrogenic *cis* and antiestrogen *trans* isomers) in the ovariectomized rats [61] concluded that clomiphene builds bone. However, the study was flawed because clomiphene is a mixture of estrogenic and antiestrogenic isomers. It may have been that the estrogenic isomer built bone in the administered mixture of clomiphene isomers. In contrast, the first study in the ovariectomized rats with the nonsteroidal antiestrogens tamoxifen and keoxifene (ie: raloxifene) only used pure compounds based on a *trans* or "antiestrogenic" conformation. Both compounds blocked estradiol-induced increases in uterine weight but retarded decreases in bone loss and did not block estradiol induced increases in bone density [21]. The results with tamoxifen were immediately confirmed by others in the rat [62, 63] and these laboratory data were used to test the concept that tamoxifen is estrogen-like in bone in the Wisconsin Tamoxifen Study. Tamoxifen maintained and built bone in postmenopausal women with node negative (low risk recurrence) breast cancer [25] This result demonstrated, for the first time in a prospective randomized clinical trial, that the principle of "selective estrogenic (bone) and antiestrogenic (breast) action" occurred in humans. Also the laboratory data suggested that the target site specificity of the 'nonsteroidal antiestrogens' was not unique to tamoxifen but was a class effect. The initial discovery with the bone building effects of tamoxifen and raloxifene [21] coupled with the demonstration of the inhibition of rat mammary carcinogenesis with either tamoxifen and raloxifene [20] prompted the description of a vision for the future use of the new class of drugs [2, 23]. However, the rat mammary carcinogenesis studies with tamoxifen and raloxifene showed that the effect of raloxifene was not superior to tamoxifen and would not be long lasting [23]. This would be demonstrated subsequently in postmenopausal women in the STAR trial [32].

SUMMARY AND CONCLUSIONS

The laboratory and clinical data which demonstrated that tamoxifen is estrogen-like by increasing rat bone density and bone density in postmenopausal women was reassuring to move forward with the chemoprevention trials with tamoxifen in the 1990's. However, the fact that keoxifene maintained bone density in the ovariectomized rat [21] (but without an estrogen-like effect in the uterus seen with tamoxifen) triggered the hypothesis that drugs of this class could be used to treat osteoporosis and atherosclerosis, and prevent breast cancer at the same time [2, 23]. The development of raloxifene was the result to prevent both osteoporosis and to reduce the incidence of breast cancer.

There is a long and sustained decrease in breast cancer incidence for a decade (at least) after tamoxifen stops [64-66]. This is not true for raloxifene in the STAR trial after treatment stops. Raloxifene is recommended to be used continuously to prevent the developing breast cancers [32].

Concepts in the Control of Coronary Heart Disease (CHD)

In the days before atorvastatin (or 'statins'; HMG CoA reductase inhibitors) was proven to reduce low density lipoprotein (LDL) cholesterol [67] and as a result reduce the risk of coronary heart disease due to atherosclerosis [68-70], a variety of drugs that interfered with cholesterol metabolism were evaluated. One such compound triparanol blocked cholesterol biosynthesis [71] but became a *cause célèbre* as the buildup in desmosterol was linked to cataract formation in young women taking the medicine [72]. The Merrell company in Cincinnati who manufactured and marketed triparanol subsequently chose to avoid development of any

drug that increases circulating desmosterol. The subsequent discovery and investigation of clomiphene by Merrell also showed an increase in desmosterol, so long term treatment with clomiphene was subsequently avoided [14].

A related compound, ICI 46,464, is the pure trans isomer of triphenylethylene but does not increase desmosterol despite the fact that circulating cholesterol is lowered in the rat [7]. A safer toxicology profile predetermined the drug as a useful antiestrogen to use in long term therapy for a disease such as breast cancer. Indeed the fact that tamoxifen lowered circulating cholesterol in the rat was included in the patent. The application for tamoxifen stated, "The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolaemic activity".

Subsequent clinical studies [24, 26, 73, 74] demonstrated a decrease in LDL cholesterol thereby holding out the promise that drugs of this class might reduce atherosclerosis and reduce the risk of CHD. Although several individual reports have noted decreases in CHD in patients taking long-term adjuvant tamoxifen [75, 76] and a recent study found that taking tamoxifen for the recommended 5 years reduces the risk of cardiovascular disease and death as a result of a cardiovascular event [77], particularly among those age 50 to 59 years, the Overview Analyses of all data does not support cardioprotection [78].

Overall, with antiestrogenic effects in the breast, estrogen-like effects in the bone, and an action that lowered circulating cholesterol, the stage was set to create a new drug group the SERMs with an evidenced based roadmap for future drug development [2].

Although tamoxifen is the pioneering SERM, raloxifene is the medicine that first exploited the "roadmap" successfully starting in 1992 [79]. Scientists at Eli Lilly [80] confirmed the concept in animal models measuring bone density, uterine weights and circulating cholesterol (tamoxifen had been patented as a hypocholesterolemia drug in the early 1960's and related compounds also affected cholesterol metabolism and biosynthesis so the Lilly scientists confirmed the class effect of the drug group) and initiated the Multiple Outcomes of Raloxifene Evaluation or MORE trial. Raloxifene would be the first SERM to be approved for two of the three properties of the "ideal SERM": reduction in the incidence of fractures from osteoporosis and the reduction in the incidence of breast cancer [29-31]. Although raloxifene lowers circulating cholesterol in postmenopausal women, raloxifene does not reduce the risk of CHD in women at high risk [81].

SUMMARY AND CONCLUSION

The tantalizing clues that the nonsteroidal antiestrogens tamoxifen and raloxifene can lower total circulating cholesterol in ovariectimized rats and LDL cholesterol in postmenopausal women did not, for these compounds translate to decreasing CHD. This goal would, however, be achieved with a new agent lasofoxifene (see section on new SERMs under investigation).

MOLECULAR MECHANISMS OF SERM ACTION

There are two ERs referred to as α and β [82-84]. Each receptor protein is encoded on different chromosomes, and have homology as members of the steroid receptor superfamily. There are distinct patterns of distribution and distinct and subtle differences in structure and ligand binding affinity [85]. The ratio of ERα and ERβ at a target site may be an additional dimension for tissue modulation. A high ERα: ERβ ratio correlates well with high levels of cellular proliferation whereas the predominance of functional ERβ over ERα correlates with repression of proliferation [86-89]. Indeed, the ratio of ERs in normal and neoplasic breast tissue could be important for the long-term success of chemoprevention with SERMs.

The functional differences between ERα and ERβ can be traced to the differences in the Activating Function 1 (AF-1) domain located in the amino terminus of the ER. The amino acid homology of AF-1 is poorly conserved between ERα and ERβ (only 20%). In contrast, the AF-2 region located at the C terminus of the ligand binding domain, differs only by one amino acid: D545 in ERα and N496 in ERβ. Together the AF-1 and AF-2 are important for the interaction with other co-regulatory proteins that control gene transcription. Studies using chimeras of ER α and β by switching the AF-1 regions demonstrates the cell and promoter specific differences in transcriptional activity [90, 91]. In general, SERMs can partially activate engineered genes regulated by an estrogen response element through ERα but not ERβ [92]. In contrast, 4-hydroxytamoxifen and raloxifene can stimulate activating protein-1 (AP-1) regulated reporter genes with both ERα and ERβ in a cell dependent fashion [93].

The simple model for estrogen action, with either ERα or ERβ initiating estrogen action in the nucleus, has now evolved to a new dimension of protein partners that modulate gene transcription (Fig. **2**). Since the first steroid receptor coactivator (SRC-1) was described by O'Malley's group [94] there are now hundreds of coactivator and corepressor molecules (Fig. **2**) [95].

The finding that there are two ERs, has resulted in the synthesis of a range of receptor specific ligands to switch on or switch off a particular receptor [96]. It is, however, the external shape of the resulting complex that becomes the catalyst for changing the response to a SERM at a tissue target. Kraichely and co-workers[97] demonstrated the important observation that agonists for ERα and ERβ produce subtle quantitative differences with the interaction of members of the SRC family (SRC 1, 2 and 3) and that the coactivator can enhance ligand affinity for the ER.

It is reasonable to ask how the ligand programs the receptor complex to interact with other proteins? X-ray crystallography of estrogens or antiestrogens locked in the ligand binding domains of the ER demonstrates the mechanics where ligands promote coactivator binding or prevent coactivator binding based on the shape of the estrogen or antiestrogen receptor complex [98, 99]. Evidence has now accumulated to document that the broad spectrum of ligands that bind to the ER can create a broad range of ER complexes that are either fully estrogenic or antiestrogenic at a particular target site [100]. Thus a mechanistic model of

estrogen action and antiestrogen action (Fig. **2**) has emerged based on the shape of the ligand that programs the complex for future action. But how is the response initiated?

Not surprisingly, the coactivator model of steroid hormone action has now become enhanced into multiple layers of complexity thereby amplifying the molecular mechanisms of modulation. It appears that coactivators are not simply protein partners that connect one site to another in a complex [101]. The coactivators actively participate in modifying the activity of the complex. Post translational modification of coactivators *via* multiple kinase pathways initiated by cell surface growth factor receptors (e.g. epidermal growth factor receptor, insulin-like growth factor receptor 1 and ERBB2, also known as HER2) can result in a dynamic model of steroid hormone action. The core coactivator e.g. SRC3 (Fig. **2**) first recruits a specific set of co-coactivators e.g. p300 and ubiquitin-conjugating ligases under the direction of numerous protein remodelers (e.g. the peptidyl-prolyl isomerase Pin1, heat shock proteins and proteasome ATPases) to form a multi-protein coactivator complex that interacts with the phosphorylated ER at the specific gene promoter site [101]. Most importantly, the proteins assembled by the core coactivator as the core coactivated complex have individual enzymatic activities to acetylate or methylate adjacent proteins. Multiple cycles of the reaction can polyubiquitinate a substrate i.e. ER or a CoA, or, depending on the ubiquitin-ubiquitin linkage proteins can either to be activated further (K63 linkage) or degraded by the 26S proteasome (K48 linkage) [102].

Thus for effective gene transcription, programmed and targeted by the shape and phosphorylation status of the ER and coactivators, a dynamic and cyclic process of remodeling capacity is required for transcriptional assembly [103] that is immediately followed by the routine destruction of transcription complexes by the proteasome. Estrogen and SERM-ER complexes have distinct accumulation patterns in the target cell nucleus [104, 105] because they are destroyed at different rates [106].

These fundamental mechanisms [101, 107] in physiology also apply to the development of acquired drug resistance to SERMs in breast cancer. Model systems have demonstrated the conversion of the tamoxifen ER complex from an anti-estrogenic signal to an estrogenic signal in an environment enhanced for phosphorylation by overexpression of the ERBB2cell surface receptor and an increase in SRC3 (AIB1) [108, 109]. The enhanced level of coactivators and its enhanced phosphorylation state derived from an activated ERBB2 phosphorylation pattern will enhance the estrogen-like activity of tamoxifen at the ER. Clearly, issues of SERM action at target tissues and the eventual development of acquired drug resistance in breast cancer will be amplified for tumor cell survival as the duration of SERM use extends from a few years to perhaps decades [52].

THE CURRENT AND NEXT GENERATION OF SERMS

Tamoxifen and Raloxifene

There are currently 2 main chemical classes of SERMs approved for clinical use: the first-generation triphenylethylene derivatives, tamoxifen [110] and toremifene [111, 112], which are used in the treatment and in the case of tamoxifen in the prevention of breast cancer [65, 113]; and raloxifene, a second-generation benzothiopene derivative indicated for the treatment and prevention of osteoporosis [29] and the reduction of breast cancer incidence in high risk post-menopausal women [31]. All 3 compounds also have beneficial effects on serum lipids, but are still associated with adverse effects such as hot flushes and an increase in the risk of venous thromboembolism (VTE). Raloxifene is the only SERM compound approved worldwide for the prevention and treatment of postmenopausal osteoporosis and fragility fractures. The pivotal registration MORE (Multiple Outcomes of Raloxifene Evaluation) trial was a multicentered, randomized, blinded, placebo-controlled trial that included 7705 women aged 31-80 years from 25 countries. Results of the trial showed significantly reduced vertebral fractures in the raloxifene group (RR 0.60; 95% CI 0.50 to 0.70; $p < 0.01$) [29]. Raloxifene did not significantly reduce nonvertebral fractures with either 60 or 120 mg/day [29]. BMD increased by 0.4 to 1.20% at the lumbar spine; these effects have been documented further for at least 7 years in the CORE (Continuing Outcomes Relevant to Evista) trial [114]. All participants received 500 mg of calcium and 400-600 IU of vitamin D each day, in addition to study treatments. It is also important to stress that continuous treatment with raloxifene effectively controls the development of breast cancer [115].

Raloxifene lacks estrogenic activity in the uterus and has not demonstrated tamoxifen-like effects in the uterus either histopathologically or ultrasonographically [116], but it has been associated with adverse effects such as VTE and vasomotor symptoms, including hot flushes. In addition, both preclinical and clinical reports suggest that these ER agonists are considerably less potent than estrogen for the treatment of osteoporosis. The goal, therefore, became to create a “Designer Estrogen” [117] and enhance the value of the new multifunctional medicines. Newer generation SERMs being investigated for the prevention and treatment of osteoporosis in postmenopausal women include ospemifene (Ophena; QuatRx Pharmaceuticals), lasofoxifene (Fablyn; Pfizer), bazedoxifene (Viviant; Wyeth Pharmaceuticals), and Arzoxifene (LY353381, Lilly) which are in Phase III clinical trials or have undergone regulatory review (Fig. **3**, Table **1**). Other SERMS have had clinical trials suspended prematurely: levormeloxifene, for causing urinary incontinence and uterine prolapse, and idoxifene, for producing increased endometrial thickness on ultrasonography but without significant histologic abnormalities [116].

The four SERMs we will consider in detail have all achieved significant clinical evaluation. Some have moved forward to be approved in some countries, others have not been advanced. It is, however, important from a drug development perspective to state the idea for each structure was an improvement on the original discovery of the core structure, in some cases, 50 years ago. The links with the original pharmacologic discoveries is illustrated in Fig. (**4**), but the goal is to find the ideal SERM (Fig. **5**). Ospemifene is the direct result of the discovery of a weak anti-estrogenic metabolite of tamoxifen Metabolite Y, formed by

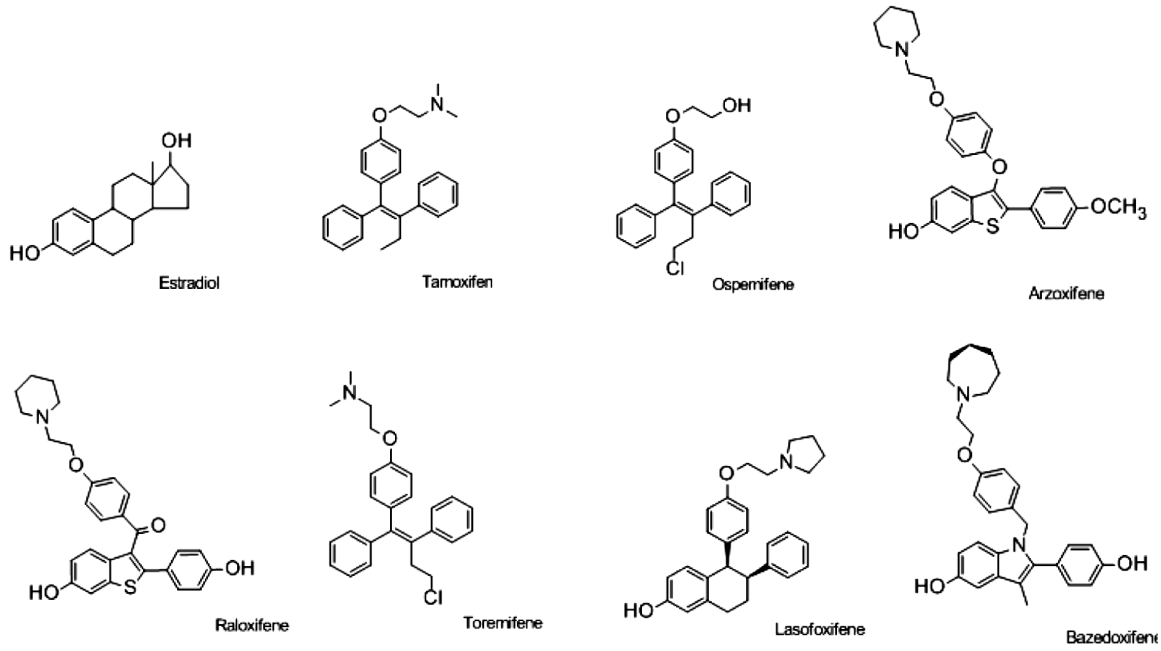

Fig. (3). Chemical structure of estradiol and selective estrogen receptor modulators (SERMs); raloxifene, tamoxifen, toremifene, ospemifene, lasofoxifene, arzoxifene and bazedoxifene.

Table 1. Current Status of New SERMs

Drug Name	Category (Structure)	Effects	Preclinical Results	Clinical Status
Ospemifene*	Tamoxifen-like	Vaginal atrophy treatment Osteoporosis treatment Breast cancer prevention	Estrogenic effects on vaginal epithelium that is not observed with tamoxifen or raloxifene [130, 131, 134] Inhibits tumor growth in animal models as effective as tamoxifen [137, 138]	Phase III trial (826 women) relieves vaginal dryness Phase II trial (118 women): Comparable to or slightly better than raloxifene [135] Phase III trial planned (detail not available) Not available
Arzoxifene* (LY353381)	Raloxifene-like	Breast cancer treatment Breast cancer prevention	Antiestrogenic in breast and endometrium, estrogenic in bone and lipids [172] Effective to prevent ER-positive and ER-negative mammary tumors especially in combination with LG100268 [138, 216]	Phase III trial (200 patients) inferior to tamoxifen [217] Phase I trials (50 and 76 women) low toxicity and favorable biomarker profile [218]
Lasofoxifene* (CP-336156, Fablyn)	Raloxifene-like	Osteoporosis treatment and prevention Vaginal atrophy treatment Breast cancer treatment and prevention Heart disease prevention	Higher potency than tamoxifen and raloxifene [139]; higher oral bioavailability than raloxifene [54] Effects similar to tamoxifen to prevent and treat NMU-induced mammary tumor in rats [219]	Phase III trial (1,907 women) significantly increases bone mineral density compared to placebo, no endometrial effects, no association with thromboembolic disorder [142] Phase III trial to compare with raloxifene (CORAL trial, details not available) Phase III trail (445 patients) improves vaginal atrophy compared to placebo Phase III trial (PEARL trial with 8,556 women), reduces ER-positive breast cancer incidence compared to placebo; slightly decreases major coronary disease risk; reduces vertebral and non-vertebral fractures; increases risks of venous thromboembolic events but not stroke; no endometrial effects [SABCS 2008, abstract 11]

Table 1. contd….

Drug Name	Category (Structure)	Effects	Preclinical Results	Clinical Status
Bazedoxifene* (TSE-424 WAY-140424)	Raloxifene-like	Osteoporosis treatment and prevention Breast cancer prevention	Increases bone density with little uterine or vasomotor effects Inhibits estrogen-stimulated breast cancer cells growth [154]	Phase III trial (7,492 women) reduces vertebral and non-vertebral fracture incidences, while raloxifene is not effective against non-vertebral fracture [160] Phase III trial (497 women) reduces endometrial thickness, unique property among known SERMs [220] Not available

*Ospemifene- not approved by the FDA, *Arzoxifene- not approved by the FDA, trials terminated by Eli Lilly, *Lasofoxifene- not approved by the FDA, approved in the EU, *Bazedoxifene- not approved by the FDA, approved in the EU.

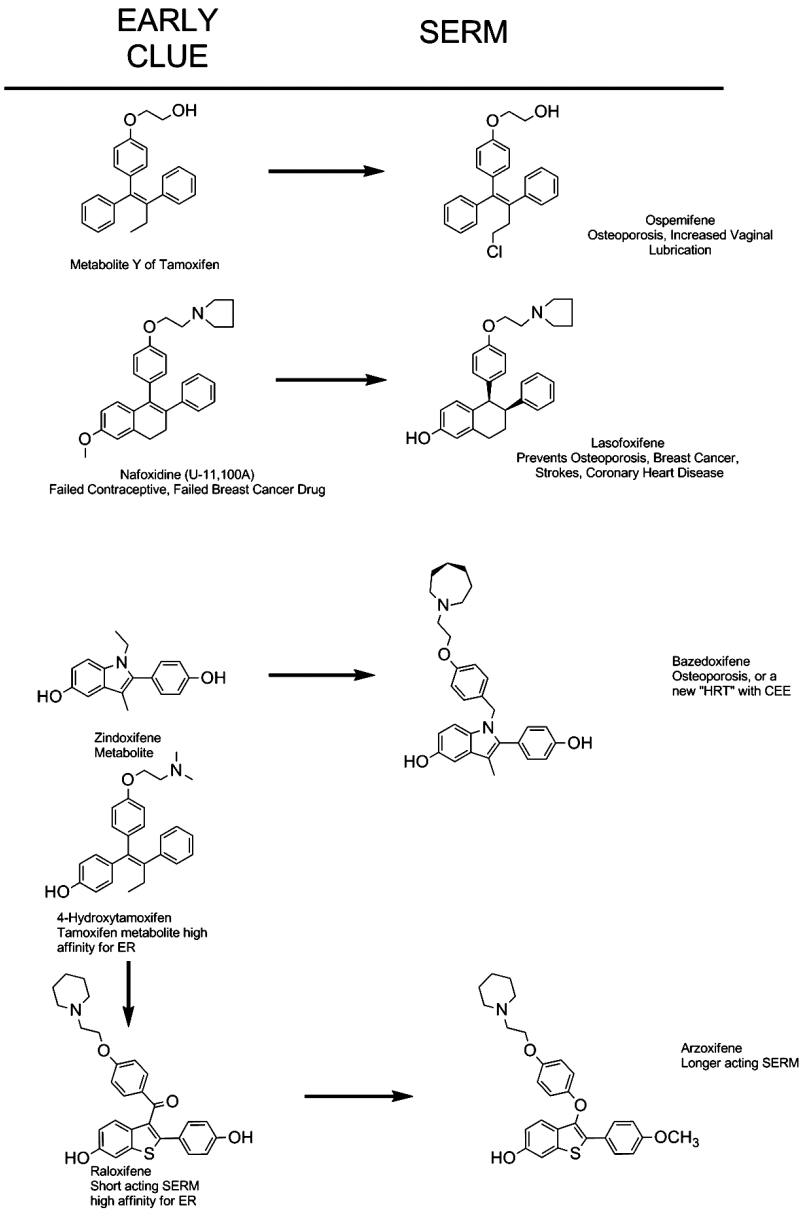

Fig. (4). Origins of current selective ER modulators for earlier nonsteroidal antiestrogens. Ospemifene is a known metabolite of the breast metabolite of the breast cancer drug toremifene. The metabolite of toremifene was found because an analogous metabolite Y was discovered for tamoxifen in the early 1980's [119]. Lasofoxifene has its origins with failed antifertility agent discovered in the early 1960's U-11, 100A [121]. The compound renamed nafoxidine was tested as a drug for the treatment of breast cancer but again failed because of serious side effects [123]. Bazedoxifene is an adaptation of an estrogenic metabolite from a failed breast cancer drug Zindoxifene [124]. Arzoxifene is the final compound in the lineage to find the optimal long acting SERM from the discovery that the hydroxylated metabolite of tamoxifen 4-hydroxytmaoxifen has a very high binding affinity for ER [35]. Raloxifene was a direct result of this discovery which became a successful SERM in clinical practice.

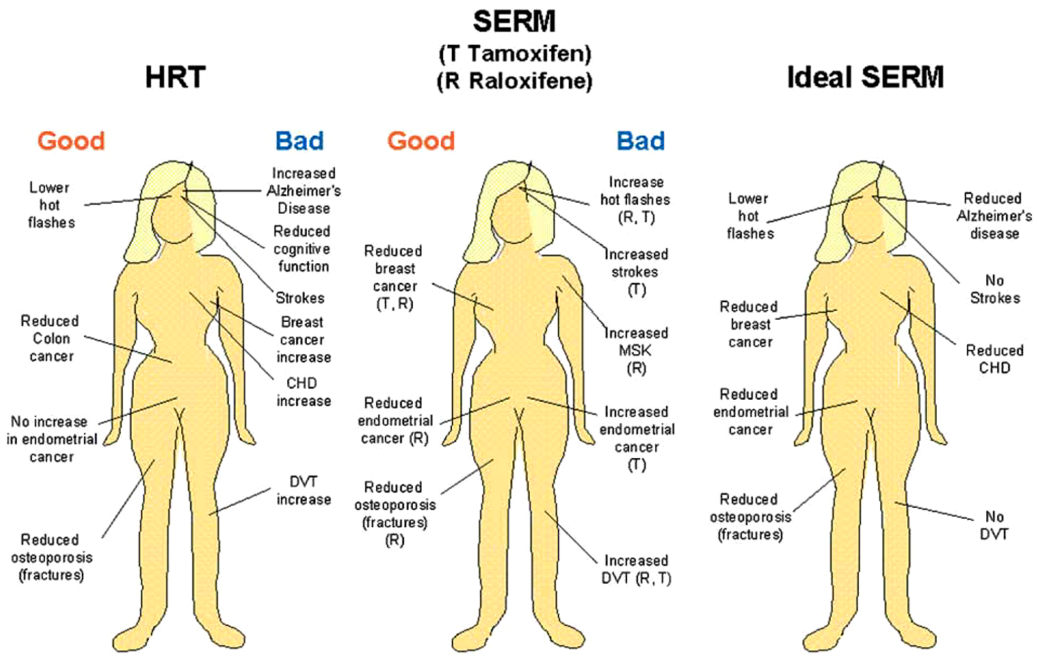

Fig. (5). Progress toward an ideal SERM. The overall good or bad aspects of administering hormone replacement therapy to postmenopausal women compared with the observed site-specific actions of the selective estrogen receptor modulators tamoxifen and raloxifene. The known beneficial or negative actions of SERMs have opened the door for drug discovery to create the ideal SERM or targeted SERMs to either improve quality of life or prevent diseases associated with aging in women. This figure is published with permission from Elsevier. Jordan, V.C. Selective estrogen receptor modulation: Concept and consequences in cancer. Cancer Cell, 2004 Mar; 5(3): 207-213.

demethylation, and deamination to a glycol side chain [118, 119]. The analogous metabolite was found for toremifene and became ospemifene. Unlike tamoxifen toremifine is not a rat hepatocarcinogen [120] so ospemifene would be a safer SERM. Lasofoxifene is derived from nafoxidine (U11, 100A) which was discovered as an antifertility compound in rodents [121, 122], that evolved to be an experimental breast cancer drug but was too toxic [123]. Basedoxifene is related to a metabolite of a failed breast cancer drug zindoxifene [124] and arzoxifene is the end product in the line of 4-hydroxytamoxifen [35], the antiestrogen is a metabolite of tamoxifen with high affinity for the ER but poor antitumor activity [37], to raloxifene (also with a poor antitumor activity [125]) and then to arzoxifene in an attempt to improve pharmacokinetics and develop a better breast cancer drug. We will consider the clinical evaluation of each.

Ospemifene

Ospemifene, is an antiestrogenic triphenylethylene derivative structurally similar to tamoxifen and toremifene. The story of the structure is of interest. In 1982/83 a new metabolite of tamoxifen was reported and shown to be a weak antiestrogen [38, 118]. Subsequently, the related metabolite of toremifene was found and reported. This metabolite is now known as ospemifene. Ospemifene was initially designed to treat vaginal atrophy in postmenopausal women; however, it may also be useful for the prevention and treatment of osteoporosis. Ospemifene binds to both ERs, though binds to the ERα more strongly. Similar to 17β-estradiol and tamoxifen, its estrogen-like effects are noted to occur in bone *via* enhanced osteoblastic proliferation and differentiation, but not osteoclast apoptosis. Raloxifene, in contrast, is noted to induce osteoclast apoptosis. Increased mineralization and bone nodule formation have been demonstrated in bone marrow cultures [126]. In an ovariectomised rat model, ospemifene's role in improved bone strength and density has been compared to estradiol and other SERMs, and at a dose of 10mg/kg, ospemifene has been found to prevent bone loss and increase bone strength on the femoral neck and lumbar vertebrae similar to the bone agonist effects observed in estradiol (at 50 μg/kg), raloxifene (3 mg/kg) and droloxifene (10 mg/kg) [127].

In the immature rat uterus, ospemifene has been shown to be of the order of 200- to 1000-fold less estrogenic than estradiol [127]. Notably, even at doses sufficient to prevent bone loss, ospemifene was found to induce weak antagonistic activity in the uterus and may even preserve normal endometrium. At doses 5-10 times higher than that required to prevent bone loss, however, ospemifene does appear to have estrogenic effects at the uterus similar to that seen with 1mg/kg of tamoxifen [127].

Tamoxifen appears to induce liver carcinogenesis *via* the creation of DNA adduct, but this does not occur with ospemifene in rats. This fact has led to the belief that ospemifene's carcinogenic potential is lower than that noted in tamoxifen [127, 128].

Data pooled from at least seven clinical trials have shown ospemifene has a favorable toxicity profile and is generally well tolerated [129-135]. Headache was the most commonly reported adverse event, with rates similar to that of placebo (15% and 12.8%, respectively) [129]. Likewise, endometrial

effects produced by ospemifene are comparable to that seen with raloxifene, and are less than that observed with tamoxifen [130, 131, 134]. In the vagina, however, ospemifene does have more estrogenic effects, thereby improving vaginal dryness more effectively than either raloxifene or tamoxifen [130, 134]. Similarly, ospemifene has been shown to have a positive, or at least neutral effect on hot flashes. Moreover, even at doses far exceeding that used in phase II and III clinical trials, phase 1 data has shown no significant toxicity.

Despite promising data in the ovariectomized mouse model, long-term data on the bone-protective effect in humans with ospemifene are lacking. A short-term, 3-month, phase II comparative study found ospemifene at doses 30, 60, or 90 mg/day compared with raloxifene, had similar to slightly better effects on bone as measured by markers of bone resorption, and comparable efficacy in lowering LDL-cholesterol [135]. The effects on bone varied across the groups, potentially due to the non-osteoporotic nature of the study population and to the short period of both treatment and follow-up [135]. A second phase II trial demonstrated that varying doses of ospemifene administration for three months did, in a dose-dependent manner, reduce markers for bone turnover compared with placebo [133]. Notably, however, the long-term prevention of bone loss and the prevention of osteoporotic fractures in women treated with ospemifene are not under study.

Data *in vitro* and *in vivo* suggest that ospemifene may have breast chemopreventive activity in breast tissue in much the same way as toremifene or raloxifene [127, 128, 136-138], but randomized clinical trials have not addressed this issue.

Lasofoxifene

Collaborative effort of Pfizer and Ligand Pharmaceuticals to synthesize novel SERMs with good oral bioavalability and higher potency for treatment of vaginal atrophy and osteoporosis resulted in the discovery of lasofoxifene. Lasofoxifene is a naphthalene derivative, a third generation SERM with high selective affinity for both the ERα and ERβ subtypes. IC50 of lasofoxifene is similar to that of estradiol, and 10 times higher than that of raloxifene and 4-hydroxytamoxifen. Lasofoxifene is able to inhibit osteoclastogenesis, reduced bone turnover, and prevented bone loss in preclinical studies [139, 140]. Lasofoxifene causes significant improvement in markers of bone turnover and bone mineral density in preclinical studies, as well as phase II and III trials [141-144]. One particular phase II study, which enrolled 394 healthy postmenopausal women, lasofoxifene 0.017, 0.05, 0.15, and 0.5 mg/day was compared with supplementation with calcium and vitamin D [145]. After six months of therapy, women receiving the two highest doses of lasofoxifene were noted to have statistically significant improvement in maintenance or gain of bone mineral density compared with the calcium plus vitamin D arm ($p<0.01$), and at one year of treatment all groups of lasofoxifene had significant improvement over the calcium plus vitamin D cohort. Across groups, 85-98% of women treated with lasofoxifene either had no loss of, or had improvement in BMD after one year.

Three separate phase III studies have also been completed. The first, OPAL (Older People And n-3 Long-chain polyunsaturated fatty acids), was actually a collection of multiple trials [146, 147]. In this study, 1907 nonosteoporotic postmenopausal women with lumbar spine T-scores from 0 to -2.5, all of whom received calcium and vitamin D supplementation, were randomized to receive lasofoxifene 0.025, 0.25, or 0.5 mg/day or placebo for 2 years. At six, twelve, and twenty-four months, lasofoxifene at all doses were shown to increase bone mineral density compared with a decrease observed in the placebo group, and at six and twenty-four months decrease bone turnover was observed compared with placebo. The groups treated with lasofoxifene also underwent bone biopsies which showed normal quality bones.

CORAL, a 2-year randomized, double-blind, placebo-controlled, and active treatment-controlled study, enrolled 410 women with lumbar spine BMD between +2 and -2.5 standard deviations of age-matched controls (Z-score) and compared indices of bone health in groups treated with lasofoxifene at either 0.25 or 1 mg/day, raloxifene 60 mg/day, or placebo [148]. All groups received calcium and vitamin D supplementation. Evaluated endpoints included percent change from baseline BMD in the lumbar-spine at 2 years (primary endpoint), as well as total hip BMD, LDL-cholesterol, safety, and biochemical markers of bone turnover including N-telopeptide, deoxypyridinoline crosslinks, bone-specific alkaline phosphatase, and osteocalcin. Lasofoxifene at both doses was superior to raloxifene and placebo at increasing lumbar spine BMD, though lasofoxifene at both doses and raloxifene were similar in increasing total hip BMD compared with placebo. Both agents decreased biochemical markers of bone turnover compared with placebo, though lasofoxifene did so to a greater extent. An editorial written by Goldstein considered lasofoxifene, therefore, superior to raloxifene to increase BMD and decrease markers of bone turnover [116].

PEARL, a large, 8556 women, 5-year, randomized, double blind, placebo-controlled, parallel-assignment study that evaluated safety and efficacy of 0.25mg/day and 0.5mg/day of lasofoxifene combined with 1000 mg calcium and 400-800 IU vitamin D daily [149]. Patients were women with osteoporosis with lumbar spine or femoral neck BMD <2.5 SD or less and the study evaluated efficacy in preventing new vertebral fractures. Though initially due to be completed in March 2006, the trial was extended to early 2008 in order to include 2 additional coprimary endpoints, nonvertebral fracture and ER-positive breast cancer. Results of the study were notable as the 0.2mg/day dose was found to reduce only vertebral fractures ($p < 0.001$) but the higher dose 0.5mg/day significantly decreased both vertebral ($p < 0.001$) and nonvertebral fractures ($p = 0.002$). Importantly, the lasofoxifene 0.5 mg dose also showed decreased risk of ER positive breast cancer [150], coronary heart disease, and stroke, though an increased risk for VTE, and long term data confirms the safety and efficacy of the agent [55].

Lasofoxifene has shown decrease in bone turnover markers, coronary heart disease, serum lipids, and stroke incidence [55]. Lasofoxifene, unlike many other SERMs, has been shown to reduce vaginal pH and decrease vaginal

dryness [151], but over 5 years it has been shown to be associated with endometrial hypertrophy, a finding which warrants close monitoring [55]. Long-term efficacy data comparing lasofoxifene with raloxifene and hormone-replacement therapy to elucidate whether lasofoxifene is superior for the prevention and treatment of postmenopausal osteoporosis and osteoporosis-related fractures is still lacking. Further studies should also be completed to elucidate whether it ought to play a role in menopause symptom control.

Bazedoxifene

Bazedoxifene (BZA, TSE-424), an indole-based ER ligand which has been carefully selected for its better side effect profile compared with its predecessors, is being developed for use both alone for the prevention and treatment of osteoporosis in postmenopausal women, and in combination with conjugated equine estrogens for menopausal symptoms [152-154]. Already approved by the European Union in April, 2009, it is in the late phases of review by the US FDA. It binds to both ERα and ERβ, though with slightly higher affinity for ERα, is less selective for ERα than raloxifene, and in fact has a nearly 10-fold lower affinity for ERα than 17β-estradiol [152, 154]. It is tissue-specific, and in both *in vitro* and *in vivo* preclinical models, has been shown to positively affect lipid profiles and skeletal-related markers *via* antiresorptive affects, and displays estrogen receptor interaction without stimulating the endometrium, causing breast cancer cell proliferation, or negatively affecting the central nervous system.

Even at low doses, bazedoxifene maintains bone mass, and reaches maximal significant efficacy at a dose of 0.3mg/kg/day, and this dose has been shown to maintain vertebral compressive strength better than or equivalent to sham-operated animals [152, 154]. Efficacy on maintaining skeletal parameters have been shown to be similar among bazedoxifene, raloxifene, and lasofoxifene [80, 139], and recently, bazedoxifene has been shown in ovariectomized monkeys to partially preserve bone densimetry- measured bone mass, as well as preserve bone strength and reduce bone turnover at a dose up to 25mg/kg/day for 18 months [155]. Further, in preclinical *in vivo* studies, an improved uterine profile for bazedoxifene compared with raloxifene was noted, as well as lack of adverse effect on plasma lipids or reproductive tract histology [152]. Bazedoxifene is well tolerated, and both increases endothelial nitric oxide synthase activity and does not antagonize the effect of 17β-estradiol on vasomotor symptoms, both of which are improvements over raloxifene [152-154].

When bazedoxifene was coadministered with CEEs such as Premarin® or human parathyroid hormone (hPTH), preclinical studies utilizing ovariectomized mice noted that at doses 7- to 10-fold higher than the bone efficacious dose, bazedoxifene antagonized the uterine stimulation by Premarin® but did not change the uterine weight compared with ovariectomized controls [156]. Further, BMD and cancellous bone compartments were similar between animals treated with bazedoxifene 3 mg/kg/day and Premarin® 2.5 mg/kg/day versus sham-operated animals. When combined with bone efficacious doses of CEEs, bazedoxifene, compared with raloxifene and lasofoxifene, showed no difference in skeletal parameters [157]. Further, lasofoxifene 0.1 mg/kg/day has been shown in another study to enhance reversal of osteopenia when coadministered with hPTH 10 μg/kg/day similarly to bazedoxifene, raloxifene, or risedronic acid and greater than hPTH monotherapy [158].

Taken together, bazedoxifene may then emerge as a promising new treatment for osteoporosis, either as monotherapy or combined with conjugate estrogens, with an improved side effect profile given the reduced uterine and vasomotor effects over SERMs currently available. In fact, bazedoxifene has been studied in the prevention and treatment of postmenopausal osteoporosis. Two phase III trials showed bazedoxifene at varying doses to improve skeletal parameters [159-161]. The first found that in postmenopausal women at risk for osteoporosis, the drug (at 10, 20, and 40mg) prevented bone loss and reduced bone turnover, with a favorable endometrial, breast, and ovarian safety profile [159, 160]. The second study recruited postmenopausal women who already had osteoporosis, showed bazedoxifene at 20 and 40 mg significantly reduced the risk of new vertebral fractures compared with placebo without any evidence of endometrial or breast stimulation, and in a higher risk group, bazedoxifene 20 mg significantly decreased the risk of nonvertebral fracture compared with both placebo and raloxifene 60mg [160]. In studies that followed women for five years, no breast or endometrial stimulation was seen at either 3 or 5 years and generally the medication was well tolerated, with rates of adverse events and discontinuations due to adverse events similar to placebo [162]. However, hot flushes and leg cramps, most of which were mild and did not lead to cessation of the medication, were noted more frequently at 5 years in patients treated with bazedoxifene compared with placebo [160].

The major adverse effect of bazedoxifene is venous thromboembolism, the majority of which occur in the first two years [163]. The increased risk of VTE with bazedoxifene over five years is similar to that seen with longterm evaluation with raloxifene [164]. Raloxifene [81, 164] has a much higher risk of VTE in the first two years than bazedoxifene. Additionally, there is a slightly increased risk for fatal stroke when raloxifene is compared with placebo over 5.6 years of followup, though the overall stroke risk is not statistically different from placebo [81]. Similarly, the risk of PE or RVT, as well as cardiac events is similar among the bazedoxifene and placebo groups.

Multiple studies have demonstrated favorable breast and endometrial safety profiles over 5 years [163]. In fact, not only is the incidence of breast and endometrial-related adverse effects similar between placebo and bazedoxifene, but there were fewer cases of endometrial carcinoma in the bazedoxifene group compared with placebo. Incidence of breast cancer and fibrocystic breast disease was not different between bazedoxifene [31] and placebo groups [162, 163], though the risk of breast cancer is decreased with tamoxifen and raloxifene [31].

Therefore, bazedoxifene has shown favorable effects on bone parameters in postmenopausal women, and has been shown to be relatively safe and well tolerated. It exhibits no

breast or endometrial stimulation and the small increase in VTE is better in the first two years, and similar in the longer-term to other SERMs.

Arzoxifene

Arzoxifene is a benzothiophene analogue in which the carbonyl hinge of raloxifene has been replaced by an ether (Fig. **3**). Additionally, there is a protective methyl ether on one of the phenolic hydroxyls. These features lead to increased antiestrogen properties, greater bioavailability, and increased binding affinity for the ERα compared with raloxifene [165-177]. Preclinical data has shown favorable estrogenic effects on bone and lipid metabolism, while exerting antiestrogen effects on breast and uterine tissue [174]. In fact, preclinical studies which compared equivalent doses of arzoxifene, tamoxifen, and raloxifene showed arzoxifene inhibits tumor growth to a greater extent than the other two agents [170, 172, 177, 178].

Phase I data has shown that in patients with metastatic breast cancer, arzoxifene at varying dosages (10, 20, 50 or 100 mg/day) was tolerated well, had no dose limiting toxicities, and was even found to decrease osteocalcin, which suggested a bone health benefit [179]. The drug was even tolerated well in women with liver disease, and the most common side effect was hot flashes, reported in 56% of women regardless of the dose taken. In a study of patients with advanced hormone receptor positive endometrial cancer, 34% of women treated with arzoxifene 20mg daily showed favorable response with minimal toxicity [180]. Further, data from healthy volunteers showed doses as low as 10 mg/day is biologically active, and doses from 25 to 100 mg daily showed similar effects on bone markers, lipoprotein levels, and gonadotropin levels [172].

In ovariectomized rats, long-term treatment with arzoxifene showed a protective effect on cancellous bone mass, architecture, and strength and did not stimulate endometrium proliferation [181]; in young rats, it entirely inhibited uterine growth [168]. At bone protective doses of 0.1 and 0.5 mg/kg/day, arzoxifene also exerts a positive effect on serum lipids [181]. Further, in ovariectomized mice, arzoxifene plus PTH increased bone mass at trabecular bone sites both more quickly and to a greter extent than PTH alone, PTH plus equine estrogens, or PTH plus raloxifene [182].

Recent data has shown that in postmenopausal women with osteoporosis and invasive breast cancer, treatment with arzoxifene for 4 years significantly reduced the risk of vertebral fractures. Neither raloxifene, bazedoxifene, nor arzoxifene reduced the risk of nonvertebral fractures in the same study [160]. Lasofoxifene 0.5 mg/day did reduce the risk of nonvertebral fractures, but it reduced markers of bone turnover to a similar amount as arzoxifene in the same study [55].

A different phase II study found that during 6 months of arzoxifene, lumbar spine bone mineral density showed dose response relationships [183], though this was not seen with raloxifene. Further, a phase III study of postmenopausal women with osteoporosis found improved bone turnover markers and increased spine and hip bone density in patients treated with arzoxifene 20 mg/day [184]. Two larger studies, FOUNDATION [185] and GENERATIONS [184] found that in women with at-risk or low bone density, arzoxifene 20mg/daily significantly increased BMD and reduced bone turnover markers compared with placebo. Data taken from the GENERATIONS study note that arzoxifene, however, has no improved clinical efficacy in preventing fractures over raloxifene as arzoxifene has some vertebral, but not nonvertebral fracture risk-reduction. All antiresorptive agents seem to exert non-vertebal fracture risk reduction, but only alendronate, risedronate, zoledronic acid, lasofoxifene, and denosumab have demonstrated some nonvertebral risk-reduction in postmenopausal women with osteoporosis [55, 186-189]. It is hypothesized that arzoxifene, despite improved BMD and markers of bone turnover over raloxifene, may not have enough antiresorptive potency to significantly improve non-vertebral fractures in patients enrolled in the GENERATIONS trial.

Along a different vein, with the exception of bazedoxifene, SERMs as a class have been shown to reduce the risk of invasive breast cancer, as arzoxifene, tamoxifen, raloxifene, and 0.5 mg/day of lasofoxifene have all been shown to reduce invasive breast cancer risk [30, 55, 81, 113, 150, 190].

Arzoxifene, like raloxifene, does not seem to have adverse effects on cardiovascular health in postmenopausal women [183, 184]. Additionally, lasofoxifene has even been shown to decrease the incidence of coronary events and stroke compared with placebo [55]. However, tibolone and tamoxifen increase the risk of stroke, and CEE with medroxyprogesterone increases the risk of Coronary Artery Disease (CAD) and stroke [113, 191, 192]. Perhaps the reason for this difference in effect is related to differences on the agents' effect on inflammation as the agents influence C-reactive protein (CRP) differently. Estrogen and tibolone increase levels of CRP [192], raloxifene and arzoxifene have no effect on CRP levels, and lasofoxifene decreases CRP levels [55]. All decrease LDL levels. Major side effects of arzoxifene include VTE (a side effect common among all agents with any estrogen receptor agonist effects), hot flushes, muscle cramps, vaginal discharge, vulvovaginitis, and increased reports of endometrial cancer and hyperplasia, though the last two failed to reach statistical significance [185]. Also, several SERMs, including arzoxifene, increase the risk of cholecystitis as estrogen has known lithogenic effects on bile [193]. Further, increased pulmonary complications including coughing, pneumonia, increased reports of upper respiratory infections, and serious COPD related events have been reported with treatment with arzoxifene [190]. Although previous trials of SERMs, estrogen, and tibolone have not reported increased pulmonary complications, bronchial epithelium and alveolar macrophages do express ER [194, 195]. Therefore, inhibition of ER increases expression of inflammatory lung markers, including tumor necrosis factor α (TNF-α) [194, 195]. In fact, there was a small increased risk of lung metastases, but not primary lung tumors, with treatment with arzoxifene, though given the lack of biologic basis for pulmonary susceptibility to metastases, this finding may be due to chance alone [190].

Arzoxifene in similar to other SERMs in that it reduces the risk of invasive breast cancer, reduces bone resorption, increase BMD modestly, and decrease the risk of vertebral, but not nonvertebral fractures [190]. Yet it increases the risk of venous thromboembolic events and adverse gyenocologic events. Results from a five year clinical study were released by Lilly in 2009 that arzoxifene met its primary endpoints of reduction in vertebral fractures and breast cancer in postmenopausal women [185]. However, due to lack of successfully meeting the study's planned secondary endpoints including reduction in non-vertebral fractures and cardiovascular events and improvements in cognitive function, Lilly announced they were discontinuing development of the drug and would not seek regulatory approval.

Tissue Selective Estrogen Complex (TSEC)

Currently, research is advancing to establish the optimal balance between ER agonist and antagonist activity for an ideal menopausal therapy. An approach, termed the tissue-selective estrogen complex, blends tissue-selective activities of a SERM with an estrogen. For example, bazedoxifene in combination with conjugated equine estrogens (CEE) has been studied for the treatment of both hot flushes and vulvar vaginal atrophy, with positive results on both menopausal symptoms [196, 197].

One study involving 3397 women either 1-5 years post menopause or >5 years post menopause enrolled in the Osteoporosis Prevention I and II Substudies aimed to evaluate the efficacy of the tissue-selective estrogen complex bazedoxifene/CEE to prevent osteoporosis [198]. The study used bazedoxifene (10, 20, or 40 mg) with CEEs (0.625 or 0.45 mg), raloxifene (60 mg), or placebo, and was administered daily for 2 years. The primary outcome was change in bone mineral density at the lumbar spine, though hip bone mineral density was also measured.

For women 1-5 years postmenopause, all bazedoxifene/CEE treatment groups showed greater percent increase in lumbar spine BMD from baseline to 2 years compared with raloxifene ($p < 0.05$). BMD significantly improved relative to raloxifene ($p < 0.05$) with both lower doses of bazedoxifene/CEE doses for women >5 years. In substudy I, mean percent increases in total hip BMD were significantly higher from baseline to month 24 with bazedoxifene (10 mg)/CEEs (0.625 or 0.45 mg) and bazedoxifene (20 mg)/CEEs (0.625 mg) compared with raloxifene. Further, total hip BMD was significantly higher with all doses of bazedoxifene/CEE doses from baseline at months 12 and 24 compared with decreases observed with placebo [198].

In substudy II, total hip BMD was higher in all bazedoxifene/CEE doses compared with placebo at both months 12 and 24, and for femoral neck BMD, the same superiority of bazedoxifene/CEE doses over placebo was true except for bazedoxifene (40 mg)/CEEs (0.45 mg) at month 12 [198]. Additionally, at both time points, median percent changes from baseline in serum osteocalcin and C-telopeptide were significantly greater with all bazedoxifene/CEE doses than with placebo ($p <0.001$). Total hip BMD was significantly better ($p < 0.05$) for bazedoxifene (10 mg)/CEEs (0.625 or 0.45 mg) over raloxifene, and bazedoxifene (20 mg)/CEEs (0.45 mg) at month 24 over raloxifene. In terms of side effects, rates of serious side effects including myocardial infarction, venous thromboembolism, superficial thrombosis or phlebitis, coronary artery disease, and breast pain were all similar between azedoxifene/CEEs groups and placebo [198]. This study highlighted the potential for a SERM/CEE combination that may provide the benefits of hormone therapy in a symptomatic postmenopausal woman with her uterus without the need for a progestin.

CONCLUSIONS

The original SERM idea [2] has now been proven in clinical trial to have benefit for women in routine clinical practice. The past 50 years has seen the rise and fall of hormone replacement therapy (HRT) [191, 199, 200] as the answer to postmenopausal women's health (Fig. **5**). In its place, the development of first tamoxifen and then the first true SERM raloxifene advanced the concept towards the ideal SERM (Fig. **5**). The agents currently in development or the process of approval and launch each edge towards an optimal multifunctional medicine for postmenopausal women's health.

Tamoxifen, the pioneering medicine that led the transition from "nonsteroidal antiestrogen" to become the first SERM in clinical practice, was the gold standard for the antihormonal therapy for two decades [14, 110] and pioneered chemoprevention [65, 113]. Nevertheless, the discovery and development of the aromatase inhibitors [201], resulted in improvements in adjuvant therapy outcomes and a reduction in side effects for postmenopausal breast cancer patients [202]. Now tamoxifen remains the standard of care for the premenopausal patients and for risk reduction in both premenopausal and postmenopausal women. Raloxifene is available for risk reduction in postmenopausal women with or without a uterus [203, 204], but unlike tamoxifen that is used for 5 years, raloxifene must be given indefinitely [32]. It should be mentioned that an aromatase inhibitor exemestane has been successfully tested to reduce breast cancer risk in postmenopausal women [205]. However, unlike the promise of a reduction of breast cancer incidence with SERMs, exemestane decreases bone density [206].

The development of novel SERMs targeted to the ER in recent years has led to significant progress in the identification of therapeutic agents for the management of postmenopausal conditions related to estrogen deficiency, particularly osteoporosis. The possibility of designing a single molecule that has all of the desired characteristics of an ideal SERM (Fig. **5**) seems to be unlikely, but progress has clearly been achieved with lasofoxifene [55] and the TSEC proposal is also innovative.

The benefits of tamoxifen use outweigh the associated risks in women who have already been diagnosed with breast cancer [110]. However, endometrial safety concerns outweigh the bone protection offered by SERMs in the development of postmenopausal osteoporosis. Because raloxifene has a good record of endometrial safety it is currently the only SERM approved for the prevention and treatment of postmenopausal osteoporosis, having demonstrated efficacy in preventing bone loss and fractures, with the added benefit of preventing breast cancer.

Clinical data on newer SERMs in development (Fig. **3**) indicate that these compounds may, or may not, have attributes that represent an improvement relative to currently available SERMs. Other SERMs have shown promise in treating the symptoms of menopause, such as vaginal atrophy, and are also undergoing investigation as possible agents for the prevention of breast cancer. A common adverse event associated with SERMs to date seems to be an increased incidence of hot flushes and warrants further study to determine a solution. There are several novel agents being evaluated to address hot flashes [207-210]. Bazedoxifene has been shown to maintain or increase BMD, reduce bone turnover, and decrease the risk of new vertebral fracture in postmenopausal women without evidence of endometrial or breast stimulation in large, prospective phase III studies [196-198]. In the global placebo- and active-controlled osteoporosis treatment study, bazedoxifene showed a significant reduction in nonvertebral fracture risk in a subgroup of more than 1,700 women at higher risk for fracture relative to both placebo and raloxifene. The TSEC containing bazedoxifene/CEEs had an acceptable endometrial profile, suggesting an alternative to the addition of a progestin to estrogens for endometrial protection [197]. The beneficial effects of bazedoxifene/CEEs on menopausal symptoms and bone loss as well as the bleeding profile and overall safety data may indicate a suitable option for symptomatic postmenopausal women. Clarification of other safety concerns (i.e., venous thromboembolic events) is needed to appropriately determine the benefit/risk balance of SERMs in development.

For the future, basic research is essential for further progress in exploiting this drug group. Basic knowledge of mechanisms must advance the original SERM concept [2, 23]. The subsets of ERα and ERβ specific agonists can be used to further define targets in other pathologic states [211-214]. Finally, we must embrace the molecular biology of coactivator/corepressor action in the molecular pharmacology drug discovery process [101, 211, 213, 214]. Forty years ago it would have been impossible to achieve the current clinical advances without laboratory findings to transform an orphan drug group the "nonsteroidal antiestrogens" [16] into the SERMs [2, 23]. This "road map" proved to be particularly prophetic and significantly advanced women's health in numerous disease states throughout the world.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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REFERENCES

[1] Lacassagne A. Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer 1936; 27: 217-25.
[2] Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 1990; 50: 4177-89.
[3] Lerner LJ, Holthaus FJ Jr., Thompson CR. A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol. Endocrinology 1958; 63: 295-318.
[4] Greenblatt RB, Barfield WE, Jungck EC, Ray AW. Induction of ovulation with MRL/41. Preliminary report. JAMA 1961; 178: 101-4.
[5] Greenblatt RB, Roy S, Mahesh VB. Induction of ovulation. Am J Obstet Gynecol 1962; 84: 900-12.
[6] Clark JH, Markaverich BM. The agonistic-antagonistic properties of clomiphene: a review. Pharmacol Ther 1981; 15: 467-519.
[7] Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 1967; 13: 101-19.
[8] Harper MJ, Walpole AL. Mode of action of I.C.I. 46,474 in preventing implantation in rats. J Endocrinol 1967; 37: 83-92.
[9] Klopper A, Hall M. New synthetic agent for the induction of ovulation: preliminary trials in women. Br Med J 1971; 1: 152-4.
[10] Williamson JG, Ellis JD. The induction of ovulation by tamoxifen. J Obstet Gynaecol Br Commonw1973; 80: 844-7.
[11] Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer 1971; 25: 270-5.
[12] Ward HW. Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J 1973; 1: 13-4.
[13] Jordan VC. Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 2006; 147(Suppl 1): S269-76.
[14] Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2003; 2: 205-13.
[15] Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 2008; 44: 30-8.
[16] Jordan VC. Biochemical pharmacology of antiestrogen action. Pharmacol Rev 1984; 36: 245-76.
[17] Powles TJ, Hardy JR, Ashley SE, *et al.* A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. Bri J cancer 1989; 60: 126-31.
[18] Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 1988; 48: 812-5.
[19] Fornander T, Rutqvist LE, Cedermark B, *et al.* Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1989; 1: 117-20.
[20] Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987; 47: 4020-4.
[21] Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987; 10: 31-5.
[22] Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc 1987; 46: 1870-4.
[23] Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 1988; 6: 589-95.
[24] Love RR, Wiebe DA, Newcomb PA, *et al.* Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann Intern Med 1991; 115: 860-4.
[25] Love RR, Mazess RB, Barden HS, *et al.* Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 1992; 326: 852-6.
[26] Love RR, Newcomb PA, Wiebe DA, *et al.* Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 1990; 82: 1327-32.

[27] Jordan VC, Koch R, Mittal S, Schneider MR. Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis *in vitro*. Br J Pharmacol 1986; 87: 217-23.

[28] Lieberman ME, Gorski J, Jordan VC. An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens *in vitro*. J Biol Chem 1983; 258: 4741-5.

[29] Ettinger B, Black DM, Mitlak BH, *et al*. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 1999; 282: 637-45.

[30] Cummings SR, Eckert S, Krueger KA, *et al.* The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA 1999; 281: 2189-97.

[31] Vogel VG, Costantino JP, Wickerham DL, *et al.* Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 2006; 295: 2727-41.

[32] Vogel VG, Costantino JP, Wickerham DL, *et al.* Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. Cancer Prev Res (Phila Pa) 2010; 3: 696-706.

[33] Kenakin T. Inverse, protean, and ligand-selective agonism: matters of receptor conformation. FASEB J 2001; 15: 598-611.

[34] Miller CP. SERMs: evolutionary chemistry, revolutionary biology. Curr Pharm Des 2002; 8: 2089-111.

[35] Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 1977; 75: 305-16.

[36] Allen KE, Clark ER, Jordan VC. Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 1980; 71: 83-91.

[37] Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 1980; 16: 239-51.

[38] Jordan VC, Bain RR, Brown RR, Brown RR, Gosden B, Santos MA. Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient during therapy for advanced breast cancer. Cancer Res 1983; 43: 1446-50.

[39] Johnson MD, Zuo H, Lee KH, *et al.* Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. Breast Cancer Res Treat 2004; 85: 151-9.

[40] Lim YC, Desta Z, Flockhart DA, Skaar TC. Endoxifen (4-hydroxy-N-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen. Cancer Chemother Pharmacol 2005; 55: 471-8.

[41] Lim YC, Li L, Desta Z, *et al.* Endoxifen, a secondary metabolite of tamoxifen, and 4-OH-tamoxifen induce similar changes in global gene expression patterns in MCF-7 breast cancer cells. J Pharmacol Exp Ther 2006; 318: 503-12.

[42] Stearns V, Johnson MD, Rae JM, *et al.* Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. J Natl Cancer Inst 2003; 95: 1758-64.

[43] Jin Y, Desta Z, Stearns V, *et al.* CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. J Natl Cancer Inst 2005; 97: 30-9.

[44] Borges S, Desta Z, Li L, *et al.* Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. Clin Pharmacol Ther 2006; 80: 61-74.

[45] Goetz MP, Rae JM, Suman VJ, *et al.* Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. J Clin Oncol 2005; 23: 9312-8.

[46] Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea- induced rat mammary carcinoma model. Cancer Res 1987; 47: 4020-4.

[47] Jordan VC, Gosden B. Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 1983; 113: 463-8.

[48] Snyder KR, Sparano N, Malinowski JM. Raloxifene hydrochloride. Am J Health Syst Pharm 2000; 57: 1669-75; quiz 76-78.

[49] Kemp DC, Fan PW, Stevens JC. Characterization of raloxifene glucuronidation *in vitro*: contribution of intestinal metabolism to presystemic clearance. Drug Metab Dispos 2002; 30: 694-700.

[50] Jeong EJ, Lin H, Hu M. Disposition mechanisms of raloxifene in the human intestinal Caco-2 model. J Pharmacol Exp Ther 2004; 310: 376-85.

[51] Falany JL, Pilloff DE, Leyh TS, Falany CN. Sulfation of raloxifene and 4-hydroxytamoxifen by human cytosolic sulfotransferases. Drug Metab Dispos 2006; 34: 361-8.

[52] Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nat Rev Cancer 2007; 7: 46-53.

[53] Prakash C, Johnson KA, Schroeder CM, Potchoiba MJ. Metabolism, distribution, and excretion of a next generation selective estrogen receptor modulator, lasofoxifene, in rats and monkeys. Drug Metab Dispos 2008; 36: 1753-69.

[54] Rosati RL, Da Silva Jardine P, *et al.* Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryltetrahydronaphthalene. J Med Chem 1998; 41: 2928-31.

[55] Cummings SR, Ensrud K, Delmas PD, *et al.* Lasofoxifene in postmenopausal women with osteoporosis. N Engl J Med 2010; 362: 686-96.

[56] Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res 1997; 57: 3071-8.

[57] Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 1983; 32: 1031-6.

[58] Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 1990; 50: 3189-92.

[59] Jordan VC, Lababidi MK, Mirecki DM. Anti-oestrogenic and anti-tumour properties of prolonged tamoxifen therapy in C3H/OUJ mice. Eur J Cancer 1990; 26: 718-21.

[60] Jordan VC, Lababidi MK, Langan-Fahey S. Suppression of mouse mammary tumorigenesis by long-term tamoxifen therapy. J Natl Cancer Inst 1991; 83: 492-6.

[61] Beall PT, Misra LK, Young RL, Spjut HJ, Evans HJ, LeBlanc A. Clomiphene protects against osteoporosis in the mature ovariectomized rat. Calcif Tissue Int 1984; 36: 123-5.

[62] Turner RT, Wakley GK, Hannon KS, Bell NH. Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. J Bone Miner Res 1987; 2: 449-56.

[63] Turner RT, Wakley GK, Hannon KS, Bell NH. Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. Endocrinology 1988; 122: 1146-50.

[64] Cuzick J, Forbes JF, Sestak I, *et al.* Long-term results of tamoxifen prophylaxis for breast cancer--96-month follow-up of the randomized IBIS-I trial. J Natl Cancer Inst 2007; 99: 272-82.

[65] Fisher B, Costantino JP, Wickerham DL, *et al.* Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 2005; 97: 1652-62.

[66] Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. J Natl Cancer Inst 2007; 99: 283-90.

[67] Nawrocki JW, Weiss SR, Davidson MH, *et al.* Reduction of LDL cholesterol by 25% to 60% in patients with primary hypercholesterolemia by atorvastatin, a new HMG-CoA reductase inhibitor. Arterioscler Thromb Vasc Biol 1995; 15: 678-82.

[68] Gould AL, Rossouw JE, Santanello NC, Heyse JF, Furberg CD. Cholesterol reduction yields clinical benefit: impact of statin trials. Circulation 1998; 97: 946-52.

[69] LaRosa JC, He J, Vupputuri S. Effect of statins on risk of coronary disease: a meta-analysis of randomized controlled trials. JAMA 1999; 282: 2340-6.

[70] Pignone M, Phillips C, Mulrow C. Use of lipid lowering drugs for primary prevention of coronary heart disease: meta-analysis of randomised trials. BMJ 2000; 321: 983-6.

[71] Steinberg D, Avigan J, Feigelson EB. Effects of Triparanol (Mer-29) on Cholesterol Biosynthesis and on Blood Sterol Levels in Man. J Clin Invest 1961; 40: 884-93.

[72] Kirby TJ. Cataracts produced by triparanol. (MER-29). Trans Am Ophthalmol Soc 1967; 65: 494-543.

[73] Love RR, Mamby CC, Feyzi JM. Tamoxifen-induced decreases in total cholesterol with 2 weeks of treatment. J Natl Cancer Inst 1993; 85: 1344-5.

[74] Love RR, Wiebe DA, Feyzi JM, Newcomb PA, Chappell RJ. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women after 5 years of treatment. J Natl Cancer Inst 1994; 86: 1534-9.

[75] Rutqvist LE, Mattsson A. Cardiac and thromboembolic morbidity among postmenopausal women with early-stage breast cancer in a randomized trial of adjuvant tamoxifen. The Stockholm Breast Cancer Study Group. J Natl Cancer Inst 1993; 85: 1398-406.

[76] McDonald CC, Alexander FE, Whyte BW, Forrest AP, Stewart HJ. Cardiac and vascular morbidity in women receiving adjuvant tamoxifen for breast cancer in a randomised trial. The Scottish Cancer Trials Breast Group. BMJ 1995; 311: 977-80.

[77] Hackshaw A, Roughton M, Forsyth S, *et al.* Long-term benefits of 5 years of tamoxifen: 10-year follow-up of a large randomized trial in women at least 50 years of age with early breast cancer. J Clin Oncol 2011; 29: 1657-63.

[78] Esteva FJ, Hortobagyi GN. Comparative assessment of lipid effects of endocrine therapy for breast cancer: implications for cardiovascular disease prevention in postmenopausal women. Breast 2006; 15: 301-12.

[79] Lewis L, Jordan V, 103-21. Comprehensive Medicinal Chemistry. In: Taylor J, Triggle D, Eds. Case Histories: Raloxifene. Oxford, UK: Elsevier Limited 2006; pp. 103-21.

[80] Black LJ, Sato M, Rowley ER, *et al.* Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 1994; 93: 63-9.

[81] Barrett-Connor E, Mosca L, Collins P, *et al.* Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 2006; 355: 125-37.

[82] Jensen EV, Jacobson HI. Basic guides to the mechanism of estrogen action. Recent Progr Hormone Res 1962; 18: 387-414.

[83] Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J. Sequence and expression of human estrogen receptor complementary DNA. Science 1986; 231: 1150-4.

[84] Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 1996; 93: 5925-30.

[85] Kuiper GG, Carlsson B, Grandien K, *et al.* Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 1997; 138: 863-70.

[86] Roger P, Sahla ME, Makela S, Gustafsson JA, Baldet P, Rochefort H. Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. Cancer Res 2001; 61: 2537-41.

[87] Shaaban AM, O'Neill PA, Davies MP, *et al.* Declining estrogen receptor-beta expression defines malignant progression of human breast neoplasia. Am J Surg Pathol 2003; 27: 1502-12.

[88] Paruthiyil S, Parmar H, Kerekatte V, Cunha GR, Firestone GL, Leitman DC. Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. Cancer Res 2004; 64: 423-8.

[89] Acconcia F, Totta P, Ogawa S, *et al.* Survival versus apoptotic 17beta-estradiol effect: role of ER alpha and ER beta activated non-genomic signaling. J Cell Physiol 2005; 203: 193-201.

[90] Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J, Nilsson S. Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. Mol Pharmacol 1998; 54: 105-12.

[91] McInerney EM, Weis KE, Sun J, Mosselman S, Katzenellenbogen BS. Transcription activation by the human estrogen receptor subtype beta (ER beta) studied with ER beta and ER alpha receptor chimeras. Endocrinology 1998; 139: 4513-22.

[92] Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 1999; 140: 5566-78.

[93] Paech K, Webb P, Kuiper GG, *et al.* Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites [see comments]. Science 1997; 277: 1508-10.

[94] Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 1995; 270: 1354-7.

[95] Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocrine Rev 2004; 25: 45-71.

[96] Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. J Med Chem 2003; 46: 1081-111.

[97] Kraichely DM, Sun J, Katzenellenbogen JA, Katzenellenbogen BS. Conformational changes and coactivator recruitment by novel ligands for estrogen receptor-alpha and estrogen receptor-beta: correlations with biological character and distinct differences among SRC coactivator family members. Endocrinology 2000; 141: 3534-45.

[98] Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. The structural basis of estrogen receptor/co-activator recognition and the antagonism of this interaction by tamoxifen. Cell 1998; 95: 927-37.

[99] Brzozowski AM, Pike AC, Dauter Z, *et al.* Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997; 389: 753-8.

[100] Wijayaratne AL, Nagel SC, Paige LA, *et al.* Comparative analyses of mechanistic differences among antiestrogens. Endocrinology 1999; 140: 5828-40.

[101] Lonard DM, O'Malley BW. The expanding cosmos of nuclear receptor coactivators. Cell 2006; 125: 411-4.

[102] Sun Y. E3 Ubiquitin Ligases as Cancer Targets and Biomarkers. Neoplasia 2006; 8: 645-54.

[103] Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 2000; 103: 843-52.

[104] Jordan VC, Dix CJ, Rowsby L, Prestwich G. Studies on the mechanism of action of the nonsteroidal antioestrogen tamoxifen (I.C.I. 46,474) in the rat. Mol Cell Endocrinol 1977; 7: 177-92.

[105] Horwitz KB, McGuire WL. Nuclear mechanisms of estrogen action. Effects of estradiol and anti- estrogens on estrogen receptors and nuclear receptor processing. J Biol Chem 1978; 253: 8185-91.

[106] Wijayaratne AL, McDonnell DP. The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. J Biol Chem 2001; 276: 35684-92.

[107] Wu RC, Smith CL, O'Malley BW. Transcriptional Regulation by Steroid Receptor Coactivator Phosphorylation. Endocrine Rev 2006; 26: 393-9.

[108] Osborne CK, Bardou V, Hopp TA, *et al.* Role of the estrogen receptor coactivator AIB1 (SRC3) and HER2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst 2003; 95: 353-61.

[109] Shou J, Massarweh S, Osborne CK, *et al.* Mechanisms of Tamoxifen Resistance: Increased Estrogen Receptor-HER2/neu Cross-Talk in ER/HER2–Positive Breast Cancer. J Natl Cancer Inst 2004; 96: 926-35.

[110] Davies C, Godwin J, Gray R, *et al.* Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 2011; 378: 771-84.

[111] Sawaki M, Wada M, Sato Y, *et al.* High-dose toremifene as first-line treatment of metastatic breast cancer resistant to adjuvant aromatase inhibitor: A multicenter phase II study. Oncol Lett 2012; 3: 61-5.

[112] Holli K, Valavaara R, Blanco G, *et al.* Safety and efficacy results of a randomized trial comparing adjuvant toremifene and tamoxifen in postmenopausal patients with node-positive breast cancer. Finnish Breast Cancer Group. J Clin Oncol 2000; 18: 3487-94.

[113] Fisher B, Costantino JP, Wickerham DL, *et al.* Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998; 90: 1371-88.

[114] Siris ES, Harris ST, Eastell R, *et al.* Skeletal effects of raloxifene after 8 years: results from the continuing outcomes relevant to Evista (CORE) study. J Bone Miner Res 2005; 20: 1514-24.

[115] Martino S, Cauley JA, Barrett-Connor E, *et al.* Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 2004; 96: 1751-61.

[116] Goldstein SR. Not all SERMs are created equal. Menopause 2006; 13: 325-7.

[117] Jordan VC. Designer estrogens. Sci Am 1998; 279: 60-7.

[118] Bain RR, Jordan VC. Identification of a new metabolite of tamoxifen in patient serum during breast cancer therapy. Biochem Pharmacol 1983; 32: 373-5.

[119] Jordan VC, Bain RR, Brown RR, Gosden B, Santos MA. Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. Cancer Res 1983; 43: 1446-50.

[120] Hard GC, Iatropoulos MJ, Jordan K, *et al.* Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. Cancer Res 1993; 53: 4534-41.

[121] Lednicer D, Lyster SC, Aspergren BD, Duncan GW. Mammalian antifertility agents. 3. 1-Aryl-2-phenyl-1,2,3,4-tetrahydro-1-naphthols, 1-aryl-2-phenyl-3,4-dihydronaphthalenes, and their derivatives. J Med Chem 1966; 9: 172-6.

[122] Lednicer D, Lyster SC, Duncan GW. Mammalian antifertility agents. IV. Basic 3,4-dihydronaphthalenes and 1,2,3,4-tetrahydro-1-naphthols. J Med Chem 1967; 10: 78-84.

[123] Legha SS, Slavik M, Carter SK. Nafoxidine--an antiestrogen for the treatment of breast cancer. Cancer 1976; 38: 1535-41.

[124] Robinson SP, Koch R, Jordan VC. *In vitro* estrogenic actions in rat and human cells of hydroxylated derivatives of D16726 (zindoxifene), an agent with known antimammary cancer activity *in vivo*. Cancer Res 1988; 48: 784-7.

[125] Gradishar W, Glusman J, Lu Y, Vogel C, Cohen FJ, Sledge GW Jr. Effects of high dose raloxifene in selected patients with advanced breast carcinoma. Cancer 2000; 88: 2047-53.

[126] Qu Q, Harkonen PL, Vaananen HK. Comparative effects of estrogen and antiestrogens on differentiation of osteoblasts in mouse bone marrow culture. J Cell Biochem 1999; 73: 500-7.

[127] Qu Q, Zheng H, Dahllund J, *et al.* Selective estrogenic effects of a novel triphenylethylene compound, FC1271a, on bone, cholesterol level, and reproductive tissues in intact and ovariectomized rats. Endocrinology 2000; 141: 809-20.

[128] Hellmann-Blumberg U, Taras TL, Wurz GT, DeGregorio MW. Genotoxic effects of the novel mixed antiestrogen FC-1271a in comparison to tamoxifen and toremifene. Breast Cancer Res Treat 2000; 60: 63-70.

[129] DeGregorio MW, Wurz GT, Taras TL, Erkkola RU, Halonen KH, Huupponen RK. Pharmacokinetics of (deaminohydroxy)toremifene in humans: a new, selective estrogen-receptor modulator. Eur J Clin Pharmacol 2000; 56: 469-75.

[130] Voipio SK, Komi J, Kangas L, Halonen K, DeGregorio MW, Erkkola RU. Effects of ospemifene (FC-1271a) on uterine endometrium, vaginal maturation index, and hormonal status in healthy postmenopausal women. Maturitas 2002; 43: 207-14.

[131] Rutanen EM, Heikkinen J, Halonen K, Komi J, Lammintausta R, Ylikorkala O. Effects of ospemifene, a novel SERM, on hormones, genital tract, climacteric symptoms, and quality of life in postmenopausal women: a double-blind, randomized trial. Menopause 2003; 10: 433-9.

[132] Ylikorkala O, Cacciatore B, Halonen K, *et al.* Effects of ospemifene, a novel SERM, on vascular markers and function in healthy, postmenopausal women. Menopause 2003; 10: 440-7.

[133] Komi J, Heikkinen J, Rutanen EM, Halonen K, Lammintausta R, Ylikorkala O. Effects of ospemifene, a novel SERM, on biochemical markers of bone turnover in healthy postmenopausal women. Gynecol Endocrinol 2004; 18: 152-8.

[134] Komi J, Lankinen KS, Harkonen P, *et al.* Effects of ospemifene and raloxifene on hormonal status, lipids, genital tract, and tolerability in postmenopausal women. Menopause 2005; 12: 202-9.

[135] Komi J, Lankinen KS, DeGregorio M, *et al.* Effects of ospemifene and raloxifene on biochemical markers of bone turnover in postmenopausal women. J Bone Miner Metab 2006; 24: 314-8.

[136] Taras TL, Wurz GT, DeGregorio MW. *In vitro* and *in vivo* biologic effects of Ospemifene (FC-1271a) in breast cancer. J Steroid Biochem Mol Biol 2001; 77: 271-9.

[137] Namba R, Young LJ, Maglione JE, *et al.* Selective estrogen receptor modulators inhibit growth and progression of premalignant lesions in a mouse model of ductal carcinoma in situ. Breast Cancer Res 2005; 7: R881-9.

[138] Wurz GT, Read KC, Marchisano-Karpman C, *et al.* Ospemifene inhibits the growth of dimethylbenzanthracene-induced mammary tumors in Sencar mice. J Steroid Biochem Mol Biol 2005; 97: 230-40.

[139] Ke HZ, Paralkar VM, Grasser WA, *et al.* Effects of CP-336,156, a new, nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. Endocrinology 1998; 139: 2068-76.

[140] Maeda T, Ke HZ, Simmons H, Thompson D. Lasofoxifene, a next generation estrogen receptor modulator: preclinical studies. Clin Calcium 2004; 14: 85-93.

[141] Gennari L. Lasofoxifene (Pfizer). Curr Opin Investig Drugs 2005; 6: 1067-78.

[142] Gennari L. Lasofoxifene: a new type of selective estrogen receptor modulator for the treatment of osteoporosis. Drugs Today (Barc) 2006; 42: 355-67.

[143] Gennari L, Merlotti D, Martini G, Nuti R. Lasofoxifene: a third-generation selective estrogen receptor modulator for the prevention and treatment of osteoporosis. Expert Opin Investig Drugs 2006; 15: 1091-103.

[144] Goldstein SR. Not all selective estrogen response modulators are created equal: update on lasofoxifene. Int J Gynecol Cancer 2006; 16 Suppl 2: 504.

[145] Moffett A, Ettinger M, Bolognese M, *et al.* Lasofoxifene, a next generation SERM, is effective in preventing loss of BMD and reducing LDL-C in postmenopausal women. J Bone Min Res 2004; 19: S96.

[146] Davidson M, Moffett A, Welty F, *et al* Extraskeletal effects of lasofoxifene on postmenopausal women. J Bone Min Res 2005; 20: S173.

[147] McClung M, Siris E, Cummings S. Lasofoxifene increased BMD of the spine and hip and decreased bone turnover markers in postmenopausal women with low or normal BMD. J Bone Min Res 2005; 20: F429.

[148] McClung MR, Siris E, Cummings S, *et al.* Prevention of bone loss in postmenopausal women treated with lasofoxifene compared with raloxifene. Menopause 2006; 13: 377-86.

[149] Cummings S, Eastell R, Ensrud K. The effects of lasofoxifene on fractures and breast cancer: 3 year results from the PEARL trial. J Bone Miner Res 2008; 23: S81.

[150] LaCroix AZ, Powles T, Osborne CK, *et al.* Breast cancer incidence in the randomized PEARL trial of lasofoxifene in postmenopausal osteoporotic women. J Natl Cancer Inst 2010; 102: 1706-15.

[151] Gennari L. Lasofoxifene, a new selective estrogen receptor modulator for the treatment of osteoporosis and vaginal atrophy. Expert Opin Pharmacother 2009; 10: 2209-20.

[152] Miller CP, Collini MD, Tran BD, *et al.* Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. J Med Chem 2001; 44: 1654-7.

[153] Gruber C, Gruber D. Bazedoxifene (Wyeth). Curr Opin Investig Drugs 2004; 5: 1086-93.

[154] Komm BS, Kharode YP, Bodine PV, Harris HA, Miller CP, Lyttle CR. Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity. Endocrinology 2005; 146: 3999-4008.

[155] Smith S, Minck D, Jolette J, *et al.* Bazedoxifene prevents ovariectomy-induced bone loss in the Cynomolgus monkey. J Bone Miner Res 2005; 20: S174.

[156] Komm B, Kharode Y, Bodine P, *et al.* Bazedoxifene + conjugated estrogens: a balanced combination to provide optimal estrogenic "safety" and efficacy. J Bone Miner Res 2003; 18: S272.

[157] Komm B, Kharode Y, Bodine P, *et al.* Combining a SERM with conjugated estrogens (CE) to improve the SERM profile: not all SERMs may succeed. J Bone Miner Res 2003; 25: S273.

[158] Kharode Y, Green P, Marzolf J, *et al.* Comparison of the effects of bazedoxifene, raloxifene, lasofoxifene and risedronate, co-treatment on h-PTH-induced reversal of established osteopenia in ovariectomized rats. J Bone Miner Res 2003; 18: S273.

[159] Miller PD, Chines AA, Christiansen C, *et al.* Effects of bazedoxifene on BMD and bone turnover in postmenopausal women: 2-yr results of a randomized, double-blind, placebo-, and active-controlled study. J Bone Miner Res 2008; 23: 525-35.

[160] Silverman SL, Christiansen C, Genant HK, *et al.* Efficacy of bazedoxifene in reducing new vertebral fracture risk in postmenopausal women with osteoporosis: results from a 3-year, randomized, placebo-, and active-controlled clinical trial. J Bone Miner Res 2008; 23: 1923-34.

[161] Pinkerton JV, Archer DF, Utian WH, *et al.* Bazedoxifene effects on the reproductive tract in postmenopausal women at risk for osteoporosis. Menopause 2009; 16: 1102-8.

[162] Archer DF, Pinkerton JV, Utian WH, *et al.* Bazedoxifene, a selective estrogen receptor modulator: effects on the endometrium, ovaries, and breast from a randomized controlled trial in osteoporotic postmenopausal women. Menopause 2009; 16: 1109-15.

[163] de Villiers TJ, Chines AA, Palacios S, *et al.* Safety and tolerability of bazedoxifene in postmenopausal women with osteoporosis: results of a 5-year, randomized, placebo-controlled phase 3 trial. Osteoporos Int 2011; 22: 567-76.

[164] Grady D, Ettinger B, Moscarelli E, *et al.* Safety and adverse effects associated with raloxifene: multiple outcomes of raloxifene evaluation. Obstet Gynecol 2004; 104: 837-44.

[165] Dodge JA, Lugar CW, Cho S, *et al.* Evaluation of the major metabolites of raloxifene as modulators of tissue selectivity. J Steroid Biochem Mol Biol 1997; 61: 97-106.

[166] Grese TA, Cho S, Finley DR, *et al.* Structure-activity relationships of selective estrogen receptor modulators: modifications to the 2-arylbenzothiophene core of raloxifene. J Med Chem 1997; 40: 146-67.

[167] Grese TA, Sluka JP, Bryant HU, *et al.* Molecular determinants of tissue selectivity in estrogen receptor modulators. Proc Natl Acad Sci USA 1997; 94: 14105-10.

[168] Palkowitz AD, Glasebrook AL, Thrasher KJ, *et al.* Discovery and synthesis of [6-hydroxy-3-[4-[2-(1-piperidinyl)ethoxy]phenoxy]-2-(4-hydroxyphenyl)]b enzo[b]thiophene: a novel, highly potent, selective estrogen receptor modulator. J Med Chem 1997; 40: 1407-16.

[169] Grese TA, Pennington LD, Sluka JP, *et al.* Synthesis and pharmacology of conformationally restricted raloxifene analogues: highly potent selective estrogen receptor modulators. J Med Chem 1998; 41: 1272-83.

[170] Sato M, Turner CH, Wang T, Adrian MD, Rowley E, Bryant HU. LY353381.HCl: a novel raloxifene analog with improved SERM potency and efficacy *in vivo*. J Pharmacol Exp Ther 1998; 287: 1-7.

[171] Bryant HU, Glasebrook AL, Yang NN, Sato M. An estrogen receptor basis for raloxifene action in bone. J Steroid Biochem Mol Biol 1999; 69: 37-44.

[172] Munster PN. Arzoxifene: the development and clinical outcome of an ideal SERM. Expert Opin Investig Drugs 2006; 15: 317-26.

[173] Dardes RC, Bentrem D, O'Regan RM, Schafer JM, Jordan VC. Effects of the new selective estrogen receptor modulator LY353381.HCl (Arzoxifene) on human endometrial cancer growth in athymic mice. Clin Cancer Res 2001; 7: 4149-55.

[174] Suh N, Glasebrook AL, Palkowitz AD, *et al.* Arzoxifene, a new selective estrogen receptor modulator for chemoprevention of experimental breast cancer. Cancer Res 2001; 61: 8412-5.

[175] Detre S, Riddler S, Salter J, A'Hern R, Dowsett M, Johnston SR. Comparison of the selective estrogen receptor modulator arzoxifene (LY353381) with tamoxifen on tumor growth and biomarker expression in an MCF-7 human breast cancer xenograft model. Cancer Res 2003; 63: 6516-22.

[176] Licun W, Tannock IF. Selective estrogen receptor modulators as inhibitors of repopulation of human breast cancer cell lines after chemotherapy. Clin Cancer Res 2003; 9: 4614-8.

[177] Freddie CT, Larsen SS, Bartholomaeussen M, Lykkesfeldt AE. The effect of the new SERM arzoxifene on growth and gene expression in MCF-7 breast cancer cells. Mol Cell Endocrinol 2004; 219: 27-36.

[178] Morello KC, Wurz GT, DeGregorio MW. SERMs: current status and future trends. Crit Rev Oncol Hematol 2002; 43: 63-76.

[179] Munster PN, Buzdar A, Dhingra K, *et al.* Phase I study of a third-generation selective estrogen receptor modulator, LY353381.HCL, in metastatic breast cancer. J Clin Oncol 2001; 19: 2002-9.

[180] McMeekin DS, Gordon A, Fowler J, *et al.* A phase II trial of arzoxifene, a selective estrogen response modulator, in patients with recurrent or advanced endometrial cancer. Gynecol Oncol 2003; 90: 64-9.

[181] Ma YL, Bryant HU, Zeng Q, *et al.* Long-term dosing of arzoxifene lowers cholesterol, reduces bone turnover, and preserves bone quality in ovariectomized rats. J Bone Miner Res 2002; 17: 2256-64.

[182] Sato M, Zeng GQ, Rowley E, Turner CH. LY353381 x HCl: an improved benzothiophene analog with bone efficacy complementary to parathyroid hormone-(1-34). Endocrinology 1998; 139: 4642-51.

[183] Downs RW Jr, Moffett AM, Ghosh A, Cox DA, Dowsett SA, Harper K. Effects of arzoxifene on bone, lipid markers, and safety parameters in postmenopausal women with low bone mass. Osteoporos Int 2010; 21: 1215-26.

[184] Kendler DL, Palacios S, Cox DA, *et al.* Arzoxifene versus raloxifene: effect on bone and safety parameters in postmenopausal women with osteoporosis. Osteoporos Int 2012; 23(3): 1091-109.

[185] Bolognese M, Krege JH, Utian WH, *et al.* Effects of arzoxifene on bone mineral density and endometrium in postmenopausal women with normal or low bone mass. J Clin Endocrinol Metab 2009; 94: 2284-9.

[186] Black DM, Cummings SR, Karpf DB, *et al.* Randomised trial of effect of alendronate on risk of fracture in women with existing vertebral fractures. Fracture Intervention Trial Research Group. Lancet 1996; 348: 1535-41.

[187] Harris ST, Watts NB, Genant HK, *et al.* Effects of risedronate treatment on vertebral and nonvertebral fractures in women with postmenopausal osteoporosis: a randomized controlled trial. Vertebral Efficacy With Risedronate Therapy (VERT) Study Group. JAMA 1999; 282: 1344-52.

[188] Pols HA, Felsenberg D, Hanley DA, *et al.* Multinational, placebo-controlled, randomized trial of the effects of alendronate on bone density and fracture risk in postmenopausal women with low bone mass: results of the FOSIT study. Fosamax International Trial Study Group. Osteoporos Int 1999; 9: 461-8.

[189] Black DM, Delmas PD, Eastell R, *et al.* Once-yearly zoledronic acid for treatment of postmenopausal osteoporosis. N Engl J Med 2007; 356: 1809-22.

[190] Cummings SR, McClung M, Reginster JY, *et al.* Arzoxifene for prevention of fractures and invasive breast cancer in postmenopausal women. J Bone Miner Res 2011; 26: 397-404.

[191] Rossouw JE, Anderson GL, Prentice RL, *et al.* Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA 2002; 288: 321-33.

[192] Cummings SR, Ettinger B, Delmas PD, *et al.* The effects of tibolone in older postmenopausal women. N Engl J Med 2008; 359: 697-708.

[193] Cirillo DJ, Wallace RB, Rodabough RJ, *et al.* Effect of estrogen therapy on gallbladder disease. JAMA 2005; 293: 330-9.

[194] Ivanova MM, Mazhawidza W, Dougherty SM, Minna JD, Klinge CM. Activity and intracellular location of estrogen receptors alpha and beta in human bronchial epithelial cells. Mol Cell Endocrinol 2009; 305: 12-21.

[195] Vegeto E, Cuzzocrea S, Crisafulli C, *et al.* Estrogen receptor-alpha as a drug target candidate for preventing lung inflammation. Endocrinology 2010; 151: 174-84.

[196] Archer DF, Lewis V, Carr BR, Olivier S, Pickar JH. Bazedoxifene/conjugated estrogens (BZA/CE): incidence of uterine bleeding in postmenopausal women. Fertil Steril 2009; 92: 1039-44.

[197] Pinkerton JV, Stovall DW. Bazedoxifene when paired with conjugated estrogens is a new paradigm for treatment of postmenopausal women. Expert Opin Investig Drugs 2010; 19: 1613-21.

[198] Lindsay R, Gallagher JC, Kagan R, Pickar JH, Constantine G. Efficacy of tissue-selective estrogen complex of bazedoxifene/conjugated estrogens for osteoporosis prevention in at-risk postmenopausal women. Fertil Steril 2009; 92: 1045-52.

[199] Crandall CJ, Aragaki AK, Cauley JA, *et al.* Breast tenderness and breast cancer risk in the estrogen plus progestin and estrogen-alone women's health initiative clinical trials. Breast Cancer Res Treat 2012; 132: 275-85.

[200] Narod SA. Hormone replacement therapy and the risk of breast cancer. Nat Rev Clin Oncol 2011; 8: 669-76.

[201] Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007; 72: 7-25.

[202] Dowsett M, Cuzick J, Ingle J, *et al.* Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 2010; 28: 509-18.

[203] Vogel VG. Raloxifene: a selective estrogen receptor modulator for reducing the risk of invasive breast cancer in postmenopausal women. Womens Health (Lond Engl) 2007; 3: 139-53.

[204] Vogel VG, Costantino JP, Wickerham DL, *et al.* Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. Cancer Prev Res (Phila) 2010; 3: 696-706.

[205] Goss PE, Ingle JN, Ales-Martinez JE, Cheung AM, Chlebowski RT, Wactawski-Wende J, *et al.* Exemestane for breast-cancer prevention in postmenopausal women. N Engl J Med 2011; 364: 2381-91.

[206] Mieog JS, Morden JP, Bliss JM, Coombes RC, van de Velde CJ. Carpal tunnel syndrome and musculoskeletal symptoms in postmenopausal women with early breast cancer treated with exemestane or tamoxifen after 2-3 years of tamoxifen: a retrospective analysis of the Intergroup Exemestane Study. Lancet Oncol 2012.

[207] Watanabe N, Ikeno A, Minato H, Nakagawa H, Kohayakawa C, Tsuji J. Discovery and preclinical characterization of (+)-3-[4-(1-piperidinoethoxy)phenyl]spiro[indene- 1,1'-indane]-5,5'-diol hydrochloride: a promising nonsteroidal estrogen receptor agonist for hot flush. J Med Chem 2003; 46: 3961-4.

[208] Wallace OB, Lauwers KS, Dodge JA, *et al.* A selective estrogen receptor modulator for the treatment of hot flushes. J Med Chem 2006; 49: 843-6.

[209] Jain N, Xu J, Kanojia RM, *et al.* Identification and structure-activity relationships of chromene-derived selective estrogen receptor modulators for treatment of postmenopausal symptoms. J Med Chem 2009; 52: 7544-69.

[210] Jain N, Kanojia RM, Xu J, *et al.* Novel chromene-derived selective estrogen receptor modulators useful for alleviating hot flushes and vaginal dryness. J Med Chem 2006; 49: 3056-9.

[211] Meegan MJ, Lloyd DG. Advances in the science of estrogen receptor modulation. Curr Med Chem 2003; 10: 181-210.

[212] Arevalo MA, Santos-Galindo M, Lagunas N, Azcoitia I, Garcia-Segura LM. Selective estrogen receptor modulators as brain therapeutic agents. J Mol Endocrinol 2011; 46: R1-9.

[213] Nilsson S, Gustafsson JA. Estrogen receptors: therapies targeted to receptor subtypes. Clin Pharmacol Ther 2011; 89: 44-55.

[214] Rosano C, Stec-Martyna E, Lappano R, Maggiolini M. Structure-based approach for the discovery of novel selective estrogen receptor modulators. Curr Med Chem 2011; 18: 1188-94.

[215] O'Malley BW. Molecular biology. Little molecules with big goals. Science 2006; 313: 1749-50.

[216] Liby K, Rendi M, Suh N, *et al.* The combination of the rexinoid, LG100268, and a selective estrogen receptor modulator, either arzoxifene or acolbifene, synergizes in the prevention and treatment of mammary tumors in an estrogen receptor-negative model of breast cancer. Clin Cancer Res 2006; 12: 5902-9.

[217] Deshmane V, Krishnamurthy S, Melemed AS, Peterson P, Buzdar AU. Phase III double-blind trial of arzoxifene compared with tamoxifen for locally advanced or metastatic breast cancer. J Clin Oncol 2007; 25: 4967-73.

[218] Fabian CJ, Kimler BF, Anderson J, *et al.* Breast cancer chemoprevention phase I evaluation of biomarker modulation by arzoxifene, a third generation selective estrogen receptor modulator. Clin Cancer Res 2004; 10: 5403-17.

[219] Cohen LA, Pittman B, Wang CX, Aliaga C, Yu L, Moyer JD. LAS, a novel selective estrogen receptor modulator with chemopreventive and therapeutic activity in the N-nitroso-N-methylurea-induced rat mammary tumor model. Cancer Res 2001; 61: 8683-8.

[220] Ronkin S, Northington R, Baracat E, *et al.* Endometrial effects of bazedoxifene acetate, a novel selective estrogen receptor modulator, in postmenopausal women. Obstet Gynecol 2005; 105: 1397-404.

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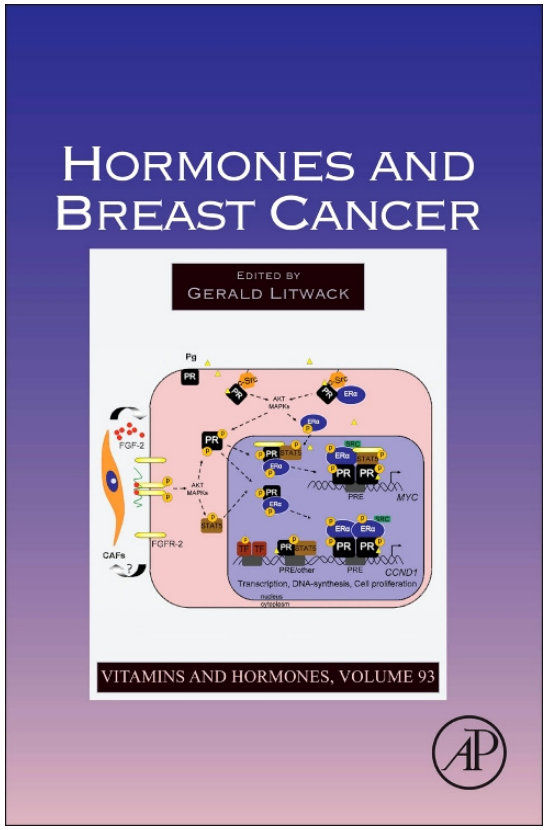

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CHAPTER ONE

Estrogen-Mediated Mechanisms to Control the Growth and Apoptosis of Breast Cancer Cells: A Translational Research Success Story

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Abstract

The treatment and prevention of solid tumors have proved to be a major challenge for medical science. The paradigms for success in the treatment of childhood leukemia, Hodgkin's disease, Burkett's lymphoma, and testicular carcinoma with cytotoxic chemotherapy did not translate to success in solid tumors—the majority of cancers that kill. In contrast, significant success has accrued for patients with breast cancer with antihormone treatments (tamoxifen or aromatase inhibitors) that are proved to enhance survivorship, and remarkably, there are now two approved prevention strategies using either tamoxifen or raloxifene. This was considered impossible 40 years ago. We describe the major clinical advances with nonsteroidal antiestrogens that evolved into selective estrogen receptor modulators (SERMs) which successfully exploited the ER

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target selectively inside a woman's body. The standard paradigm that estrogen stimulates breast cancer growth has been successfully exploited for over 4 decades with therapeutic strategies that block (tamoxifen, raloxifene) or reduce (aromatase inhibitors) circulating estrogens in patients to stop breast tumor growth. But this did not explain why high-dose estrogen treatment that was the standard of care to treat postmenopausal breast cancer for 3 decades before tamoxifen caused tumor regression. This paradox was resolved with the discovery that breast cancer resistance to long-term estrogen deprivation causes tumor regression with physiologic estrogen through apoptosis. The new biology of estrogen action has been utilized to explain the findings in the Women's Health Initiative that conjugated equine estrogen alone given to postmenopausal women, average age 68, will produce a reduction of breast cancer incidence and mortality compared to no treatment. Estrogen is killing nascent breast cancer cells in the ducts of healthy postmenopausal women. The modulation of the ER using multifunctional medicines called SERMs has provided not only significant improvements in women's health and survivorship not anticipated 40 years ago but also has been the catalyst to enhance our knowledge of estrogen's apoptotic action that can be further exploited in the future.

1. INTRODUCTION

Translational research is a conversation between the laboratory and clinical practice. Pharmacology has always been by definition translational research. The goal in the laboratory is to discover a weakness in the disease that can be exploited selectively to kill the infection (or at least stop disease progression and the death of the host), but without injuring the normal tissue. The key word here is "selectively," as the proposed strategy for disease treatment leaves the safety of the laboratory to enter the uncertain world of treating patients.

At the outset, we will consider the disease to be controlled and the relentless threat to the patient the disease presents. Breast cancer is unique with its most important drug target, the estrogen receptor (ER). What is unique is the fact that the ER is not tumor specific. The ER is ubiquitous in one form or another (ERα or ERβ) within a woman's body. Nevertheless, the most progress during the past 40 years in patient survivorship has been made by targeting the ER in breast cancer. We will examine two ideas that have been essential to reduce the death rate from breast cancer: first, how do we develop drugs to treat disease? Second, how do we ensure selectivity, that is, kill the disease and not the patient. The story will advance rapidly through the twentieth century, but as with all journeys of discovery, surprises were in store along the way and dogma destroyed. These surprises

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are at the heart of our conversation with nature that is necessary for progress in medical science to save lives.

We will first describe the stages of breast cancer and its incidence in various countries. This is important not only to appreciate the extent of the disease worldwide but also to provide a basis to understand how fashions in treatment have evolved. The first fashion was to treat what could be seen, that is, metastatic breast cancer (stage IV) by endocrine ablative surgery or the empirical use of high-dose hormone therapy (Kennedy, 1965a). Endocrine therapy was palliative and no significant gains were anticipated. After the palliative use of endocrine approaches to treat stage IV breast cancer for 70 years, by the 1970s, nobody cared about palliative endocrine therapy. By the 1960s, combination cytotoxic chemotherapy was showing dramatic promise for the treatment of stage IV breast cancer so combination cytotoxic chemotherapy was used as an adjuvant to destroy micrometastases (stages I and II) that could not be seen but were predicted to grow and cause a recurrence of the disease. Regrettably, success was modest and cures elusive. However, the change in fashion to embrace long-term adjuvant therapy with antihormones saved millions of lives worldwide. The subsequent discovery and development of selective estrogen receptor modulators (SERMs) (Jordan, 2001) was the key step in developing a practical approach to reduce the incidence of breast cancer but, at the same time, maintained a hope to be able to reduce the morbidity produced by other diseases such as osteoporosis, coronary heart disease, strokes, and endometrial cancer. It has therefore been possible over the past 40 years to address effectively the targeted treatment of all stages of breast cancer and prevent the disease. As a result prognosis, survivorship has been enhanced and breast cancer incidence can now be reduced not only in the high-risk population but also in the general population.

2. CLINICAL PRESENTATION OF BREAST CANCER

Of the 275,370 American women that are estimated to die in 2012 from cancer, 39,510 of them (or approximately 14%) are projected to die from cancer of the breast (Howlader et al., 2009). Of the baby girls born today, 12.38% will be diagnosed with breast cancer at some point in their lifetime; 2.76% will die from breast cancer (Howlader et al., 2009). With the exception of skin cancers, breast cancer is the most common of all cancers in women, accounting for about one-third of all diagnoses in the United States (Breast Cancer Facts & Figures, 2011–2012). In recent

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years, 124.3 out of 100,000 women per year have been diagnosed with invasive breast cancer in the United States (31.4 out of 100,000 women per year have been diagnosed with *in situ* breast cancer; 23 out of 100,000 died (Howlander et al., 2009)). The District of Columbia has had the highest number of deaths due to invasive breast cancer in women with 27.96 out of 100,000 (Howlader et al., 2009). Louisiana, New Jersey, Ohio Mississippi, Missouri, Maryland, and Virginia all have relatively high death rates (above 24.18 per 100,000) (Howlader et al., 2009). While White American women have the highest rate of breast cancer diagnosis, African American women have an increased mortality rate from breast cancer, with 31.6 out of 100,000 dying (Howlader et al., 2009).

According to the American Cancer Society, 89% of women with breast cancer will still be living 5 years after their diagnosis (Breast Cancer Facts & Figures, 2011–2012). In fact, as of 2008, there were about 2.6 million women alive in America who had at one time been diagnosed with breast cancer (Breast Cancer Facts & Figures, 2011–2012).

Breast cancer also accounts for about 14% of cancer deaths among Canadian women, second only to lung cancer (Canadian Cancer Statistics, 2012). In Canada, in 2012, there will be an estimated 96 cases of breast cancer per 100,000 women or about 22,700 new diagnoses, with Ontario and Nova Scotia having the highest incidences. Five thousand one-hundred Canadian women will die in 2012 from breast cancer—out of 36,000 total female cancer deaths—with Prince Edward Island having the highest breast cancer mortality rate (Canadian Cancer Statistics, 2012).

In Brazil, in 2008, there were 49,400 new cases of breast cancer with 50.7 cases per 100,000 women (EISRCM, 2006) representing 28% of cancers in women (INCA, 2006). In 2006, there were 10,834 deaths due to breast cancer (INCA, 2006). Malignant breast cancer is the seventh leading cause of death in Brazilian women (INCA, 2006). In the European Union, breast cancer represented about 30% of cancer incidences in women (Ferlay, Parkin, & Steliarova-Foucher, 2010), and about 16.6% of all female cancer deaths (Ferlay et al., 2010). In China, 168,013 new cases of breast cancer in women were estimated in 2005 (Yang, Parkin, Ferlay, Li, & Chen, 2005).

Breast cancer cases are divided into several stages, depending on the development of the disease. The population distribution of this relentlessly moving target, as it first occurs in the breast and subsequently breaks out, is illustrated in Fig. 1.1. Invasive breast cancer—or cancer cells from the breast that have overrun tissue beyond their origin, be it breast or other parts of the body—is divided into four stages. Potentially cancerous, abnormally

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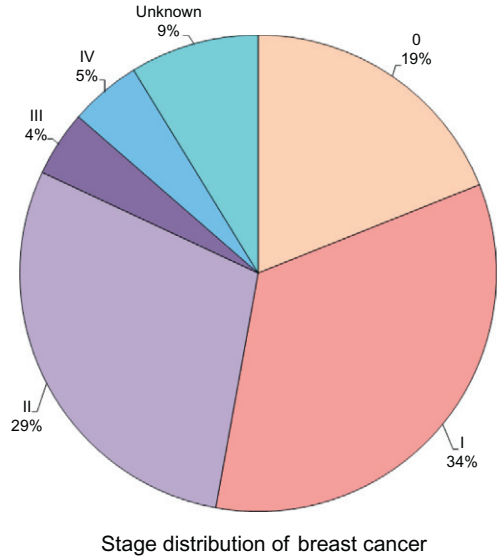

Figure 1.1 Percentage of each stage of breast cancer as recorded by SEER between 2002 and 2008 (Howlader et al., 2009; Ries, Eisner, & Kosary, 2001).

growing cells in the wall of the breast duct called ductal carcinoma *in situ*, or DCIS, is often referred to as stage 0 (Breast Cancer Survival Rates by Stage, 2011).

Stage I breast cancer is the first stage where the cancerous cells have spread into breast tissue away from the duct. This type of tumor is confined to the breast, and its diameter is no more than 2 cm. Stage II breast tumors have either spread to the lymph nodes under the arm or grown to be more than 2 cm in diameter (Breast Cancer Survival Rates by Stage, 2011).

Stage III breast cancer is known as "locally advanced cancer" and is divided into three subsections. Stage IIIA is when the tumor spreads to underarm lymph nodes that are attached to other bodily features (including other lymph nodes). Stage III also comprises tumors of greater than 5 cm diameter that have spread to isolated underarm lymph nodes. Stage IIIB is any breast tumor that has grown into the skin of the breast or into the chest wall. The size of the tumor is unimportant in stage IIIB classification. Stage IIIC tumors have either spread to the lymph nodes above or below the collarbone, or spread to the lymph nodes under the arm and behind the breastbone (Breast Cancer Survival Rates by Stage, 2011).

Metastatic breast cancer is known as stage IV. This cancer has spread from the breast to other organs. The brain, bones, and liver are frequent locations

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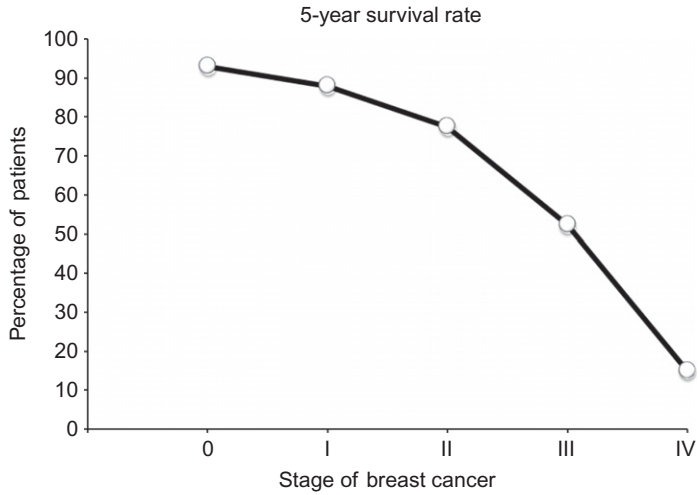

Figure 1.2 Five-year survival rates among the various stages of breast cancer (Breast Cancer Survival Rates by Stage, 2011).

for secondary breast cancers. Stage IV breast cancer has a poor prognosis with a 15% 5-year survival rate (Breast Cancer Survival Rates by Stage, 2011).

It is, therefore, important to stress that all "breast cancer" is not the same. We now know this from the molecular fingerprints from individual tumors that can be classified into subgroups (Hu et al., 2006; Perou et al., 2000; Sorlie et al., 2003). But personalized medicine has not yet arrived. Early detection and staging remain essential for survival (Fig. 1.2). Treatments with endocrine therapies have proved to be more successful the sooner they are deployed. But how did this happen?

3. TARGETED THERAPY

3.1. Foundations of chemical therapy

In 1908, Professor Paul Ehrlich was awarded the Nobel Prize for Medicine. In his Nobel Prize Lecture (Baumler, 1984), he described his work on antitoxins for diphtheria toxin and alluded to his side chain theory of receptors. However, he also alluded to his new studies on arsenicals (Baumler, 1984). He stated, "I want to show you that we are approaching the problem of obtaining an insight into the nature of the effects produced by drugs by following these points systematically, it will be easier than before to develop planned synthesis for pharmaceuticals targeted to requirements" (Baumler, 1984). He died of a heart attack and kidney failure on the afternoon of

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August 20th, 1915, so he was not to receive his second Nobel Prize for his discovery that changed pharmacology and the treatment of disease forever. Based upon his early experience discovering dyes that stain bacteria but not human cells, he conceived of the idea that chemicals could be synthesized to kill the disease-causing bacteria specifically. Through his research, he created the process of synthesizing analogues of known toxic chemicals, testing the efficacy and safety of chemicals in appropriate animal models of human disease, and a suitable candidate could then be tested in clinical trial.

Sahachiro Hata, in Erlich's team, created the appropriate animal models of disease and ultimately discovered that chemical 606 was completely effective against laboratory models of syphilis. Ehrlich approached Hoescht to enter into mass production for clinical trials. These trials worked spectacularly to cure a fatal disease and Salvarsan became the first specific chemical therapy (or chemotherapy). Professor Ehrlich had created the roadmap for drug discovery by the pharmaceutical industry, but he also turned from the treatment of infections to cancer research. In 1909, the press announced, "The beginning of the end of the cancer problem is in sight," and an editorial in *Scientific American* in 1912 stated, "Unquestionably, their [Ehrlich and Wasseman's] investigations justify the hope of a cure for human cancer" (Schrek, 1960). However, in 1915, Ehrlich admitted defeat and stated, "I have wasted 15 years of my life in experimental cancer" (Schrek, 1960). So it would remain for the next 30 years, but this stagnation would change with the first successful use of a chemical therapy to treat metastatic breast cancer (stage IV) (Haddow, Watkinson, Paterson, & Koller, 1944).

3.2. The first chemical therapy to treat cancer

The link between estrogen and the growth of breast cancer is a fascinating tale. The interconnected research ventures in endocrinology and chemistry during the first 40 years of the twentieth century would create a new dimension in therapeutics, result in the use of high doses of synthetic estrogens to treat some metastatic breast cancers successfully, but also create a paradox. If ovarian estrogens fuel the growth of breast cancer, why does a high dose of estrogen kill breast cancer cells in postmenopausal women? This paradox has only recently been solved and we will use this chapter to illustrate how the twists and turns of endocrine therapy have both revolutionized patient care and exposed a new biology of estrogen action: estrogen-induced apoptosis.

In 1896, George Beatson reported the first case of oophorectomy as a treatment for breast cancer (Beatson, 1896). Although it is often said that he performed the operation empirically, he actually relied on his knowledge

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that farmers had discovered there was a link between the ovary and the lactating mammary gland. In 1900, Boyd (1900) collected all-known cases of oophorectomy from hospitals around Britain and discovered there was a 30% response rate. This is perhaps the first "clinical trial" and gave the medical community new knowledge that has stood the test of time. The response rate to any endocrine therapy is 30%. The work during the early decades of the twentieth century on laboratory mouse models of breast cancer by Lathrop and Loeb (1916) and Lacassagne (1933) would be valuable to advance knowledge about hormones and breast cancer growth. However, an understanding of why oophorectomy was beneficial to treat breast cancer and which tumor would be responsive would remain a mystery until the 1960s. The first clues that the ovaries contained a substance that causes responses in a target organ were reported by Allen and Doisey (1923). They named their substance in pig ovary estrogen. They determined the biological effect by ovariectomizing mice to stop the estrous cycles and discovered that the vaginal epithelium would undergo replication and cornification when pig ovarian extract was injected. The animal model in the mouse (referred to henceforth as the "Allen–Doisy test") would be the essential test system to discover synthetic estrogens a decade later during the 1930s.

The story of the discovery of potent nonsteroidal estrogens is remarkable (Jordan, Mittal, Gosden, Koch, & Lieberman, 1985). With only a few early clues that simple synthetic molecules could initiate mouse vaginal cornification, two major groups of potent estrogenic compounds were described in the 1930s: the stilbenes (Dodds, 1938; Dodds, Goldberg, Lawson, & Robinson, 1938) of which diethylstilbestrol (Fig. 1.3) would become a key compound and used clinically, and the longer acting triphenylethylenes (Robson, 1937; Robson & Schonberg, 1942; Robson, Schonberg, & Fahim, 1938; Thompson & Werner, 1953). These two classes of compounds would be the essential tools with which to change breast cancer therapy but most of the therapeutic advances over the decades between 1930 and 1980 would be almost by chance. Remarkably, the successful translational research would enhance survival from breast cancer and significantly improve women's health. Two practical facts emerged during this period: estrogens support mammary and breast tumorigenesis and growth; but, estrogen was used routinely to treat and cause regression of some metastatic breast cancers. This paradox would lie dormant until its rediscovery during the past decade.

Lacassagne (1936a, 1936b), Shimkin and Wyman (1945, 1946), and Shimkin, Wyman, and Andervont (1946) contributed evidence that estrogens could increase mouse mammary tumorigenesis. Lacassagne (1936b)

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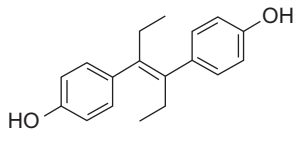
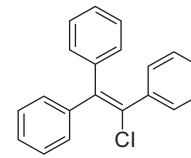
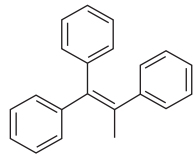


Diethylstilbestrol

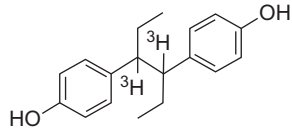


Triphenylchlorethylene

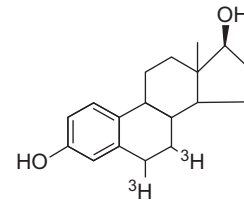


Triphenylpropene

High-specific activity radiolabeled estrogens to identify estrogen target tissues



[^{3}H] Hexestrol



6,7 [^{3}H] Estradiol

Figure 1.3 Compounds used by Haddow as the first "chemical therapy for cancer" (Haddow et al., 1944) and tritiated DES (hexestrol) and estradiol used in the first studies of retention of the estrogen in target tissues (Glascock & Hoekstra, 1959; Jensen & Jacobson, 1962).

went one step further at the Annual Meeting of the American Association of Cancer Research in Boston in 1936 by stating that

> *If one accepts the consideration of adenocarcinoma of the breast as the consequence of a special hereditary sensibility to the proliferative actions of oestrone, one is led to imagine a therapeutic preventative for subjects predisposed by their heredity to this cancer. It would consist—perhaps in the very near future when the knowledge and use of hormones will be better understood—in the suitable use of a hormone antagonistic or excretory, to prevent the stagnation of oestrone in the ducts of the breast.*

Unfortunately, there would be no "therapeutic antagonist" to use clinically until tamoxifen started its journey as an antiestrogen for the treatment of breast cancer (Jordan, 2003c, 2008b) some 40 years later!

In the first half of the twentieth century, breast cancer treatment was severe and unsuccessful. Radical mastectomy was the standard of care, radiation

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therapy was advancing from an art to a science, and nonspecific cytotoxic chemotherapy started to be introduced to treat cancer in general after the end of the Second World War. Prospects for the patient in general were abysmal and the examination of the state-of-the-art breast cancer treatment in 1977 (Stoll, 1977b) was not too much more hopeful. Nevertheless, with the wisdom of insight, one counterintuitive observation in the 1940s was to act as a catalyst for the eventual discovery of targeted cancer therapies. Alexander Haddow, conducting laboratory studies, discovered that carcinogenic polycyclic hydrocarbons actually caused tumor regression in animals but clearly one could not apply this "translational therapy" to patient care. However, he reasoned that the polycyclic synthetic estrogens had a similar sort of structure as the carcinogens (scary but true!), so following testing in the laboratory he compared and contrasted high-dose DES and triphenylethylenes (Fig. 1.3) as treatments for prostate cancer, breast cancer, and "other cancers." Prostate cancer responded as did metastatic breast cancer (stage IV) (30%) but none of the "other cancers" responded (Haddow et al., 1944). The application of high-dose estrogen therapy to provide palliative treatment for some postmenopausal women with metastatic breast cancer was the first chemical therapy to treat any cancer successfully. This approach became the standard of medical care in both the United Kingdom and the United States of America (Kennedy, 1965b; Kennedy & Nathanson, 1953) for the next 30 years until the resurrection of the triphenylethylene-based antiestrogen tamoxifen (Jordan, 2003c). In 1970, Sir Alexander Haddow FRS, during the inaugural Karnofsky (Haddow, 1970) lecture (the highest honor bestowed by the American Society for Clinical Oncology), stated his concerns for the future of specific and effective cancer therapy.

> *In the first place, the fact that the cancer cell is but a modification of the normal somatic cell holds out little prospect of a chemotherapiaspecifica in Ehrlich's sense, whereby chemical substances which, on the one hand, are taken up by certain parasites and are able to kill them, are, on the other hand, tolerated well by the organism itself, or at any rate without too great damage.*
>
> *(Haddow, 1970)*

In his Karnofsky lecture, Haddow also mentioned the importance of the few breast tumors that just melted away during high-dose estrogen therapy. However, he stated,

> *. . . the extraordinary extent of tumour regression observed in perhaps 1% of postmenopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us . . .*
>
> *(Haddow, 1970)*

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It is also important to stress that, at the time of Haddow's Karnofsky lecture in 1970, bacteria were routinely grown in the laboratory for testing antibiotic sensitivity; the right antibiotic could then be used appropriately to treat the right disease. No such tests existed for cancer. Practice was to give the drug and hope it might work. Therefore, the definition of the anticancer mechanism of DES in some breast tumors was the essential first step to determine which tumors will respond and which will not. What is the target for drug sensitivity or in Ehrlich's terms—the receptor? One study in 1949 by Walpole and Paterson (1949) declared defeat but the answer to the question "why" was to come ultimately from DES itself. The stilbene can be hydrogenated with tritium across the double bond to produce high-specific activity [^{3}H] hexestrol (Fig. 1.3). Hexestrol is a potent estrogen. Glascock and Hoekstra (1959) in fact showed the binding of [^{3}H] hexestrol in the estrogen target tissues of sheep and goats. The idea was subsequently translated to a clinical study in patients with metastatic breast cancer. Those patients whose breast tumor retained [^{3}H] hexestrol were more likely to respond to endocrine ablation (Folca, Glascock, & Irvine, 1961). These very preliminary findings were refined first by Jensen and Jacobson (1962) using [^{3}H] estradiol (Fig. 1.3) to describe the binding and retention of estradiol in the estrogen target tissues (uterus, vagina, pituitary gland) of the immature rat. Tritiated estradiol was bound initially, but not retained in tissues (muscle, lung) that were not targets of estrogen action. Gorski's group subsequently extracted and identified the soluble ER from the immature rat uterus (Toft & Gorski, 1966; Toft, Shyamala, & Gorski, 1967). These data were rapidly translated to identify the ER in breast tumors (Jordan, Wolf, Mirecki, Whitford, & Welshons, 1988) and there was a spectrum of none to a lot. Gorski's group discovered (Toft et al., 1967) that the extracted ER from target tissues could subsequently be liganded with [^{3}H] estradiol *in vitro*, so there was no need to inject radioactive estrogens into patients. The Jensen group went on to establish sucrose density gradient analysis as the method of choice to identify the breast tumor ER in the United States. In 1974 (McGuire, Carbone, & Vollmer, 1975), an NCI conference to consider the value of the ER assay to predict responsiveness of metastatic breast cancer to endocrine ablation or DES concluded that the absence of ER in a breast tumor predicted that the tumor would not respond to endocrine ablation or DES. If ER was present, there was about a 60% probability of an objective response. Thus, patients with ER-negative tumors should not be treated with endocrine ablation surgery; it would be worthless. At that time, in the mid-1970s, medical practice changed in America with a requirement that all patients with a diagnosis of breast cancer should have an ER assay on their tumor

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tissue. By the end of the 1970s, ER assay laboratories were springing up at most academic institutions (V.C.J. was involved in establishing one at the Worcester Foundation for Experimental Biology, Massachusetts in the early 1970s and was director of the steroid receptor laboratory at the Ludwig Unit in Bern, Switzerland (1979), organizing international quality control for the Ludwig clinical trials group, and the steroid receptor laboratory at the University of Wisconsin Clinical Cancer Center in the 1980s).

It should again be stressed that during the 1960s and 1970s the therapeutics of breast cancer was primitive. Only metastatic disease (stage IV) was addressed with therapy and this stage is fatal within a few years (Fig. 1.2). But the therapeutic options slowly evolved and this story again has its origins in the interest in synthetic estrogens. The synthetic estrogens, stilbenes or triphenylethylenes, used by Haddow in the 1940s (Haddow et al., 1944) (Fig. 1.3) were synthesized by Imperial Chemical Industries (ICI) Ltd. (now Astra Zeneca) but they were not alone in their interest in estrogens. Numerous pharmaceutical companies during the 1950s were interested in synthetic estrogens primarily because of the revolution in therapeutics that occurred with the development of the oral contraceptive that emanated from the vision of Gregory Pincus at the Worcester Foundation (Speroff, 2009). His chemical method stopped ovulation in the woman. No egg—no baby. It was reasoned by chemists in the pharmaceutical industry that if only another novel chemical method of contraception could be discovered, then the use of chemicals to prevent pregnancy, which was not a disease, could be expanded.

Leonard Lerner, a young scientist in the pharmaceutical industry in the 1950s, would take the next conceptual advance in reproduction research; that step would fail, but open the door for others to create the first targeted therapy for any cancer, the first endocrine therapy to save hundreds of thousands of women's lives, and the first chemical therapy approved to reduce the incidence of breast cancer in women of high risk for the disease. This did not occur because there was a specific plan by the pharmaceutical industry. The advance with tamoxifen would come from ICI Pharmaceuticals Division where their fertility control program would discover and then abandon ICI 46,474 to be resurrected and advanced by individuals with close friendships and who were in the right place at the right time and ready to exploit a unique opportunity.

3.3. Nonsteroidal antiestrogens

Leonard Lerner was tasked within the William S. Merrell Company to study nonsteroidal estrogens. At the time, the company marketed

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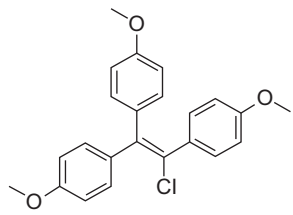
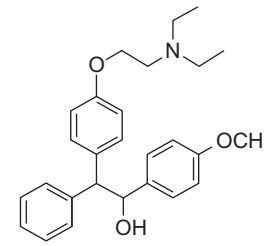
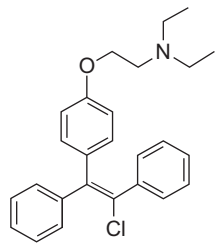
trianisylchlorethylene (TACE) (Fig. 1.4), but Lerner noticed a compound in the cardiovascular program was similar in structure—MER25 (Fig. 1.4) (Lerner, 1981). He tested the triphenylethanol and could detect no estrogenic activity in any species tested but it was a weak blocker of estrogen action (Lerner, Holthaus, & Thompson, 1958). However, what electrified the pharmaceutical industry was that MER25 and its successor clomiphene (Fig. 1.4) were postcoital antifertility agents in rats. Unfortunately, in clinical trial, the nonsteroidal antiestrogens were effective in inducing ovulation in subfertile women, so hopes of making a blockbuster drug disappeared. Clomiphene was tested as a breast cancer drug in metastatic disease (Hecker et al., 1974), as was nafoxidine (Legha, Slavik, & Carter, 1976), but development was abandoned because of concerns about toxic side effects (Fig. 1.4). No one was recommending careers in failed antifertility drugs or cancer therapy. Arthur Walpole was the head of the Fertility Control Program at ICI Pharmaceuticals Division in Alderley Park, Cheshire. He was interested in cancer research but was tasked to improve the toxicology profile of clomiphene that increased circulating desmosterol. Desmosterol was



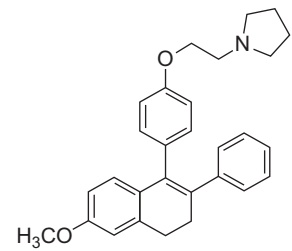
Trianisylchlorethylene (TACE)



Ethamoxytriphetol (MER25)



Clomiphene (mixture of *cis*- and *trans*-isomers)



Nafoxidine

Figure 1.4 Structures of nonsteroidal estrogens and antiestrogens mentioned in the text.

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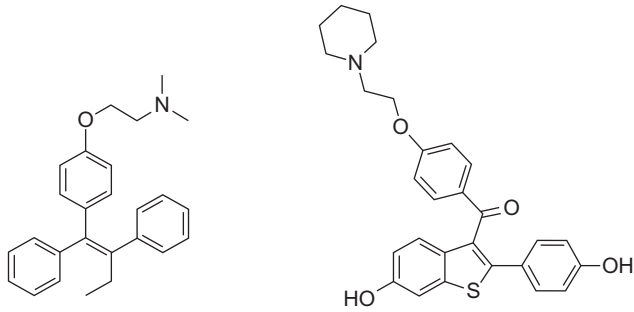
associated with cataract formation in women (Laughlin & Carey, 1962). The result of the antifertility program at Alderley Park in the 1960s was ICI 46,474, the *trans*-isomer of a substituted triphenylethylene (Fig. 1.5) that was antiestrogenic with postcoital antifertility properties in the rat (Harper & Walpole, 1967a, 1967b). The patent application read,

> *The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolaemic activity (Jordan, 2003c).*

Preliminary clinical studies demonstrated modest anticancer activity in metastatic breast cancer in postmenopausal women (Cole, Jones, & Todd, 1971) and the induction of ovulation in subfertile women (Klopper & Hall, 1971). However, after a review of all the data at Alderley Park in 1972, the Research Director decided to terminate clinical development—there was no financial future in ICI 46,474 (Jordan, 2006). However, Walpole reasoned that the company should put ICI 46,474 on the market as an orphan drug for the treatment of metastatic breast cancer and the induction of ovulation for subfertile women and "outsource" work to discover a strategy for the clinical use of tamoxifen. Walpole had recently met and examined the Ph.D. thesis of a young graduate student, Craig Jordan, in the Department of Pharmacology at the University of Leeds. Jordan was now spending 2 years as a visiting scientist as the Worcester foundation. Why not sponsor his research with an unrestricted grant? Let Jordan develop a clinical strategy for a nonsteroidal antiestrogen for the treatment of breast cancer.



Tamoxifen
(ICI 46,474 a failed
"morning after pill")

Raloxifene
(formerly keoxifene a
failed breast cancer drug)

Figure 1.5 Structures of tamoxifen and raloxifene.

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Scholarships were made available for Jordan students, technician's salaries were provided, and hundreds of laboratory rats were chauffeured from Alderley Park to the University of Leeds. This personal story has recently been told elsewhere (Poirot, 2011), but it is time now to focus on the pioneering medicine tamoxifen and how it not only changed breast cancer therapy but also acted as the catalyst to create new knowledge about the pharmacology of nonsteroidal antiestrogens that sequentially led to selective ER modulators, chemoprevention, the science of acquired drug resistance to antihormone therapy, and the new biology of estrogen-induced apoptosis.

4. TRANSITION TO TAMOXIFEN

Tamoxifen is unique in the annals of cancer therapeutics. While it is the first targeted therapy to treat cancer (a nonsteroidal antiestrogen targeted to the ER to stop estrogen-stimulated growth), the selective toxicity of tamoxifen was lucky. There are ERs all around the postmenopausal women's body, but as it turned out, these ERs do not appear to play a significant role in physiological homeostasis. Indeed, it was lucky that tamoxifen was also an antitumor agent in the premenopausal women without significant actions on normal physiology. Tamoxifen is approved by the FDA for the treatment of all stages of breast cancer, DCIS, male breast cancer, and for the reduction of breast cancer risk in both high-risk pre- and postmenopausal women. No other cancer therapy has such a spectrum of approved applications. At the outset of the translational research studies in the early years of the 1970s, it could not have been anticipated that a palliative medicine, FDA approved in December 1977 for the short-term (1–2 years) disease control of one in three postmenopausal patients with metastatic breast cancer, could so dramatically change the prognosis and survivorship for millions of women with ER-positive early breast cancer. During the 1970s, a laboratory strategy was put in place that would ultimately revolutionize thinking about the approach to treating breast cancer by targeting the tumor, killing the cancer cells not the patient, and treating earlier stages of the disease or even women only at risk for developing breast cancer (Jordan, 2008b). In the 1970s, the new fashion in therapeutics was combination cytotoxic chemotherapy that declared victory in childhood leukemia and was in the process of mopping up Hodgkin's disease (Stoll, 1977a). Justifiably, cytotoxic chemotherapy was king and only the appropriate acronym of drugs had now to be discovered to cure breast cancer. By contrast, no one in the

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pharmaceutical industry or clinical oncology was advocating a massive effort in endocrine therapy—or in fact any effort. Few cared.

The three publications that presaged the future clinical promise of tamoxifen as a pioneering medicine were all published in the *European Journal of Cancer* (Jordan, 2008b). The idea that tamoxifen blocked estrogen-stimulated breast cancer growth through blocking estradiol binding to the ER was controversial but was demonstrated both biochemically (Jordan & Koerner, 1975) and in cell culture (Lippman & Bolan, 1975). However, although these data were embraced in the United States, the same was not true for the United Kingdom where no clear clinical correlations between ER and tumor response could be demonstrated in clinical trial for the next 15 years (NATO, 1983; SCTO, 1987). Conceptually, this was important because the Europeans tended toward palliative applications with endocrine therapy, whereas in the United States, the goal was cure with combination cytotoxic chemotherapy. Simply stated, nobody cared about the mechanism of tamoxifen action but the good news was that in the United Kingdom everyone with breast cancer was to receive tamoxifen. This inadvertent policy was perhaps the correct decision for the wrong reason that ensured survivorship for tens of thousands of women in the United Kingdom.

The second conceptual advance was the finding that two sustained release subcutaneous injections of tamoxifen at the same time—as oral administration of dimethylbenzanthracene (DMBA) to 50-day-old female Sprague–Dawley rats, would prevent the initiation and growth of mammary carcinogenesis (Jordan, 1976). This observation was expanded (Jordan, Allen, & Dix, 1980; Jordan, Naylor, Dix, & Prestwich, 1980) and subsequently used as important laboratory evidence by Dr. Trevor Powles to explore the potential of tamoxifen to be used in the chemoprevention of breast cancer in high-risk women (Powles et al., 1989). The new dimension of the chemoprevention of breast cancer arrived in 1998 with the FDA approval of the pioneer tamoxifen for reducing the incidence of breast cancer in pre- and postmenopausal women at high risk (Fisher et al., 1998; Powles et al., 1998; Veronesi et al., 1998).

The third paper and advance that translated to clinical trial ultimately extended the survivorship of perhaps millions of women receiving long-term adjuvant tamoxifen therapy to prevent the recurrence of ER-positive breast cancer in patients with node-positive or node-negative breast cancer (stages I and II). In the early 1970s, the dilemma was when to use combination cytotoxic chemotherapy in the treatment plan for

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breast cancer. There was great enthusiasm that the use of combination cytotoxic chemotherapy would eventually lead to the cure of breast cancer. Very good results had been noted during the late 1960s (Cooper, 1969) and now a new strategy was considered: adjuvant therapy to destroy micrometastatic disease that had spread systemically after the woman had a mastectomy and local radiation. The strategy seemed sound that combination cytotoxic would cure patients with a low tumor burden. Regrettably, early results were modest (Bonadonna et al., 1976; Fisher et al., 1975) with the best effect noted in premenopausal patients. However, subsequent work demonstrated that cytotoxic chemotherapy destroys the ovary so the treatment could reasonably be interpreted as an aggressive ovarian ablation (Jordan, 1998). With the slow development of the antiestrogen tamoxifen during the 1970s, attentions started to focus not on the palliative use of tamoxifen for metastatic breast cancer but on the idea that tamoxifen might have potential as an adjuvant therapy. In the laboratory, the DMBA rat mammary carcinoma model was considered to be "state of the art" for the study of the endocrine treatment of breast cancer. Huggins, Grand, and Brillantes (1961) first showed that a single oral administration of 20 mg DMBA to 50-day-old female Sprague–Dawley rats would produce multiple mammary carcinomas in all rats within 150 days after DMBA treatment. The development of tumors was endocrine dependent; the tumors contained ER and regressed in response to ovariectomy (Welsch, 1985). In the absence of any other experimental options, other than the DMBA model, to explore adjuvant therapy with tamoxifen, different durations of tamoxifen were used (or its potent metabolite 4-hydroxytamoxifen discovered around this time(Jordan, Collins, Rowsby, & Prestwich, 1977; Jordan, Dix, Naylor, Prestwich, & Rowsby, 1978) as tamoxifen actions was the sum of its antiestrogenic metabolites) to determine if a short course of the antiestrogen for a month (equivalent to a year in women as adjuvant therapy) would be cidal (Lippman & Bolan, 1975) or whether longer durations would be necessary to prevent tumor development. The idea was to destroy the early transformed cells, not unlike adjuvant therapy. The profound conclusion was that longer adjuvant therapy was going to be a better clinical strategy (Jordan, 1978; Jordan & Allen, 1980; Jordan, Allen, et al., 1980; Jordan, Dix, & Allen, 1979). The laboratory studies also derived another conclusion that was to have ramifications for the later use of polar nonsteroidal antiestrogen for the treatment of breast cancer. Tamoxifen was metabolically activated to 4-hydroxytamoxifen (Jordan et al., 1977, 1978). This was not a requirement for antiestrogenic activity

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but an advantage (Jordan & Allen, 1980). Polar nonsteroidal antiestrogens may be better at blocking estrogen actions at the ER but they had poor bioavailability and were rapidly excreted (Jordan & Allen, 1980). The subsequent idea that tamoxifen needed to be metabolically activated by hydroxylation of the primary metabolite *N*-desmethyltamoxifen to endoxifen was to preoccupy pharmacogenomics research on tamoxifen during the past decade with arguments both for and against the critical role of different CYP2D6 genotypes (Brauch et al., 2013; Dieudonne et al., 2009; Kiyotani et al., 2010; Lammers et al., 2010; Lash et al., 2011; Madlensky et al., 2011; Rae et al., 2012; Regan et al., 2012; Schroth et al., 2009). Simply stated, if CYP2D6 was aberrant then there is low metabolism to endoxifen and a lower probability of a response of the patients tumor to tamoxifen. Be that as it may, the fundamental issue in the 1970s was to select an appropriate duration of adjuvant tamoxifen therapy to test in breast cancer clinical trials.

The clinical community selected a 1-year course of adjuvant tamoxifen therapy in all early clinical trials (LBCSG, 1984; Rose et al., 1985). This was an obvious choice based on the limited effectiveness of tamoxifen to treat metastatic breast cancer. Tamoxifen is only effective for about 1 year (Ingle et al., 1981) so there was an understandable concern that longer adjuvant tamoxifen therapy would precipitate early drug resistance and recurrent disease that would now be fatal. But the studies with the DMBA rat mammary carcinoma model did not comply with clinical "predictions" based on the treatment of metastatic breast cancer. Short-term therapy (1 month equivalent to a year in a patient) was unable to control tumorigenesis in the rat but continuous therapy for six months (6 years in a patient) was 90% effective in controlling tumorigenesis (Jordan, Allen, et al., 1980). The DMBA rat model was to be proved to predict accurately subsequent clinical trials data. Five years of adjuvant tamoxifen therapy became the standard of care for the treatment of breast cancer for 20 years and remains so for the premenopausal patient.

There are several notable features of adjuvant tamoxifen therapy that were exposed during clinical trials and these data were enhanced and amplified by the regular review of ongoing adjuvant clinical trials through the Oxford Overview Analysis process. The survival advantage for these women taking long-term tamoxifen therapy is profound, whereas short term (1 year of treatment) is not of significance in premenopausal patients (Davies et al., 2011; EBCTCG, 1998, 2005). Most importantly, and we will examine this clinical observation in more detail during the discussions of acquired tamoxifen

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resistance, is the sustained decrease in mortality noted *after* 5 years of adjuvant tamoxifen. This was a surprising observation that now has a plausible scientific explanation. The science will be considered in Section 7.

The next step in the tamoxifen tale was the evaluation of its worth to prevent breast cancer in high-risk women. The evidence to support this decision to test the hypothesis in clinical trial was solid. The expanding database on tamoxifen as the endocrine adjuvant therapy of choice during 1980s and 1990s was reassuring for clinicians. Most important in this regard was the use of adjuvant tamoxifen therapy for the treatment of node-negative breast cancer because 80% of patients are cured by surgery and local radiotherapy, which meant that an increasing proportion of "cured" patients were already being treated with 5 years of adjuvant therapy (Fisher et al., 1989; SCTO, 1987). The fact that adjuvant tamoxifen reduced contralateral breast cancer (primary breast cancer) by 50% (Cuzick & Baum, 1985) was proof of principle: primary prevention would be successful and the earlier knowledge that tamoxifen prevented mammary tumorigenesis in rodents (Jordan, 1976) enhanced the opportunities for the clinical trials community.

Overall, the placebo-controlled clinical trials of chemoprevention demonstrated a significant decrease in the incidence of breast cancer following tamoxifen therapy that was sustained even when the drug treatment was terminated (Cuzick, Forbes, & Howell, 2006; Fisher et al., 2005, 1998; Powles, Ashley, Tidy, Smith, & Dowsett, 2007). However, the strategy was flawed as only a few women (2–5 per thousand per year) has their breast cancer prevented but hundreds of women per thousand would experience significant side effects such as menopausal symptoms and there would be an increased risk of deep vein thrombosis in postmenopausal women. Perhaps more serious was the finding in the laboratory that tamoxifen increased the growth of human endometrial cancer implanted in athymic mice but did block estrogen-stimulated growth of breast cancer completely in the same athymic mouse (Gottardis, Robinson, Satyaswaroop, & Jordan, 1988). These observations moved rapidly from the laboratory to clinical care within 3 years once the laboratory findings were confirmed in a placebo-controlled clinical trial (Fornander et al., 1989). Clinical findings demonstrated a three- to fivefold increase in the risk of developing endometrial cancer in postmenopausal women who now, as a treatment population, would have regular gynecological examinations when using tamoxifen. Although endometrial cancer was not significant for the treatment of breast cancer as the decreases in mortality were profound (EBCTCG, 2005), for the well women, this was a troubling side effect. It was said "one cancer was being substituted for

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another." In the prevention setting, this and the emerging new laboratory knowledge during the early 1990s that tamoxifen was a hepatocarcinogen in rats (Greaves, Goonetilleke, Nunn, Topham, & Orton, 1993; Hard et al., 1993) (this laboratory observation has never translated to patient populations—fortunately) mandated that a profoundly different strategy was essential, if chemoprevention was ever to be accepted as a reality in clinical practice.

5. SELECTIVE ESTROGEN RECEPTOR MODULATION

Up until the mid-1970s, the nonsteroidal antiestrogens were initially potential and then failed postcoital contraceptives. The antiestrogens became agents of interest to be exploited in gynecology. Both clomiphene and tamoxifen were successful for the induction of ovulation in subfertile women. A review by Lunan and Klopper (1975) focuses almost entirely on the potential applications in gynecology and there is only passing references to breast cancer treatment. By the mid-1980s, with tamoxifen FDA approved in December 1977 and adjuvant clinical trials well underway it was now time to consolidate all the information about the nonsteroidal antiestrogens as pharmacological agents (Jordan, 1984), so that further effective translational research could help patients. It was time also to review all that was known about tamoxifen (Furr & Jordan, 1984). After all, tamoxifen was, and is, the only nonsteroidal antiestrogen to be approved for the therapeutics of all stages of breast cancer and chemoprevention. It is, however, of interest to mention that tamoxifen had not been granted patent protection in the United States because of the perceived primacy of the earlier Merrel patents in the 1960s (Jordan, 2003c). That all changed in 1986 almost exactly at the time that long-term adjuvant tamoxifen therapy was the treatment strategy of choice for patients with ER-positive breast cancer (Consensus conference, Adjuvant chemotherapy for breast cancer, 1985). But it was the move toward using tamoxifen to prevent breast cancer in high-risk populations of women that now became the driving force behind understanding the "good, bad, and the ugly" of tamoxifen pharmacology. A surprise was in store.

It was reasoned at the time that, if estrogen was important to maintain bone density, then a nonsteroidal antiestrogen may prevent breast cancer in the few but create osteoporosis in the majority. The same argument was articulated about coronary heart disease and atherosclerosis, but it was already known in tamoxifen's patent (earlier described in Section 3.3) that circulating cholesterol was lowered by the drug (Harper & Walpole, 1967b).

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The question of bone loss with nonsteroidal antiestrogen was addressed in the ovariectomized and intact rat using tamoxifen and the failed breast cancer drug keoxifene (Fig. 1.5), also a nonsteroidal antiestrogen (Black, Jones, & Falcone, 1983). Both nonsteroidal antiestrogens actually prevented bone loss from ovariectomy and a combination with estrogen further improved bone density (Jordan, Phelps, & Lindgren, 1987). These breakthrough data were confirmed (Turner, Evans, & Wakley, 1993; Turner, Wakley, Hannon, & Bell, 1987, 1988; Turner et al., 1998), but initially, these data in the refereed literature were ignored by the pharmaceutical industry. They did, however, act as preliminary data to initiate a prospective placebo-controlled clinical trial with tamoxifen in postmenopausal breast cancer patients with node-negative breast cancer. This trial was initiated at a time when node-negative breast cancer patients did not receive adjuvant therapy as a standard of care. The Wisconsin Tamoxifen Study demonstrated that tamoxifen lowered low-density lipoprotein (bad) cholesterol, did not substantially reduce high-density lipoprotein (good) cholesterol (Love et al., 1990, 1991), and improved bone density measured by dual photon absorptiometry (Love et al., 1992).Thus, not only did the animal studies unexpectedly translate to potential clinical benefit but also a new concept and vision was about to change medicine.

The laboratory studies with keoxifene and tamoxifen on bone density showed estrogen-like actions, but parallel studies at the same time demonstrated that tamoxifen and keoxifene could prevent rat mammary carcinogenesis (Gottardis & Jordan, 1987), an antiestrogenic effect. Thus, this class of compounds including clomiphene (Fig. 1.4), which was mixed isomers that are estrogenic or antiestrogenic (Beall et al., 1985), had all shown a similar effect on bone in the rat. So the potential new drug group had the potential to turn on and turn off sites around the body. At this time, it was already known that tamoxifen was more estrogenic in the rodent uterus (Harper & Walpole, 1967b) and human endometrial cancer would grow with tamoxifen (Gottardis et al., 1988) so this again illustrated the target site specific actions. The complex of the "antiestrogen" with the ER was being interpreted differently at different sites around the body (Jordan & Robinson, 1987). The endometrial cancer issue clearly was a "bad" for tamoxifen but others in the class, like keoxifene, were less estrogen-like in the uterus (Black et al., 1983), less likely to stimulate endometrial cancer in patients (Gottardis, Ricchio, Satyaswaroop, & Jordan, 1990), and were already known to maintain or build bone (Jordan et al., 1987). A road map for industry was proposed and simply stated (Lerner & Jordan, 1990):

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Is this the end of the possible applications for antiestrogens? Certainly not. We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer.

In 1993, keoxifene was renamed raloxifene (Fig. 1.5) with patent protection to treat and prevent osteoporosis in postmenopausal women. The pivotal registration trial call Multiple Outcomes Relative to Evista was to demonstrate that raloxifene simultaneously could prevent fractures of the lumbar spine by about 50% (Ettinger et al., 1999) and reduce the incidence of ER-positive breast cancer by about 80% (Cummings et al., 1999) with no increase in endometrial cancer. Raloxifene became the first multifunctional medicine in women's health because of the positive results of the Study of Tamoxifen and Raloxifene (Vogel et al., 2006) where both drugs, now called selective ER modulators or SERMs, reduced breast cancer incidence in high-risk postmenopausal women by 50%. Two diseases, osteoporosis and breast cancer, were controlled by one drug. However, it was later shown (Vogel et al., 2010) that a 5-year course of raloxifene is not sufficient to maintain long-term benefit for the prevention of breast cancer-like tamoxifen. Raloxifene is approved by the FDA for indefinite administration for the prevention and treatment of osteoporosis, and breast cancer reduction is sustained during extended treatment (Martino et al., 2004). Again, the unanticipated merits of tamoxifen to sustain antitumor actions in chemoprevention (Powles et al., 2007) would raise the question why? A plausible answer would occur through serendipity and the examination of acquired drug resistance to SERMs in the laboratory.

6. ACQUIRED DRUG RESISTANCE AND THE SURPRISE OF SERMS

During the 1970s, the concept of acquired resistance to antihormone therapy was simple. Breast tumors were considered to be a mixture of cells: some were ER negative and some ER positive. The concentration of ER in a tumor was therefore an average of total tumor ER per unit protein, for

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example, 150 femtomoles ER per mg tumor protein. This was measured by extracting the unoccupied tumor ER, and following some competitive binding assay with tritiated estradiol plus/minus a massive excess of non-radioactive ligand, which was either an estrogen or an antiestrogen (Jordan et al., 1988), the total ER tumor concentrations was established. Based on the 1974 Conference in Bethesda (McGuire et al., 1975), it was decided that tumors would be classified as either ER positive (above 10 femtomoles/mg cytosol protein) or ER negative (below 10 femtomoles/mg cytosol protein). Tumors that were ER positive would most likely respond to endocrine ablation or high-dose estrogen therapy but ER-negative tumors were unlikely to respond (McGuire et al., 1975).

Failure of endocrine therapy (Ingle et al., 1981) usually occurs after about a year or two of treatment in metastatic breast cancer (stage IV). The received wisdom was that the ER-positive cells were dying and the tumor was being repopulated with ER-negative cells. However, this did not explain the fact that clinicians could identify an endocrine therapy responsive tumor that would respond and fail but then respond again to a different endocrine therapy. This could continue for several cycles and is referred to as the "endocrine cascade." Clearly some other mechanism of acquired resistance was occurring. The adaptations of the tumor to the environment during treatment were illustrated by the responses of some tumors to high-dose DES therapy. We noted earlier that Haddow observed that some tumors melted away, but during the 1960s and 1970s, Basil Stoll showed that some tumors would regress but they would regrow during DES therapy only to regress again once DES treatment was stopped (Stoll, 1977b). This was called a "withdrawal response." There was no explanation for all these events.

During the 1980s, with the general acceptance by the clinical community that clinical trials had to be started to test long-term adjuvant tamoxifen therapy, it became clear that there was a need for realistic laboratory models of acquired drug resistance to tamoxifen. These would be necessary to assess mechanisms of resistance and subvert the process, but more importantly, in the short term, to discover effective second-line therapies for patients that prematurely recur during adjuvant tamoxifen treatment.

Tamoxifen blocks estradiol-stimulated MCF-7 tumor growth when cells are inoculated into athymic mice (Osborne, Hobbs, & Clark, 1985). However, tamoxifen cannot control tumor growth indefinitely; eventually, MCF-7 tumors grow despite continuing tamoxifen treatment (Osborne, Coronado, & Robinson, 1987). This situation was examined from another

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perspective using serial transplantations of MCF-7 tumors with acquired tamoxifen resistance. Remarkably, the growth of tumors with acquired tamoxifen resistance is dependent upon tamoxifen (Gottardis & Jordan, 1988; Gottardis, Wagner, Borden, & Jordan, 1989) or indeed any SERM such as raloxifene or toremifene (O'Regan et al., 2002). Physiologic estrogen treatment also caused tumors to grow so the ER mechanism was reconfigured in the breast cancer cells to grow with either estrogen or tamoxifen as the binding ligand. No treatment or treatment with a pure steroidal antiestrogen ICI 164,384 (Gottardis, Jiang, Jeng, & Jordan, 1989) (the lead compound for the series that became the clinically approved drug fulvestrant) would therefore be predicted to be an appropriate clinical treatment strategy. These data in the laboratory presaged the subsequent clinical findings that either an aromatase inhibitor (no estrogen) or fulvestrant would be appropriate second-line therapies following treatment failure with tamoxifen (Howell et al., 2004; Osborne et al., 2002).

The issue of the development of acquired resistance to tamoxifen within a year or two when used for the treatment of metastatic breast cancer (stage IV) appeared to be replicated in the laboratory (Gottardis & Jordan, 1988), but the fact that adjuvant tamoxifen treatment could be continued for 5 years without rapid early treatment recurrences was not explained by the laboratory models developed in the 1980s. Again serendipity intervened with a chance observation that opened up the study of a new biology of estrogen-induced apoptosis.

7. ESTROGEN-INDUCED APOPTOSIS: BACK TO THE BEGINNING

The MCF-7 tumor model of acquired resistance to tamoxifen was a significant advance for the study of SERM resistance, but the tumor biology could only be retained *in vivo*, through repeated transplantation into generations of athymic mice every 4 or 5 months. Cell culture models of antihormone therapy were becoming available (Sweeney, McDaniel, Maximov, Fan, & Jordan, 2012) once it was realized that the MCF-7 cell line, that had actually been derived from a patient treated with high-dose DES, was subsequently grown and propagated *in vitro* in a media rich in an estrogen as a contaminant of the phenol red redox indicator (Berthois, Katzenellenbogen, & Katzenellenbogen, 1986; Bindal, Carlson, Katzenellenbogen, & Katzenellenbogen, 1988; Bindal & Katzenellenbogen, 1988). Studies removing all estrogens from media

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initially cause MCF-7 cells to die but remaining cells adapt and grow independent of estrogen but retain the ER (i.e., do not become ER negative) (Katzenellenbogen, Kendra, Norman, & Berthois, 1987; Welshons & Jordan, 1987). These early studies would replicate the action of aromatase inhibitor on the ER-positive tumor. During the next decade, numerous cell lines would be created (Herman & Katzenellenbogen, 1994; Jiang, Wolf, Yingling, Chang, & Jordan, 1992; Masamura, Santner, Heitjan, & Santen, 1995; Pink, Jiang, Fritsch, & Jordan, 1995; Shim et al., 2000) that would yield further insights into estrogen action in the twenty-first century. However, the breakthrough in the understanding of the evolution of acquired resistance to tamoxifen was to come from the years of retransplantation of MCF-7 tumors into tamoxifen-treated athymic mice. Continuous retransplantation into tamoxifen-treated mice over a 5-year period changes the tumor cell response to physiological estrogen treatment from a survival signal to a trigger of apoptosis (Wolf & Jordan, 1993; Yao et al., 2000). Small tumors do not grow with physiologic estradiol treatment but melt away completely. Large tumors undergo dramatic regression but eventually start to regrow vigorously with continuing estradiol treatment. Retransplantation of the growing tumors into new athymic mice demonstrates growth is dependent upon estrogen treatment, no treatment results in no tumor growth, and tamoxifen again inhibits estradiol-stimulated growth (Fig. 1.6). The estrogen destroys cells with acquired resistance to tamoxifen with the remaining tumor tissue again responsive to tamoxifen treatment. These laboratory findings were reproducible and exhibited a cyclical pattern of sensitivity and resistance indicating a plasticity in the tumor cell population (Balaburski et al., 2010; Yao et al., 2000). They also suggested a mechanism to explain the sustained and enhanced antitumor effect of tamoxifen *after* a long duration of the SERM had been administered (at least 5 years). It was proposed that acquired drug resistance evolves through Phase I resistance where both estrogen and tamoxifen stimulate tumor growth and then the survival mechanisms are reconfigured so that, in Phase II that occurs before 5 years, only tamoxifen supports the survival of micrometastases; physiologic estrogen causes tumor cell death (Fig. 1.6) (Jordan, 2004). The use of adjuvant tamoxifen for 5 years prepares the micrometastatic disease to be destroyed by the woman's own estrogen once the tamoxifen is stopped (Wolf & Jordan, 1993). Mortality continues to decrease as micrometastatic disease is eradicated (EBCTCG, 2005). The same events would explain the sustained effects of long-term tamoxifen treatment in the chemoprevention setting (Cuzick et al., 2006; Fisher et al., 2005; Powles et al., 2007).

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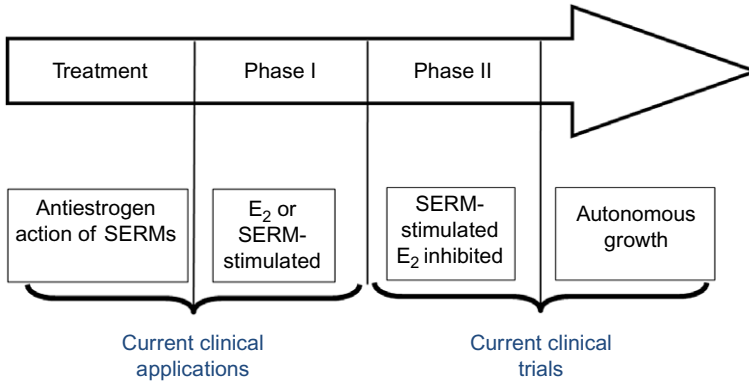

Figure 1.6 The evolution of drug resistance (Jordan, 2004).

Table 1.1 Response rates to high-dose DES treatment of breast cancer patients in Lonning et al. (2001)

Response		
Complete	**Partial**	**Stable disease**
4[a]/32	6/32	2/32

[a]One patient remains disease-free 10 years and 6 months after commencing DES treatment.

These laboratory data (Wolf & Jordan, 1993; Yao et al., 2000) also proposed the potential use of high- or low-dose estrogen treatment as a salvage therapy for patients who had received exhaustive (i.e., the endocrine cascade) antihormone therapy. This strategy has been evaluated clinically. Lonning et al. (2001) noted an overall 30% response rate to high-dose DES (15 mg daily) (Table 1.1) and one patient had a remarkable response continuing to last now for over 10 years.

> *One of the patients (AO) who achieved a complete response of a 16 × 16 mm cytological confirmed chest wall relapse, received DES treatment for five years, where after she been subject to regular follow-up without active treatment. To this day, she remains disease-free 10 years and six months after commencing DES treatment.*
>
> *(Lonning, 2009)*

Recently, Ellis et al. (2009) have tested the "low-dose" estrogen therapy hypothesis (Yao et al., 2000) and noted a similar clinical benefit (~29%) for women receiving either 6 mg estradiol daily or 30 mg estradiol daily, after failing an aromatase inhibitor. Responses were not as profound in the Ellis study (Ellis et al., 2009) compared with the Lonning study

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(Lonning et al., 2001) probably because the patients in the Ellis study were not treated "exhaustively" with antihormones and had therefore not evolved to Phase II endocrine resistance.

The "Phase II" tamoxifen resistance model also taught another interesting lesson. The pure antiestrogen fulvestrant produced tumoristasis, whereas physiologic estrogen causes profound tumor regression starting after about 1 week (Osipo, Gajdos, Liu, Chen, & Jordan, 2003). However, a combination of fulvestrant plus physiologic estrogen causes dramatic tumor growth (Osipo et al., 2003). These data imply that a combination of fulvestrant and aromatase inhibitor as endocrine therapy following failure of long-term tamoxifen treatment may produce better tumor control than fulvestrant alone (presupposing one does not use physiologic estrogen to treat patients alone first!) Although results are not exactly optimal, two large treatment trials have recently been published using similar dosage regimens. One shows significant PFS and survival advantages for the combination (Mehta et al., 2012), whereas the other does not (Bergh et al., 2012). However, neither trial uses optimal fulvestrant therapy, that is, 500 mg, or twice the recommended monthly dose of 250 mg (Di Leo et al., 2010).

With the relentless rise of interest in the development of an aromatase inhibitor to replace tamoxifen as the long-term adjuvant therapy of choice for postmenopausal women, studies of resistance moved naturally to study the effect of estrogen withdrawal on ER-positive cells *in vitro*. The experimental results (Song et al., 2001) were to dovetail nicely into results from prior studies with tamoxifen *in vivo* (Yao et al., 2000). Long-term estrogen-deprived (LTED) MCF-7 cells could initially gain a "supersensitivity" to estrogen in the environment once the main source of estrogen had been removed. In other words, the original studies (Katzenellenbogen et al., 1987; Welshons & Jordan, 1987) that demonstrated initial cell death when MCF-7 cells were exposed to an estrogen-free environment, but then a population of cells grew spontaneously. This "estrogen-free growth" was interpreted as the cells being selected that were "hypersensitive" to extremely low estrogen concentrations (Masamura et al., 1995; Shim et al., 2000). But further examinations of concentration response relationship showed that estrogen-induced apoptosis occurred in these cells (Song et al., 2001), but not just at high concentrations but at low concentrations as predicted by the MCF-7 tamoxifen-resistant model *in vivo* (Jordan, Liu, & Dardes, 2002). Specific clones of MCF-7 cells generated from populations of LTED MCF-7 cells (Jiang et al., 1992; Pink et al., 1995) can undergo immediate estrogen-induced apoptosis (MCF-7:5C)

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(Lewis et al., 2005) or apoptosis induced by estrogen a week later (MCF-7:2A) (Ariazi et al., 2011).

Several facts are emerging to understand the new biology of estrogen-induced apoptosis. The molecular events to trigger apoptosis with physiologic estrogen initiate the mitochondrial or intrinsic pathway first and then for the final execution there is recruitment of the extrinsic pathway (Lewis et al., 2005). This process is fundamentally different to cytotoxic chemotherapy that immediately causes a G1 blockade with a commitment to program cell death within 12 h. Massive DNA disruption requires immediate action by the cell.

What then is the physiologic trigger for estrogen-induced apoptosis? Apoptosis caused by estrogen can be modulated and is dependent upon the shape of the ligand ER complex. Estrogens are classified (Jordan et al., 2001) into Class I or planar estrogens such as estradiol or DES and Class II or angular estrogens such as hydroxylated triphenylethylenes. Both classes of estrogen *cause* cell replication but only Class I estrogens, which permit the ligand to be sealed within the ligand-binding domain (Brzozowski et al., 1997; Shiau et al., 1998), can initiate immediate estrogen-induced apoptosis in the correctly configured estrogen-deprived breast cancer cell. Coactivators must bind to the ER complex for growth or apoptosis (Hu et al., 2011). By contrast, an estrogenic triphenylethylene in Class II alters the shape of the ER complex "so that it temporarily adapts to the shape of an antiestrogenic ER complex" (Maximov et al., 2011) which cannot adequately bind coactivators to delay estrogen-induced apoptosis. These data dramatically illustrate the promiscuous nature of cell replication and survival with almost any signal input that is minimally adequate to bind to the ER. The signal for death must be precise because it is final for the cell.

One obvious application for the discovery of the cellular mechanisms that *prevent* estrogen-induced apoptosis is to deploy a companion therapy to neutralize resistance to apoptosis and enhance responsiveness to estrogen. Looked at simply, it would be an advantage to enhance apoptosis and convert clinical responses of 30% for estrogen-treated patients following exhaustive antihormone therapy to over 50%. Two approaches have addressed the goal of enhancing response rates to physiologic estrogen-induced apoptosis. First, the MCF-7:2A cells have a delayed response to estrogen-induced apoptosis and also have an enhanced glutathione synthetic pathway (Ariazi et al., 2011). Glutathione protects against oxidative stress. The administration of buthioninesulphoximine that blocks the synthesis of glutathione causes rapid estrogen-induced apoptosis (Lewis-Wambi, Swaby, Kim, & Jordan, 2009; Lewis-Wambi et al., 2008). Second, it was believed that blocking the cSrc

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oncogene, which is present in 70% of human breast cancer, would further enhance estrogen-induced apoptosis of the breast cancers treated exhaustively with antihormone therapy. In fact, blocking cSrc actually also blocked estrogen-induced apoptosis (Fan et al., 2012). This was not the anticipated result, but this is new knowledge that must, in the future, be considered when dissecting the trigger mechanism of estrogen-induced apoptosis. It could not have been predicted that cSrc was essential for estrogen-induced apoptosis. The discovery of the precise triggering mechanism for estrogen-induced apoptosis will, because it is biologically unique, provide additional approaches to discover new targeted therapies.

8. THE LEGACY OF TAMOXIFEN

In 1970, there was no tamoxifen, only ICI 46,474, a failed "morning after pill," that was abandoned by the pharmaceutical industry in 1972 (Jordan, 2003c, 2006). By a series of fortunate friendships and the key individuals being in the right place at the right time, the first target drug in breast cancer therapy, tamoxifen, was reinvented (Jordan, 2008b) to become a life-saving medicine, the first SERM, the first chemopreventive drug to reduce the risk of any cancer and the drug that would throw light on the "mechanism of estrogen-induced apoptosis" solving Haddow's paradox when he deployed the first chemical therapy, high-dose estrogen, to treat breast cancer successfully (Jordan, 2008a).

There are two additional therapeutic advances that tamoxifen catalyzed: the aromatase inhibitors and the development of the SERM principle as a multifunctional drug group.

Angela Brodie's dedicated and pioneering work (Brodie & Longcope, 1980; Brodie, Marsh, & Brodie, 1979; Brodie, Schwarzel, Shaikh, & Brodie, 1977; Coombes, Goss, Dowsett, Gazet, & Brodie, 1984) was essential as proof of principle that a selective aromatase inhibitor could be discovered with clinical efficacy. The problem with her discovery, 4-hydroxyandrostenedione, was that it was an injectable rather than a more convenient oral preparation. However, the fact that the failed "morning after pill" ICI 46,474 was transformed successfully into the "gold standard" tamoxifen for the adjuvant treatment of breast cancer provided a new target (the aromatase enzyme) to improve antihormonal therapy in breast cancer. With profits expanding from sales of tamoxifen in the United States after 1990, the key issue for the successful drug development of an aromatase inhibitor would be satisfied: profits. The patent from tamoxifen would

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run out in America, and aromatase inhibitors be substituted. Three orally active third-generation aromatase inhibitors were subsequently successfully developed for adjuvant therapy: anastrazole, letrozole, and exemestane. Each was demonstrated to have a small but consistent improvement over 5 years of tamoxifen alone whether given instead of tamoxifen in postmenopausal patients, after 5 years of tamoxifen or switching after a couple of years of tamoxifen (Baum et al., 2002; Boccardo et al., 2005; Coates et al., 2007; Coombes et al., 2004; Goss et al., 2003, 2005; Howell et al., 2005; Thurlimann et al., 2005). There has even been a successful trial of exemestane as a preventive in postmenopausal high-risk women (Goss et al., 2011). However, it is hard to see how this approach would be superior to a sophisticated third-generation SERM functioning as a multifunctional medicine in women's health.

The advantages of aromatase inhibitors for postmenopausal patients are clear in large population trials and for health-care systems. Patents for aromatase inhibitors are running out or have run out and cheap generics are becoming available. (The aromatase inhibitors were initially priced extremely high compared to tamoxifen to compensate for each only securing about one-third of the original tamoxifen market.) A disease-free survival advantage is noted for adding an aromatase inhibitor to the treatment plan compared to tamoxifen alone (Dowsett et al., 2010) and concerns about endometrial cancer and blood clots are diminished. Current clinical studies to improve endocrine response rates seek to exploit emerging knowledge about the molecular mechanisms of antihormone resistance to aromatase inhibitors (Roop & Ma, 2012). Combinations of letrozole and lapatinib, an inhibitor of the HER2 pathway, show some advantages over letrozole alone in ER-positive and HER-positive metastatic breast cancer (Riemsma et al., 2012). A similar improvement in responsiveness to aromatase inhibitors is noted with a combination with the mTor inhibitor everolimus (Bachelot et al., 2012; Baselga et al., 2012, 2009).

The second major advance in therapeutics catalyzed by tamoxifen is the SERM group of medicines. The cluster of laboratory findings in the 1980s that described the fact that the "nonsteroidal antiestrogens" were actually targeted estrogens and antiestrogens in select estrogen target tissues (Jordan, 2001) prompted a significant effort by the pharmaceutical industry to exploit the concept with new SERMs (Jordan, 2003a, 2003b). This, in large measure, was because both tamoxifen and raloxifene were so successful economically. The osteoporosis market is much bigger than the endocrine treatment of breast cancer.

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Today considerable scientific success has been achieved with new SERMs, but it remains a challenge to create a drug with absolute safety guarantees for all women. The bar is now very high by necessity as prevention of multiple diseases implies that subjects who are the target population are in fact currently well. We will comment briefly on three compounds: lasofoxifene, basedoxifene, and ospemifene, but first it is worth mentioning that the molecules each have a "history" (Fig. 1.7). Lasofoxifene started its

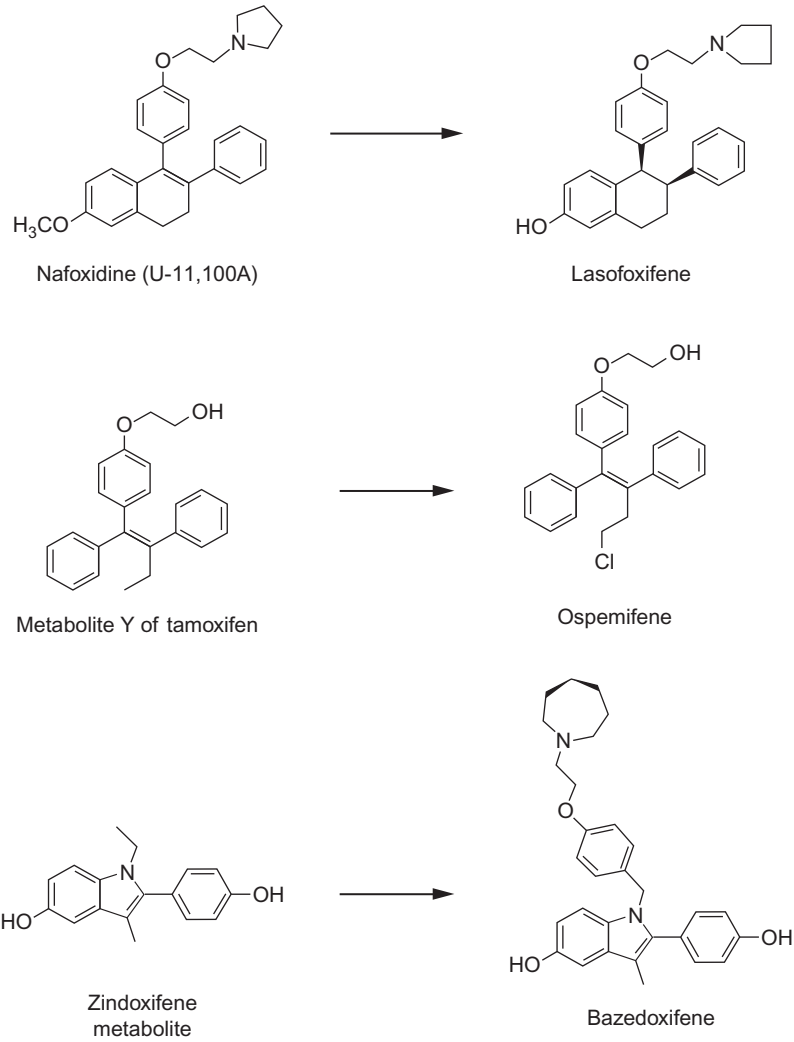

Figure 1.7 The historical origins of the new SERMs.

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molecular odyssey from its origins in the fertility control program at Upjohn in Kalamazoo in the early 1960s (Lednicer, Emmert, Duncan, & Lyster, 1967; Lednicer, Lyster, & Duncan, 1967). The antiestrogen/postcoital contraceptive, U11,100A, was initially discovered by the fertility control program. But U11,100A was reinvented to become nafoxidine for the treatment of breast cancer (Legha et al., 1976). Unfortunately, this program was abandoned because of the severe side effect of photophobia. Lasofoxifene is a very potent SERM with an effective dose of 0.5 mg daily being recommended for the treatment and prevention of osteoporosis (Cummings et al., 2010). This is 1/100th the recommended daily dose of raloxifene (60 mg daily). Medicinal chemists discovered that the levorotatory enantiomer is resistant to glucuronidation so that the molecule is not readily excreted (Rosati et al., 1998). The registration trial of postmenopausal evaluation and risk-reduction with lasofoxifene documented significant decreases in ER-positive breast cancer, coronary heart disease, strokes, and endometrial cancer (Cummings et al., 2010). Basically, all that the original roadmap (Lerner & Jordan, 1990) predicted for a SERM to prevent breast cancer (and endometrial cancer) as beneficial side effect for the prevention of osteoporosis and coronary heart disease. Lasofoxifene is approved in the European Union, but not in the United States.

Bazedoxifene (Fig. 1.7) (Gruber & Gruber, 2004; Komm et al., 2005; Miller et al., 2001), evolved from the metabolite of an earlier compound, zindoxifene, that failed to have antitumor activity in clinical trial (Stein et al., 1990), actually showed estrogen-like activity in laboratory studies (Robinson, Koch, & Jordan, 1988). Introduction of the appropriate phenylalkylaminoethoxy side chain created an important new SERM. Bazedoxifene is of interest as it has not only been tested as a SERM for the prevention of osteoporosis (Kawate & Takayanagi, 2011; Silverman et al., 2012) but also has been evaluated as a new kind of hormone replacement therapy, that is, bazedoxifene plus conjugated equine estrogens (CEE) (Kagan, Williams, Pan, Mirkin, & Pickar, 2010; Lindsay, Gallagher, Kagan, Pickar, & Constantine, 2009; Pinkerton, Pickar, Racketa, & Mirkin, 2012). There is an additive effect on bone density, but the SERM blocks breast and endometrial actions of estrogen. Clearly, this is an innovation application of SERMs that clearly avoids the tumorigenic effect of both CEE and synthetic progestin (Crandall et al., 2012).

Last, there is ospemifene (Fig. 1.7). The history of ospemifene is interesting as it has also evolved from a previously researched predecessor. A new metabolite of tamoxifen (metabolite Y) was reported in 1982–1983 and was

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shown to have weak antiestrogenic properties (Bain & Jordan, 1983; Jordan, Bain, Brown, Gosden, & Santos, 1983). Later, a similar metabolite was found for another antiestrogen toremifene, and this metabolite is now known as ospemifene. Originally, ospemifene was developed to treat vaginal atrophy in postmenopausal women, but it also can be useful for the prevention and treatment of osteoporosis. In clinical trials, ospemifene was shown to be well tolerated and have a safe toxicity profile (DeGregorio et al., 2000; Rutanen et al., 2003; Voipio et al., 2002). However, there is still not enough data from the trials to assess the effectiveness of ospemifine in regard with osteoporosis or breast cancer prevention.

However, with all the SERMs, the principal issue can be the quality of life for the patient. Hormone replacement therapy solves the menopausal symptom of hot flashes and night sweats. This is an important issue for those with severe symptoms. The SERMs, at present, are known to exacerbate rather than resolve this issue. However, prompted by potential markets, pharmaceutical chemists are attempting to decipher the complexities of this important SERM side effect to allow therapeutic compliance with SERMs to become optimal (Jain et al., 2006, 2009; Wallace et al., 2006; Watanabe et al., 2003).

So how far can the SERM concept go? Already medicinal chemists have created selective agonist/antagonist for all members of the nuclear receptor superfamily (Fan & Jordan, 2013), and there is the promise of the further understanding of selective ERα/ERβ modulators (Sengupta & Jordan, 2013). The products, should they find applications in the clinic, hold the promise of treating diseases selectively that could never have been imagined 40 years ago.

But all is not resolved with SERMs and one discovery in the early 1980s remains a work in progress. The availability of [^{3}H] tamoxifen allowed the identification of an "antiestrogen-binding protein" by Sutherland et al. (1980). It was hypothesized that it could be linked with antiestrogen action, but in recent years, compelling evidence has been presented that it plays a role in cholesterol metabolism (Payre et al., 2008) and is identified in mice as membranous epoxide hydrolase. The biology is complex but there are suggestions that this may be a mechanism for tumoricidal action (Delarue et al., 1999; Payre et al., 2008).

In coming to the end of our story, we return to the beginning of chemical therapy for cancer. Sir Alexander Haddow FRS, it is fair to state, actually became the catalyst for change in the chemical treatment of cancer. In 1970, there were no tests to establish the sensitivity of a tumor to chemical therapy.

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It was empirical medicine of trial and error in patients based on often suspect clinical experience rather than rigorously controlled clinical trials. Haddow advanced clinical certainty during his career from individual experience by organizing a small clinical trial to obtain preliminary data (Haddow et al., 1944), with a subsequent large multicentered trial to ensure a valid result was being promoted to improve clinical practice. To prove the validity and reproducibility of these preliminary data, a collaborative clinical trial was organized with a dozen centers throughout the United Kingdom organized by the Royal Society of Medicine (Haddow was the President of the Section of Oncology at the Royal Society of Medicine). He stated his discovery during his 1970 Karnofsky lecture:

> *When the various reports were assembled at the end of that time, it was fascinating to discover that rather general impression, not sufficiently strong from the relatively small numbers in any single group, became reinforced to the point of certainty; namely, the beneficial responses were three times more frequent in women over the age of 60 years than in those under that age; that oestrogens may, on the contrary, accelerate the course of mammary cancer in younger women, and that their therapeutic use should be restricted to cases 5 years beyond the menopause. Here was an early and satisfying example of the advantages which may accrue from cooperative clinical trial.*
>
> *(Haddow, 1970)*

A similar conclusion was noted by Stoll (1977b) through a review of his lifetime experience with 407 postmenopausal patients with stage IV breast cancer treated with high-dose estrogen (Table 1.2). It is clear a prolonged period of estrogen deprivation after the menopause is needed for the optimal apoptotic activity of estrogen to develop.

These early data have relevance to solve a current paradox in women's health that has major significance. The Women's Health Initiative (WHI) Study of combination CEE and the synthetic progestin medroxyprogesterone acetate (HRT) (to prevent endometrial cancer) was initiated to assess the effects of HRT on improving women's health, that is, preventing fractures, coronary heart disease, and Alzheimer's, and balancing

Table 1.2 Objective response rates in postmenopausal women with metastatic breast cancer using high-dose estrogen therapy

Age since menopause	Patient #	Regression (%)
Postmenopausal 0–5 years	63	9
Postmenopausal >5 years	344	35

The 407 patients are divided in relation to menopause (Stoll, 1977b).

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this with the known side effects of increasing the incidence of breast cancer and thromboembolic disorders. The study did show a decrease in osteoporotic fractures but no benefit for coronary heart disease or for Alzheimer's disease (Rossouw et al., 2002). Breast cancer incidence was increased (Chlebowski et al., 2003; Shumaker et al., 2003). However, the examination of the second WHI trial of CEE alone versus placebo in hysterectomized postmenopausal women showed an initial decrease in breast cancer incidence (Anderson et al., 2004; Prentice et al., 2008), and then a further decrease that was sustained for 5 years after CEE treatment was terminated (LaCroix et al., 2011). A recent analysis demonstrates rather remarkably not only a sustained decrease in breast cancer incidence but also all cancers and a significant decrease in mortality (Anderson et al., 2012). The population of women were aged an average of 68 years, that is, following a long period of estrogen deprivation CEE causes a tumoricidal action which fits nicely with the Haddow/Stoll explanation of needing an "estrogen holiday" to create the correct antitumor sensitivity to estrogen. In other words, estrogen should not be given alone straight after menopause as an ERT. These data obtained in the modern era close the circle on our current understanding of estrogen action in the life and death of breast cancer cells (Jordan, 2008a). The saga of SERMs not only advanced women's health, dramatically improving survivorship and preventing both breast cancer and osteoporosis but also created the opportunity to discover the new biology of estrogen-induced apoptosis. This natural mechanism is programmed in a completely different way than the cellular response to cytotoxic therapy. Our ability to decipher the actual trigger of estrogen-induced apoptosis may open up new opportunities in targeted cancer therapeutics.

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REFERENCES

Allen, E., & Doisey, E. A. (1923). An ovarian hormone: Preliminary reports on its localization, extraction and partial purification and action in test animals. *Journal of the American Medical Association*, *81*(10), 819–821.

Anderson, G. L., Chlebowski, R. T., Aragaki, A. K., Kuller, L. H., Manson, J. E., Gass, M., et al. (2012). Conjugated equine oestrogen and breast cancer incidence and mortality in

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postmenopausal women with hysterectomy: Extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *The Lancet Oncology*, *13*(5), 476–486.

Anderson, G. L., Limacher, M., Assaf, A. R., Bassford, T., Beresford, S. A., Black, H., et al. (2004). Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: The Women's Health Initiative randomized controlled trial. *Journal of the American Medical Association*, *291*(14), 1701–1712.

Ariazi, E. A., Cunliffe, H. E., Lewis-Wambi, J. S., Slifker, M. J., Willis, A. L., Ramos, P., et al. (2011). Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(47), 18879–18886.

Bachelot, T., Bourgier, C., Cropet, C., Ray-Coquard, I., Ferrero, J. M., Freyer, G., et al. (2012). Randomized phase II trial of everolimus in combination with tamoxifen in patients with hormone receptor-positive, human epidermal growth factor receptor 2-negative metastatic breast cancer with prior exposure to aromatase inhibitors: A GINECO study. *Journal of Clinical Oncology*, *30*(22), 2718–2724.

Bain, R. R., & Jordan, V. C. (1983). Identification of a new metabolite of tamoxifen in patient serum during breast cancer therapy. *Biochemical Pharmacology*, *32*(2), 373–375.

Balaburski, G. M., Dardes, R. C., Johnson, M., Haddad, B., Zhu, F., Ross, E. A., et al. (2010). Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: A unifying concept in anti-hormone resistance. *International Journal of Oncology*, *37*(2), 387–398.

Baselga, J., Campone, M., Piccart, M., Burris, H. A., 3rd., Rugo, H. S., Sahmoud, T., et al. (2012). Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. *The New England Journal of Medicine*, *366*(6), 520–529.

Baselga, J., Semiglazov, V., van Dam, P., Manikhas, A., Bellet, M., Mayordomo, J., et al. (2009). Phase II randomized study of neoadjuvant everolimus plus letrozole compared with placebo plus letrozole in patients with estrogen receptor-positive breast cancer. *Journal of Clinical Oncology*, *27*(16), 2630–2637.

Baum, M., Budzar, A. U., Cuzick, J., Forbes, J., Houghton, J. H., Klijn, J. G., et al. (2002). Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: First results of the ATAC randomised trial. *The Lancet*, *359*(9324), 2131–2139.

Baumler, E. (1984). *Paul Ehrlich: Scientist for life*. (1st ed.). New York: Holmes & Meier Publishers.

Beall, P. T., Misra, K. L., Young, R. L., Spjut, H. J., Evan, H. J., & LeBlanc, A. (1985). Clomiphene protects against osteoporosis in the mature ovariectomized rat. *Calcified Tissue International*, *36*, 123–125.

Beatson, G. T. (1896). On the treatment of inoperable cases of carcinoma of the mamma: Suggestions for a new method of treatment, with illustrative cases. *The Lancet*, *148*, 104–107.

Bergh, J., Jonsson, P. E., Lidbrink, E. K., Trudeau, M., Eiermann, W., Brattstrom, D., et al. (2012). FACT: An open-label randomized phase III study of fulvestrant and anastrozole in combination compared with anastrozole alone as first-line therapy for patients with receptor-positive postmenopausal breast cancer. *Journal of Clinical Oncology*, *30*(16), 1919–1925.

Berthois, Y., Katzenellenbogen, J. A., & Katzenellenbogen, B. S. (1986). Phenol red in tissue culture media is a weak estrogen: Implications concerning the study of estrogen-responsive cells in culture. *Proceedings of the National Academy of Sciences of the United States of America*, *83*(8), 2496–2500.

Bindal, R. D., Carlson, K. E., Katzenellenbogen, B. S., & Katzenellenbogen, J. A. (1988). Lipophilic impurities, not phenolsulfonaphthalein, account for the estrogenic activity in commercial preparations of phenol red. *Journal of Steroid Biochemistry*, *31*(3), 287–293.

Author's personal copy

Bindal, R. D., & Katzenellenbogen, J. A. (1988). Bis(4-hydroxyphenyl)[2-(phenoxysulfonyl) phenyl]methane: Isolation and structure elucidation of a novel estrogen from commercial preparations of phenol red (phenolsulfonphthalein). *Journal of Medicinal Chemistry*, *31*(10), 1978–1983.

Black, L. J., Jones, C. D., & Falcone, J. F. (1983). Antagonism of estrogen action with a new benzothiophene derived antiestrogen. *Life Sciences*, *32*(9), 1031–1036.

Boccardo, F., Rubagotti, A., Puntoni, M., Guglielmini, P., Amoroso, D., Fini, A., et al. (2005). Switching to anastrozole versus continued tamoxifen treatment of early breast cancer: Preliminary results of the Italian Tamoxifen Anastrozole Trial. *Journal of Clinical Oncology*, *23*(22), 5138–5147.

Bonadonna, G., Brusamolino, E., Valagussa, P., Rossi, A., Brugnatelli, L., Brambilla, C., et al. (1976). Combination chemotherapy as an adjuvant treatment in operable breast cancer. *The New England Journal of Medicine*, *294*(8), 405–410.

Boyd, S. (1900). On oophorectomy in cancer of the breast. *British Medical Journal*, *2*, 1161–1167.

Brauch, H., Schroth, W., Goetz, M. P., Mürdter, T. E., Winter, S., Ingle, J. N., et al. (2013). Tamoxifen use in postmenopausal breast cancer: CYP2D6 matters. *Journal of Clinical Oncology*, *31*, 176–180.

Breast Cancer Facts and Figures (2011–2012). Atlanta, American Cancer Society: American Cancer Society, Inc.

Breast Cancer Survival Rates by Stage (2011). American Cancer Society.

Brodie, A. M., & Longcope, C. (1980). Inhibition of peripheral aromatization by aromatase inhibitors, 4-hydroxy- and 4-acetoxy-androstene-3,17-dione. *Endocrinology*, *106*(1), 19–21.

Brodie, A. M., Marsh, D., & Brodie, H. J. (1979). Aromatase inhibitors. IV. Regression of hormone-dependent, mammary tumors in the rat with 4-acetoxy-4-androstene-3,17-dione. *Journal of Steroid Biochemistry*, *10*(4), 423–429.

Brodie, A. M., Schwarzel, W. C., Shaikh, A. A., & Brodie, H. J. (1977). The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. *Endocrinology*, *100*(6), 1684–1695.

Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., et al. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*, *389*(6652), 753–758.

Canadian Cancer Statistics (2012). Canadian Cancer Society.

Chlebowski, R. T., Hendrix, S. L., Langer, R. D., Stefanick, M. L., Gass, M., Lane, D., et al. (2003). Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: The Women's Health Initiative Randomized Trial. *Journal of the American Medical Association*, *289*(24), 3243–3253.

Coates, A. S., Keshaviah, A., Thurlimann, B., Mouridsen, H., Mauriac, L., Forbes, J. F., et al. (2007). Five years of letrozole compared with tamoxifen as initial adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer: Update of study BIG 1-98. *Journal of Clinical Oncology*, *25*(5), 486–492.

Cole, M. P., Jones, C. T., & Todd, I. D. (1971). A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. *British Journal of Cancer*, *25*(2), 270–275.

Consensus conference (1985). Adjuvant chemotherapy for breast cancer. *Journal of the American Medical Association*, *254*(24), 3461–3463.

Coombes, R. C., Goss, P., Dowsett, M., Gazet, J. C., & Brodie, A. (1984). 4-Hydroxyandrostenedione in treatment of postmenopausal patients with advanced breast cancer. *The Lancet*, *2*(8414), 1237–1239.

Coombes, R. C., Hall, E., Gibson, L. J., Paridaens, R., Jassem, J., Delozier, T., et al. (2004). A randomized trial of exemestane after two to three years of tamoxifen therapy in

Author's personal copy

postmenopausal women with primary breast cancer. *The New England Journal of Medicine*, *350*(11), 1081–1092.
Cooper, R. G. (1969). Combination chemotherapy in hormone resistant breast cancer. *Proceeding of the American Association for Cancer Research*, *10*, 15.
Crandall, C. J., Aragaki, A. K., Cauley, J. A., McTiernan, A., Manson, J. E., Anderson, G., et al. (2012). Breast tenderness and breast cancer risk in the estrogen plus progestin and estrogen-alone women's health initiative clinical trials. *Breast Cancer Research and Treatment*, *132*(1), 275–285.
Cummings, S. R., Eckert, S., Krueger, K. A., Grady, D., Powles, T. J., Cauley, J. A., et al. (1999). The effect of raloxifene on risk of breast cancer in postmenopausal women: Results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *Journal of the American Medical Association*, *281*(23), 2189–2197.
Cummings, S. R., Ensrud, K., Delmas, P. D., LaCroix, A. Z., Vukicevic, S., Reid, D. M., et al. (2010). Lasofoxifene in postmenopausal women with osteoporosis. *The New England Journal of Medicine*, *362*(8), 686–696.
Cuzick, J., & Baum, M. (1985). Tamoxifen and contralateral breast cancer. *The Lancet*, *2*(8449), 282.
Cuzick, J., Forbes, J. F., & Howell, A. (2006). Re: Tamoxifen for the prevention of breast cancer: Current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *Journal of the National Cancer Institute*, *98*(9), 643, author reply 643–644.
Davies, C., Godwin, J., Gray, R., Clarke, M., Cutter, D., Darby, S., et al. (2011). Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: Patient-level meta-analysis of randomised trials. *The Lancet*, *378*(9793), 771–784.
DeGregorio, M. W., Wurz, G. T., Taras, T. L., Erkkola, R. U., Halonen, K. H., & Huupponen, R. K. (2000). Pharmacokinetics of (deaminohydroxy)toremifene in humans: A new, selective estrogen-receptor modulator. *European Journal of Clinical Pharmacology*, *56*(6–7), 469–475.
Delarue, F., Kedjouar, B., Mesange, F., Bayard, F., Faye, J. C., & Poirot, M. (1999). Modifications of benzylphenoxy ethanamine antiestrogen molecules: Influence affinity for antiestrogen binding site (AEBS) and cell cytotoxicity. *Biochemical Pharmacology*, *57*(6), 657–661.
Di Leo, A., Jerusalem, G., Petruzelka, L., Torres, R., Bondarenko, I. N., Khasanov, R., et al. (2010). Results of the CONFIRM phase III trial comparing fulvestrant 250 mg with fulvestrant 500 mg in postmenopausal women with estrogen receptor-positive advanced breast cancer. *Journal of Clinical Oncology*, *28*(30), 4594–4600.
Dieudonne, A. S., Lambrechts, D., Claes, B., Vandorpe, T., Wildiers, H., Timmerman, D., et al. (2009). Prevalent breast cancer patients with a homozygous mutant status for CYP2D6*4: Response and biomarkers in tamoxifen users. *Breast Cancer Research and Treatment*, *118*(3), 531–538.
Dodds, E. (1938). Biological effects of the synthetic oestrogenic substance 4:4′-dihydroxy-alpha:beta-diethylstilbene. *The Lancet*, *232*(6015), 1389–1391.
Dodds, E. C., Goldberg, L., Lawson, W., & Robinson, R. (1938). Oestrogenic activity of esters of diethyl stilboestrol. *Nature*, *142*(3587), 211–212.
Dowsett, M., Cuzick, J., Ingle, J., Coates, A., Forbes, J., Bliss, J., et al. (2010). Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. *Journal of Clinical Oncology*, *28*(3), 509–518.
EBCTCG, (1998). Tamoxifen for early breast cancer: An overview of the randomised trials. Early Breast Cancer Trialisits' Collaborative Group. *The Lancet*, *351*(9114), 1451–1467.
EBCTCG, (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. *The Lancet*, *365*(9472), 1687–1717.

Author's personal copy

EISRCM, (2006). *O Câncer de Mama no Brasil Situacão epidemiológica e rastreamento*. Rio de Janeiro: Encontro Internacional Sobre Rastreamento de Cancer de Mama.

Ellis, M. J., Gao, F., Dehdashti, F., Jeffe, D. B., Marcom, P. K., Carey, L. A., et al. (2009). Lower-dose vs. high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: A phase 2 randomized study. *Journal of the American Medical Association*, *302*(7), 774–780.

Ettinger, B., Black, D. M., Mitlak, B. H., Knickerbocker, R. K., Nickelsen, T., Genant, H. K., et al. (1999). Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: Results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. *Journal of the American Medical Association*, *282*(7), 637–645.

Fan, P., & Jordan, V. C. (2013). An emerging principle: Selective nuclear receptor modulators. In V. C. Jordan (Ed.), *Estrogen actions, SERMs, and women's health*. London: Imperial College Press.

Fan, P., McDaniel, R. E., Kim, H. R., Clagett, D., Haddad, B., & Jordan, V. C. (2012). Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines. *European Journal of Cancer*, *48*, 3488–3498.

Ferlay, J., Parkin, D. M., & Steliarova-Foucher, E. (2010). Estimates of cancer incidence and mortality in Europe in 2008. *European Journal of Cancer*, *46*, 765–781.

Fisher, B., Carbone, P., Economou, S. G., Frelick, R., Glass, A., Lerner, H., et al. (1975). 1-Phenylalanine mustard (L-PAM) in the management of primary breast cancer. A report of early findings. *The New England Journal of Medicine*, *292*(3), 117–122.

Fisher, B., Costantino, J., Redmond, C., Poisson, R., Bowman, D., Couture, J., et al. (1989). A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. *The New England Journal of Medicine*, *320*(8), 479–484.

Fisher, B., Costantino, J. P., Wickerham, D. L., Cecchini, R. S., Cronin, W. M., Robidoux, A., et al. (2005). Tamoxifen for the prevention of breast cancer: Current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *Journal of the National Cancer Institute*, *97*(22), 1652–1662.

Fisher, B., Costantino, J. P., Wickerham, D. L., Redmond, C. K., Kavanah, M., Cronin, W. M., et al. (1998). Tamoxifen for prevention of breast cancer: Report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *Journal of the National Cancer Institute*, *90*(18), 1371–1388.

Folca, P. J., Glascock, R. F., & Irvine, W. T. (1961). Studies with tritium-labelled hexoestrol in advanced breast cancer. Comparison of tissue accumulation of hexoestrol with response to bilateral adrenalectomy and oophorectomy. *The Lancet*, *2*(7206), 796–798.

Fornander, T., Rutqvist, L. E., Cedermark, B., Glas, U., Mattsson, A., Silfversward, C., et al. (1989). Adjuvant tamoxifen in early breast cancer: Occurrence of new primary cancers. *The Lancet*, *1*(8630), 117–120.

Furr, B. J., & Jordan, V. C. (1984). The pharmacology and clinical uses of tamoxifen. *Pharmacology and Therapeutics*, *25*(2), 127–205.

Glascock, R. F., & Hoekstra, W. G. (1959). Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep. *The Biochemical Journal*, *72*, 673–682.

Goss, P. E., Ingle, J. N., Ales-Martinez, J. E., Cheung, A. M., Chlebowski, R. T., Wactawski-Wende, J., et al. (2011). Exemestane for breast-cancer prevention in postmenopausal women. *The New England Journal of Medicine*, *364*(25), 2381–2391.

Goss, P. E., Ingle, J. N., Martino, S., Robert, N. J., Muss, H. B., Piccart, M. J., et al. (2003). A randomized trial of letrozole in postmenopausal women after five years of tamoxifen

Author's personal copy

therapy for early-stage breast cancer. *The New England Journal of Medicine*, *349*(19), 1793–1802.
Goss, P. E., Ingle, J. N., Martino, S., Robert, N. J., Muss, H. B., Piccart, M. J., et al. (2005). Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: Updated findings from NCIC CTG MA.17. *Journal of the National Cancer Institute*, *97*(17), 1262–1271.
Gottardis, M. M., Jiang, S. Y., Jeng, M. H., & Jordan, V. C. (1989). Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal anti-estrogens. *Cancer Research*, *49*(15), 4090–4093.
Gottardis, M. M., & Jordan, V. C. (1987). Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Research*, *47*(15), 4020–4024.
Gottardis, M. M., & Jordan, V. C. (1988). Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Research*, *48*(18), 5183–5187.
Gottardis, M. M., Ricchio, M. E., Satyaswaroop, P. G., & Jordan, V. C. (1990). Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. *Cancer Research*, *50*(11), 3189–3192.
Gottardis, M. M., Robinson, S. P., Satyaswaroop, P. G., & Jordan, V. C. (1988). Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Research*, *48*(4), 812–815.
Gottardis, M. M., Wagner, R. J., Borden, E. C., & Jordan, V. C. (1989). Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro. *Cancer Research*, *49*(17), 4765–4769.
Greaves, P., Goonetilleke, R., Nunn, G., Topham, J., & Orton, T. (1993). Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. *Cancer Research*, *53*(17), 3919–3924.
Gruber, C., & Gruber, D. (2004). Bazedoxifene (Wyeth). *Current Opinion in Investigational Drugs*, *5*(10), 1086–1093.
Haddow, A. (1970). David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer*, *26*(4), 737–754.
Haddow, A., Watkinson, J. M., Paterson, E., & Koller, P. C. (1944). Influence of synthetic oestrogens on advanced malignant disease. *British Medical Journal*, *2*(4368), 393–398.
Hard, G. C., Latropoulos, M. J., Jordan, K., Radi, L., Kaltenberg, O. P., Imondi, A. R., et al. (1993). Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. *Cancer Research*, *53*(19), 4534–4541.
Harper, M. J., & Walpole, A. L. (1967a). Mode of action of I.C.I. 46,474 in preventing implantation in rats. *The Journal of Endocrinology*, *37*(1), 83–92.
Harper, M. J., & Walpole, A. L. (1967b). A new derivative of triphenylethylene: Effect on implantation and mode of action in rats. *Journal of Reproduction and Fertility*, *13*(1), 101–119.
Hecker, E., Vegh, I., Levy, C. M., Magin, C. A., Martinez, J. C., Loureiro, J., et al. (1974). Clinical trial of clomiphene in advanced breast cancer. *European Journal of Cancer*, *10*(11), 747–749.
Herman, M. E., & Katzenellenbogen, B. S. (1994). Alterations in transforming growth factor-alpha and -beta production and cell responsiveness during the progression of MCF-7 human breast cancer cells to estrogen-autonomous growth. *Cancer Research*, *54*(22), 5867–5874.
Howell, A., Cuzick, J., Baum, M., Buzdar, A., Dowsett, M., Forbes, J. F., et al. (2005). Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *The Lancet*, *365*(9453), 60–62.

Author's personal copy

Howell, A., Robertson, J. F., Abram, P., Lichinitser, M. R., Elledge, R., Bajetta, E., et al. (2004). Comparison of fulvestrant versus tamoxifen for the treatment of advanced breast cancer in postmenopausal women previously untreated with endocrine therapy: A multinational, double-blind, randomized trial. *Journal of Clinical Oncology*, *22*(9), 1605–1613.

Howlader, N., Noone, A. M., Krapcho, M., Neyman, N., Aminou, R., Altekruse, S. F., et al. (2009). *SEER cancer statistics review, 1975–2009 (Vintage 2009 populations)*. Bethesda, MD: National Cancer Institute. *http://seer.cancer.gov/csr/1975_2009_pops09/*.

Hu, Z., Fan, C., Oh, D. S., Marron, J. S., He, X., Qaqish, B. F., et al. (2006). The molecular portraits of breast tumors are conserved across microarray platforms. *BMC Genomics*, *7*, 96.

Hu, Z. Z., Kagan, B. L., Ariazi, E. A., Rosenthal, D. S., Zhang, L., Li, J. V., et al. (2011). Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. *PLoS One*, *6*(6), e20410.

Huggins, C., Grand, L. C., & Brillantes, F. P. (1961). Mammary cancer induced by a single feeding of polymucular hydrocarbons, and its suppression. *Nature*, *189*, 204–207.

INCA (2006). *A Situacao do Cancer no Brasil*. Rio de Janeiro: Instituto Nacional de Cancer.

Ingle, J. N., Ahmann, D. L., Green, S. J., Edmonson, J. H., Bisel, H. F., Kvols, L. K., et al. (1981). Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *The New England Journal of Medicine*, *304*(1), 16–21.

Jain, N., Kanojia, R. M., Xu, J., Jian-Zhong, G., Pacia, E., Lai, M. T., et al. (2006). Novel chromene-derived selective estrogen receptor modulators useful for alleviating hot flushes and vaginal dryness. *Journal of Medicinal Chemistry*, *49*(11), 3056–3059.

Jain, N., Xu, J., Kanojia, R. M., Du, F., Jian-Zhong, G., Pacia, E., et al. (2009). Identification and structure-activity relationships of chromene-derived selective estrogen receptor modulators for treatment of postmenopausal symptoms. *Journal of Medicinal Chemistry*, *52*(23), 7544–7569.

Jensen, E. V., & Jacobson, H. I. (1962). Basic guides to the mechanism of estrogen action. *Recent Progress in Hormone Resistence*, *18*, 387–414.

Jiang, S. Y., Wolf, D. M., Yingling, J. M., Chang, C., & Jordan, V. C. (1992). An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Molecular and Cellular Endocrinology*, *90*(1), 77–86.

Jordan, V. C. (1976). Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. *European Journal of Cancer*, *12*(6), 419–424.

Jordan, V. C. (1978). Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen treatment as a potential adjuvant therapy. *Reviews on Endocrine-Related Cancer*, (October Suppl.), 49–55.

Jordan, V. C. (1984). Biochemical pharmacology of antiestrogen action. *Pharmacological Reviews*, *36*(4), 245–276.

Jordan, V. C. (1998). Chemotherapy is antihormonal therapy-how much proof doOncologists need? *European Journal of Cancer*, *34*(5), 606–608.

Jordan, V. C. (2001). Selective estrogen receptor modulation: A personal perspective. *Cancer Research*, *61*(15), 5683–5687.

Jordan, V. C. (2003a). Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 1. Receptor interactions. *Journal of Medicinal Chemistry*, *46*(6), 883–908.

Jordan, V. C. (2003b). Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *Journal of Medicinal Chemistry*, *46*(7), 1081–1111.

Jordan, V. C. (2003c). Tamoxifen: A most unlikely pioneering medicine. *Nature Reviews. Drug Discovery*, *2*(3), 205–213.

Author's personal copy

Jordan, V. C. (2004). Selective estrogen receptor modulation: Concept and consequences in cancer. *Cancer Cell*, *5*(3), 207–213.
Jordan, V. C. (2006). Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. *British Journal of Pharmacology*, *147*(Suppl. 1), S269–S276.
Jordan, V. C. (2008a). The 38th David A. Karnofsky lecture: The paradoxical actions of estrogen in breast cancer—Survival or death? *Journal of Clinical Oncology*, *26*(18), 3073–3082.
Jordan, V. C. (2008b). Tamoxifen: Catalyst for the change to targeted therapy. *European Journal of Cancer*, *44*(1), 30–38.
Jordan, V. C., & Allen, K. E. (1980). Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *European Journal of Cancer*, *16*(2), 239–251.
Jordan, V. C., Allen, K. E., & Dix, C. J. (1980). Pharmacology of tamoxifen in laboratory animals. *Cancer Treatment Reports*, *64*(6–7), 745–759.
Jordan, V. C., Bain, R. R., Brown, R. R., Gosden, B., & Santos, M. A. (1983). Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. *Cancer Research*, *43*(3), 1446–1450.
Jordan, V. C., Collins, M. M., Rowsby, L., & Prestwich, G. (1977). A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *The Journal of Endocrinology*, *75*(2), 305–316.
Jordan, V. C., Dix, C. J., & Allen, K. E. (1979). The effectiveness of longterm treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In S. E. Salmone & S. E. Jones (Eds.), *Adjuvant therapy of cancer II* (pp. 19–26). New York: Grune and Stratton.
Jordan, V. C., Dix, C. J., Naylor, K. E., Prestwich, G., & Rowsby, L. (1978). Nonsteroidal antiestrogens: Their biological effects and potential mechanisms of action. *Journal of Toxicology and Environmental Health*, *4*(2–3), 363–390.
Jordan, V. C., & Koerner, S. (1975). Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *European Journal of Cancer*, *11*(3), 205–206.
Jordan, V. C., Liu, H., & Dardes, R. (2002). Re: Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 beta-estradiol and the two faces of Janus: Sex steroids as mediators of both cell proliferation and cell death. *Journal of the National Cancer Institute*, *94*(15), 1173, author reply 1173–1175.
Jordan, V. C., Mittal, S., Gosden, B., Koch, R., & Lieberman, M. E. (1985). Structure-activity relationships of estrogens. *Environmental Health Perspectives*, *61*, 97–110.
Jordan, V. C., Naylor, K. E., Dix, C. J., & Prestwich, G. (1980). Anti-oestrogen action in experimental breast cancer. *Recent Results in Cancer Research*, *71*, 30–44.
Jordan, V. C., Phelps, E., & Lindgren, J. U. (1987). Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Research and Treatment*, *10*(1), 31–35.
Jordan, V. C., & Robinson, S. P. (1987). Species-specific pharmacology of antiestrogens: Role of metabolism. *Federation Proceedings*, *46*(5), 1870–1874.
Jordan, V. C., Schafer, J. M., Levenson, A. S., Liu, H., Pease, K. M., Simons, L. A., et al. (2001). Molecular classification of estrogens. *Cancer Research*, *61*(18), 6619–6623.
Jordan, V. C., Wolf, M., Mirecki, D. M., Whitford, D., & Welshons, W. V. (1988). Hormone receptor assays: Clinical usefulness in the management of carcinoma of the breast. *CRC Critical Reviews in Clinical Laboratory Sciences*, *26*, 97–152.
Kagan, R., Williams, R. S., Pan, K., Mirkin, S., & Pickar, J. H. (2010). A randomized, placebo- and active-controlled trial of bazedoxifene/conjugated estrogens for treatment of moderate to severe vulvar/vaginal atrophy in postmenopausal women. *Menopause*, *17*(2), 281–289.
Katzenellenbogen, B. S., Kendra, K. L., Norman, M. J., & Berthois, Y. (1987). Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer

Author's personal copy

cells grown in the short-term and long-term absence of estrogens. *Cancer Research*, *47*(16), 4355–4360.
Kawate, H., & Takayanagi, R. (2011). Efficacy and safety of bazedoxifene for postmenopausal osteoporosis. *Clinical Interventions in Aging*, *6*, 151–160.
Kennedy, B. J. (1965a). Hormone therapy for advanced breast cancer. *Cancer*, *18*(12), 1551–1557.
Kennedy, B. J. (1965b). Systemic effects of androgenic and estrogenic hormones in advanced breast cancer. *Journal of the American Geriatrics Society*, *13*, 230–235.
Kennedy, B. J., & Nathanson, I. T. (1953). Effect of intensive sex steroid hormone therapy in advanced breast cancer. *Journal of the American Medical Association*, *152*, 1135–1141.
Kiyotani, K., Mushiroda, T., Imamura, C. K., Hosono, N., Tsunoda, T., Kubo, M., et al. (2010). Significant effect of polymorphisms in CYP2D6 and ABCC2 on clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients. *Journal of Clinical Oncology*, *28*(8), 1287–1293.
Klopper, A., & Hall, M. (1971). New synthetic agent for the induction of ovulation: Preliminary trials in women. *British Medical Journal*, *1*(5741), 152–154.
Komm, B. S., Kharode, Y. P., Bodine, P. V., Harris, H. A., Miller, C. P., & Lyttle, C. R. (2005). Bazedoxifene acetate: A selective estrogen receptor modulator with improved selectivity. *Endocrinology*, *146*(9), 3999–4008.
Lacassagne, A. (1933). Influence d'un facteur familial dans la production par la folliculine, de cancers mammaires chez la souris male. *Comptes Rendus des Seances **de** la Societe de Biologie et des ses Filiales (Paris)*, *114*, 427–429.
Lacassagne, A. (1936a). A comparative study of the carcinogenic action of certain oestrogenic hormones. *American Journal of Cancer*, *28*, 735–740.
Lacassagne, A. (1936b). Hormonal pathogenesis of adenocarcinoma of the breast. *American Journal of Cancer*, *27*(2), 217–228.
LaCroix, A. Z., Chlebowski, R. T., Manson, J. E., Aragaki, A. K., Johnson, K. C., Martin, L., et al. (2011). Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: A randomized controlled trial. *Journal of the American Medical Association*, *305*(13), 1305–1314.
Lammers, L. A., Mathijssen, R. H., van Gelder, T., Bijl, M. J., de Graan, A. J., Seynaeve, C., et al. (2010). The impact of CYP2D6-predicted phenotype on tamoxifen treatment outcome in patients with metastatic breast cancer. *British Journal of Cancer*, *103*(6), 765–771.
Lash, T. L., Cronin-Fenton, D., Ahern, T. P., Rosenberg, C. L., Lunetta, K. L., Silliman, R. A., et al. (2011). CYP2D6 inhibition and breast cancer recurrence in a population-based study in Denmark. *Journal of the National Cancer Institute*, *103*(6), 489–500.
Lathrop, A. E., & Loeb, L. (1916). Further investigations on the origin of tumors in mice. III. On the part played by internal secretion in the spontaneous development of tumors. *Journal of Cancer Research*, *1*(1), 1–19.
Laughlin, R. C., & Carey, T. F. (1962). Cataracts in patients treated with triparanol. *Journal of the American Medical Association*, *181*, 369–370.
LBCSG (1984). Randomised trial of chemo-endocrine therapy, endocrine therapy, and mastectomy alone in postmenopausal patients with operable breast cancer and axillary node metastasis. *The Lancet*, *1*(8384), 1256–1260.
Lednicer, D., Emmert, D. E., Duncan, G. W., & Lyster, S. C. (1967). Mammalian antifertility agents. V. 5,6-Diarylhodronaphtalenes. *Journal of Medicinal Chemistry*, *10*(6), 1051–1054.
Lednicer, D., Lyster, S. C., & Duncan, G. W. (1967). Mammalian antifertility agents. IV. Basic 3,4-dihydronaphthalenes and 1,2,3,4-tetrahydro-1-naphthols. *Journal of Medicinal Chemistry*, *10*(1), 78–84.

Author's personal copy

Legha, S. S., Slavik, M., & Carter, S. K. (1976). Nafoxidine—An antiestrogen for the treatment of breast cancer. *Cancer*, *38*(4), 1535–1541.

Lerner, L. J. (1981). The first nonsteroidal antiestrogen—MER25. In R. L. Sutherland & V. C. Jordan (Eds.), *Nonsteroidal antiestrogens, molecular pharmacology and antitumor activity* (pp. 1–16). Sydney: Academic Press.

Lerner, L. J., Holthaus, F. J., Jr., & Thompson, C. R. (1958). A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol. *Endocrinology*, *63*(3), 295–318.

Lerner, L. J., & Jordan, V. C. (1990). Development of antiestrogens and their use in breast cancer: Eighth Cain memorial award lecture. *Cancer Research*, *50*(14), 4177–4189.

Lewis, J. S., Meeke, K., Osipo, C., Ross, E. A., Kidawi, N., Li, T., et al. (2005). Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *Journal of the National Cancer Institute*, *97*(23), 1746–1759.

Lewis-Wambi, J. S., Kim, H. R., Wambi, C., Patel, R., Pyle, J. R., Klein-Szanto, A. J., et al. (2008). Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Research*, *10*(6), R104.

Lewis-Wambi, J. S., Swaby, R., Kim, H., & Jordan, V. C. (2009). Potential of l-buthionine sulfoximine to enhance the apoptotic action of estradiol to reverse acquired antihormonal resistance in metastatic breast cancer. *The Journal of Steroid Biochemistry and Molecular Biology*, *114*(1–2), 33–39.

Lindsay, R., Gallagher, J. C., Kagan, R., Pickar, J. H., & Constantine, G. (2009). Efficacy of tissue-selective estrogen complex of bazedoxifene/conjugated estrogens for osteoporosis prevention in at-risk postmenopausal women. *Fertility and Sterility*, *92*(3), 1045–1052.

Lippman, M. E., & Bolan, G. (1975). Oestrogen-responsive human breast cancer in long term tissue culture. *Nature*, *256*(5518), 592–593.

Lonning, P. E. (2009). Additive endocrine therapy for advanced breast cancer—Back to the future. *Acta Oncologica*, *48*(8), 1092–1101.

Lonning, P. E., Taylor, P. D., Anker, G., Iddon, J., Wie, L., Jorgensen, L. M., et al. (2001). High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Research and Treatment*, *67*(2), 111–116.

Love, R. R., Mazess, R. B., Barden, H. S., Epstein, S., Newcomb, P. A., Jordan, V. C., et al. (1992). Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *The New England Journal of Medicine*, *326*(13), 852–856.

Love, R. R., Newcomb, P. A., Wiebe, D. A., Surawicz, T. S., Jordan, V. C., Carbone, P. P., et al. (1990). Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. *Journal of the National Cancer Institute*, *82*(16), 1327–1332.

Love, R. R., Wiebe, D. A., Newcomb, P. A., Cameron, L., Leventhal, H., Jordan, V. C., et al. (1991). Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Annals of Internal Medicine*, *115*(11), 860–864.

Lunan, C. B., & Klopper, A. (1975). Antioestrogens. A review. *Clinical Endocrinology*, *4*(5), 551–572.

Madlensky, L., Natarajan, L., Tchu, S., Pu, M., Mortimer, J., Flatt, S. W., et al. (2011). Tamoxifen metabolite concentrations, CYP2D6 genotype, and breast cancer outcomes. *Clinical Pharmacology and Therapeutics*, *89*(5), 718–725.

Martino, S., Cauley, J. A., Barrett-Connor, E., Powles, T. J., Mershon, J., Disch, D., et al. (2004). Continuing outcomes relevant to Evista: Breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *Journal of the National Cancer Institute*, *96*(23), 1751–1761.

Masamura, S., Santner, S. J., Heitjan, D. F., & Santen, R. J. (1995). Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. *The Journal of Clinical Endocrinology and Metabolism*, *80*(10), 2918–2925.

Author's personal copy

Maximov, P., Sengupta, S., Lewis-Wambi, J. S., Kim, H. R., Curpan, R. F., & Jordan, V. C. (2011). The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: A hypothesis. *Hormonal Molecular Biology and Clinical Investigation*, *5*(1), 27–34.

McGuire, W. L., Carbone, P. P., & Vollmer, E. P. (Eds.), (1975). *Estrogen receptors in human breast cancer*. New York: Raven Press.

Mehta, R. S., Barlow, W. E., Albain, K. S., Vandenberg, T. A., Dakhil, S. R., Tirumali, N. R., et al. (2012). Combination anastrozole and fulvestrant in metastatic breast cancer. *The New England Journal of Medicine*, *367*(5), 435–444.

Miller, C. P., Collini, M. D., Tran, B. D., Harris, H. A., Kharode, Y. P., Marzolf, J. T., et al. (2001). Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. *Journal of Medicinal Chemistry*, *44*(11), 1654–1657.

NATO (1983). Controlled trial of tamoxifen as adjuvant agent in management of early breast cancer. Interim analysis at four years by Nolvadex Adjuvant Trial Organisation. *The Lancet*, *1*(8319), 257–261.

O'Regan, R. M., Gajdos, C., Dardes, R. C., De Los Reyes, A., Park, W., Rademaker, A. W., et al. (2002). Effects of raloxifene after tamoxifen on breast and endometrial tumor growth in athymic mice. *Journal of the National Cancer Institute*, *94*(4), 274–283.

Osborne, C. K., Coronado, E. B., & Robinson, J. P. (1987). Human breast cancer in the athymic nude mouse: Cytostatic effects of long-term antiestrogen therapy. *European Journal of Cancer & Clinical Oncology*, *23*(8), 1189–1196.

Osborne, C. K., Hobbs, K., & Clark, G. M. (1985). Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. *Cancer Research*, *45*(2), 584–590.

Osborne, C. K., Pippen, J., Jones, S. E., Parker, L. M., Ellis, M., Come, S., et al. (2002). Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: Results of a North American trial. *Journal of Clinical Oncology*, *20*(16), 3386–3395.

Osipo, C., Gajdos, C., Liu, H., Chen, B., & Jordan, V. C. (2003). Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. *Journal of the National Cancer Institute*, *95*(21), 1597–1608.

Payre, B., de Medina, P., Boubekeur, N., Mhamdi, L., Bertrand-Michel, J., Terce, F., et al. (2008). Microsomal antiestrogen-binding site ligands induce growth control and differentiation of human breast cancer cells through the modulation of cholesterol metabolism. *Molecular Cancer Therapeutics*, *7*(12), 3707–3718.

Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., et al. (2000). Molecular portraits of human breast tumours. *Nature*, *406*(6797), 747–752.

Pink, J. J., Jiang, S. Y., Fritsch, M., & Jordan, V. C. (1995). An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Research*, *55*(12), 2583–2590.

Pinkerton, J. V., Pickar, J. H., Racketa, J., & Mirkin, S. (2012). Bazedoxifene/conjugated estrogens for menopausal symptom treatment and osteoporosis prevention. *Climacteric*, *15*(5), 411–418.

Poirot, M. (2011). Four decades of discovery in breast cancer research and treatment—An interview with V. Craig Jordan. *The International Journal of Developmental Biology*, *55*, 703–712.

Powles, T. J., Ashley, S., Tidy, A., Smith, I. E., & Dowsett, M. (2007). Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. *Journal of the National Cancer Institute*, *99*(4), 283–290.

Powles, T., Eeles, R., Ashley, S., Easton, D., Chang, J., Dowsett, M., et al. (1998). Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomised chemoprevention trial. *The Lancet*, *352*(9122), 98–101.

Author's personal copy

Powles, T. J., Hardy, J. R., Ashley, S. E., Farrington, G. M., Cosgrove, D., Davey, J. B., et al. (1989). A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *British Journal of Cancer*, *60*(1), 126–131.

Prentice, R. L., Chlebowski, R. T., Stefanick, M. L., Manson, J. E., Langer, R. D., Pettinger, M., et al. (2008). Conjugated equine estrogens and breast cancer risk in the Women's Health Initiative clinical trial and observational study. *American Journal of Epidemiology*, *167*(12), 1407–1415.

Rae, J. M., Drury, S., Hayes, D. F., Stearns, V., Thibert, J. N., Haynes, B. P., et al. (2012). CYP2D6 and UGT2B7 genotype and risk of recurrence in tamoxifen-treated breast cancer patients. *Journal of the National Cancer Institute*, *104*(6), 452–460.

Regan, M. M., Leyland-Jones, B., Bouzyk, M., Pagani, O., Tang, W., Kammler, R., et al. (2012). CYP2D6 genotype and tamoxifen response in postmenopausal women with endocrine-responsive breast cancer: The breast international group 1-98 trial. *Journal of the National Cancer Institute*, *104*(6), 441–451.

Riemsma, R., Forbes, C. A., Amonkar, M. M., Lykopoulos, K., Diaz, J. R., Kleijnen, J., et al. (2012). Systematic review of lapatinib in combination with letrozole compared with other first-line treatments for hormone receptor positive (HR +) and HER2 + advanced or metastatic breast cancer (MBC). *Current Medical Research and Opinion*, *28*(8), 1263–1279.

Ries, L. A. G., Eisner, M. P., & Kosary, C. L. (2001). *SEER cancer statistics review, 1973–1998*. Bethesda, Maryland: National Cancer Institute.

Robinson, S. P., Koch, R., & Jordan, V. C. (1988). In vitro estrogenic actions in rat and human cells of hydroxylated derivatives of D16726 (zindoxifene), an agent with known antimammary cancer activity in vivo. *Cancer Research*, *48*(4), 784–787.

Robson, J. M. (1937). Oestrous reactions, including mating, produced by triphenyl ethylene. *Nature*, *140*(3535), 196.

Robson, J. M., Schonberg, A., & Fahim, H. A. (1938). Duration of action of natural and synthetic oestrogens. *Nature*, *142*, 292–293.

Robson, J. M., & Schonberg, A. (1942). A new synthetic oestrogen with prolonged action when given orally. *Nature*, *150*(3792), 22–23.

Roop, R. P., & Ma, C. X. (2012). Endocrine resistance in breast cancer: Molecular pathways and rational development of targeted therapies. *Future Oncology*, *8*(3), 273–292.

Rosati, R. L., Da Silva Jardine, P., Cameron, K. O., Thompson, D. D., Ke, H. Z., Toler, S. M., et al. (1998). Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryltetrahydronaphthalene. *Journal of Medicinal Chemistry*, *41*(16), 2928–2931.

Rose, C., Thorpe, S. M., Andersen, K. W., Pedersen, B. V., Mouridsen, H. T., Blichert-Toft, M., et al. (1985). Beneficial effect of adjuvant tamoxifen therapy in primary breast cancer patients with high oestrogen receptor values. *The Lancet*, *1*(8419), 16–19.

Rossouw, J. E., Anderson, G. L., Prentice, R. L., LaCroix, A. Z., Kooperberg, C., Stefanick, M. L., et al. (2002). Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results from the Women's Health Initiative randomized controlled trial. *Journal of the American Medical Association*, *288*(3), 321–333.

Rutanen, E. M., Heikkinen, J., Halonen, K., Komi, J., Lammintausta, R., & Ylikorkala, O. (2003). Effects of ospemifene, a novel SERM, on hormones, genital tract, climacteric symptoms, and quality of life in postmenopausal women: A double-blind, randomized trial. *Menopause*, *10*(5), 433–439.

Schrek, R. (1960). Fashions in cancer research. In W. B. Wartman (Ed.), *1959–1960 Year book* (pp. 26–39). Chicago: The Year Book Publishers.

Schroth, W., Goetz, M. P., Hamann, U., Fasching, P. A., Schmidt, M., Winter, S., et al. (2009). Association between CYP2D6 polymorphisms and outcomes among women

Author's personal copy

with early stage breast cancer treated with tamoxifen. *Journal of the American Medical Association*, *302*(13), 1429–1436.
SCTO (1987). Adjuvant tamoxifen in the management of operable cancer: The Scottish Trial. Report from the Breast Cancer Trials Committee, Scottish Cancer Trials Office (MRC), Edinburgh. *The Lancet*, *2*(8852), 171–175.
Sengupta, S., & Jordan, V. C. (2013). Novel selective estrogen receptor modulators. In V. C. Jordan (Ed.), *Estrogen actions, SERMs, and women's health*. London: Imperial College Press.
Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., et al. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell*, *95*(7), 927–937.
Shim, W. S., Conaway, M., Masamura, S., Yue, W., Wang, J. P., Kmar, R., et al. (2000). Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. *Endocrinology*, *141*(1), 396–405.
Shimkin, M. B., & Wyman, R. S. (1945). Effect of adrenalectomy and ovariectomy on mammary carcinogenesis in strain C3H mice. *Journal of the National Cancer Institute*, *6*, 187–189.
Shimkin, M. B., & Wyman, R. S. (1946). Mammary tumors in male mice implanted with estrogen-cholesterol pellets. *Journal of the National Cancer Institute*, 7(2), 71–75.
Shimkin, M. B., Wyman, R. S., & Andervont, H. B. (1946). Mammary tumors in mice following transplantation of mammary tissue. *Journal of the National Cancer Institute*, 7(2), 77.
Shumaker, S. A., Legault, C., Rapp, S. R., Thal, L., Wallace, R. B., Ockene, J. K., et al. (2003). Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: The Women's Health Initiative Memory Study: A randomized controlled trial. *Journal of the American Medical Association*, *289*(20), 2651–2662.
Silverman, S. L., Chines, A. A., Kendler, D. L., Kung, A. W., Teglbjaerg, C. S., Felsenberg, D., et al. (2012). Sustained efficacy and safety of bazedoxifene in preventing fractures in postmenopausal women with osteoporosis: Results of a 5-year, randomized, placebo-controlled study. *Osteoporosis International*, *23*(1), 351–363.
Song, R. X., Mor, G., Naftolin, F., McPherson, R. A., Song, J., Zhang, Z., et al. (2001). Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *Journal of the National Cancer Institute*, *93*(22), 1714–1723.
Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., et al. (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(14), 8418–8423.
Speroff, L. (2009). *A good man, Gregory Goodwin Pincus: The man, his story, the birth control pill*. Portland, Oregon: Arnica Publishing.
Stein, R. C., Dowsett, M., Cunningham, D. C., Davenport, J., Ford, H. T., Gazet, J. C., et al. (1990). Phase I/II study of the anti-oestrogen zindoxifene (D16726) in the treatment of advanced breast cancer. A Cancer Research Campaign Phase I/II Clinical Trials Committee study. *British Journal of Cancer*, *61*(3), 451–453.
Stoll, B. (1977a). *Breast cancer management early and late*. London: William Herman Medical Books Ltd.
Stoll, B. A. (1977b). Palliation by castration or by hormone administration. In B. A. Stoll (Ed.), *Breast cancer management early and late* (pp. 133–146). London: W. Heinemann Medical Books Ltd.
Sutherland, R. L., Murphy, L. C., San Foo, M., Green, M. D., Whybourne, A. M., & Krozowski, Z. S. (1980). High-affinity anti-oestrogen binding site distinct from the oestrogen receptor. *Nature*, *288*(5788), 273–275.

Author's personal copy

Sweeney, E. E., McDaniel, R. E., Maximov, P. Y., Fan, P., & Jordan, V. C. (2012). Models and mechanisms of acquired antihormone resistance in breast cancer: Significant clinical progress despite limitations. *Hormonal Molecular Biology and Clinical Investigation*, *9*(2), 143–163.

Thompson, C. R., & Werner, H. W. (1953). Fat storage of an estrogen in women following orally administered tri-p-anisyl chloroethylene. *Proceedings of the Society for Experimental Biology and Medicine*, *84*, 491–492.

Thurlimann, B., Keshaviah, A., Coates, A. S., Mouridsen, H., Mauriac, L., Forbes, J. F., et al. (2005). A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *The New England Journal of Medicine*, *353*(26), 2747–2757.

Toft, D., & Gorski, J. (1966). A receptor molecule for estrogens: Isolation from the rat uterus and preliminary characterization. *PNAS*, *55*(6), 1574–1581.

Toft, D., Shyamala, G., & Gorski, J. (1967). A receptor molecule for estrogens: Studies using a cell-free system. *Proceedings of the National Academy of Sciences of the United States of America*, *57*(6), 1740–1743.

Turner, R. T., Evans, G. L., Sluka, J. P., Adrian, M. D., Bryant, H. U., Turner, C. H., et al. (1998). Differential responses of estrogen target tissues in rats including bone to clomiphene, enclomiphene, and zuclomiphene. *Endocrinology*, *139*(9), 3712–3720.

Turner, R. T., Evans, G. L., & Wakley, G. K. (1993). Mechanism of action of estrogen on cancellous bone balance in tibiae of ovariectomized growing rats: Inhibition of indices of formation and resorption. *Journal of Bone and Mineral Research*, *8*(3), 359–366.

Turner, R. T., Wakley, G. K., Hannon, K. S., & Bell, N. H. (1987). Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. *Journal of Bone and Mineral Research*, *2*(5), 449–456.

Turner, R. T., Wakley, G. K., Hannon, K. S., & Bell, N. H. (1988). Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. *Endocrinology*, *122*(3), 1146–1150.

Veronesi, U., Maisonneuve, P., Costa, A., Sacchini, V., Maltoni, C., Robertson, C., et al. (1998). Prevention of breast cancer with tamoxifen: Preliminary findings from the Italian randomised trial among hysterectomised women. Italian Tamoxifen Prevention Study. *The Lancet*, *352*(9122), 93–97.

Vogel, V. G., Costantino, J. P., Wickerham, D. L., Cronin, W. M., Cecchini, R. S., Atkins, J. N., et al. (2006). Effects of tamoxifen vs. raloxifene on the risk of developing invasive breast cancer and other disease outcomes: The NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *Journal of the American Medical Association*, *295*(23), 2727–2741.

Vogel, V. G., Costantino, J. P., Wickerham, D. L., Cronin, W. M., Cecchini, R. S., Atkins, J. N., et al. (2010). Update of the national surgical adjuvant breast and bowel project study of tamoxifen and raloxifene (STAR) P-2 trial: Preventing breast cancer. *Cancer Prevention Research (Philadelphia, Pa.)*, *3*(6), 696–706.

Voipio, S. K., Komi, J., Kangas, L., Halonen, K., DeGregorio, M. W., & Erkkola, R. U. (2002). Effects of ospemifene (FC-1271a) on uterine endometrium, vaginal maturation index, and hormonal status in healthy postmenopausal women. *Maturitas*, *43*(3), 207–214.

Wallace, O. B., Lauwers, K. S., Dodge, J. A., May, S. A., Calvin, J. R., Hinklin, R., et al. (2006). A selective estrogen receptor modulator for the treatment of hot flushes. *Journal of Medicinal Chemistry*, *49*(3), 843–846.

Walpole, A. L., & Paterson, E. (1949). Synthetic oestrogens in mammary cancer. *The Lancet*, *2*(6583), 783–786.

Watanabe, N., Ikeno, A., Minato, H., Nakagawa, H., Kohayakawa, C., & Tsuji, J. (2003). Discovery and preclinical characterization of (+)-3-[4-(1-piperidinoethoxy)phenyl]

Author's personal copy

spiro[indene-1,1′-indane]-5,5′-diol hydrochloride: A promising nonsteroidal estrogen receptor agonist for hot flush. *Journal of Medicinal Chemistry*, *46*(19), 3961–3964.

Welsch, C. W. (1985). Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: A review and tribute to Charles Brenton Huggins. *Cancer Research*, *45*(8), 3415–3443.

Welshons, W. V., & Jordan, V. C. (1987). Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *European Journal of Cancer & Clinical Oncology*, *23*(12), 1935–1939.

Wolf, D. M., & Jordan, V. C. (1993). A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results in Cancer Research*, *127*, 23–33.

Yang, L., Parkin, D., Ferlay, J., Li, L., & Chen, Y. (2005). Estimates of cancer incidence in China for 2000 and projections for 2005. *Cancer Epidemiology, Biomarkers & Prevention*, *14*(1), 243–249.

Yao, K., Lee, E. S., Bentrem, D. J., England, G., Schafer, J. I., O'Regan, R. M., et al. (2000). Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clinical Cancer Research*, *6*(5), 2028–2036.

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The evolution of nonsteroidal antiestrogens to become selective estrogen receptor modulators

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ABSTRACT

The discovery of the first nonsteroidal antiestrogen ethamoxytriphetol (MER25) in 1958, opened the door to a wide range of clinical applications. However, the finding that ethamoxytriphetol was a "morning after" pill in laboratory animals, energized the pharmaceutical industry to discover more potent derivatives. In the wake of the enormous impact of the introduction of the oral contraceptive worldwide, contraceptive research was a central focus in the early 1960's. Numerous compounds were discovered e.g., clomiphene, nafoxidine, and tamoxifen, but the fact that clinical studies showed no contraceptive actions, but, in fact, induced ovulation, dampened enthusiasm for clinical development. Only clomiphene moved forward to pioneer an application to induce ovulation in subfertile women. The fact that all the compounds were antiestrogenic made an application in patients to treat estrogen responsive breast cancer, an obvious choice. However, toxicities and poor projected commercial returns severely retarded clinical development for two decades. In the 1970's a paradigm shift in the laboratory to advocate long term adjuvant tamoxifen treatment for early (non-metastatic) breast cancer changed medical care and dramatically increased survivorship. Tamoxifen pioneered that paradigm shift but it became the medicine of choice in a second paradigm shift for preventing breast cancer during the 1980's and 1990's. This was not surprising as it was the only medicine available and there was laboratory and clinical evidence for the eventual success of this application. Tamoxifen is the first medicine to be approved by the Food and Drug Administration (FDA) to reduce the risk of breast cancer in women at high risk. But it was the re-evaluation of the toxicology of tamoxifen in the 1980's and the finding that there was both carcinogenic potential and a significant, but small, risk of endometrial cancer in postmenopausal women that led to a third paradigm shift to identify applications for selective estrogen receptor (ER) modulation. This idea was to establish a new group of medicines now called selective ER modulators (SERMs). Today there are 5 SERMs FDA approved (one other in Europe) for applications ranging from the reduction of breast cancer risk and osteoporosis to the reduction of menopausal hot flashes and improvements in dyspareunia and vaginal lubrication. This article charts the origins of the current path for progress in women's health with SERMs.

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1. Introduction

Today tamoxifen (Fig. 1) is part of the fabric of our society: almost everyone knows someone who is alive today because of their treatment with this antiestrogen used to prevent breast cancer recurrence. But this medicine is not only a pioneering breast cancer treatment and chemopreventive, but also a drug so thoroughly researched in the laboratory (I (VCJ) would always say this was to reveal "the Good, the Bad, and the Ugly") that a whole new group of medicines, the selective estrogen receptor modulators (SERMs) was created to address specific tasks in therapeutics. This saga, that first started in 1958 [1] with the report of the first nonsteroidal antiestrogen MER25 (Fig. 1) will twist and turn as fashions and priorities in medical research changed. Many nonsteroidal antiestrogens were synthesized initially when it was thought that there was great potential for their use as "morning after pills" but this application was not to occur as the compounds guaranteed what they were designed to prevent in women! The long gestation period for nonsteroidal antiestrogens resulted in clomiphene (Fig. 1), the first medicine to induce ovulation in women [2] in the 1960's, and then the orphan drug tamoxifen

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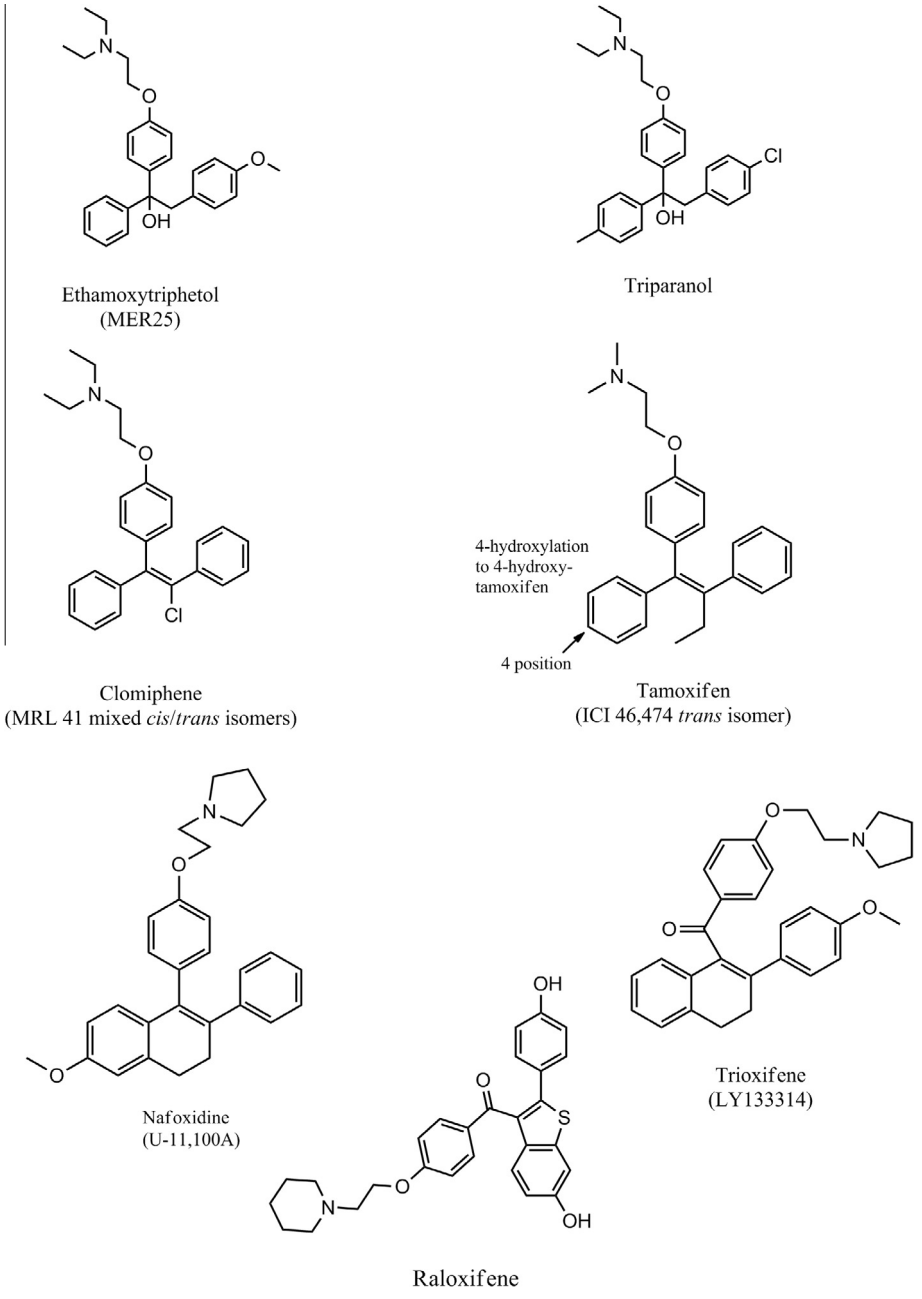

Fig. 1. Compounds described in the text.

would be approved almost by chance, for the treatment of metastatic breast cancer in the 1970's. Tamoxifen stood the test of time as a pioneering breast cancer treatment used ubiquitously to treat all stages of the disease, ductal carcinoma in situ, and as a pioneering chemopreventive. No other cancer drug has achieved this status. But with the description of SERMs in the 1980's, a whole new era in women's health was born. This is that story which will in the future encompass all members of the nuclear receptor superfamily.

2. Discovery of nonsteroidal antiestrogens

The discovery of the antiestrogenic properties of MER25 (ethamoxytriphetol) (Fig. 1) [1] was in part chance but an example of serendipity i.e., an unanticipated advance in knowledge. Dr. Leonard Lerner was a young reproductive endocrinologist at the William S. Merrell Company in Cincinnati in the mid 1950's, charged with the investigation of nonsteroidal estrogens for clinical applications. Lerner was glancing through compounds to be tested in the cardiovascular program and notice that one MER25 had a structure resembling triphenylethylene-like estrogens. He asked for the compound to test but unexpectedly he found no estrogen-like activity in any species tested [3]. Instead he noted weak but consistent antiestrogenic action in all animal models [1]. The compound was also structurally similar to triparanol (Fig. 1), a drug originally marketed by the Merrell Company to reduce circulating cholesterol levels, but was withdrawn because triparanol increases circulating desmosterol levels (Fig. 2) which was thought to be responsible for the rapid onset of cataracts in young women [4,5]. These observations were to be essential for the future drug development of nonsteroidal antiestrogens when they would eventually be required to be given for up to a decade [6].

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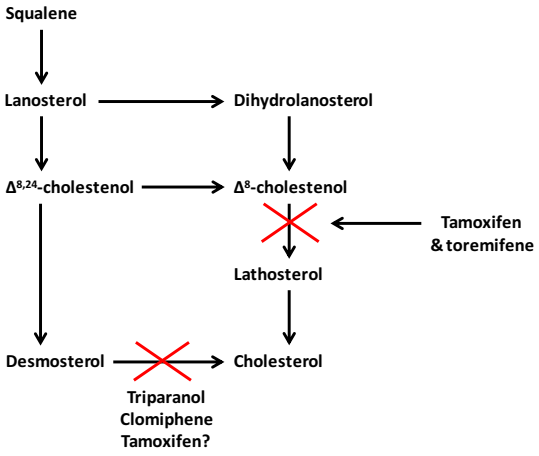

Fig. 2. The inhibition of cholesterol biosynthesis by triparanol, clomiphene, tamoxifen, and the β-chlorinated derivative of tamoxifen toremifene.

Clinical trials with MER25 were conducted [3] but not reported in the literature. By contrast, clinical trials with clomiphene (Fig. 1), a mixture of estrogenic and antiestrogenic geometric isomers of a triphenylethylene was tested extensively for the regulation of fertility but shown to induce ovulating in subfertile women [2] and showed modest activity as a breast cancer therapy [7]. However, the breast cancer treatment option was discontinued as clomiphene produces an increase in desmosterol Fig. 2.

The Upjohn Company focused a huge synthetic effort on the antifertility properties of indene and naphthalene [8] derivatives thereby solving the issue of separating the isomers of triphenylethylenes, the landscape of which had actually been defensively patented by Merrell in the 1960's. The Upjohn Company discovered a compound, U-11, 100A (Fig. 1) that would be unsuccessfully developed as a breast cancer drug [9]. The compound, renamed nafoxidine was effective at controlling the growth of 30% of breast cancers for about a year but severe side effects such as photophobia precluded further clinical development. Nevertheless, nafoxidine was the structural basis for a new SERM lasofoxifene examined 20 years later (see last section). The fact that Merrell defensively patented triphenylethylenes as breast cancer drugs prevented patent security for tamoxifen in the United States until 1985 of their original patent submitted in the 1960's! The defensive patenting of triphenylethylenes by Merrell was actually to turn out to be a stimulus for innovation in medicinal chemistry and after 1985 was to create a significant unanticipated financial windfall for ICI Pharmaceuticals Division that had now undergone a metamorphosis to Zeneca. In 1984, an NCI panel declared long term adjuvant tamoxifen therapy the antihormone treatment of choice for the treatment of ER positive breast cancer [10] and Zeneca now had 20 years patent protection. This provided profits to invest in chemoprevention and fund the development of a range of other leading and innovative antihormonal therapies: bicalutamide, anastrozole, and fulvestrent at Zeneca.

The patenting restrictions led Eli Lilly to explore chemistry described originally in India at the Central Drug Research Institute in Lucknow, India [11] to link the bulky antiestrogenic group by a ketone bridge to the ER ligand binding moiety [12]. The result was trioxifene (Fig. 1) that was to fail against tamoxifen in clinical trials to treat breast cancer. Nevertheless, the structural advance gave the world the high affinity antiestrogens LY117018 [13] and LY156758 [14] after it was discovered that tamoxifen was metabolically activated to the high affinity antiestrogen 4-hydroxytamoxifen [15,16]. During the late 1960's and throughout the 1970's tamoxifen was being developed glacially throughout the world (including the United States without patent protection) by ICI Pharmaceuticals Division. Why was that and how did the opportunity to change that, significantly advance women's health?

3. The tamoxifen tale

During the early years of the 1960's, Arthur Walpole, Mike Harper, and Dora Richardson were the key members of the Fertility Control program at ICI Pharmaceuticals Division, Alderley Park, near Macclesfield, Chesire. Walpole was the senior scientist and head of the program, Harper was the experimental reproductive endocrinologist and Richardson the synthetic organic chemist. The team was tasked with advancing the goal of discovering a safe and effective "post coital" contraceptive and the work on reproduction would be continued by Labhsetwar into the 1970's [17–20] despite the fact that the fertility program was going nowhere. The principal achievements of the team was the discovery that the geometric isomers of a substituted triphenylethylene were estrogenic or antiestrogenic: the cis isomer ICI 47,699 was an estrogen [21] and the trans isomer ICI 46,474 was an antiestrogen with antifertility properties in the rat by preventing implantation that was found to be an estrogen dependent process [22,23]. Most importantly, for the future development of ICI 46,474, as a long term anticancer agent, the antiestrogen did not increase desmosterol levels in rats [22].

Although Walpole had an interest in cancer research [24] no studies were conducted at ICI Pharmaceuticals Division but Walpole did initiate clinical studies outside the company to demonstrate activity as an anticancer agent in metastatic breast cancer [25,26] and like clomiphene, the induction of ovulation in subfertile women [27]. However, in the spring of 1972, a meeting was held at ICI Pharmaceuticals Division to review all clinical progress with ICI 46,474 and the decision was subsequently made to terminate clinical development. Fortunately for me (VCJ), and probably for my future career, Arthur Walpole was the examiner of my PhD on "failed contraceptives" entitled: *A Study of the Oestrogenic and Anti-oestrogenic Activities of Some Substituted Triphenylethylenes and Triphenylethanes*. I passed my PhD examination and I was appointed as a faculty member at Leeds University in mid-1972 but was required to obtain my "Been to America" (BTA). My chairman in the Pharmacology Department Mike Barrett (formerly of ICI Pharmaceuticals) and Walpole recommended I spend two years at the Worcester Foundation (the home of the oral contraceptive), with Mike Harper who was now heading a research team to develop a once-a-month pill. Mike Harper had published all of the antifertility properties of ICI 46,474 in the mid 1960's [21–23] when he was at ICI Pharmaceuticals Division, Alderley Park.

When I (VCJ) got to the Foundation in September 1972, Harper had left to accept an appointment at the World Health Organization in Geneva and I was told I could do anything I wanted as long as some of it involved contraception. A phone call to Walpole at ICI Pharmaceuticals Division to discuss the idea that ICI 46,474 should be developed as a breast cancer drug, resulted in an unrestricted research grant to study the anticancer properties of ICI 46,474 in the laboratory, an appointment to be an ICI Americas consultant on the project and act as an advisor to them with clinical trial cooperative groups in America. What I did not know was that Walpole had tendered his resignation in 1972, but he agreed to remain at ICI Pharmaceuticals Division if ICI 46,474 was put on the market as an orphan drug. He suggested that funds be made available for me (VCJ) to discover the best strategy for the clinical use of ICI 46,474 as a breast cancer drug. ICI Americas/ICI Pharmaceuticals Division/and the Yorkshire Cancer Campaign would fund my (VCJ) laboratory first at the

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Worcester Foundation and then at Leeds University throughout the 1970's. That decade resulted in publications to support three strategic applications of tamoxifen (formerly ICI 46,474): target the ER in the tumor where tamoxifen and its metabolites block estrogen stimulated growth [15,28], tamoxifen for the prevention of mammary carcinogenesis [29,30] and the idea of long term adjuvant tamoxifen therapy would be the appropriate strategy to prevent tumor recurrence [31–33].

During the next 30 years, clinical studies established unequivocally that long term adjuvant tamoxifen therapy using 5 or more years of treatment produced major survival advantages for patients with as ER positive breast tumor [6,34–36]. However, it was the paradigm shift from treatment to chemoprevention during the 1980's and 1990's that opened up new opportunities in women's health.

4. The chemoprevention of breast cancer in high risk women

The idea that breast cancer can be prevented is not new. In 1936, Professor Antoine Lacassagne [37] presented the following strategy at the Annual Meeting of the American Association for Cancer Research in Boston.

> "If one accepts the consideration of adenocarcinoma of the breast as the consequence of a special hereditary sensibility to the proliferative actions of oestrone, one is led to imagine a therapeutic preventative for subjects predisposed by their heredity to this cancer. It would consist – perhaps in the very near future when the knowledge and use of hormones will be better understood – in the suitable use of a hormone antagonistic or excretory, to prevent the stagnation of oestrone in the ducts of the breast."

However, at that time there were no "antiestrogenic" compounds and neither was there a target at which to aim. The compounds were to develop from the serendipitous discovery of MER25 [38]. The main compounds were all discovered and developed on the evidence of a bioassay *in vivo*: the inhibition of postcoital implantation in rodents! The target was to be discovered using high specific activity tritiated estrogen in whole animal distribution studies with the tritiated estrogen binding in and being retained in estrogen target tissues i.e., uterus, vagina, pituitary gland [39,40]. The ER was first identified as an extractable protein from immature rat uteri [41,42]. From there, translation to clinical applications in breast cancer flowed with the ER assay to determine estrogen dependent growth in breast tumors as a predictive test for ablative surgery in advanced disease [43] and then transformed into a target for antiestrogen action to treat breast cancer [44].

In 1986, Professor Trevor Powles took the initiative to be the first recruit a vanguard study of high risk women for what was to become the "Royal Marsden Study". He based his plan on the fact that tamoxifen prevented rat mammary carcinogenesis [29,30,45] and adjuvant tamoxifen reduced the risk of contralateral breast cancer [46]. His early results [47] proved provocative as there was maintained compliance vs. placebo but the spectre of carcinogenesis with tamoxifen was already apparent and this had to be addressed in any future trials. Nevertheless, substantial recruitment and compliance continued and a decrease in breast cancer incidence was noted at a 20 year follow-up [48]. It was clear from studies in athymic mice with transplantable ER positive tumors, that a target site specificity with tamoxifen was occurring. Since Harper and Walpole's [21,22] first publications on ICI 46,474, there was known species specificity; tamoxifen was classified as an antiestrogen in the immature rat uterus but an estrogen in the ovariectomized mouse uterus and vagina. However, studies of metabolic differences did not prove the obvious – tamoxifen was an estrogen in the mouse because it is metabolized to an estrogen [49]. Studies in the athymic mouse were to demonstrate that tamoxifen did not support the growth of ER + MCF-7 tumors but stimulated the uterus to grow [50]. It was stated "these studies strongly support the concept that the drug (tamoxifen) can selectively stimulate or inhibit events in target in tissues of different species without metabolic intervention". "The drug-estrogen receptor complex is perceived as a stimulatory or inhibitory signal" [50]. Subsequent studies in the high incidence mammary tumor strain of mice (C3HOUJ) demonstrated that tamoxifen prevented mammary tumor carcinogenesis and was superior to oophorectomy [51]. The original prediction by Lacassagne was correct [37]. Nevertheless, the "breakthrough" experiment that had major ramifications for clinical medicine and patient care was the finding that athymic mice bitransplanted with an ER positive breast tumor (MCF-7) and an ER positive endometrial cancer (EnCa 101) would exhibit "antiestrogenic" actions on the breast tumor to stop growth but "estrogenic" action in the endometrial cancer to promote growth [52] (Fig. 3). These data were presented to the clinical community [53,54] with the concern "a large cohort of patients under long term tamoxifen therapy (>5 years) needs to be monitored for the occurrence of tamoxifen-stimulated endometrial tumors" [52].

Retrospective analysis of clinical trials data confirmed there was a low but significant increase in endometrial cancer incidence in postmenopausal women receiving long term adjuvant tamoxifen treatment [55,56]. Not only was this finding important for patient care in general practice but also this knowledge was essential to ensure safety for the trials that were planned to test the worth of tamoxifen to prevent breast cancer in high risk women [57–59]. However, the surprise was the toxicological finding that high dose tamoxifen treatment for the life-time of a rat would initiate hepatocellular carcinoma [60–62]. Fortunately these data did not translate to clinical practice. The Oxford Overview of clinical trials did not show an increase in hepatocellular carcinoma in patients receiving adjuvant therapy but it is clear that if tamoxifen had been tested for carcinogeneity in 1973 when the first animal studies for adjuvant therapy and chemoprevention were started, tamoxifen would not have been developed by the pharmaceutical industry [63]. Hundreds of thousands of women would have died and the aromatase inhibitors would have been abandoned as these new antihormonal agents were only developed because the strategy of long term adjuvant tamoxifen was shown to be successful financially [63]!

This is not the place to review the results of the tamoxifen trials of chemoprevention. Suffice to say they were successful overall [48,57–59,64,65] and tamoxifen was approved by the FDA in 1998 as the pioneer for the reduction of breast cancer incidence in pre and post-menopausal women with a high risk.

What is important to stress is the fact that a more transparent understanding of tamoxifen's pharmacology and long term safety was needed in the 80's if tamoxifen was to advance in the 90's for broad clinical testing as a chemopreventive. The question was straight forward: "if tamoxifen is classified as an antiestrogen but estrogen is necessary to maintain bone density and (as was thought at the time) to decrease the risk of coronary heart disease, what advantage would there be in preventing half a dozen breast cancers per 1000 women per year if 300 women developed osteoporosis and there were more women dying of heart attacks?" Unexpectedly, a series of laboratory studies was to provide reassurances that tamoxifen was not "just an antiestrogen" but it was selectively estrogenic and antiestrogenic in different estrogen target tissues around a woman's body. Most importantly, the laboratory finding all translated to successful clinical trials and a new paradigm was conceived with the creation of a new group of medicines – the selective estrogen receptor modulators or SERMs.

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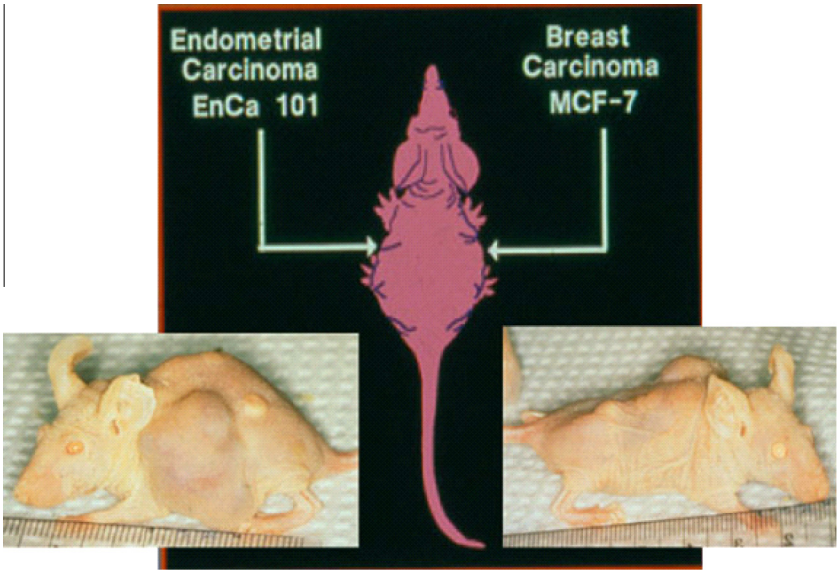

Fig. 3. The pioneering bitransplantation study by Gottardis [72] with an ER-positive breast tumor (MCF-7) implanted in one axilla and an ER-positive endometrial tumor (EnCa 101) in the other axilla. Tamoxifen blocks estrogen-stimulated growth of the breast tumor (right), but tamoxifen encourages the growth of the endometrial tumor (left). These data were transmitted immediately to the clinical community [53,54], confirmed in clinical trials [55,56] to change clinical practice.

5. Nonsteroidal antiestrogens were "born" but SERMs were "conceived"

Nonsteroidal antiestrogens had initially been developed and failed in their primary application as "morning after" pills but in the 1960's and 70's both clomiphene and tamoxifen succeed in a secondary application. The fact that subfertile women could now induce ovulation and successfully give birth to children was a pioneering advance but not, at that time, a significant market. Another secondary application was the treatment of metastatic breast cancer, but this too was an insignificant market for a palliative drug such as tamoxifen. By contrast, what happened over 30 years was the confirmation that long term adjuvant tamoxifen therapy was the best strategy for clinical trials [66] and would be found to save perhaps millions of lives. The FDA approval of tamoxifen for chemoprevention in 1998 would now result in another blockbuster drug resurrected through the development of the new and novel strategy [38] of using a SERM (raloxifene) to prevent multiple diseases in women.

Serendipity took control with an initial investigation of the effects of tamoxifen and a failed breast cancer drug keoxifene on ovarectomized rat bone loss [67]. The findings were not anticipated; what was anticipated was that these two nonsteroidal antiestrogens would increase bone loss. What was found was that the opposite occurred and that ovarectomized rats treated with the antiestrogen plus estrogen had no bone loss. By contrast, the antiestrogens blocked estrogen induced increases in uterine weight [67]. There was target site specificity for nonsteroidal antiestrogens. This was not unlike the estrogen-like effects of tamoxifen in the athymic mouse uterus vs. the prevention of estrogen stimulated growth of an implanted breast tumor [50] or the stimulation of endometrial cancer growth against the inhibition of growth of a breast tumor implanted in the same athymic mouse [52]. All results had been observed at the same time in our Tamoxifen Team laboratory in Wisconsin – it was a principle! This was the preliminary data used to fund and advance subsequently successful clinical trials [68–70]. With this knowledge, and the fact that tamoxifen caused a decrease in circulating cholesterol in rats (Fig. 2) [22] which, incidentally, caused ICI Pharmaceuticals Division to place a "hypocholesterolanemic" indication in their patent application 20 years earlier [71], it was now possible to consider a new approach to preventing breast cancer by developing multifunctional medicines for women's health. This was a prescient concept because the carcinogenic problems with tamoxifen, once they surfaced, [55,61] would not go away and would preclude broad applications for the medicine in women's health. The new concept [38] was stated simply and directly based on laboratory data i.e., before the publication of the results of ongoing clinical trials at the time with tamoxifen or initiation of new trials with other SERMs, as a roadmap for the pharmaceutical industry to follow.

> "Is this the end of the possible applications for antioestrogens? Certainly not. We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be post-menopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer."

Raloxifene (Fig. 1) was the result. It was actually the obvious choice (the compound had been tested clinically previously and was a failed breast cancer treatment under its former name keoxifene) as it was now known to preserve bone density in the laboratory [67], prevent carcinogen induced mammary cancer in rats [72], be less uterotrophic then tamoxifen in rats [14,73,74] and inhibit tamoxifen stimulated endometrial cancer growth [75]. These findings were subsequently confirmed by others in the laboratory [76], in clinical trials for osteoporosis [77,78] and trials to evaluate the reduction of risk in breast cancer in high risk postmenopausal women [79,80]. Raloxifene is now FDA approved for the treatment and prevention of osteoporosis and the chemoprevention of breast cancer in postmenopausal high risk women. The SERMs had travelled from concept [38,81] to a clinically

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proven "cluster", of medicines: tamoxifen (and the related compound toremifene – a safer SERM in rats [62] but used to treat breast cancer[82]) and raloxifene that succeeded despite their original development plan as a breast cancer drug which failed. It has taken about 15 years of clinical gestation since tamoxifen (breast cancer risk reduction) and raloxifene (prevention of osteoporosis) were FDA approved for use in women at risk for disease but there has been a recent flurry of SERM approvals that deserve special comment. The new SERMs are innovative reinventions of early molecules in medicinal chemistry as the science has become more sophisticated and novel target for improvements in women's health more imaginative.

6. New developments

Current progress in the FDA approvals of the new SERMs bazedoxifene (Fig. 4) for the prevention of osteoporosis and (in combination with conjugated estrogen) for the amelioration of postmenopausal hot flashes and ospemiphene (Fig. 4) for the improvement of atrophic vaginal symptoms and vaginal lubrication has been presented earlier [83,84]. The summary of FDA approved SERMs to enhance and cement the market are illustrated in Fig. 4 but the figure also includes lasofoxifene that was approved in the European Union but with no plans for launching the product for the treatment and prevention of osteoporosis. Approval has lapsed. Despite this deficit, the medicine is worthy of comment because of the advance in pharmacology as a multifunctional medicine in women health.

Bazedoxifene, ospemifene, and lasofoxifene each are compounds derived from prior pharmalogical knowledge (Fig. 5). The principal structural feature of basedoxifene that binds to the ER is a potential metabolite of a failed breast cancer drug called zindoxifene that was found to actually be an estrogen [85]. The core ligand was married to a predictable bulky antiestrogenic side chain to create the new SERM bazedoxifene [86]. Ospemiphene is a known metabolite of the SERM toremifene [82] that was studied in detail twenty years ago when tamoxifen was found to have the potential to be carcinogenic in rat liver at high doses [62]. It seems that tamoxifen is hydroxylated in the α position on the ethyl substitution at the ethylene bond and this is the metabolite that caused adduct formation in the rat liver DNA. Toremifene has a β chlorine so α hydroxylation does not occur [62] and it is a safer SERM in rat liver. However, this metabolic transformation has no toxicological relevance in patients. The "antiestrogenic" side chain of ospemiphene is a glycol formed by the deamination of the dealkylated toremifene side chain. This metabolic transformation was first noted for tamoxifen [87,88] in patient sera and the metabolite, metabolite Y was confirmed as a weakly antiestrogenic compound with partial estrogen-like actions [88].

We have met the origins of lasofoxifene earlier. It is the compound U-11,100A or nafoxidine (Fig. 1), discovered at the Upjohn research laboratories in their search for antifertility agents [8] but developed as a potential breast cancer drug that failed because of severe toxicities [9]. Lasofoxifene (Fig. 5) is a miracle of medicinal chemistry. With demethylation of nafoxidine, the resulting molecule has high affinity for the ER but as a result, the molecule also has rapid clearance because of phase II metabolism and increased excretion. This principle was first illustrated by 4-hydroxytamoxifen [15,16,32] and noted in raloxifene analogs [74]. However, reduction of the lone double bond in the non-aromatic ring of nafoxidine results in a possibility of two diasterioisomers. One isomer is used that is protected from conjugation and phase II metabolism. As a result lasofoxifene is used at a daily dose of 0.5 mg for the treatment and prevention of osteoporosis [89]. This contrasts with raloxifene used at a 60 mg daily dose either for the treatment and prevention of osteoporosis or the prevention of breast cancer [77,79,90].

Once the SERM concept was conceived [38,81] clinical development advanced effectively with raloxifene as the molecule was known to be free from endometrial cancer in animals and did not produce rat liver carcinogenesis. The drug would be safely used for the prevention of osteoporosis in otherwise healthy women! However, despite the fact raloxifene reduces cholesterol levels in rats [76], there was no evidence of any benefit by a reduction of coronary heart disease in high risk women [91]. However, tamoxifen and raloxifene were both two "repurposed" drugs [92] and

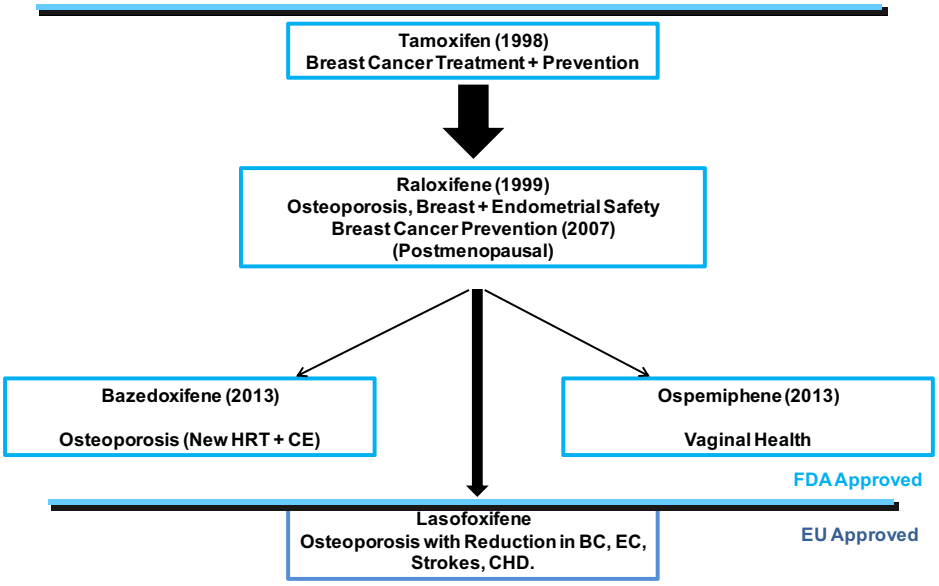

Fig. 4. The approvals of individual selective oestrogens receptor modulators (SERMs) in the United States of America through the evaluation system of the Food and Drug Administration (FDA). Approvals were specifically for indications at the highest level of toxicologic safety for women without disease but as a new hormone replacement therapy with conjugated estrogen (HRT + CE) to prevent disease i.e., chemoprevention of osteoporosis, breast cancer (BC), menopausal symptoms or dyspareunia. One SERM, lasofoxifene, was approved for use in the European Union (EU) but was never launched or marketed despite the fact that clinical trials demonstrated a reduction in breast cancer (BC), osteoporosis fracture, strokes, endometrial cancer (EC) and coronary heart disease (CHD) [89].

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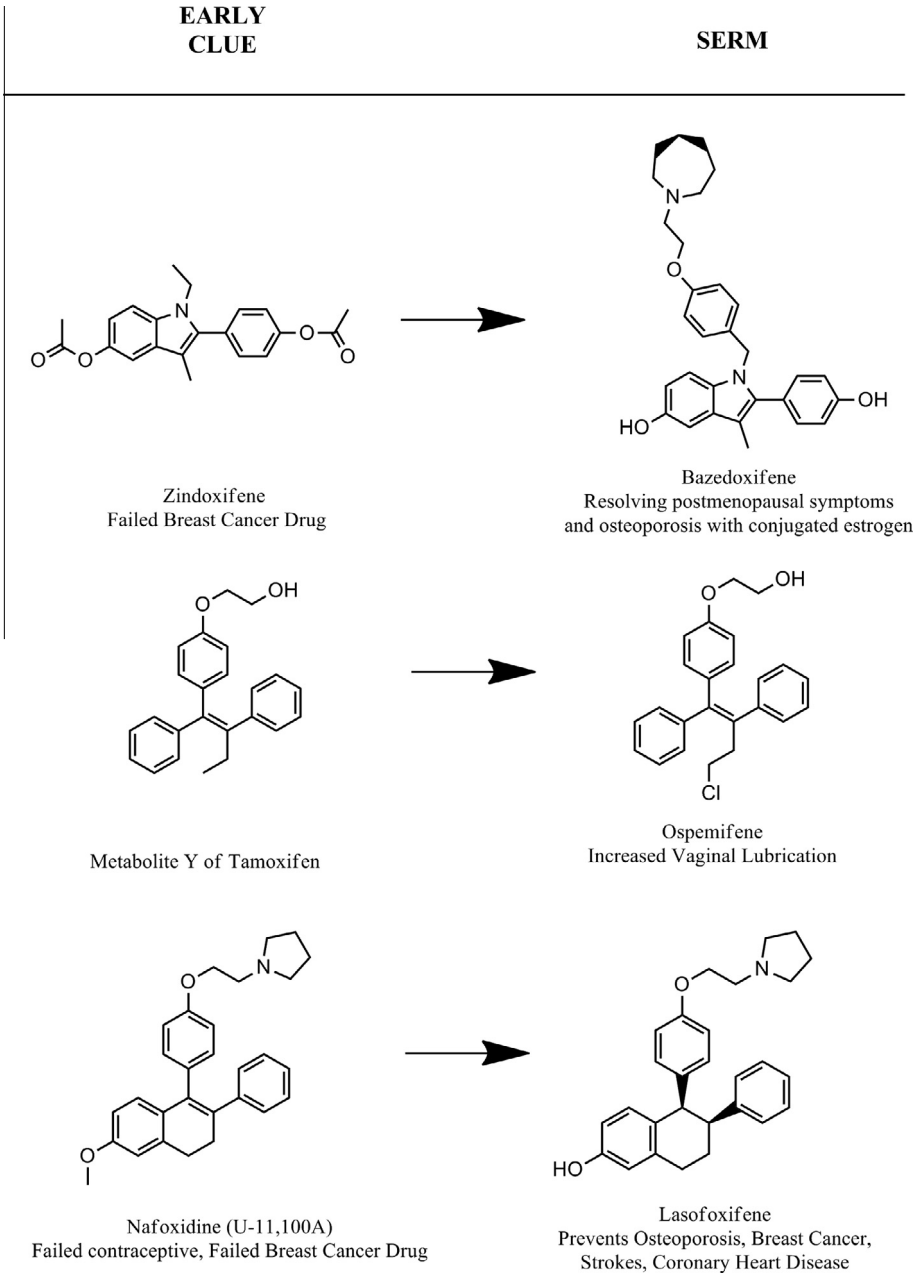

Fig. 5. Origins of current selective ER modulators from earlier nonsteroidal antiestrogens. The discovery that the metabolite of tamoxifen, 4-hydroxytamoxifen (Fig. 1) has a very high binding affinity for the ER [15] acted as a catalyst for the design of the majority of future SERMs. The raloxifene drug development "odyssey" throughout the 1980's [97] is a replay of the tamoxifen tale [71]. During the 70's [71], interestingly enough the work was done in the same laboratory but on different continents! The repurposing [92] and repatenting [97] of a failed breast cancer drug (keoxifene) resulted in raloxifene (Fig. 1), the same SERM, to establish a principle in translational research. Bazedoxifene is an adaptation of an estrogenic metabolite from a failed breast cancer drug Zindoxifene [85]. Ospemifene is a known metabolite of the breast cancer drug toremifene. The metabolite of toremifene was found because an analogous metabolite Y was discovered for tamoxifen in the early 1980's [88]. Lasofoxifene has its origins with failed antifertility agent discovered in the early 1960's U-11, 100A [8]. The compound renamed nafoxidine was tested as a drug for the treatment of breast cancer but again failed because of serious side effects [9].

there was still a long way to go to discover the "ideal SERM" as a multifunctional medicine (Fig 6). Lasofoxifene, the nafoxidine derivative, was to produce a few surprises. The PEARL trial of lasofoxifene in postmenopausal women at risk for osteoporosis used 0.25 and 0.5 mg daily doses against a placebo control to determine the prevention of osteoporosis [89]. Fractures were decreased, and breast cancer incidence was also reduced [93]. The surprise was a decrease in coronary heart disease and also a decrease in strokes [89]. The incidence of endometrial cancer was not increased but there was an estrogen-like increase in deep vein thrombosis. Lasofoxifene has demonstrated that medicinal chemistry and a commitment to large well organized clinical trial can provide much valuable information about the potential of selective modulation of the nuclear receptor superfamily.

If there is a message from the past 40 years of drug discovery, it is that a failure in one application can be a discovery in another [38]. There were a lot of "failures" but translational research was advanced to benefit women's health. In the closing years of the 19th century, the French author Jules Verne wrote: *whatever one man is capable of conceiving, other men are capable of achieving.* The SERMs were *conceived* [38] based on a cluster of interlocking experiments conducted by the Tamoxifen Team at the University

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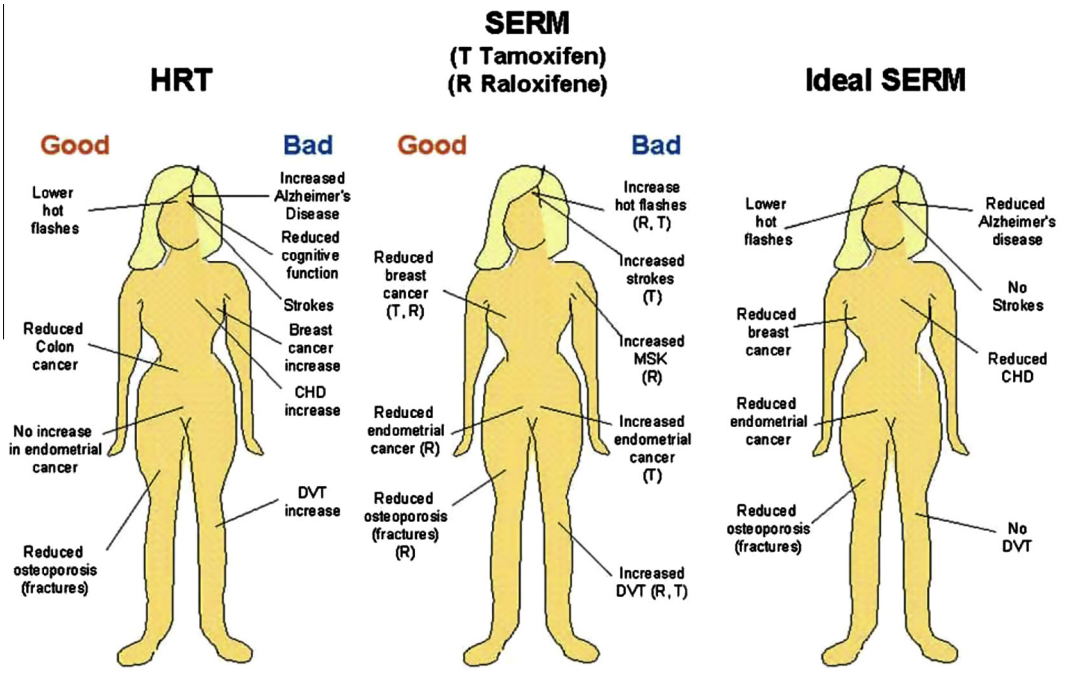

Fig. 6. Progress toward an ideal SERM. The overall good or bad aspects of administering hormone replacement therapy (HRT) to postmenopausal women compared with the observed site-specific actions of the SERMs tamoxifen and raloxifene. The known beneficial or negative actions of SERMs have opened the door for drug discovery to create the ideal SERM or targeted SERMs to either improve quality of life or prevent diseases associated with aging in women. This figure is published with permission from Elsevier. Jordan, V.C. Selective estrogen receptor modulation: Concept and consequences in cancer. Cancer Cell, 2004 Mar; 5[3]: 207–213.

of Wisconsin Comprehensive Cancer Center (1980–1993) [49–52,67,72–74,85,87,88,94]. A more detailed survey of SERMs and their origins for women's health can be found elsewhere [95,96]. Today, this particular special issue of STEROIDS provides opportunities for the next generation of men and women medical scientists to achieve success in their professional careers with the discovery of new modulating medicines in human health targeting the nuclear receptor superfamily.

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References

[1] Lerner LJ, Holthaus Jr FJ, Thompson CR. A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol. Endocrinology 1958 Sep;63(3):295–318.
[2] Greenblatt RB, Barfield WE, Jungck EC, Ray AW. Induction of ovulation with MRL/41. Preliminary report. JAMA 1961 Oct;14(178):101–4.
[3] Lerner LJ. The first nonsteroidal antioestrogen – MER25. In: Sutherland RL, Jordan VC, editors. Non-steroidal antioestrogens: molecular pharmacology and antitumour activity. Sydney and New York: Academic Press; 1981. p. 1–16.
[4] Avigan J, Steinberg D, Vroman HE, Thompson MJ, Mosettig E. Studies of cholesterol biosynthesis. I. The identification of desmosterol in serum and tissues of animals and man treated with MER-29. J Biol Chem 1960 Nov;235:3123–6.
[5] Laughlin RC, Carey TF. Cataracts in patients treated with triparanol. JAMA 1962 Jul;28(181):339–40.
[6] Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V, et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. Lancet 2013 Mar 9;381(9869):805–16.
[7] Herbst AL, Griffiths CT, Kistner RW. Clomiphene Citrate (NSC-35770) in disseminated mammary carcinoma. Cancer Chemother Rep 1964 Dec;43:39–41.
[8] Lednicer D, Lyster SC, Aspergren BD, Duncan GW. Mammalian antifertility agents. 3. 1-Aryl-2-phenyl-1,2,3,4-tetrahydro-1-naphthols, 1-aryl-2-phenyl-3,4-dihydronaphthalenes, and their derivatives. J Med Chem 1966 Mar;9(2):172–6.
[9] Legha SS, Slavik M, Carter SK. Nafoxidine – an antiestrogen for the treatment of breast cancer. Cancer 1976 Oct;38(4):1535–41.
[10] Consensus development conference. Report: adjuvant chemotherapy of breast cancer. JAMA 1985 Oct 18;254(15):3461–3.
[11] Iyer RN, Gopalachari R, Kamboj VP, Kar AB. Anti-implantation effect of 2,3-diphenylacrylophenones. Indian J Exp Biol 1967 Jul;5(3):169–70.
[12] Jones CD, Suarez T, Massey EH, Black LJ, Tinsley FC. Synthesis and antiestrogenic activity of [3,4-dihydro-2-(4-methoxyphenyl)-1-naphthalenyl][4-[2-(1-pyrrolidinyl)ethoxy]-phenyl]methanone, methanesulfonic acid salt. J Med Chem 1979 Aug;22(8):962–6.
[13] Black LJ, Goode RL. Uterine bioassay of tamoxifen, trioxifene and a new estrogen antagonist (LY117018) in rats and mice. Life Sci 1980 Apr 28;26(17):1453–8.
[14] Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 1983 Feb 28;32(9):1031–6.
[15] Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 1977 Nov;75(2):305–16.
[16] Allen KE, Clark ER, Jordan VC. Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 1980;71(1):83–91.
[17] Labhsetwar AP. Role of oestrogen in spontaneous ovulation demonstrated by use of an antagonist of oestrogen, ICI 46,474. Nature 1970 Jan 3;225(5227):80–1.
[18] Labhsetwar AP. Role of estrogens in ovulation: a study using the estrogen-antagonist, I.C.I. 46,474. Endocrinology 1970 Sep;87(3):542–51.
[19] Labhsetwar AP. Effects of an antioestrogen on the corpus luteum of rabbits and rats. J Reprod Fertil 1971 May;25(2):295–7.
[20] Labhsetwar AP. Role of estrogens in spontaneous ovulation: evidence for the positive feedback in hamsters. Endocrinology 1972 Apr;90(4):941–6.
[21] Harper MJ, Walpole AL. Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. Nature 1966 Oct 1;212(5057):87.

Please cite this article in press as: Craig Jordan V et al. The evolution of nonsteroidal antiestrogens to become selective estrogen receptor modulators. Steroids (2014), http://dx.doi.org/10.1016/j.steroids.2014.06.009

[22] Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 1967 Feb;13(1):101–19.
[23] Harper MJ, Walpole AL. Mode of action of I.C.I. 46,474 in preventing implantation in rats. J Endocrinol 1967 Jan;37(1):83–92.
[24] Jordan VC. The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. Breast Cancer Res Treat 1988 Jul;11(3): 197–209.
[25] Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer 1971 Jun;25(2): 270–5.
[26] Ward HW. Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J 1973 Jan 6;1(5844):13–4.
[27] Klopper A, Hall M. New synthetic agent for the induction of ovulation: preliminary trials in women. Br Med J 1971 Jan 16;1(5741):152–4.
[28] Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 1975 Mar;11(3):205–6.
[29] Jordan VC. Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethyl benzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 1974;5:354.
[30] Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 1976 Jun;12(6):419–24.
[31] Jordan VC. Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. Rev Endocr Relat Cancer 1978;(October supplement):49–55.
[32] Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 1980 Feb;16(2):239–51.
[33] Jordan VC, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In: Salmon S, Jones S, editors. Adjuvant Therapy of Cancer II. New York: Grune & Stratton Inc; 1979. p. 19–26.
[34] Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 1998 May 16;351(9114):1451–67.
[35] Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005 May 14-20;365(9472):1687–717.
[36] Davies C, Godwin J, Gray R, Clarke M, Cutter D, Darby S, et al. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 2011 Aug 27;378(9793):771–84.
[37] Lacassagne A. Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer 1936;27(2):217–28.
[38] Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 1990 Jul 15;50(14):4177–89.
[39] Glascock RF, Hoekstra WG. Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep. Biochem J 1959 Aug;72:673–82.
[40] Jensen EV, Jacobson HI. Basic guides to the mechanism of estrogen action. Recent Prog Horm Res 1962;18:387–414.
[41] Toft D, Gorski J. A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc Natl Acad Sci USA 1966 Jun;55(6):1574–81.
[42] Toft D, Shyamala G, Gorski J. A receptor molecule for estrogens: studies using a cell-free system. Proc Natl Acad Sci USA 1967 Jun;57(6):1740–3.
[43] McGuire WL, Carbone PP, Vollmer EP, editors. Estrogen Receptors in Human Breast Cancer, Raven Press; 1975.
[44] Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. Clin Cancer Res 2003 Jun;9(6):1980–9.
[45] Jordan VC, Naylor KE, Dix CJ, Prestwich G. Anti-oestrogen action in experimental breast cancer. Recent Results Cancer Res 1980;71:30–44.
[46] Cuzick J, Baum M. Tamoxifen and contralateral breast cancer. Lancet 1985 Aug 3;2(8449):282.
[47] Powles TJ, Hardy JR, Ashley SE, Farrington GM, Cosgrove D, Davey JB, et al. A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. Br J Cancer 1989 Jul;60(1):126–31.
[48] Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. J Natl Cancer Inst 2007 Feb 21;99(4):283–90.
[49] Lyman SD, Jordan VC. Metabolism of tamoxifen and its uterotrophic activity. Biochem Pharmacol 1985 Aug 1;34(15):2787–94.
[50] Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc 1987 Apr;46(5):1870–4.
[51] Jordan VC, Lababidi MK, Langan-Fahey S. Suppression of mouse mammary tumorigenesis by long-term tamoxifen therapy. J Natl Cancer Inst 1991 Apr 3;83(7):492–6.
[52] Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 1988 Feb 15;48(4):812–5.
[53] Hardell L. Tamoxifen as risk factor for carcinoma of corpus uteri. Lancet 1988 Sep 3;2(8610):563.
[54] Jordan VC. Tamoxifen and endometrial cancer. Lancet 1988 Oct 29;2(8618):1019.
[55] Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1989 Jan 21;1(8630):117–20.
[56] Fisher B, Costantino JP, Redmond CK, Fisher ER, Wickerham DL, Cronin WM. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. J Natl Cancer Inst 1994 Apr 6;86(7):527–37.
[57] Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998 Sep 16;90(18):1371–88.
[58] Powles T, Eeles R, Ashley S, Easton D, Chang J, Dowsett M, et al. Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomised chemoprevention trial. Lancet 1998 Jul 11;352(9122):98–101.
[59] Cuzick J, Forbes J, Edwards R, Baum M, Cawthorn S, Coates A, et al. First results from the International Breast Cancer Intervention Study (IBIS-I): a randomised prevention trial. Lancet 2002 Sep 14;360(9336):817–24.
[60] Williams GM, Iatropoulos MJ, Djordjevic MV, Kaltenberg OP. The triphenylethylene drug tamoxifen is a strong liver carcinogen in the rat. Carcinogenesis 1993 Feb;14(2):315–7.
[61] Greaves P, Goonetilleke R, Nunn G, Topham J, Orton T. Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. Cancer Res 1993 Sep 1;53(17):3919–24.
[62] Hard GC, Iatropoulos MJ, Jordan K, Radi L, Kaltenberg OP, Imondi AR, et al. Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. Cancer Res 1993 Oct 1;53(19):4534–41.
[63] Jordan VC. What if tamoxifen (ICI 46,474) had been found to produce rat liver tumors in 1973? A personal perspective. Ann Oncol 1995 Jan;6(1):29–34.
[64] Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 2005 Nov 16;97(22):1652–62.
[65] Cuzick J, Forbes JF, Sestak I, Cawthorn S, Hamed H, Holli K, et al. Long-term results of tamoxifen prophylaxis for breast cancer–96-month follow-up of the randomized IBIS-I trial. J Natl Cancer Inst 2007 Feb 21;99(4):272–82.
[66] Jordan C. Tamoxifen as the first targeted long term adjuvant therapy for breast cancer. Endocr Relat Cancer 2014 Mar 21.
[67] Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987 Oct;10(1):31–5.
[68] Love RR, Newcomb PA, Wiebe DA, Surawicz TS, Jordan VC, Carbone PP, et al. Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 1990 Aug 15;82(16):1327–32.
[69] Love RR, Wiebe DA, Newcomb PA, Cameron L, Leventhal H, Jordan VC, et al. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann Intern Med 1991 Dec 1;115(11):860–4.
[70] Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, et al. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 1992 Mar 26;326(13):852–6.
[71] Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2003 Mar;2(3):205–13.
[72] Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987 Aug 1;47(15):4020–4.
[73] Jordan VC, Gosden B. Differential antiestrogen action in the immature rat uterus: a comparison of hydroxylated antiestrogens with high affinity for the estrogen receptor. J Steroid Biochem 1983 Sep;19(3):1249–58.
[74] Jordan VC, Gosden B. Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 1983 Aug;113(2):463–8.
[75] Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 1990 Jun 1;50(11):3189–92.
[76] Black LJ, Sato M, Rowley ER, Magee DE, Bekele A, Williams DC, et al. Raloxifene (LY139481 HCl) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 1994 Jan;93(1):63–9.
[77] Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple outcomes of raloxifene evaluation. JAMA 1999 Jun 16;281(23):2189–97.
[78] Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, et al. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 2004 Dec 1;96(23):1751–61.
[79] Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 2006 Jun 21;295(23):2727–41.
[80] Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Update of the national surgical adjuvant breast and bowel project Study of Tamoxifen and Raloxifene (STAR) P-2 trial: preventing breast cancer. Cancer Prev Res 2010 Jun;3(6):696–706.

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[81] Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 1988;6(5):589–95.
[82] Wiseman LR, Goa KL. Toremifene. a review of its pharmacological properties and clinical efficacy in the management of advanced breast cancer. Drugs 1997 Jul;54(1):141–60.
[83] Maximov PY, Lee TM, Jordan VC. The discovery and development of selective estrogen receptor modulators (SERMs) for clinical practice. Curr Clin Pharmacol 2013 May;8(2):135–55.
[84] Jordan VC. A(nother) scientific strategy to prevent breast cancer in post-menopausal women by enhancing estrogen-induced apoptosis?. Menopause [ePub].
[85] Robinson SP, Koch R, Jordan VC. In vitro estrogenic actions in rat and human cells of hydroxylated derivatives of D16726 (zindoxifene), an agent with known antimammary cancer activity in vivo. Cancer Res 1988 Feb 15;48(4):784–7.
[86] Miller CP, Collini MD, Tran BD, Harris HA, Kharode YP, Marzolf JT, et al. Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. J Med Chem 2001 May 24;44(11):1654–7.
[87] Bain RR, Jordan VC. Identification of a new metabolite of tamoxifen in patient serum during breast cancer therapy. Biochem Pharmacol 1983 Jan 15;32(2):373–5.
[88] Jordan VC, Bain RR, Brown RR, Gosden B, Santos MA. Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. Cancer Res 1983 Mar;43(3):1446–50.
[89] Cummings SR, Ensrud K, Delmas PD, LaCroix AZ, Vukicevic S, Reid DM, et al. Lasofoxifene in postmenopausal women with osteoporosis. N Engl J Med 2010 Feb 25;362(8):686–96.
[90] Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 1999 Aug 18;282(7):637–45.
[91] Barrett-Connor E, Mosca L, Collins P, Geiger MJ, Grady D, Kornitzer M, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 2006 Jul 13;355(2):125–37.
[92] Jordan VC. Repurposing failed pharmaceuticals as the first targeted medicines for the treatment and prevention of breast cancer. Hamdan Med J. in press.
[93] LaCroix AZ, Powles T, Osborne CK, Wolter K, Thompson JR, Thompson DD, et al. Breast cancer incidence in the randomized PEARL trial of lasofoxifene in postmenopausal osteoporotic women. J Natl Cancer Inst 2010 Nov 17;102(22):1706–15.
[94] Gottardis MM, Jiang SY, Jeng MH, Jordan VC. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res 1989 Aug 1;49(15):4090–3.
[95] Maximov PY, McDaniel RE, Jordan VC. Tamoxifen: pioneering medicine in breast cancer. Milestones in drug therapy. Basel: Springer; 2013.
[96] Jordan VC, editor. Estrogen action, selective estrogen receptor modulators and women's health: progress and promise. London: Imperial College Press; 2013.
[97] Lewis JS, Jordan VC. Case histories: raloxifene. In: Taylor J, Triggle D, editors. Comprehensive medicinal chemistry II. Oxford: Elsevier Limited; 2006. p. 103–21.

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Selective estrogen-induced apoptosis in breast cancer

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ABSTRACT

Antihormone therapy remains the gold standard of care in the treatment of estrogen receptor (ER) positive breast cancer. However, development of acquired long term antihormone resistance exposes a vulnerability to estrogen that induces apoptosis. Laboratory and clinical studies indicate that successful therapy with estrogens is dependent on the duration of estrogen withdrawal and menopausal status of a woman. Interrogation of estradiol (E_2) induced apoptosis using molecular studies indicate treatment of long term estrogen deprived MCF-7 breast cancer cells with estrogen causes an endoplasmic reticulum stress response that induces an unfolded protein response signal to inhibit protein translation. E_2 binds to the ER and mediates apoptosis through the classical genomic pathway. Furthermore, the induction of apoptosis by estrogens is dependent on the conformation of the estrogen–ER complex. In this review, we explore the mechanism and the processes involved in the paradox of estrogen induced apoptosis and the new selectivity of estrogen action on different cell populations that is correctly been deciphered for clinical practice.

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1. Introduction

Ovarian estrogen in premenopausal patients or estrogens produced by peripheral aromatization of adrenal androgenic precursors in postmenopausal patients support the growth of breast cancer. As a result of this knowledge, treatment practices evolved throughout the 20th century to either remove the source of estrogen synthesis by ablative surgery (oophorectomy, adrenalectomy or hypophysectomy) or block the actions of estrogen which stimulates tumor growth through the breast tumor estrogen receptor (ER)-signal transduction system [1]. Two clinical approaches to breast cancer therapy have proved to be successful [2]: either the development of nonsteroidal antiestrogens that block estrogen binding to the ER or the development of aromatase inhibitors which block the peripheral aromatase enzyme system that convert steroidal precursors from the adrenals to estrogens. Both therapeutic advances have resulted in dramatic increases in patient survival if the nonsteroidal antiestrogen tamoxifen or an aromatase inhibitor is given for extended periods (5–10 years) as an adjuvant therapy [3–5].

There is compelling support for the proposition that estrogen is an essential component for the development of breast cancer and is essential for the promotion and replication of breast cancer cells. The first evidence that there was a link between estrogen and the development of breast cancer was presented at the annual meeting of the American Association for Cancer Research in Boston in 1936. Professor Antoine Lacassagne [6] presented his vision of the prevention of breast cancer in the future based on the results he had obtained in laboratory animals by either administering estrogens to develop mammary cancer [7] or removing estrogen through ovariectomy to prevent mammary cancer in high incident strains: *"if one accepts the consideration of adenocarcinoma of the breast as the consequences of a special hereditary sensibility to the proliferative actions of oestrone, one is led to imagine a therapeutic preventive for subjects predisposed by their heredity to this cancer. It would consist- in the near future when knowledge and use of hormones will be better understood- in the suitable use of a hormone antagonist to prevent the stagnation of estrone in the ducts of the breast."* This visionary strategy became a reality with the development of the nonsteroidal antiestrogen, tamoxifen for the treatment of breast cancer [8] and the successful testing of its worth in high risk women to reduce the incidence of breast cancer [9,10]. Thus, the use of an "antiestrogen" to prevent the development of breast cancer was further proof of the critical role of estrogen in the process of breast carcinogenesis. Today, two selective ER modulators (SERMs) tamoxifen and raloxifene are available for the chemoprevention of breast cancer in both the United States and United Kingdom [11,12].

The final proof of the direct role of estrogen to stimulate breast cancer cell proliferation came from the laboratory. Initially it was

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difficult to demonstrate that estrogen directly caused the replication of breast cancer cells *in vitro* but growth of ER positive tumors could be demonstrated *in vivo* if the same ER positive MCF7 breast cancer cells were inoculated into athymic (immune deficient mice) and treated with estrogen [13]. It was to remain a mystery why exogenous estrogen could not stimulate MCF-7 breast cancer cells to grow *in vitro* until Berthois and colleagues [14] made the landmark discovery that MCF-7 cells had been routinely maintained for more than a decade in media containing high concentrations of phenol red as a pH indicator. Phenol red or a contaminant was actually an estrogen, so cells were already growing maximally. Removal of the phenol red from the media stopped cell replication and the cells now had a robust proliferation in response to exogenous estrogen. The evidence of the relevance of the critical role of endogenous estrogen being necessary for breast cancer development and growth was overwhelming but there was an unexplained paradox lurking in the historical record of therapeutics. The first successful therapy used to treat any cancer was the use of high dose estrogen to treat metastatic breast cancer in postmenopausal patients [15]. The encouraging initial trial was the result of laboratory studies, so the treatment strategy was based on translational research. However, the clinical research went a step further. Haddow used this preliminary data [15] to conduct a multicentric clinical trial through the Royal Society of Medicine. He made a discovery: "*When the various reports were assembled at the end of that time, it was fascinating to discover that rather general impression, not sufficiently strong from the relatively small numbers in any single group, became reinforced to the point of certainty; namely, the beneficial responses were three times more frequent in women over the age of 60 years than in those under that age; that estrogens may, on the contrary, accelerate the course of mammary cancer in younger women, and that their therapeutic use should be restricted to cases 5 years beyond the menopause. Here was an early and satisfying example of the advantages which may accrue from cooperative clinical trial*" [16]. Dr. Basil Stoll [17] was able to quantify this finding within his own clinical practice and demonstrated that patients more than 5 years beyond menopause had a high probability of a response to high dose estrogen therapy but those less than five years from menopause were unlikely to have a responsive tumor (Table 1).

High dose estrogen therapy for the treatment of metastatic breast cancer in postmenopausal women became the standard of care for 30 years [18–20] until the advent of tamoxifen. Response rates to tamoxifen for postmenopausal patients with metastatic breast cancer were similar at 30% [21,22], but side effects with tamoxifen were much less severe. This allowed tamoxifen to advance as long term adjuvant therapy and ultimately be shown to save lives. However, in 1970 at the dawn of interest ICI 46, 474 (to become tamoxifen) as an experimental antiestrogen for the treatment of breast cancer, Sir Alexander Haddow was selected to present the inaugural Karnofsky lecture at the American Society of Clinical Oncology [16]. His article paints a gloomy picture for the future of targeted cancer therapies and the remote prospects of success for anticancer agents as had been achieved with the selective toxicity of antibiotics for the cure of infectious disease. He did however highlight a potential glimmer of hope with his statement that reflected upon the pioneering success he had achieved with his discovery of high dose estrogen treatment as the first "chemical therapy" in cancer. "*The extraordinary extent of tumour regression observed in perhaps 1% of post-menopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanism continues to elude us……*" [16].

However, resolution of the paradox of the antitumor actions of estrogen was to be discarded and dismissed with the refocusing on the accepted paradigm of the obvious understanding of the antitumor action of antiestrogen, tamoxifen. It is therefore ironic that through the clinical development of tamoxifen as a long term adjuvant therapy and the necessity to examine the evolution of acquired resistance to long term tamoxifen, that the veil should be lifted on Haddow's paradox and the new biology of estrogen-induced apoptosis be discovered.

Table 1
Objective response rates in postmenopausal women with metastatic breast cancer using high dose estrogen therapy. The 407 patients are divided in relation to menopausal status [17]. The objective remission rate of breast cancer tumors was higher in women more than 5 years postmenopausal. Reprinted with permission from Obiorah I and Jordan VC. Menopause 2013; 20: 372–382.

Age since menopause	Patient number	% Regression
Postmenopausal 0–5 Years	63	9
Postmenopausal >5 Years	344	35

2. The evolution of acquired resistance to SERMs

The first clinically relevant models of acquired resistance to tamoxifen were developed by inoculating MCF-7 cells into ovarectomized athymic mice and initially treating with estrogen for a short time to establish palpable tumors. Continuous tamoxifen treatment of the tumor bearing mice resulted in the growth of tumors despite tamoxifen treatment [23]. However, the finding through retransplantation studies that tumors grew because of tamoxifen treatment and also continued to respond to estrogen for growth [24,25], recapitulated acquired resistance to tamoxifen therapy in the treatment of metastatic breast cancer. Tamoxifen treatment fails in a year or two [21,22], tumors exhibit a "withdrawal" response from tamoxifen [26,27] so this is tamoxifen stimulated growth. Finally estrogen can still maintain tumor growth following the cessation of tamoxifen. Second line therapies with either an aromatase inhibitor to prevent estrogen synthesis or a pure antiestrogen fulvestrant to destroy ER, are effective second line therapies [28,29]. However, the characteristics of the model of acquired resistance did not explain why tamoxifen could be given effectively as a long term adjuvant therapy. If this model was true for all acquired resistance, micrometastatic disease would fail to be controlled for more than two years of adjuvant therapy. It was the retransplantation of tumors into new generations of athymic mice for at least 5 years that was to result in the discovery of a new biology of estrogen action: estrogen induced apoptosis.

3. The antitumor action of physiologic estrogen

The finding that acquired resistance to tamoxifen evolves through phases also demonstrates that the cell selection pressure of tamoxifen and its metabolites exposes a vulnerability in cell populations that struggle to survive in a long term tamoxifen (antiestrogen environment) environment. Estrogen triggers apoptosis after 5 years of retransplantation in tamoxifen-treated mice [30,31]. Numerous publications *in vivo* with SERM (tamoxifen or raloxifene) stimulated tumors [32–35] were of value to document biological control mechanisms. The key to advancing the mechanistic understanding of estrogen induced apoptosis and the reasons for selectivity in breast cancer occurred with the development of long term estrogen deprived cell models to replicate resistance to aromatase inhibitors during adjuvant therapy.

4. Cellular models of estrogen deprivation *in vitro*

To decipher the mechanism involved in antihormone resistance following long term treatment, antiestrogen resistant clonal

variants of MCF-7 cells: MCF7:5C and MCF7:2A were established after long-term culture in estrogen free media [36,37] or long term estrogen deprived (LTED) MCF-7 cell populations examined [38]. The variant clone, MCF7:5Ccells express wild type ER but have drastically reduced levels of progesterone receptor (PR) when compared to the parent MCF-7 cells. Using DNA quantification assays 17β estradiol (E_2) drastically reduced the growth of the MCF7:5C cells in a time dependent manner that resulted in 90% inhibition after six days of treatment [39]. The observed E_2 induced inhibition in cell proliferation was confirmed to be apoptosis using annexin v-FITC and DNA binding dye, DAPI staining methods [39,40]. Although fulvestrant partially inhibited the growth of the MCF7:5C cells, this biological effect was not due to apoptosis. On the other hand, these cells are resistant to 4-hydroxytamoxifen (4OHT), while fulvestrant caused 40% growth inhibition. The induction of E_2 induced apoptosis *in vitro* raised the question of its ability to induce tumor regression *in vivo*. MCF7:5C cells injected into athymic nude mice resulted in detectable spontaneously growing tumors within 4 weeks. Treatment of the MCF7:5C tumors with E_2resulted in complete regression after 4 weeks of therapy. Involvement of apoptosis in the E_2 induced tumor reduction was determined using TUNEL assay.

Another clone, MCF7:2A was identified and characterized from long term estrogen deprived MCF-7 breast cancer cells [37]. Significant growth inhibition is observed in the second week of treatment with E_2 [41]. Similar to the MCF7:5C cells, the MCF7:2A cells grow maximally in the absence of estrogens [37,41]. However, the MCF7:2A are inhibited by both antiestrogens 4OHT and fulvestrant and these cells are both ER/PR positive [37]. Because the MCF7:2A cells were initially resistant to E_2 induced apoptosis with proapoptotic genes activated much later than in MCF7:5C cells [42], potential mechanisms of action for this resistance were explored. Glutathione (GSH), a tripeptide has been implicated in the tumorigenesis and progression of breast cancer [43]. Elevated levels of GSH were observed in MCF7:2A cells and microarray studies show high levels of glutathione synthetase and glutathione peroxidase 2 [41,42,44]. Both enzymes are involved in GSH synthesis. Depletion of the cells of GSH using L-buthionine sulfoximine (BSO), a GSH inhibitor, sensitized the MCF7:2A cells to E_2 induced apoptosis [42,44,45]. Therefore, utilization of BSO with estrogen in patients with ER positive metastatic breast cancer in the context of a clinical trial could potentially inhibit disease progression in patients with exhaustive antihormone resistance.

5. Differential effects of estrogens in MCF7:5C cells

Based on the fact that the ER is the major signaling pathway for breast cancer growth and apoptosis, a series of planar and angular estrogens (Fig. 1) were evaluated for their ability to trigger apoptosis in the MCF7:5C cells. Estrogens can be classified into class I (planar) and class II (angular) estrogens [46,47] based on the reported crystal structure of the ligand binding domain (LBD) ER with estrogens (E_2, diethylstilbestrol) and antiestrogens (4OHT and raloxifene) [48,49]. The planar estrogens are sealed within the LBD by helix 12 to induce estrogenic action, whereas the bulky side chains of 4OHT and raloxifene prevent helix 12 from sealing the LBD resulting in antiestrogen action. We previously synthesized a range of estrogenic angular triphenylethylenes (TPEs) which are structurally similar to 4OHT. The TPEs (bisphenol (BP), ethoxytriphenylethylene (EtOX) and trihydroxytriphenylethylene (3OHTPE) cause proliferation of MCF-7 cells [50] at higher concentrations when compared to the planar estrogens. First, we compared the ability of bisphenol A (BPA), a planar estrogen to stimulate growth in MCF7 cells and induce apoptosis in the MCF7:5C cells to BP, an angular TPE [51]. The TPE, BP is a more potent estrogen than BPA in stimulating MCF-7 cell growth. Despite the fact that BPA is only a weak estrogen, it inhibited the growth of MCF7:5C cells at high concentration at the end of a 7 day assay, whereas BP did not readily induce apoptosis at this time point but rather blocked E_2 induced apoptosis. Planar estrogens, E_2 and BPA induce similar apoptosis related genes by 48 h of treatment, whereas BP did not induce apoptosis genes but rather the pattern of genes down regulated by BP resembles the pattern observed with E_2 and BPA. To further investigate the paradox of how BP which is fully estrogenic in MCF-7 cells but appeared to have antiestrogen-like in MCF7:5C cells [51], we examined the relationship whereby the structure of an estrogenic ligand can affect their ability to induce apoptosis by using a range of compounds [52]. Planar estrogens, which included E_2, DES and constituents of conjugated equine estrogens (equilin, estrone and equilenin), prevented the growth and induced apoptosis of MCF7:5C cells even though they all caused cell proliferation of the MCF-7 cells [52,53]. This corresponds with what is observed in clinical practice. Clinical studies [54,55] have shown that thirty percent of postmenopausal patients with metastatic breast cancer show an objective response with estrogen therapy after undergoing exhaustive antihormone therapy. Similarly a persistent decrease was noted in the incidence and mortality of breast cancer in postmenopausal women from the Women Health Initiative trial, who were treated with conjugated equine estrogen (CEE) when compared to those on placebo [56,57]. In contrast, all TPEs, BP, EtOX and 3OHTPE did not readily induce apoptosis at the end of the first week but rather blocked E_2 induced apoptosis in a similar manner as the selective estrogen receptor modulators (SERMS) [52]. However the TPEs were all able to induce apoptosis after 14 days of treatment, whereas the SERMS remain antiestrogenic in the MCF7:5C cells, and do not trigger apoptosis.

We previously observed that E_2 induced apoptosis is a slow process [41], therefore we sought to compare its antiproliferative effects to that of a classic cytotoxic chemotherapy [58]. Paclitaxel caused 50% growth reduction and almost 100% growth inhibition of the MCF7:5C cells by 24 h and 48 h respectively [58]. By contrast, inhibition of cell proliferation was not observed until after 72 h of E_2 treatment and 80% growth inhibition was seen at 120 h [58]. Apoptosis was quantified using annexin v staining after 12 h of treatment with paclitaxel, while an apoptotic response was only detected with E_2 treatment after 72 h. Interestingly in a related study, we found that BP was only able to significant prevent cell proliferation after 9 days of treatment, whereas apoptosis was determined to be starting after 6 days of treatment [59]. It is evident that the initial response of the MCF7:5C cells to E_2 and BP is cell proliferation and this was confirmed using cell cycle flow cytometry studies. Both estrogens caused a persistent increase in S phase in the first 96 h following treatment. This contrasted with paclitaxel which causes a rapid G2/M blockade and apoptosis by 12 h. It became important to determine the point at which apoptosis is triggered for planar estrogen E_2 [58] and angular TPE, BP [59] i.e. the time after which the cell is committed to apoptosis. The critical trigger point of apoptosis for E_2 occurred between 24 h and 36 h, while that of BP occurred after 4 days of treatment. The delayed biological actions indicate that estrogen induced apoptosis involves a multidynamic process that is dramatically distinct from that of a chemotherapeutic drug such as paclitaxel. The conformation of the ER complex regulates the time at which the cell commits to apoptosis.

6. Importance of the conformation of the ligand-ER complex involved in estrogen induced apoptosis

The angular TPEs possess short-term antiestrogenic properties in MCF7:5C cells similar to those of the 4OHT, which indicate that

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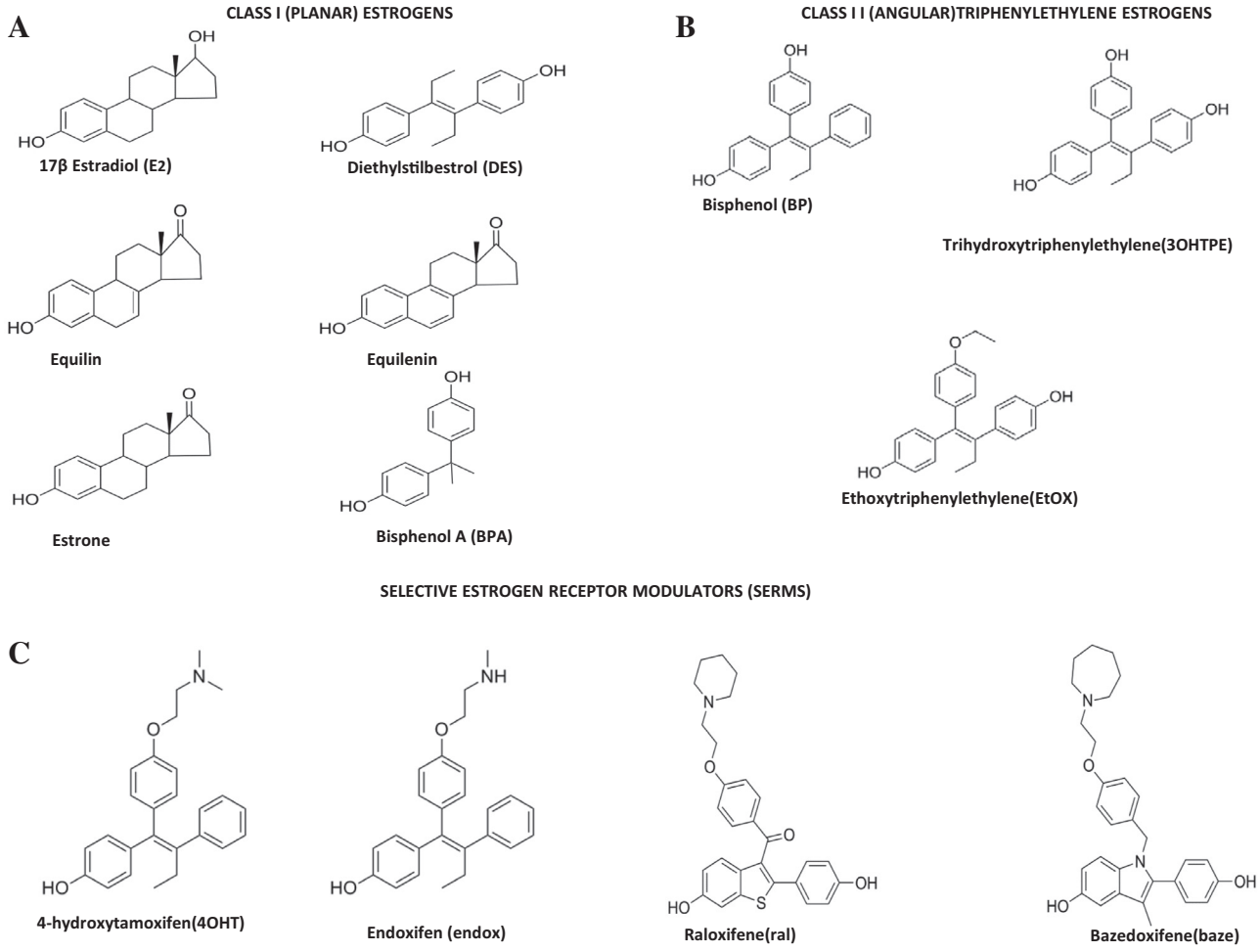

Fig. 1. Chemical structures of compounds.(A)Planar estrogens (B) Angular Triphenylethylenes**(C)** Selective estrogen receptor modulators.

they make an antiestrogenic like conformation with the ER. The recruitment of ER α or coactivator, SRC3 (AIB1) at the promoter of pS2 (TFF1) gene, an estrogen responsive gene, by the planar and angular estrogens was determined using chromatin immunoprecipitation assays in MCF-7 and MCF7:5C cells. Planar estrogens, E_2 and BPA readily recruited high levels of ERα to the pS2 promoter [51,52]. On the other hand, TPEs were about 50% efficient as the planar estrogen in the recruitment of ERα, whereas 4OHT showed much lower levels of ERα recruitment to the promoter. SRC3 plays a key role in the transcriptional regulation of E_2 induced growth [60–62] and apoptosis [63] in breast cancer cells. Recruitment of SRC3 shows a similar pattern as the ERα following treatment with the planar estrogens, TPEs and 4OHT. Planar estrogens show high levels of SRC recruitment, whereas 4OHT show no recruitment to the pS2 promoter and the TPEs show variable low levels of recruitment that lie between that of the planar estrogens and 4OHT. This indicates that the conformation of the TPE-ER complex results in a moderate reduction of ERα binding to the ERE region of the pS2 promoter and a severe inhibition of SCR3 binding when compared to the planar estrogens. The reduction of SCR3 recruitment observed with the TPEs correlates with another study, where Bourgoin-Voillard and colleagues [64] discovered that class II estrogens such as BP had a reduced tendency to induce recruitment of coactivators containing LxxLL motif, thus suggesting that the TPE:ER complex appears to be "antiestrogen-like" when compared to 4OHT. Furthermore molecular modelling data indicate that TPEs would bind to the ER in an antagonist conformation in a similar manner to that observed with 4OHT based on X-ray crystallography [49]. These data suggest that the antiestrogenic conformation may be responsible for the initial retardation of TPE induced apoptosis. Nevertheless, the molecular dynamics of the TPE:ER complex must eventually create accumulated cellular damage to trigger apoptosis.

7. The modulation of c-Src on estrogen induced apoptosis

c-Src is a non-receptor tyrosine kinase that plays a crucial role in signaling cascades that control cell growth, angiogenesis, invasion adhesion and metastasis and act as an adaptor protein in the crosstalk between the ER and growth factors such as the EGFR family [65,66]. Many of the proliferative actions of estrogen are dependent on c-Src [66]. The multiple involvement of c-Src in many intracellular signaling pathways, such as the mitogen- activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways makes it a potential therapeutic target in breast cancer cells. Elevated c-Src activity has been noted in tamoxifen resistant breast cancer cells and treatment of these cells with a c-Src inhibitor suppresses growth, invasion and motility of the endocrine resistant cells [67,68]. However, treatment with therapeutic c-Src inhibitors shows either modest or limited activity in patients with advanced breast cancer [69–71]. Because c-Src alone is not sufficient to cause oncogenic transformation, improvement in the value of a c-Src inhibitor could be achieved in combination with other targeted therapies. Due to the fact that we have previously shown that E_2 induces apoptosis in the MCF7:5C cells, we reasoned that combination of PP2, an experimental c-Src inhibitor,

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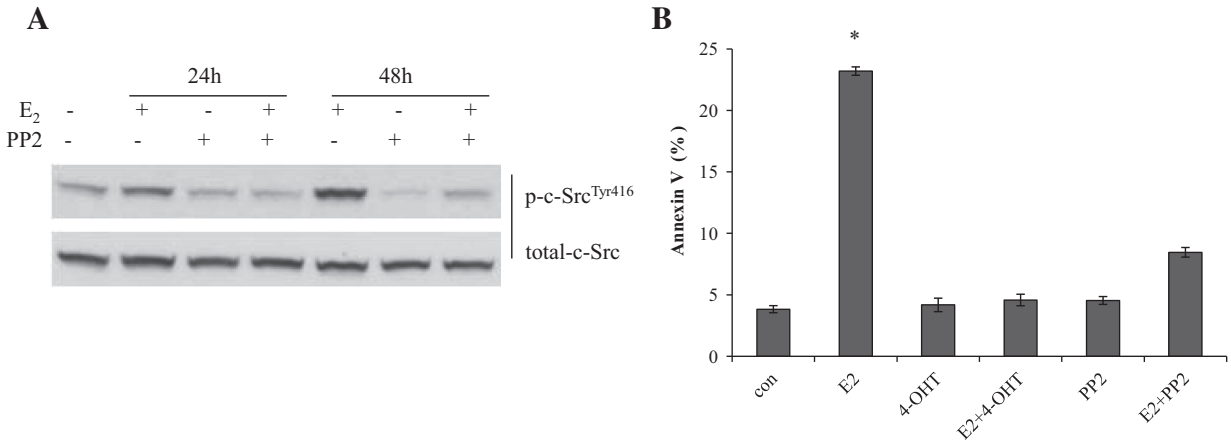

Fig. 2. The c-Src inhibitor blocked E_2 activated nongenomic pathway. (A) E_2 stimulated c-Src after 24 h treatment. (B) MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2, 4-OHT, E2 plus 4-OHT, PP2, E2 plus PP2 respectively for 72 h and the cells were harvested for Annexin V binding assay through flow cytometry. The percentage of Annexin V binding was compared with control. $P < 0.05$, *compared with control. Reprinted with permission from Fan et al. Cancer Research 2013; 73: 4510–4520.

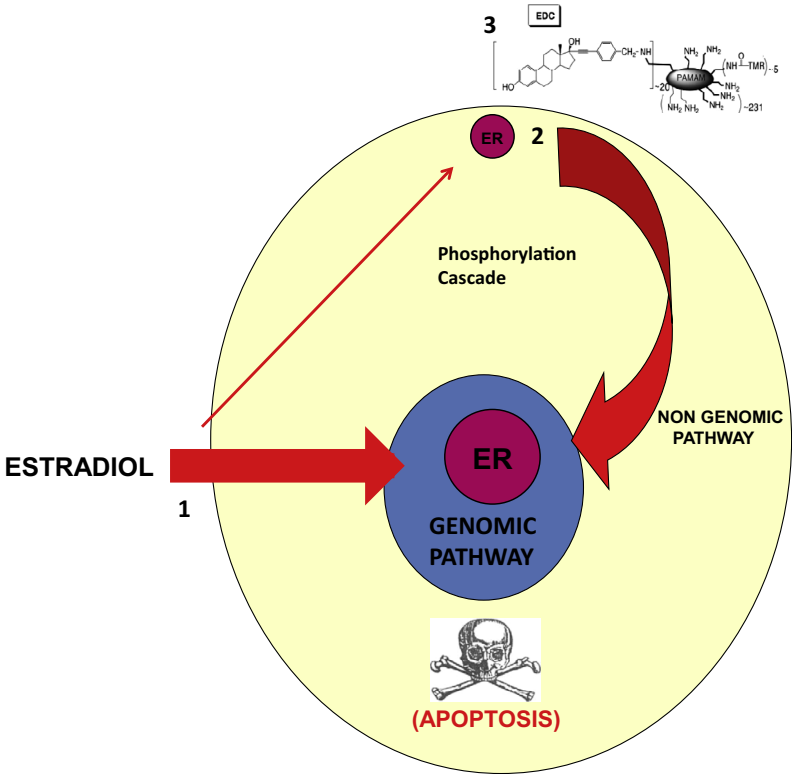

Fig. 3. E_2 induced estrogen receptor signaling. 1. The genomic mechanism of ER signaling is by estrogen binding to the nuclear ER and then binding to hormone response elements in the promoters of target genes (classic) or through protein–protein tethering with nuclear DNA-binding transcription factors (non-classic) to alter gene transcription. 2. E_2 can act through nongenomic signaling by activating cell surface membrane localized extranuclear ER. 3. estrogen dendrimer conjugate(EDC) specifically activate the nongenomic signaling of ER action.

and E_2 will potentiate the apoptotic effect of E_2. Surprisingly, although PP2 was able to block E_2 induction of c-Src (Fig. 2A), PP2 failed to induce apoptosis but rather blocked E_2 apoptosis (Fig. 2B) [72], which was confirmed using siRNA to knockdown c-Src in MCF7:5C cells which resulted in a reduction of E_2 induced apoptosis [73]. These data indicates that E_2 may trigger apoptosis via the nongenomicc-Src signaling pathway. This hypothesis, though unlikely due to the long delay in estrogen induced apoptosis observed previously [58], was addressed using a novel reagent supplied by Dr. John Katzenellenbogen [74].

8. E_2 induced apoptosis is through the genomic pathway in MCF7:5C cells

Estrogens can exert their effects by either classically binding to the nuclear ER and hormone response elements to alter gene transcription (genomic pathway) or by acting through nongenomic signaling via cell surface membrane localized extranuclear ER (Fig. 3). The role of the nongenomic pathway in ER signaling was evaluated in E_2 induced apoptosis. Estrogen dendrimer conjugate (EDC), a synthetic ligand that only interacts with the extra-nuclear ER to

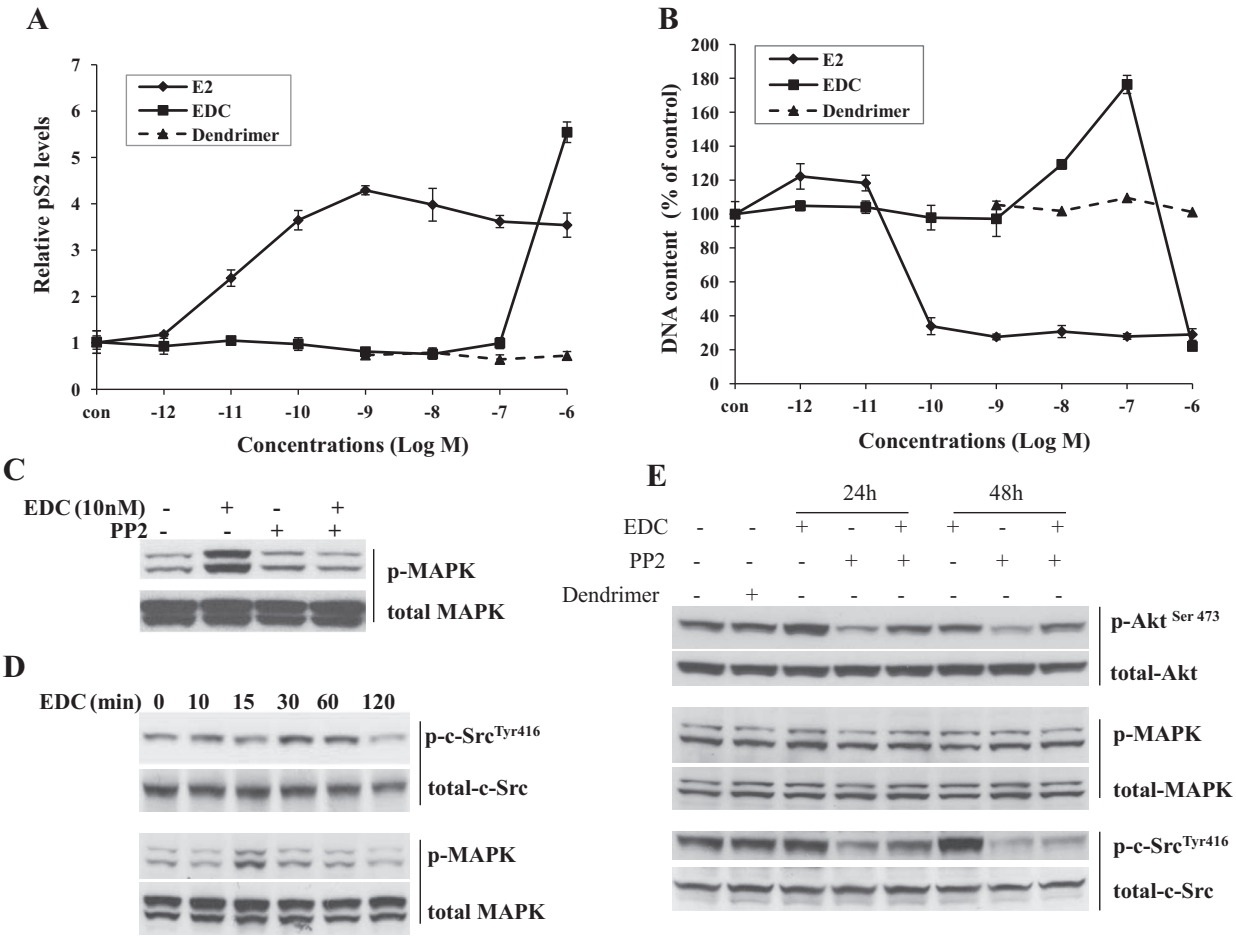

Fig. 4. Effect of estrogen dendrimer conjugate(EDC) on estrogen induced apoptosis. (A) E_2,activates pS2, an estrogen responsive gene, but EDC and the empty dendrimer does not. (B) Cell growth was inhibited by E_2 treatment whereas growth was enhanced by EDC and unaffected by the empty dendrimer. (C) The c-Src inhibitor blocked EDC activated nongenomic pathway via inhibition of EDC induced phosphorylation of MAPK. (D) EDC rapidly activated MAPK and c-Src in MCF-7:5C cells. (E) EDC activated signaling pathways after 24 h. Reprinted with permission from Fan et al. Cancer Research 2013; 73: 4510–4520.

induce nongenomic signaling was used to activate the nongenomic pathway [74]. EDC was neither able to activate the estrogen responsive gene, pS2 (Fig. 4A) nor induce apoptosis in the MCF7:5C cells (Fig. 4B). Nevertheless, EDC did activate the nongenomic pathway via induction of phosphorylated c-Src, MAPK and AKT in the MCF7:5C cells and PP2 blocked the EDC activated nongenomic pathway (Fig. 4C–E). This suggests that the nongenomic signaling pathway is not crucial for E_2 induced apoptosis but that E_2immediately activates the nongenomic pathway within minutes with subsequent activation of the genomic pathway. On the other hand, E_2 induces ERE activity which can be blocked by 4OHT but not by PP2. Inhibition of c-Src increased expression of classic ER targeted genes such as pS2, by increasing the accumulated ER. C-Src is important for phosphorylation and degradation of ER [73]. Selective induction of AP-1 complexes, consisting of c-Fos, c-Jun and Jund, were activated by E_2 in MCF7:5C cells suggesting that AP-1 may play an important role in E_2 mediated apoptosis.

9. Estrogen induced endoplasmic reticulum stress and unfolded protein response

Differential regulation of global gene expression and identification of genes and potential signaling pathways associated with E_2 induced apoptosis was interrogated using Agilent microarray studies. The major groups of MCF7:5C specific genes overrepresented include estrogen signaling, endoplasmicreticulum stress (ERS) and inflammatory response genes [41] and functional testing indicate that ERS and inflammatory stress response led to apoptosis. Endoplasmic reticulum is the key site for the synthesis and folding of proteins. Disturbances of the homeostasis within the endoplasmic reticulum can lead to accumulation of unfolded proteins that result in ERS. In order to overcome, a number of responses occur within the endoplasmic reticulum [75,76]. The first response is the synthesis of new proteins and prevention of accumulation of unfolded proteins [77]. Next chaperone proteins, such as BiP/GRP78, trigger an unfolded protein response (UPR) to relieve the ERS. Under normal conditions, BiP binds to unfolded proteins, PERK, ATF6 and IRE1 and maintains them in an inactive state. Under stress conditions, BiP dissociates from the UPR proteins and allows their oligomerization and autophosphorylation to initiate a UPR signal that serves to prevent protein synthesis and induce transportation of malfolded proteins to the cytosol for degradation. The UPR signal causes activation of PERK, ATF6 and IRE1. Activation of PERK induces phosphorylation of eIF2α resulting in inhibition of protein synthesis and translocation into the lumen of the endoplasmic reticulum [75,76]. Dissociation of BiP from ATF6 results in its transport to the golgi apparatus where it undergoes cleavage and translocate to the nucleus to induce transcription of UPR genes [75,78] such as XBP-1. Activated IRE1 induces splicing of XBP-1 which can now efficiently activate UPR [79,80]. Under severe or prolonged ERS, the UPR signal switches from cell survival to apoptosis. The regulation of UPR genes was evaluated

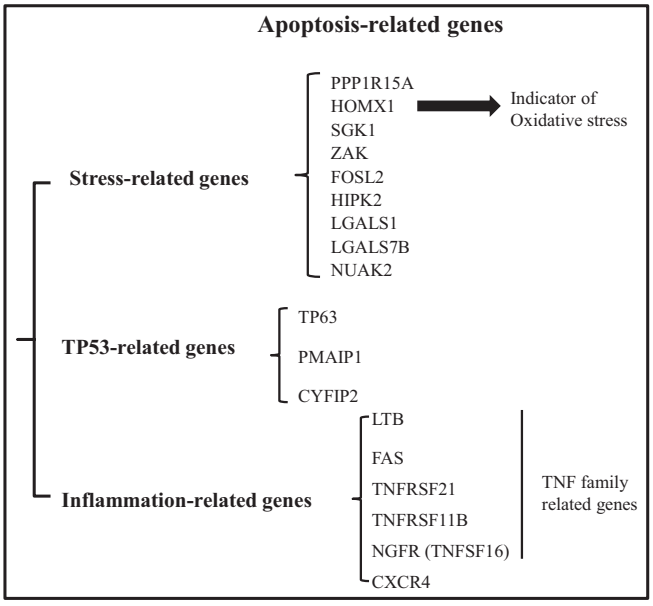

Fig. 5. Classification of E_2-induced apoptosis-related genes selected by RNA-seq in MCF-7:5C cells. E_2 induced stress related genes, TP53 related genes and inflammation related genes. Reprinted with permission from Fan et al. Cancer Research 2013; 73: 4510–4520.

in the MCF7:5C cells in response to E_2. Significant induction of UPR sensors, IRE1α and PERK/eIF2α by E_2 was detected after 24 h of treatment which further increased by prolongation of treatment to 72 h [73]. Treatment of the MCF7:5C cells with a PERK inhibitor blocked phosphorylation of eIF2α and blocked E_2 induced apoptosis, thus confirming that ERS is necessary for E_2 triggered apoptosis [73].

10. Apoptotic pathways mediated by estrogens

We previously reported that MCF7:5C cells respond to E_2 by suppressing ERα signaling leading to activation of ERS and inflammatory stress [41]. One of the apoptosis pathways associated with ERS mediated apoptosis is via activation of DDIT3/CHOP [75,81]. Studies suggest that PERK/eIF2α and IRE1α/ATF6 signaling are necessary for maximum induction of DDIT3 [82–84]. Overexpression of DDIT3 leads to a decrease in bcl-2 protein and translocation of Bax protein from the cytosol to the mitochondria [85,86]. Puthalakath and colleagues [87] reported that ERS induced by diverse stimuli required Bim for initiating apoptosis in a variety of cell lines including MCF-7 cells. Knockdown of Bim expression resulted in protection from ERS induced apoptosis. Increased Bim levels noted with ERS induction was dependent on transcriptional activation of DDIT3 [87]. Therefore prolonged ERS potentially leads to activation of BCL2L11/Bim and Bax. The involvement of the intrinsic pathway in E_2 induced apoptosis was first reported by

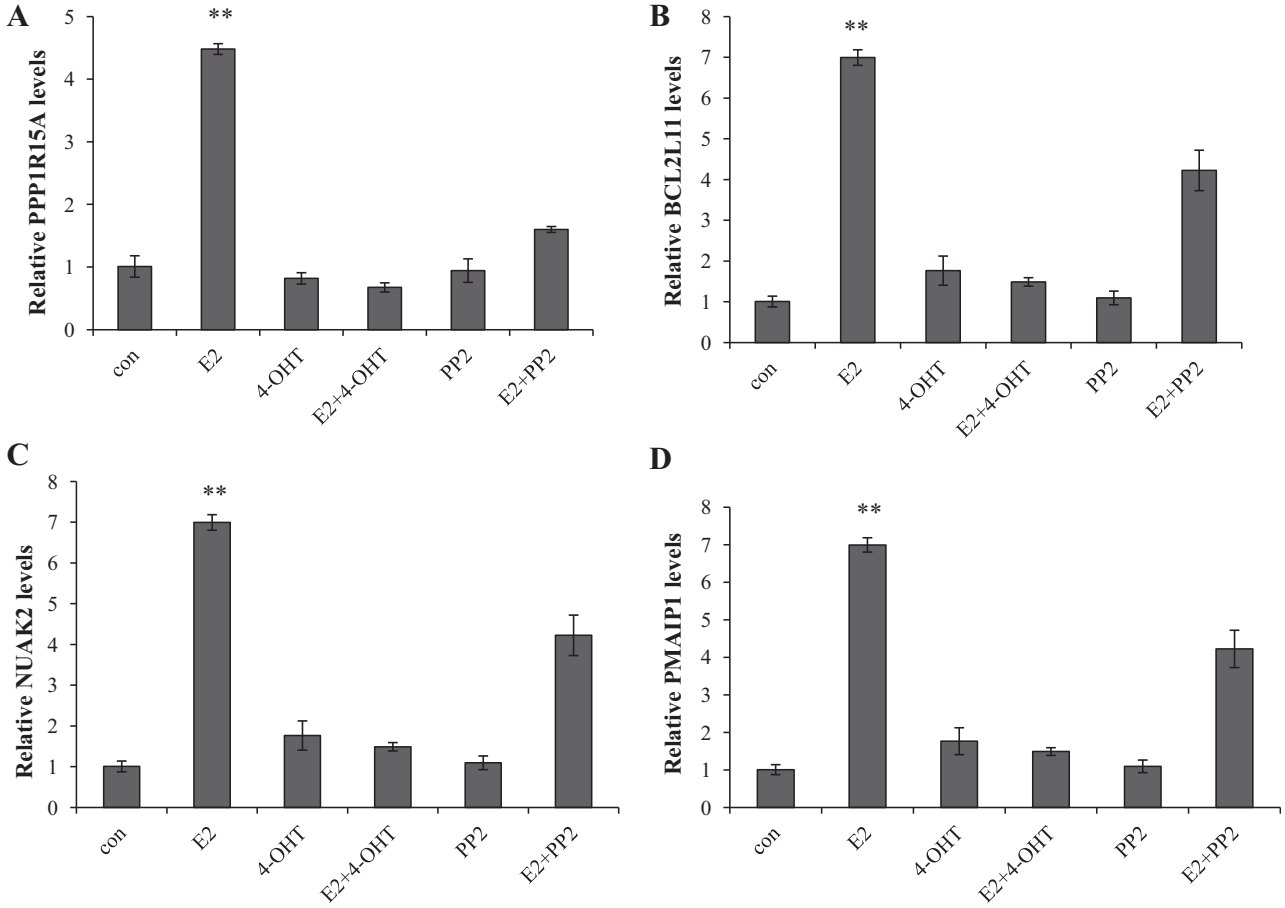

Fig. 6. The c-Src inhibitor blocked apoptosis-related genes induced by E_2. Apoptosis related genes: (A) PPP1R15A (GADD34) gene, (B) BCL2L11 (Bim) gene, (C) NUAK2 gene, (D) PMAIP1 (Noxa) gene, identified with RNA-seq analysis were confirmed using real-time PCR. $P < 0.001$, ** compared with control. Reprinted with permission from Fan et al. Cancer Research 2013; 73: 4510–4520.

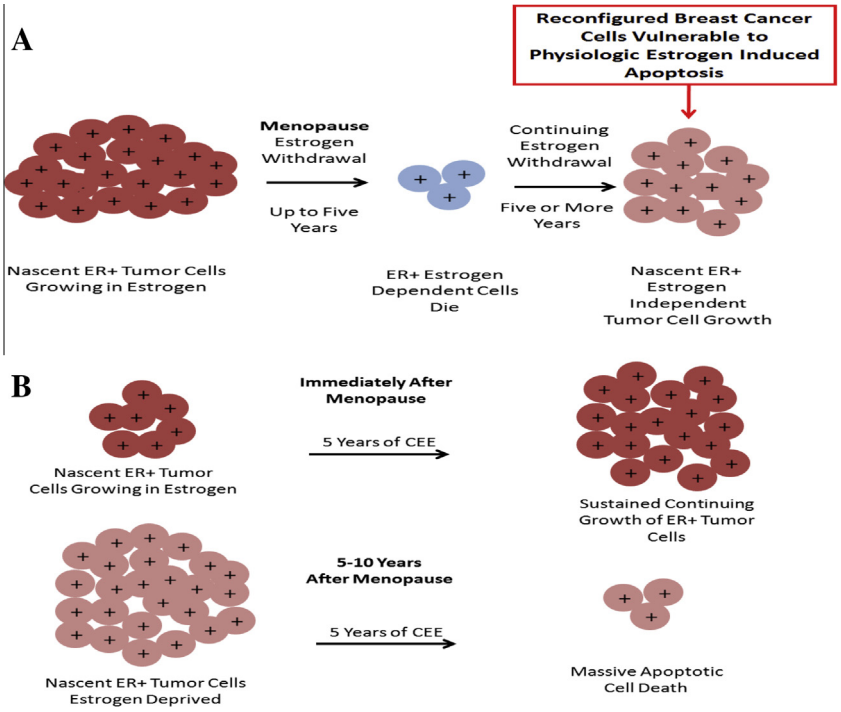

Fig. 7. The success of estrogen replacement therapy is dependent on menopausal status of a woman. A. Estrogen withdrawal in postmenopausal women causes ER positive dependent cells to die but some cells continue to grow independent of estrogen. B Treatment of women immediately after menopause with CEE results in sustained growth of nascent ER positive tumors, whereas treatment 5 years after menopause causes apoptotic cell death. Reprinted with permission from Obiorah I and Jordan VC. Menopause 2013; 20: 372–382.

Lewis and colleagues [40] who showed that E_2 treatment increased expression of proapoptotic proteins including, Bax, Bak, Bim, Noxa, Puma and p53. Depletion of Bim and Bax using short interfering RNAs (siRNAs) reversed the apoptotic effect of E_2. Furthermore mitochondrial pathway activity was determined by loss of mitochondrial potential, increase in cytochrome c release and cleavage of caspase 9 and poly ADP ribose polymerase (PARP) protein. The Fas/Fasl signaling (extrinsic) pathway has been implicated in E_2 induced apoptosis. Song and colleagues [38] reported elevated levels of Fas in long term estrogen deprived (LTED)MCF-7 cells and a marked increase of FasL noted with E_2 treatment. This correlated with the report from Osipo et al. [32] which showed that E_2 induced reduction of tamoxifen resistant breast cancer tumors, by activating Fas expression and suppressing NF-kB and HER2/neu activity**.** A similar observation was noted in raloxifene resistant MCF7 cells [33]. The growth of cells *in vitro* and *in vivo* was repressed by E_2 by increasing induction of Fas expression and reducing expression of NF-kB. Although there is obvious involvement of both intrinsic and extrinsic pathways in E_2 induced apoptosis, none of the previous studies investigated a time course of the sequence of activation of the apoptotic pathways in estrogen induced apoptosis.

Because the trigger of E_2 induced apoptosis was observed to occur after 24 h [58], differential regulation of apoptotic gene expression was interrogated at 36 h and 48 h in response to E_2using polymerase chain reaction (PCR) arrays. At 36 h, E_2 induced ERS and proinflammatory related genes [58]. DDIT3 was one of the highest inducible genes during E_2 mediated ERS. This is similar to what is observed in several microarray or PCR array studies [51,84] that analyzed differential gene expression associated with ERS. As expected, IRE1α was also upregulated at 36 h, giving further evidence of its involvement in the UPR induced by ERS. In addition, Bim expression was increased indicating an early involvement of the mitochondrial pathway possibly mediated by DDIT3. The gene expression expanded to involve the TNF family of genes such as, Fas and TNF and continued increased expression of Bim, ERS and proinflammmatory genes at 48 h of E_2 treatment [58]. However, a prolonged induction of ERS and inflammatory stress response was observed in the first week of treatment with BP and induction of caspase 4, an inflammatory caspase and downstream target of ERS, after 5 days of treatment [59]. Induction of both mitochondrial and extramitochondrial apoptotic related genes were activated after 7 days of treatment. The delay in induction of genes correlates with the more pronounced delayed apoptosis noted with BP when compared to that of E_2 [59]. Furthermore, RNA-seq analysis of genes regulated by E_2 [73] revealed a range of apoptosis-related genes functionally classified into: TP53-related genes, stress related genes and inflammatory response genes (Fig. 5). The majority of these apoptosis-related genes observed with the RNA-seq experiments were confirmed with real-time-PCR (Fig. 6).

11. Conclusions

Low physiologic concentrations of estrogen induce apoptosis in LTED MCF-7 cells. The laboratory phenomenon translates well with observation in clinical treatment of postmenopausal women with advanced antihormone resistant breast cancer. In postmenopausal women with metastatic breast cancer and acquired resistance to aromatase inhibitors, a daily dose of 6 mg of estradiol provided a similar clinical benefit rate (28% vs. 29%) as 30 mg [54,55]. Loning and colleagues [55] found an objective response in 30 percent of postmenopausal breast cancer patients with previous exhaustive antihormone therapy who received high dose diethylstilbestrol (15 mg).

Estrogen deprivation is necessary to sensitize breast cancer tumor cell to estrogen treatment and subsequent tumor reduction. Estrogens induce cell proliferation of fully estrogenised MCF-7 cells after 3 days of culture in estrogen free medium because the cell population has adapted to an estrogen rich (phenol red) environment and will grow with a resupply of estrogens

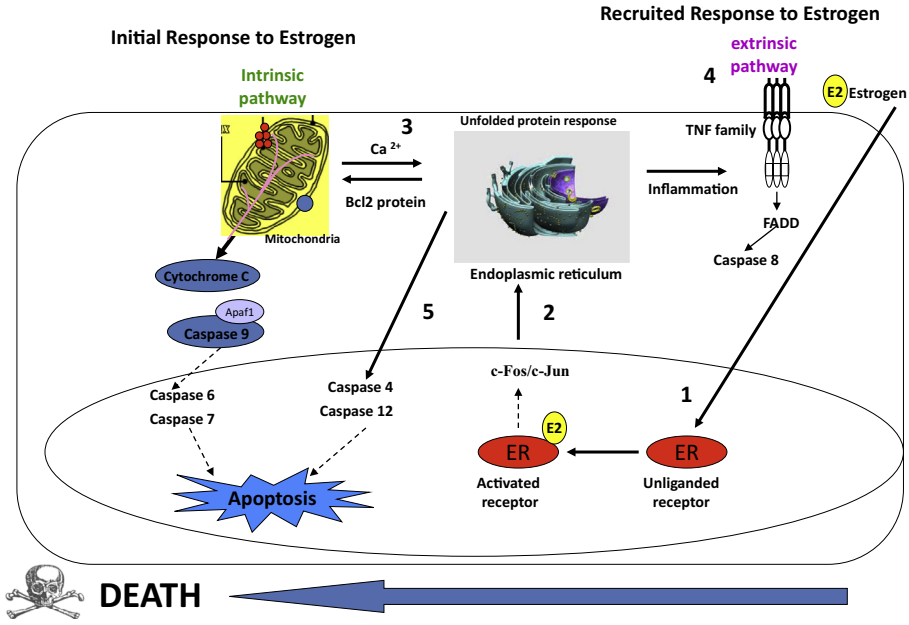

Fig. 8. The mechanism of E_2 induced apoptosis. 1. Activation of ER by E_2 induces activation AP-1 complex. 2. Endoplasmic reticulum stress caused accumulation of unfolded proteins that stimulates a UPR signal. 3. Failure to combat ERS induces apoptosis via induction of the mitochondrial pathway. 4. Subsequent activation of the extrinsic pathway of apoptosis occurs through the TNF family of proapoptotic genes. 5. Apoptosis can occur independent of the intrinsic and extrinsic pathway through activation of caspase 4.

(Fig. 7B). Therefore the ability of estrogens to induce apoptosis to treat or prevent breast tumors is dependent on the menopausal state of a woman and the duration of estrogen deprivation [53]. Beneficial responses with estrogen administration were noted in women over the age of 60 [15,57] and 35% remission rate of breast tumors was observed in women more than 5 years postmenopausal when compared to women who were less than 5 years postmenopausal (9%) [17]. The cells vulnerable to E_2 mediated apoptosis have been selected because estrogen deprivation that occurs at menopause causes death of estrogen dependent nascent breast cancer cells (Fig. 7A). The surviving cells grow independent of estrogens and may induce breast cancer tumors unless exogenous estrogens induce apoptotic death. Therefore it may be best to use estrogens as a preventive therapy after 5 years of menopause.

The mechanism of E_2 induced apoptosis has been extensively investigated (Fig. 8). EDC was not able to activate estrogen targeted genes, nor was it able induces apoptosis in the MCF7:5C cells confirming that the nongenomic pathway was not critical for E_2 triggered apoptosis. E_2 appears to induce apoptosis through the genomic pathway. Treatment of the MCF7:5C cells with estrogens [58,59] induces an initial reaction of cell growth. However, there is a subsequent induction of ERS within 24 h of treatment with E_2. In order to relieve ERS, UPR sensors PERK, ATF6 and IRE1α are activated. PERK serves to prevent protein translation via phosphorylation of eIF2α, whereas ATF6 and IRE1α act to upregulate UPR related genes. When UPR fails to contain ERS, apoptosis is activated in a number a ways (Fig. 8). Severe ERS induces apoptosis through activation of Bim which initiates the signaling cascade associated with the intrinsic mitochondrial pathway. Subsequent induction of the extrinsic pathway of apoptosis occurs through the TNF family of apoptosis related genes. ERS also induces apoptosis via activation of caspase 4. Inhibition of caspase 4 using a specific caspase 4 inhibitor abolished both E_2 [41] and BP [59] induced apoptosis.

Estrogens have been classified based on the conformation they create with the ER [46,47]. Planar estrogens are sealed within the LBD of the ER and coactivators are readily recruited resulting in a more rapid induction of apoptosis in the MCF7:5C cells. TPEs adopt an antiestrogen-like conformation with ER with reduced coactivator recruitment which may be responsible to their ability to initially block E_2 induced apoptosis. This correlates with the observed prolonged ERS with BP treatment and induction of apoptosis via caspase 4 after 5 days of treatment and subsequent induction of intrinsic and extrinsic pathway after 7 days of treatment. The fact that TPEs, though structurally similar to 4OHT, finally induce apoptosis is reassuring because TPEs were among the successful chemical therapies used by Haddow [15] to treat postmenopausal women with advanced breast cancer.

In conclusion, estrogen induced apoptosis is a delayed process when compared to that of a classic cytotoxic chemotherapy. However, the multi-faceted but relentless process involved ultimately results in the paradoxical induction of cell death induced by estrogens. This mechanism plays a potential role in the chemoprevention of breast cancer with estrogen therapy alone leads to increased patient survival if used at least five years after menopause [53,57]. Here is the key to selective estrogen induced apoptosis in breast cancer. Occult breast cancer cells grow robustly in a replete environment of estrogen but die quickly at menopause when estrogen is decreased. Only cells that are adapted by trial and error will grow in an estrogen depleted environment (Fig. 7A). Estrogen given immediately after menopause (within 5 years) will maintain the cell replication of populations adapted to a replete environment but administration of estrogen to populations adapted to growing in an estrogen deprived environment will trigger estrogen induced apoptosis (Fig. 7B). Estrogen is selective in its action based on the environmental adaptation of the population of breast cancer cells.

Acknowledgements

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References

[1] Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 2009;69:1243–54.
[2] Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007;72:7–25.
[3] Davies C, Godwin J, Gray R, Clarke M, Cutter D, Darby S, McGale P, Pan HC, Taylor C, Wang YC, et al. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 2011;378:771–84.
[4] Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V, et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. Lancet 2013;381:805–16.
[5] Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, Bliss J, et al. Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 2010;28:509–18.
[6] Lacassagne A. Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer 1936;27:217–28.
[7] Lacassagne A. A comparative study of the carcinogenic action of certain oestrogenic hormones. Am J Cancer 1936;28:735–40.
[8] Jordan VC. The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. Breast Cancer Res Treat 1988;11:197–209.
[9] Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998;90:1371–88.
[10] Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, et al. Tamoxifen for the prevention of breast cancer: current status of the national surgical adjuvant breast and bowel project P-1 study. J Natl Cancer Inst 2005;97:1652–62.
[11] Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 2006;295:2727–41.
[12] Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Update of the national surgical adjuvant breast and bowel project study of Tamoxifen and Raloxifene (STAR) P-2 trial: preventing breast cancer. Cancer Prev Res (Phila) 2010;3:696–706.
[13] Shafie S. Estrogen and the growth of breast cancer: new evidence suggests indirect action. Science 1980;209:701–2.
[14] Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 1986;83:2496–500.
[15] Haddow A, Watkinson JM, Patterson E. Influence of synthetic oestrogens upon advanced malignant disease. BMJ 1944;2:393–8.
[16] Haddow A, David A. Karnosky memorial lecture: thoughts on chemical therapy. Cancer Res 1970;26:737–54.
[17] Stoll B. Palliation by castration or by hormone administration. In: Stoll B, editor. Breast cancer management early and late. London, UK: William Herman Medical Books Ltd.; 1977. p. 133–46.
[18] Kennedy BJ. Hormone therapy for advanced breast cancer. Cancer 1965;18:1551–7.
[19] Kennedy BJ, Brown JH. Combined estrogenic and androgenic hormone therapy in advanced breast cancer. Cancer 1965;18:431–5.
[20] Kennedy BJ. Endocrine therapy of breast cancer. JAMA 1967;200:971–2.
[21] Cole M, Jones C, Todd I. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer 1971;25:270–5.
[22] Ingle J, Ahmann D, Green S, Edmonson J, Bisel H, Kvols L, et al. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. N Engl J Med 1981;304:16–21.
[23] Osborne CK, Coronado EB, Robinson J. Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. Eur J Cancer Clin Oncol 1987;23:1189–96.
[24] Gottardis MM, Jordan VC. Develoment of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term anti-estrogen administration. Cancer Res 1988;48:5183–7.
[25] Gottardis MM, Wagner RJ, Borden EC, Jordan VC. Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro. Cancer Res 1989;49:4765–9.
[26] Howell A, Dodwell DJ, Anderson H, Redford J. Response after withdrawal of tamoxifen and progestogens in advanced breast cancer. Ann Oncol 1992;3:611–7.
[27] Canney PA, Griffiths T, Latief TN, Priestman TJ. Clinical significance of tamoxifen withdrawal response. Lancet 1987;329:36.
[28] Howell A, Robertson JFR, Quaresma Albano J, Aschermannova A, Mauriac L, Kleeberg UR, et al. Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. J Clin Oncol 2002;20:3396–403.
[29] Osborne CK, Pippen J, Jones SE, Parker LM, Ellis M, Come S, et al. Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. J Clin Oncol 2002;20:3386–95.
[30] Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 1993;127:23–33.
[31] Yao K, Lee E, Bentrem D, England G, Schafer J, O'Regan R, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000;6:2028–36.
[32] Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 2003;95:1597–608.
[33] Liu H, Lee E, Gajdos C, Pearce S, Chen B, Osipo C, et al. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 2003;95:1586–97.
[34] Osipo C, Meeke K, Cheng D, Weichel A, Bertucci A, Liu H, et al. Role for HER2/neu and HER3 in fulvestrant-resistant breast cancer. Int J Oncol 2007;30:509.
[35] Balaburski GM, Dardes RC, Johnson M, Haddad B, Zhu F, Ross EA, et al. Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. Int J Oncol 2010;37:387–98.
[36] Jiang SY, Wolf D, Yingling J, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 1992;90:77–86.
[37] Pink JJ, Jiang S-Y, Fritsch M, Jordan VC. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. Cancer Res 1995;55:2583–90.
[38] Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 2001;93:1714–23.
[39] Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 2005;94:131–41.
[40] Lewis JS, Meeke K, Osipo C, Ross E, Kidawi N, Li T, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 2005;97:1746–59.
[41] Ariazi E, Cunliffe H, Lewis-Wambi JS, Slifker M, Willis A, Ramos P, et al. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci USA 2011;108:18879–86.
[42] Sweeney E, Fan P. Jordan VC. Mechanisms underlying differential response to estrogen-induced apoptosis in long-term estrogen-deprived breast cancer cells, Int J Oncol 2014;44:1529–38.
[43] Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. Biomed Pharmacother 2003;57:145–55.
[44] Lewis-Wambi J, Kim H, Wambi C, Patel R, Pyle J, Klein-Szanto A, et al. Buthionine sulfoximine sensitizes anti-hormone-resistant human breast cancer cells to estrogen-induced apoptosis. Breast Cancer Res 2008;10:R104.
[45] Lewis-Wambi JS, Swaby R, Kim H, Jordan VC. Potential of l-buthionine sulfoximine to enhance the apoptotic action of estradiol to reverse acquired antihormonal resistance in metastatic breast cancer. J Steroid Biochem Mol Biol 2009:33–9.
[46] Jordan VC, Schafer JM, Levenson AS, Liu H, Pease KM, Simons LA, et al. Molecular classification of estrogens. Cancer Res 2001;61:6619–23.
[47] Schafer JM, Liu H, Bentrem DJ, Zapf JW, Jordan VC. Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. Cancer Res 2000;60:5097–105.
[48] Brzozowski AM, Pike ACW, Dauter Z, Hubbard RE, Bonn T, Engstrom O, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997;389:753–8.
[49] Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;95:927–37.
[50] Maximov PY, Myers CB, Curpan RF, Lewis-Wambi JS, Jordan VC. Structure–function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. J Med Chem 2010;53:3273–83.
[51] Sengupta S, Obiorah I, Maximov PY, Curpan R, Jordan VC. Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. B J Pharmacol 2013;169:167–78.
[52] Obiorah I, Sengupta S, Curpan R, Jordan VC. Defining the conformation of the estrogen receptor complex that controls estrogen induced apoptosis in breast cancer. Mol Pharmacol 2014;85:789–99.
[53] Obiorah I, Jordan VC. 2012 NAMS/PFIZER- WULF H. UTIAN ENDOWED LECTURE. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. Menopause; 2013.
[54] Ellis M, Gao F, Dehdashti F, Jeffe D, Marcom P, Carey L, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 2009;302:774–80.
[55] Lønning P, Taylor P, Anker G, Iddon J, Wie L. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat 2001;67:111–6.
[56] Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, et al. Effects of conjugated equine estrogen in postmenopausal women with

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hysterectomy: the Women's Health Initiative randomized controlled trial. JAMA 2004;291:1701–12.
[57] Anderson G, Chlebowski R, Aragaki A, Kuller L, Manson J, Gass M, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. Lancet Oncol 2012;13:476–86.
[58] Obiorah I, Surojeet S, Fan P, Jordan VC. Delayed triggering of estrogen induced apoptosis that contrasts with rapid paclitaxel induced breast cancer cell death. Br J Cancer 2014;110:1488–96.
[59] Obiorah I. Jordan VC. Differences in the rate of oestrogen-induced apoptosis in breast cancer by estradiol and the triphenylethylene bisphenol, Br J Pharmacol 2014. http://dx.doi.org/10.1111/bph.12762 [E pub ahead of print].
[60] Font de Mora J, Brown M. AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. Mol Cell Biol 2000;20:5041–7.
[61] List H-J, Lauritsen KJ, Reiter R, Powers C, Wellstein A, Riegel AT. Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 Is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells. J Biol Chem 2001;276:23763–8.
[62] Lahusen T, Henke RT, Kagan BL, Wellstein A, Riegel AT. The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer. Breast Cancer Res Treat 2009;116:225–37.
[63] Hu ZZ, Kagan BL, Ariazi EA, Rosenthal DS, Zhang L, Li JV, et al. Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. PLoS One 2011;6:27.
[64] Bourgoin-Voillard S, Gallo D, Laïos I, Cleeren A, Bali LE, Jacquot Y, et al. Capacity of type I and II ligands to confer to estrogen receptor alpha an appropriate conformation for the recruitment of coactivators containing a LxxLL motif—relationship with the regulation of receptor level and ERE-dependent transcription in MCF-7 cells. Biochem Pharmacol 2010;79:746–57.
[65] Finn RS. Targeting Src in breast cancer. Ann Oncol 2008;19:1379–86.
[66] Shupnik MA. Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. Oncogene 2004;23:7979–89.
[67] Hiscox S, Morgan L, Green T, Barrow D, Gee J, Nicholson RI. Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. Breast Cancer Res Treat 2006;97:263–74.
[68] Zhao Y, Planas-Silva MD. Mislocalization of cell-cell adhesion complexes in tamoxifen-resistant breast cancer cells with elevated c-Src tyrosine kinase activity. Cancer Lett 2009;275:204–12.
[69] Saad F, Lipton A. SRC kinase inhibition: targeting bone metastases and tumor growth in prostate and breast cancer. Cancer Treat Rev 2010;36:177–84.
[70] Finn RS, Bengala C, Ibrahim N, Roche H, Sparano J, Strauss LC, et al. Dasatinib as a single agent in triple-negative breast cancer: results of an open-label phase 2 study. Clin Cancer Res 2011;17:6905–13.
[71] Mayer EL, Baurain JF, Sparano J, Strauss L, Campone M, Fumoleau P, et al. A phase 2 trial of dasatinib in patients with advanced HER2-positive and/or hormone receptor-positive breast cancer. Clin Cancer Res 2011;17:6897–904.
[72] Fan P, McDaniel RE, Kim HR, Clagett D, Haddad B, Jordan VC. Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines. Eur J Cancer 2012;48:3488–98.
[73] Fan P, Griffith OL, Agboke F, Anur P, Zou X, McDaniel RE, et al. C-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. Cancer Res 2013;73:4510–20.
[74] Harrington WR, Kim SH, Funk CC, Madak-Erdogan Z, Schiff R, Katzenellenbogen JA, et al. Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. Mol Endocrinol 2006;20:491–502.
[75] Oyadomari S, Mori M. Roles of CHOP//GADD153 in endoplasmic reticulum stress. Cell Death Differ 2003;11:381–9.
[76] Ron D. Translational control in the endoplasmic reticulum stress response. J Clin Invest 2002;110:1383–8.
[77] Harding HP, Calfon M, Urano F, Novoa I, Ron D. Transcriptional and translational control in the Mammalian unfolded protein response. Annu Rev Cell Dev Biol 2002;18:575–99.
[78] Haze K, Yoshida H, Yanagi H, Yura T, Mori K. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell 1999;10:3787–99.
[79] Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 2001;107:881–91.
[80] Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, et al. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature 2002;415:92–6.
[81] Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev 1999;13:1211–33.
[82] Scheuner D, Song B, McEwen E, Liu C, Laybutt R, Gillespie P, et al. Translational control is required for the unfolded protein response and in vivo glucose homeostasis. Mol cell 2001;7:1165–76.
[83] Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D. Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol Cell 2000;5:897–904.
[84] Okada T, Yoshida H, Akazawa R, Negishi M, Mori K. Distinct roles of activating transcription factor 6 (ATF6) and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) in transcription during the mammalian unfolded protein response. Biochem J 2002;366:585–94.
[85] Matsumoto M, Minami M, Takeda K, Sakao Y, Akira S. Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells. FEBS Lett 1996;395:143–7.
[86] McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 2001;21:1249–59.
[87] Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND, et al. ER stress triggers apoptosis by activating BH3-only protein Bim. Cell 2007;129:1337–49.

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Acquired resistance to selective estrogen receptor modulators (SERMs) in clinical practice (tamoxifen & raloxifene) by selection pressure in breast cancer cell populations

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ABSTRACT

Tamoxifen, a pioneering selective estrogen receptor modulator (SERM), has long been a therapeutic choice for all stages of estrogen receptor (ER)-positive breast cancer. The clinical application of long-term adjuvant antihormone therapy for the breast cancer has significantly improved breast cancer survival. However, acquired resistance to SERM remains a significant challenge in breast cancer treatment. The evolution of acquired resistance to SERMs treatment was primarily discovered using MCF-7 tumors transplanted in athymic mice to mimic years of adjuvant treatment in patients. Acquired resistance to tamoxifen is unique because the growth of resistant tumors is dependent on SERMs. It appears that acquired resistance to SERM is initially able to utilize either E_2 or a SERM as the growth stimulus in the SERM-resistant breast tumors. Mechanistic studies reveal that SERMs continuously suppress nuclear ER-target genes even during resistance, whereas they function as agonists to activate multiple membrane-associated molecules to promote cell growth. Laboratory observations *in vivo* further show that three phases of acquired SERM-resistance exists, depending on the length of SERMs exposure. Tumors with Phase I resistance are stimulated by both SERMs and estrogen. Tumors with Phase II resistance are stimulated by SERMs, but are inhibited by estrogen due to apoptosis. The laboratory models suggest a new treatment strategy, in which limited-duration, low-dose estrogen can be used to purge Phase II-resistant breast cancer cells. This discovery provides an invaluable insight into the evolution of drug resistance to SERMs, and this knowledge is now being used to justify clinical trials of estrogen therapy following long-term antihormone therapy. All of these results suggest that cell populations that have acquired resistance are in constant evolution depending upon selection pressure. The limited availability of growth stimuli in any new environment enhances population plasticity in the trial and error search for survival.

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1. Introduction

The estrogen receptor (ER), including estrogen receptor alpha (ERα) and estrogen receptor (ERβ), mediates the biological effects of estrogen for the development and progression of breast cancer, and serves as an important diagnostic and therapeutic target for the prevention and treatment. Targeted therapy to ERα is the most successful strategy in breast cancer treatment and prevention. These endocrine therapies include the aromatase inhibitors (AIs) that indirectly target the ER by blocking the synthesis of estrogen from androgen in peripheral tissues, show improved efficacy in postmenopausal breast cancer patients [1,2]. Another strategy option is to use pure antiestrogens (also called selective estrogen receptor down-regulators, SERDs), such as fulvestrant, which have no agonist activity and cause degradation of ER [3]. Fulvestrant has been approved to treat advanced breast cancer after tamoxifen failure [4,5]. The most widely used therapy for ER-positive breast cancer are the selective estrogen receptor modulators (SERMs), which are synthetic molecules that bind to ER and can modulate its transcriptional capabilities in different estrogen target tissues. Tamoxifen (Fig. 1), the pioneering SERM, is extensively used for targeted therapy of ER-positive breast cancers [6] and is also approved as the first chemo-preventive agent for lowering breast cancer incidence in high risk women [7]. The therapeutic and preventive efficacy of tamoxifen was initially proven by a series of experiments in the laboratory which laid the foundation of its clinical applications [8]. A 5-year course (long-term) of adjuvant

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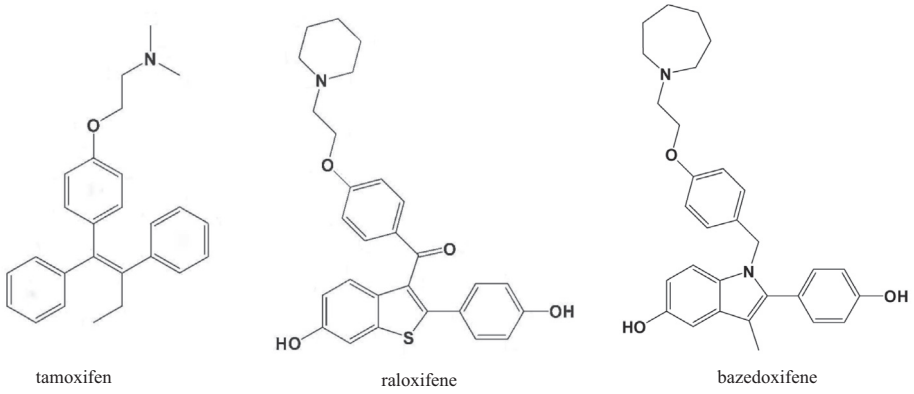

Fig. 1. Chemical structures of three SERMs—tamoxifen, raloxifene, and bazedoxifene.

tamoxifen treatment in patients is superior to 1–2 years of treatment, demonstrating that longer is better [9–11]. This illustrates the translation of the earlier laboratory principle [12]. Currently, 5 years of adjuvant tamoxifen is recommended to be optimal, however, two recent trials of 5 versus 10 years of adjuvant tamoxifen therapy show 10-year is superior [13,14]. Unfortunately, the use of tamoxifen is associated with *de novo* and acquired resistance and some undesirable side effects, such as thromboembolic events and increased rates of endometrial cancer [15]. However, this latter effect is only noted in postmenopausal patients. The study of the molecular mechanisms of resistance provides an opportunity to precisely understand the mechanism of SERMs action which may further help in designing new and improved SERMs. Clinical studies demonstrate that another SERM, raloxifene (Fig. 1), which is primarily used to treat postmenopausal osteoporosis; simultaneously reduces the risk of breast cancer [16–18]. Raloxifene is as effective as tamoxifen in preventing breast cancer in postmenopausal women but with fewer side effects [19,20]. The third generation SERM, bazedoxifene (Fig. 1), administrated with conjugated estrogens, represents a promising alternative to hormone therapy for the prevention of osteoporosis and the treatment of postmenopausal symptoms in non-hysterectomized postmenopausal women [21,22]. Overall, these findings open a new horizon for SERMs as a class of drug which can not only be used for therapy and prevention of breast cancer, but also for various other diseases and disorders. We will provide a basic background of SERMs, the current progress of the SERMs, and focus in detail on the evolution of acquired resistance to endocrine therapy by selection pressure on cell populations.

2. Basic molecular mechanisms of SERM action

The antitumor effects of SERMs are thought to be due to its antiestrogenic activity, mediated by competitive inhibition of estrogen binding to ER [23]. SERMs are antiestrogenic in the breast but estrogen-like in the bones and reduce circulating cholesterol levels. This discovery in the laboratory suggests the clinical application to simultaneously prevent osteoporosis, coronary heart disease, and breast cancer [24,25]. However, SERMs also have different degrees of estrogenicity in the uterus. Tamoxifen exhibits partial agonistic activity thought to be associated with an increased risk of endometrial cancer [26–30], but raloxifene and bazedoxifene do not [30–32]. Coregulators are crucial in determining the final tissue outcome in terms of transcriptional activation or repression mediated by SERMs [33–36]. Now dozens of coactivators are known, and corepressor molecules also exist to prevent the gene transcription by unliganded receptors [33–36]. X-ray crystallography of the ligand binding domains (LBD) of the ER liganded with either estrogens or antiestrogens show the potential of ligands to promote or prevent coactivator binding based on the shape of the estrogen or anti-ER complex [37,38]. Evidence has accumulated that the broad spectrum of ligands that bind to the ER can create a broad range of ER complexes that are either fully estrogenic or antiestrogenic at a particular target site [39]. Thus, a mechanistic model of estrogen action and antiestrogen action has emerged [40,41] based on the shape of the ligand that programs the complex to adopt a particular shape that ultimately interacts with coactivators or corepressors in target cells to determine the estrogenic or antiestrogenic response, respectively.

The three homologous members of the p160 SRC family (SRC1, SRC2 and SRC3) mediate the transcriptional functions of nuclear receptors and other transcription factors, and are the most studied of all the transcriptional co-activators [42]. The relative abundance of SRC1 in uterine cells is responsible for the agonistic activity of tamoxifen, whereas the low SRC1 level in breast cancer cells [33]. However, raloxifene does not recruit SRC1 even in the uterine cells [33], suggesting that the interaction with each specific ligand elicits a unique conformation of the receptor that is critical for the interaction of co-regulators. Our finding indicates that tamoxifen does not recruit SRC3 to the promoter of ER-target gene, pS2 and acts as an estrogen antagonist in wild-type breast cancer cells [43]. These observations further provide an explanation for the earlier studies, where tamoxifen has been reported to induce growth of endometrial cancer cells but not of breast cancer cells in athymic mice [29] and also that the agonistic property of raloxifene is less in endometrial cancer cells [30]. Currently, there is no direct evidence about the interaction between bazedoxifene and coactivators. Molecular modeling studies demonstrate that bazedoxifene binds to ERα in an orientation similar to raloxifene [44]. Tamoxifen, raloxifene, and bazedoxifene all exhibit antiestrogenic activity to inhibit ER-target genes and modulate the ER signal transduction pathway in breast cancer [45]. However, bazedoxifene is distinct from other SERMs in its ability to inhibit antihormone resistant breast cancer growth *in vitro* and *in vivo* [44,45]. In our SERM-resistant cell line, bazedoxifene reduces levels of ERα protein and blocks stimulation induced by 4-hydroxytamoxifen.

3. Drug resistance to SERMs

There are three types of resistance to SERMs: metabolic resistance, *de novo* resistance and acquired resistance [46]. Metabolic [47] and *de novo* [46] resistance have been extensively reviewed and will not be considered further because they do not influence the modulation or inhibition of long-term growth in breast cancer. The fact that SERMs initially act as estrogen antagonists in breast cancer to switch off growth during the successful treatment of

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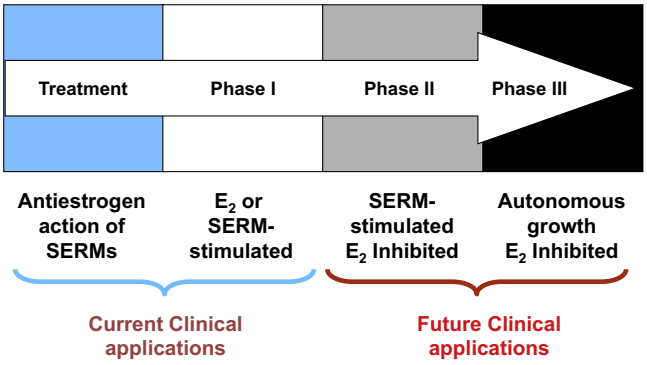

Fig. 2. The evolution of resistance to SERMs after long-term therapy. Phase I acquired resistance develops after a year or two of therapy of ER positive metastatic breast cancer. Phase II acquired resistance occurs after 5 years of SERM treatment in the laboratory or potentially as occult disease during 5 years adjuvant tamoxifen therapy. Phase III acquired resistance potentially develops after indefinite therapy for ER positive breast cancer.

breast cancer, but then cause SERM-stimulated breast cancer growth is a unique form of acquired drug resistance. The cell populations are clearly being modulated over years of therapy so that those cells that can adapt and grow in an antiestrogenic environment now dominate. Understanding this process provides an opportunity to save more lives.

The aforementioned studies (Section 2) describe the mechanics of antiestrogen action, but do not define the modulation of estrogenic properties of individual SERMs at the ER or in fact, how ER-positive tumor cells grow spontaneously in an estrogen-deprived environment. An early study of acquired resistance to tamoxifen identified a D351Y mutation *in vivo*, in some but not all, tamoxifen-stimulated tumors grown in athymic mice [48,49]. Subsequent studies, in engineered cells, showed that this amino acid could modulate estrogenic action of SERMs at an estrogen response gene target [50–52]. Indeed, the D351Y would convert the antiestrogenic raloxifene-ER complex to an estrogenic complex [53,54]. This was the first natural mutation to convert an antiestrogenic to an estrogenic complex. The significance of the D351Y amino acid has recently been illustrated in five reports [55–59] which find that mutations in amino acid D537 and Y538 are associated with acquired resistance to antihormone therapy in breast cancer metastases. It is argued that the unoccupied receptor is now able to close helix 12 and trigger growth by anchoring at D351.

The mutations in the ER is an interesting and significant finding in patient metastases, although none or very fewer are noted in primary tumors [55–59]. So the question arises how the development of acquired resistance occurs to subvert an effective antiestrogenic therapy like tamoxifen for the long-term treatment of breast cancer. The evidence all points to the evolution of cell populations that initially respond to therapy, but through Darwinian principles of survival, cells with chance mutation in growth pathway replicate in the face of adversity. The plasticity of cell populations ebb and flow like the tide with each new antihormonal treatment. This results in cell lines that eventually thrive in each new environment. It is the story of cellular changes that occur over years in patients that can only be understood by creating selection studies over years in the laboratory. We will present the story so far.

3.1. Models of acquired resistance to antihormone therapy

The evolution of acquired resistance to SERM treatment was primarily discovered using MCF-7 tumors transplanted in athymic mice to mimic years of adjuvant treatment in patients [60–62]. Acquired resistance to SERMs is unique because the growth of

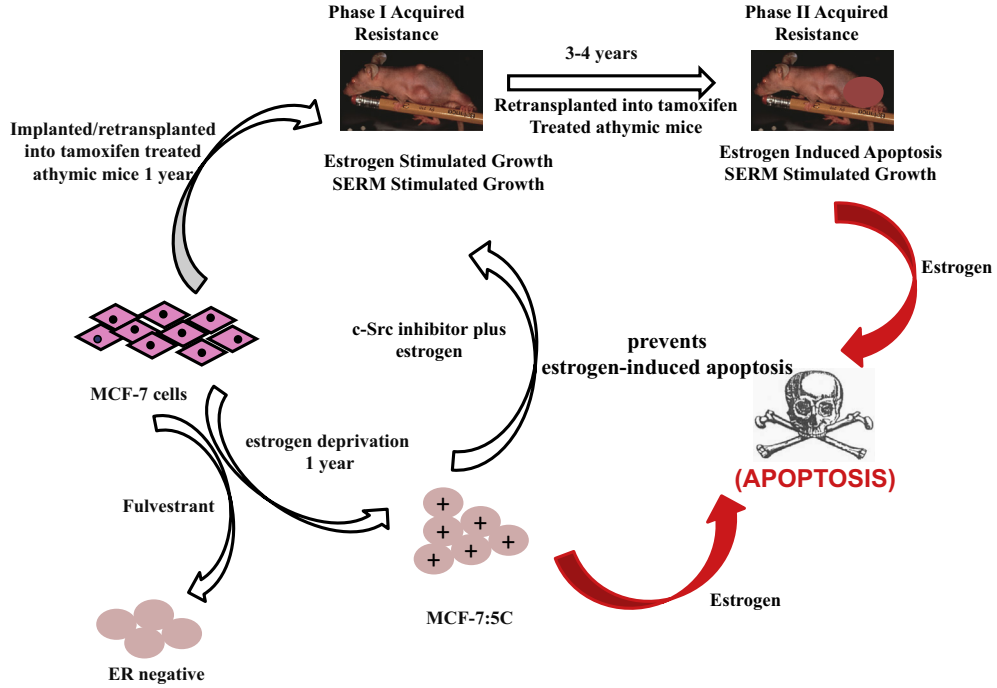

Fig. 3. The melding of model systems. During the past 25 years, the MCF-7 breast cancer cell line has been used to recapitulate an evolving model *in vivo* of acquired tamoxifen resistance (62) observed in clinical breast cancer. In parallel, the same cell line has been used to recapitulate models *in vitro* of estrogen deprivation using either fulvestrant, that destroys the ER protein, or aromatase inhibitors that create a long-term estrogen-deprived state. The cells derived from estrogen deprivation with fulvestrant loose the ER (90), but estrogen deprivation in an estrogen-free environment *in vitro* increases the ER level. Clones grow out that are sensitive to estrogen-induced apoptosis (86). A c-Src inhibitor blocks estrogen-induced apoptosis in the short-term (94), but long-term (2 months) treatment with estrogen plus a c-Src inhibitor results in a new populations of cells (MCF-7:PF) (96) that recapitulates *in vitro* Phase I resistance to SERMs *in vivo*. These data, accumulated over decades, illustrate the plasticity of cell populations in that successful attempt to adapt to hostile environment.

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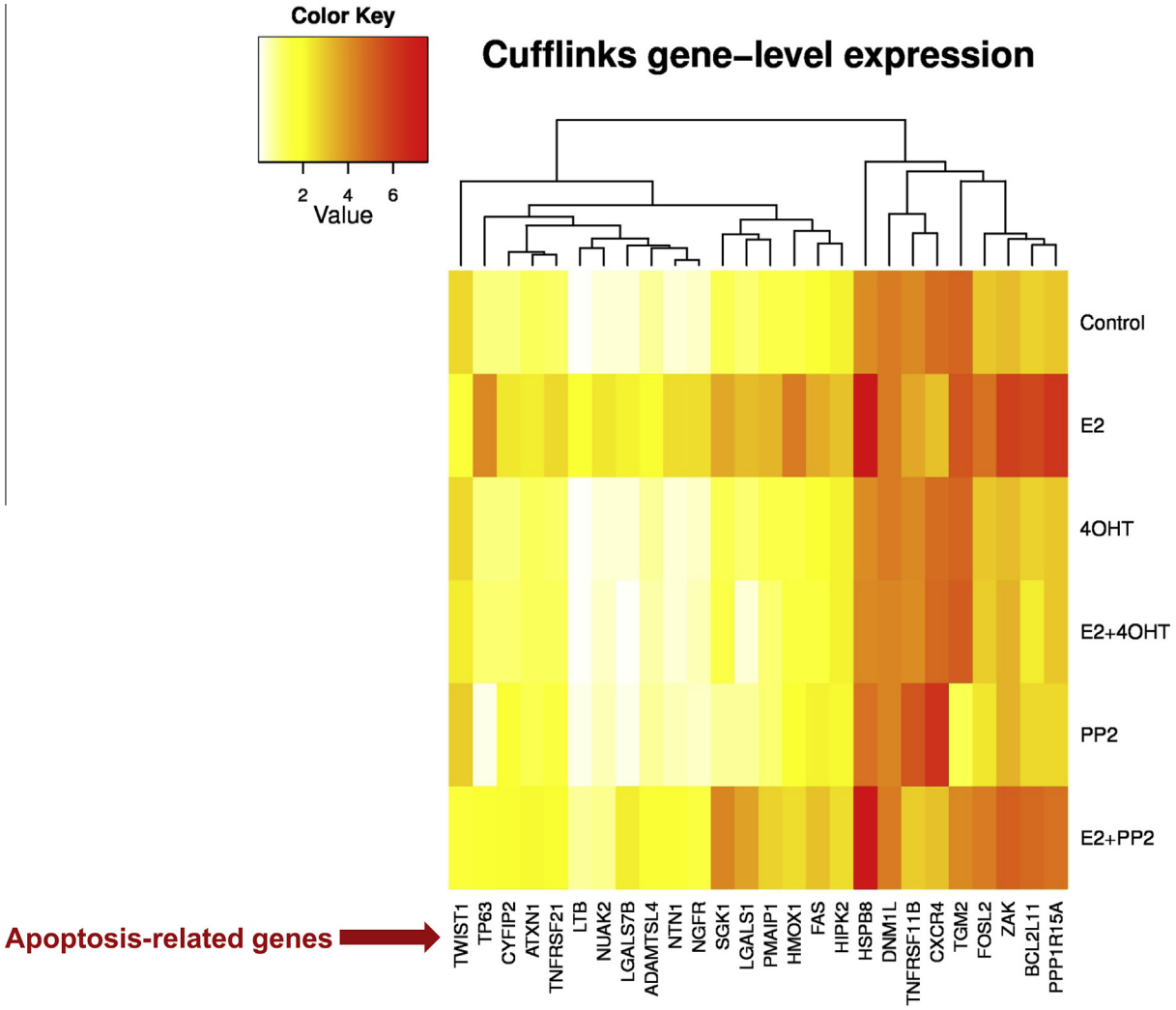

Fig. 4. The c-Src inhibitor and 4-hydroxytamoxifen block apoptosis-related genes induced by estrogen in MCF-7:5C cells. This result has been reported in reference 94. MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9} mol/L), 4-OHT (10^{-6} mol/L), E_2 (10^{-9} mol/L) plus 4-OHT (10^{-6} mol/L), PP2 (5×10^{-6} mol/L), E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) respectively for 72 h. Cells were harvested in TRIzol for RNA-sequence analysis.

resistant tumors is dependent on SERMs [60–62]. Thus, it appears that acquired resistance to SERMs is initially able to utilize either E_2 or a SERM as the growth stimulus in the ER-positive SERM-resistant breast tumors [63,64]. However, no mechanism has been established to explain this paradox. Laboratory observations further show that three phases of acquired tamoxifen-resistance exists (Fig. 2), which depend on the length of tamoxifen exposure [65]. Tumors with Phase I resistance are stimulated by estrogen and tamoxifen but inhibited by AIs and fulvestrant [63,64]. This form of acquired resistance to tamoxifen takes about a year or two to develop during retransplantation of tumors into tamoxifen-treated athymic mice. Subsequent clinical studies more than a decade later showed that both AIs and fulvestrant were equivalent options following tamoxifen failure in metastatic breast cancer [4,5]. Tumors with Phase II resistance are stimulated by tamoxifen but are inhibited by estrogen due to apoptosis [65]. The results of laboratory models [62,66] suggested new treatment strategies, in which limited-duration, low-dose estrogen can be used to purge Phase II-resistant breast cancer cells and act as a salvage therapy following long-term antihormone therapy. This discovery provides an invaluable insight into the evolution of drug resistance to SERMs [67] and this knowledge has been used to justify clinical trials of estrogen therapy following long-term antihormone therapy [68–70].

However, the rapid development of Phase I resistance to tamoxifen begs to question "why is long-term (5 years or more) adjuvant tamoxifen therapy so effective as an adjuvant treatment? There is a 50% decrease in mortality compared to historical no treatment controls in the 10 years of tamoxifen has stopped [14]. If the athymic mouse model replicates human disease [71], the clinical studies of more than 2 years of adjuvant tamoxifen would all have failed." But micrometastatic disease is clearly not established metastatic breast cancer. There is not the same bulk or vascularity, nor is there the same genetic variation and cellular plasticity eager to survive. The answer emerged from laboratory studies of the retransplantation of tamoxifen-stimulated tumors over a 5 year period. What emerged was the serendipitous finding that physiological estrogen is now a trigger for death, but tamoxifen therapy is the signal to sustain cancer cell homeostasis. Phase II acquired resistance evolved from Phase I acquired resistance to tamoxifen and exposes a vulnerability in breast cancer. Estrogen, the signal for breast cancer cell survival and growth, could also become the signal for apoptosis to be triggered in a prepared population of cells only able to grow in estrogen-deprived conditions. The ER is paradoxically selective either in the cells that have been previously programmed for growth in an estrogen-rich environment or prepared for execution in those cells that cling to their survival after year in a long-term estrogen-free environment.

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A similar story has also occurred over the past 20 years with the development of models to study antihormone resistance to AIs. This followed the vigorous evaluation of multiple AIs for the treatment of breast cancer, and AIs became the antihormone therapy of choice for adjuvant therapy in postmenopausal patients with ER-positive disease (2). The discovery that breast cancer cells, in particular MCF-7 cells, had been grown routinely in the redox indicator phenol red [72] that contains a contaminant with estrogenic activity, revolutionized options to solve the question of what happens to hormone-responsive cells once starved of estrogen i.e. AI therapy. Short-term estrogen withdrawal results in expansion of the ER pool in ER-positive MCF-7 cells and following a crises period during the first month, a population of cells grows out that is hormone-independent for growth [73,74]. The Santen's group provided compelling evidence that the long-term estrogen-deprived cells (LTED) had acquired hypersensitivity to low estrogen concentration in the local environment [75,76]. He proposed that this was a reasonable explanation for the failure of AI therapy i.e. the drug could not prevent the synthesis of all estrogen and there is also estrogen in the environment e.g. phytoestrogens or endocrine disruptors. In other words, the cells survive with a minimal survival system; the population grows successfully with what is available. However, in the wake of findings [62] that physiological estrogen causes tamoxifen (estrogen withdrawal) resistant MCF-7 tumor regression in athymic mice after LTED (5 years of tamoxifen), some other process must be occurring; the growth stimulus returns–cells die! Long-term estrogen-deprived MCF-7 cells in culture have a concentration related apoptotic response to exogenous estrogen [77]. High concentrations kill all cells thereby providing an explanation for Haddow's 1944 earlier observation that estrogen can be used at high doses to treat breast cancer a decade after menopause [78]. However, the trigger actually is initiated in LTED cells by low concentrations of estrogen in the physiological range [77,79]. However, Santen had been using a population of LTED MCF-7 cells and another approach to answer the question occurred in parallel by others. Two clones of LTED MCF-7 breast cancer cells were derived, characterized, and stored without further study following their initial reports in the 1990s [80,81]. The MCF-7:5C clone [80] was originally described as being completely refractory to either estrogen or antiestrogen despite in fact it was ER-positive and progesterone receptor (PgR)-negative. By contrast, the MCF-7:2A clone [81] was unaffected by estrogen in a 7 day growth assay, but antiestrogens inhibited spontaneous growth; estrogen increased PgR synthesis in these ER-positive cells.

However, re-evaluation of these clones (2A and 5C) a decade later created a surprise. The MCF-7:2A cells had a delayed apoptotic response based on increased glutathione levels; slow cell death could be triggered by physiological estrogen after 7 days and enhanced apoptosis facilitated using the blocker of glutathione synthesis, buthionine sulfoximine (BSO) [82–84]. Changing media conditions caused early catastrophic apoptosis with physiological estrogen in MCF-7:5C cells within a week [85,86]. Cells grew spontaneously in ovariectomized athymic mice and physiological estrogen caused complete tumor regression within 14 days [86]. Our supersensitive MCF-7:WS8 cells that grew with minute, radioimmunologically undetectable levels of estrogen, MCF-7:5C and MCF-7:2A cells all became the conduit for understanding estrogen-induced apoptosis as a consequence of acquired resistance to both AIs and phase II tamoxifen- or raloxifene-resistant growth [87–97].

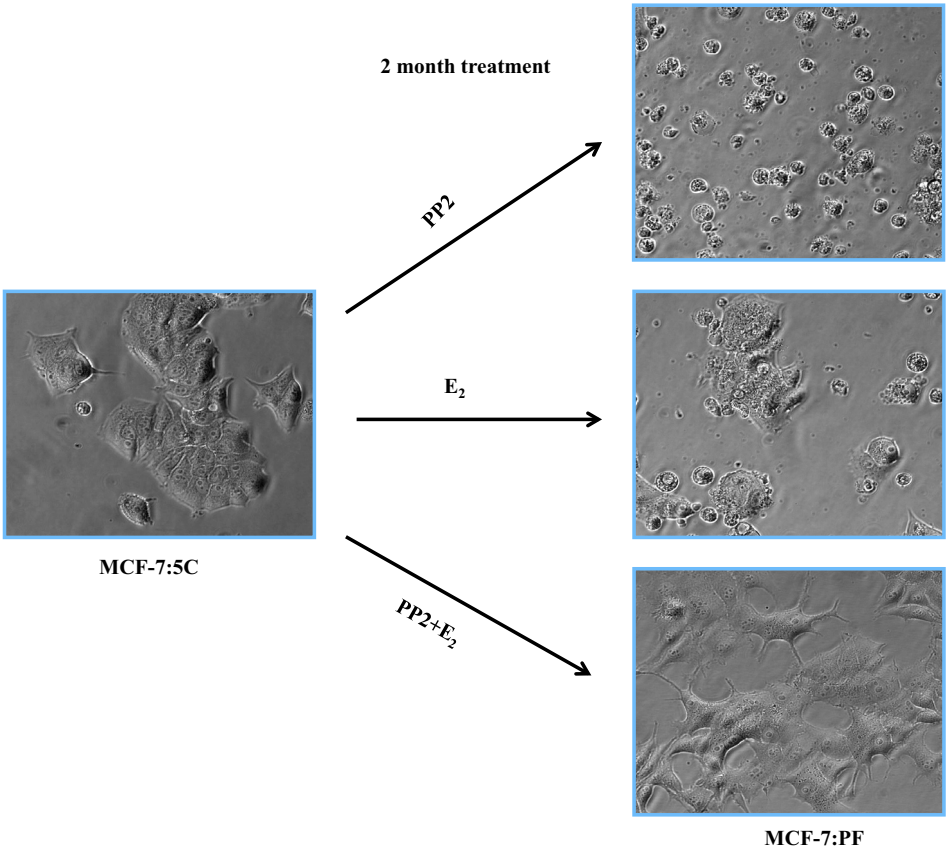

Fig. 5. The c-Src inhibitor completely blocks E_2-induced apoptosis after long-term treatment. MCF-7:5C cells were long-term treated with vehicle (0.1% EtOH), PP2 (5×10^{-6} mol/L), E_2 (10^{-9} mol/L), and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) in T_{25} flasks for 8 weeks. Cells were photographed under bright field illumination at (20×) magnification (Zeiss).

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3.2. Melding the models

By creating selection pressure over months or years, the development of acquired resistance to antihormone resistance is forced to evolve and create new surviving cell populations based on the restrictions in the environment. In Fig. 3, we have illustrated how models *in vivo* of acquired resistance to tamoxifen that requires 5 years to evolve and consolidate, can potentially be linked to models of AI resistance *in vitro* (MCF-7:5C).

Our recent publications establish that estrogen induces apoptosis through endoplasmic reticulum stress and oxidative stress [84,87,94]. A variety of apoptosis-related genes are activated by estrogen in MCF-7:5C and MCF-7:2A cells [84,94] and (Fig. 4). The finding that the c-Src tyrosine kinase is increased in MCF-7:5C cells [94] and inhibition of c-Src in short-term (7 days) experiments will reversibly block estrogen-induced apoptosis [94,95], created an opportunity to determine what long-term inhibition of c-Src in the presence of estrogen would do to the biological properties of the cell populations [96]. A two month period of selection pressure was chosen, as this is the time period used clinically to evaluate tumor response to therapy. The results of the three long-term therapies are illustrated in Fig. 5 based on analysis of the resulting populations. Estrogen alone causes early catastrophic apoptosis but the resulting cell population that results after 2 months of continuous treatment consists of a balance of apoptosis and growth thereby restricting the outgrowth of cells. The c-Src inhibitor is a reversible blocker and the cells revert to the original MCF-7:5C cell phenotype with estrogen-induced apoptosis upon wash out [96]. However, the cell populations (MCF-7:PF) that grow out under the pressure of estrogen plus the c-Src inhibitor is particularly interesting as, for the first time, it replicates Phase I acquired resistance to SERMs *in vitro*. The cells grow robustly with estrogen but also SERMs will stimulate growth *in vitro* based on their individual intrinsic estrogenic efficacy as partial agonists.

Further investigation suggests that ER is a major driver of growth utilized by both E_2 and SERMs in resistant models *in vivo* [60,63] and *in vitro* [96]. In contrast to E_2 that activates classical ER-target genes, SERMs continue to act as effective antiestrogens to inhibit classical ER-target genes, even at the time of growth stimulation. This result is consistent with our previous finding *in vivo* that growth of tumors by tamoxifen or fulvestrant is potentially independent of ER transcriptional activity, as evidenced by lack of induction of E_2-responsive genes [90]. Other groups have reported similar observations with tamoxifen suppressing classical ERE-regulated genes despite acquired resistance *in vitro* [98] or *in vivo* [99]. A significant alteration of ER function observed in SERM-resistant cells is the activation of multiple membrane-associated molecules including focal adhesion molecules, adapter proteins, and growth factor receptor (Fig. 6). All of these integral adaptations contribute to SERM-resistance.

4. Challenges and conclusions

Over the past 40 years, we have witnessed a dramatic improvement in the survivorship of the majority of patients with a diagnosis of ER-positive breast cancer [100,101]. Although extensive studies have advanced the understanding of the mechanisms underlying the acquired SERM-resistance, acquired resistance to SERMs is not one-dimensional with a simple solution. Resistant cell populations are in constant evolution depending upon selection pressure and the availability of growth stimuli that enhances population plasticity and survival of new clones. Breast cancer cells have the potential to integrally modulate a variety of membrane-associated molecules to subvert long-term nuclear pressure

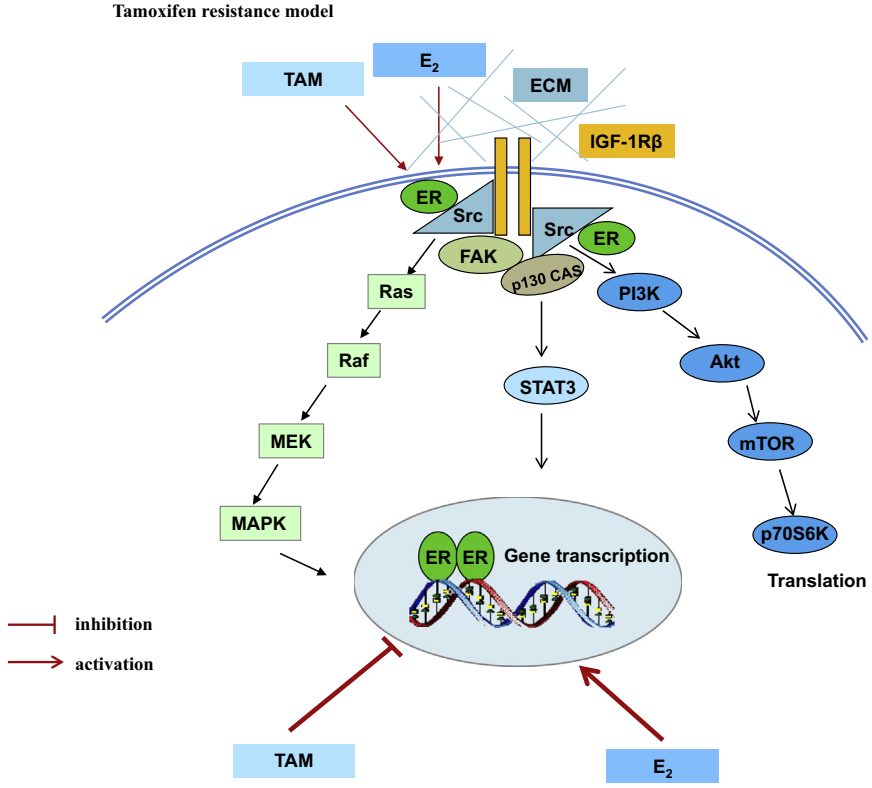

Fig. 6. Genomic and nongenomic signal transduction pathways in tamoxifen-resistant model. Estrogen (E_2) and tamoxifen (TAM) exert differential functions on nuclear ER. E_2 activates classical ER-target genes but TAM acts to block gene activation. Both E_2 and TAM increases the non-genomic activity of ER through membrane-associated molecules such as extracellular matrix (ECM), c-Src, insulin-like growth factor-1 receptor beta (IGF-1Rβ), and focal adhesion kinase (FAK) to enhance downstream signaling cascades.

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exerted by SERMs. This results in the promotion of cell growth (Fig. 6). These functional alterations lead to acquired SERM resistance. As a result, the strategic definition of molecular mechanisms driving the development of endocrine resistance is an important and proactive first step to improve therapy. How to prioritize and advance individualized treatment is another challenge to improve the therapeutic efficacy of antihormone therapy [102].

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References

[1] Jordan VC, Brodie AMH. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007;72:7–25.
[2] Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, Bliss J, et al. Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 2010;28:509–18.
[3] Ariazi EA, Lewis-Wambi JS, Gill SD, Pyle JR, Ariazi JL, Kim HR, et al. Emerging principles for the development of resistance to antihormonal therapy: implications for the clinical utility of fulvestrant. J Steroid Biochem Mol Biol 2006;102:128–38.
[4] Osborne CK, Pippen J, Jones SE, Parker LM, Ellis M, Come S, et al. Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. J Clin Oncol 2002;20:3386–95.
[5] Howell A, Robertson JF, Quaresma Albano J, Aschermannova A, Mauriac L, Kleeberg UR, et al. Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. J Clin Oncol 2002;20:3396–403.
[6] Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. Clin Cancer Res 2003;9:1980–9.
[7] Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998;90:1371–88.
[8] Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 2008;44:30–8.
[9] Gelber RD, Cole BF, Goldhirsch A, Rose C, Fisher B, Osborne CK, et al. Adjuvant chemotherapy plus tamoxifen compared with tamoxifen alone for postmenopausal breast cancer: meta-analysis of quality-adjusted survival. Lancet 1996;347:1066–71.
[10] Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005; 365: p. 1687–717.
[11] Davies C, Godwin J, Gray R, Clarke M, Cutter D, Darby S, et al. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 2011;378:771–84.
[12] Jordan VC. Laboratory studies to develop general principles for the adjuvant treatment of breast cancer with antiestrogens: problems and potential for future clinical applications. Breast Cancer Res Treat 1983;3:S73–86.
[13] The aTTom Collaborative Group. aTTom: Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years in 6,953 women with early breast cancer. J Clin Oncol 2013; 31 [supplement].
[14] Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V, et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. Lancet 2013;381:805–16.
[15] Cuzick J, Powles T, Veronesi U, Forbes J, Edwards R, Ashley S, et al. Overview of the main outcomes in breast cancer prevention trials. Lancet 2003;361:296–300.
[16] Jordan VC. The rise of raloxifene and the fall of invasive breast cancer. J Natl Cancer Inst 2008;100:831–3.
[17] Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple outcomes of raloxifene evaluation. JAMA 1999;281:2189–97.
[18] Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 1999;282:637–45.
[19] Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. The study of tamoxifen and raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. JAMA 2006;295:2727–41.
[20] Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Update of the National Surgical Adjuvant Breast and Bowel Project Study of tamoxifen and raloxifene (STAR) P-2 Trial: preventing breast cancer. Cancer Prev Res 2010;3:696–706.
[21] Jordan VC. A(nother) scientific strategy to prevent breast cancer in postmenopausal women by enhancing estrogen-induced apoptosis? Menopause 2014; [Epub ahead of print].
[22] Gallagher JC, Shi H, Mirkin S, Chines AA. Changes in bone mineral density are correlated with bone markers and reductions in hot flush severity in postmenopausal women treated with bazedoxifene/conjugated estrogens. Menopause 2013;20:1126–32.
[23] Jordan VC. Tamoxifen (ICI 46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 2006;147:S269–76.
[24] Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 1990;50:4177–89.
[25] Jordan VC. SERMs: meeting the promise of multifunctional medicines. J Natl Cancer Inst 2007;99:350–6.
[26] O'Regan RM, Cisneros A, England GM, MacGregor JI, Muenzner HD, Assikis VJ, et al. Effects of the antiestrogens tamoxifen, toremifene, and ICI 182,780 on endometrial cancer growth. J Natl Cancer Inst 1998;90:1552–8.
[27] Grese TA, Sluka JP, Bryant HU, Cullen GJ, Glasebrook AL, Jones CD, et al. Molecular determinants of tissue selectivity in estrogen receptor modulators. Proc Natl Acad Sci USA 1997;94:14105–10.
[28] Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfverswärd C, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1989;1:117–20.
[29] Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 1988;48:812–5.
[30] Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimu lated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 1990;50:3189–92.
[31] Pinkerton JV, Harvey JA, Lindsay R, Pan K, Chines AA, Mirkin S, et al. Effects of bazedoxifene/conjugated estrogens on the endometrium and bone: a randomized trial. J Clin Endocrinol Metab 2014;99:E189–98.
[32] Mirkin S, Komm BS, Pan K, Chines AA. Effects of bazedoxifene/conjugated estrogens on endometrial safety and bone in postmenopausal women. Climacteric 2013;16:338–46.
[33] Shang Y, Brown M. Molecular determinants for the tissue specificity of SERMs. Science 2002;295:2465–8.
[34] Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 1995;270:1354–7.
[35] Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev 2004;25:45–71.
[36] Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 2000;103:843–52.
[37] Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, et al. The structural basis of estrogen receptor/co-activator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;95:927–37.
[38] Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engström O, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997;389:753–8.
[39] Wijayaratne AL, Nagel SC, Paige LA, Christensen DJ, Norris JD, Fowlkes DM, et al. Comparative analyses of mechanistic differences among antiestrogens. Endocrinology 1999;140:5828–40.
[40] Schaufele F, Chang CY, Liu W, Baxter JD, Nordeen SK, Wan Y, et al. Temporally distinct and ligand-specific recruitment of nuclear receptor-interacting peptides and cofactors to subnuclear domains containing the estrogen receptor. Mol Endocrinol 2000;14:2024–39.
[41] Hall JM, McDonnell DP, Korach KS. Allosteric regulation of estrogen receptor structure, function, and coactivator recruitment by different estrogen response elements. Mol Endocrinol 2002;16:469–86.
[42] Xu J, Wu RC, O'Malley BW. Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer 2009;9:615–30.
[43] Sengupta S, Obiorah I, Maximov PY, Curpan R, Jordan VC. Molecular mechanism of action of bisphenol and bisphenol a mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. Br J Pharmacol 2013;169:167–78.
[44] Lewis-Wambi JS, Kim H, Curpan R, Grigg R, Sarker MA, Jordan VC. The selective estrogen receptor modulator bazedoxifene inhibits hormone-independent breast cancer cell growth and down-regulates estrogen receptor α and cyclin D1. Mol Pharmacol 2011;80:610–20.
[45] Wardell SE, Nelson ER, Chao CA, McDonnell DP. Bazedoxifene exhibits antiestrogenic activity in animal models of tamoxifen-resistant breast cancer:

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implications for treatment of advanced disease. Clin Cancer Res 2013;19:2420–31.
[46] Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 2007;25:5815–24.
[47] Brauch H, Schwab M. Prediction of tamoxifen outcome by genetic variation of CYP2D6 in post-menopausal women with early breast cancer. Br J Clin Pharmacol 2014;77:695–703.
[48] Wolf DM, Jordan VC. The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 1994;31:129–38.
[49] Wolf DM, Jordan VC. Characterization of tamoxifen stimulated MCF-7 tumor variants grown in athymic mice. Breast Cancer Res Treat 1994;31:117–27.
[50] Liu H, Park WC, Bentrem DJ, McKian KP, Reyes Ade L, Loweth JA, et al. Structure-function relationships of the raloxifene-estrogen receptor-alpha complex for regulating transforming growth factor-alpha expression in breast cancer cells. J Biol Chem 2002;277:9189–98.
[51] Liu H, Lee ES, Deb Los Reyes A, Zapf JW, Jordan VC. Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor alpha complex. Cancer Res 2001;61:3632–9.
[52] Schafer JM, Liu H, Tonetti DA, Jordan VC. The interaction of raloxifene and the active metabolite of the antiestrogen EM-800 (SC 5705) with the human estrogen receptor. Cancer Res 1999;59:4308–13.
[53] Levenson AS, Catherino WH, Jordan VC. Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. J Steroid Biochem Mol Biol 1997;60:261–8.
[54] Levenson AS, Jordan VC. The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. Cancer Res 1998;58:1872–5.
[55] Jeselsohn R, Yelensky R, Buchwalter G, Frampton G, Meric-Bernstam F, Gonzalez-Angulo AM, et al. Emergence of constitutively active estrogen receptor-α mutations in pretreated advanced estrogen receptor-positive breast cancer. Clin Cancer Res 2014;20:1757–67.
[56] Merenbakh-Lamin K, Ben-Baruch N, Yeheskel A, Dvir A, Soussan-Gutman L, Jeselsohn R, et al. D538G mutation in estrogen receptor-α: a novel mechanism for acquired endocrine resistance in breast cancer. Cancer Res 2013;73:6856–64.
[57] Li S, Shen D, Shao J, Crowder R, Liu W, Prat A, et al. Endocrine-therapy-resistant ESR1 variants revealed by genomic characterization of breast-cancer-derived xenografts. Cell Rep 2013;4:1116–30.
[58] Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. Nat Genet 2013;45:1446–51.
[59] Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, et al. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. Nat Genet 2013;45:1439–45.
[60] Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 1988;48:5183–7.
[61] Gottardis MM, Wagner RJ, Borden EC, Jordan VC. Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro. Cancer Res 1989;49:4765–9.
[62] Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000;6:2028–36.
[63] Gottardis MM, Jiang SY, Jeng MH, Jordan VC. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res 1989;49:4090–3.
[64] Lee ES, Schafer JM, Yao K, England G, O'Regan RM, De Los Reyes A, et al. Cross-resistance of triphenylethylene-type antiestrogens but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000;6:4893–9.
[65] Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. Cancer Cell 2004;5:207–13.
[66] Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 1993;127:23–33.
[67] Jordan VC. The 38th David A. Karnofsky Lecture. The paradoxical actions of estrogen in breast cancer: survival or death? J Clin Oncol 2008;26:3073–82.
[68] Jordan VC, Lewis JS, Osipo C, Cheng D. The apoptotic action of estrogen following exhaustive antihormonal therapy: a new clinical treatment strategy. Breast 2005;14:624–30.
[69] Lønning PE, Taylor PD, Anker G, Iddon J, Wie L, Jørgensen LM, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat 2001;67:111–6.
[70] Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 2009;302:774–80.
[71] Howell A, Dodwell DJ, Anderson H, Redford J. Response after withdrawal of tamoxifen and progestogens in advanced breast cancer. Ann Oncol 1992;3:611–7.
[72] Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 1986;83:2496–500.
[73] Welshons WV, Jordan VC. Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. Eur J Cancer Clin Oncol 1987;23:1935–9.
[74] Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. Cancer Res 1987;47:4355–60.
[75] Masamura S, Santner SJ, Heitjan DF, Santen RJ. Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. J Clin Endocrinol Metab 1995;80:2918–25.
[76] Yue W, Wang JP, Conaway M, Masamura S, Li Y, Santen RJ. Activation of the MAPK pathway enhances sensitivity of MCF-7 breast cancer cells to the mitogenic effect of estradiol. Endocrinology 2002;143:3221–9.
[77] Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 2001;93:714–23.
[78] Haddow A, Watkinson JM, Paterson E, Koller PC. Influence of synthetic oestrogens upon advanced malignant disease. BMJ 1944;23:393–8.
[79] Jordan VC, Liu H, Dardes R. Re: Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 beta-estradiol and the two faces of Janus: sex steroids as mediators of both cell proliferation and cell death. J Natl Cancer Inst 2002;94:1173.
[80] Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 1992;90:77–86.
[81] Pink JJ, Jiang SY, Fritsch M, Jordan VC. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. Cancer Res 1995;55:2583–90.
[82] Lewis-Wambi JS, Kim HR, Wambi C, Patel R, Pyle JR, Klein-Szanto AJ, et al. Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. Breast Cancer Res 2008;10:R104.
[83] Lewis-Wambi JS, Swaby R, Kim H, Jordan VC. Potential of l-buthionine sulfoximine to enhance the apoptotic action of estradiol to reverse acquired antihormonal resistance in metastatic breast cancer. J Steroid Biochem Mol Biol 2009;114:33–9.
[84] Sweeney EE, Fan P, Jordan VC. Mechanisms underlying differential response to estrogen-induced apoptosis in long-term estrogen-deprived breast cancer cells. Int J Oncol 2014;44:1529–38.
[85] Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 2005;94:131–41.
[86] Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 2005;97:1746–59.
[87] Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, et al. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci USA 2011;108:18879–86.
[88] Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 2003;95:1597–608.
[89] Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, et al. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 2003;95:1586–97.
[90] Osipo C, Meeke K, Cheng D, Weichel A, Bertucci A, Liu H, et al. Role for HER2/neu and HER3 in fulvestrant-resistant breast cancer. Int J Oncol 2007;30:509–20.
[91] Obiorah IE, Sengupta S, Curpan R, Jordan VC. Defining the conformation of the estrogen receptor complex that controls estrogen induced apoptosis in breast cancer. Mol Pharmacol 2014;85:789–99.
[92] I Obiorah, Sengupta S, Fan P, Jordan VC. Delayed triggering of oestrogen induced apoptosis that contrasts with rapid paclitaxel-induced breast cancer cell death. Br J Cancer 2014;110:1488–96.
[93] Obiorah I, Jordan VC. Scientific rationale for postmenopause delay in the use of conjugated equine estrogens among postmenopausal women that causes reduction in breast cancer incidence and mortality. Menopause 2013;20:372–82.
[94] Fan P, Griffith OL, Agboke FA, Anur P, Zou X, McDaniel RE, et al. C-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. Cancer Res 2013;73:4510–20.
[95] Fan P, McDaniel RE, Kim HR, Clagett D, Haddad B, Jordan VC. Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines. Eur J Cancer 2012;48:3488–98.
[96] Fan P, Agboke FA, McDaniel RE, Sweeney EE, Zou X, Creswell K, et al. Inhibition of c-Src blocks oestrogen-induced apoptosis and restores oestrogen-stimulated growth in long-term oestrogen-deprived breast cancer cells. Eur J Cancer 2014;50:457–68.
[97] Balaburski GM, Dardes RC, Johnson M, Haddad B, Zhu F, Ross EA, et al. Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. Int J Oncol 2010;37:387–98.
[98] Hutcheson IR, Knowlden JM, Madden TA, Barrow D, Gee JM, Wakeling AE, et al. Oestrogen receptor-mediated modulation of the EGFR/MAPK pathway in tamoxifen-resistant MCF-7 cells. Breast Cancer Res Treat 2003;81:81–93.

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[99] Massarweh S, Osborne CK, Creighton CJ, Qin L, Tsimelzon A, Huang S, et al. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. Cancer Res 2008;68:826–33.
[100] Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2003;2:205–13.
[101] Jordan VC, Obiorah I, Fan P, Kim HR, Ariazi E, Cunliffe H, et al. The St. Gallen Prize Lecture 2011: evolution of long-term adjuvant anti-hormone therapy: consequences and opportunities. Breast 2011;20:S1–S11.
[102] Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 2009;69:1243–54.

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EDITORIAL

Problems With the Progesterone Receptor in Practice?

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The estrogen receptor (ER) has proven to be the best target for the treatment and prevention of breast cancer.[1] The link between ER status and response to endocrine ablation originally was observed in women with metastatic breast cancer[2] long before tamoxifen was first marketed in the United States in 1978. The development of tamoxifen for long-term adjuvant therapy[3] and the evaluation of tamoxifen efficacy in worldwide randomized clinical trials led to a substantial increase in disease-free survival and overall survival, but only in the patients with ER-positive tumors.[4,5] Unfortunately, as with all targets in cancer, not all ER-positive tumors respond, despite the fact that initially, the assay was rigorously quality controlled in cooperative groups.

The solution seemed so easy because estrogen is necessary to induce the progesterone receptor (PgR), and therefore, those patients with ER+/PgR+ breast tumors should be more likely to respond to tamoxifen therapy.[6,7] Extrapolation of these data from the metastatic breast cancer setting to adjuvant therapy has been less rewarding. There was initial promise that PgR status correlated well with disease-free and overall survivorship in stage II breast cancer.[8] However, the Early Breast Cancer Trialists' Collaborative Group Overview analysis of randomized clinical trials has found strong correlation between ER status and response to adjuvant tamoxifen but no further benefit associated with positive PgR status.[4,5]

The development of therapeutic agents targeted specifically to block the aromatase enzyme, thereby creating a "no estrogen state," has introduced a new dimension in breast cancer therapeutics.[9] A multitude of recent clinical studies have compared and contrasted several new aromatase inhibitors to adjuvant tamoxifen.

In this issue of the *Journal*, Goss et al[10] report an analysis of ER/PgR status and breast cancer responsiveness to extended adjuvant antihormonal therapy. Following the successful completion of 5 years of adjuvant tamoxifen treatment, the MA.17 trial evaluated 5 additional years of letrozole compared with a placebo control. Patients with ER+/PgR+ breast tumors constituted 73% of the patient population, whereas patients with ER+/PgR− tumors constituted 12% of the study population. The authors found that patients whose tumors are ER+/PgR+ are more likely to benefit from an additional 5 years of letrozole than are those with ER+/PgR− tumors. Should we be surprised?

The authors contend that their result is controversial in light of the fact that a recent retrospective analysis of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial[11] showed that patients with ER+/PgR− tumors are more likely to benefit from anastrozole than tamoxifen. However, the BIG (Breast International Group) 1-98 trial[12] showed that the PgR status did not influence the magnitude of benefit of letrozole compared with tamoxifen.

Patients with advanced breast cancer have a higher response rate to endocrine therapy if they have ER+/PgR+ tumors, compared with ER+/PgR− tumors.[13,14] There is also a strong inverse relationship between S phase fraction (SPF) and steroid receptor classification. Tumors with both ER and PgR expression have low SPF, and SPF increases significantly with loss of PgR.[15] What is the mechanism? We have known for nearly two decades that enhanced epidermal growth factor signaling reduces PgR levels[16] and these ER+ tumor cells respond less completely to antiestrogen treatment than ER+/PgR+ tumor cells.[17,18] These concepts have recently been confirmed and extended with reference to human epidermal growth factor receptor 2–*neu*[19] and insulin-like growth factor receptor signaling.[20] Laboratory studies also suggest that when drug resistance develops during long-term tamoxifen treatment[21] and treatment is stopped, the undetected nascent tumors will still respond to either estrogen or tamoxifen for growth.[22] These data explain the effectiveness of the letrozole after tamoxifen treatment was stopped in MA.17.

The controversy arises when the sequential adjuvant study MA.17 is compared with adjuvant antihormone treatments that are initiated immediately after surgery. Unlike either the comparative ATAC or BIG 1-98 study populations, the MA.17 study investigates responsiveness in an enriched population after tamoxifen. The enrichment is evidenced by the high proportion of ER+/PgR+ tumors (73%) compared to either the ATAC (62%) or BIG 1-98 studies (63%). In other words, the ER+/PgR− tumors are more likely to recur during tamoxifen treatment. A patient can only be included in MA.17 if tamoxifen therapy is successful.

The controversy really centers on the apparent conflict in outcomes of subgroup analyses between ATAC and BIG 1-98. Based on a small study of neoadjuvant therapy[23] in which aromatase inhibitors performed better than tamoxifen in a growth factor–rich environment, the analysis of the ATAC data according to ER/PgR status[11] supported the hypothesis that PgR− status could be used to select a cohort of patients with ER+ disease who would benefit most from adjuvant aromatase inhibitors. The

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observation in BIG 1-98 that the degree of benefit from letrozole compared with tamoxifen did not differ according to PgR status at this point seemed to be counterintuitive. Central pathology review of ER and PgR in tumor samples for 58% of randomized cases[24] (subsequently increased to 79% [M. Regan, personal communication, January 2007]) confirmed this lack of enhanced effectiveness of letrozole compared with tamoxifen for the ER+/PgR− cohort in BIG 1-98. In fact, the benefit of letrozole compared with tamoxifen was numerically larger for the ER+/PgR+ cohort compared with the ER+/PgR− cohort based on the centrally reviewed receptor values. Furthermore, 2.3% of the BIG 1-98 cases enrolled on the basis of locally determined receptor-positive breast cancer were found to have no expression of either ER or PgR on central review. This small cohort of false-positive receptor cases had a substantially worse disease-free survival compared with the centrally reviewed receptor-positive cohort, and did not benefit from letrozole compared with tamoxifen. Thus, misclassification of true receptor status can influence observed results. Obtaining quality-controlled quantification of ER and PgR values is essential to assure that patients enrolled in endocrine therapy trials have the targeted disease and also that those cared for outside of clinical trials receive proper adjuvant therapy.

Recently, Dowsett and Allred[25] presented time-to-recurrence results according to a centrally reviewed assessment of ER and PgR conducted on 32% of patients enrolled on the monotherapy arms in the ATAC trial. In contrast to the original ATAC report, which was based on data provided on the case report forms,[11] the centrally reviewed ER+/PgR− cohort did not demonstrate a differentially greater benefit of anastrozole. Notably, the centrally reviewed cases were highly selected according to geographic region, with almost 80% of United Kingdom cases included, but with less that 10% of the cases from the United States and other non-United Kingdom centers submitted for central review. It is possible, therefore, that the method of PgR determination in different parts of the world could have influenced the results.

It is also quite reasonable to conclude that the apparent differences in outcome between ATAC and BIG 1-98 reported initially are due primarily to the play of chance. The importance of PgR− status to predict markedly superior response to aromatase inhibitor compared with tamoxifen may have been exaggerated in the original ATAC subgroup analyses. The current report from MA.17 shows that the ER+/PgR+ cohort benefits more from letrozole following tamoxifen than the ER+/PgR− cohort, while BIG 1-98 suggests little difference in the magnitude of the letrozole effect according to PgR status. We fully support the recommendation of Goss et al[10] who, in their study in this issue of the *Journal*, "caution against using these results for clinical decision-making."

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REFERENCES

1. Jensen EV, Jordan VC: The estrogen receptor: A model for molecular medicine—The Dorothy P: Landon AACR Prize for Translational Research. Clin Cancer Res 9:1980-1989, 2003

2. McGuire WL, Carbone PP, Sears ME, et al: Estrogen receptors in human breast cancer: An overview, in: McGuire WL, Carbone PP, Volmer EP (eds): Estrogen Receptor in Human Breast Cancer. Raven Press, New York, NY, 1975, pp 1-7

3. Jordan VC: Tamoxifen: A most unlikely pioneering medicine. Nat Rev Drug Discov 2:205-213, 2003

4. Tamoxifen for early breast cancer: An overview of the randomised trials. Lancet 351:1451-1467, 1998

5. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. Lancet 365:1687-1717, 2005

6. Horwitz KB, McGuire WL, Pearson OH, et al: Predicting response to endocrine therapy in human breast cancer: A hypothesis. Science 189:726-727, 1975

7. Elledge RM, Green S, Pugh R, et al: Estrogen receptor (ER) and progesterone receptor (PgR), by ligand-binding assay compared with ER, PgR and pS2, by immuno-histochemistry in predicting response to tamoxifen in metastatic breast cancer: A Southwest Oncology Group Study. Int J Cancer 89:111-117, 2000

8. McGuire WL, Clark GM, Dressler LG, et al: Role of steroid hormone receptors as prognostic factors in primary breast cancer. NCI Monogr 19-23, 1986

9. Jordan VC, Brodie AMH: Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 72:7-25, 2007

10. Goss PE, Ingle JN, Martino S, et al: Efficacy of letrozole extended adjuvant therapy according to estrogen receptor and progesterone receptor status on the primary tumor. J Clin Oncol 25:2006-2011, 2007

11. Dowsett M, Cuzick J, Wale C, et al: Retrospective analysis of time to recurrence in the ATAC trial according to hormone receptor status: An hypothesis-generating study. J Clin Oncol 23:7512-7517, 2005

12. Thurlimann B, Keshaviah A, Coates AS, et al: A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. N Engl J Med 353:2747-2757, 2005

13. Bloom ND, Robin EH, Schreibman B, et al: The role of progesterone receptor in the management of advanced breast cancer. Cancer 45:2992-2997, 1980

14. Osborne CK, Yochmowitz MG, Knight WA, et al: The value of estrogen and progesterone receptors in the treatment of breast cancer. Cancer 46:2884-2888, 1980

15. Wenger CR, Beardslee S, Owens MA, et al: DNA ploidy, S-phase, and steroid receptors in more than 127,000 breast cancer patients. Breast Cancer Res Treat 28:9-20, 1993

16. Cormier EM, Wolf MF, Jordan VC: Decrease in estradiol-stimulated progesterone receptor production in MCF-7 cells by epidermal growth factor and possible clinical implication for paracrine-regulated breast cancer growth. Cancer Res 49:576-580, 1989

17. Cormier EM, Jordan VC: Contrasting ability of antiestrogens to inhibit MCF-7 growth stimulated by estradiol or epidermal growth factor. Eur J Cancer Clin Oncol 25:57-63, 1989

18. Robinson SP, Jordan VC: The paracrine stimulation of MCF-7 cells by MDA-MB-231 cells: Possible role in antiestrogen failure. Eur J Cancer Clin Oncol 25:493-497, 1989

19. Osborne CK, Bardou V, Hopp TA, et al: Role of the estrogen receptor coactivator AIB1 (SRC3) and HER2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst 95:353-361, 2003

20. Cui X, Zhang P, Deng W, et al: Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: Progesterone receptor as a potential indicator of growth factor activity in breast cancer. Mol Endocrinol 17:575-588, 2003

21. Jordan VC: Selective estrogen receptor modulation: Concept and consequences in cancer. Cancer Cell 5:207-213, 2004

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22. Gottardis MM, Jordan VC: Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 48:5183-8187, 1988

23. Ellis MJ, Coop A, Singh B, et al: Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: Evidence from a Phase III randomized trial. J Clin Oncol 19:3808-3816, 2001

24. Viale G, Regan M, Dell'Orto P, et al: Central review of ER, PgR and HER-2 in BIG 1-98 evaluating letrozole versus tamoxifen as adjuvant endocrine therapy for postmenopausal women with receptor-positive breast cancer. Breast Cancer Res Treat 94:S13, 2005 (suppl 1; abstr 44)

25. Dowsett M, Allred D, on behalf of the TransATAC Investigators: Relationship between quantitative ER and PgR expression and HER2 status with recurrence in the ATAC trial. Breast Cancer Res Treat 100:S21, 2006 (suppl 1; abstr 48)

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Estrogen Receptors in BRCA1-Mutant Breast Cancer: Now You See Them, Now You Don't

V. Craig Jordan

Estrogen receptor (ER) protein is expressed in estrogen target tissues (1,2). The binding of exogenous estrogen to ER orchestrates many important responses throughout a woman's body to maintain the optimal homeostasis for successful reproduction. Without estrogen, there would be no human race. However, estrogen is also involved in the development and growth of breast and endometrial cancers and, as a result, has recently earned a bad reputation in women's health (3,4).

The measurement of ER expression in breast tumors was originally used to identify which women were likely to respond to endocrine ablation therapy (5). Patients whose tumor expressed no ER were unlikely to respond to endocrine ablative surgery, whereas patients whose tumors had a detectable level of ER had improved chances of responding to ablative surgery (6). However, during the early 1970s ER was recognized as a therapeutic target for improving treatment rather than as a predictive test to recommend short-term palliation from endocrine ablative surgery (7,8). The antiestrogen tamoxifen was reinvented from being a failed contraceptive to the first targeted therapy in breast cancer (7,8). This conceptual shift led to the current recognition that the ER is perhaps the most important target identified thus far in cancer medicine. Hundreds of thousands of breast cancer patients' lives have been improved and lengthened with the application of long-term adjuvant tamoxifen therapy (9). Although the aromatase inhibitors are now improving response rates and the side-effect profile of long-term adjuvant therapy in postmenopausal women, tamoxifen remains the antiestrogenic treatment of choice for premenopausal women and those high-risk women who choose to reduce their chances of developing breast cancer (10).

Despite the prominence of the ER as a target in breast cancer, many aspects concerning its origins and its efficacy as a therapeutic target have remained a mystery. Questions about how ER synthesis and regulation are accomplished, whether ER-negative breast cancers are derived from ER-positive breast cancers, and whether ER expression can be regenerated in ER-negative breast cancers have remained central issues in endocrinology and cancer biology for the past 40 years.

In this issue of the Journal, Hosey et al. (11) provide a fascinating insight into these issues by presenting a unifying hypothesis for the regulation of ER synthesis in breast cancer. They approached these questions by integrating prior clinical observations that have shown that BRCA1-mutant breast cancers express little ER compared with spontaneous breast tumors (12) and then deployed breast cancer cell lines, nucleic acid transfection technology, chromatin precipitation assays, and, most importantly, the power of short-interfering RNA technology to knock down expression of BRCA1. They found that BRCA1 is a central player in the regulation of ER synthesis in breast cancer.

Overall, the current success by Hosey et al. (11) in answering the questions about ER regulation is best summarized by a statement taken from the book Trilobite! by Richard Fortey (13): "Central … is the notion of science as a web of knowledge where the apparently peripheral can suddenly become pivotal." Hosey et al. (11) have answered questions that could not have been answered 15 years ago. For example, the identification of the BRCA1 gene (14) and its mutations in familial breast cancer initially appeared to be unrelated to the ER, but the finding that breast tumors occur early during the premenopausal years of a woman's life and may have a hormonal component to their growth

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control (15,16) but, paradoxically, are ER-negative (12) provided a crucial piece of information necessary to solve the riddle of ER regulation. The question then became "what does a BRCA1 mutation have to do with the ER system?"

A connection between BRCA1 expression and ER has already been made by others. For example, Rosen's group (17,18) has demonstrated that the transient transfection of the wild-type BRCA1 gene into MCF-7 breast cancer cells inhibits signaling by the ER complex (17) and that BRCA1 protein interacts directly with ER (18). More recently, Rosen's group has shown that the repression of ER activity by BRCA1 is mediated through phosphatidylinositol-3 kinase signaling (19), which increases ER phosphorylation at serine 167 located in the activating function-1 domain of ER. All of these studies are interesting, but none directly addresses what a BRCA1 mutation has to do with the ER system.

Hosey et al. (11) took a direct approach to this question. They used three breast cancer cell lines: HCC1937 cells (20), which are homozygous for the BRCA1 5382insC mutation (which causes the last 34 amino acids of the BRCA1 protein to be missing) and are essentially ER negative, and the two ER-positive cell lines, MCF-7 (21) and T47D, which have different ER regulatory systems (22). Simply stated, Hosey et al. (11) showed that transfection of the wild-type BRCA1 gene into HCC1937 cells reactivates ER production and that the knockdown of BRCA1 expression with short-interfering RNAs in ER-positive cells eliminates expression of ER. They provide convincing evidence that BRCA1 protein directly regulates the synthesis of ER through binding to the ESR1 promoter and that the ubiquitous transcription factor Oct-1 also plays an important role in the regulation of ER expression. Finally, Hosey et al. (11) demonstrate that knockdown of BRCA1 expression in ER-positive cells abrogates the growth inhibitory response of the cells to the pure antiestrogen drug fulvestrant (23,24). They nicely show that expression of exogenous ER in BRCA1-depleted cells reactivates fulvestrant sensitivity. However, it would have been interesting to examine the effects of BRCA1 expression on the sensitivity of the cells to tamoxifen, a more clinically relevant antiestrogen drug. Fulvestrant is usually used as a second- or third-line antihormone therapy and is not really used to treat premenopausal patients, i.e., patients who tend to carry BRCA1 mutations. The fact that tamoxifen substantially enhances the development of mammary tumors in BRCA1 co/co MMTV-CRE/p53+/− mice and is more estrogen-like in cells with no full-length BRCA1 knockdown (25) suggests that this valuable observation should be pursued because of its clinical relevance.

Despite the large size of BRCA1, many mutations that alter the functions of the BRCA1 protein have been identified across the entire gene. The 5382insC mutation in the HCC1937 cells used by Hosey et al. (11), which is located in the terminal transactivation domain of BRCA1, and the 185delAG mutation are the two most common mutations found in the Ashkenazi Jewish population. Mutations for the BRCA1 gene occur with a combined frequency of about 100× higher in Ashkenazi Jews than in an unselected white population (26,27). Because 185delAG and 5382insC are the most severe mutations (i.e., they are associated with more aggressive, ER-negative breast cancers), the decision by Hosey et al. (11) to study a cell line that has the 5382insC mutation was a wise one. However, it is possible that other mutations in the BRCA1 gene may explain why some BRCA1 mutant breast tumors remain ER positive and actually respond to tamoxifen treatment (16). This possibility would be interesting to test.

On the basis of their results, Hosey et al. (11) developed a plausible model to explain the formation of an ER-negative tumor through 1) the loss of ER expression after the wild-type BRCA1 allele is lost by a mechanism involving loss of heterozygosity and 2) the loss of BRCA1 expression in sporadic tumors by mechanisms involving loss of heterozygosity and epigenetic inactivation. Their model can now be rigorously investigated and validated so that the mystery of ER regulation can be settled once and for all.

In summary, the study by Hosey et al. (11) exemplifies the "notion of science as a web of knowledge where the apparently peripheral can suddenly become central" (13). The results of Hosey et al. (11) provide justifiable optimism that the current technology can be used to solve biologic questions. However, this is only one of the lessons to be learned from the advance made by Hosey et al. (11). The other lessons are that models are needed to solve mechanisms in biology and that there needs to be an integrated approach with different medical disciplines to address current research problems in biology and medicine. The discovery of mutations in the BRCA1 gene was clearly peripheral to the discovery of a plausible mechanism to explain the regulation of ER synthesis. The use of a breast cancer cell line (20) that was derived from a BRCA1 mutation carrier was critical for the demonstration that wild-type BRCA1 plays a role in ER synthesis. Perhaps most importantly, however, it is the financial investment in individual nondirected research that has provided the most powerful tools for investigators to solve problems. For example, Fire et al. (28) and Mello (29) studied the development of *Caenorhabditis elegans*, a transparent worm, and made the unanticipated discovery that a certain form of RNA would silence or interfere with the expression of genes. This discovery created and commercialized short-interfering RNAs for the whole human genome that ultimately allowed Hosey et al. (11) to silence genes selectively. They switched off ER synthesis by silencing the BRCA1 gene in two widely used ER-positive cell lines MCF-7 and T47D. Now you see the ER and now you don't. We do not live simply in interesting times; we live in exciting times.

References

(1) Jensen EV, Jacobson HI. Basic guides to the mechanism of estrogen action. Recent Prog Horm Res 1962;18:387–414.

(2) Toft D, Gorski J. A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc Natl Acad Sci USA 1966; 55:1574–81.

(3) Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. JAMA 2002;288: 321–33.

(4) Chlebowski RT, Hendrix SL, Langer RD, Stefanick ML, Gass M, Lane D, et al. Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative randomized trial. JAMA 2003;289:3243–53.

(5) McGuire WL, Carbone PP, Sears ME, Escher GC. Estrogen receptors in human breast cancer: an overview. In: McGuire WL, Carbone PP, Volmer EP, editors. Estrogen receptor in human breast cancer. New York: Raven Press; 1975. p. 1–7.

(6) Jensen EV, Block GE, Smith S, Kyser K, DeSombre ER. Estrogen receptors and breast cancer response to adrenalectomy. Natl Cancer Inst Monogr 1971;34:55–70.
(7) Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2003;2:205–13.
(8) Jordan VC, Brodie AMH. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007;72:7–25.
(9) Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687–1717.
(10) Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 2005;97:1652–62.
(11) Hosey AM, Gorski JJ, Murray MM, Quinn JE, Chung WY, Stewart GE, et al. Molecular basis for estrogen receptor α deficiency in BRCA1-linked breast cancer. J Natl Cancer Inst 2007;99:1683–94.
(12) Verhoog LC, Brekelmans CT, Seynaeve C, van den Bosch LM, Dahmen G, van Geel AN, et al. Survival and tumour characteristics of breast-cancer patients with germline mutations of BRCA1. Lancet 1998;351:316–21.
(13) Fortey R. Tribolite! Eyewitness to evolution. London: Harper Collins; 2000.
(14) Futreal PA, Liu Q, Shattuck-Eidens D, Cochran C, Harshman K, Tavtigian S, et al. BRCA1 mutations in primary breast and ovarian carcinomas. Science 1994;266:120–2.
(15) Rebbeck TR, Levin AM, Eisen A, Snyder C, Watson P, Cannon-Albright L, et al. Breast cancer risk after bilateral prophylactic oophorectomy in BRCA1 mutation carriers. J Natl Cancer Inst 1999;91:1475–9.
(16) Narod SA, Brunet JS, Ghadirian P, Robson M, Heimdal K, Neuhausen SL, et al. Tamoxifen and risk of contralateral breast cancer in BRCA1 and BRCA2 mutation carriers: a case-control study. Hereditary Breast Cancer Clinical Study Group. Lancet 2000;356:1876–81.
(17) Fan S, Wang JA, Yuan R, Ma Y, Meng Q, Erdos MR, et al. BRCA1 inhibition of estrogen receptor signaling in transfected cells. Science 1999;284:1354–6.
(18) Fan S, Ma YX, Wang C, Yuan RQ, Meng Q, Wang JA, et al. Role of direct interaction of BRCA1 inhibition of estrogen receptor activity. Oncogene 2001;20:77–87.
(19) Ma Y, Hu C, Riegel AT, Fan S, Rosen EM. Growth factor signaling pathways modulate BRCA1 repression of estrogen receptor-*a* activity. Mol Endocrinol 2007;21:1905–23.
(20) Tomlinson GE, Chen TT, Stastny VA, Virmani AK, Spillman MA, Tonk V, et al. Characterization of a breast cancer cell line derived from a germline BRCA1 mutation carrier. Cancer Res 1998;58:3237–42.
(21) Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res 1997;57:3071–8.
(22) Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. Cancer Res 1996;56:2321–30.
(23) Howell A. Pure oestrogen antagonists for the treatment of advanced breast cancer. Endocr Relat Cancer 2006;13:689–706.
(24) Lower EE, Esparaz BT, Garnett SA, Wade JL 3rd. Evaluation of fulvestrant in clinical practice: use of an electronic data registry. Clin Breast Cancer 2007;7:565–9.
(25) Jones LP, Li M, Halama ED, Ma Y, Lubet RA, Grubbs CJ, et al. Promotion of mammary development by tamoxifen in a mouse model of BRCA-1 mutation-related breast cancer. Oncogene 2005;24:3554–62.
(26) Struewing JP, Hartge P, Wacholder S, Baker SM, Berlin M, McAdams M, et al. The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. N Engl J Med 1997;336:1401–8.
(27) Ford D, Easton DF, Peto J. Estimates of the gene frequency of BRCA1 and its contribution to breast and ovarian cancer incidence. Am J Hum Genet 1995;57:1457–62.
(28) Fire A, SiQun X, Montgomery MK, Kostas SA, Driver SE, Mellow CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 1998;391:806–11.
(29) Mello CC. Return to the RNAi world: rethinking gene expression and evolution [Nobel lecture]. Angew Chem Int Ed Engl 2006;46:6985–94.

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The Rise of Raloxifene and the Fall of Invasive Breast Cancer

V. Craig Jordan

Bernard Fisher has recently stated, "A clinical trial is just a mechanism by which to evaluate what you have done in the laboratory" (*Oncology News International*, March 2008). In this issue of the Journal, Grady et al. (1) have analyzed the incidence of invasive breast cancer in a clinical trial of women treated with raloxifene with the intention of reducing their risk of dying from coronary heart disease. To the casual observer, an analysis of this nature would seem to be unusual, if not a bit bizarre, but the fact is that raloxifene is a selective estrogen receptor modulator (SERM) that has estrogen-like activity to reduce low-density lipid (LDL) cholesterol (2) and to reduce the risk of fractures in osteoporosis (3), and antiestrogenic properties to block the growth of breast cancers (4). When the Raloxifene Use for the Heart (RUTH) trial started, raloxifene was approved for the prevention of osteoporosis in high-risk postmenopausal women, and it was known from clinical trials that raloxifene produced a decrease in invasive breast cancer (5). So where did all the ideas come from to examine these qualities of raloxifene, which had previously failed its original application as a breast cancer drug (6)? The answer is the laboratory.

Raloxifene started life in the laboratories at Eli Lilly as Y156758, a nonsteroidal antiestrogen (7) with a high affinity for the estrogen receptor (ER) (8) and a primary application as a treatment for breast cancer. Regrettably, this polyhydroxylated class of drugs has a very short biological half-life (9) and subsequent clinical studies with the drug under the name keoxifene also showed virtually no activity in patients who had failed tamoxifen treatment (10). Further development as a breast cancer therapy was abandoned in the late 1980s. However, at this time, selective ER modulation was recognized (11–13) for "nonsteroidal antiestrogens" (tamoxifen and raloxifene are members of this class) and a new opportunity occurred for clinical development (14). This opportunity was based on the laboratory finding that tamoxifen and keoxifene (aka raloxifene) simultaneously maintained bone density in ovariectomized rats (12) and inhibited rat mammary carcinogenesis (15). These findings rapidly translated into the hypothesis that perhaps one could reduce the risk of breast cancer by treating women with a drug that maintained bone density, thereby reducing the risk of osteoporosis. It was well known that this class of drugs lowered circulating cholesterol in laboratory

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animals; in fact, tamoxifen had a patent as a potential hypocholesteremic agent since the 1960s (16,17). Based on all of these laboratory data, a clinical development strategy was simply stated that was to eventually resurrect and catalyze the rise of raloxifene (18):

> Nevertheless there is a real concern about being able to target the right population [for prevention]. We cannot predict who will develop breast cancer; we can only guess at the probability. Furthermore "high risk" women are, in fact, only a minority of those who will develop breast cancer so any success must be balanced against as yet unknown accumulative toxicities. Is this the end of the possible applications for antiestrogens? Certainly not. We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer.

In the late 1980s and early 1990s, clinical studies were exploring the pharmacology of tamoxifen as a prelude to its use in high-risk women as a potential chemopreventive agent. What was found was that tamoxifen lowered LDL cholesterol in postmenopausal women but did not affect high-density lipid cholesterol (19,20). More importantly, tamoxifen enhanced spinal bone density compared with placebo in a randomized clinical study of postmenopausal women (21). It was about this time that scientists at Eli Lilly confirmed (22) the findings of the earlier laboratory studies that raloxifene had potential for maintaining bone density (12) and also lowered circulating cholesterol. The scene was therefore set to test raloxifene as a SERM to prevent fractures from osteoporosis in the Multiple Outcomes of Raloxifene Evaluation (MORE) trial (1994) and, subsequently, to initiate the RUTH trial (1998). Both trials naturally evaluated the original hypothesis that multiple diseases could potentially be controlled with a SERM, thereby enhancing public health (14,18). It is now clear based on clinical trials data, however, that raloxifene is not effective to reduce the risk of coronary heart disease (23,24). It could be that patients recruited to the RUTH trial have disease that is too far advanced for the modest reductions in LDL cholesterol to have any impact on pathology. The SERM approach may work only in patients who have very early atherosclerotic lesions so that long-term therapy can effectively retard the development of pathology. In the years to come, it may be impossible to answer this question by examining populations of women who are using raloxifene to prevent osteoporosis because of the widespread use of statins to reduce LDL cholesterol.

One interesting aspect of the study of Grady et al. (1) is the 44% reduction in invasive breast cancer, which also comprises a 55% reduction in invasive ER-positive breast cancer. This placebo-controlled study can be compared with the Study of Tamoxifen and Raloxifene (STAR), where raloxifene was noted to be equivalent to tamoxifen at reducing the risk of breast cancer (25). Although the STAR, was not placebo controlled, in Fisher's pioneering placebo-controlled tamoxifen study, the National Surgical Adjuvant Breast and Bowel Project P-1 trial, there was a 50% reduction in invasive breast cancer and a 69% reduction in ER-positive breast cancer (26,27). Overall, these data contrast with the MORE trial, in which there was a 76% decrease in invasive breast cancer and 90% decrease in ER-positive breast cancer. The question is why? One plausible explanation for the greater reduction in invasive breast cancer in the MORE trial than in the RUTH and STAR trials could be the low circulating levels of estradiol in postmenopausal women at risk for osteoporosis compared with those in women in both the RUTH and STAR trials. The polyphenolic compounds related to raloxifene are competitive inhibitors of estrogen action, and it is also known that raloxifene has only a 2% bioavailability, with rapid excretion (28). Once patients become noncompliant about taking raloxifene, there would be no protection for the development of invasive breast cancer. Although the numbers are very small in the study of Grady et al. (1) and the MORE trial (5), raloxifene appears to be poor at controlling the risk of developing noninvasive carcinomas. Indeed, tamoxifen seems to be marginally superior to raloxifene in controlling noninvasive breast cancer in the STAR trial (25).

Overall, clinical evidence is accumulating that the SERMs hold great promise in being able to control multiple diseases (29). This is the good news because, until recently, it was generally believed that hormone replacement therapy was the answer to controlling the development of coronary heart disease and osteoporosis, but at the price of an enhanced risk of invasive breast cancer (30,31). For the future, this is no longer acceptable and the SERMs may be one way of further advancing targeted public health.

References

1. Grady D, Cauley JA, Geiger MJ, et al. Reduced incidence of invasive breast cancer with raloxifene among women at increased coronary risk. *J Natl Cancer Inst.* 2008;100(12):854–861.
2. Walsh BW, Kuller LH, Wild RA, et al. Effects of raloxifene on serum lipids and coagulation factors in healthy postmenopausal women. *JAMA.* 1998; 279(18):1445–1451.
3. Ettinger B, Black DM, Mitlak BH, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators [erratum in *JAMA.* 1999;282(22):2124]. *JAMA.* 1999;282(7):637–645.
4. Gradishar W, Glusman J, Lu Y, Vogel C, Cohen FJ, Sledge GW Jr. Effects of high dose raloxifene in selected patients with advanced breast carcinoma. *Cancer.* 2000;88(9):2047–2053.
5. Cummings SR, Eckert S, Krueger KA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA.* 1999;281(23):2189–2197.
6. Lewis JS, Jordan VC. In Case histories: raloxifene. In: John Taylor, David Triggle, eds. *Comprehensive Medicinal Chemistry II.* Vol. 8. Oxford, UK: Elsevier Limited; 2006:103–121.
7. Jones CD, Jevnikar MG, Pike AJ, et al. Antiestrogens. 2. Structure-activity studies in a series of 3-aroyl-2-arylbenzo[b]thiophene derivatives leading to [6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thien-3-yl] [4-[2-(1-piperidinyl)ethoxy]-phenyl]methanone hydrochloride (LY156758), a remarkably effective estrogen antagonist with only minimal intrinsic estrogenicity. *J Med Chem.* 1984;27(8):1057–1066.
8. Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. *Life Sci.* 1983;32(9):1031–1036.
9. Jordan V, Gosden B. Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. *Endocrinology.* 1983; 113(2):463–468.

10. Buzdar A, Marcus C, Holmes F, Hug V, Hortobagyi G. Phase II evaluation of Ly156758 in metastatic breast cancer. *Oncology.* 1988;45(5):344–345.
11. Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. *Fed Proc.* 1987;46(5):1870–1874.
12. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat.* 1987;10(1):31–35.
13. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res.* 1988;48(4):812–815.
14. Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest.* 1988;6(5):589–595.
15. Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res.* 1987;47(15):4020–4024.
16. Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. *J Reprod Fertil.* 1967;13(1):101–119.
17. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov.* 2003;2(3):205–213.
18. Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res.* 1990;50(14):4177–4189.
19. Love RR, Newcomb PA, Wiebe DA, et al. Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. *J Natl Cancer Inst.* 1990;82(16):1327–1332.
20. Love RR, Wiebe DA, Newcomb PA, et al. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Ann Intern Med.* 1991; 115(11):860–864.
21. Love RR, Mazess RB, Barden HS, et al. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med.* 1992;326(13):852–856.
22. Black LJ, Sato M, Rowley ER, et al. Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. *J Clin Invest.* 1994;93(1):63–69.
23. Early Breast Cancer Trialists Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet.* 2005;365(9472): 1687–1717.
24. Barrett-Connor E, Mosca L, Collins P, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Engl J Med.* 2006;355(2):125–137.
25. Vogel VG, Costantino JP, Wickerham DL, et al. The study of tamoxifen and raloxifene (STAR): report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA.* 2006;295(23): 2727–2741.
26. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst.* 1998;90(18): 1371–1388.
27. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst.* 2005;97(22): 1652–1662.
28. Snyder KR, Sparano N, Malinowski JM. Raloxifene hydrochloride. *Am J Health Syst Pharm.* 2000;57(18):1669–1675.
29. Jordan VC. SERMS: meeting the promise of multifunctional medicines. *J Natl Cancer Inst.* 2007;99(5):350–356.
30. Chlebowski RT, Hendrix SL, Langer RD, et al. Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. *JAMA.* 2003; 289(24):3243–3253.
31. Million Women Study Collaborators. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet.* 2003;362(9382): 419–427.

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Cancer Prevention Research

Paradoxical Clinical Effect of Estrogen on Breast Cancer Risk: A "New" Biology of Estrogen-induced Apoptosis

V. Craig Jordan and Leslie G. Ford

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Cancer Prevention Research

Insight

Paradoxical Clinical Effect of Estrogen on Breast Cancer Risk: A "New" Biology of Estrogen-induced Apoptosis

V. Craig Jordan[1] and Leslie G. Ford[2]

Abstract

Administration of estrogen replacement therapy (ERT) decreases the incidence of breast cancer, as shown in a double-blind, placebo-controlled randomized trial of the *Women's Health Initiative* (WHI) in 10,739 postmenopausal women with a prior hysterectomy. Although paradoxical because estrogen is recognized to stimulate breast cancer growth, laboratory data support a mechanism of estrogen-induced apoptosis under the correct environmental circumstances. Long-term antiestrogen treatment or estrogen deprivation causes the eventual development and evolution of antihormone resistance. Cell populations emerge with a vulnerability, as estrogen is no longer a survival signal but is an apoptotic trigger. The antitumor effect of ERT in estrogen-deprived postmenopausal women is consistent with laboratory models. *Cancer Prev Res; 4(5); 633–7. ©2011 AACR.*

Introduction

It is widely held that estrogen can be carcinogenic in breast tissue (1) and is the "fuel for the fire" to stimulate the growth of estrogen receptor (ER)-positive breast cancer cells (2). This knowledge, supported by an enormous body of laboratory data, provides the conceptual basis for the successful development of antihormonal strategies to treat breast cancer (3). Selective ER modulators (SERMs), for example, tamoxifen, block estrogen-stimulated tumor growth at the ER, and aromatase inhibitors prevent peripheral estrogen synthesis in postmenopausal patients, thereby creating estrogen deprivation to stop tumor growth (3). The successful treatment strategy for breast cancer with SERMs was subsequently translated into reducing the risk of breast cancer in high-risk women. SERMs are available to reduce the incidence of breast cancer in pre- and postmenopausal (tamoxifen) or postmenopausal (raloxifene) women (4–6). As predicted by the mechanism of action of SERMs as anticancer agents, only ER-positive breast cancer is reduced. In practice, preventing estrogen action prevents breast tumor initiation and growth. Paradoxically, the recent analysis of estrogen replacement therapy (ERT) in the *Women's Health Initiative* (WHI) double-blind, placebo-controlled randomized trial in 10,739 postmenopausal women with a prior hysterectomy (ages 50–79; ref. 7) actually showed a decrease in invasive breast cancer, which was sustained for 5 years after ERT was stopped. This result seems to run counter to the perceived wisdom of the role of estrogen in breast carcinogenesis, was significant in women of all ages, and was similar in every age group.

When the WHI was initiated in 1993, their present clinical result of a reduction in breast cancer was unanticipated (7) but is consistent nevertheless with parallel laboratory studies completed over the past 20 years. Estrogen-induced apoptosis is a plausible molecular mechanism to support an antitumor action of physiologic estrogen (8). The key to our understanding of estrogen-induced apoptosis is the finding that breast cancer cell populations adapt to estrogen deprivation, but these populations are dynamic, and resistance to estrogen deprivation evolves over time (5 years). This evolution of resistance to estrogen deprivation causes a reconfiguration of cellular survival pathways, which in turn exposes a vulnerability of breast cancer cell survival. Physiologic estrogen causes apoptosis and does not act as a survival signal (8).

We will weigh the laboratory and clinical evidence to support the proposition that physiologic estrogen can cause apoptosis in breast cancer cells following long-term estrogen deprivation. Our objective is to make a case based on scientific observations to support our proposition that nascent breast cancer cells could have the same apoptotic response to ERT after estrogen deprivation caused by menopause. We will present the evidence in chronological order (Box 1).

Evidence from the Historical Use of Estrogens to Treat Metastatic Breast Cancer

The application of high-dose estrogen therapy for the treatment of metastatic breast cancer was the first use of a chemical therapy to treat any cancer successfully (9). Estrogen therapy became the standard of care to treat metastatic breast cancer in postmenopausal patients until

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BOX 1. Cumulative evidence to support low dose estrogen-induced apoptosis in long-term estrogen-deprived nascent breast cancer?

1. Historical use of estrogens to treat breast cancer.
2. Physiologic estrogen as an antitumor agent in SERM-resistant breast cancer models *in vivo*.
3. Estrogen-induced apoptosis in estrogen-deprived ER-positive cell lines *in vitro*.
4. A current evaluation of estrogen to treat acquired antihormone resistance in metastatic breast cancer.
5. The extrapolation of the concept that physiologic estrogen kills breast cancer cells to adjuvant antihormone therapy.

the introduction of tamoxifen (late 1970s in the United States), a nonsteroidal antiestrogen (10). Tamoxifen became the "gold standard" for the treatment of ER-positive (estrogen stimulated) breast cancer for the next 20 years. Estrogen was all but abandoned as a treatment option, but Ingle and colleagues completed a provocative trial of tamoxifen versus the synthetic estrogen diethylstilbestrol (DES; high-dose) in metastatic breast cancer (11). Responses were equivalent with fewer side effects with tamoxifen, but a re-analysis years later demonstrated that survival was significantly improved with DES (12).

Towards the end of his distinguished career, Professor Sir Alexander Haddow FRS reflected (during the inaugural Karnofsky Memorial Lecture; ref. 13) on the remarkable responses noted with estrogen in some tumors, often when treatment was more than a decade past menopause: "*The extraordinary extent of tumour regression observed in perhaps 1% of post-menopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us.*"

Although laboratory research to address Haddow's estrogen paradox essentially ceased for the next 20 years, at least 1 animal model transplanted with a human breast tumor replicated the antitumor action of high-dose estrogen therapy for breast cancer (14, 15). The question could have been addressed. However, the breakthrough in our understanding of a mechanism for estrogen-induced apoptosis came with a study of continuous long-term SERM treatment in transplantable SERM-resistant breast cancer in athymic mice. As often happens in science, a discovery in an apparently unrelated area becomes the required breakthrough to create transparency in nature.

Physiologic Estrogen Is an Antitumor Agent in SERM-Resistant Breast Cancer *In Vivo*

In the 1980s, the first athymic animal models of tamoxifen-induced antihormone resistance were reported, but the acquired resistance surfaced within 2 years as tamoxifen-stimulated growth (2). This replicated the use of tamoxifen in the treatment of metastatic ER-positive breast cancer but did not explain the astonishing success of 5 years of adjuvant tamoxifen therapy in reducing recurrences by 50% and mortality by 30%. Most important, the gains obtained during therapy are maintained (and mortality further reduced) for the next 15 years. We were missing a vital clue about the evolution of antihormone resistance in micrometastatic breast cancer.

Five years of re-transplantation of tumors into tamoxifen-treated athymic mice revealed a vulnerability in breast cancer that would subsequently be exploited in clinical trial. Physiologic estradiol does not promote tumor growth, but small tumors undergo rapid and complete regression (16). It was suggested (16) that following the cessation of adjuvant tamoxifen, a woman's own estrogen would exert an antitumor action and enhance survivorship. Further studies (17) subsequently demonstrated that following tumor regression with physiologic estradiol, any remaining tumor that re-grows in the estrogen environment is again responsive to tamoxifen as an antitumor agent. Continuing studies demonstrated that the principle of physiological estrogen therapy causing apoptosis in SERM-resistant disease was also true for raloxifene (18, 19). These data provided a scientific rationale for subsequent clinical studies.

Estrogen Induces Apoptosis in Estrogen-deprived ER-positive Breast Cancer Cell Lines

Song and colleagues (20) first showed in cell culture that high concentrations of estrogen could induce cellular apoptosis directly through a FAS/FASL pathway. However, the discovery that physiologic concentrations of estradiol could induce apoptosis (21) in both cell culture and animal models was the advance pertinent to the clinical observation that ERT reduces the incidence of breast cancer in postmenopausal women (7). This is now a consistent experimental observation with new knowledge emerging about the molecular mechanisms of estrogen-induced apoptosis. Figure 1 summarizes much of the current data on molecular mechanisms of estrogen-induced apoptosis, the topic of a forthcoming mini-review in *Cancer Prevention Research* later this year.

Despite the significant body of laboratory data to support the proposition that physiologic estrogen can induce apoptosis in long-term estrogen-deprived breast cancer cells, only the translation to patients tests the veracity of the experimental approach as a conversation with nature and a general principle.

Current Evaluation of Estrogen to Treat Acquired Antihormone Resistance in Metastatic Breast Cancer

Lonning and colleagues (22) studied the efficacy of high dose of DES on the responsiveness of metastatic breast

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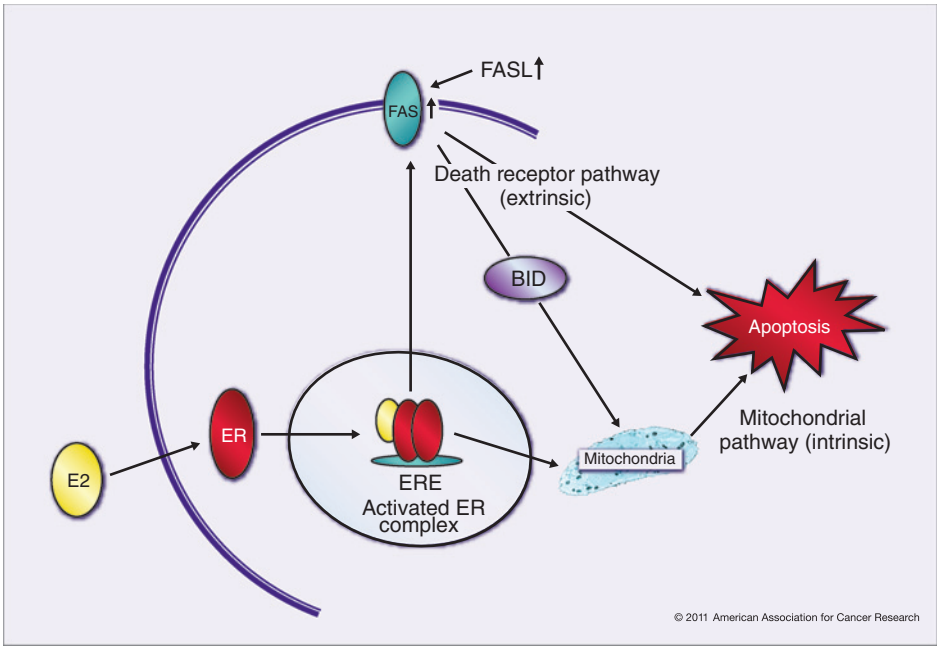

Figure 1. The 2 main pathways involved in estrogen-induced apoptosis regulation. This apoptosis can be triggered either through the extrinsic death-receptor pathway with an increase in Fas ligand (20) or Fas (27) or via the intrinsic pathway of mitochondrial disruption and release of cytochrome *C* (28). E2, estradiol (the most potent estrogen in women); ERE, estrogen response element; BID, Bcl-2–interacting domain.

cancer following exhaustive treatment with antihormone therapies (tamoxifen, aromatase inhibitors, etcetera). A remarkable 4 of 32 patients had complete responses (22), and 1 patient, who was treated for 5 years, had no recurrence of her disease 6 years after stopping DES (23). The question, however, is whether physiologic estrogen has efficacy as an antitumor agent in the appropriately prepared estrogen-deprived breast tumor. Ellis and colleagues (24) addressed this question and found an equivalent clinical benefit for high (30 mg daily) and low (6 mg daily) dose of estradiol in metastatic breast cancer patients who had failed aromatase inhibitor therapy, that is, long-term estrogen deprivation. Their clinical advance was that low-dose estrogen was as efficacious as high-dose estrogen for antitumor therapy in breast cancer (for the appropriate tumor that had been estrogen deprived), but there were fewer side effects with low-dose therapy. The target, estrogen-deprived breast cancer, is vulnerable to physiologic estrogen.

The Extrapolation of the Concept that Physiologic Estrogen Kills Breast Cancer to Adjuvant Antihormone Therapy

The result from the WHI Trial of ERT in hysterectomized women (7), which showed a sustained reduction in the incidence of breast cancer, provides additional evidence that the strategy to decipher the mechanism of physiologic estrogen to induce apoptosis (8, 25, 26) has significance for both treatment and prevention. Indeed, the idea that a woman's own estrogen was responsible for enhanced survivorship by causing apoptosis of the appropriately prepared and vulnerable micrometastases (16) followed the completion of long-term adjuvant tamoxifen therapy and now is incorporated into the Study of Letrozole Extension (SOLE) Trial. This extended adjuvant antihormone treatment study (Fig. 2) is addressing the question of whether regular drug holidays will decrease recurrence rates compared with continuous therapy. For initial safety reasons, a women's own estrogen during the drug holiday is hypothesized to be adequate as an apoptotic trigger because rigorous prior antihormone therapy will have selected vulnerable cell populations as the waiting target. Subsequent trials may have to use ERT for a few weeks to trigger apoptosis.

We have presented an integrated approach to support the proposition that ERT could induce apoptosis and reduce the incidence of breast cancer. The important issue for the decision of breast cancer cells to survive or die in response to estradiol depends entirely on the cell populations present in an estrogenized environment or following estrogen deprivation. Based on laboratory data, the decision is survival or death, respectively. The role of estrogen deprivation, either pharmacologic with antihormones or physiologic with menopause, is to select populations of cells that can survive without physiologic estrogen. These cells choose to die through a natural process when re-exposed to pharmacologic or physiologic estrogen. The genetics are the same, but different epigenetic events based on the well-established property of cancer cells to be able to adapt to any environment and survive remains true. As the WHI study of ERT shows (7), physiologic estrogen has delivered what the scientific database would now predict.

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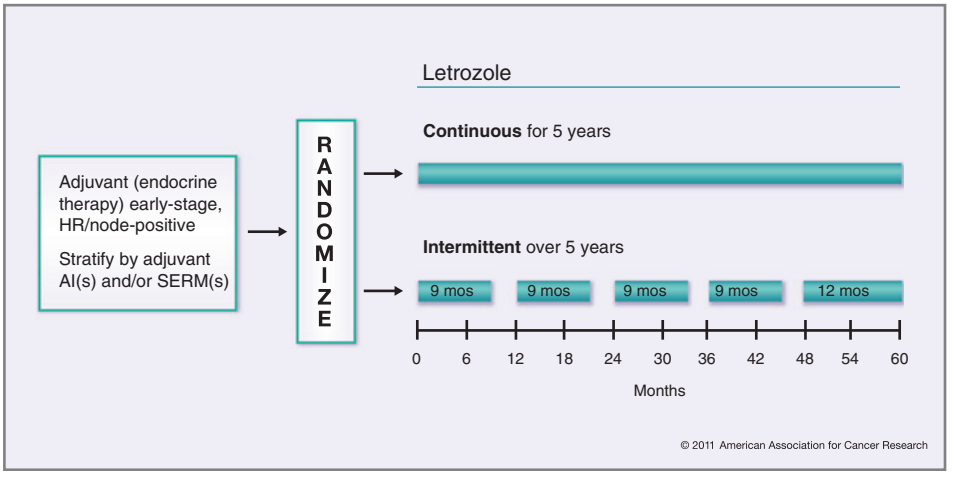

Figure 2. Schema for the Study of Letrozole Extension (SOLE; IBCSG 35-07) conducted by the International Breast Cancer Study Group (IBCSG). Upon completing 4 to 6 years of prior adjuvant endocrine therapy with a SERM(s) and/or aromatase inhibitor(s) (AI), patients were randomly assigned to continuous or intermittent letrozole (3-month drug holidays per year) for 5 years. The rationale for this approach was that the woman's own estrogen in the intermittent arm would trigger apoptosis in long-term estrogen-deprived breast cancer and reduce recurrence rates. Adapted from *International Breast Cancer Study Group* - Study of Letrozole Extension (www.ibcsg.org).

Disclosure of Potential Conflicts of Interest

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References

1. Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. N Engl J Med 2006;354:270–82.
2. Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res 1997;57:3071–8.
3. Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 2009;69:1243–54.
4. Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 2005;97:1652–62.
5. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 2006;295:2727–41.
6. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: preventing breast cancer. Cancer Prev Res 2010;3:696–706.
7. La Croix AZ, Chlebowski RT, Manson JE, Aragaki AK, Johnson KC, Martin L, et al. Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. JAMA 2011;305:1305–14.
8. Jordan VC. The 38th David A. Karnofsky Lecture: the paradoxical actions of estrogen in breast cancer—survival or death? J Clin Oncol 2008;26:3073–82.
9. Haddow A, Watkinson JM, Paterson E, Koller PC. Influence of synthetic oestrogens on advanced malignant disease. Br Med J 1944;2:393–8.
10. Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: Eighth Cain Memorial Award Lecture. Cancer Res 1990;50:4177–89.
11. Ingle JN, Ahmann DL, Green SJ, Edmonson JH, Bisel HF, Kvols LK, et al. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. N Engl J Med 1981;304:16–21.
12. Peethambaram PP, Ingle JN, Suman VJ, Hartmann LC, Loprinzi CL. Randomized trial of diethylstilbestrol vs. tamoxifen in postmenopausal women with metastatic breast cancer. An updated analysis. Breast Cancer Res Treat 1999;54:117–22.
13. Haddow A, David A. Karnofsky memorial lecture. Thoughts on chemical therapy. Cancer 1970;26:737–54.
14. Brunner N, Spang-Thomsen M, Vindelov L, Nielsen A. Effect of 17 beta-oestradiol on growth curves and flow cytometric DNA distribution of two human breast carcinomas grown in nude mice. Br J Cancer 1983;47:641–7.
15. Brunner N, Bastert GB, Poulsen HS, Spang-Thomsen M, Engelholm SA, Vindelov L, et al. Characterization of the T61 human breast carcinoma established in nude mice. Eur J Cancer Clin Oncol 1985;21:833–43.
16. Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 1993;127:23–33.

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17. Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000;6:2028–36.
18. Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, et al. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 2003;95:1586–97.
19. Balaburski GM, Dardes RC, Johnson M, Haddad B, Zhu F, Ross EA, et al. Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. Int J Oncol 2010;37:387–98.
20. Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 2001;93:1714–23.
21. Jordan VC, Liu H, Dardes R. Re: Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 beta-estradiol and the two faces of Janus: sex steroids as mediators of both cell proliferation and cell death. J Natl Cancer Inst 2002;94:1173–5.
22. Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jorgensen LM, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat 2001;67:111–6.
23. Lonning PE. Additive endocrine therapy for advanced breast cancer—back to the future. Acta Oncol 2009;48:1092–101.
24. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 2009;302:774–80.
25. Maximov PY, Lewis-Wambi JS, Jordan VC. The paradox of oestradiol-induced breast cancer cell growth and apoptosis. Curr Signal Transduct Ther 2009;4:88–102.
26. Lewis-Wambi JS, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? Breast Cancer Res 2009;11:206.
27. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 2003;95:1597–608.
28. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 2005;97:1746–59.

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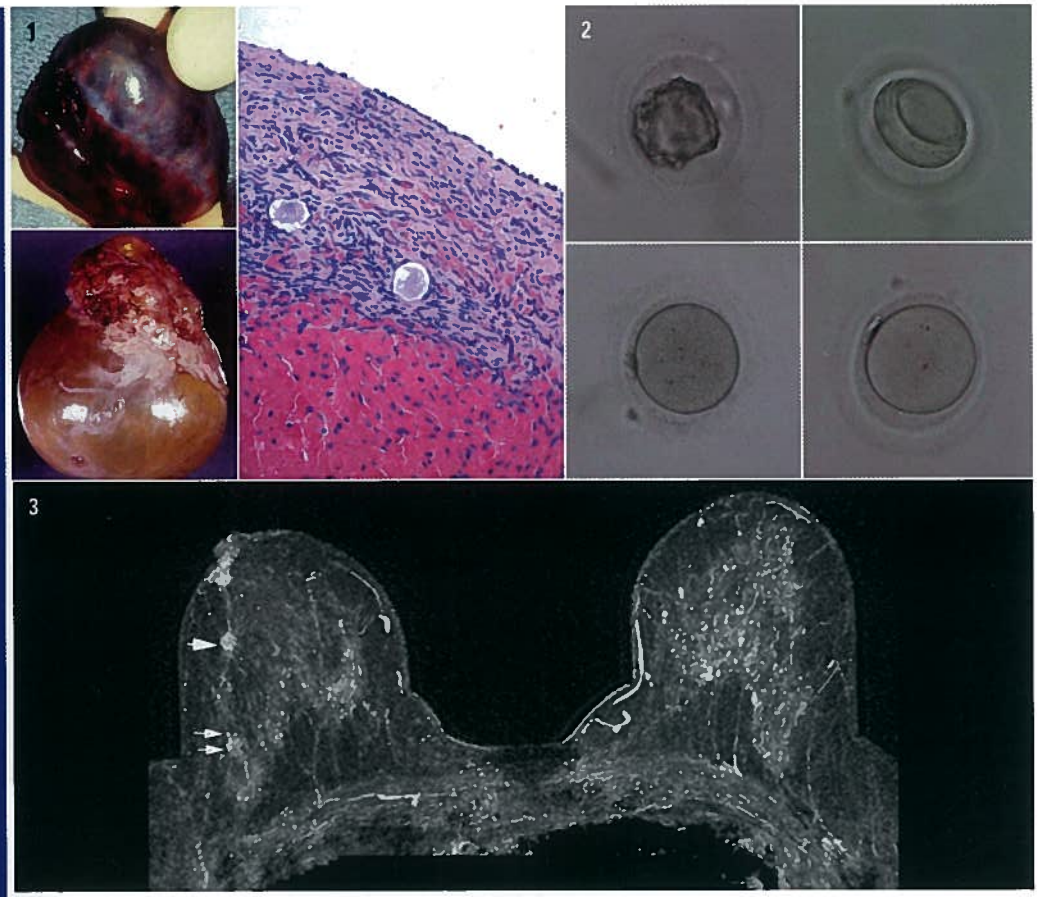

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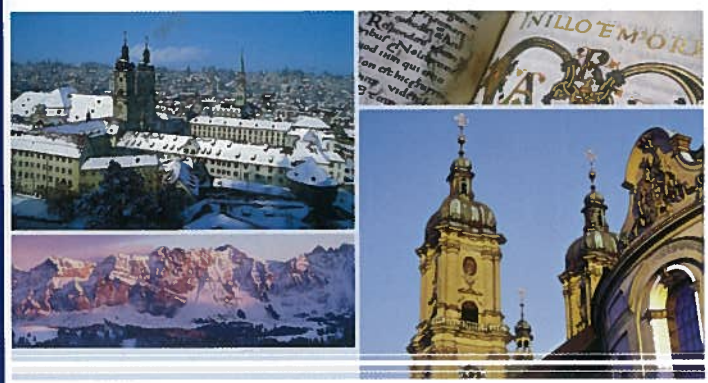

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ÉDITORIAL

Decades of Discovery: The Selective Estrogen Receptor Modulator (SERM) Story: The St. Gallen Prize

"I have but one lamp by which my feet are guided, and that is the lamp of experience. I know no way of judging of the future but by the past."
(Patrick Henry, the First Elected Governor of Virginia, 1775)

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Selective Estrogen Receptor Modulators (SERMs) are a well-established drug group in medicine. The SERMs are also unique, as their mechanisms depend on differentially switching on and switching off target sites around a woman's body – selectively [1]! Tamoxifen is the pioneering SERM [2] and the first medicine to be tested and approved for the reduction of risk of any cancer [3]. The problem to be solved was how to identify and treat the appropriate high risk women to reduce or eliminate their risk of developing breast cancer. The population based models [4] could focus down on a few thousand high risk women, but breast carcinogenesis would only be subverted in a few dozen. These lucky few did not know who they were, amongst the thousands who were treated and who would never get breast cancer. Unfortunately, the strategy to apply the pioneering SERM, tamoxifen, for population based chemoprevention was flawed at the outset, as laboratory and clinical evidence predicted that there was an elevated risk of an increase in endometrial cancer for postmenopausal women [5-6]. This was a slight, but significant risk. Women worried. A range of other side effects (e.g. blood clots, cataracts, menopausal symptoms) would also be experienced by the many to benefit the few. The situation changed dramatically with the discovery that the two "lead" SERMs, tamoxifen and raloxifene, maintained bone density in laboratory animals, but also prevented mammary carcinogenesis [7-8]. Raloxifene was also less uterotrophic than tamoxifen. Would there be no endometrial risk? With the recognition of SERMs in the 1980's, a unique public health strategy was possible.

The new strategy was stated (twice) in the literature, which provided a simple roadmap for the pharmaceutical industry to follow (eventually!). *Are we looking in the wrong place? The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused upon the identification of a population of high-risk women to test "chemopreventive" agents. But are resources being used less than optimally? The problem is much greater than the current horizon. Indeed, even if we had the best chemopreventive for a minority of selected women, the overall impact on the disease might be negligible. An alternative would be to seize upon the developing clues provided by an extensive clinical investigation of available antiestrogens. Could analogs be developed to treat osteoporosis or even retard the development of atherosclerosis? If this proved to be true, then a majority of women in general could be treated for these conditions as soon as menopause occurred. Should the agent also retain antibreast tumor actions, then it might be expected to act as a chemosuppressive on all developing breast cancers if these have an evolution from hormone-dependent to hormone-independent disease. A bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century* [9].

And subsequently: *Is this the end of the possible applications for anti-estrogens? Certainly not! We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer* [10].

Today, new SERMs hold the promise of fulfilling the stated prediction from two decades ago. Lasofoxifene [11] for example, is approved in the E.U. for the prevention of osteoporosis in os-

* See biography page 392.

teopenic women, but at the same time, lasofoxifene reduces the incidence of breast cancer, coronary heart events, strokes and endometrial cancer. Raloxifene, the pioneering SERM to prevent both breast cancer and osteoporosis [12-13] is not as robust in its SERM pharmacology (there is no effect on coronary events or strokes) as lasofoxifene. Remarkably, lasofoxifene is 100 times more potent as a SERM; raloxifene is recommended at 60 mg daily but lasofoxifene is effective at 0.5 mg daily!

With this background of the current success of SERMs, my goal is to guide the reader through an evolution of ideas. History is often written as the achievement of Dynasties. But as with Dynasties, the dogma of the preceding Dynasty in medicine must be overcome, not by sudden force, but by unrelenting pressure and the reason of evidence. Only tenacity can change medicine through ideas as the standard of care is maintained and jealously guarded by the Dynasty.

My early catalytic role in the evolution of our story is well-documented in the refereed literature [2, 14]. Suffice to say as a pharmacologist, I had a passion to develop drugs to treat cancer. But where to start? By a series of accidents, I met the right people at the right time, but the career choice to study the pharmacology of nonsteroidal antiestrogen for my Ph.D. was then seen as a dead end. They were failed contraceptives and of only academic interest. But this was the point of a Ph.D. in Britain – training in research method with a Medical Research Scholarship. Thus, we enter the first of our 4 decades.

The 1970's: The re-invention of tamoxifen as the "gold standard" for the treatment and prevention of breast cancer

The Dynasty to be defeated in the opening years of the 1970's was combination cytotoxic chemotherapy. Chemotherapy was king, fresh from the victory over childhood leukemia and poised to "MOPP" up Hodgkin's Disease. It was reasoned by the Dynasty, if only the right combination of agents could be found in the lexicon of options, a cure was assured. No one was advocating antihormone (or as it was described, "hormone therapy"!) research and treatment.

I saw an opportunity to develop a failed contraceptive, ICI 46,474, further than was believed originally it could go. In 1972, ICI 46,474 was abandoned by the pharmaceutical industry for continuing clinical testing because there was no profit to be made. Nevertheless, the meeting between me and the Head of the Fertility Control Program, Arthur Walpole (or "Walop" as he was affectionately known), proved to be critical to our story. He examined my Ph.D. at Leeds University, but ensured I had the resources at the Worcester Foundation in Shrewsbury, MA, USA and Leeds University, to create a clinical strategy for this orphan drug. He ensured it was put on the market, now tell us how to use it! The strategy I conceived and implemented is in Fig. 1.

The strategy was based on 3 principles:

1) target the tumor ER with tamoxifen;

2) give long term adjuvant tamoxifen therapy;

3) plan for chemoprevention.

All these principles were unpopular at the outset, but persistence and hundreds of evidence-based lectures around the world to my clinical colleagues slowly defeated the Dynasty of combination cytotoxic chemotherapy to cure breast cancer. "Antihormone therapy" became the treatment of choice with long term adjuvant tamoxifen therapy targeted to the tumor ER (the first targeted therapy).

How bad was the first Decade of Discovery? If I may be so bold at this point to tell a story of my friend and colleague, Steven E. Jones, M.D. When I started my international journey to advocate my principles for adjuvant antihormone therapy, Steven was in Arizona, the co-Director of the Adjuvant Therapy of Cancer Meeting in the 1970's. I was setting up a Ludwig Institute in Bern, Switzerland and was invited to present my new ideas about the use of tamoxifen at their 1979 meeting. There I was, sandwiched between the greats of cyotoxic chemotherapy, Vince DeVita and Bernie Fisher. I, in contrast, was advocating a stealth attack on breast cancer with tamoxifen that by comparison had no side effects. Little hope, one would think, but the plan succeeded. Two decades later, Steven Jones rose at a meeting in Washington and started his talk by declaring, "Craig Jordan was correct." Through the clinical trials mechanism, it is now proven that long term (5 years) adjuvant tamoxifen treatment targeted to the tumor ER has enhanced the survival of millions of women worldwide [15]. An orphan drug that is cheap and easy to administer has and continues to save hundreds of thousands of lives annually.

Now our story changes to the second decade with the "new" fashion in oncology – chemoprevention.

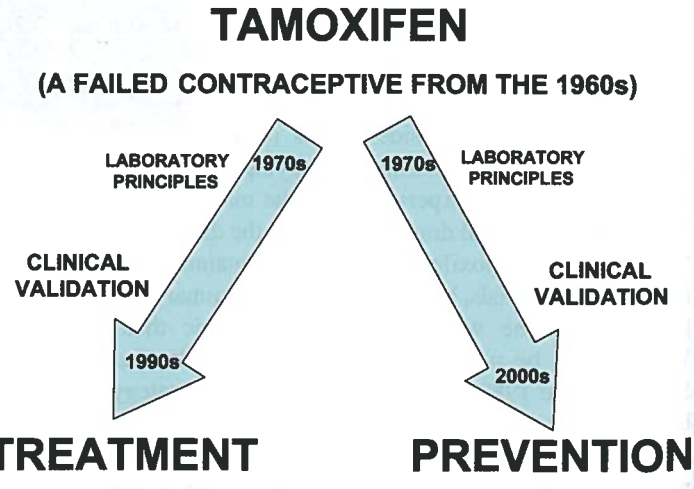

Fig. 1. The translational development of tamoxifen for breast cancer. Tamoxifen, was originally a failed oral contraceptive, ICI 46,474, that was abandoned by the pharmaceutical industry in 1972. The successful development of tamoxifen required three key components: 1) target the tumor ER with tamoxifen 2) give long term adjuvant tamoxifen therapy 3) plan for chemoprevention. As a result of targeting the tumor ER with tamoxifen, antihormone therapy became the treatment of choice for long term adjuvant tamoxifen therapy. This has been successfully validated through clinical trials [15] and in turn has saved the lives of millions of women around the world.

Table 1. Decades of Discovery

- The 1970's: The re-invention of tamoxifen as the "gold standard" for the treatment and prevention of breast cancer.
- The 1980's: SERMs surface.
- The 1990's: Raloxifene's Promise is a Reality.
- The 2000's: Estrogen-induced apoptosis?

The 1980's: SERMs surface

The idea of preventing cancer became popular in the 1980's. This is a noble goal and one of the primary goals of cancer research, but the goal has proved hard to address. The idea as applied to breast cancer has its origin with the French Scientist, Professor Antoine Lacassagne, who stated, at his lecture at the Annual Meeting of the American Association for Cancer Research in Boston (1936): *"If one accepts the consideration of adenocarcinoma of the breast as a consequence of a special hereditary sensibility to the proliferative action of oestrone, one is led to imagine a therapeutic preventive for subjects predisposed by their heredity to this cancer..."* [16]. However, Lacassagne's evidence was based on oophorectomy of mice from strains that develop a high incidence of mammary cancer and there were no mechanisms or compounds to advance and address the question. This would have to wait another quarter century with the serendipitous discovery of the nonsteroidal antiestrogens [10].

Tamoxifen was advanced for testing as a potential chemopreventive for breast cancer in the early 1980's based on three facts:

1) There was laboratory evidence that tamoxifen would prevent rat mammary carcinogenesis [17-19].

2) Tamoxifen was becoming widely used in medicine to treat breast cancer so, it was argued that side effects were known and anticipated. This was not really true, as it took translational research [5] to draw the attention of the clinical community of the small risk of endometrial cancer [6].

3) Tamoxifen, when used as an adjuvant therapy reduced the incidence of contralateral breast cancer [20].

Nevertheless, there was a major toxicological (and ethical issue) with treating well women with a drug classified as a "nonsteroidal antiestrogen" [21]. If, as was believed at the time, estrogen was good to build bone and to reduce the risk of coronary heart disease, what would be the value of the chemoprevention strategy that prevents breast cancer but condemns women to an elevated risk of crushing osteoporosis or fatal coronary heart disease. To address the concern, laboratory studies were initiated to evaluate the pharmacology of tamoxifen on estrogen target tissues.

Studies in rats demonstrated that both tamoxifen and the failed and discontinued breast cancer drug, raloxifene (then known as keoxifene) [22], both maintained bone density in ovariectomized rats [7] and prevented rat mammary carcinogenesis [8]. However, raloxifene was not as effective as tamoxifen, probably because of poor pharmacodynamics, i.e. raloxifene does not accumulate, is rapidly excreted and there is only 2% bioavailability by the oral route of administration [23]. This pharmacological fact was to recur clinically following clinical trials 20 years later (see next section).

A pattern was emerging in the mid 1980's concerning the pharmacology of the nonsteroidal antiestrogens clomiphene, tamoxifen and raloxifene. The facts that lead to the SERM concept being described in my laboratory can now be summarized.

1) Clomiphene, a mixture of estrogenic *cis* and antiestrogenic *trans* geometric isomers, has bone preserving properties in the ovariectomized rat [24]. Clomiphene had been tested as a breast cancer drug in patients [25], but the manufacturer declined to advance development based on potential problems with cholesterol metabolism and a concern about cataracts. The drug remained the gold standard for the induction of ovulation where only give day courses were given [26].

2) The fact that clomiphene was an impure mixture of estrogenic and antiestrogenic isomers made the bone preserving effects uncertain. The estrogenic isomer might have been the favored pharmacologic agent at bone. In contrast, tamoxifen is the pure antiestrogenic trans isomer that preserves bone [7] and raloxifene is a fixed ring structure that is exclusively antiestrogenic (very weakly estrogenic) in the uterus, but estrogenic in bone [7].

3) Both tamoxifen and raloxifene are antitumor agents in rat mammary carcinogenesis [8].

4) Tamoxifen stimulates endometrial cancer growth (and mouse uterine growth) but blocks estradiol-stimulated growth of breast cancer transplanted in the same immune deficient animal [5, 27]. This experiment demonstrates target site specificity.

5) Tamoxifen lowers circulating cholesterol in the rat [28] and this property was included in the initial patent application which read: *"The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolaemic activity"* [2].

The claims as a breast cancer drug were denied and required to be omitted in the United States until eventually a patent was awarded in 1985 in the Court of Appeals. In other words, tamoxifen was tested and marketed in America initially without patent protection for a dozen years. But, nobody cared, as there was little possibility of success, either as a therapy or commercially (or so everybody thought!).

Thus, based on all these data, primarily from my laboratory, the SERM concept surfaced and the roadmap for clinical development started as noted previously [9-10]. These data were the scientific basis of the Wisconsin Tamoxifen Study initiated in the late 1980's to evaluate the pharmacology of tamoxifen on bone density and circulating cholesterol. It was the proven clinical translation of the tamoxifen (SERM) concept to preserve bone density [29] and lower circulating cholesterol [30-31] that awakened the sleeping pharmaceutical industry to develop raloxifene to prevent and treat osteoporosis in postmenopausal women. This started with the "magical" patenting in 1992 of raloxifene for this indication [22] and the publication of laboratory studies confirming my work on the SERM actions of raloxifene in rats [32].

The 1990's: Raloxifene's Promise is a Reality

During the 1990's, I transitioned from my focus on laboratory investigations with SERMs to a role of "scientific resource" for major clinical trials. I was invited by Eli Lilly to chair their Oncology Advisory Committee, which had responsibilities to adjudicate breast cancer detection in their initial osteoporosis trial, Multiple Outcomes with Raloxifene Evaluation (MORE). Subsequently, Dr. Norman Wolmark would invite me to be the scientific chair of the largest breast chemoprevention study – the Study of Tamoxifen and Raloxifene (STAR).

The MORE trial recruited 7,705 postmenopausal women with osteoporosis to be randomized to placebo, 60 or 120 mg raloxifene daily. Raloxifene reduced fractures of the spine by 40% over the initial 3 year evaluation period [12]. In our parallel evaluation of the incidence of breast cancer, there was a significant decrease in the incidence of ER-positive breast cancer by 70% with no increase in endometrial cancer [12]. The laboratory concept of SERMs [10] translated to the clinic. Women being treated for osteoporosis would develop less breast cancer if they took raloxifene. But here was an important pharmacological point – it was proved that they must keep taking raloxifene to obtain benefit. This laboratory principle noted with rapidly excreted SERMs in the 1980's [8, 18] was to emerge as a clinical fact from the STAR trial after treatment stopped (see later).

What happened to tamoxifen in chemoprevention, Professor Trevor Powles initiated the first pilot toxicity study of tamoxifen in high risk women in the early 1980's [33], but it was Dr. Bernard Fisher who successfully conducted the first randomized placebo controlled clinical chemoprevention trial of tamoxifen in women at high risk for breast cancer. All preclinical predictors were confirmed-tamoxifen reduced the incidence of breast cancer, increased the incidence of endometrial in postmenopausal women and there was a decrease, though not significant in fracture rate [3, 34]. Unanticipated information (though prior clinical studies suggested an effect) was an increase in operations for cataracts. The other fact consistent with the overview analysis of clinical trial for adjuvant therapeutic tamoxifen [15] was that tamoxifen alone caused a long term beneficial effect to suppress the development of breast cancer more than a decade after tamoxifen therapy stopped [35]. We will return to the science behind this observation later. The fact, as we noted, that tamoxifen increased endometrial cancer in postmenopausal women, now caused a turn to raloxifene, that had no increased endometrial cancer in MORE [12].

The STAR Trial pitted tamoxifen 20 mg daily against raloxifene 60 mg daily for 5 years to compare and contrast efficacy and side effects for the reduction of breast cancer incidence in high risk postmenopausal women. As an aside, I was often asked how I would feel if raloxifene was found to be superior to tamoxifen. Happy – as the science of both drugs came from my laboratory and both drugs had to be reinvented as useful medicine after being essentially discarded by industry: tamoxifen, a failed contraceptive and raloxifene, a failed breast cancer drug. The first analysis of the STAR Trial showed equivalent efficacy to reduce the incidence of breast cancer by 50% [13]. However, side effects were reduced with raloxifene. In particular, there was less endometrial hyperplasia with raloxifene and fewer hysterectomies. Operations for cataracts were lower on raloxifene. This analysis was conducted during raloxifene therapy [13], but the subsequent analysis conducted after therapy had stopped [36] demonstrated tamoxifen had a sustained antitumor action whereas there was a reduced (75%) efficacy for raloxifene. The drugs were different with their pharmacology and raloxifene must be given indefinitely.

We conclude that the fact that raloxifene is a drug with low bioavailability and therefore the pharmacodynamics to concentrate at the target site – the effect on the breast tissue is reduced. If sustained, local concentrations of tamoxifen and raloxifene are different and the elevated concentrations of tamoxifen drive cell population to evolve differently than those exposed to low levels of raloxifene, there will be, therefore, consequences for tumorigenesis and the evolution of drug resistance. We hypothesize that the low levels of raloxifene remain therapeutically "antiestrogenic" for the duration of therapy, but the endogenous estrogen from the woman's own body causes nascent tumor regrowth.

In contrast, the sustained high concentrations of tamoxifen locally in the breast causes a change in the evolution of the breast cancer cell population that in some way leaves an "antitumor memory" for years after therapy stops – but how? This leads us to the final decade of discovery: estrogen-induced apoptosis.

The 2000's: Estrogen-induced apoptosis?

The first chemical therapy to treat any cancer successfully was the use of high dose estrogen therapy to treat metastatic breast cancer in postmenopausal patients [37]. High dose estrogen therapy became the standard of care until the introduction of tamoxifen in the 1970's [10, 38]. At the end of his career, Sir Alexander Haddow FRS, presented the inaugural Karnofsky Lecture, where he expressed his disappointment about the lack of progress in understanding mechanisms: *"...the extraordinary extent of tumour regression observed in perhaps 1% of post-menopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us..."* [39]. What was known was that the high dose estrogen therapy was more effective as a breast cancer treatment the further away the patient was from the menopause, but why?

The advance in our understanding was to await an examination of model systems in the laboratory to decipher the mechanisms of antihormone drug resistance (Fig. 2). The whole topic has recently been summarized [40], but the facts must be stated to illustrate how transparency in nature can occur through unanticipated results in another area of research.

The first transplantable model of resistance to long term tamoxifen therapy demonstrated unique qualities. Acquired resistance is evidenced by tamoxifen-stimulated (actually SERM-stimulated as it turns out) growth. Tumors grow because of tamoxifen, not in spite of tamoxifen, as occurs with all other anticancer agents. What was even more surprising was the fact that when tamoxifen treatment is stopped, then estrogen again can

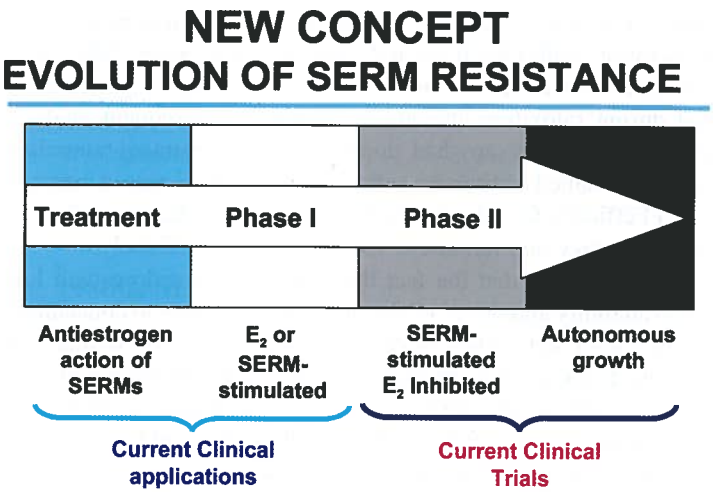

Fig. 2. The evolution of drug resistance to SERMs. Acquired resistance occurs during long-term treatment with a SERM and is evidenced by SERM-stimulated breast tumor growth. Tumors also continue to exploit estrogen for growth when the SERM is stopped, so a dual signal transduction process develops. The pure antiestrogen, fulvestrant, destroys the ER and prevents tumor growth in SERM-resistant disease. This phase of drug resistance is referred to as Phase I resistance. Continued exposure to a SERM results in continued SERM-stimulated growth (Phase II), but eventually autonomous growth occurs that is unresponsive to fulvestrant or aromatase inhibitors. The event that distinguishes Phase I from Phase II acquired resistance is a remarkable switching mechanism that now causes apoptosis, rather than growth, with physiologic levels of estrogen. These distinct phases of laboratory drug resistance have their clinical parallels and this new knowledge is being integrated into the treatment plan.

stimulate growth. This model replicates tamoxifen resistance during the treatment of ER-positive metastatic breast cancer: resistance occurs within a couple of years, estrogen or tamoxifen is required for continued growth and estrogen withdrawal or fulvestrant (the pure antiestrogen that causes destruction of the ER) is an appropriate second line therapy. What the model of acquired resistance did not do was explain how it was possible to use 5 years of adjuvant tamoxifen therapy to treat patients selectively. If the laboratory model was correct, and had been available at the time long term adjuvant therapy was planned as a treatment strategy, then no one would consider treatment longer than a year for adjuvant antihormone therapy. It would obviously be dangerous for patients. The same argument was used in the 1970's by the clinical community. Tamoxifen cannot control metastatic breast cancer on average, more than 2 years, so one cannot give long term (greater than 5 years) adjuvant tamoxifen. We were missing something fundamental about the biology of micrometastatic breast cancer exposed to long term tamoxifen therapy.

The breakthrough in understanding came through serendipity and as always, with outstanding graduate students with exceptional laboratory skills. The model of acquired resistance to tamoxifen could only only be maintained by serial transplantation in successive generations of tamoxifen-treated athymic mice. We were unable to transfer the tumors to cell culture for study, so the expense of preserving the only naturally developed model of resistance to tamoxifen had to be born. That, as it turned out, was the good, new and an opportunity for future discovery.

The acquired drug resistance to tamoxifen evolves in an environment of tamoxifen (Fig. 2). Retransplantation of tumor into further tamoxifen treated mice causes adapted cell populations to develop rather than rely entirely on tamoxifen for growth (Phase I), but as the survival networks become reconfigured, a vulnerability emerges (Phase II). After 5 years of exposure to tamoxifen, the resulting tumor no longer sees estrogen as a survival signal, but as an apoptotic trigger (Phase II).

In 1992, these data were presented for the first time at the St. Gallen Breast Cancer Conference [41]. The hypothesis advanced was that the termination of tamoxifen, at the correct time, was important for the woman's own estrogen to destroy the microfoci of appropriately prepared target cells. This new biology of physiologic estrogen causing apoptosis was the reason for the enhanced survivorship of patients treated with a full 5 years of tamoxifen. As a result, a shift in thinking occurred and the clinical trials community subsequently exploited the concept, now published in the refereed literature [42], that therapeutic estrogen or indeed "physiologic estrogen" in the form of low dose estrogen replacement therapy (ERT) could cause the correctly configured tumors with acquired antihormone resistance to regress [43-44]. The Estrogen Dynasty originally deposed, struck back. Nature answered as well. Today, there is much interest in the paradoxical actions of physiologic estradiol in breast cancer [45-46]. Recent results from the Women's Health Initiative demonstrate a reduction in the incidence of breast cancer for hysterectomized, postmenopausal women who take long term estrogen replacement therapy [47]. Like our tamoxifen story, the effects persist for years following stopping ERT [48]. Practical advances, not only in the therapy of cancer, but preemptively in "natural" chemoprevention may result from these findings in the future.

In summary, I have mapped out the Decades of Discovery that emerged from a single quest some 40 years ago – to develop a drug useful for the treatment and prevention of breast cancer. At the time, I could count on the fingers of one hand, the people who were interested in the quest. No one cared, and it was not going to happen. But science is not like that; as in politics, ideas have their time but it is really about people and a passion to keep the flame of truth alight. I am immensely grateful to Professor Hans-Jörg Senn and his Committee for selecting me to receive the St. Gallen Prize for Advances in Breast Cancer Research. Thanks also go to my friends and colleagues Aron Goldhirsch and Richard Gelber. We all started our personal journeys together in Bern, Switzerland in the late 1970's and we remain older friends and colleagues to this day. Most importantly, I thank the 40 years of "Tamoxifen Teams" that worked and trained with me in my laboratories in Leeds University (UK), WFEB (US), Ludwig Institute for Cancer Research, Bern (Switzerland), University of Wisconsin (Madison), Northwestern University (Chicago), Fox Chase Cancer Center (Philadelphia) and the Lombardi Comprehensive Cancer Center (Washington, DC). I had the privilege to guide their lives and they turned the ideas we conceived into lives saved around the world.

The quotation in the heading of this Editorial was the one I used to open my Prize Lecture in St. Gallen, 16 March 2011: *"I have but one lamp by which my feet are guided, and that is the*

lamp of experience. I know no way of judging of the future but by the past" (Patrick Henry, the First Elected Governor of Virginia, 1775). My lamp was tamoxifen. However, this journey, as I hope I have illustrated, is so much more than the successful development of tamoxifen for the adjuvant treatment of breast cancer. It is about a way of constructing a conversation with nature with the goal of defeating a powerful enemy within us – cancer.

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Références

1. Jordan VC (2001) Selective estrogen receptor modulation: a personal perspective. Cancer Res 61: 5683-7
2. Jordan VC (2003) Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2: 205-13
3. Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90: 1371-88
4. Gail MH, Brinton LA, Byar DP, Corle DK, Green SB, Schairer C, Mulvihill JJ (1989) Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. J Natl Cancer Inst 81: 1879-86
5. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48: 812-5
6. Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, Skoog L, Somell A, Theve T, Wilking N, et al. (1989) Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1: 117-20
7. Jordan VC, Phelps E, Lindgren JU (1987) Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10: 31-5
8. Gottardis MM, Jordan VC (1987) Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 47: 4020-4
9. Jordan VC (1988) Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 6: 589-95
10. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50: 4177-89
11. Cummings SR, Ensrud K, Delmas PD, LaCroix AZ, Vukicevic S, Reid DM, Goldstein S, Sriram U, Lee A, Thompson J, Armstrong RA, Thompson DD, Powles T, Zanchetta J, Kendler D, Neven P, Eastell R (2010) Lasofoxifene in postmenopausal women with osteoporosis. N Engl J Med 362: 686-96
12. Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, Norton L, Nickelsen T, Bjarnason NH, Morrow M, Lippman ME, Black D, Glusman JE, Costa A, Jordan VC (1999) The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA 281: 2189-97
13. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER, Jr., Wade JL, 3rd, Robidoux A, Margolese RG, James J, Lippman SM, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N (2006) Effects of tamoxifen *vs* raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 295: 2727-41
14. Jordan VC (2006) Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 147 (Suppl 1): S269-76
15. EBCTCG (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 365: 1687-717
16. Lacassagne A (1936) Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer 27: 217-25
17. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12: 419-24
18. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16: 239-51
19. Jordan VC, Allen KE, Dix CJ (1980) Pharmacology of tamoxifen in laboratory animals. Cancer Treat Rep 64: 745-59
20. Cuzick J, Baum M (1985) Tamoxifen and contralateral breast cancer. Lancet 2: 282
21. Jordan VC (1984) Biochemical pharmacology of antiestrogen action. Pharmacol Rev 36: 245-76
22. Lewis JS, Jordan VC (2006) Case Histories: Raloxifene. Comprehensive Medicinal Chemistry II 8: 103-21
23. Snyder KR, Sparano N, Malinowski JM (2000) Raloxifene hydrochloride. Am J Health Syst Pharm 57: 1669-75; quiz 76-8
24. Beall PT, Misra LK, Young RL, Spjut HJ, Evans HJ, LeBlanc A (1984) Clomiphene protects against osteoporosis in the mature ovariectomized rat. Calcif Tissue Int 36: 123-5
25. Herbst AL, Griffiths CT, Kistner RW (1964) Clomiphene Citrate (Nsc-35770) in Disseminated Mammary Carcinoma. Cancer Chemother Rep 43: 39-41
26. ASRM PC (2006) Use of clomiphene citrate in women. Fertil Steril 86: S187-93
27. Jordan VC, Robinson SP (1987) Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc 46: 1870-4
28. Harper MJ, Walpole AL (1967) A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 13: 101-19
29. Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, Carbone PP, DeMets DL (1992) Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 326: 852-6
30. Love RR, Wiebe DA, Newcomb PA, Cameron L, Leventhal H, Jordan VC, Feyzi J, DeMets DL (1991) Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann Intern Med 115: 860-4
31. Love RR, Newcomb PA, Wiebe DA, Surawicz TS, Jordan VC, Carbone PP, DeMets DL (1990) Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 82: 1327-32
32. Black LJ, Sato M, Rowley ER, Magee DE, Bekele A, Williams DC, Cullinan GJ, Bendele R, Kauffman RF, Bensch WR, et al. (1994) Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 93: 63-9
33. Powles TJ, Hardy JR, Ashley SE, Farrington GM, Cosgrove D, Davey JB, Dowsett M, McKinna JA, Nash AG, Sinnett HD, et al. (1989) A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. Br J Cancer 60: 126-31
34. Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, Atkins JN, Margolese RG, Runowicz CD, James JM, Ford LG, Wolmark N (2005) Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97: 1652-62

35. Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M (2007) Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. J Natl Cancer Inst 99: 283-90
36. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER, Wade JL, 3rd, Robidoux A, Margolese RG, James J, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N (2010) Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. Cancer Prev Res (Phila) 3: 696-706
37. Haddow A, Watkinson JM, Paterson E, Koller PC (1944) Influence of Synthetic Oestrogens on Advanced Malignant Disease. Br Med J 2: 393-8
38. Jordan VC (2009) A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 69: 1243-54
39. Haddow A (1970) Thoughts on Chemical Therapy. Cancer 26: 737-54
40. Jordan VC, Ford LG (2011) Paradoxical Clinical Effect of Estrogen on Breast Cancer Risk: A "New" Biology of Estrogen-Induced Apoptosis. Cancer Prev Res (Phila): Epub (Ahead of print)
41. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127: 23-33
42. Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, Jordan VC (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6: 2028-36
43. Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jorgensen LM, Mella O, Howell A (2001) High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat 67: 111-6
44. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A, Jamalabadi-Majidi S, Crowder R, Siegel BA (2009) Lower-dose *vs* high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 302: 774-80
45. Lewis-Wambi JS, Jordan VC (2009) Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? Breast Cancer Res 11: 206
46. Maximov PY, Lewis-Wambi JS, Jordan VC (2009) The Paradox of Oestradiol-Induced Breast Cancer Cell Growth and Apoptosis. Curr Signal Transduct Ther 4: 88-102
47. Stefanick ML, Anderson GL, Margolis KL, Hendrix SL, Rodabough RJ, Paskett ED, Lane DS, Hubbell FA, Assaf AR, Sarto GE, Schenken RS, Yasmeen S, Lessin L, Chlebowski RT (2006) Effects of conjugated equine estrogens on breast cancer and mammography screening in postmenopausal women with hysterectomy. JAMA 295: 1647-57
48. LaCroix AZ, Chlebowski RT, Manson JE, Aragaki AK, Johnson KC, Martin L, Margolis KL, Stefanick ML, Brzyski R, Curb JD, Howard BV, Lewis CE, Wactawski-Wende J (2011) Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. JAMA 305: 1305-14

Biography

V. Craig Jordan OBE, PhD, DSc, FMedSci, is the Scientific Director at the Lombardi Comprehensive Cancer Center, Georgetown University, Washington DC, USA. He is the Vincent T. Lombardi Professor of Translational Cancer Research, and Professor of Pharmacology and Oncology at the Georgetown University Medical Center. Previously, he was the Diana, Princess of Wales Professor of Cancer Research at Northwestern University, Chicago IL (1999-2004) and the Alfred G. Knudson Professor of Cancer Research at the Fox Chase Cancer Center, Philadelphia PA (2004-2009). He was the Director of the Breast Cancer Research and Treatment Program at the Wisconsin Comprehensive Cancer Center in Madison WI (1987-1992), Director of the Lynn Sage Breast Cancer Research Program at the Robert H. Lurie Comprehensive Cancer Center at Northwestern University (1993-2004), and Vice President and Research Director of Medical Science at the Fox Chase Cancer Center (2004-2009).

Jordan's four decades of translational research described the scientific principles to be used for the effective use of long term adjuvant tamoxifen and the laboratory basis for the use of tamoxifen as a chemopreventive. He first described the concept of selective oestrogen receptor modulation and the so-called SERMs are now a well-established drug group for the treatment and prevention of osteoporosis and breast cancer. He did the initial work on raloxifene in the laboratory, and helped to guide it through pivotal clinical trials. He was the Scientific Chair of the Study of Tamoxifen and Raloxifene (STAR). For more than twenty years, he has developed and studied the evolution of drug resistance to SERMs and aromatase inhibitors and his current work on the apoptotic actions of physiological oestrogen in antihormone resistant breast cancer is finding clinical applications.

Jordan has received international recognition for his pioneering studies in the adjuvant treatment and prevention of breast cancer, and his major prizes include the Karnofsky Award (2008), the American Cancer Society Chemoprevention Award (2006), the Charles F. Kettering Prize (2003), the American Cancer Society Medal of Honor (2002), the Bristol Myers Squibb Award (2002), and the European Institute of Oncology Breast Cancer Therapy Award (2001). In 2002, her Majesty the Queen appointed him Officer of the Most Excellent Order of the British Empire for his contributions to International Breast Cancer Research. His contributions to science and medicine have been recognized by prestigious professional societies, with election to the National Academy of Sciences (2009) in the United States, Fellowship of the Academy of Medical Sciences (2009) in the United Kingdom, and an honorary Fellowship of the Royal Society of Medicine and the Jephcott Gold Medal (2008). The St. Gallen Prize for Clinical Breast Cancer Research (2011).

Two other studies conducted in Germany—one a randomized trial of similar design[8] and the other an observational analysis[9]—included approximately 300 recipients of autologous stem-cell transplantation and noted almost no cases of severe, grade 4 haemorrhage in the 'therapeutic' transfusion group. The authors of these two trials concluded that this approach is safe and feasible for this patient population. I would submit that the results of the present study by Stanworth *et al.*[7] are also consistent with this conclusion.

> "...serious haemorrhage is remarkably low when patients are cared for by experienced clinicians..."

By contrast, there was a higher rate of significant haemorrhage in the German studies in patients with AML who did not receive prophylactic transfusions, including a few patients experiencing intracerebral haemorrhage. However, some bleeding events in both the Wandt and Stanworth trials occurred at platelet counts >10,000/μl and might not have been prevented by prophylactic transfusions. Taken together, both studies would seem to support the current approach of prophylactic transfusions at counts of <10,000/μl in patients with acute leukaemia receiving active treatment.

Importantly, both studies only included patients who were, in general, clinically stable and without active bleeding or associated coagulopathy, and very closely monitored in a setting in which transfusions were immediately available if needed. Certainly, decisions need to be individualized and some patients should receive prophylactic transfusions if they have counts higher than 10,000/μl in the presence of bleeding, invasive procedures, hyperleukocytosis, coagulopathy, sepsis, etc. By contrast, prophylactic transfusions are not necessarily indicated at any platelet count level in patients with aplastic anaemia or myelodysplastic syndrome who are clinically stable without significant bleeding and not receiving active therapy.[6]

Thus, although excellent clinical trials provide overall guidance, circumstances in individual patients, which can vary from day to day, remain critical in deciding when to administer platelet transfusion. An important overriding message is that when using any approach, the incidence of serious haemorrhage is remarkably low when patients are cared for by experienced clinicians with access to modern transfusion technology. Lastly, important questions remain about the need to achieve specific platelet counts so that surgical procedures can be done safely in thrombocytopenic patients.[10] Currently, practice is driven by consensus statements based more on opinion than evidence, and it would be wonderful if these well organized groups could address this very important clinical issue in a prospective trial.

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Competing interests
The author declares no competing interests.

1. Gaydos, L. A., Freireich, E. J. & Mantel, N. The quantitative relation between platelet count and hemorrhage in patients with acute leukemia. *N. Engl. J. Med.* **266**, 905–909 (1962).
2. The Trial to Reduce Alloimmunization to Platelets Study Group. Leukocyte reduction and ultraviolet B irradiation of platelets to prevent alloimmunization and refractoriness to platelet transfusions. *N. Engl. J. Med.* **337**, 1861–1869 (1997).
3. Heckman, K. D. *et al.* Randomized study of prophylactic platelet transfusion threshold during induction therapy for adult acute leukemia: 10,000/microL versus 20,000/microL. *J. Clin. Oncol.* **15**, 1143–1149 (1997).
4. Rebulla, P. *et al.* The threshold for prophylactic platelet transfusions in adults with acute myeloid leukemia. Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto. *N. Engl. J. Med.* **337**, 1870–1875 (1997).
5. Wandt, H. *et al.* Safety and cost effectiveness of a 10×10(9)/L trigger for prophylactic platelet transfusions compared with the traditional 20×10(9)/L trigger: a prospective comparative trial in 105 patients with acute myeloid leukemia. *Blood* **91**, 3601–3606 (1998).
6. Schiffer, C. A. *et al.* Platelet transfusion for patients with cancer: clinical practice guidelines of the American Society of Clinical Oncology. *J. Clin. Oncol.* **19**, 1519–1538 (2001).
7. Stanworth, S. J. *et al.* A no-prophylaxis platelet-transfusion strategy for hematologic cancers. *N. Engl. J. Med.* **368**, 1771–1780 (2013).
8. Wandt, H. *et al.* Therapeutic platelet transfusion versus routine prophylactic transfusion in patients with haematological malignancies: an open-label, multicentre, randomised study. *Lancet* **380**, 1309–1316 (2012).
9. Wandt, H., Schaefer-Eckart, K., Frank, M., Birkmann, J. & Wilhelm, M. A therapeutic platelet transfusion strategy is safe and feasible in patients after autologous peripheral blood stem cell transplantation. *Bone Marrow Transplant.* **37**, 387–392 (2006).
10. Schiffer, C. A. What to do if there is no evidence? The issue of surgical procedures in patients with thrombocytopenia. *Transfusion* **51**, 2262–2264 (2011).

TARGETED THERAPIES

Any surprises from selective oestrogen-receptor modulators?

V. Craig Jordan

The marriage of medicinal chemistry, molecular biology and medicine is perhaps best exemplified by the evolution of selective oestrogen-receptor modulators (SERMs). Translational studies might be useful for predicting the myriad clinical responses to SERMs, contributing to improvements in women's health.

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In their recently published meta-analysis, Cuzick and colleagues[1] contribute a comprehensive analysis of the clinical trials on four selective oestrogen-receptor modulators (SERMs): tamoxifen, raloxifene, arzoxifene and lasofoxifene. The authors focus on the reduction of the incidence of breast cancer in their analysis, despite the comparative data on serious adverse effects (such as endometrial cancer) and other beneficial effects (such as a reduction in bone fractures). They conclude that the performance of SERMs remains as predicted some two decades ago by translational researchers[2]—that the incidence of oestrogen receptor (ER)-positive breast cancer is reduced when women take SERMs. Although all SERMs act on the ER, they are not all the same. Tamoxifen is approved to prevent breast cancer in premenopausal and postmenopausal women, whereas raloxifene is approved for this use in postmenopausal women only. Raloxifene is also approved to prevent osteoporosis in postmenopausal women. Arzoxifene and lasofoxifene were tested to prevent osteoporosis, but have been either abandoned, or approved but not marketed, respectively.

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All clinical meta-analyses tend to 'lump' numbers together and make generalizations, a practice that should be avoided in the field of clinical pharmacology. All SERMs are not the same and neither are their test populations. Although the SERMS share structural similarities (they are, broadly, branched phenolic compounds),[2] the subtle differences in their molecular structures impose differences in the external shape of the enveloping ER complex, which determines stimulatory or inhibitory effects at various target sites (Figure 1). For example, in a postmenopausal woman, SERMs switch on oestrogen-like responses to reduce circulating cholesterol, build bone and—for tamoxifen and arzoxifene—increase the incidence of endometrial cancer. By contrast, SERMs uniformly reduce the incidence of ER-positive invasive breast cancer, but only tamoxifen reduces the incidence of ductal carcinoma *in situ*.[3]

Tamoxifen, the pioneering SERM, was originally classified as a nonsteroidal antioestrogen designed to be used as a contraceptive pill. When this primary application failed, the drug was subsequently reintroduced as the antihormonal adjuvant therapy of choice in breast cancer, owing to its chemopreventive potential.[4] However, tamoxifen required rigorous toxicological and pharmacological investigation if the bold move to prevent breast cancer in high-risk women was to be embraced.[3,5] It was during this process that the pharmacology of SERMs surfaced in the late 1980s, and a roadmap for the pharmaceutical industry was developed[2] based on findings with tamoxifen and other SERMs in the laboratory.

At the time, many believed that if oestrogen was necessary to protect women from coronary heart disease and osteoporosis, little advantage could be gained if tamoxifen prevented breast cancer, but increased heart attacks and fractures. Unexpectedly, tamoxifen was found to modulate oestrogen target tissues in all the right places. Well, almost! Tamoxifen reduced circulating cholesterol, built bone and also prevented breast cancer. However, translational research[2] alerted the clinical community to the fact that tamoxifen increased the risk of endometrial cancer during adjuvant therapy for breast cancer[6] and in women at risk of developing breast cancer.[3] However, this important effect is only of significance in postmenopausal women, for whom venous thrombosis is also a pertinent issue. On the basis of these data, the roadmap stated

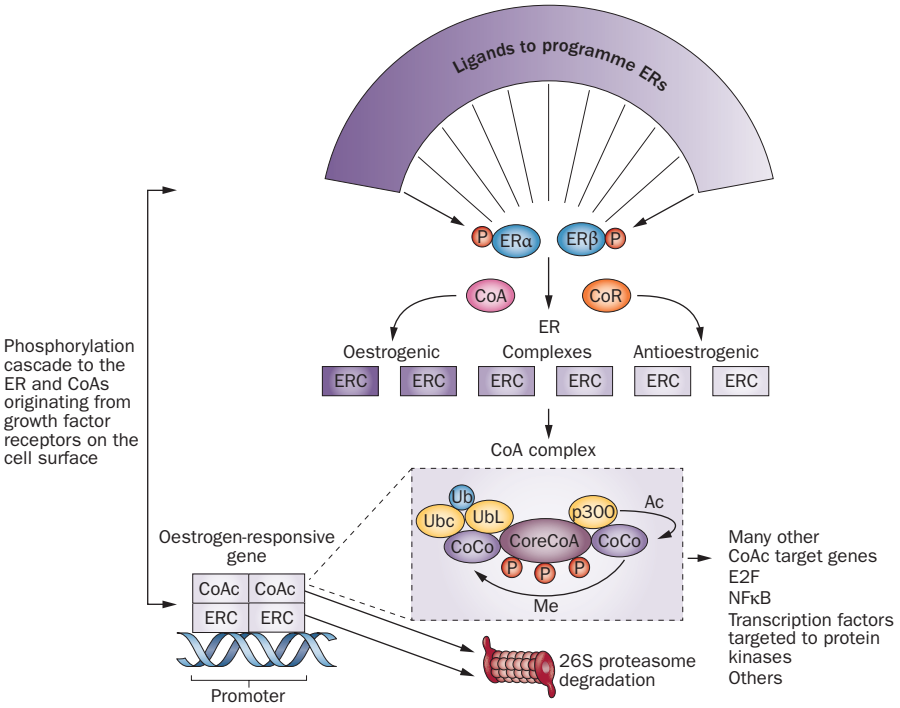

Figure 1 | Molecular networks potentially influence the expression of SERM action in a target tissue. The target tissue is programmed to express a spectrum of responses—between full oestrogen action and antioestrogen action—on the basis of the shape of the ligand and the sophistication of the tissue-modulating network. Abbreviations: CoA, co-activator; CoAc, co-activator complex; CoCo, co-co-activator; CoR, co-repressor; ER, oestrogen receptor; ERC, oestrogen receptor complex; SERM, selective oestrogen-receptor modulator. Permission obtained from Nature Publishing Group © Jordan, V. C. *Nat. Rev. Cancer* **7**, 46–53 (2007).

simply that if tamoxifen can maintain bone density and prevent breast cancer, safer compounds should be found to prevent osteoporosis and prevent breast cancer simultaneously.[2] Tamoxifen is, incidentally, the only FDA-approved SERM for breast cancer chemoprevention in premenopausal high-risk women.

Raloxifene was subsequently advanced to address the concerns about endometrial cancer in postmenopausal women who are treated with tamoxifen. Indeed, raloxifene was the first SERM for the prevention of osteoporosis that had the beneficial effects of preventing breast cancer without increasing the risk of endometrial cancer.[7] Tamoxifen (20 mg daily) and raloxifene (60 mg daily) were subsequently assessed head-to-head in the study of tamoxifen against raloxifene (STAR) to prevent breast cancer. Each SERM was given for 5 years to postmenopausal high-risk women and both were found to decrease breast cancer incidence by 50% during treatment;[8] however, raloxifene was progressively less effective than tamoxifen at controlling breast cancer incidence after therapy was stopped.[9] This effect was also predicted 20 years earlier in the laboratory[2]—raloxifene was shown to be short-acting because of poor bioavailability and rapid excretion. Furthermore, tamoxifen imposes a selection pressure on nascent breast cancer cells, which are microscopic clones of tamoxifen-resistant cells that are vulnerable to the apoptotic effects of the patient's own oestrogen when therapy stops.[10] One possible solution is to give raloxifene indefinitely,[9] a practice-changing approach that has been approved by the FDA for preventing osteoporosis. Indeed, clinical trial data has demonstrated a maintained reduction in breast cancer for 10 years in this patient population.

Tamoxifen and raloxifene are the only SERMs approved and recommended by the FDA and the UK National Institute of Health and Clinical Excellence (NICE) for the prevention of breast cancer. However, if drugs initially discarded by the pharmaceutical industry but with considerably different pharmacologies can have so much clinical value by chance,[4] can medicinal chemists create 'designer oestrogens' for the control of multiple diseases in women?[10] In the synthesis of arzoxifene, medicinal chemistry was logically applied to design a drug more robust than raloxifene—that is, with enhanced bioavailability—but with

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Key points

- Raloxifene must not be used to prevent breast cancer in high-risk premenopausal women
- Tamoxifen is the only selective oestrogen-receptor modulator available to prevent breast cancer in premenopausal women

a maintained pharmacological profile. Although envisaged to be a treatment for breast cancer and osteoporosis, the drug failed as a breast cancer therapy. As Cuzick and colleagues point out,[1] arzoxifene also increases the incidence of endometrial cancer; it will not be used for treating osteoporosis. By contrast, lasofoxifene is arguably a marvel of medicinal chemistry. Developed from a failed, and toxic, breast cancer drug from the 1970s called nafoxidine, lasofoxifene is a hydroxylated SERM that would be anticipated to have metabolic conjugation and be rapidly excreted. Although lasofoxifene has two stereoisomers, one isomer is not readily metabolically conjugated and this is the one used clinically. The lack of metabolic conjugation effectively lowers the daily dose to 0.5 mg, about 100-fold lower than of that of raloxifene. Importantly, lasofoxifene is effective at reducing fractures, breast cancer, endometrial cancer, stroke and coronary heart disease;[2] however, increased blood clots seem to be a problem.[1]

Winston Churchill once said, "Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning." Indeed, in 1970, there was no tamoxifen, SERMs or chemoprevention, but through the application of medicinal chemistry and the marriage of molecular biology with medicine, these strategies are now being used to good effect.[2] As our science continues to improve, so too will women's health.

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Competing interests
The author declares no competing interests.

1. Cuzick, J. *et al.* Selective oestrogen receptor modulators in prevention of breast cancer: an updated meta-analysis of individual participant data. *Lancet* **381**, 25–31 (2013).
2. Maximov, P. Y., Lee, T. M. & Jordan, V. C. The discovery and development of selective estrogen receptor modulators (SERMs) for clinical practice. *Curr. Clin. Pharmacol.* **8**, 135–155 (2013).
3. Fisher, B. *et al.* Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J. Natl Cancer Inst.* **90**, 1371–1388 (1998).
4. Jordan, V. C. Tamoxifen: a most unlikely pioneering medicine. *Nat. Rev. Drug Discov.* **2**, 205–213 (2003).
5. Powles, T. J. *et al.* A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *Br. J. Cancer* **60**, 126–131 (1989).
6. Fornander, T. *et al.* Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* **1**, 117–120 (1989).
7. Cummings, S. R. *et al.* The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* **281**, 2189–2197 (1999).
8. Vogel, V. G. *et al.* Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* **295**, 2727–2741 (2006).
9. Vogel, V. G. *et al.* Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 trial: preventing breast cancer. *Cancer Prev. Res. (Phila.)* **3**, 696–706 (2010).
10. Jordan, V. C. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* **5**, 207–213 (2004).

EPIDEMIOLOGY

Biorepositories for cancer research in developing countries

Sandipan Ray, Aliasgar Moiyadi and Sanjeeva Srivastava

Well-documented biorepositories are essential for cancer research. Currently, major biobanks are located in the developed world, which represents the minority global population; however, countries with low-resource settings contribute more than 50% of the global cancer burden. Therefore, there is an urgent need to establish next-generation biorepositories in developing countries.

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Biospecimens are a precious and irreplaceable resource for cancer research, which requires a large number of specimens in the form of well-annotated biobanks or biorepositories.[1] Biobanking is the organized collection and storage of biospecimens and clinicopathological information, and is undoubtedly highly useful for the study of complex human diseases such as cancer. In addition to primary tumour specimens, biopsy of metastatic cancer is now becoming a standard procedure before second-line and salvage therapy. The specimens from these metastatic tumours are increasingly regarded as an invaluable resource for cancer research to accelerate the knowledge of intrinsic and acquired resistance to treatment.[2]

The establishment of next-generation biobanks (storing primary and metastatic samples with the relevant clinical annotation) in developing countries is crucial to build comprehensive and globally inclusive biorepositories and provide an unbiased platform for cancer research, especially for genomic-based translational studies. The existence of heterogeneous patterns of cancer, owing to diverse ethnic populations in countries such as India, provides a highly attractive source for research materials. Moreover, cancers of the cervix, stomach, liver, lip and oral cavity are predominantly found in the population of developing countries, but are rare in resource-rich countries.[3] Consequently, repositories of biological specimens collected from limited geographical regions might not accurately reflect the complexity and heterogeneity of cancers in a global context. This limitation severely constrains the extrapolation of data emerging for tumours, particularly those of specific geoethnic background or similar cancers in different parts of the world, and might account for the seemingly variable patterns of treatment response among patient populations. To obtain an inclusive spectrum of cancer pathobiology, and accelerate cancer research and management, there is an indispensable need to encourage biobanking activities across the globe. This need is particularly acute in developing and resource-limited regions, where

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PERSONAL PERSPECTIVE

A(nother) scientific strategy to prevent breast cancer in postmenopausal women by enhancing estrogen-induced apoptosis?

Virgil Craig Jordan, OBE, PhD, DSc, FMedSci

Abstract

The innovation of combining bazedoxifene with conjugated estrogens provides a new opportunity for women's health. The finding by the Women's Health Initiative—that the administration of conjugated equine estrogens alone to women in their 60s who have had hysterectomy results in a decrease in breast cancer incidence and a drop in mortality—was unanticipated but can now be exploited for another gain in women's health. The issue to be considered is how postmenopausal women can improve their lifestyle to take advantage of conjugated equine estrogens–alone therapy. Food and Drug Administration approval of the combination of bazedoxifene and conjugated estrogens now provides an opportunity for postmenopausal women to reduce hot flashes and to potentially selectively sensitize occult breast cancer cells to the apoptotic actions of estrogen. Clinical trials are proposed to advance women's health and to reduce the incidence of breast cancer.

Key Words: Breast cancer – Apoptosis – Hormone replacement therapy – Chemoprevention.

Two recent clinical advances are good news for women's health. The first advance is the Food and Drug Administration approval of the combination of conjugated equine estrogens (CEE) with the selective estrogen receptor modulator (SERM) bazedoxifene for the treatment of moderate to severe vasomotor symptoms (hot flashes) associated with menopause and for the prevention of osteoporosis. The combination of bazedoxifene/CEE is an innovation. The second advance is the report that CEE alone administered to hysterectomized women in their 60s, as one of the clinical trials in the Women's Health Initiative, actually causes a decrease in the incidence of breast cancer,[1] a decrease in mortality from breast cancer, and an overall decrease in mortality.[2,3] Women were treated for about 6 years, but benefits remained during the 6 years after CEE was stopped.[3] It has been proposed that CEE triggers estrogen-induced apoptosis in vulnerable estrogen-deprived estrogen receptor (ER)–positive breast cancer cells that are present in the breast ducts 5 to 10 years after menopause.[4] The question is whether researchers can build on these separate clinical advances and perform translational research on the new biology of estrogen-induced apoptosis to conceive another paradigm for preventing breast cancer in postmenopausal women.

There are currently two Food and Drug Administration–approved options for the chemoprevention of breast cancer—the SERMs tamoxifen and raloxifene[5,6] (Fig. 1). However, F1 adverse effects and compliance have proved to be major issues for healthy women, with exacerbation of menopausal symptoms and reduced compliance observed. There is a low risk of breast cancer (6 per 1,000 women per year) even in high-risk groups; thus, the benefit (prevention of recurrence) for some is offset by menopausal adverse effects on many women. Thus, AQ1 researchers must get smart if noncompliance is to be solved to maintain long-term SERM therapy. Menopausal adverse effects must be controlled especially among women in their 50s, in whom quality of life is an issue. A SERM will block estrogen-stimulated breast cancer cell growth in menopause but may also exacerbate menopausal symptoms. A SERM plus CEE could be the answer.

Bazedoxifene (Fig. 2) is a new SERM[7,8] derived from an estrogenic derivative of the failed breast cancer drug zindoxifene.[9] F2 The addition of a strategically placed bulky side chain makes bazedoxifene selectively estrogenic or antiestrogenic at target sites around a woman's body.[10] Bazedoxifene's pharmacology is more similar to that of raloxifene than to that of tamoxifen, as bazedoxifene displays both breast and uterine safety.[11] Indeed, bazedoxifene (Fig. 2) looks more like raloxifene than tamoxifen (Fig. 1). However, the addition of CEE

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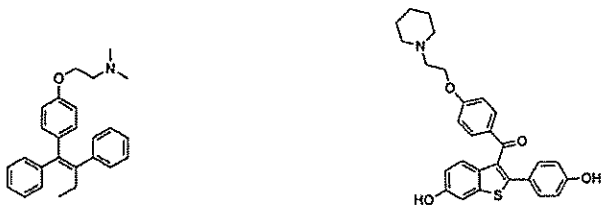

Tamoxifen
(ICI46,474)

Failed Contraceptive

Raloxifene
(LY156,758)

Failed Breast Cancer Drug

Repurposing

Pioneer for the adjuvant treatment and prevention of breast cancer

First targeted therapy for breast cancer–target: ER.

Pioneering medicine for the prevention of both breast cancer and osteoporosis

First SERM therapy for multiple diseases–target: ER.

FIG. 1. Early origins and repurposing of the two pioneering selective estrogen receptor modulators (SERMs) tamoxifen and raloxifene; ER, estrogen receptor.[7]

to bazedoxifene ameliorates hot flashes by 85%, enhancing bone building, but the SERM blocks estrogen action in the uterus. Thus, a subtle pharmacological balance between target sites for the SERM and target sites for the CEE or, in fact, a combined estrogenic effect of both must be achieved. Additive estrogenic effects on bone were first seen with both tamoxifen and raloxifene in a long-term ovariectomized rat model, which was used to demonstrate that the combination of SERMs adds to the estrogen effect on bone building but blocks proliferation in the uterus.[12] This is exactly what bazedoxifene/CEE does in women, with the added benefit of preventing hot flashes.

The value of CEE treatment in reducing the incidence of breast cancer seems to depend on a 5-year gap after menopause, when the ovary stops producing hormones and releasing eggs.[4,13-15] Estrogen deprivation is necessary in preparing occult breast cancer cells, through clonal selection, to become vulnerable to estrogen-induced apoptosis (Fig. 3). Breast cancer cells survive and grow based on the availability of the growth signal estrogen. The dramatic decrease in estrogen during menopause causes most ER-positive cells (which depend on estrogen to replicate) to die. It is a simple Darwinian principle: reduced resources for survival (estrogen) result in decreased populations of cells. However, at a certain point in

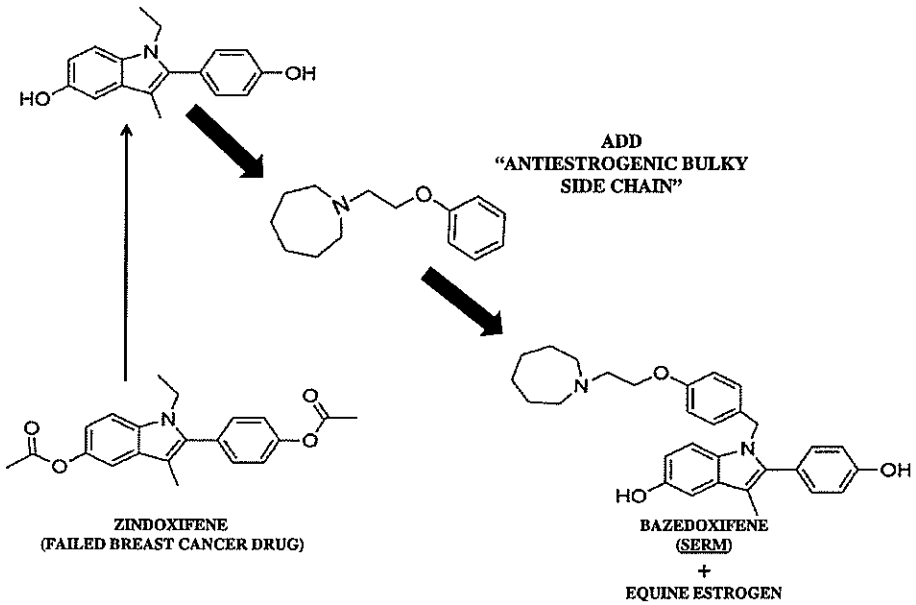

FIG. 2. Building of bazedoxifene as a selective estrogen receptor modulator (SERM) from the failed breast cancer drug zindoxifene and combination with conjugated equine estrogens to create bazedoxifene/conjugated equine estrogens.[7]

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Fig 3 4/C

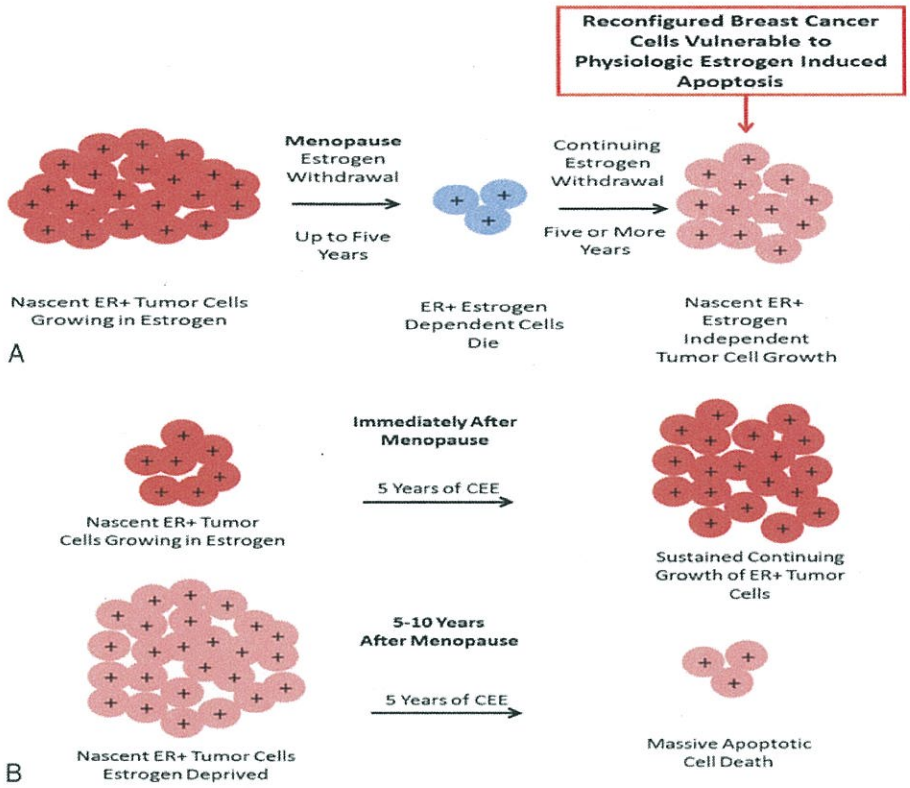

FIG. 3. Success of estrogen therapy is dependent on the menopause status of a woman. **A:** Estrogen withdrawal in postmenopausal women causes estrogen receptor (ER)–positive estrogen-dependent cells to die, but some cells continue to grow independently of estrogen. **B:** Treatment of women with conjugated equine estrogens (CEE) immediately after menopause results in sustained growth of nascent ER-positive tumors, whereas treatment 5 years after menopause causes apoptotic cell death. Reproduced with permission from Obiorah and Jordan.[4]

time, the biological necessity of cancer cell survival prevails, and adapted cells start to slowly repopulate in an estrogen-depleted environment. The process can be replicated in the laboratory, but the repopulating cells now respond to physiologic estrogen by triggering estrogen-induced apoptosis.[16,17] Surprisingly, even the long-term exposure of estrogen-deprived breast cancer cells to months of physiologic estrogen, which initially causes immediate and catastrophic cell death, only slowly permits the surviving cells to recover.[18] The cancer cells that now survive in estrogen do not expand dramatically as a population.[18] The resulting cancer cell population does not seem to respond to estrogen with growth alone, but there is now a balance of replication and apoptosis to maintain a stable population.[18] Thus, it is not implausible that this novel biological mechanism of estrogen-induced apoptosis in estrogen-deprived cells results in the destruction of microscopic tumors in women during CEE therapy.

In athymic mice, the process of drug resistance to tamoxifen (antiestrogen) evolves through two distinct phases of acquired resistance across a 5-year period[19,20] (Fig. 4). The initial phase (phase I) of drug resistance results in tamoxifen-stimulated cancer cell growth similar to that with estrogen. This process of cancer cell selection takes 1 to 2 years. In phase II of tamoxifen resistance, tamoxifen by itself promotes tumor growth and estrogen alone causes tumor regression through apoptosis. Raloxifene can result in the same acquired

Fig 4 4/C

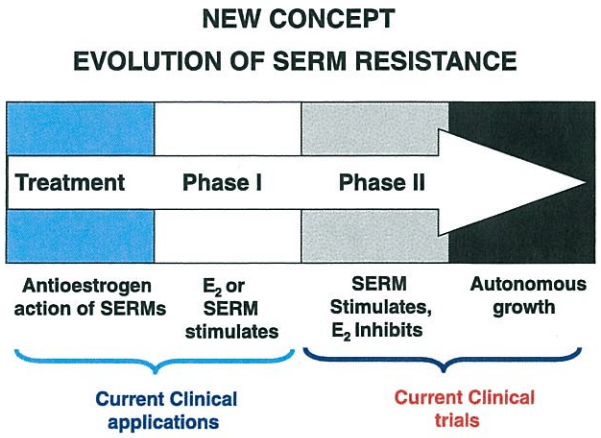

FIG. 4. Evolution of acquired selective estrogen receptor modulator (SERM) resistance. After long-term treatment with SERMs (1-2 y in vivo), initially responsive estrogen receptor–positive tumors become resistant to treatment and are stimulated by SERMs (phase I resistance) and estradiol (E_2). After long-term transplantation into a SERM-treated animal (≥5 y), breast tumor growth is inhibited by E_2, although still stimulated by SERMs (phase II of resistance). This process with SERMs in vivo is replicated with estrogen deprivation with MCF-7 breast cancer cells in vitro; cells initially start to grow spontaneously, but estrogen still induces growth (hypersensitivity). This is phase I. Long-term estrogen deprivation causes spontaneous growth in culture but apoptosis with physiologic estrogens both in vitro and in vivo (phase II). Reproduced with permission from Jordan.[21]

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resistance[22] but takes longer. Thus, the clinically available SERMs in an estrogen-deprived environment eventually distil a cancer cell population that will be vulnerable to estrogen-induced apoptosis. Small tumors disappear with estrogen therapy, but larger tumors, which have greater population plasticity to select a surviving cell, repopulate toward the estrogenic growth signal. An estrogen-stimulated tumor manifests as a recurrence.

The propositions considered here are (1) using a 5-year course of bazedoxifene/CEE to avoid menopausal symptoms and (2) building bones with uterine and breast safety. The antiestrogenic effects of bazedoxifene are anticipated to drive the selection of an estrogen-deprived ER-positive malignant cell population in the breast ducts. The long course of bazedoxifene (perhaps 5 y) would then be stopped, and a course of a few weeks of CEE alone would be used to "purge" vulnerable cells in the ducts by inducing nascent tumor eradication by triggering apoptosis. Continuous use of estrogen for years may not be necessary to prevent breast cancer in a vulnerable cell population primed to die quickly.

It is therefore reasonable to advance the aforementioned propositions with clinical trial validation because there is a concern that the dominance of bazedoxifene may be insufficient to drive cell selection for the estrogen-vulnerable population in the breast. As a polyhydroxylated molecule, bazedoxifene (Fig. 2) is very similar to raloxifene (Fig. 1). The Study of Tamoxifen and Raloxifene (STAR) trial teaches important lessons about the effectiveness of raloxifene 60 mg daily in breast chemoprevention during 5 years of treatment[5] (and after treatment cessation[6]). Although 5-year tamoxifen (20 mg daily) treatment clearly drives cell selection for estrogen-vulnerable cancer cells such as the laboratory model,[19] raloxifene does not.[6] The sustained antitumor effect does not persist in women when raloxifene stops,[6] so it must be given longer to prevent breast cancer—but how long? Laboratory studies have demonstrated that it is possible to induce the expected selection for the estrogen-vulnerable cancer cell population with raloxifene eventually.[22] Unfortunately, the principle of maintained antitumor effects has only been demonstrated clinically with tamoxifen (ie, chemoprevention was maintained after long-term tamoxifen treatment in the STAR trial, but chemoprevention failed after raloxifene was stopped).[6] Tamoxifen induced phase II resistance in microscopic tumors after 5 years of treatment, so a woman's own estrogen now kills the vulnerable microscopic breast cancer cells. By contrast, raloxifene, it seems, only caused phase I resistance, so a woman's own estrogen enhances tumor growth after raloxifene stops. Be that as it may, there is a final complication when comparing bazedoxifene with raloxifene. Bazedoxifene/CEE only uses 20 mg of the SERM daily (ie, only a third of the dose of raloxifene [60 mg daily] in the STAR trial). Also, the combination of estrogen with bazedoxifene, a competitive inhibitor of estrogen action at the ER, creates a situation where they must compete so that bazedoxifene can successfully select a vulnerable populations of cells. Although it is unknown whether bazedoxifene will be dominant, the good news is that compliance with bazedoxifene/CEE may be superior. Compliance is key to maintaining SERM selection pressure. Compliance with raloxifene at higher doses is poor because of its significant adverse effects. Rapid excretion of raloxifene, combined with poor compliance, creates a weak environment for breast cancer cells to be selected for vulnerable populations. It must be stressed, however, that laboratory studies have demonstrated that constant raloxifene in an estrogen-free environment selects cells that will be triggered to die when estrogen is reintroduced.[23]

Only clinical trials can determine the dose and timing of bazedoxifene and estrogen, but medical advances could be profound. Resolution of menopausal symptoms and a reduction in breast cancer incidence would be major advances in public health.

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REFERENCES

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1. Anderson GL, Limacher M, Assaf AR, et al. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *JAMA* 2004;291:1701-1712.
2. LaCroix AZ, Chlebowski RT, Manson JE, et al. Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. *JAMA* 2011;305:1305-1314.
3. Anderson GL, Chlebowski RT, Aragaki AK, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *Lancet Oncol* 2012;13:476-486.
4. Obiorah I, Jordan VC. 2012 NAMS/PFIZER–Wulf H. Utian Endowed Lecture. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. *Menopause* 2013;20:372-382.
5. Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* 2006;295:2727-2741.
6. Vogel VG, Costantino JP, Wickerham DL, et al. Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 trial: preventing breast cancer. *Cancer Prev Res (Phila)* 2010;3:696-706.
7. McDaniel RE, Maximov PY, Jordan VC. Estrogen-mediated mechanisms to control the growth and apoptosis of breast cancer cells: a translational research success story. In: Litwack G, editor. *Hormones and Breast Cancer*. London, England: Academic Press, 2013:1-49.
8. Maximov PY, Lee TM, Jordan VC. The discovery and development of selective estrogen receptor modulators (SERMs) for clinical practice. *Curr Clin Pharmacol* 2013;8:135-155.
9. Robinson SP, Koch R, Jordan VC. In vitro estrogenic actions in rat and human cells of hydroxylated derivatives of D16726 (zindoxifene), an agent with known antimammary cancer activity in vivo. *Cancer Res* 1988;48:784-787.
10. Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. *Nat Rev Cancer* 2007;7:46-53.
11. Pinkerton JV, Archer DF, Utian WH, et al. Bazedoxifene effects on the reproductive tract in postmenopausal women at risk for osteoporosis. *Menopause* 2009;16:1102-1108.
12. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 1987; 10:31-35.

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13. Prentice RL, Chlebowski RT, Stefanick ML, et al. Conjugated equine estrogens and breast cancer risk in the Women's Health Initiative clinical trial and observational study. *Am J Epidemiol* 2008;167:1407-1415.
14. Prentice RL, Chlebowski RT, Stefanick ML, et al. Estrogen plus progestin therapy and breast cancer in recently postmenopausal women. *Am J Epidemiol* 2008;167:1207-1216.
15. Beral V, Reeves G, Bull D, Green J. Breast cancer risk in relation to the interval between menopause and starting hormone therapy. *J Natl Cancer Inst* 2011;103:296-305.
16. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 2005;94:131-141.
17. Lewis JS, Meeke K, Osipo C, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 2005;97:1746-1759.
18. Fan P, Agboke F, McDaniel R, et al. Inhibition of c-Src blocks oestrogen-induced apoptosis and restores oestrogen-stimulated growth in long-term oestrogen-deprived breast cancer cells. *Eur J Cancer* 2014;50:457-468.
19. Yao K, Lee ES, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;6:2028-2036.
20. Jordan VC. The 38th David A. Karnofsky Lecture: the paradoxical actions of estrogen in breast cancer—survival or death? *J Clin Oncol* 2008;26:3073-3082.
21. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;5:207-213.
22. Balaburski GM, Dardes RC, Johnson M, et al. Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. *Int J Oncol* 2010;37:387-398.
23. Liu H, Lee ES, Gajdos C, et al. Apoptotic action of 17β-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. *J Natl Cancer Inst* 2003;95:1586-1597.

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Editorial

Timing is Key to Avoid the Bad and Enhance the good of Soy Supplements

V. Craig Jordan

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Epidemiology teaches us that there is an inverse relationship between soy consumption in Asian countries and a decrease in breast cancer risk. Naturally, this observation sparked a sustained interest in a potential natural approach to the chemoprevention of breast cancer, but the strategy of lifetime soy consumption from fetus to grave is not practical in Western society. However, there are many interconnected dimensions to the soy story some of which are potentially bad. This is the focus of the interesting study by Shike and coworkers(1) in this issue of the *Journal of the National Cancer Institute*.

Shike and coworkers(1) address the impact of short term (7-30 days) soy administration to a mixed population of premenopausal and early post-menopausal women with a diagnosis of breast cancer. Soy contains genistein and diadzein that were measured as known phytoestrogens in patient sera. Unfortunately, it has been

known for 3 decades that phytoestrogens have the potential to induce estrogen regulated genes through the estrogen receptor (ER) signal transduction pathway(2).

The authors now find a breast cancer genistein gene signature that is characterized by increases in cell cycle genes, which considering that women only consumed soy for 1-4 weeks, is not good. Patients may take soy for years in the belief they will be protected from either the growth of nascent breast cancers (chemoprevention) or recurrence of ER positive breast cancer during adjuvant therapy with aromatase inhibitors. They will not under the current treatment regimen, but they might with another.

The majority of patients in the study (1) were early post-menopausal and what we know from estrogen withdrawal in ER positive breast cancer cells that there is catastrophe early cell death in the new estrogen austere conditions (3, 4)and the population of cells is forced to adapt by environmental selection pressure. It is known that the new breast cancer cell population has adaptive hypersensitivity(5)to exogenous estrogen and it scavenges any estrogen through elevated ER levels(3, 4, 6). These cell populations are hypersensitive to estrogen for growth (see Figure 1). This is a characteristic of breast cancer that must replicate to survive. However, the study by Shike and coworkers (1) comes at a fortunate moment when the interlocking dimensions of estrogen action in breast cancer are being redefined and our understanding is being transformed from

random clinical and laboratory observations into a set of rules to test in clinical trials.

So what does the current paper contribute and what evidence is there in the literature to move from myths about phytoestrogens to create a foundation for future clinical study. The patients population (a total of 104) is broad consisting of a mix or pre and post-menopausal women (39.4% and 60.6% respectively) with a mean age of 56.2± 11.9 (SD) years. Though not stated, this would be the time of choice for woman to use phytoestrogen supplements to ameliorate the symptoms of menopause. As will be examined and defined, this is an appropriate population to define the risks of soy consumption, but excludes the potential benefits. Another complicating and diluting aspect of the study, in the fact that the breast cancers turn out to be 82% ER positive and 18% ER negative. A plausible reason for not having only patients with ER positive tumour is the remote possibility that low concentrations of circulating phytoestrogens have a mechanism of action other than activation or suppression of the ER. This strategy however, was not unreasonable, as the authors state it is the first study to monitor gene activation before and after the consumption of soy.

As the name suggests, phytoestrogens display estrogenic effects in laboratory tests(2) therefore it may be instructive to draw upon both clinical and laboratory

data about the actions of estrogen on breast cancers. In this way, a logical strategy for deploying phytoestrogens in future clinical trials can be formulated.

There are two sets of clinic data that illustrate the changing pharmacology of estrogen action in relation to the time from menopause in post-menopausal women with either metastatic breast cancer or occult disease in the breast. High dose synthetic estrogen therapy was the first documented chemical therapy to treat any human cancer successfully in clinical trial. Haddow(7) first reported a small series of patients with metastatic breast cancer which had a 30% response rate to high dose estrogen therapy. He used these data to complete the first multi-institutional clinical trial through the Royal Society of Medicine, London. He reported his retrospective observations during the Inaugural Karnofsky Award Lecture(8).

"When the various reports were assembled at the end of that time, it was fascinating to discover that rather general impression, not sufficiently strong from the relatively small numbers in any single group, became reinforced to the point of certainty; namely, the beneficial responses were three times more frequent in women over the age of 60 years than in those under that age; that estrogens may, on the contrary, accelerate the course of mammary cancer in younger women, and that their therapeutic use should be restricted to cases 5 years beyond the menopause. Here was an early and satisfying example of the advantages which may accrue from cooperative clinical trial."(8)

High dose estrogen treatment became the standard of care for 30 years prior to tamoxifen's approval in 1977 for the treatment of metastatic breast cancer. As a result of the ubiquitous early use of high dose estrogen the precise accumulation of response rates in relation to time of menopause occurred.

Stoll(9) noted that objective remission rate from estrogen treatment in 407 patients with metastatic breast cancer was higher in women more than 5 years past menopause (35%) when compared to women who were less than 5 years postmenopause (9%).

The second data set is the estrogen replacement therapy literature with the Million Women's Study(10) and the Women's Health Initiative (WHI) (11).

In the Million Women's Study, 1,129,025 postmenopausal women were recruited to evaluate breast cancer risk in hormone therapy users and never users. The study accrued 4.05 million women years of follow up, 15,750 incident breast cancer with a total of 7,107 breast cancer in current users of hormone therapy.

The principal conclusion relevant to our current considerations of timing and hormone type, ie: combination of estrogen and progestin (HRT) or estrogen alone (ERT) were as follows: the ERT current users had little increase in breast cancer if use was started more than 5 years after menopause (RR 1.05) but if ERT was begun straight after menopause there was increase in breast cancer (RR 1.43). The

pattern was similar for current users of HRT with an anticipated increase in breast cancer in users who start 5 years after menopause (RR 1.53) but a further elevation of risk if HRT is started immediately after menopause (RR 2.04)(10)

The WHI recruited 10,739 hysterectomized postmenopausal women into a randomized trial to receive either CEE (0.625 mg daily) or placebo. Women were aged between 50-79 years. The treatment phase of the trial was a median of 5.9 years as stop rules for stroke were invoked but an overall follow up with a median of 11.8 years. The first surprise was a finding of a lower incidence of breast cancer. At the latest analysis with 11.8 years median follow up(11), there was a lower incidence of invasive breast cancer (151 cases) compared with placebo (199 cases). Fewer women died from breast cancer in the estrogen group (6 deaths) compared with placebo (16 deaths). Indeed, few women died of any cause in the estrogen group after breast cancer diagnosis (30 deaths) than did those in the placebo group (50 deaths).

The breakthrough in understanding how one hormone estradiol, can be responsible for either the growth or death of breast cancer occurred with the realization that tumor cell populations adapt and evolve over years in response to long-term tamoxifen treatment(12). Acquired resistance to tamoxifen occurs in MCF-7 tumors implanted into tamoxifen treated athymic mice within about a year. The acquired resistance mimics acquired resistance in metastatic breast cancer and

tumors grow with either estrogen or tamoxifen. However, continuing growth for years of re-transplanted tumors into tamoxifen treated athymic mice exposes a vulnerability in evolving tumor cell populations: physiologic estrogen-induced apoptosis(13). This occurs not only in animal models *in vivo* but also in response to long-term estrogen deprivation *in vitro*(14, 15). Estrogen is no longer percieved as a survival signal through cell replication but as a trigger of apoptosis. An understanding of the ER mediated mechanism of estrogen induced apoptosis in vulnerable estrogen deprived breast cancer cells has been defined(14-16), refined(17) and interrogated (18-21). It is the timing of estrogen administration after menopause that determins tumor growth or cancer cell death (Figure 1). Clinical translation validates the significance of the new biology of estrogen-induced apoptosis.

Exhaustive anti-hormonal therapy in breast cancer, prepares acquired resistant cell populations for execution by estrogen. Lonning and coworkers(22) conducted a small study on 32 patients with four complete remissions and a 30% overall response rate. One patient had a complete response for 5 years of high dose estrogen therapy, and remained disease free for another 6 years after estrogen was stopped(23). Ellis and coworkers(24) evaluated the benefits of high (30mg daily) and low (6mg daily) dose estrogen treatment as a salvage therapy following failure of aromatase inhibitors. There was approximately a 30% clinical benefit for both

dosage group, but a significantly lower side effect rate for the low dose estrogen. In this context, the significant decrease in breast cancer, in the estrogen alone trial in the WHI trial with women in their 60's illustrates the value of low dose estrogen treatment on prepared and vulnerable estrogen deprived nascent breast cancer(11).

The general principle that emerges from both laboratory and clinical studies is that estrogen enhances growth in breast cancer cell populations maintained in estrogen but triggers apoptosis in cell populations adapted long-term estrogen deprivation. The study by Shike(1) illustrates the dangers of phytoestrogen consumption too soon around menopause but the biology of estrogen in estrogen deprived condition suggests that phytoestrogen could have benefit a decade after menopause. Recent laboratory studies support this(25) but there are two issues. Firstly, appropriate dosing of soy to create high levels of circulating phytoestrogen are needed to trigger apoptosis. Ten nanomolar concentrations with phytoestrogens stimulate cell replication in culture but a hundred nanomolar is necessary to trigger apoptosis(25). The Shike study(1) shows a huge range of cumulating levels of genestein (0-400 ng/ml) but this may be due to compliance problems or different durations of treatment (1-4 weeks). The second and most important issue is that women want to take soy products to ameliorate menopausal symptoms. It is now clear they should not but there is an alternative that could potentially act as evidenced based chemopreventive with an appropriate trial. The recent Food and

Drug Administration approval of the SERM bazedoxifene with an additional conjugated estrogen (CE) supplement(26) prevents hot flashes and menopausal symptoms while preventing breast and endometrial proliferation. It has been proposed recently(27) that 5 years of the bazedoxifene/CE combination at menopause could drive nascent breast cancer to vulnerable populations that become sensitive to estrogen-induced apoptosis. After the bazedoxifene/CE combination is stopped, a few short weeks of estrogen alone could execute vulnerable nascent breast cancers.

The clinical findings of Shike and coworkers(1) are consistent with the current rules of estrogen action in estrogen replete or estrogen deprived breast cancer cells both in the laboratory and the clinic(28) (Figure 1). No estrogen is good around the menopause and the innovative SERM/CE combinations may help ameliorate menopausal symptoms safely. However, a growing body of laboratory(25, 29) and clinical (11) evidence has now created an opportunity for evidence based clinical studies of chemoprevention with some form of estrogen, perhaps given intermittently, a decade following menopause.

References.

1. Shike M, Doane A, Russo L, et al. The effects of soy supplementation on gene expression in breast cancer: a randomized placebo-controlled study. *Journal of the National Cancer Institute*.2014 In Press.
2. Jordan VC, Koch R, Lieberman ME. Structure-activity relationships of nonsteroidal estrogens and antiestrogens. In: Jordan VC, (ed). *Estrogen/Antiestrogen Action and Breast Cancer Therapy*. Madison, WI: University of Wisconsin Press; 1986, pp. 19-41.
3. Welshons WV, Jordan VC. Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *Eur J Cancer Clin Oncol*.1987;23(12):1935-9.
4. Katzenellenbogen BS, Kendra KL, Norman MJ, et al. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res*.1987;47(16):4355-60.
5. Masamura S, Santner SJ, Heitjan DF, et al. Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. *J Clin Endocrinol Metab*.1995;80(10):2918-25.
6. Jeng MH, Shupnik MA, Bender TP, et al. Estrogen receptor expression and function in long-term estrogen-deprived human breast cancer cells. *Endocrinology*.1998;139(10):4164-74.
7. Haddow A, Watkinson JM, Paterson E, et al. Influence of synthetic oestrogens on advanced malignant disease. *Br Med J*.1944;2(4368):393-8.
8. Haddow A. David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer*.1970;26(4):737-54.
9. Stoll B. Palliation by castration or hormone ablation. In: Stoll BA, (ed). *Breast Cancer Management Early and Late*. London: William Herman Medical Books Ltd; 1977, 135-149.
10. Beral V, Reeves G, Bull D, et al. Breast cancer risk in relation to the interval between menopause and starting hormone therapy. *J Natl Cancer Inst*.2011;103(4):296-305.
11. Anderson GL, Chlebowski RT, Aragaki AK, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *Lancet Oncol*.2012;13(5):476-86.
12. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell*.2004;5(3):207-13.
13. Yao K, Lee ES, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res*.2000;6(5):2028-36.
14. Song RX, Mor G, Naftolin F, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *J Natl Cancer Inst*.2001;93(22):1714-23.
15. Lewis JS, Meeke K, Osipo C, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst*.2005;97(23):1746-59.
16. Obiorah I, Sengupta S, Fan P, et al. Delayed triggering of oestrogen induced apoptosis that contrasts with rapid paclitaxel-induced breast cancer cell death. *Br J Cancer*.2014;110(6):1488-96.
17. Ariazi E, Cunliffe H, Lewis-Wambi JS, et al. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *Proc Natl Acad Sci USA*.2011;108(47):18879-18886.
18. Fan P, Agboke FA, McDaniel RE, et al. Inhibition of c-Src blocks oestrogen-induced apoptosis and restores oestrogen-stimulated growth in long-term oestrogen-deprived breast cancer cells. *Eur J Cancer*.2014;50(2):457-68.
19. Fan P, Griffith OL, Agboke FA, et al. c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. *Cancer Res*.2013;73(14):4510-20.

20. Obiorah I, Sengupta S, Curpan R, et al. Defining the conformation of the estrogen receptor complex that controls estrogen induced apoptosis in breast cancer. *Molecular Pharmacology*.2014:Epub ahead of print.
21. Obiorah IE, Jordan VC. Differences in the rate of oestrogen-induced apoptosis in breast cancer by estradiol and the triphenylethylene bisphenol. *Br J Pharmacol*.2014.
22. Lonning PE, Taylor PD, Anker G, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat*.2001;67(2):111-6.
23. Lonning PE. Additive endocrine therapy for advanced breast cancer - back to the future. *Acta Oncol*.2009;48(8):1092-101.
24. Ellis MJ, Gao F, Dehdashti F, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *JAMA*.2009;302(7):774-80.
25. Obiorah IE, Fan P, Jordan VC. Breast cancer cell apoptosis with phytoestrogens is dependent on an estrogen deprived state. *Cancer Prev Res (Phila)*.2014. epub ahead of print.
26. Mirkin S, Komm BS. Tissue-selective estrogen complexes for postmenopausal women. *Maturitas*.2013;76:213-220.
27. Jordan VC. A(nother) scientific strategy to prevent breast cancer in postmenopausal women by enhancing estrogen-induced apoptosis? *Menopause*.2014. epub ahead of print.
28. Jordan VC. The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer--survival or death? *J Clin Oncol*.2008;26(18):3073-82.
29. Obiorah I, Jordan VC. 2012 NAMS/PFIZER- Wulf H. Utian endowed lecture. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. *Menopause*.2013;20(4):372-382.
30. Sengupta S, Obiorah I, Maximov PY, et al. Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. *Br J Pharmacol*.2013;169(1):167-78.

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Notes

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Figure 1. Rules for the change in estrogen receptor (ER) positive breast cancer cell populations as they leave an estrogen rich environment at menopause, adapt to a declining estrogen environment over a 5 year period (referred to as Gap). Estrogen independent clones then grow out that are able to survive in an estrogen austere environment. This is modeled in the laboratory with long term estrogen deprived cells that exhibit acquired hypersensitivity to estrogen for growth(6) and then estrogen induced apoptosis(14, 15). Laboratory studies illustrate that the constituents of conjugated equine estrogen (CEE)(29), the endocrine disruptor bisphenol A (30) and phytoestrogens(25) can trigger cell replication or apoptosis dependent upon the cell populations and its natural estrogen rich or austere environment.

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CLINICAL UPDATES IN

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Welcome

In this latest issue of *Clinical Updates in Breast Cancer* we begin with highlights from the recent ASCO 2007 annual meeting which took place this year in Illinois' "windy city", Chicago.

We are also delighted and proud to feature three articles written by esteemed experts in their field. The first article is by Dr V. Craig Jordan and his colleagues who write on the current clinical indications for fulvestrant, covering topics from the structure and mechanism of action, dosing, and how fulvestrant should be used in sequence with other agents.

Associate Professor Peter Graham discusses the optimal timing of radiotherapy with chemotherapy and with hormonal therapy. He also reports on the STARS trial which is anticipated to open this year.

The third article, by Professor Christobel Saunders, reports on the very important topic of MRI screening of women in Australia who are at high risk of breast cancer.

Finally, we end the issue with a paper from the *International Journal of Radiation Oncology Biology Physics* which investigates whether breast radiotherapy is beneficial in women with favourable early breast cancer treated by lumpectomy plus tamoxifen or anastrozole.

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Highlights from the 43rd American Society of Clinical Oncology (ASCO) annual meeting

Chicago, Illinois, USA
1–5 June 2007

Hypofractionation for early breast cancer: First results of the UK standardisation of breast radiotherapy (START) trials

J.A. Dewar, J.S. Haviland, R.K. Agrawal, J.M. Bliss, P. Hopwood, B. Magee, J.R. Owen, M. A. Sydenham, K. Venables, J.R. Yarnold, on behalf of the START Trials centres

J Clin Oncol 2007; **25** (18 Suppl): Abstract LBA518

First results from the START Trials (ST-A and ST-B) show that in hypofractionated post-operative radiotherapy women with completely excised invasive breast cancer (T1-3, N0-1, M0), the fractionation sensitivity of breast cancer is similar to that of late reacting normal tissue. The studies recruited 2236 (ST-A) and 2215 (ST-B) patients from 35 centres in the UK during the period 1999–2002. Follow-up occurred for a median of 5.1 years (ST-A) and 6 years (ST-B). Local-regional (LR) relapse was the primary endpoint. In the ST-A centres there were 93 LR relapses at the 5-year point (4.1%, 3.2–5.0%), and the absolute difference in LR relapse rates for 50 Gy compared to 41.6 Gy was 0.2% (-1.3%–2.6%). This difference increased to 0.9% (-0.8%–3.7%) for 50 Gy compared to 39 Gy. In the ST-B centres, LR relapses occurred in 65 cases at the 5-year point (2.8%, 2.1–3.5%). The absolute difference in LR relapse rates was -0.6% for 40 Gy compared to 50 Gy. The rate of mild to marked change in photographic breast appearance was lower in the 39 Gy group compared to the 50 Gy group for ST-A (HR 0.69, 0.52–0.91) and this result was similar for the 40 Gy compared to the 50 Gy group in ST-B (HR 0.83, 0.66–1.04). The a/ß estimates for tumour control and change in breast appearance were 5.0 Gy (-2.7–12.7) and 3.1 Gy (1.6–4.6), respectively. Induration, telangiectasia and breast oedema were reported less frequently in the 39 Gy (ST-A) and 40 Gy (ST-B) groups compared to the 50 Gy groups. Quality of life results supported the clinical findings. The study results confirm the findings of a recent pilot trial and add weight to the benefit of using hypofractionated radiotherapy schedules in patients with early breast cancer.

The effect of margin status on local recurrence following breast conservation and radiation therapy for DCIS

M.R. Kell, C. Dunne, C. Canning, M. Morrow

J Clin Oncol 2007; **25** (18 Suppl): Abstract 597

Inadequate surgical margins in patients receiving breast conserving surgery (BCS) and postoperative irradiation (RT) for ductal carcinoma in situ (DCIS) could result in high rates of local recurrence, and excessively large resections could lead to poor cosmetic outcome without oncological benefit. To examine adequate surgical margins in more detail, published trials that examined outcomes after adjuvant RT following BCS for DCIS were reviewed. Fixed and random effects methods were used to combine data. Results from 3606 patients in randomised trials showed that patients with negative margins were less likely to experience disease recurrence compared to patients with positive margins after RT (RR 0.53, 95% CI = 0.42–0.66, $p < 0.01$). When combining randomised trial data with non-randomised data (5500 patients), in cases where the margin status was close or unknown there was a significant risk of in-breast tumour recurrence (IBTR) compared to cases with a negative margin (RR = 1.68, 95% CI = 1.22–2.33, $p < 0.01$). Examining specific margin thresholds, a 2 mm margin was shown to be superior to a margin of less than 2 mm (OR = 0.67, 95% CI 0.51–0.89, $p < 0.01$). No significant difference in the rate of IBTR was observed when comparing a 2 mm margin to a margin of more than 5 mm (OR = 1.49, 95% CI 0.54–4.9, $p > 0.05$).

Locoregional control significantly reduces the risk of subsequent distant metastases in patients with locally advanced breast cancer managed with radiotherapy as the primary locoregional treatment

W. Rogowski, A. Badzio, R. Dziadziuszko, J. Madrzak, M. Welnicka-Jaskiewicz, J. Jassem, P. Barrett-Lee

J Clin Oncol 2007; **25** (18 Suppl): Abstract 11076

In order to evaluate whether locoregional control reduces the subsequent risk of distant metastases, the records of 261 patients with primarily inoperable locally advanced breast cancer treated between 1991 and 1997 at the Medical University of Gdansk, Poland or Velindre NHS Trust, Cardiff, UK, were analysed. All patients in the study received megavoltage radiotherapy to the breast with two tangential fields. Adjacent lymph node areas underwent radiation using customised fields. Radiotherapy was the only local treatment for 241 patients, with the remaining 20 patients subsequently receiving mastectomy. The majority of patients also received chemotherapy and/or endocrine therapy prior to or following radiation therapy. With a median follow-up of 37 months, the 5-year overall survival rate was 36% (95% CI 28–43%). Locoregional relapse-free survival at 5 years was 48% (95% CI 40–57%). One hundred and sixty-seven patients experienced disease recurrence (67%); local recurrence occurred in 30 patients (12%), distant metastases in 72 patients (27%) and both local and distant recurrence occurred in 65 patients (26%). In a subgroup of patients with locoregional failure, the median time to distant metastases (33 months) was significantly less compared to patients free of locoregional recurrence or progression before presenting with distant metastases (43 months, $p < 0.05$). Although the results were nowhere near satisfactory, the study concluded locoregional control in patients with locally advanced breast cancer significantly reduces the risk of subsequent distant metastases and is, thus, an important treatment goal.

Optimal use of aromatase inhibitors for adjuvant treatment of hormone-sensitive early breast cancer: up front or sequenced after tamoxifen?

J. Cuzick, P. Sasieni, A. Howell
J Clin Oncol 2007; **25** (18 Suppl): Abstract 541

In the absence of clinical trial data, it has been difficult for clinicians to decide whether to commence adjuvant treatment of early breast cancer with an aromatase inhibitor (AI) or to reserve AI therapy until after 2 to 3 years of tamoxifen therapy. Early modelling data have indicated that an upfront strategy is more favourable (Cuzick *et al. Br J Cancer* 2006: 27; 460–4). To assist clinicians, a model was previously constructed in order to compare these treatment sequences after various periods of initial treatment with tamoxifen. Using newly-reported data, the model has now been updated and recurrence rates and time lost to recurrence during the first 10 years of follow-up have been predicted (see **Table 1**). Using a 5-year carryover period, at 10 years a 17.7% recurrence rate was predicted for upfront AI therapy. This compared to a predicted recurrence rate of 22.9% for 5 years of tamoxifen therapy. The upfront strategy is predicted to result in one less recurrence for every 19 women treated upfront, and the average time lost to recurrence is predicted to reduce by 3.4 months (2.8%). With updated data, the model now also predicts that use of AI therapy upfront will dominate use of AI therapy following tamoxifen. In addition, the model shows that 10 years of tamoxifen or 5 years of tamoxifen followed by 5 years of AI therapy is superior to 5 years of tamoxifen therapy alone. Longer duration of treatment with an AI is therefore of benefit, especially in younger women.

Effect on patient outcome of residual DCIS in patients with complete eradication of invasive breast cancer after neoadjuvant chemotherapy

C. Mazouni, F. Peintinger, S. Wan-Kau, F. Andre, A. M. Gonzalez-Angulo, F. Symmans, F. Meric-Bernstam, V. Valero, G. Hortobagyi, L. Pusztai
J Clin Oncol 2007; **25** (18 Suppl): Abstract 530

To determine whether residual ductal carcinoma in situ (DCIS) affects the outcome of patients with complete eradication of invasive cancer, a retrospective database analysis of 2302 breast cancer patients treated prospectively with neoadjuvant chemotherapy at the UT MD Anderson Cancer Centre between 1980 and 2004 was undertaken. The analysis compared overall, disease-free and local recurrence-free survival in patients with histologically defined eradication of breast cancer. During a mean follow-up of 250 months, 78 (3.4%) patients had no residual invasive or in situ cancer (pCR) and 2025 (88%) had residual invasive cancer. Disease-free survival rates were similar in patients with pCR and those with no residual invasive cancer but persistent in situ disease (pCR+DCIS). At the 5-year point disease-free survival was 87.1% in both groups, and at the 10-year point disease-free survival was 81.3% versus 81.7%, respectively. Overall survival rates were 91.9% versus 92.5% at 5 years and 91.8% versus 92.5% at 10 years. These overall survival rates were significantly higher than that of patients with residual invasive cancer (74.4%, $p < 0.001$). Local-regional recurrence-free survival rates at 5 years were not different for patients with pCR or pCR+DCIS (92.8% versus 90.9%, $p = 0.63$). The study showed that in patients with complete eradication of invasive cancer in the breast and lymph nodes, residual DCIS does not have an adverse effect on survival or local recurrence rate. Including patients with residual DCIS when defining pathologic complete response is therefore justified in cases where this outcome is used as an early surrogate marker for long-term survival.

Survival with adjuvant surgical oophorectomy and tamoxifen in premenopausal women with operable breast cancer

R.R. Love, N.V. Dinh, T.T. Quy, N.D. Linh, E.M. Hade, G.S. Young, D. Jarjoura
J Clin Oncol 2007; **25** (18 Suppl): Abstract 552

During the period between 1993 and 1999, 709 premenopausal women of Vietnamese or Chinese origin with clinical stage II to III operable breast cancer were randomised to prospectively receive immediate pre-mastectomy adjuvant surgical oophorectomy followed by tamoxifen for 5 years (n = 356) or mastectomy alone with the same combination of hormonal therapy following disease recurrence (n = 353). After a median follow-up of 7 years, disease-free survival (DFS) and overall survival (OS) were significantly better in patients who received adjuvant therapy (log-rank, $p = 0.0003$ and $p = 0.0002$, respectively). At 10 years, DFS probabilities were 62% and 51% (95% CI 4–22%) for the adjuvant and observation groups, respectively. These probabilities were 70% and 52% (95% CI 6–34%) for OS. In oestrogen receptor-positive patients, DFS rates at 5 years were 83% and 61% for the adjuvant

Table 1. Recurrence rates and time lost to recurrence

10-year recurrence rates (%) using a 5-year carryover period					Time lost to recurrence in first 10 years (%) using a 5-year carryover period				
Tam 5-y	Upfront AI	Tam 2-y + AI 3-y	Tam 10-y	Tam 5-y + AI 5-y	Tam 5-y	Upfront AI	Tam 2-y + AI 3-y	Tam 10-y	Tam 5-y + AI 5-y
22.9	17.7	18.1	21.6	18.7	12.1	9.3	10.0	11.7	10.9

and observation groups, respectively. At 10 years, these rates were 66% and 47%. OS rates at 5 and 10 years in oestrogen receptor-positive patients were 88% and 74%, and 82% and 74% in the adjuvant and observation groups, respectively. The trial showed that in women with oestrogen receptor-positive operable breast cancers, DFS and OS rates at 5 and 10 years following adjuvant oophorectomy and tamoxifen are favourable compared to the use of other adjuvant regimens.

Genomic heritage of sentinel lymph node metastases: implications for clinical management of breast cancer patients

D.L. Ellsworth, R.E. Ellsworth, T.E. Becker, B. Deyarmin, H.L. Patney, J.A. Hooke, C.D. Shriver

J Clin Oncol 2007; **25** (18 Suppl): Abstract 571

Allelic imbalance (AI) was used to examine genomic relationships among metastases in the sentinel and non-sentinel axillary lymph nodes (from complete axillary dissections) in 15 patients with lymph node-positive breast cancer. Localisation of sentinel nodes occurred using standard scintigraphic and gamma probe techniques with 1.0 mCi technetium-99m sulfur colloid. Identification of pathologically positive nodes occurred through the use of H&E histology and immunohistochemistry. Following isolation by laser microdissection of primary breast tumours and metastases in sentinel and axillary nodes, AI was assessed at 26 chromosomal regions. In addition, the timing and molecular mechanisms of metastatic spread to the sentinel and axillary nodes were examined. The study results showed AI frequencies overall were significantly higher in primary breast tumours compared to lymph node metastases ($p < 0.05$). Discordance was high in patterns and frequencies of AI events between metastases in the sentinel and non-sentinel axillary nodes. Multiple genetically-divergent lineages of metastatic cells were observed to independently colonise the lymph node. Furthermore, it appeared that some lymph node metastases acquired metastatic potential early in tumourigenesis, while other metastases evolved at a later time. Significantly, observations showed that lineages colonising the sentinel nodes originated at different times and progressed by different molecular mechanisms. It is therefore possible that metastases colonising the sentinel nodes are not descendants of progenitor cells that colonise the lymph nodes early in tumourigenesis. In the sentinel nodes, metastatic growth could be due to stimulating factors from the primary tumour affecting proliferation of previously disseminated cells rather than the timing of metastatic spread.

Concurrent chemoradiotherapy (CRT) following neoadjuvant chemotherapy (NACT) in locally advanced breast cancer (LABC)

A. Alvarado-Miranda, R. Morales-Barrera, O. Arrieta, J. Zinser-Sierra, A. Gamboa-Vignole, E. Maafs-Molina, T. Ramirez-Ugalde, F. Lara-Medina

J Clin Oncol 2007; 25 (18 Suppl): Abstract 11063

A retrospective analysis of 112 patients with locally advanced breast cancer (LABC) (Stage IIB-IIIB) treated with concurrent chemoradiotherapy (CRT) after neoadjuvant chemotherapy (NACT) between January 2000 and December 2003 was undertaken in order to determine whether this modality provides good locoregional control. Patients received 5FU 500 mg/m^2, doxorubicin 50 mg/m^2 and cyclophosphamide 500mg/m^2 (FAC) or doxorubicin 50 mg/m^2 and cyclophosphamide 500 mg/m^2 (AC) administered intravenously in four cycles of 21 days. CRT involved 60 Gy whole-breast irradiation together with concurrent weekly mitomycin 5 mg, 5FU 500 mg and dexamethasone 16 mg or cisplatin 30 mg, gemcitabine 100 mg and dexamethasone 16 mg. Patients subsequently underwent surgery, and after 6–8 weeks they received two additional courses of FAC, AC or paclitaxel 90 mg per week for 12 weeks. Oestrogen receptor (ER)-positive patients received hormone therapy. The median tumour size was 5 cm, and the percentage of patients in stages IIB, IIIA and IIIB were 21.4%, 42.9% and 35.7%, respectively. Pathologic complete response (pCR) was 42% (95% CI 33.2–50.5) in breast and 29.5% (95% CI 21.4–37.5) in breast and axillary lymph nodes. Median relapse-free survival (RFS) was not reached. At 5 years, the RFS was 76.9% (95% CI 68.2–84.7) and no relationship between pCR and RFS was found. Multivariate analyses showed that negative ER was the main determinant of pCR ($p = 0.016$) and that clinical stage was the main determinant of RFS ($p = 0.03$). Local recurrence occurred in only one patient. Overall survival at 5 years was 84.2% (95% CI 75–93.2). During CRT, grade 1–2 neutropenia occurred in 32.2%, grade 1–2 anaemia in 5.2%, and grade 3 radioepithelitis in 22.4% of patients. The modality provided good locoregional control for LABC and was associated with an acceptable toxicity profile. Further investigation of this modality in patients with LABC is warranted.

The current clinical indications for fulvestrant

An article by Roshani R. Patel, Jennifer R. Pyle and Dr V. Craig Jordan

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Introduction

Fulvestrant is a novel treatment for postmenopausal women with advanced breast cancer who have previously failed tamoxifen therapy. It has been shown to be as effective as third-generation aromatase inhibitors in phase III trials.[1] The selective oestrogen receptor modulator (SERM) tamoxifen has antagonist actions in the breast but also agonist effects on other tissues including the endometrium. Such agonist activity has been shown to stimulate endometrial thickening and increase the risk of endometrial cancer.[2] Drug resistance to tamoxifen is often expressed as tamoxifen-stimulated growth via the oestrogen receptor (ER).[3] In contrast, fulvestrant is an ER antagonist that has no agonist effects.[1] Fulvestrant shows minimal side effects and is well tolerated.[4]

Structure and mechanism of action

Faslodex™ is a long-acting formulation of fulvestrant used to treat hormone receptor positive metastatic cancer. Fulvestrant inhibits oestrogen-stimulated tumour growth by first binding to the ER[5] and then promoting destruction of the complex. Fulvestrant has a chemical structure similar to that of oestradiol but it also contains a strategically placed long alkylsulphinyl side chain (**Figure 1**) at the 7α position.

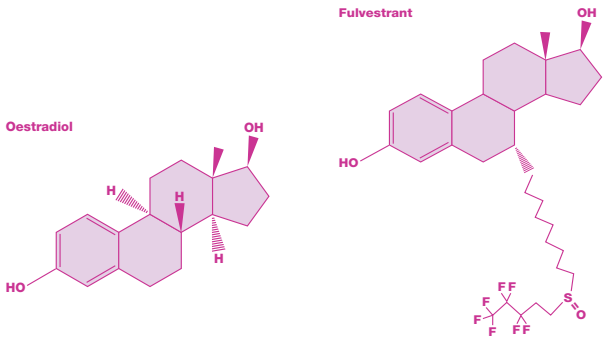

Figure 1. The chemical structures of oestradiol and fulvestrant

This structure allows fulvestrant to bind with a high affinity to the ER and thereby compete with oestradiol for ER interaction. Once the fulvestrant molecule binds to the ER, the shape of the complex is changed dramatically from the normal shape of the oestrogen-ER complex. A cascade of events then occurs, including the inhibition of activating function 1 and 2, inhibition of receptor dimerisation, and the inhibition of transcription co-activator recruitment.[6] Fulvestrant causes the ER to be tagged with ubiquitin, a small protein that latches onto damaged or mutated proteins, which results in the drug-bound ER being rapidly degraded by proteasomes (**Figure 2**).[7-9] Consequently, the cellular ER concentration is reduced thereby inhibiting the signal transduction pathway for tumour growth.

The novel mechanism of action for fulvestrant makes it a suitable agent to treat SERM-resistant advanced breast cancer.

Dosing of fulvestrant

Fulvestrant (250 mg) is given as a monthly intramuscular injection. The drug is slowly absorbed by the body from the injection site. Pharmacokinetic studies have demonstrated that regardless of the dosing schedule, there is no difference in bioavailability or release of the drug from the injection site.[10] Further clinical studies indicate that once-monthly doses of fulvestrant 250 mg result in C_{trough} values that double between the first and sixth doses. This results in a 3–6 month period before steady-state levels are achieved, due largely to the slow and sustained release of the drug.[11] As the pharmacokinetics of fulvestrant allow for once-a-month dosing, there may be benefits for patients in whom compliance with oral medication is an issue. As well as having to remember to take their medication, taking a pill each day can serve as a daily reminder of their condition. By receiving an injection once a month, compliance can be assured while the patient receives continued follow-up with their practitioners and psychosocial support from the care team.[12]

Clinical application and positioning of fulvestrant in the endocrine sequence of therapy

Currently, the clinical use of fulvestrant primarily applies to postmenopausal women with advanced ER-positive breast cancer who have failed prior endocrine therapy or have recurrent breast cancer. To understand how fulvestrant should be used in sequence with other agents, it is important to understand the results from two large phase III trials in the second-line setting and some important phase II trials regarding third-line treatment. In addition, another study has investigated the role fulvestrant could play in the first-line treatment of advanced breast cancer.

Two large multicentre, randomised trials were run in parallel to

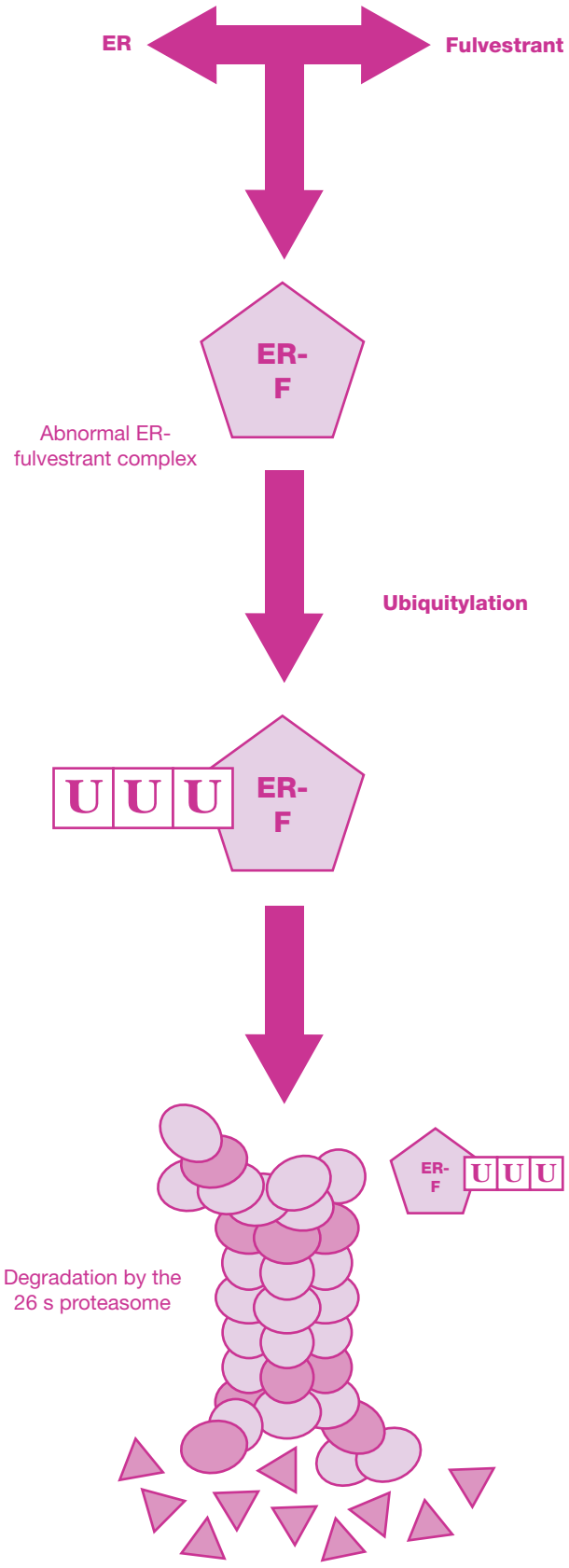

Figure 2. The cascade of events leading to degradation of the oestrogen receptor, including binding of fulvestrant, conformational change of the oestrogen receptor, and degradation of the oestrogen receptor by proteasomes.

determine whether the use of fulvestrant or anastrozole after disease progression on tamoxifen would achieve better outcomes. Trial 0020 involved Europe, Australia and South America, while trial 0021 was a North American study.

Trial 0020

Trial 0020 recruited 451 patients. Fulvestrant was administered as a single 250 mg intramuscular dose in 222 patients. Anastrozole was administered orally as a 1 mg dose to the 229 patients in the other group. Patient demographics were similar in both groups and patients were followed for an average of 14 months. Overall, there was no statistically significant difference in clinical benefit between the two groups. In the arm receiving fulvestrant, 10 (4.5%) patients had a complete response, 36 (16.2%) patients had a partial response and 53 (23.9%) patients had stable disease for more than 24 weeks. The arm receiving anastrozole had four (1.7%) patients with a complete response, 32 (14%) patients with a partial response and 67 (29.3%) patients with stable disease for more than 24 weeks. The average time to progression was 5.5 months with fulvestrant and 5.1 months with anastrozole.[13]

Trial 0021

The design of trial 0021 was very similar to trial 0020 except that fulvestrant was administered as two 125 mg injections (one in each buttock). There were 206 patients in the fulvestrant arm and 194 patients in the anastrozole arm. Average follow-up was 16.8 months. In the fulvestrant arm, 10 (4.9%) patients had a complete response, 26 (12.6%) had a partial response, and 51 (24.8%) had stable disease for more than 24 weeks. The arm receiving anastrozole had seven (3.6%) patients with a complete response, 27 (13.9%) with a partial response and 36 (18.6%) with stable disease for more than 24 weeks. On average, time to progression was 5.4 months with fulvestrant and 3.4 months with anastrazole.[4]

Both studies were combined for further analysis. As a second-line treatment, fulvestrant was similar to anastrazole in terms of overall survival. Further follow-up was obtained for patients who completely (20 in the fulvestrant group and 11 in the anastrazole group) or partially (62 in the fulvestrant group and 59 in the anastrazole group) responded to treatment. The duration of response was significantly longer in the fulvestrant group (16.7 months) compared with the anastrozole group (13.7 months). Both drugs were well tolerated and had similar side effect profiles that included hot flushes, gastrointestinal disturbances and thromboembolic disease. There were significantly more subjective complaints of joint pain in the anastrozole group compared to the fulvestrant group.[14]

Smaller clinical trials demonstrate that fulvestrant has promise after disease progression with aromatase inhibitors (AIs) in select patients. The first trial, the North Central Cancer Treatment Group Trial N0032, evaluated the use of fulvestrant in postmenopausal women who had disease progression of oestrogen receptor and/or progesterone receptor (ER/PR)-positive disease. Seventy-seven patients were previously treated with an AI or an AI plus one other antihormonal agent. Patients received anywhere from one to 10 cycles of treatment (average of two cycles because of disease progression). Of 21 patients who received only an AI in the past, six patients had a partial response and five patients had tumours that

did not progress during the 6 months they were followed. The remaining 56 patients received an AI and tamoxifen in the past. After subsequent treatment with fulvestrant in this group, 11 patients had tumours that did not progress for at least 6 months and five patients had a partial response. When evaluating the average disease progression of all 77 patients in the study, the clinical benefit rate (as defined by CR + PR + stable disease for at least 6 months) was 35.1% (90% CI, 26.0% to 45.0%).[15]

Another trial, known as SAKK 21/00, evaluated two groups of patients with advanced breast cancer. The first group (group A) initially had AI-responsive disease but progressed while on AIs (70 patients). The second group (group B) had AI-resistant disease and never responded to AIs (20 patients). All patients except two had been treated with an AI in the past. In group A, 84% had also received either tamoxifen or toremifene; in group B, 89% had received tamoxifen. Moreover, 36% of the patients in group A and 32% of those in group B had received prior chemotherapy. Patients in both groups received an average of four injections or 3.8 months of treatment with fulvestrant. In this study, only one patient in group A had a partial response. Of the remaining patients, 18 had stable disease for more than 24 weeks (11 for more than 36 weeks and six for more than a year). In group B, six patients had stable disease for more than 24 weeks, three patients had stable disease for more than 36 weeks, and one patient with metastatic lung disease had a complete response. The patient with a complete response was still on fulvestrant after 1 year.[16]

Finally, the question has been addressed as to whether fulvestrant is useful as a first-line agent in postmenopausal women instead of tamoxifen. Patients with advanced ER/PR-positive breast cancer were divided into two groups with similar demographics. The first group (313 patients) received fulvestrant as a single 250 mg intramuscular dose once a month. The second group (274 patients) was placed on 20 mg tamoxifen daily. Patients were followed for an average of 14.5 months. In patients with hormone receptor-positive tumours, tamoxifen had a non-significant benefit versus fulvestrant in terms of clinical benefit (62.7% versus 57.1%), with the overall conclusion being that fulvestrant had similar efficacy to tamoxifen in this setting.[17]

Summary

Based on the studies discussed above, it can be concluded that fulvestrant might potentially be used anywhere in the sequence of hormonal therapy. However, the likelihood of a response to fulvestrant is related to the number of prior antihormonal therapies. There is a higher probability of a response after a patient has responded to one therapy compared with after two successive therapies.

The question of what to do following fulvestrant failure is currently under investigation. Patients who initially respond to fulvestrant but subsequently have disease progression are likely to have breast cancer cells that retain their ER/PR positivity. These cancer cells are likely to respond to other endocrine treatment whether fulvestrant is used as a second-line or first-line agent.[18] Finally, because of the time required to reach a steady state with fulvestrant, questions have arisen about response times with fulvestrant and other agents. Anastrozole takes about 7 days to reach a steady state,[19] compared with fulvestrant which takes up to 6 months.[11] A retrospective study to analyse time to response in the trials that compared fulvestrant, anastrazole and tamoxifen indicated that these were similar for all three drugs.[20] New phase III trials are underway to evaluate the use of a 500 mg loading dose followed by 250 mg on days 14, 28 and monthly thereafter to see if the time to response can be decreased with fulvestrant.[20] Although fulvestrant has demonstrated that it is effective for patients who have failed prior hormonal therapy, new strategies to determine the optimum sequencing of this drug continue. Nevertheless, as our understanding of cellular signalling increases and newer targeted therapies evolve, the possibility of combining growth factor inhibitors with anti-oestrogens such as fulvestrant may offer benefits for disease control in the future.[21,22]

Acknowledgments

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References

1. Vergote I, Abram P. *Ann Oncol* 2006; **17**: 200–4.
2. Jordan VC. *Nat Rev Drug Discov* 2003; **2**: 205–13.
3. Jordan VC. *Cancer Cell* 2004; **5**: 207–13.
4. Osborne CK *et al. J Clin Oncol* 2002; **20**: 3386–95.
5. Wakeling AE *et al. Cancer Res* 1991; **51**: 3867–73.
6. Carlson RW. *Clin Breast Cancer* 2005; **6** (Suppl 1): S5–8.
7. Dauvois S *et al. Proc Natl Acad Sci U S A* 1992; **89**: 4037–41.
8. Alarid ET *et al. Mol Endocrinol* 1999; **13**: 1522–34.
9. Wijayaratne AL, McDonnell DP. *J Biol Chem* 2001; **276**: 35684–92.
10. Robertson JF, Harrison MP. *Cancer Chemother Pharmacol* 2003; **52**: 346–8.
11. Robertson JF *et al. Clin Pharmacokinet* 2004; **43**: 529–38.
12. Howell A. *Crit Rev Oncol Hematol* 2006; **57**: 265-73.
13. Howell A *et al. J Clin Oncol* 2002; **20**: 3396–403.
14. Robertson JF *et al. Cancer* 2003; **98**: 229–38.
15. Ingle JN *et al. J Clin Oncol* 2006; **24**: 1052–6.
16. Perey L *et al. Ann Oncol* 2007; **18**: 64–9.
17. Howell A *et al. J Clin Oncol* 2004; **22**: 1605–13.
18. Vergote I *et al. Breast Cancer Res Treat* 2003; **79**: 207–11.
19. Buzdar AU *et al. Cancer* 2002; **95**: 2006–16.
20. Dodwell D, Pippen J. *Clin Breast Cancer* 2006; 7: 244–7.
21. Nicholson RI *et al. Breast Cancer Res Treat* 2003; **80** (Suppl 1): S29–34. Discussion, *Breast Cancer Res Treat* 2003; **80** (Suppl 1): S35.
22. Gralow JR. *Breast Cancer Res Treat* 2005; **89** (Suppl 1): S9–S15.

Maximising local control in early breast cancer

An article by Associate Professor Peter Graham

Asscociate Professor Peter Graham

Associate Professor Peter Graham is a Radiation Oncologist from St. George Hospital, Sydney, and is the principal investigator of the TROG-STARS trial.

The management of early breast cancer requires the co-ordinated application of a number of therapeutic modalities, with many women routinely receiving breast-conserving surgery plus radiation therapy as well as adjuvant chemotherapy and/or hormonal therapy. However, despite extensive research into combinations of these therapies, the optimal sequence of adjuvant treatments remains uncertain.

Adjuvant radiotherapy, for instance, has been firmly established as the cornerstone of local disease control.[1] However, the optimal timing of radiotherapy – either concurrently with systemic adjuvant treatment or sequentially after completing systemic adjuvant treatment – remains to be fully established, particularly with respect to adjuvant hormonal therapy.

Radiotherapy and adjuvant chemotherapy

The order in which adjuvant therapy is administered remains an important consideration. It has been suggested that delaying radiotherapy following surgery may lead to increased local recurrence rates,[2,3] while delaying chemotherapy may have a detrimental effect on distant recurrence rates or survival.[3,4] Logistical issues also play an important role; in regional areas or areas in which there are waiting lists for radiotherapy, it makes sense for patients to have their chemotherapy while waiting for radiotherapy to start.

The optimal sequencing of radiation and chemotherapy was initially evaluated in a small study of 244 patients treated with breast-conserving surgery in which half the patients received radiation therapy before anthracycline-based chemotherapy and half received radiation therapy after anthracycline-based chemotherapy.[3] After a median of 58 months follow-up, the authors reported an increased risk of distant recurrence in the radiation therapy-first group (36% versus 25%, p = 0.05) and of local recurrence in the chemotherapy-first group (14% versus 5%, p = 0.07), leading to the recommendation that breast irradiation should be given after anthracycline-based chemotherapy in patients at substantial risk of systemic metastases.[3] However, longer follow-up revealed no difference in the rates of local or distant recurrence between the two treatment groups.[5]

A recent Cochrane report reviewed the available early breast cancer clinical trial evidence comparing the concurrent administration of chemotherapy and radiation therapy versus sequential administration (chemotherapy first, then radiotherapy and vice versa).[4] The review concluded that the order or synchronicity in which chemotherapy and radiation therapy is administered does not in fact have a major effect on survival or recurrence provided that both therapies are commenced within 7 months of surgery. Furthermore, the frequency and severity of side effects were similar regardless of the administration strategy.[4]

Although these results appear clear-cut, the authors do specify some caveats in drawing conclusions from this review. The first is that the treatments in the included trials were given approximately 10 years ago, and as such the radiotherapy and chemotherapy regimens, as well as the surgical technique, may be outdated compared with today. Secondly, although the length of follow-up in the included trials is sufficient to assess local failure rates, it is not yet mature enough to accurately assess mortality. Finally, the trials provided only limited information on adverse events, side effects or quality of life associated with the various strategies; therefore it is difficult to draw any conclusions regarding comparative tolerability.[4]

Radiotherapy and adjuvant hormonal therapy

Tamoxifen

Although tamoxifen has been shown to enhance local control of breast cancer compared with adjuvant radiotherapy alone,[6–8] conflicting data exist regarding the effect of tamoxifen on cancer cell radiosensitivity and its potential for increasing risk of radiation toxicities.

Tamoxifen is a cytostatic drug that arrests cells in the G1 phase of the cell cycle;[9] as such tamoxifen has the theoretical potential to compromise efficacy of radiation treatment because G1 is a relatively radioresistant phase of the cell cycle. However, in vitro studies testing this hypothesis have not provided consistent results. One study found that irradiated MCF-7 cells treated with tamoxifen were less radiosensitive,[10] while a similar study in MCF-7 cells irradiated and then incubated with 4-hydroxytamoxifen (the anti-oestrogenic metabolite of tamoxifen) showed no alteration in radiosensitivity compared with cells not exposed to tamoxifen.[11]

Similarly, conflicting clinical data exist regarding the risk of toxicities when tamoxifen and radiotherapy are administered concurrently. One study in post-mastectomy patients receiving radiotherapy and tamoxifen reported an increased risk of lung fibrosis.[12] However, in a study designed to quantify radiographic changes in the lungs of irradiated patients using computed tomography scans, no correlation of lung density changes and the use of concurrent tamoxifen was identified.[13] The practice of many clinicians as a result of these

Table 1. Local failure and overall survival rates in three studies comparing concurrent (Conc) versus sequential (Seq) radiotherapy and tamoxifen treatment[15–17]

Tamoxifen sequencing efficacy				
	% Local failure		% Overall survival	
	Conc	Seq	Conc	Seq
Ahn	10	14	84	80
Harris	3	7	81	86
Pierce	7	5	88	90

inconsistencies is to delay initiation of tamoxifen until completion of radiotherapy. No majority support was established at the St. Gallen 2007 consensus meeting on whether endocrine therapy should be delayed until after radiation therapy, or whether it should be commenced prior to radiation therapy.[14]

In January 2005, three non-randomised retrospective studies assessing the timing of tamoxifen and radiation therapy spanning 1082 patients appeared in the *Journal of Clinical Oncology*.[15–17] Two of the studies[15,16] demonstrated a 4% lower in-breast failure rate when the two modalities were administered concurrently, with the third study suggesting a 2% in-breast failure benefit when administered in sequence (**Table 1**).[17] However, the reverse was true when considering overall survival; two of the studies[16,17] demonstrated a modest overall survival advantage (5% and 2%, respectively) in favour of sequential administration, while the third study demonstrated a 4% advantage in favour of concurrent therapy.[15] The pattern of potential differences is therefore heterogeneous, and no statistically significant differences in recurrence, survival or in-breast failure were identified in any of these studies.

The logical take-home message from these studies is that tamoxifen might be administered concurrently or sequentially with radiation therapy without significantly affecting efficacy. However, due to the inherent limitations of the study designs it is not possible to draw firm conclusions from these studies. In fact each of the authors advocate the development of a suitably designed randomised trial of the timing of hormone therapy.

Aromatase inhibitors

Due to their superior efficacy and overall tolerability benefits, the aromatase inhibitors (AIs) are superseding tamoxifen as the 'gold standard' hormonal therapy for the management of early breast cancer in postmenopausal women.[18] Less information is known about the timing of radiation therapy with AIs, though preclinical data suggest that AIs might enhance radiosensitivity of breast cancer cells.

In an in vitro study using MCF-7 breast cancer cells expressing the aromatase gene, exposure to 2 Gray radiation was associated with a two-fold enhanced cell kill in cells pre-incubated with an AI (letrozole) compared with controls.[19] My laboratory has recently completed a similar study assessing the sequencing of anastrozole and radiotherapy (manuscript in press). However, in contrast to the letrozole study, we included an assessment of the addition of anastrozole both before and after irradiation.

Our study confirmed that cells pretreated with anastrozole prior to 2 Gray radiation displayed a similar degree of cell kill as that seen in the letrozole trial; furthermore, cell kill was more pronounced when cells were pretreated with anastrozole before irradiation rather than exposed to anastrozole post irradiation. Compared to 2 Gray alone, inhibition of proliferation at 7 days was 80% with pre-radiation anastrozole and 60% with post-radiation anastrozole. There was no evidence of radiation protection as a result of pre-radiotherapy anastrozole incubation.

The above results were obtained using a single fraction of radiation. In clinical practice, however, radiotherapy is delivered as a multi-fractionated course, where the results of a single fraction might be multiplied by the effect of anywhere between 16 and 25 further fractions of radiotherapy. The overall cumulative effect may therefore be a 3 to 4 log difference in cell kill. In addition, reducing oestrogen exposure has been shown to strongly diminish inter-fraction tumour cell repopulation in mouse xenograft experiments.[20]

The preclinical data outlined above, allied with the potential for reduced radiation toxicities, suggest that concurrent or sequential application of AIs with radiation therapy is a strong candidate for enhancing local control, compared to concurrent or sequential application of tamoxifen and radiation therapy. It is my belief that this supposition merits testing in a well-designed, randomised clinical trial.

TROG-STARS (STudy of Anastrozole and Radiotherapy Sequencing) Trial

STARS is an investigator-led, randomised clinical trial assessing the effect of anastrozole commenced before and continued during adjuvant radiotherapy for breast cancer versus anastrozole (and subsequent anti-oestrogen therapy) delayed until after radiotherapy.

STARS is designed to answer a key clinical question: can anastrozole enhance breast cancer cell kill induced by radiotherapy and therefore have a positive effect on local control? As such, the trial design randomises subjects with newly-diagnosed breast cancer to receive anastrozole before or after radiotherapy (**Figure 1**).

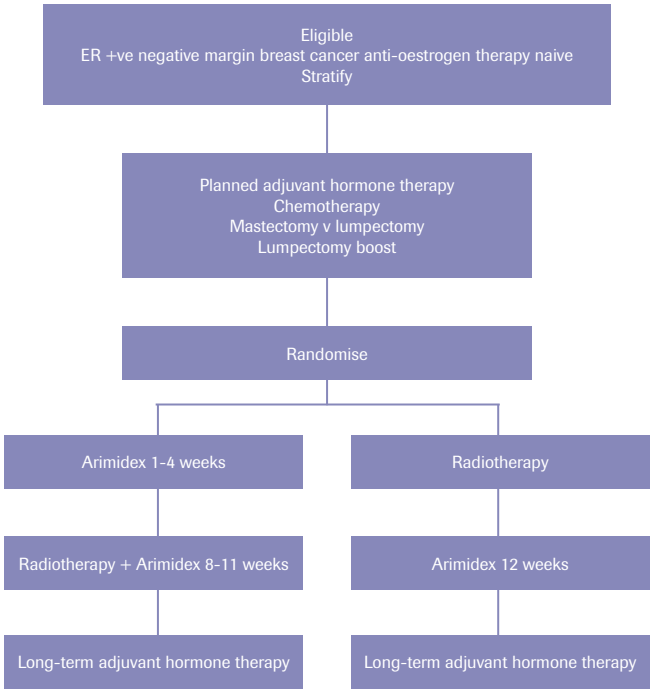

Figure 1. TROG-STARS trial schema

STARS is being run country-wide in conjunction with TROG (Trans-Tasman Research Oncology Group). The primary endpoint is local control rate, with secondary endpoints assessing survival, rib fracture rate, cosmetic outcome and pulmonary fibrosis. It is anticipated the study will open in 2007 and the aim is to randomise 2000 patients over the next 5 years; a modest pilot study has confirmed that this target is eminently achievable.

If you are interested in participating in this study or have any further questions, please contact me via email on GrahamP@SESAHS.NSW.GOV.AU.

References

1. Early Breast Cancer Triallists' Collaborative Group (EBCTCG). Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005; **366**: 2087–106.
2. Huang J, Barbera L, Brouwers M *et al.* Does delay in starting treatment affect outcomes of radiation therapy? A systematic review. *J Clin Oncol* 2003; **21**: 555–63.
3. Recht A, Come S, Henderson C *et al.* The sequencing of chemotherapy and radiation therapy after conservative surgery for early-stage breast cancer. *New Engl J Med* 1996; **334**: 1356–61.
4. Hickey BE, Francis D, Lehman MH. Sequencing of chemotherapy and radiation therapy for early breast cancer. *Cochrane Database Syst Rev* 2006, Issue 4. Art. No.: CD005212. DOI: 10.1002/14651858.CD005212.pub2.
5. Bellon JR, Come SE, Gelman RS *et al.* Sequencing of chemotherapy and radiation therapy in early stage breast cancer: Updated results of a prospective randomized trial. *J Clin Oncol* 2005; **23**: 1934–40.
6. Fowble B, Fein D, Hanlon A *et al.* The impact of tamoxifen on breast recurrence, cosmesis, complications and survival in estrogen receptor-positive early-stage breast cancer. *Int J Radiat Oncol Biol Phys* 1996; **35**: 669–77.
7. Rutqvist LE, Cedermark B, Glas U *et al.* Randomised trial of adjuvant tamoxifen in node negative postmenopausal breast cancer. *Acta Oncol* 1992; **31**: 265–70.
8. Leborgne F, Leborgne JH, Ortega B *et al.* Breast conservation treatment of early stage breast cancer: Patterns of failure. *Int J Radiat Oncol Biol Phys* 1995; **31**: 765–75.
9. Osborne C, Boldt D, Clark G *et al.* Effects of tamoxifen on human breast cancer cell cycle kinetics: Accumulation of cells in early G1 phase. *Cancer Res* 1983; **43**: 3583–5.
10. Wazer DE, Tercilla OF, Lin P-S *et al.* Modulation in the radiosensitivity of MCF-7 human breast carcinoma cells by 17α-estradiol and tamoxifen. *Br J Radiol* 1989; **62**: 1079–83.
11. Sarkaria JN, Miller EM, Parker CJ *et al.* 4-Hydroxytamoxifen, an active metabolite of tamoxifen, does not alter the radiation sensitivity of MCF-7 breast carcinoma cells irradiated in vitro. *Breast Cancer Res Treat* 1994; **30**: 159–65.
12. Bentzen S, Skoczylas J, Overgaard M et al. Radiotherapy-related lung fibrosis enhanced by tamoxifen. *J Natl Cancer Inst* 1996; **88**: 918–22.
13. Wennberg B, Gagliardi G, Sundbom L *et al.* Early response of lung in breast cancer irradiation: Radiologic density changes measured by CT and symptomatic radiation pneumonitis. *Int J Radiat Oncol Biol Phys* 2002; **52**: 1196–206.
14. Goldhirsch A, Wood WC, Gelber RD *et al.* Progress and Promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. *Ann Oncol* 2007; **18**: 1133–44.
15. Ahn PH, Vu HT, Lannin D *et al.* Sequence of radiotherapy with tamoxifen in conservatively managed breast cancer does not affect local relapse rates. *J Clin Oncol* 2005; **23**: 17–23.
16. Harris EER, Christensen VJ, Hwang W-T *et al.* Impact of concurrent versus sequential tamoxifen with radiation therapy in early-stage breast cancer patients undergoing breast conservation treatment. *J Clin Oncol* 2005; **23:** 11–6.
17. Pierce LJ, Hutchins lF, Green SR *et al.* Sequencing of tamoxifen and radiotherapy after breast-conserving surgery in early-stage breast cancer. *J Clin Oncol* 2005; **23**: 24–9.
18. ATAC Triallists' Group. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* 2005; **365**: 60–2.
19. Azria D, Larbouret C, Cunat S *et al.* Letrozole sensitizes breast cancer cells to ionizing radiation. *Breast Can Res* 2005; **7:** R156–R163.
20. Sarkaria JN, Fowler JF, Lindstrom MJ *et al.* The decreased influence of overall treatment time on the response of human breast tumor xenografts following prolongation of the potential doubling time (Tpot). *Int J Radiat Oncol Biol Phys* 1995; **31**: 833–40.

Magnetic resonance imaging in women at high risk of breast cancer

An article by Professor Christobel Saunders

Professor Christobel Saunders

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Approximately 5% of women who develop breast cancer have a known inheritable genetic mutation;[1] however, up to 15% have a strong family history in the absence of a known mutation. These mutations are usually located in the high-risk cancer susceptibility genes BRCA1[2] or BRCA2[3] and, unfortunately, many of these patients will be young at diagnosis compared to patients with sporadic breast cancer. Familial risk can be estimated by applying the National Breast Cancer Centre (NBCC) criteria for women at potentially high risk (risk category 3, see **Table 1**).[4]

However, other women can be identified who are potentially at high risk of developing breast cancer. These include women with previously diagnosed high-risk breast pathology such as atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS), those with previous mantle irradiation for a lymphoma, and those with dense breasts on mammography. Of course, there are many other environmental and physiological factors that contribute to a lesser degree to breast cancer risk, but how these interact with, for example, an inherited gene mutation remains unclear, especially for the individual woman.

Table 1. NBCC advice on familial aspects of breast cancer[4]

Category 3. Potentially high risk Covers much less than 1% of the female population

• Two 1° or 2° relatives on one side of the family diagnosed with breast or ovarian cancer **plus** one or more of the following features on the same side of the family:
  • additional relative(s) with breast or ovarian cancer
  • breast cancer diagnosed before the age of 40
  • bilateral breast cancer
  • breast **and** ovarian cancer in the same woman
  • Jewish ancestry
  • breast cancer in a male relative
• One 1° or 2° relative diagnosed with breast cancer at age 45 or younger **plus** another 1° or 2° relative on the same side of the family with sarcoma (bone/soft tissue) at age 45 or younger
• Member of a family in which the presence of a high-risk breast cancer gene mutation has been established

Lifetime risk of breast cancer: 1 in 4 to 1 in 2, or possibly higher if shown to have a high-risk mutation
Risk may be more than 3 times the population average. Individual risk may be higher or lower if genetic results are known

Population mammographic screening programmes using age as a risk criterion have been in place in Australia since 1992, and in some states a family history of breast cancer is a criterion used for more frequent mammographic screening. With the advent of DNA-based genetic testing, women at high risk due to BRCA gene mutations can be identified, but as yet there is no proven modality of screening that will reduce their risk of dying from breast cancer. In younger women at high risk for developing breast cancer, the value of mammography is limited by a higher prevalence of dense breast tissue, a low sensitivity of this imaging modality, and concerns over radiation exposure. Ultrasound has not yet been demonstrated as a useful tool in screening high-risk women – at least not as a stand-alone modality – and although clinical breast examination may improve the sensitivity of screening, a better screening tool than current convention endorses has long been sought.

In the last few years, a number of studies have demonstrated the value of breast magnetic resonance imaging (MRI) as part of imaging surveillance for high-risk patients.[5–10] All of these studies used family history or genetic status as a high-risk criterion and there is no evidence yet for MR screening in women at high risk due to other reasons such as previous high-risk pathology. MRI of the breast is reported to have high sensitivity but lower specificity than mammography.[11] A meta-analysis has found MRI to have better discrimination compared to mammography in determining whether a high-risk woman has breast cancer. Sensitivity was 80.1% (95% CI 73.3–85.8%) for MRI and 36.8% (95% CI 29.6–44.5%) for mammography, and specificity was 93.0% (95% CI 92.5–93.6%) for MRI and 97.5% (95% CI 97.1–97.8%) for mammography.[12] This decreased specificity may result in an increase in lesions detected that are ultimately benign but which require investigation – and thus poses a dilemma as lesions seen only on MRI can be difficult to localise for biopsy.

Breast MRI is performed by a limited number of units in Australia and requires not only general breast imaging expertise but a 1.5T or 3T magnet with dedicated breast coil and the ability to biopsy observed lesions. Gadolinium is used as a contrast agent and although the technical sequences used to acquire the images is beyond the scope of this paper, like mammographic screening, images should be double read and reported preferably using the BI-RADS MRI lexicon and 7-point scale.[13] Breast MRI examinations in premenopausal women are done on days 7–12 of the cycle, as the cycling breast can "light up" and make reading difficult. If a suspicious lesion is found on MR (and not seen on mammography), a targeted ultrasound should be performed to try to correlate with the MR-lesion in terms of location, size and morphology. MRI intervention tools

(MR compatible biopsy devices and MRI-guided hookwire localisations) are likely to be the best way to sample MRI-only detected lesions.[14,15] However, if they are not available, and the lesion is detected on MRI only, it is recommended the patient has short-term (3 to 6 month) follow-up MRI.

The need to correlate all imaging and clinical findings coupled with the considerable learning curve of this technique underlines the immense importance of carrying out breast MRI examination and reporting in centres with related expert multidisciplinary experience in breast surveillance, including MRI imaging, in order to gain sufficient competence in the technique and interpretation of images.

In the absence of much experience in this technique in Australia, and with no surveillance programmes using MRI up and running, we set out to explore the efficacy of three modalities of annual screening for breast cancer – mammography, MRI and high-resolution ultrasound along with clinical breast examination – within a prospective, non-randomised clinical trial from June 2002 to October 2005 (paper submitted). This study was conducted in women 50 years of age or younger at potentially high risk of developing breast cancer. In 72 participants (139 MR investigations), 15 lesions were detected, of which three were of significance: one metastatic papillary cancer in an axillary lymph node; one borderline lesion; and one solid atypical lesion. All 15 lesions were visible on MRI, with four lesions visible on MRI only. Mammography and screening ultrasound detected three of the 15 lesions each. The use of targeted ultrasound to locate lesions seen initially on MRI only successfully identified a further six lesions. Four lesions were found by MRI only. All four lesions were managed with repeat MRI scans within 6 months and remained stable. Other outcomes recorded were an overall recall rate of 10.1% in the 2-year period of the study (12.5% in the first year; in the second year this reduced to 7.5%). No women with false-positive MRI have developed breast pathology at a mean of over 3 years of follow-up.

The results of the study suggest that MRI was more accurate than mammography or ultrasound in detecting breast lesions in young women at high risk for developing breast cancer. MRI generated more findings judged as uncertain, so short-term follow-up or MR-guided biopsy techniques are required to support diagnostic MRI. In addition, we were able to show that targeted screening using MRI in a high-risk surveillance clinic is feasible. However, it does rely on close co-operation of a multidisciplinary team, access to MRI magnet time, adequate funding, assistance of a trained breast nurse (or similar) to recruit patients and collect good audit data, and finally, educational initiatives for all staff.

Following on from this work, a group of interested individuals last year submitted an application to the Medical Services Advisory Committee of the Department of Health and Ageing in Australia for a Medicare Item Number for Breast MRI to screen high-risk young women (http://www.MSAC.gov.au). This application has been supported by the Minister and the details of the item number are now under consideration, but are likely to mean there will be a rebate for the test for women under 50, referred by a specialist, with a high-risk family history on NBCC criteria, or a proven genetic mutation in a cancer susceptibility gene. Further details including how to accredit the providers of the screening tests, how to ensure the woman is part of a surveillance programme, and indeed whether the surveillance programme could be administered via BreastScreen Australia, are under discussion.

I would support the careful introduction of breast MRI in Australia in the setting of a specialist multidisciplinary clinic for management of women at high risk of breast cancer. The programme should include training of radiological and clinical personnel in these techniques, and a detailed national audit of outcomes. Controlled programmes of this nature would facilitate addressing current dilemmas as to whether MRI screening in high-risk women has an impact on mortality from this disease.

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References

1. Claus EB, Risch N, Thompson WD. *Am J Hum Genet* 1991; **48**: 232–42.
2. Hall JM, Lee MK, Newman B *et al*. *Science* 1990; **250**: 1684–9.
3. Wooster R, Neuhausen S, Mangion J *et al*. *Science* 1994; **265**: 2088–90.
4. National Breast Cancer Centre. Advice about familial aspects of breast cancer and epithelial ovarian cancer. A guide for health care professionals. February 2006.
5. Kuhl, CK, Schrading S, Leutner CC *et al*. *J Clin Oncol* 2005; **23**: 8469–76.
6. Leach MO, Boggis CR, Dixon AK *et al*. *Lancet* 2005; **365**: 1769–78. Erratum in: *Lancet* 2005; **365**: 1848.
7. Kriege M, Brekelmans CT, Boetes C *et al*. *New Engl J Med* 2004; **351**: 427–37.
8. Warner E, Plewes DB, Hill KA *et al*. *JAMA* 2004; **292**: 1317–25.
9. Podo F, Sardanelli F, Canese R *et al*. *J Exp Clin Cancer Res* 2002; **21**: 115–24.
10. Stoutjesdijk MJ, Boetes C, Jager GJ *et al*. *J Natl Cancer Inst* 2001; **93**: 1095–102.
11. Heywang-Kobrunner SH, Bick U, Bradley WG Jr *et al*. *Eur Radiol* 2001; **11**: 531–46.
12. Warner E, Messersmith H, Causer P *et al*. MRI Screening of women at high risk for breast cancer: A Clinical Practice Guideline 2007. [Online] [Accessed 4 September 2007] Available at URL: http://www.cancercare.on.ca/pdf/pebcmris.pdf
13. American College of Radiology (ACR). ACR BI-RADS® -Magnetic Resonance Imaging. In: ACR Breast Imaging Reporting and Data System, Breast Imaging Atlas. Reston, VA. American College of Radiology, 2003.
14. Perlet C, Heinig A, Prat X *et al*. *Eur Radiol* 2002; **12**: 1463–70.
15. Viehweg P, Heinig A, Amaya B *et al*. *Eur J Radiol* 2002; **42**: 32–9.

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CLINICAL INVESTIGATION **Breast**

LUMPECTOMY PLUS TAMOXIFEN OR ANASTROZOLE WITH OR WITHOUT WHOLE BREAST IRRADIATION IN WOMEN WITH FAVORABLE EARLY BREAST CANCER

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Purpose: In women with favorable early breast cancer treated by lumpectomy plus tamoxifen or anastrazole, it remains unclear whether whole breast radiotherapy is beneficial.
Methods and Material: Between January 1996 and June 2004, the Austrian Breast and Colorectal Cancer Study Group (ABCSG) randomly assigned 869 women to receive breast radiotherapy ± boost (n = 414) or not (n = 417) after breast-conserving surgery (ABCSG Study 8A). Favorable early breast cancer was specified as tumor size <3 cm, Grading 1 or 2, negative lymph nodes, positive estrogen and/or progesterone receptor status, and manageable by breast-conserving surgery. Breast radiotherapy was performed after lumpectomy with 2 tangential opposed breast fields with mean 50 Gy, plus boost in 71% of patients with mean 10 Gy, in a median of 6 weeks. The primary endpoint was local relapse-free survival; further endpoints were contralateral breast cancer, distant metastases, and disease-free and overall survival. The median follow-up was 53.8 months.
Results: The mean age was 66 years. Overall, there were 21 local relapses, with 2 relapses in the radiotherapy group (5-y rate 0.4%) vs. 19 in the no-radiotherapy group (5.1%), respectively (p = 0.0001, hazard ratio 10.2). Overall relapses occurred in 30 patients, with 7 events in the radiotherapy group (5-y rate 2.1%) vs. 23 events in the no-radiotherapy group (6.1%) (p = 0.002, hazard ratio 3.5). No significant differences were found for distant metastases and overall survival.
Conclusion: Breast radiotherapy ± boost in women with favorable early breast cancer after lumpectomy combined with tamoxifen/anastrazole leads to a significant reduction in local and overall relapse. © **2007 Elsevier Inc.**

Breast irradiation, Women with favorable early breast cancer, Lumpectomy plus hormone therapy with/without irradiation.

INTRODUCTION

In breast-conserving treatment, whole breast radiotherapy is in general recognized as being capable of significantly reducing local recurrence (1, 2) and is therefore considered a standard constituent of the interdisciplinary treatment regime for a vast majority of breast cancer patients. According to recent findings of the Early Breast Cancer Trialists'

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Conflict of interest: none.

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Table 1. Trial profile

869 randomized women in ABCSG trial 8A 38 ineligible 831 women included in Intention to treat analysis	
Radiotherapy Group (RT) (n = 414)	No Radiotherapy Group (no RT) (n = 417)
Assigned to be treated with surgery, hormone therapy (tamoxifen/anastrozole) and radiotherapy 4 refused radiotherapy	Assigned to be treated with surgery and hormone therapy (tamoxifen/anastrozole) 13 received radiotherapy

Abbreviation: ABCSG = Austrian Breast and Colorectal Study Group.

Collaborative Group (EBCTCG), whole-breast radiotherapy applying modern techniques may even have an impact on long-term survival (3). However, because of logistic and economic burdens and adverse side effects associated with breast irradiation, there have been several attempts to identify a subgroup of patients who might not need radiotherapy to achieve an acceptable level of local control. Because adjuvant endocrine treatment not only improves disease-free survival but also provides local control, such a subgroup will most likely comprise endocrine-responsive disease. Selective estrogen receptor modulators, such as tamoxifen, have been proven to significantly reduce local breast recurrence (4, 5). More recently, third-generation aromatase inhibitors (*e.g.*, anastrozole) have been tested against tamoxifen alone and have been shown to significantly reduce any disease recurrence and slightly improve overall survival (6–8).

In women with favorable early breast cancer (postmenopausal status, small-size tumors, lymph node-negative, Grade 1 and 2, hormone-responsive disease), it has been an issue of controversial debates whether patients benefit from additional whole-breast radiotherapy, in particular when adjuvant long-term hormonal treatment is applied.

More recently, randomized trials of women with favorable early breast cancer treated with tamoxifen for 5 y (20 mg/day) have demonstrated a significant effect on breast recurrence when whole-breast radiotherapy was applied (9), even in patients older than 70 years (10).

The role of switching from tamoxifen to anastrozole has been tested in a randomized trial conducted by the Austrian Breast and Colorectal Cancer Study Group (ABCSG trial 8) and others (11), and the switch to anastrozole showed beneficial effects on event-free survival (6).

Within this trial of postmenopausal women treated with breast-conserving surgery and hormonal treatment including tamoxifen and anastrozole (50%), a favorable subgroup of patients with early disease was selected prospectively on the basis of a retrospective analysis of highly selected patients treated at Vienna General Hospital from 1983 to 1994 (12). This subgroup of patients with a very favorable risk profile was invited to participate in a randomized trial to evaluate whether whole-breast irradiation is still beneficial with regard to local relapse-free survival, disease-free survival, and overall survival.

METHODS AND MATERIALS

Patients

The ABCSG trial 8A was a prospective, multicenter, randomized trial for a favorable subgroup of patients participating in ABCSG-8 (6).

Between January 1996 and June 2004, 869 women were assigned to radiotherapy vs. no radiotherapy.

Eligible patients were postmenopausal women with histologically verified, locally radically treated invasive or minimally invasive breast cancer who had received no previous chemotherapy, hormone therapy, or radiotherapy.

Postmenopausal status was assumed for patients whose last menstruation took place at least 12 months before study entry, for those who had undergone bilateral ovarectomy, or those for whom follicle-stimulating hormone and luteinizing hormone concentrations indicated postmenopausal status. All patients had a tumor 3 cm or less in diameter, pathologic stage T1 or early T2, a G1 or G2 ductal carcinoma, or a Gx lobular tumor. Important inclusion criteria were the absence of both positive lymph nodes and organ metastases. All patients had endocrine-responsive tumors, with positive estrogen and/or positive progesterone receptors.

Thirty-eight women were ineligible (because of positive lymph nodes, tumor diameter >3 cm, nonbreast-conserving surgery, no preclusion of distant metastases, no R0 resection, proven existence of carcinoma before randomization, no informed consent, premenopausal status, or violation of other inclusion criteria).

The intention-to-treat analysis included 831 randomized patients.

Eligible patients were randomized into 2 groups (Table 1): the radiotherapy (RT) group (n = 414) receiving whole-breast radiotherapy ± boost to the tumor bed after surgery plus adjuvant hormone therapy, and the no-radiotherapy (no-RT) group (n = 417) given exclusively endocrine treatment post surgery. Seventeen women did not receive the treatment of the group into which they were randomized: 4 patients refused radiotherapy in the RT group and 13 patients received radiotherapy in the no-RT group.

All patients provided written informed consent. The study was approved by the relevant ethics committees in Austria.

Patient characteristics

Patients received a physical examination and were monitored for safety and tolerance. Monitoring took place at 3-monthly intervals throughout the first 3 years, at 6-monthly intervals in the fourth and fifth years, and yearly thereafter.

Gynecological examinations, chest wall radiographs, bone scintigraphy, and standard mammography were done as appropriate to identify the presence of disease recurrence.

Table 2. Baseline patient characteristics

	Radiotherapy group (414)	No Radiotherapy group (417)
	Number of women (%)	
Age at diagnosis (y)		
<50	9 (2)	5 (1)
50–59	115 (28)	115 (28)
60–69	145 (35)	149 (36)
>70	145 (35)	148 (35)
Tumor size		
pT1b	143 (35)	136 (33)
pT1c	228 (55)	246 (59)
pT2	43 (10)	35 (8)
Pathological grade		
G1	135 (33)	138 (33)
G2	257 (62)	259 (62)
Gx	22 (5)	20 (5)
Estrogen receptor status		
+++	252 (61)	278 (67)
++/+	156 (38)	134 (32)
Negative	6 (1)	5 (1)
Progesterone receptor status		
+++	122 (29)	128 (31)
++/+	207 (50)	208 (50)
Negative	85 (21)	79 (19)
Unknown	0	2 (<1)

The majority of women in both groups had a tumor stage pT1c and G2 disease (Table 2).

The treatment groups were well balanced in terms of age, tumor stage and grade, estrogen receptor and progesterone receptor status, and by the type of systemic therapy (RT/no-RT group: 50.5%/49.1% tamoxifen and 49.5%/50.9% anastrozole).

The mean age at the time of diagnosis was 65.7 years (RT group = 65.4, no-RT group = 66.1). The youngest patient was 46 years old, and the oldest was 80 years. All 831 eligible women were lymph node-negative.

Treatment

Surgery and hormone therapy. All women underwent breast-conserving surgery, lumpectomy or wide resection with appropriate margins (aim = 10 mm). Classical quadrantectomy was used only in occasional cases when R0 resection was not achievable otherwise.

Axillary lymph node dissection was performed, with a minimum of 10 lymph nodes removed as prerequisite for inclusion into the trial.

In 2001, an amendment of the study protocol was introduced, allowing for sentinel lymph node biopsy after extensive quality control in individual centers.

Invasive ductal carcinoma grade G1 or G2 and lobular tumors were the predominant pathohistological characteristics.

Surgery was followed by adjuvant hormone therapy for 5 years, which started within 6 weeks after surgery. After 2 years of adjuvant oral tamoxifen therapy (20 mg/day), the women who were randomly assigned (before the beginning of hormone therapy) switched to 1 mg anastrozole once daily for 3 years.

Radiotherapy. Radiotherapy to the whole breast was to be given within 6 weeks post surgery in the radiotherapy group. Three different options of irradiation were applied (Table 3): external beam whole-breast photon radiotherapy alone, external beam whole-breast photon radiotherapy plus electron, or the same plus iridium 192 boost. Whole-breast dose, boost dose, fractionation, and technique of radiotherapy were at the discretion of the participating center, which followed its traditional treatment schedule for adjuvant radiotherapy in this patient group.

The mean dose of whole-breast irradiation was 51 Gy (± 4 Gy), given in daily fractions to the breast and adjacent chest wall over a period of 39 days (±7 days). A parallel-opposed pair of tangential fields was treated daily, Monday to Friday, and photons or cobalt 60 gamma rays were used.

In 269 women, whole-breast radiotherapy was followed by an electron boost with a mean dose of 10 Gy (±2 Gy) to the tumor bed.

The target volume was determined on the basis of the preoperative mammogram, operative notes, clinical assessment and location of clips, if available.

Twenty women were treated with an iridium 192 boost of mean 9 Gy (±2 Gy) in high-dose-rate afterloading technique.

End points

The major end point was local relapse-free survival defined as time between randomization and occurrence of local relapse. Other points of interest were disease-free survival (defined as time between randomization and the first occurrence of local relapse or distant metastasis), overall survival (time between randomization and death of any cause), and incidence of contralateral breast cancer and distant metastasis, respectively.

Statistics

Randomization for the study was done centrally at the ABCSG randomization center in Vienna, Austria. Patients were allocated to the treatment groups according to the method of Pocock and Simon (13), stratifying for the following prognostic factors: age, tumor stage, tumor grade, treatment (tamoxifen or anastrozole), and participating centers grouped into federal states.

Analyses were by intention to treat. Additional sensitivity anal-

Table 3. Radiation dose, technique, and duration in RT group (n = 414)

	Mean dose	Mean overall treatment time
Whole-breast radiotherapy alone (n = 118)	51 Gy (± 4 Gy)	39 d (± 7)
Whole-breast radiotherapy (plus boost) (n = 289)	50 Gy (± 2 Gy)	46 d (± 8)
Electron boost (n = 269)	10 Gy (± 2 Gy)	46 d (± 7)
Iridium 192 boost (HDR brachytherapy) (n = 20)	9 Gy (± 2 Gy)	43 d (± 9)

Abbreviations: HDR = high-dose-rate; RT = radiotherapy.

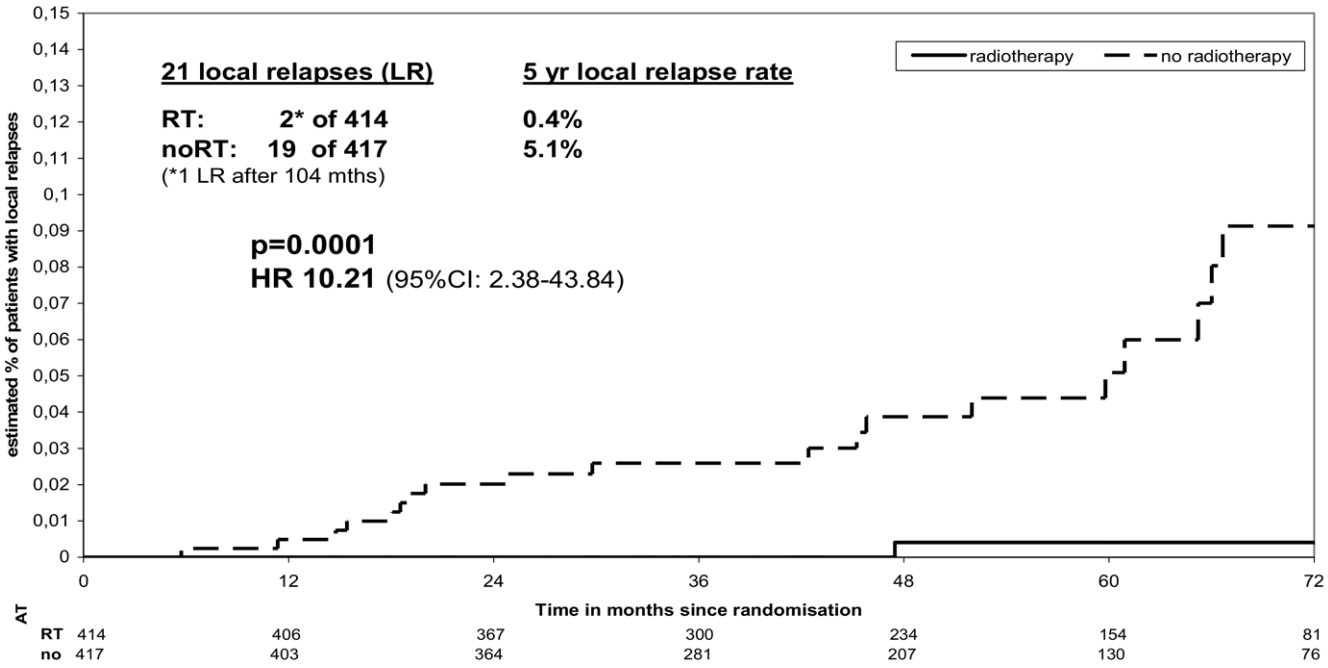

Fig. 1. Local relapse (n = 831). HR = Hazards Ratio; RT = radiotherapy.

yses were performed by allocating patients to treatment groups (RT/no-RT) according to their actual treatment. Data are presented in absolute numbers, percentages, and Kaplan-Meier curves (14). Data were tested by log-rank tests (15, 16). Hazard ratios (HR) and their corresponding 95% confidence intervals (CI) were estimated by the proportional-hazards regression model of Cox (17).

All p values are two-sided, and a p value < 0.05 was significant.

The ABCSG statistician analyzed all data using the statistical software package SAS (version 8.02; SAS Institute, Cary, NC).

Quality control: Because local relapse was the primary end point in this trial, the surgery and pathology reports of all patients suffering a local relapse were centrally reviewed by a pathologist and surgeon blinded for treatment allocation to assess the quality of primary treatment.

RESULTS

A total of 869 women were randomized in ABCSG trial 8A to either radiotherapy after lumpectomy and adjuvant hormone therapy or only lumpectomy and adjuvant hormone therapy.

The median follow-up was 53.8 months after randomization.

The results show a significant reduction in breast recurrence among the patients who received radiation. Two local relapses were observed in the RT group (414 women) compared with 19 in the no-RT group (417 women). One relapse in the RT group occurred after 104 months.

This corresponds to a hazard ration of 10.21 (95% CI, 2.38–43.84) with a p value of 0.0001 (Fig. 1).

Overall, 10 women presented with distant metastases, 5 in the RT group vs. 5 in the no-RT group.

With regard to disease-free survival, there were 7 events in the RT group vs. 23 events in the no-RT group (overall relapse). One woman presented with 2 events simultaneously, both local relapse and distant metastasis. The HR for disease-free survival is 3.48 (95%CI, 1.49–8.12) with a p value of 0.0021 (Fig. 2).

There were 2 women with contralateral breast cancer in the RT group vs. 5 in the no-RT group.

The 5-year survival rate (overall 96.2%) was slightly higher in patients who were treated with radiotherapy (97.9%) than in those who received lumpectomy and adjuvant hormone therapy alone (94.5%). There were 11 deaths in the RT group and 18 deaths in the no-RT group, respectively; the difference was not statistically significant (p = 0.18). In the RT and no-RT groups, 2 deaths occurred each after a preceding recurrence (total deaths = 4). There were 9 deaths in the RT group and 16 deaths in the no-RT group without preceding recurrence.

All results derived from the intention-to-treat analysis are robust with respect to patients who did not receive their randomized treatment. If such patients are allocated to treatment groups according to their actual treatment, results remain unchanged.

DISCUSSION

This trial demonstrates that the addition of radiotherapy to tamoxifen and/or anastrazole significantly reduces the rate of local relapse, even in this prognostically favorable group of women with early breast cancer characterized by postmenopausal status, hormone responsiveness, small tumor size, good differentiation, and negative lymph node

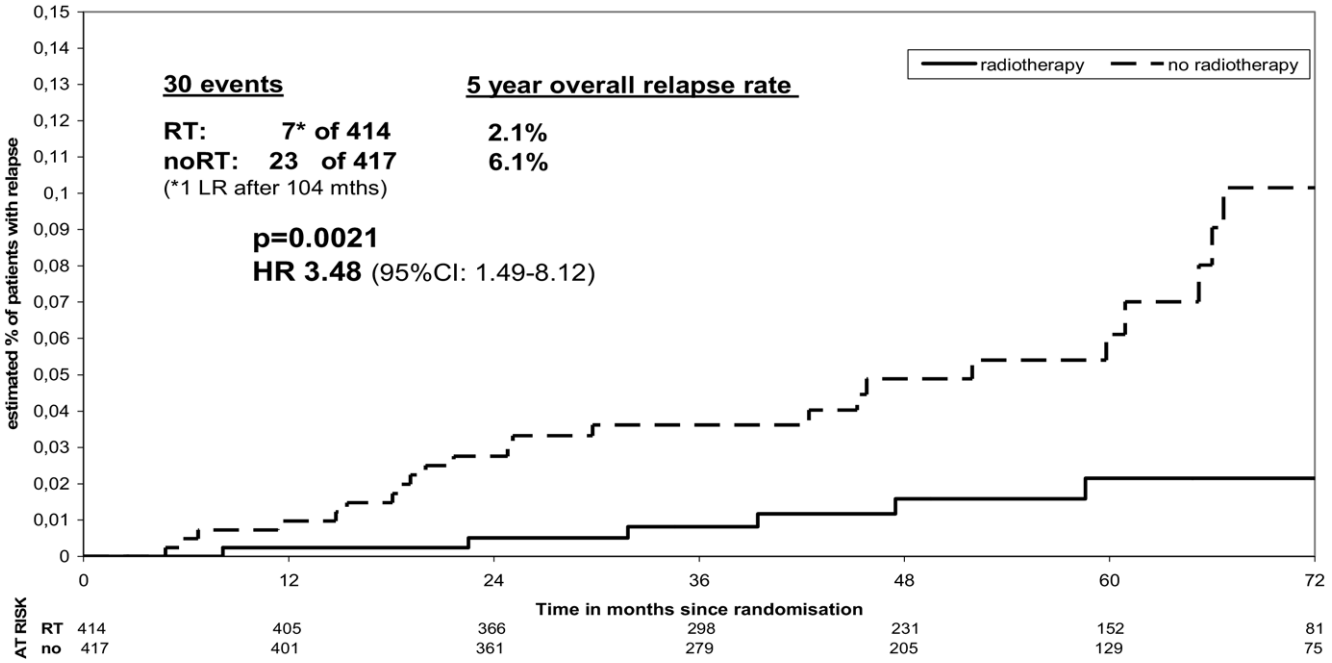

Fig. 2. Overall relapse (n = 831). HR = Hazard Ratio; RT = radiotherapy.

status. The estimated 5-year actuarial rate of 0.4%, after a median follow-up of 54 months, is to be considered as outcome of excellent local control and is significantly different from the rate of 5.1% for those without radiotherapy (Fig. 1). The trial thus failed to prospectively identify a subgroup of women with early breast cancer not benefiting from adjuvant radiotherapy.

Our overall results are comparable with 5-year actuarial local relapse rates as recently reported by Fyles *et al.* and Hughes *et al.*, with 0.6% vs. 7.7% and 1% vs. 4% (9, 10). The patient selection criteria in both studies were comparable to our trial. However, there was a minimum age of 70 years in the trial reported by Hughes *et al.* (10).

Moreover, the large difference in local relapse rates translated into a significant difference in overall relapse rate at 5 years, with 2.1% vs. 6.1% in favor of the radiotherapy group (Fig. 2).

From the natural history of favorable breast cancer, a slow evolution of recurrent disease is well known (3). With longer follow-up, more recurrences will likely occur in both groups, with a somewhat higher probability for the group administered 5 years of hormonal treatment and then stopped (Fig. 1). Similar recurrence rates as observed during the first years of follow-up (1% vs. 0.1% per year) will probably occur during longer follow-up (3, 18). We do not yet know if the long-term difference (>5 years) will become larger, smaller, or will remain the same (compare Fig. 1). At present, our Kaplan-Meier estimates suggest that the difference in absolute numbers is growing with time: 0.4% vs. 9% at 6 years. This is in accordance with the estimates reported by Fyles *et al.* (3.5% vs. 17.6% at 8 years) (9), Hughes *et al.* (1% vs. 7% at 7 years) (10), and Fisher *et al.* (2.8% vs. 16.5% at 8 years) (5). Longer follow-up is needed before any solid conclusions can be drawn. It will be interesting to observe whether a "carry-over" effect of adjuvant therapy can be observed after the end of endocrine treatment.

On the other hand, subgroups may be defined with a very low rate of recurrence, even in the group of patients not receiving radiotherapy. At present, the additional key factors seem to be age and tumor size. However in clinical trial research, a prospective investigation requiring a very large number of patients in a very small subcohort of patients to detect a very small difference in local relapse rate may not prove feasible because it would call for an enormous number of participating centers over a very long time period.

The overall low risk of local relapse at 5 years (5.1%) in the surgery-alone group has to be discussed separately. First, it has to be stated that this local relapse rate is far lower than local relapse rates in historical randomized trials with 35% at 8 years (19) and 24% at 10 years (2). Selection of patients in these trials was less precise and hormonal status had not been included in the selection criteria. Furthermore, the results at 5 years in the surgery-alone group can be regarded as acceptable in terms of absolute numbers (n = 19/417). It can be argued that the patients who have a local recurrence have a second chance to be cured because the majority can successfully be salvaged. These patients would then actually not have had major benefit from upfront radiotherapy, balancing advantages against disadvantages (*e.g.*, logistics, side effects).

Clinical practice, reflecting everyday problems, may still be discussed as outweighing the advantages and drawbacks of radiotherapy on an individual basis. This is particularly

true for aging or frail patients. We were able to demonstrate that in the group without radiotherapy, 94.9% would not have had any benefit from radiotherapy at 5 years. However, for putting into practice such individual decision-based strategy on indication of radiotherapy, excellent surgical and histopathologic quality control is a precondition, which has to be integrated into an accurate assessment of the individual patient within multidisciplinary tumor boards.

Axillary recurrence was seen in 1 woman only (n = 1/417). Negative findings from axillary node dissection and sentinel-node biopsy, respectively (in recent years), were reported for all women. This underlines clearly that in this favorable group with proven negative findings in the axilla, there is no evident risk for recurrence, which was also reported by Hughes *et al.* (10).

There was no difference in distant metastases and no significant difference in overall survival. Such a difference can only be expected after long-term follow-up (*e.g.*, at about 15 years), when local failure may then translate into overall failure as shown by the EBCTCG in their recent meta-analysis of breast-conserving treatment (3). However, this may also be questioned taking into account the low frequency of events observed until now and given that local relapse can be successfully salvaged and thus is frequently nonlethal.

In conclusion, for patients with favorable early breast cancer as addressed in this randomized trial, further evidence is provided to indicate that whole-breast radiotherapy remains the major integral part of adjuvant treatment in breast-conserving treatment, even if tamoxifen is replaced partly by aromatase inhibitors. It is a matter of future research, to investigate whether even more favorable subgroups can be prospectively defined, in whom radiotherapy is not beneficial, or whether whole-breast radiotherapy can be replaced by partial-breast irradiation, as currently investigated by different European and North American trial groups (20, 21).

REFERENCES

1. Fisher B, Anderson S, Bryant J, *et al.* Twenty-year follow-up of a randomized trial comparing total mastectomy, lumpectomy, and lumpectomy plus irradiation for the treatment of invasive breast cancer. *N Engl J Med* 2002;347:1233–1241.
2. Liljegren G, Holmberg L, Bergh J, *et al.*, and the Uppsala-Örebro Breast Cancer Study Group. 10-year results after sector resection with or without postoperative radiotherapy for stage I breast cancer: A randomized trial. *J Clin Oncol* 1999; 17:2326–2333.
3. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: An overview of the randomised trials. *Lancet* 2005;366:2087–2106.
4. Fisher B, Costantino J, Redmond C, *et al.* A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. *N Engl J Med* 1989;320:479–484.
5. Fisher B, Bryant J, Dignam J, *et al.* Tamoxifen, radiation therapy, or both for prevention of ipsilateral breast tumor recurrence after lumpectomy in women with invasive breast cancers of one centimeter or less. *J Clin Oncol* 2002;20:4141–4149.
6. Jakesz R, Jonat W, Gnant M, *et al.*, on behalf of the ABCSG and the GABG. Switching of postmenopausal women with endocrine-responsive early breast cancer to anastrozole after 2 years adjuvant tamoxifen: Combined results of ABCSG trial 8 and ARNO 95 trial. *Lancet* 2005;366:455–462.
7. Coombes RCh, Hall E, Gibson LJ, *et al.*, for the Intergroup Exemestane Study. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. *N Engl J Med* 2004;350: 1081–1092.
8. The Breast International Group (BIG) 1–98 Collaborative Group. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med* 2005; 353:2747–2757.
9. Fyles AW, McCready D, Manchul L, *et al.* Tamoxifen with or without breast irradiation in women 50 yeas of age or older with early breast cancer. *N Engl J Med* 2004;351:963–970.
10. Hughes K, Schnaper L, Berry D, *et al.* Lumpectomy plus tamoxifen with or without irradiation in women 70 years of age or older with early breast cancer. *N Engl J Med* 2004;351:971–977.
11. Boccardo F, Rubagotti A, Puntoni M, *et al.* Switching to anastrozole versus continued tamoxifen treatment of early breast cancer: Preliminary results of the Italian Tamoxifen Anastrozole Trial. *J Clin Oncol* 2005;23:5138–5147.
12. Grünberber Th, Gorlitzer M, SolmanTh, *et al.* It is possible to omit postoperative irradiation in a highly selected group of elderly breast cancer patients. *Breast Cancer Res Treatment* 1998;50:37–46.
13. Pocock SJ, Simon R. Sequential treatment assignment with balancing for prognostic factors in the controlled clinical trial. *Biometrics* 1975;31:103–115.
14. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958;53:457–481.
15. Peto R, Peto J. Asymptotically efficient rank invariant procedures. *J R Stat Soc* 1972;135:185–207.
16. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother Rep* 1966;50:163–170.
17. Cox DR. Regression models and life tables. *J R Stat Soc* 1972;34:187–220.
18. Fisher B, Jeong J-H, Anderson S, Bryant J, Fisher ER, Wolmark N. Twenty-five-year follow-up of a randomized trail comparing radical mastectomy, total mastectomy and total mastectomy followed by irradiation. *N Engl J Med* 2002;347: 567–575.
19. Clark RM, Whelan T, Levine M, *et al.*, for the Ontario Clinical Oncology Group. Randomized clinical trial of breast irradiation following lumpectomy and axillary dissection for node-negative breast cancer: An update. *J Natl Cancer Inst* 1996;88:1659–1664.
20. Kuske RR, Winter K, Arthur DW, *et al.* Phase II trial of brachytherapy alone after lumpectomy for select breast cancer: Toxicity analysis of RTOG 95–17. *Int J Radiat Oncol Biol Phys* 2006;65:45–51.
21. Ott OJ, Hildebrandt G, Pötter R, *et al.* Accelerated partial breast irradiation with multi-catheter brachytherapy: Local control, side effects and cosmetic outcome for 274 patients. Interim results of the German-Austrian multi-centre trial. *Radiother Oncol*. 2006 Nov 24 [Epub ahead of print].

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Original article

Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients

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Abstract

The ubiquitous application of selective oestrogen receptor modulators (SERMs) and aromatase inhibitors for the treatment and prevention of breast cancer has created a significant advance in patient care. However, the consequence of prolonged treatment with antihormonal therapy is the development of drug resistance. Nevertheless, the systematic description of models of drug resistance to SERMs and aromatase inhibitors has resulted in the discovery of a vulnerability in tumour homeostasis that can be exploited to improve patient care. Drug resistance to antihormones evolves, so that eventually the cells change to create novel signal transduction pathways for enhanced oestrogen (GPR30 + OER) sensitivity, a reduction in progesterone receptor production and an increased metastatic potential. Most importantly, antihormone resistant breast cancer cells adapt with an ability to undergo apoptosis with low concentrations of oestrogen. The oestrogen destroys antihormone resistant cells and reactivates sensitivity to prolonged antihormonal therapy. We have initiated a major collaborative program of genomics and proteomics to use our laboratory models to map the mechanism of subcellular survival and apoptosis in breast cancer. The laboratory program is integrated with a clinical program that seeks to determine the minimum dose of oestrogen necessary to create objective responses in patients who have succeeded and failed two consecutive antihormonal therapies. Once our program is complete, the new knowledge will be available to translate to clinical care for the long-term maintenance of patients on antihormone therapy.
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Keywords: Aromatase inhibitors; Tamoxifen; Raloxifene; Gene array analysis

Introduction

The translation and application of long-term antihormonal strategies, aimed at the tumour oestrogen receptor (OER), has significantly improved the prognosis of patients with breast cancer.[1] Long-term adjuvant tamoxifen treatment not only enhances survival and disease-free survival in patients with OER positive tumours during treatment but also reduces mortality for at least 10 years after treatment has stopped.[2,3] Building on the success of long-term tamoxifen therapy, a number of aromatase inhibitors have been shown to improve prognosis and reduce side effects (blood clots and endometrial cancer) if given instead of tamoxifen[4–6] or after tamoxifen treatment.[7,8] Thus, the original scientific strategy[9] of long-term antihormonal adjuvant therapy targeted to patients with OER positive disease[10,11] has emerged as the standard of care for breast cancer patients worldwide.

The new dimension of chemoprevention has advanced significantly during the past decade.[12] Preliminary studies were initiated in the 1980s to explore the safety and suitability of administering tamoxifen to women only at risk for breast cancer.[13–15] The rationale of these studies was based on the wide clinical experience using tamoxifen to treat all stages of breast cancer, the reduction of contralateral breast cancer noted in patients receiving adjuvant tamoxifen treatment[16–18] and laboratory studies that repeatedly demonstrated that tamoxifen can prevent mammary cancer in animal models.[19–22]

The current status and results of the worldwide efforts to quantitate and evaluate the value of tamoxifen as a

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chemopreventive have been summarized recently[23] but it is the P-1 trial completed by Fisher and the National Surgical Adjuvant Breast and Bowel Project (NSABP)[24,25] that is considered to be the landmark.[26] The results can be summarized simply. Tamoxifen reduced the incidence of breast cancer by 50%[24] in pre and postmenopausal women at high risk.[27] Side effects noted were increases in early stage low grade endometrial cancer, blood clots, and cataracts[24,25] but only in postmenopausal women receiving long-term tamoxifen treatment. Tamoxifen is available in the United States for risk reduction in pre and postmenopausal women. However, the consensus today is that tamoxifen is better deployed as a chemopreventive for premenopausal women to reduce the risk of OER positive breast cancer.[28–32] There are no increases in the side effects of endometrial cancer or blood clots but tamoxifen keeps preventing breast cancer long after treatment stops[31] consistent with earlier treatment results.[3]

The concern that tamoxifen was going to be associated with the risk of endometrial cancer[33] and the recognition that the drugs called nonsteroidal antioestrogens[34] were in fact selective OER modulators (SERMs) led to a paradigm change for chemoprevention. SERMs were oestrogenic in ovariectomized rat bone[35] but at the same time prevented mammary cancer.[21] These data led to the evidence-based hypothesis that SERMs could prevent breast cancer as a beneficial side effect during the treatment and prevention of osteoporosis.[36,37] Based on this laboratory-based hypothesis, raloxifene was subsequently shown to reduce fractures in postmenopausal women with or at high risk for osteoporosis[38] but at the same time caused a 75% reduction in the incidence of breast cancer.[39] A follow-up trial P-2 by the NSABP[40] established that raloxifene was equivalent to tamoxifen at preventing invasive breast cancer in high risk postmenopausal women but with significantly fewer side effects (hysterectomies, cataracts, overall thrombolic events). However, although lower numbers of endometrial cancer were noted in raloxifene treated women compared to tamoxifen treated women, this was not significant because of a higher hysterectomy rate.[40] Nevertheless, a related trial called Raloxifene use for the Heart or RUTH, showed no increase in endometrial cancers during raloxifene treatment compared to placebo arm.[41]

Thus from this brief introduction, it can be appreciated that significant clinical advances have been made through the application of the principle of long-term antihormone therapy[9,36] for the treatment and prevention of breast cancer. All of the advances can now be applied in clinical practice to improve patient care. Nevertheless, despite these advances through the use of sustained administration of antihormonal drugs, there are consequences for the tumour with the eventual development of drug resistance. In the case of SERMs, the type of resistance is unique and is expressed as SERM stimulated growth.[42] But, it is the consistent study of the process of drug resistance to antihormones that resulted in the discovery[43] of a weakness in the mechanisms of antihormonal drug resistance that has potential for the future exploitation in clinical practice.

Classification of SERM resistance

During the past 20 years we have focused our laboratory research program on developing models of SERM resistance in vivo to replicate events that could potentially occur clinically. The models were initially developed in vivo to avoid problems with cell culture where cells that become resistant to short term SERM treatment do not develop the essential requirements for angiogenesis that are necessary to survive and grow in patients. We now have a range of models that have been evaluated for growth in vivo (athymic mice) and that have been passaged in vivo for more than 5–10 years to replicate the long-term antihormonal therapy routinely used to treat patients (Table 1).

Initial studies of resistance to tamoxifen treatment demonstrated the unique feature of SERM stimulated growth. Resistant tumours that develop in athymic mice from both OER positive breast and endometrial cells grow in response to either a SERM or estradiol.[33,44] This is why an aromatase inhibitor or the pure antioestrogen fulvestrant (that binds to OER and facilitates the rapid destruction of the complex)[45] are successful second line therapies.[46,47] This form of resistance is referred to as Phase I resistance.[42]

However, these models represent only a few years of SERM treatment which is inconsistent with clinical experience of 5 years of adjuvant tamoxifen or possibly 10 years or more of raloxifene treatment to maintain bone density. The discovery that long-term SERM treatment exposes a vulnerability in the cancer cell that could have potential therapeutic applications was first reported at the St. Gallen meeting in the early 1990s.[43] Simply stated, long-term SERM treatment creates an absolute dependency on the SERM for tumour growth but small physiologic doses of oestradiol cause tumour cell death. Small tumours respond more readily to the apoptotic action of oestrogen but when tumours regrow during continuous oestrogen

Table 1
The available SERM resistant OER positive tumours used to investigate drug resistance in our laboratory.

Phase	Organ site	SERM	Cell line	Reference
I	Breast	tamoxifen	MCF-7	44, 67, 68
	Breast	tamoxifen	T47D	69
	Endometrial	tamoxifen	human tumour	33
	Endometrial	tamoxifen	ECC-1	70
II	Breast	tamoxifen	MCF-7	43, 48, 71
	Breast	raloxifene	MCF-7	72
	Endometrial	raloxifene	ECC-1	(unpublished)

Phase I resistance refers to tumours that can be stimulated to grow into oestrogen or a SERM whereas Phase II resistance refers to tumours stimulated to grow only with a SERM. Oestrogen causes Phase II tumors to undergo apoptosis and regress.[42]

therapy, the tumours again respond to the SERM or no treatment[48] (equivalent to treatment with an aromatase inhibitor for patients). This form of resistance is referred to as Phase II resistance.[42] The models for SERM resistance are summarized in Table 1. Thus, it is plausible to consider a clinical strategy whereby limited duration, low dose oestrogen treatment could be used to purge and destroy Phase II resistant breast cancer cells but then patients could be treated again with antihormonal therapy to control tumour growth. However, a case could be made that the ubiquitous use of tamoxifen is declining and over the next decade the standard of care will be long-term treatment with one of several aromatase inhibitors. The question we have addressed in the laboratory is whether long-term oestrogen deprivation of breast cancer cells will expose the vulnerability to the apoptotic actions of oestrogen.

Table 2
The basic characteristics of the MCF-7 cell lines developed from long-term oestrogen deprivation.

	Cell Line	
	MCF-7:2A	MCF-7:5C
OER	++	++
Oestrogen induced PgR	++	–
GPR30	++++	++++
Growth inhibitory response to SERMs	++	–
Growth inhibitory response to fulvestrant	+++	++
Invasion proteins	++	++++

Results are replicate (5) data from the affymetrix U-133 gene arrays relative to wild type MCF-7 cells.However, the biology of responses to antioestrogens are based on cell growth experiments where no effect is–and 100% response is ++++.

Resistance of breast cancer to oestrogen deprivation

There are two laboratory approaches to developing models of drug resistance to aromatase inhibitors. The traditional model is to study the impact of oestrogen withdrawal on the growth of OER positive breast cancer cells. In contrast, there is a model in vivo employing athymic mice transplanted with MCF-7 cells stably transfected with the aromatase enzyme. Without oestrogen tumours do not grow but when animals are treated with the enzyme substrate androstenedione to make oestrogen, tumour growth occurs. Simultaneous treatment with a number of aromatase inhibitors results in initial control of oestrogen-stimulated tumour growth but then the inhibitors fail and tumour growth occurs despite continuing treatment. This approach has been most instructive about strategies for antihormonal sequencing and the rationale of avoiding a combination of a SERM and an aromatase inhibitor for breast cancer therapy.[49,50]

The traditional approach of oestrogen withdrawal using breast cancer cells not engineered in any way, was not possible until Berthois and coworkers[51] discovered that cell culture media contained significant quantities of oestrogen found to increase the growth rate of MCF-7 cells. In other words, despite the fact that investigators were adding charcoal stripped serum to remove endogenous oestrogen, the media already contained oestrogenic chemical contaminants from the phenol red pH indicator.

Initial studies of the short and long-term effects of oestrogen deprivation of MCF-7[52,53] and T47D[54] breast cancer cells noted some interesting differences based on the regulation of OER in the different cell types.[55] The MCF-7 cells that are obtained following long-term oestrogen

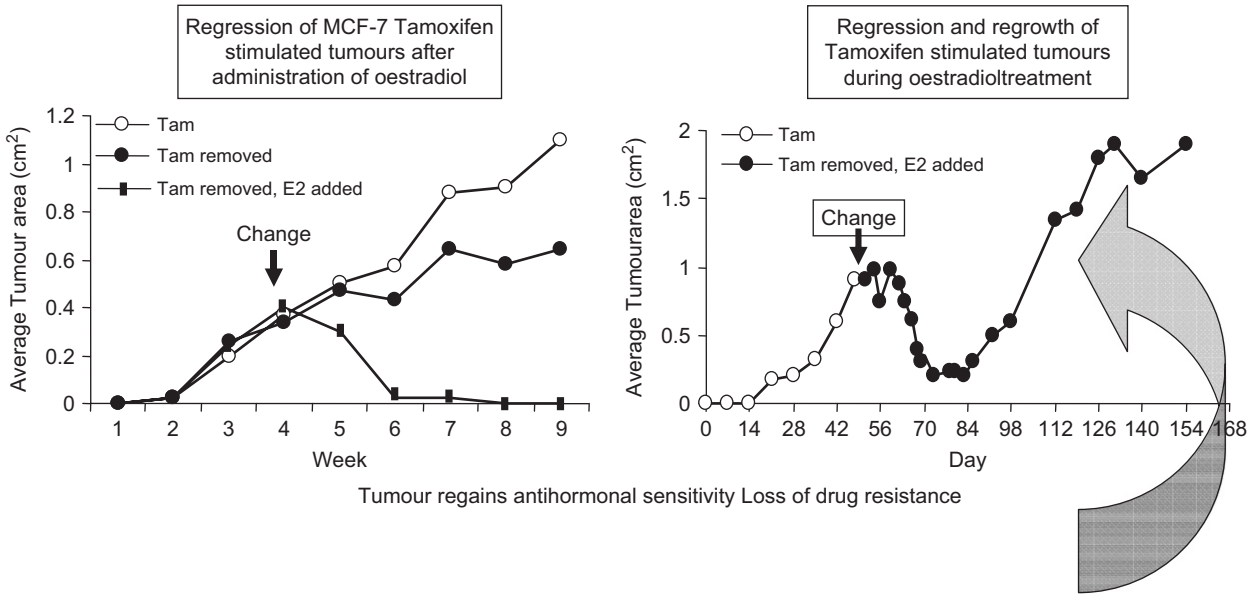

Fig. 1. Diagrammatic representation of the actions of physiologic oestradiol (E2) on the growth of small phase II MCF-7 tamoxifen resistant tumors in ovariectomized athymic mice. A larger tumour will regress with oestrodiol treatment but will eventually display oestrogen-stimulated growth. If tumours are retransplanted into a new generation of ovariectomized athymic mice and treated with oestradiol, tamoxifen will block oestrogen-stimulated tumour growth.[48] First presented in St. Gallen, 1993.[43]

deprivation remain OER positive (Table 2) whereas the T47D lose the OER.[56] The levels of OER increase in the oestrogen deprived MCF-7 cells (Table 2) and also there are increases in GPR30[57] noted in our gene array data. Thus, the oestrogen-deprived cells have an enhanced signal transduction pathway to support survival. Since breast cancers seem to rarely lose the OER efforts to study antihormonal drug resistance have focused on the MCF-7 line.

Our program to develop MCF-7 cell lines resistant to oestrogen withdrawal successfully described two clones of cells: the MCF-7:5C and the MCF-7:2A line. The MCF-7:5C line[58] is OER positive but progesterone receptor (PgR) negative and unresponsive to both oestrogen and SERM treatment. In contrast, the MCF-7:2A cell line[59] did respond to SERM therapy with a reduction in growth rate but oestrogen did not affect the growth rate, except at high concentrations. We have known for nearly 20 years that activation of growth factor receptor pathways can create intrinsic SERM resistance[60,61] and a down regulation of PgR induction.[62] These data would be consistent with the finding for the MCF-7:5C cells (Table 2). The laboratory observation that deactivation of the OER signal transduction pathway with fulvestrant is consistent with clinical observation that fulvestrant produces reasonable control of aromatase resistant breast cancer.[63] However, the models of oestrogen deprivation we developed in the early 1990s were to take center stage once the SERM resistant models were found to be reproducible[48] and worthy of further development (Table 1). The key to the value of the two MCF-7 clones (5C, 2A) was that they could be studied in vitro to understand the mechanism of oestrogen-induced apoptosis using genomics.

The new biology of oestrogen action

A re-examination of MCF-7 clones 5C and 2A occurred at the time when clinical investigators were re-examining the value of high dose oestrogen therapy in those patients who had been treated exhaustively with successive antihormonal therapies.[64] The clinical studies demonstrated that high dose oestrogen therapy could cause tumour regression or stasis (30%) in patients treated exhaustively with antihormones.[64] Additionally, high concentrations of oestrogen could induce apoptosis in long-term oestrogen deprived cells in culture.[65] In contrast, we pursued our original hypothesis that the apoptotic supersensitization of breast cancer cells by long-term antihormonal therapy could occur with physiologic or a very low concentration of oestrogen treatment.[43,48]

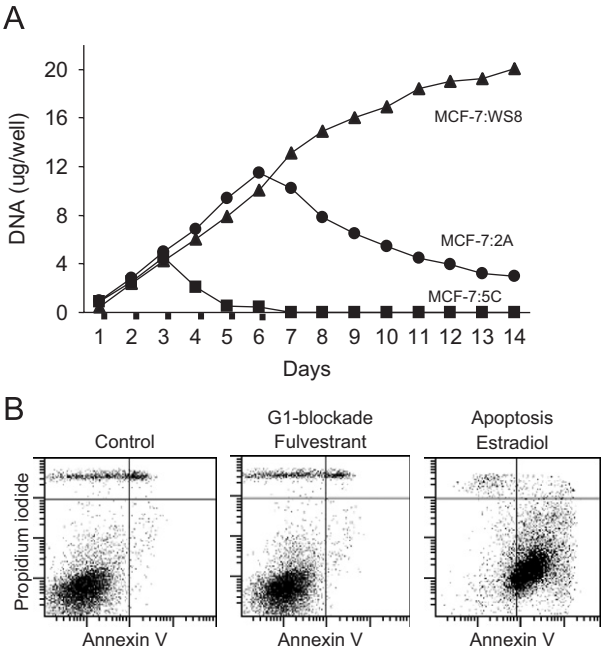

Fig. 3. The action of oestradiol (1 nM) on the growth of wild type MCF-7 cells (WS8) or long-term oestrogen-deprived MCF-7 cells (5C and 2A). In Panel A the MCF-7:5C cells undergo rapid apoptosis during the first few days of oestradiol exposure whereas the MCF-7:2A cells slowly initiate apoptosis during the days after 6 of oestradiol treatment. In panel B MCF07:5C cells respond to fulvestrant (1 μM) with a G1 blockade at 72 h whereas oestradiol (1 nM) causes massive and complete apoptosis. These results were obtained using flow cytometry.

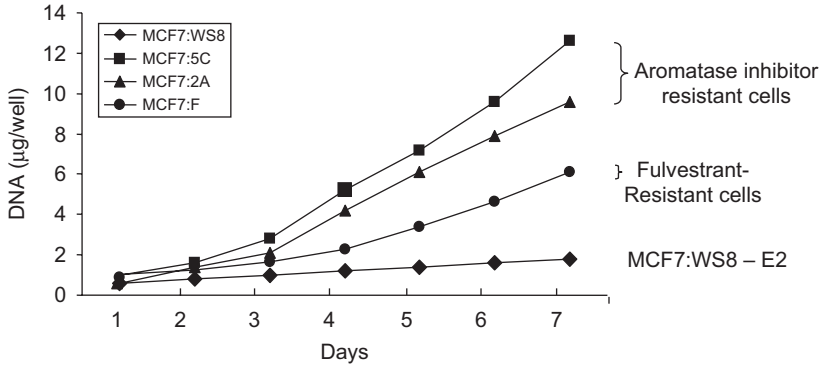

Fig. 2. The growth of wild type MCF-7 cells (WS8) and various antihormonally resistant sublines in an oestrogen-free environment. The cells MCF-7:5C and 2A grow spontaneously and could be considered to represent aromatase inhibitor resistant cells. These remain OER positive. In contract, MCF-7F are fulvestrant resistant (MCF-7 cells grown for over a year in an oestrogen-deprived environment containing fulvestrant). These cells grow spontaneously but have no OER.

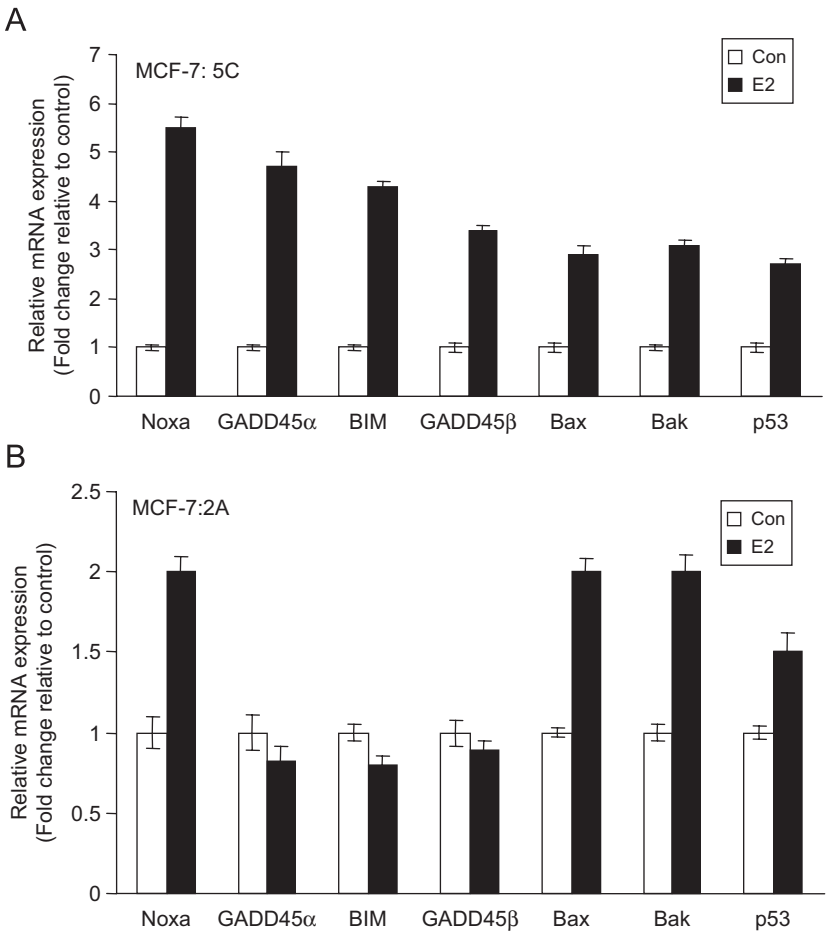

Fig. 4. Oestrogenic regulation of apoptotic genes in long term estrogen-deprived MCF-7:5C and MCF-7:2A breast cancer cells as determined by Affymetrix gene microarrays. For experiment, cells were treated with 1 nM oestradiol for 48 h and total RNA was prepared using the Qiagen Rneasy Mini kit. cRNA was generated, labeled, and hybridized to the Affymetrix Human Genome U133 plus 2l0 arrays containing 54,300 probe sets. Chips were then scanned and analysed using the Affymetrix Microarray Analysis Suite version 5.0. Assessment of data quality was conducted following default guidelines in the Affymetrix's GeneChip® Expression Analysis Data Analysis Fundamentals Training Manual. Global scaling for average signal intensity for all arrays was set to 500. Four biological replicates from each of the two cell lines were arrayed to determine consistent and reproducible patterns of gene expression. The above figure shows that oestradiol treatment caused 3 to 6 fold induction of the proapoptotic genes NOXA, GADD45α, GADD45β, BIM, BAX, BAK and p53 in (A) MCF-7:5C cells but only a 2–fold induction of NOXA, BAX, and BAK in (B) MCF-7:2A cells.

Two important observations, that were made during the re-evaluation of the MCF-7:5C and 2A cells, reinforced the view that oestrogen-induced apoptosis could be applied to reverse resistance to aromatase inhibitors. The first observation occurred by changing the charcoal stripped serum from the original 5% charcoal stripped calf serum[58] to 10% developed stripped fetal bovine serum.[66] This caused a dramatic increase in the growth rate of the 5C cells to be comparable to the MCF-7:2A cells (Figs. 1 and 2). Remarkably, physiologic oestradiol (lnM) now caused a massive apoptotic response in the MCF-7:5C cells (Fig. 3A,B). The MCF-7:2A cells had previously[59] been found to be responsive to antioestrogens by inhibiting growth and oestrogen by inducing progesterone receptor synthesis. The 2A cells, however, only weakly responded to the growth inhibitory effects of high concentrations 1 μM oestradiol. This original assumption is not true if the time course is extended (Fig. 3A). The 2A cells appear to have a survival mechanism that is able to protect them initially from the apoptotic actions of oestradiol. Nevertheless, this survival mechanism eventually fails. Overall, our models now create an interesting opportunity to interrogate the time courses with genomics and proteomics to find the precise oestrogen-induced mechanisms for protecting the cell from apoptosis.

Analysis of apoptotic pathways

A number of U-133 Affymetrix gene arrays were completed using the MCF-7, MCF-7:5C and 2A cell lines to define the early events of oestrogen action. A 48 h time point was used in our preliminary studies and five replicates were analysed to ensure statistical veracity. All gene array analyses were completed at Translational Genomics, AZ. Results illustrated in Fig. 4 show the 48 h increase in proapoptotic genes that are activated by oestrogen in the MCF-7:5C cells. This is consistent with the time course for the apoptotic death response of the

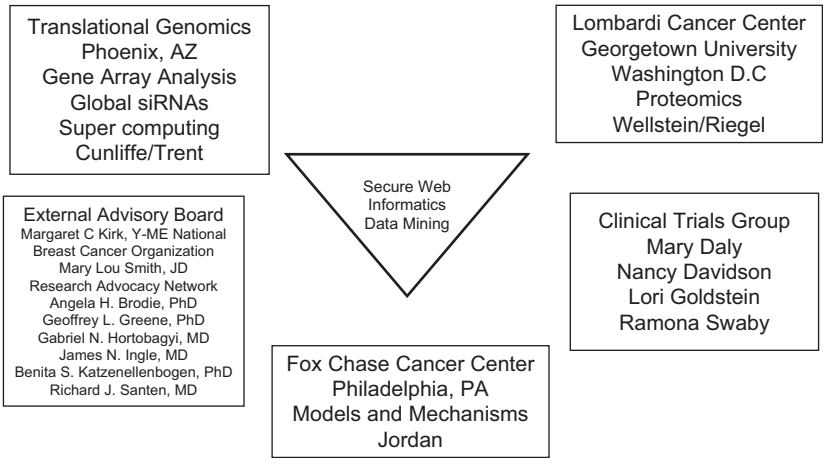

Fig. 5. The organization of our Department of Defence Center of Excellence Grant entitled "A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis." The model systems to study the survival and apoptosis induced with oestrogen are used for time course experiments at the Fox Chase Cancer Center. The materials are distributed to Translational Genomics for siRNA analysis or gene array and the Vincent T. Lombardi Cancer Center is involved to conduct proteomics. All results are uploaded into a shared secure web for data processing and target identification by our informatics and biostatistical group. Each laboratory is able to validate emerging pathways and study individual genes of interest. Our program is integrated with a clinical trials program that provides patient samples for validation of apoptotic or survival pathways. We are grateful to our external advisory board of Patient Advocates and professional colleagues for their continuing advice and support.

MCF-7:5C cells noted in Fig. 3. In contrast, oestrogen had not yet activated the full apoptotic response in MCF-7:2A cells that become apoptotic over a much longer time course (Fig. 3).

Overall, we have confirmed our novel observations that breast cancer and endometrial cancer cells (unpublished observation) become resistant to long-term antihormonal interventions by reconfiguring the oestrogen signal transduction pathway to induce an apoptotic response rather than enhancing survival and further growth. These data plus the emerging anecdotal results of clinical case reports (James Ingle, MD and Mr. Michael Dixon personal communications) prompted us to develop a multicenter program to explore our unique model systems systematically so that we can describe the mechanisms of oestrogen-induced survival and apoptosis in breast cancer. Completion of these studies would then provide an invaluable database to translate to patient care. The goal would be to determine the lowest dose of oestrogen necessary to cause apoptosis in a significant number of women whose tumours no longer respond to antihormonal therapy. This would reverse antihormone resistance in a significant proportion of patients.

Translation of laboratory results to patient care

We have established a multi-center collaborative translational research grant with headquarters at the Fox Chase Cancer Center (FCCC) (Figs. 5 and 6). The five year program is sponsored by the US Department of Defense Breast Cancer Program BC050277 entitled "A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis."

Our goal is to create maps of the survival and apoptotic responses to oestrogen noted in our models in vivo and in vitro. Biological samples from our time course experiments using our models at the FCCC are being distributed to Translational Genomics in Arizona for Agilent gene array analysis, CGH and CpG methylation arrays. Total human genome siRNA analysis is also being completed on our cell lines. Additionally, samples for proteomics are being dispatched to Georgetown University (Vincent T. Lombardi Cancer Center, PIs Anton Wellstein and Anna T. Riegel). All processed data are then being uploaded into a secure website for data mining and target identification, so that verification and validation studies can occur at each of the collaborating sites. A clinical program is exploring the clinical applications of our laboratory observation with two successive protocols:

(1) A single arm phase II study of pharmacologic dose oestrogen in postmenopausal women with hormone receptor-positive metastatic breast cancer after failure of sequential endocrine therapies.
(2) Reversal of anti-estrogen resistance with sequential dose de-escalation of pharmacologic oestrogen in a single arm phase II study of postmenopausal women with hormone receptor-positive metastatic breast cancer after failure of sequential endocrine therapies.

Our clinical studies are in place (1) to confirm the clinical finding[64] that high dose oestrogen treatment following exhaustive antihormonal treatment of OER positive breast cancer will give a 30% response rate and (2) to determine the lowest dose of oestrogen that will induce an equivalent tumour regression as high dose oestrogen (30 mg. oestradiol daily). All patients will be monitored weekly using the Apoptosense® serum assay to detect apoptotic markers in responding and non-responding patients. Additionally, where possible, patients will have biopsies of accessible

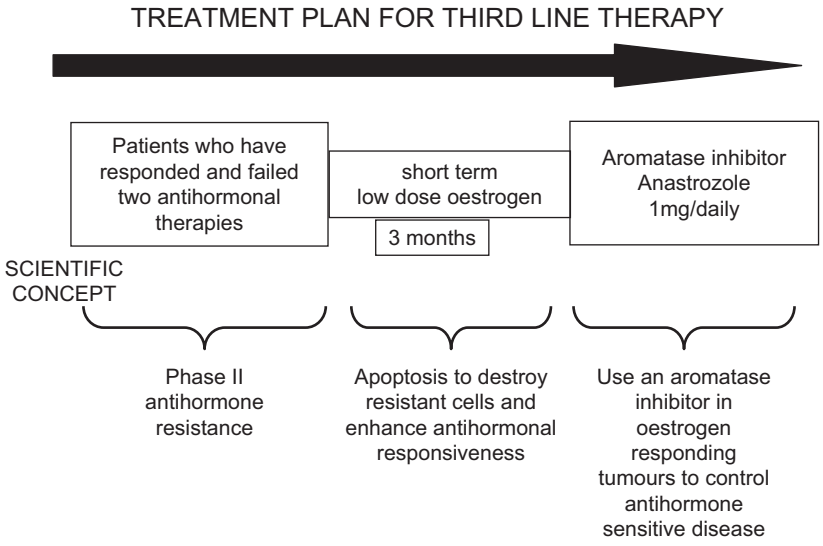

Fig. 6. An anticipated treatment plan for third line endocrine therapy. Patients must have responded and failed two successive antihormonal therapies to be eligible for a course of low dose oestradiol therapy for 3 months. The anticipated response rate is 30%[64] and responding patients will be treated with anastrozole until relapse. Validation of the treatment plan via the Center of Excellence grant (Fig. 5) will establish a platform to enhance response rates with apoptotic oestrogen by integrating known inhibitors of tumour survival pathways into the 3 month debulking treatment plan. The overall goal is to increase response rates and maintain patients for longer on antihormonal strategies before chemotherapy is required.

tumour tissue before and after 12 weeks of oestrogen therapy (or shorter if patients rapidly progress). Responding patients will be retreated with 1 mg anastrozole daily until progression.

Overall, the map of survival and apoptotic pathways we create from our laboratory models will be invaluable to guide our selection of target genes in biopsies using real time RTPCR. This will provide clues as to our future strategy of improving response rates with agents that selectively block survival pathways which can then be used in combination with our apoptotic oestrogen purge. It is our long term goal to improve oestrogen-induced response rates in patients refractory to antihormonal therapies. In so doing, select patients with metastatic breast cancer can anticipate longer disease control before chemotherapy is necessary. Most importantly, the new knowledge will provide an in silico platform to identify the apoptotic target so effectively located by the OER.

Conflict of Interest

None declared.

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References

1. Jordan VC, Brodie AMH. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* 2007;**72**:7–25.
2. EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;**354**:1451–67.
3. EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005;**365**:1687–717.
4. Howell A, Cuzick J, Baum M, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* 2005;**365**:60–2.
5. Thurlimann B, Keshaviah A, Coates AS, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med* 2005;**353**:2747–57.
6. Coates AS, Keshaviah A, Thurlimann B, et al. Five years of letrozole compared with tamoxifen as initial adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer: update of study BIG 1-98. *J Clin Oncol* 2007;**25**(5):486–92.
7. Coombes RC, Hall E, Gibson LJ, et al. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. *N Engl J Med* 2004;**350**(11):1081–92.
8. Goss PE, Ingle JN, Martino S, et al. Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17. *J Natl Cancer Inst* 2005;**97**:1262–71.
9. Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 1980;**16**:239–51.
10. Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *Eur J Cancer* 1975;**11**:205–6.

11. Jordan VC, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. *Adjuvant Therapy of Cancer* 1979;**2**:19–26.
12. Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. *Nat Rev Cancer* 2007;**7**:46–53.
13. Powles TJ, Hardy JR, Ashley SE, et al. A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *Br J Cancer* 1989;**60**(1):126–31.
14. Love RR, Mazess RB, Barden HS, et al. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med* 1992;**326**(13):852–6.
15. Love RR, Wiebe DA, Newcomb PA, et al. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Ann Intern Med* 1991;**115**(11):860–4.
16. Cuzick J, Baum M. Tamoxifen and contralateral breast cancer letter. *Lancet* 1985;**2**(8449):282.
17. Fornander T, Rutqvist LE, Cedermark B, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* 1989;**1**(8630):117–20.
18. Fisher B, Costantino J, Redmond C, et al. A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor- positive tumors. *N Engl J Med* 1989;**320**(8):479–84.
19. Jordan VC. Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. *J Steroid Biochem* 1974;**5**:354.
20. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA- induced rat mammary carcinoma. *Eur J Cancer* 1976;**12**: 419–24.
21. Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea- induced rat mammary carcinoma model. *Cancer Res* 1987;**47**(15):4020–4.
22. Jordan VC, Lababidi MK, Langan-Fahey S. Suppression of mouse mammary tumorigenesis by long-term tamoxifen therapy. *J Natl Cancer Inst* 1991;**83**(7):492–6.
23. Cuzick J, Powles T, Veronesi U, et al. Overview of the main outcomes in breast-cancer prevention trials. *Lancet* 2003;**361**:296–300.
24. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 1998;**90**: 1371–88.
25. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* 2005;**97**:1652–62.
26. 〈http://www.nature.com/milestones/milecancer/full/milecancer05.html〉.
27. Gail MH, Brinton LA, Byar DP, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst* 1989;**81**(24):1879–86.
28. Gail MH, Costantino JP, Bryant J, et al. Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J Natl Cancer Inst* 1999;**91**(21):1829–46.
29. Jordan VC. Optimising endocrine approaches for the chemoprevention of breast cancer. Beyond the Study of Tamoxifen and Raloxifene (STAR) Trial. *Eur J Cancer* 2006;**42**:2909–13.
30. Veronesi U, Maisonneuve P, Decensi A. Tamoxifen: an enduring star. *J Natl Cancer Inst* 2007;**99**(4):258–60.
31. Cuzick J, Forbes JF, Sestak I, et al. Long-term results of tamoxifen prophylaxis for breast cancer—96-month follow-up of the randomized IBIS-I trial. *J Natl Cancer Inst* 2007;**99**(4):272–82.
32. Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. *J Natl Cancer Inst* 2007; **99**(4):283–90.
33. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 1988;**48**:812–5.
34. Jordan VC. Biochemical pharmacology of antiestrogen action. *Pharmacol Rev* 1984;**36**(4):245–76.
35. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 1987; **10**(1):31–5.
36. Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 1988;**6**(5):589–95.
37. Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res* 1990;**50**:4177–89.
38. Ettinger B, Black DM, Mitlak BH, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial multiple outcomes of raloxifene evaluation (MORE) investigators [see comments] [published erratum appears in JAMA 1999 Dec 8;282(22): 2124]. *J Am Med Assoc* 1999;**282**(7):637-45.
39. Cummings SR, Eckert S, Krueger KA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple outcomes of raloxifene evaluation. *J Am Med Assoc* 1999;**281**:2189–97.
40. Vogel VG, Costantino JP, Wickerham DL, et al. The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *J Am Med Assoc* 2006;**295**:2727–41.
41. Barrett-Connor E, Mosca L, Collins P, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Engl J Med* 2006;**355**(2):125–37.
42. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;**5**:207–13.
43. Wolf DM, Jordan VC. *A laboratory model to explain the survival advantage observed in patients taking adjuvent tamoxifen therapy. Recent results in cancer research*, 127. Heidelberg: Springer; 1993 23–33.
44. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 1988;**48**:5183–8187.
45. Wijayaratne AL, McDonnell DP. The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. *J Biol Chem* 2001;**276**:35684–92.
46. Osborne CK, Pippen J, Jones SE, et al. Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. *J Clin Oncol* 2002;**20**(16):3386–95.
47. Howell A, Robertson JFR, Quaresma Albano J, et al. Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. *J Clin Oncol* 2002;**20**:3396–403.
48. Yao K, Lee ES, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;**6**(5):2028–36.
49. Jelovac D, Macedo L, Goloubeva OG, Handratta V, Brodie AMH. additive antitumor effect of aromatase inhibitor letrozole and antiestrogen fulvestrant in a postmenopausal breast cancer model. *Cancer Res* 2005;**65**:5439–44.
50. Long BJ, Jelovac D, Handratta V, et al. therapeutic strategies using the aromatase inhibitor letrozole and tamoxifen in a breast cancer model. *JNCI* 2004;**96**:456–65.
51. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci USA* 1986;**83**(8):2496–500.
52. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res* 1987;**47**:4355–60.

53. Welshons WV, Jordan VC. Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *Eur J Cancer* 1987; **232**:1935–9.
54. Murphy CS, Meisner LF, Wu SQ, Jordan VC. Short- and long-term estrogen deprivation of T47D human breast cancer cells in culture. *Eur J Cancer Clin Oncol* 1989;**25**(12):1777–88.
55. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 1996;**56**(10):2321–30.
56. Pink JJ, Bilimoria MM, Assikis J, Jordan VC. Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. *Br J Cancer* 1996;**74**(8):1227–36.
57. Bologa CG, Revankar CM, Young SM, et al. Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* 2006;**2**(4):207–12.
58. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endo* 1992;**90**:77–80.
59. Pink JJ, Jiang SY, Fritsch M, Jordan VC. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* 1995;**55**:2583–90.
60. Cormier EM, Jordan VC. Contrasting ability of antiestrogens to inhibit MCF-7 growth stimulated by estradiol or epidermal growth factor. *Eur J Cancer Clin Oncol* 1989;**25**(1):57–63.
61. Robinson SP, Jordan VC. The paracrine stimulation of MCF-7 cells by MDA-MB-231 cells: possible role in antiestrogen failure. *Eur J Cancer Clin Oncol* 1989;**25**:493–7.
62. Cormier EM, Wolf MF, Jordan VC. Decrease in estradiol-stimulated progesterone receptor production in MCF-7 cells by epidermal growth factor and possible clinical implication for paracrine-regulated breast cancer growth. *Cancer Res* 1989;**49**:576–80.
63. Ingle JN, Suman VJ, Rowland KM, et al. Fulvestrant in women with advanced breast cancer after progression on prior aromatase inhibitor therapy: North Central Cancer Treatment Group Trial N0032. *J Clin Oncol* 2006;**24**(7):1052–6.
64. Lonning PE, Taylor PD, Anker G, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 2001;**67**:111–6.
65. Song RX, Mor G, Naftolin F, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *J Natl Cancer Inst* 2001;**93**:1714–23.
66. Lewis JS, Osipo C, Meeke K, Jordan VC. Estradiol induced apoptosis in a breast cancer cell line resistant to estrogen deprivation. *J Steroid Biochem* 2005;**94**(1–3):131–41.
67. Gottardis MM, Jiang SY, Jeng MH, Jordan VC. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. *Cancer Res* 1989;**49**(15):4090–3.
68. Wolf DM, Langan-Fahey SM, Parker CJ, McCague R, Jordan VC. Investigation of the mechanism of tamoxifen-stimulated breast tumor growth with nonisomerizable analogues of tamoxifen and metabolites. *J Natl Cancer Inst* 1993;**85**(10):806–12.
69. MacGregor-Schafer JI, Lee E-S, O'Regan RM, Yao K, Jordan VC. Rapid development of tamoxifen stimulated mutant p53 breast tumors (T47D) in athymic mice. *Clin Cancer Res* 2000;**6**:4373–7380.
70. Osipo C, Meeke K, Liu H, et al. Trastuzumab therapy for tamoxifen-stimulated endometrial cancer. *Cancer Res* 2005;**65**(18):8504–13.
71. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant on estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* 2003;**95**:1597–607.
72. Liu H, Lee ES, Gajdos C, et al. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. *J Natl Cancer Inst* 2003;**95**(21):1586–97.

Short communication

By looking back we can see the way forward: enhancing the gains achieved with antihormone therapy

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Sir Alexander Haddow discovered the first chemical therapy to treat cancer [1]. Based on Paul Ehrlich's pioneering work that resulted in chemical therapy or chemotherapy to treat bacterial infections [2], Haddow investigated the therapeutic potential of numerous polycyclic hydrocarbons to cause tumour regression in experimental animals. Some compounds were effective, but the fact that they were known carcinogens prohibited further exploration in humans. Nevertheless, the triphenylethylene-based oestrogens [3] have a structural similarity to polycyclic hydrocarbons and they were also observed to cause tumour regression in animals. This was the translational basis of Haddow's landmark clinical experiments to evaluate the efficacy of high-dose oestrogen on the growth of breast and prostate cancer. Responses were noted but Haddow later commented [4] in 1970 during the inaugural David A Karnofsky lecture that, 'The extraordinary extent of tumour regression observed in perhaps 1% of postmenopausal cases has always been regarded as of major theoretical importance and it is a matter of some disappointment that so much of the underlying mechanisms continue to elude us.'

High-dose oestrogen therapy was introduced into clinical care during the 1950s [5] for the treatment of postmenopausal women with metastatic breast cancer. This approach complemented the use of ovarian ablation (using radiation at that time) in premenopausal patients, but the observation that high-dose oestrogen was an effective treatment for one in three elderly postmenopausal breast cancer patients remained a mechanistic paradox until recently [6].

Through serendipity, a young endocrinologist, Leonard Lerner at Merrell Dowe Pharmaceuticals in the USA, recognized that a triphenylethanolic compound being tested as a cardiovascular drug had a structure similar to the triphenylethylenes [7]. He asked to test the compound but found that there was no oestrogenic activity in any species tested, only anti-oestrogen activity. The compound, MER25 or ethamoxytriphetol, was the first nonsteroidal anti-oestrogen [8]. However, it was the fact that nonsteroidal anti-oestrogens were postcoital antifertility agents in rats that drove the structural evolution of triphenylethylene-based oestrogens to become a whole range of novel anti-oestrogenic compounds [9]. Regrettably, the promise of preventing pregnancy was premature because the compounds actually induced ovulation [10]. Also, drug toxicities noted during the 1960s and 1970s retarded any serious consideration of the non-steroidal anti-oestrogens as therapeutic agents for indications such as breast cancer therapy [10]. Only ICI 46,474, the *trans* isomer of a substituted triphenylethylene [11], took a tenuous path to clinical testing in breast cancer [10,12] and was subsequently kept on life support to be reinvented [13] as a potential targeted therapy for the long-term adjuvant treatment and prevention for oestrogen receptor positive breast cancer.

Today, the advance with the clinical implementation of the scientific strategy is profound [14,15], and the practice of oncology has progressed significantly over the past three decades [6]. However, the consequences of long-term antihormonal therapy is drug resistance, and it is the laboratory study of the drug resistance of tamoxifen and subsequently the aromatase inhibitors that has provided the opportunity to solve the paradox of high-dose oestrogen therapy in breast cancer. Solving this mystery has had the potential to show the way forward for future advances in cancer care.

Models to study the development of drug resistance to long-term tamoxifen resistance were first reported 20 years ago [16,17]. Drug resistance to tamoxifen develops within about a year in MCF-7 breast cancer cells. Inoculated cells grow into transplantable tamoxifen-stimulated tumours in ovariectomized athymic mice [16], and drug resistance (subsequently also noted for raloxifene [18,19]) is consistent with clinical

VEGFR = vascular endothelial growth factor receptor.

experience. However, it should be stressed that tamoxifen-stimulated growth is a unique form of drug resistance. Tumours stop growing when tamoxifen is withdrawn, but oestrogen also stimulates tumours to grow. This is the scientific basis for the use of an aromatase inhibitor or fulvestrant, the pure anti-oestrogen, after the development of tamoxifen resistance [20]. However, the finding that tamoxifen resistance actually evolves into new phases [21] provided an experimental basis for solving the mystery of the mechanism of high-dose oestrogen therapy and an opportunity to enhance the effectiveness of antihormonal therapy in patients rendered refractory to multiple anti-oestrogenic treatments.

Tamoxifen-stimulated MCF-7 breast tumours can only be maintained as a model of human disease by serial transplantation into tamoxifen-treated athymic mice; no appropriate cellular model is available. However, the realization that the model does not replicate adjuvant treatment with tamoxifen (5 or more years) raised the question of what occurs under these clinical circumstances. The discovery that physiological oestrogen causes rapid tumour regression of long-term (5 plus years) tamoxifen-resistant MCF-7 tumours [22] and the subsequent finding that the oestrogen-stimulated regrowth of regressed tumour would again respond to the anti-oestrogen tamoxifen [23] indicated a new strategic approach to cancer care. Simply stated, for the first time there was a novel method for killing antihormone-resistant breast cancer cells and then effectively retreating with tamoxifen to maintain responding patients for longer periods. The development of mechanistic studies and the important observations that the principle of oestrogen-stimulated tumour cell regression and apoptosis also applied to oestrogen-deprived cells (aromatase inhibitor resistant) [24-26] enhanced the overall relevance of the observations and provided opportunities for further mechanism based clinical trials.

The important study conducted by Lønning and coworkers [27] provides the laboratory-to-clinic translation of the fact that high-dose oestrogen treatment can produce a response rate of up to 30% among patients who have been treated with exhaustive antihormone therapy. The question now being addressed in multiple clinical studies is whether low-dose oestrogen therapy will be as effective in treating patients with a sensitized breast tumour.

With the evolution of thinking about oestrogen action following Haddow's success with the first chemical therapy [1], it is reasonable to examine how we can improve the efficacy of long-term antihormonal therapy and the putative 30% response rate of low-dose oestrogen therapy in metastatic breast cancer. We are pursuing two paths. To improve long-term antihormone therapy, we are investigating the value of long-term vascular endothelial growth factor receptor (VEGFR)2 inhibitors [28] to block residual oestrogen or selective oestrogen receptor modulator induced VEGF secretion [29]. The recent report that VEGF creates drug resistance to tamoxifen [30] implies that dual long-term adjuvant treatment with tamoxifen and a VEFGR2 inhibitor will have potential clinical merit. However, the key to success, we believe, is the use of low-dose VEGFR2 inhibitor with the adjuvant antihormone to avoid toxicity during long-term therapy.

To improve the value of low-dose oestrogen therapy treatment after exhaustive antihormonal therapy, we believe that the real question is why do 70% of tumours in the clinic not respond to oestrogen induced apoptosis? We have developed cell lines that either respond rapidly or have a delayed response to oestrogen. Using this approach, we have examined the inhibitor of glutathione synthesis buthionine sulfoximine, which has previously been evaluated in the clinic to improve responses to chemotherapy [31]. In preliminary studies, buthionine sulfoximine dramatically enhanced the response of refractory antihormone resistant cells to the early apoptotic actions of oestrogen.

We suggest that there is now a clinical opportunity to use our proposed clinical trial [6,32] design that employs a yet to be determined 12-week course of low-dose oestradiol therapy to treat patients after exhaustive antihormonal therapy. A succession of combined antisurvival agents could potentially improve response rates to well above the 30% rate in metastatic breast cancer rendered refractory by exhaustive antihormonal therapy. The novel test platform is rapid and has tumour response as the end-point. We believe that new combinations of agents could subsequently be employed in much larger trials without oestrogen once its apoptotic efficacy is established.

In closing, it is gratifying that the story of oestrogen action through the oestrogen receptor has continued to offer surprises in each decade since Haddow's report in 1944 [1]. By looking back, we have been able to plan a way forward to benefit patients.

Competing interests

The authors declare that they have no competing interests.

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References

1. Haddow A, Watkinson JM, Paterson E: **Influence of synthetic oestrogens upon advanced malignant disease.** *BMJ* 1944, **2:** 393-398.

2. Baumler E: *Paul Ehrlich, Scientist for Life*. New York, NY: Holmes & Meier; 1984.
3. Robson JM, Schonberg A: **Oestrous reactions including mating produced by triphenylethylene.** *Nature* 1937, **140:**196.
4. Haddow A: **David A. Karnofsky memorial lecture. Thoughts on chemical therapy.** *Cancer* 1970, **26:**737-754.
5. Kennedy BJ, Nathanson IT: **Effects of intensive sex steroid hormone therapy in advanced breast cancer.** *JAMA* 1953, **152:** 1135-1141.
6. Jordan VC: **The 38th David A. Karnofsky Lecture. The paradoxical actions of estrogen in breast cancer: survival or death?** *J Clin Oncol* 2008, **26:**3073-3082.
7. Lerner LJ: **The first non-steroidal antiestrogen-MER 25**. In *Non-steroidal Antioestrogens: Molecular Pharmacology and Antitumour Activity.* Edited by Sutherland RL, Jordan VC. Sydney, Australia: Sydney Academic Press; 1981:1-6.
8. Lerner LJ, Holthaus JF, Thompson CR: **A non-steroidal estrogen antagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenylethanol.** *Endocrinology* 1958, **63:**295-318.
9. Jordan VC: **Biochemical pharmacology of antiestrogen action.** *Pharmacol Rev* 1984, **36:**245-276.
10. Jordan VC: **Tamoxifen: a most unlikely pioneering medicine.** *Nat Rev Drug Discov* 2003, **2:**205-213.
11. Harper MJ, Walpole AL: **A new derivative of triphenylethylene: effect on implantation and mode of action in rats.** *J Reprod Fertil* 1967, **13:**101-119.
12. Cole MP, Jones CT, Todd ID: **A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474.** *Br J Cancer* 1971, **25:**270-275.
13. Jordan VC: **Tamoxifen: Catalyst for the change to targeted therapy.** *Eur J Cancer* 2008, **44:**30-38.
14. Early Breast Cancer Trialists' Collaborative Group: **Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials.** *Lancet* 2005, **365:**1687-1717.
15. Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, Atkins JN, Margolese RG, Runowicz CD, James JM, Ford LG, Wolmark N: **Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study.** *J Natl Cancer Inst* 2005, **97:**1652-1662.
16. Gottardis MM, Jordan VC: **Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration.** *Cancer Res* 1988, **48:**5183-5187.
17. Gottardis MM, Wagner RJ, Borden EC, Jordan VC: **Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro.** *Cancer Res* 1989, **49:**4765-4769.
18. O'Regan RM, Gajdos C, Dardes RC, De Los Reyes A, Park W, Rademaker AW, Jordan VC: **Effects of raloxifene after tamoxifen on breast and endometrial tumor growth in athymic mice.** *J Natl Cancer Inst* 2002, **94:**274-283.
19. Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, Loweth J, McKian K, De Los Reyes A, Wing L, Jordan VC: **Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo.** *J Natl Cancer Inst* 2003, **95:**1586-1597.
20. Howell A, Robertson JFR, Quaresma Albano J, Aschermannova A, Mauriac L, Kleeberg UR, Vergot I, Erikstein B, Webster A, Morris C: **Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment.** *J Clin Oncol* 2002, **20:**3396-3403.
21. Jordan VC: **Selective estrogen receptor modulation: concept and consequences in cancer.** *Cancer Cell* 2004, **5:**207-213.
22. Wolf DM, Jordan VC: **A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy.** In *Recent Results in Cancer Research*, vol 127. Heidelberg, Germany: Springer-Verlag; 1993:23-33.
23. Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, Jordan VC: **Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice.** *Clin Cancer Res* 2000, **6:**2028-2036.
24. Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, Yue W, Wang J, Santen RJ: **Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol.** *J Natl Cancer Inst* 2001, **93:**1714-1723.
25. Lewis JS, Osipo C, Meeke K, Jordan VC: **Estrogen induced apoptosis in breast cancer model resistant to long-term estrogen withdrawal.** *J Steroid Biochem Mol Biol* 2005, **94:**131-141.
26. Lewis JS, Meeke K, Osipo C, Bell E, Kidawi N, Chandel NS, Jordan VC: **Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation.** *J Natl Cancer Inst* 2005, **97:**1746-1759.
27. Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jorgensen LM, Mella O, Howell A: **High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy.** *Breast Cancer Res Treat* 2001, **67:**111-116.
28. Bhide RS, Cai ZW, Zhang YZ, Qian L, Wei D, Barbosa S, Lombardo LJ, Borzilleri RM, Zheng X, Wu LI, Barrish JC, Kim SH, Leavitt K, Mathur A, Leith L, Chao S, Wautlet B, Mortillo S, Jeyaseelan R Sr, Kukral D, Hunt JT, Kamath A, Fura A, Vyas V, Marathe P, D'Arienzo C, Derbin G, Fargnoli J: **Discovery and preclinical studies of (R)-1-(4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5- methylpyrrolo[2,1-f][1,2,4]triazin-6-yloxy)propan-2-ol (BMS-540215), an in vivo active potent VEGFR-2 inhibitor.** *J Med Chem* 2006, **49:**2143-2146.
29. Takei H, Lee ES, Jordan VC: **In vitro regulation of vascular endothelial growth factor by estrogens and antiestrogens in estrogen-receptor positive breast cancer.** *Breast Cancer* 2002, **9:**39-42.
30. Qu Z, Van Ginkel S, Roy AM, Westbrook L, Nasrin M, Maxuitenko Y, Frost AR, Carey D, Wang W, Li R, Grizzle WE, Thottassery JV, Kern FG: **Vascular endothelial growth factor reduces tamoxifen efficacy and promotes metastatic colonization and desmoplasia in breast tumors.** *Cancer Res* 2008, **68:**6232-6240.
31. Bailey HH, Ripple G, Tutsch KD, Arzoomanian RZ, Alberti D, Feierabend C, Mahvi D, Schink J, Pomplun M, Mulcahy RT, Wilding G: **Phase I study of continuous-infusion L-S,R-buthionine sulfoximine with intravenous melphalan.** *J Natl Cancer Inst* 1997, **89:**1789-1796.
32. Jordan VC, Lewis-Wambi J, Kim H, Cunliffe H, Ariazi E, Sharma C, Shupp HA, Swaby R: **Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients.** *Breast* 2007, **16 (suppl 2):**105-113.

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Original Article

New hypotheses and opportunities in endocrine therapy: amplification of oestrogen-induced apoptosis

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SUMMARY

Aims: To outline the progress being made in the understanding of acquired resistance to long term therapy with the selective oestrogen receptor modulators (SERMs, tamoxifen and raloxifene) and aromatase inhibitors. The question to be addressed is how we can amplify the new biology of oestrogen-induced apoptosis to create more complete responses in exhaustively antihormone treated metastatic breast cancer.

Methods and Results: Three questions are posed and addressed. (1) Do we know how oestrogen works? (2) Can we improve adjuvant antihormonal therapy? (3) Can we enhance oestrogen-induced apoptosis?

The new player in oestrogen action is GPR30 and there are new drugs specific for this target to trigger apoptosis. Similarly, anti-angiogenic drugs can be integrated into adjuvant antihormone therapy or to enhance oestrogen-induced apoptosis in Phase II antihormone resistant breast cancer. The goal is to reduce the development of acquired antihormone resistance or undermine the resistance of breast cancer cells to undergo apoptosis with oestrogen respectively. Finally, drugs to reduce the synthesis of glutathione, a subcellular molecule compound associated with drug resistance, can enhance oestradiol-induced apoptosis.

Conclusions: We propose an integrated approach for the rapid testing of agents to blunt survival pathways and amplify oestrogen-induced apoptosis and tumour regression in Phase II resistant metastatic breast cancer. This Pharma platform will provide rapid clinical results to predict efficacy in large scale clinical trials.

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Introduction

Tamoxifen (ICI46,474) was not hailed as an impressive breakthrough in the early 1970's when it was marketed as an orphan drug that produced modest responses in the treatment of metastatic breast cancer in post menopausal women.[1] Only one in three tumours responded to treatment for about a year. Nevertheless, side effects with tamoxifen were less than other available endocrine therapies (diethylstilboestrol (DES) or androgens).[2–4]

Despite initial disinterest in endocrine therapy, significant progress was subsequently made in the treatment and chemoprevention of breast cancer through the clinical application of laboratory principles for the antihormonal therapy of breast cancer.[5] Today antihormonal therapies (tamoxifen and aromatase inhibitors) target the oestrogen receptor (ER) present in the majority of breast cancers and long term adjuvant therapy with tamoxifen increases patient survival.[6,7] Aromatase inhibitors that are used exclusively in postmenopausal patients improve disease-free survival when compared to tamoxifen, and reduced the risk of endometrial cancer and blood clots noted with tamoxifen.[8] Additionally, the application of SERMs for the chemoprevention of breast cancer either directly with tamoxifen and raloxifene[9,10] or indirectly with raloxifene for the prevention of osteoporosis[11–13] will surely reduce the incidence of breast cancer in select populations over the next decade.

The critical strategy that led to the success of endocrine therapy for the treatment and prevention of breast cancer was the implementation of the laboratory principle of extended durations of treatment.[14,15] However, the consequence of long term treatment is the development of antihormonal drug resistance. Numerous laboratory models of antihormonal drug resistance have been developed over the past 20 years and several valuable principles have emerged. Drug resistance with SERMs evolves through at least two distinct phases: Phase I and Phase II[16] (Fig. 1). Phase I resistance to tamoxifen treatment is characterized by either tamoxifen or oestradiol-stimulated growth. Both ligands can exploit the ER signal transduction pathway to aid tumour cell survival. This phase of drug resistance has a clinical equivalent in metastatic breast cancer. When treatment fails during tamoxifen therapy, the tumour has a withdrawal response or regression upon withdrawal of tamoxifen

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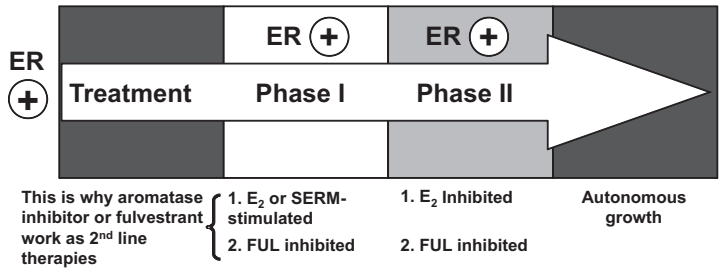

Fig. 1. The evolution of drug resistance to SERMs. Acquired resistance occurs during long-term treatment with a SERM and is evidenced by SERM-stimulated breast tumour growth. Tumours also continue to exploit oestrogen for growth when the SERM is stopped, so a dual signal transduction process develops. The aromatase inhibitors prevent tumour growth in SERM-resistant disease and fulvestrant that destroys the ER is also effective. This phase of drug resistance is referred to as Phase I resistance. Continued exposure to a SERM results in continued SERM-stimulated growth (Phase II), but eventually autonomous growth occurs that is unresponsive to fulvestrant or aromatase inhibitors. The event that distinguishes Phase I from Phase II acquired resistance is a remarkable switching mechanism that now causes apoptosis, rather than growth, with physiologic levels of oestrogen. These distinct phases of laboratory drug resistance[17,18] have their clinical parallels and this new knowledge is being integrated into the treatment plan.

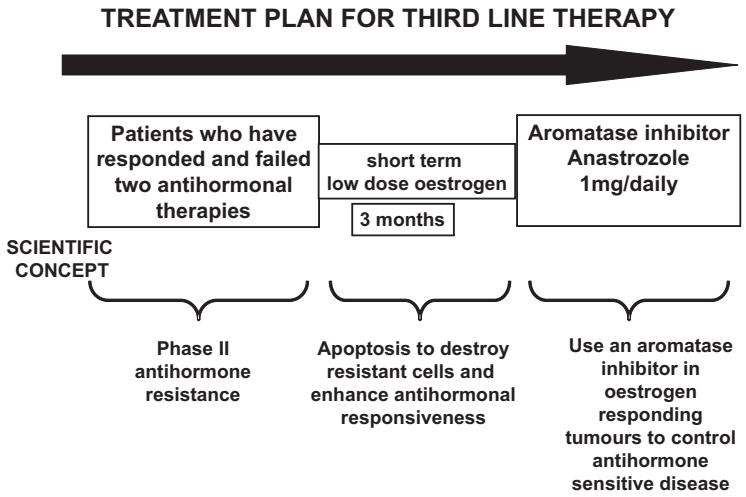

Fig. 2. Clinical protocol to investigate the efficacy of oestradiol induced apoptosis in long-term endocrine refractory breast cancer. An anticipated treatment plan for third-line endocrine therapy. Patients must have responded and experience treatment failure with two successive antihormone therapies to be eligible for a course of low-dose oestradiol therapy for 3 months. The anticipated response rate is 30% and responding patients will be treated with anastrozole until relapse. Validation of the treatment plan will establish a platform to enhance response rates with apoptotic oestrogen by integrating known inhibitors of tumour survival pathways into the 3-month debulking "oestrogen purge". The overall goal is to increase response rates and maintain patients for longer on antihormone strategies before chemotherapy is required.

treatment.[19] Second line therapy following tamoxifen treatment failure is with either an aromatase inhibitor or fulvestrant.[20]

The description of Phase II resistance to tamoxifen was first presented at the St. Gallen Breast Cancer Conference in 1992.[21] Re-transplantation of tamoxifen-resistant MCF-7 breast tumours into tamoxifen treated athymic mice for 5 or more years causes the signaling networks through the ER, that normally act as a survival network, to become reconfigured to be activated by physiological oestradiol that causes rapid apoptosis and triggers tumour regression.[17] The fact that these laboratory data pertaining to the evolution of drug resistance to tamoxifen also applies to antihormonal resistance to raloxifene,[22] and oestrogen withdrawal[23–25] creates a valid general principle in breast cancer that can now be exploited in the clinic to enhance patient survivorship.[18,26] Indeed, it has been suggested that these current data[23,27] can explain the effectiveness of high dose oestrogen therapy to treat metastatic breast cancer in post menopausal women before tamoxifen was available.[28] High dose DES produces a 35% response rate in unselected patients[29] and interestingly enough Lonning and colleagues[30] reported a 30% response rate for high dose DES in a population of women who have received exhaustive anti-hormonal therapy to treat metastatic breast cancer. Remarkably, one woman has had a complete remission for more than 8 years after starting DES treatment (Per Lonning, personal communication).

We now choose to amplify the clinical potential of short term low oestradiol therapy to treat breast cancer in those patients whose ER positive tumour has responded and failed at least two consecutive antihormonal therapies. Based on the emerging clinical experience and on an expanding laboratory data base we anticipate a 30% clinical benefit.[30] We address the question of why tumour cell survival signaling prevents 70% of Phase II tumours from responding to low dose oestradiol and we will advance short and long term solutions to apply pharmacological interventions to sensitize refractory breast cancers to oestradiol's apoptotic actions.

A clinical model to evaluate oestrogen induced apoptosis

We have previously proposed a clinical test bed to define the molecular biology and breast tumour responsiveness to both high doses (30 mg daily) and low dose (6 mg daily) oestradiol. That strategy is based on the translation of our laboratory description of the evaluation of anti-hormone resistance through phase I to phase II resistance where oestradiol switches from being a survival signal to an apoptotic trigger.[16,27,31] The schema for the trial is illustrated in Fig. 2. Breast cancer patients who are eligible for recruitment to the trial must have responded and failed two consecutive anti-hormonal therapies e.g. fail tamoxifen adjuvant therapy during year 3–5 and subsequently respond and fail an aromatase inhibitor during the treatment of metastatic breast cancer. In our first protocol of high dose oestradiol therapy (30 mg daily), we are treating for 12 weeks and then responding patients will be treated with an aromatase inhibitor (anastrazole 1 mg daily)

until progression.[32] Several other investigators have initiated similar clinical trials but with less rigorous entry criteria concerning failure of repeated endocrine therapies. Nevertheless, we contend that the moment is right to address the issue of the regulation of apoptosis and advance the idea that other agents may be synergistic with oestradiol to trigger apoptosis in the predicted 70% of patients that do not respond to short term oestradiol therapy.

Opportunities in the endocrine regulation of breast cancer

Naturally, it is not possible to consider all of the opportunities that could be exploited for patient benefit but we pose three questions that will be answered with an example of current research from our laboratory. The questions will be addressed as an integrated translational research scheme summarized in Fig. 3.

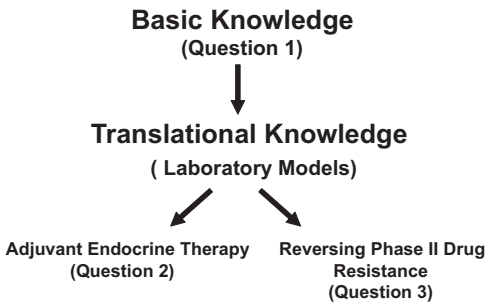

Fig. 3. The interactive translational research model employed to address new hypotheses and opportunities to amplify oestrogen-induced apoptosis for the treatment of Phase II endocrine resistant breast cancer. The questions posed are described in the text.

Question 1: Do we know how oestrogen works in target tumours?

The ER with its modulation through co-activators and co-repressors[33] has been investigated extensively through the structure function relationships of ligands that create novel folding of the receptor complex.[34] However the array of SERMs is only able to add marginally to advancing cancer therapeutics. We are beginning to understand the cross talk between the ER pathway and growth factor receptor pathways but our basic knowledge is in its infancy. There is an increasing menu of medicines to block growth factor pathways, but the challenge is to place the right targeted agent in the endocrine treatment paradigms. We will return to this challenge in our summary.

Our confidence in the position that "we understand how oestradiol works" has been challenged twice in recent times firstly with the discovery of a second ER referred to as ER-β (ER-α is the classical ER), and secondly with the discovery of the G protein-coupled receptor GPR30, an oestrogen-, SERM- and fulvestrant-binding protein. The role of ER-β in breast cancer is controversial but there is evidence that overexpression of ER-β can inhibit proliferation[35] and cause apoptosis.[36] However, ER-β specific ligands have yet to find a role in cancer therapeutics. The G protein-coupled receptor GPR30 is the latest putative receptor that can modulate oestrogen action specifically.[37] The molecule, a seven-pass transmembrane receptor located in the endoplasmic reticulum, mediates rapid non-genomic actions of oestradiol to initiate mobilization of intracellular Ca^{++} stores.

Based on high through put screening assays a new class of molecules specific for GPR30 has been identified,[38] and one compound G1 (Fig. 4) is available for laboratory investigations. We have addressed the question of whether the GPR30 agonist G1 is a stimulator or blocker of oestradiol-stimulated growth

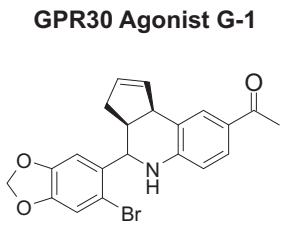

Fig. 4. G1, the first of a new class of agents that act as selective agonists of GPR30. A range of antagonists is also being developed.

in the wild-type ER-positive MCF-7 breast cancer cell line and whether G1 can provoke apoptosis in our oestrogen deprivation-resistant cell lines MCF-7:5C[24] and MCF-7:2A.[39] Data shown in Fig. 5 illustrates the fascinating pharmacology of the new drug group. G1 is anti-oestrogenic in the wild type MCF-7 cell line, and enhances apoptosis in both the MCF-7:5C and MCF-7:2A cell lines. Most importantly, G1 induces apoptosis in the MCF-7:2A cells more rapidly than oestradiol. This is important as it provides evidence that in endocrine resistant breast cancer cells, which are initially refractory to the immediate apoptotic actions of oestradiol, there is the potential to circumvent survival and initiate apoptosis quickly via a new pathway. The mechanism of action of G1 in all breast cancer cell lines is the rapid mobilization of high levels of Ca^{++} from intracellular stores. This increase of Ca^{++} is cytotoxic, thus, the new drug group has potential to enhance apoptosis in anti-hormone resistant cell lines and further development of these agents may find an application for short term treatment of patients whose tumours are Phase II anti-hormone resistant.

Question 2: Can we improve adjuvant antihormonal therapy?

We have probably reached a zenith with what can be achieved with adjuvant antihormonal therapy. Nevertheless, significant increases in efficacy can be achieved by improving compliance for long term adjuvant therapy or selecting out those patients that have variant CYP2D6 that does not metabolize tamoxifen to the active metabolite endoxifen.[40] What is required is a new initiative that can significantly enhance the effectiveness of antihormonal therapy and reduce the development of acquired drug resistance and possibly block intrinsic resistance. It could be, that the 40% of ER positive breast cancers that do not respond initially to antihormones could be encouraged to do so by pharmacologic intervention.

Angiogenesis is critical for the growth of tumours and the establishment of metastatic lesions.[41] However, antiangiogenic drugs must be integrated into the cancer treatment plan as there are no advantages to monotherapy. As a result there is increasing interest in combining antiangiogenic drugs with cytotoxic chemotherapy with the goal of achieving better tumour responses.[42] There has however, been little interest in combining antiangiogenic agents with antihormonal therapy primarily because such long term treatments are required and the effective doses of antiangiogenic drugs have significant side effects that are often life threatening.

The development of acquired resistance to SERMs implies that angiogenic mechanisms must be activated in cancer cells to permit SERM stimulated growth. Indeed, recent research has demonstrated that an autocrine Vascular Endothelial Growth Factor (VEGF) VEGF receptor 2 (VEGFR2) and P38 signaling loop confers resistance to 4-hydroxytamoxifen in MCF-7 breast cancer cells.[43] Thus, the rationale of combining antihormonal therapy with antiangiogenic therapy has conceptual merit.

We have addressed the idea that low doses of an inhibitor of the VEGFR2 tyrosine kinase could be synergistic with tamoxifen to enhance the control of tumour cell growth in vivo. There is merit to using low doses of small molecule inhibitors of VEGFR2 in treatment regimens as side effects will be reduced and the drug may be sufficient to block the modest, but significant, angiogenic

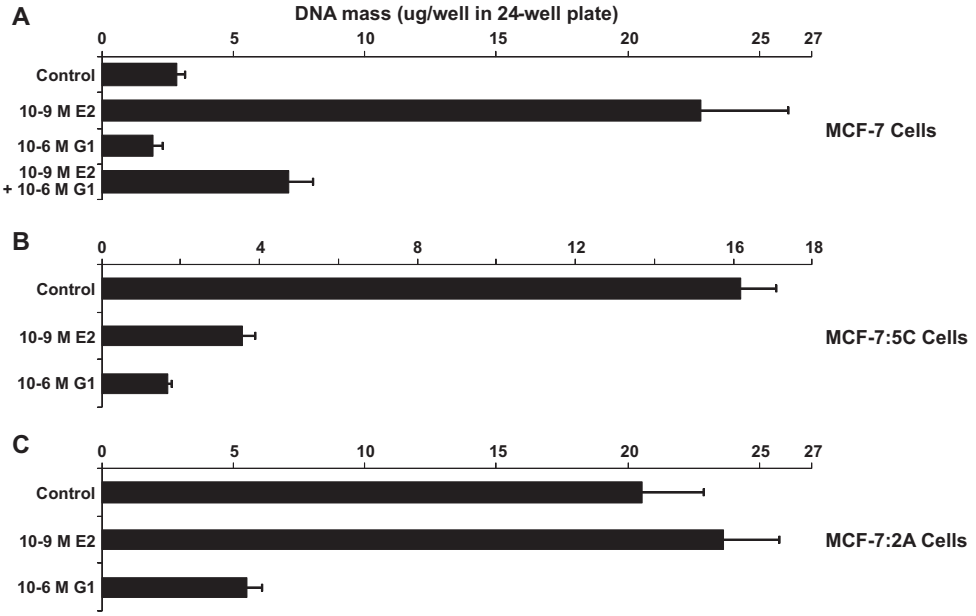

Fig. 5. The selective GPR30 agonist G1 inhibits growth of **(A)** wild-type MCF-7 cells and of oestrogen deprivation-resistant **(B)** MCF-7:5C and **(C)** MCF-7:2A cells. Cells were cultured under oestrogen-free conditions for 4 days, and then seeded into 24-well plates. Wild-type MCF-7 cells were seeded at 15,000 cells per well, MCF-7:5C cells at 25,000 cells per well, and MCF-7:2A cells at 30,000 cells per well. Beginning 24 hours after seeding (day 0) and every 2 days thereafter up to 6 days (days 2, 4, and 6), the cells were treated with 1 nM E_2, 1 μM G1, 1 nM E_2 + 1 μM G1, or Control (0.1% EtOH)-treated. The experiment was stopped on day 7. As a measure of proliferation, the amount of DNA per well was determined using a fluorescence-based DNA quantitation assay (CyQuant GR, Invitrogen, Carlsbad, CA). Data are shown as the mean of 8 replicate wells per group ± SD. **(A)** In wild-type MCF-7 cells, G1 significantly inhibited E_2-stimulated growth by 78% (E_2 vs. E_2+G1, $P < 0.0001$), and inhibited growth relative to control-treated cells (control vs. G1, $P = 0.0003$). **(B)** In estrogen deprivation-resistant MCF-7:5C cells, E_2 induced apoptosis as expected leading to a 78% reduction in growth (control vs. E_2, $P < 0.0001$). G1 also significantly inhibited growth by 90% (control vs. G1, $P < 0.0001$), and further, was more potent than E_2 (G1 vs. E_2 $P < 0.0001$). **(C)** The oestrogen deprivation-resistant MCF-7:2A cells grew independently of E_2 within the 7 day course of the experiment, as expected, yet G1 significantly inhibited growth by 73% ($P < 0.0001$).

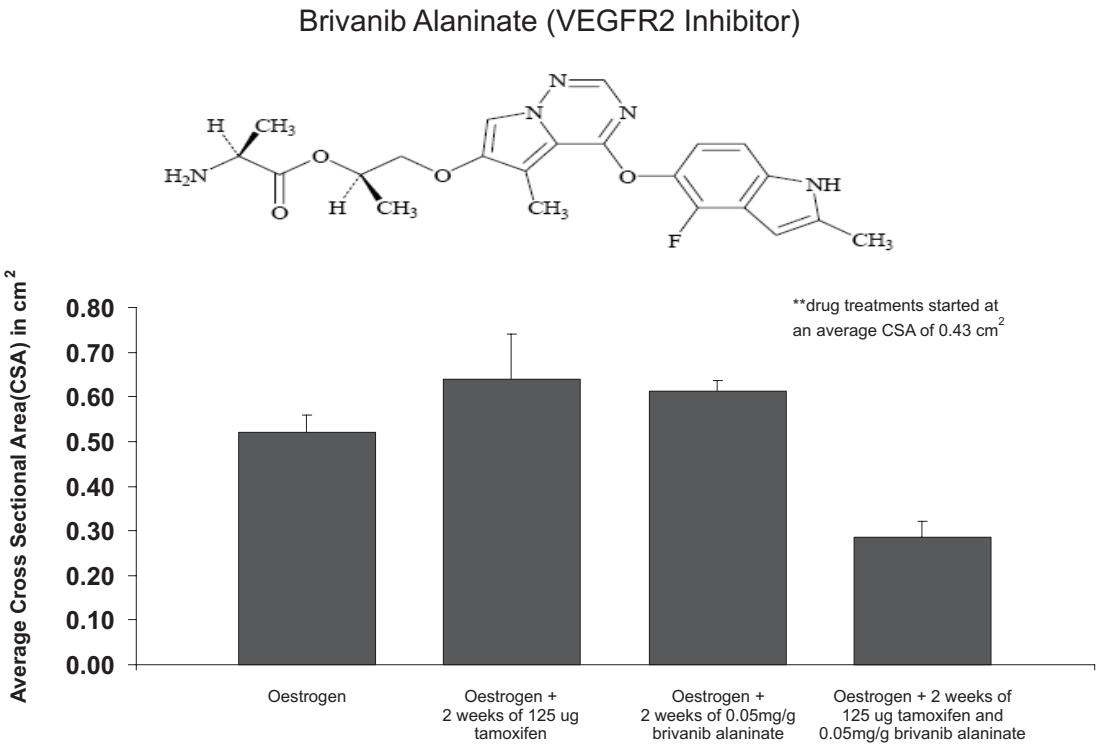

Fig. 6. Established MCF-7 E2 tumours and their response to various drug treatments. Tumours were implanted bilaterally into the mammary fat pads of athymic mice and 0.3 cm estradiol capsules were implanted subcutaneously into the dorsum of each mouse. Tumours were grown to 0.43 cm^2 and then drug treatments were initiated. Tumours that were treated with 125 ug of tamoxifen or 0.05 mg/g brivanib alaninate were unable to overcome oestradiol stimulated growth ($p = 0.65$, $p = 0.21$). Tumours continued to grow in the presence of oestrogen. When 125 ug of tamoxifen was combined with 0.05 mg/g brivanib alaninate, the effect was synergistic ($p = 0.009$) and the tumours decreased in size. The tumours were 38% smaller than the oestrogen treated tumours, even though the observed difference was not significant ($p = 0.16$). However, the decrease in average cross sectional area was significant when comparing the combination treatment to tamoxifen treated tumours ($p = 0.01$) or those treated with brivanib alaninate ($p = 0.007$).

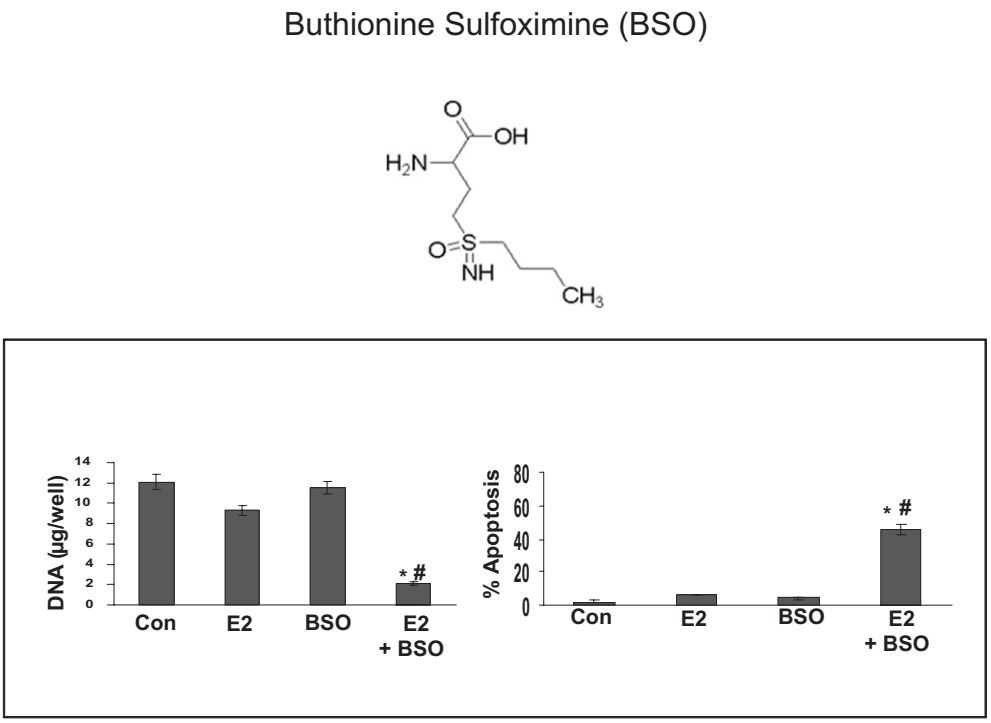

Fig. 7. The combination treatment of BSO plus oestradiol inhibits the growth of antihormone-resistant MCF-7:2A breast cancer cells. MCF-7:2A cells (30,000/well) were seeded in 24-well plates and after 24 hours were treated with <0.1% ethanol vehicle (control), 1 nM E2 (E2), 100 μM BSO (chemical structure shown above), or 100 μM BSO plus 1 nM E2 for 7 days. At the indicated time point, cells were harvested and total DNA (μg/well) was quantitated. These data represent the mean of three independent experiments; *bars*, ±SE. $^{**}P < 0.001$ compared with control cells; $^{\#\#}P < 0.001$ compared with oestradiol-treated cells. Annexin V staining for apoptosis was performed in MCF-7:2A cells following BSO plus E2 treatment. Quantitation of apoptosis (percent of control) in the different treatment groups is shown on the *right*. bars, ±SEs. $^{*}P < 0.05$ compared with control cells; $^{\#}P < 0.01$ compared with oestradiol-treated cells.

action of tamoxifen. In preliminary studies, we show (Fig. 6) that a combination of tamoxifen and a VEGFR2 inhibitor brivanib alaninate is superior to tamoxifen alone at inhibiting oestradiol induced tumour growth in athymic animals. The low dose of brivanib alaninate used does not significantly affect oestradiol-stimulated tumour growth when used alone. We conclude that the angiogenic signal from oestradiol is too strong but that the inhibition of the cell cycle with tamoxifen and the antiangiogenic brivanib alaninate in combination is synergistic.

The issue to be addressed is how to test the concept before committing to large scale adjuvant trials. One approach would be to evaluate efficacy and safety in our proposed model of oestradiol induced apoptosis in Phase II resistant breast cancer (Fig. 2). The goal would be to evaluate short term antiangiogenesis treatment by limiting toxicity during the 12 week treatment period and to assess improvements in response rates to physiologic (6 mg dose) oestradiol treatment alone. This would also address the third question we pose.

Question 3: Can we enhance oestrogen-induced apoptosis?

An effective treatment strategy for breast cancer must have a clear goal with the aim of enhancing patient survivorship. The progress[44] being made by translating the laboratory studies[45] of low dose apoptotic oestradiol therapy into clinical practice must be amplified to bring further benefits to a select group of patients. Those patients with Phase II resistant metastatic breast cancer are a significant proportion of all those who respond initially to adjuvant endocrine therapy. The goal is to harness the apoptotic trigger and create an enhanced sensitivity to oestrogen so that a higher proportion of tumours have a complete response to treatment. An application of general pharmacologic principles can be seen as a first step in amplifying oestrogen-induced apoptosis. Inhibitors of angiogenesis would be a logical innovation to aid oestrogen-induced apoptosis. By denying the ability of resistant cells to grow by restricting angiogenesis, may result in cellular instability and to enhance sensitivity to apoptosis. However, it is the critical players in the inhibition of apoptosis that need to be targeted in a broad strategy of combination therapy. It is generally agreed that Bcl-2 plays a central role in preventing the intrinsic apoptosis trigger through the mitochondrial pathway of cytochrome C release. One mechanism by which Bcl-2 may function is as an anti-oxidant by up-regulation of glutathione leading to rapid detoxification of reactive oxygen species and inhibition of free-radical mediated mitochondrial damage.

Glutathione is a water soluble tripeptide composed of glutamine, cysteine, and glycine. Elevated levels prevent apoptotic cell death whereas depletion of glutathione facilitates apoptosis.[46] L-Buthionine sulfoximine (BSO) (Fig. 7) is a specific inhibitor of glutanylcysteine synthase that blocks the rate limiting step of glutathione biosyntheses.

Recent laboratory studies demonstrate[39] that oestrogen deprived MCF7 cells that are initially refractory to oestradiol induced apoptosis are sensitized to the immediate apoptotic action of oestrogen by BSO at concentrations that can be achieved clinically[47] (Fig. 7). Since there is an extensive clinical experience with BSO it would not be unreasonable to integrate the antioxidant concept into the clinical test model.[48]

An integrated clinical strategy to target survival pathways in Phase II breast cancer

Overall, we are making significant progress towards understanding oestrogen-induced apoptosis and there is evidence that a new drug group based on GPR30 agonists could be developed to provide additional specificity and induce apoptosis in breast cancer. This approach could overcome some resistance in tumour cells observed

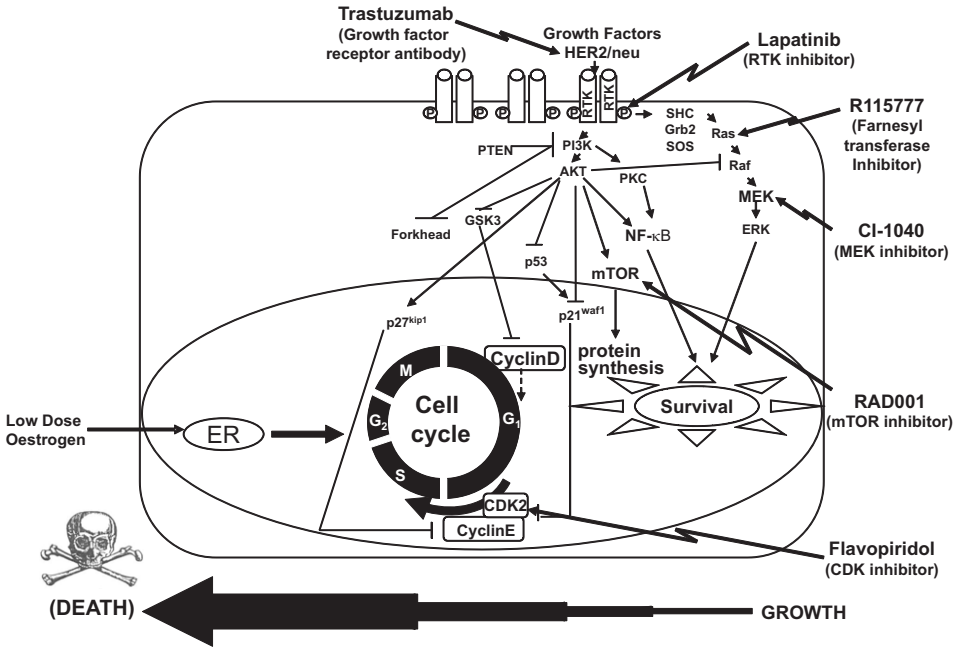

Fig. 8. Hypothetical apoptosis enhancement strategy to amplify the tumouricidal action of low dose oestradiol treatment. The strategy is to employ targeted agents from the pharmaceutical industry to block several pathways and shift the cellular equilibrium to apoptosis in oestrogen refractory Phase II resistant cells. The diagram illustrates candidate drugs to create a cocktail in the proposed Pharma platform. Drugs would be tested singly with oestradiol against oestradiol alone or in increasing combinations of survival inhibitors.

with oestradiol alone. This, however, is a long term goal and builds on an evolving understanding of the complexities of oestrogen action in cancer. Similarly the anti-angiogenic drugs that block the tyrosine kinase activity of VEGFR2 could undermine the survival of tumours that are refractory to the apoptotic oestrogen trigger. But in practical terms, the application of BSO with an apoptotic oestrogen trigger has immediate clinical applications in our clinical test model (Fig. 2). We have presented developing laboratory evidence to support each of these pharmacologic strategies to amplify the apoptotic oestrogen therapy in Phase II resistant breast cancer. However, the mechanism based clinical test model is more than a translational research tool.

Rapid clinical results can be developed through mechanism based targeting of several pathways that have the potential to amplify the apoptotic oestrogen trigger to create a significant increase in complete tumour responses. In Fig. 8 are examples of agent classes that could potentially be tested rapidly in the 12 week model against oestrogen alone. This will establish efficacy of a new targeted agent as a clinically useful drug.

The hypothetical, yet systematic, strategy to evaluate selectively the inhibition of survival signals has a foundation in laboratory science. The obvious strategy of blocking the growth factor receptor signal cascade using either trastuzumab or the tyrosine kinase inhibitor lapatinib as an immediate practical approach in the 12 week test model. Recent studies demonstrate that antihormone responsiveness can be restored by aromatase resistant cells using trastuzumab[49] and lapatinib[50] is showing promise in clinical trials of breast cancer with chemotherapy.[51]

The mammalian target-of-rapamycin (mTOR) is emerging as an important target for therapeutic intervention in multiple cancer tissue types including breast cancer. mTOR integrates signals from multiple pathways to sense cellular nutrient and energy levels. mTOR is a serine/threonine kinase downstream of PI3K/Akt that, in the presence of mitogenic stimulation and sufficient nutrients, promotes protein translation by activating 40S ribosomal protein S6 kinases (S6K1-2) and inhibiting the eukaryotic initiation factor 4E binding proteins (4E-BP1-3).[52] RAD001 (everolimus) is an orally available mTOR inhibitor that alone and synergistically in combination with the aromatase inhibitor letrozole[53] blocks proliferation and induced apoptosis in MCF-7 and T47D breast cancer cells stably expressing aromatase.[53] These and other data have lead to the evaluation of RAD001 in combination with letrozole in a recently completed Phase I clinical trial in patients with advanced breast cancer.[54] RAD001 is also currently under evaluation in 15 other breast cancer clinical trials (search of clinicaltrials.gov on 2/26/2009) either as a single agent or in combination with various chemotherapeutics, fulvestrant, aromatase inhibitors, and agents which target EGFR and HER2/ErbB2.

We evaluated RAD001 in an MCF-7 breast cancer xenograft tumour model (MCF-7/E2) grown in athymic mice (Fig. 9A) that represents the therapeutic stage of antihormone-based therapy, and in a SERM-resistant (MCF-7/RAL1) xenograft tumour model (Fig. 9B) that was selected *in vivo* by continuous treatment with the SERM raloxifene for greater than 3 years.[31,55] RAD001 inhibited MCF-7/E2 tumour growth in the presence of E_2 (E_2 + RAD001 vs E_2 alone). Additionally, RAD001 in the absence of E_2, a situation comparable to combination therapy of RAD001 plus an aromatase inhibitor in the clinic, further reduced MCF-7/E2 growth (RAD001 alone vs. E_2 + RAD001). These MCF-7/RAL1 tumours can be considered cross-resistant to oestrogen deprivation (or aromatase inhibitors). However, RAD001 was still effective at blocking growth despite resistance to oestrogen deprivation (RAD001 vs. vehicle). Fulvestrant can be used clinically as a second-line therapy after failure of a first-line antihormone therapy, as illustrated here by fulvestrant inhibiting growth in the MCF-7/RAL1 tumours (fulvestrant vs. vehicle or RAL). Yet the combination of RAD001 plus fulvestrant was superior at blocking growth than either agent alone (Fulvestrant + RAD001 vs. fulvestrant alone and vs. RAD001 alone). Taken together, RAD001 represents a promising therapeutic for use in antihormone-sensitive, and importantly, in antihormone-resistant breast cancer, especially in combination with fulvestrant.

It is clear that other inhibitors of signal transduction pathways (Fig. 8) may be useful to enhance estrogen-induced apoptosis such as the MEK inhibitor CI-1040,[56] the farnesyl transferase inhibitor lonafarnib[57] and the cyclin-dependent kinase inhibitor flavopiridol.[58] Indeed, inhibitors of CDK may have merit as a

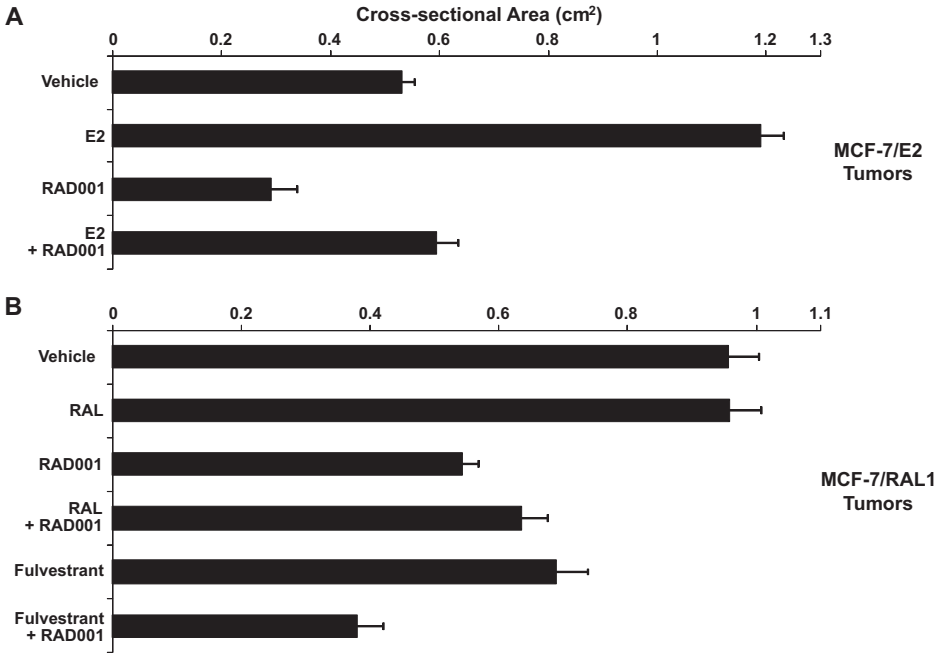

Fig. 9. Growth inhibition of naïve MCF-7/E_2 tumours and SERM-resistant MCF-7/RAL1 tumours in response to RAD001 (everolimus). **(A)** *RAD001 inhibition of MCF-7/E_2 tumour growth.* Twenty ovariectomized athymic nude mice were bilaterally transplanted with MCF-7/ E_2 tumour pieces 1 mm^3 in size in the axillary mammary fat pads, and implanted with a 0.3 cm E_2 silastic capsule *sc*. Once the tumours grew to an average cross-sectional area of 0.39 cm^2, the animals were randomized into 4 treatment groups of 5 mice per group (10 tumours per group) corresponding to Vehicle (of the RAD001 formulation), E_2 (0.3 cm E_2 capsule *sc*), RAD001 [40 mg/kg/day (6.25 mg/day) RAD001 given 5 days/week], and E_2 + RAD001 (0.3 cm E_2 capsule *sc* plus 6.25 mg/day RAD001 given 5 days/week). The average cross-sectional area of RAD001-treated MCF-7/E_2 tumours was significantly smaller than Vehicle-treated tumours ($P = 0.0066$, T test). Similarly, the average cross-sectional area of E_2 + RAD001-treated tumours was significantly smaller than E_2 alone-treated tumours ($P < 0.0001$). **(B)** *RAD001 inhibition of MCF-7/RAL1 tumour growth.* Thirty ovariectomized athymic nude mice were bilaterally implanted in the axillary mammary fat pads with 1 mm^3 MCF-7/RAL1 tumour pieces. Mice were treated with 1.5 mg/day RAL *po* until the MCF-7/RAL1 tumours grew to an average cross-sectional area of 0.26 cm^2, and then the animals were separated into 6 treatment groups of 5 mice each (10 tumours per group) corresponding to Vehicle (of the RAD001 formulation), 1.5 mg/day RAL *po*, RAD001 (6.25 mg/day RAD001 given 5 days/week), RAL + RAD001 (1.5 mg/day RAL *po* plus 6.25 mg/day RAD001 given 5 days/week), Fulvestrant (2 mg/day *sc* of the clinically used Faslodex preparation given 5 days/week), Fulvestrant + RAD001 (2 mg/day Faslodex *sc* plus 6.25 mg/day RAD001 given 5 days/week). The average cross-sectional areas of RAD001-treated and Fulvestrant-treated tumours were each significantly smaller than Vehicle-treated tumours ($P < 0.0001$ and $P = 0.0015$, respectively). Similarly, RAL + RAD001-treated tumours were significantly smaller than RAL-treated tumours ($P = 0.0002$). Additionally, Fulvestrant + RAD001-treated tumors were significantly smaller than RAD001 alone-treated tumors ($P = 0.0026$) or Fulvestrant alone-treated tumors ($P = 0.0004$). The data shown represent the average cross-sectional tumour area (cm^2) per group ± SE. Tumour cross-sectional area was calculated using the equation $(l/2) \times (w/2) \times \pi$. The cross-sectional areas of MCF-7/E2 tumours were compared at day 41, and of MCF-7/RAL1 tumours at day 54.

short term blocking strategy to enhance apoptosis. The cyclin-dependent kinase inhibitory drugs such as flavopiridol that have been tested clinically and causes apoptosis through an intrinsic pathway dependent on BAX and BAK[58] would be of significant interest in combination with oestradiol to amplify apoptosis.

In summary a whole spectrum of new compounds can now be tested to enhance tumour response to oestrogen with the added advantage that this testing platform can document rapid tumour responses. Combinations could create an optimal cocktail for individual tumours to predict a complete response triggered by oestrogen.

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References

1. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2003;**2**:205–13.
2. Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. *Br J Cancer* 1971;**25**:270–5.
3. Ward HW. Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. *Br Med J* 1973;**1**:13–4.
4. Ingle JN, Ahmann DL, Green SJ, Edmonson JH, Bisel HF, Kvols LK, et al. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *N Engl J Med* 1981;**304**: 16–21.
5. Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 2008;**44**:30–8.
6. EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;**354**:1451–67.
7. EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005;**365**:1687–717.
8. Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, Forbes JF, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* 2005;**365**:60–2.
9. Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* 2005;**97**:1652–62.
10. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA* 2006;**295**:2727–41.
11. Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* 1999;**281**:2189–97.
12. Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, et al.;

for the CORE Investigators. Continuing Outcomes Relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J Natl Cancer Inst* 2004;**96**:1751–61.
13. Jordan VC. The rise of raloxifene and the fall of invasive breast cancer. *J Natl Cancer Inst* 2008;**100**:831–3.
14. Jordan VC, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. *Adjuvant Ther Cancer* 1979;**2**:19–26.
15. Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 1980;**16**:239–51.
16. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;**5**:207–13.
17. Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;**6**:2028–36.
18. Lewis JS, Cheng D, Jordan VC. Targeting oestrogen to kill the cancer but not the patient. *British J Cancer* 2004;**90**:822–32.
19. Howell A, Dodwell DJ, Anderson H, Redford J. Response after withdrawal of tamoxifen and progestogens in advanced breast cancer. *Ann Oncol* 1992;**3**:611–7.
20. Howell A, Robertson JFR, Quaresma Albano J, Aschermannova A, Mauriac L, Kleeberg UR, et al. Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. *J Clin Oncol* 2002;**20**:3396–403.
21. Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. In: Recent Results in Cancer Research. Heidelberg: Springer-Verlag; 1993. p. 23–33.
22. Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, et al. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. *J Natl Cancer Inst* 2003;**95**:1586–97.
23. Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *J Natl Cancer Inst* 2001;**93**:1714–23.
24. Lewis JS, Osipo C, Meeke K and Jordan VC. Estradiol induced apoptosis in a breast cancer cell line resistant to estrogen deprivation. *J Steroid Biochem* 2005; **94**:131–41.
25. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 2005;**97**:1746–59.
26. Maximov PY, Lewis-Wambi JS, Jordan VC. The paradox of oestradiol-induced breast cancer cell growth and apoptosis. *Curr Signal Transduct Ther* 2009;**4**:88–108.
27. Jordan VC. The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer – survival or death? *J Clin Oncol* 2008;**26**:3073–82.
28. Haddow A, Watkinson JM, Paterson E. Influence of synthetic oestrogens upon advanced malignant disease. *Br Med J* 1944;**2**:393–8.
29. Kennedy BJ. Hormone therapy for advanced breast cancer. *Cancer* 1965;**18**: 1551–7.
30. Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jorgensen LM, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 2001;**67**:111–6.
31. Ariazi EA, Lewis-Wambi JS, Gill SD, Pyle JR, Ariazi JL, Kim HR, et al. Emerging principles for the development of resistance to antihormonal therapy: implications for the clinical utility of fulvestrant. *J Steroid Biochem Mol Biol* 2006;**102**:128–38.
32. Jordan VC, Lewis-Wambi J, Kim H, Cunliffe H, Ariazi E, Sharma CG, et al. Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients. *Breast* 2007;**16**(Suppl 2):S105–13.
33. Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocrine Rev* 2004;**25**:45–71.
34. Ariazi EA, Jordan VC. Estrogen receptors as therapeutic targets in breast cancer. In: Ottow E, Weinmann H, editors. Nuclear Receptors as Drug Targets. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim; 2008, p. 127–99.
35. Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc Natl Acad Sci USA* 2004;**101**:1566–71.
36. Helguero LA, Faulds MH, Gustafsson JA, Haldosen LA. Estrogen receptors alpha (ERalpha) and beta (ERbeta) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line HC11. *Oncogene* 2005;**24**: 6605–16.
37. Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, Hathaway HJ. Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. *Annu Rev Physiol* 2008;**70**:165–90.
38. Bologa CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, Kiselyov AS, et al. Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* 2006;**2**:207–12.
39. Lewis-Wambi JS, Kim HR, Wambi C, Patel R, Pyle JR, Klein-Szanto AJ, et al. Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* 2008;**10**:R104.
40. Goetz MP, Rae JM, Suman VJ, Safgren SL, Ames MM, Visscher DW, et al. Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. *J Clin Oncol* 2005;**23**:9312–8.
41. Folkman J. Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov* 2007;**6**:273–86.
42. Ma J, Waxman DJ. Combination of antiangiogenesis with chemotherapy for more effective cancer treatment. *Mol Cancer Ther* 2008;**7**:3670–84.
43. Aesoy R, Sanchez BC, Norum JH, Lewensohn R, Viktorsson K, Linderholm B. An autocrine VEGF/VEGFR2 and p38 signaling loop confers resistance to 4-hydroxytamoxifen in MCF-7 breast cancer cells. *Mol Cancer Res* 2008;**6**:1630–8.
44. Ellis M, Dehdahti F, Kommareddy A, Jamalabadi-Majidi S, Crowder R, Jeffe DB, Gao F, Fleming G, Silverman P, Dickler M, et al. A randomized phase 2 trial of low dose (6 mg daily) versus high dose (30 mg daily) estradiol for patients with estrogen receptor positive aromatase inhibitor resistant advanced breast cancer. *Cancer Res* 2009;**69**(Suppl):67s.
45. Swaby RF, Jordan VC. Low-dose estrogen therapy to reverse acquired antihormonal resistance in the treatment of breast cancer. *Clin Breast Cancer* 2008;**8**:124–33.
46. Schnelldorfer T, Gansauge S, Gansauge F, Schlosser S, Beger HG, Nussler AK. Glutathione depletion causes cell growth inhibition and enhanced apoptosis in pancreatic cancer cells. *Cancer* 2000;**89**:1440–7.
47. Bailey HH, Ripple G, Tutsch KD, Arzoomanian RZ, Alberti D, Feierabend C, et al. Phase I study of continuous-infusion L-S,R-buthionine sulfoximine with intravenous melphalan. *J Natl Cancer Inst* 1997;**89**:1789–96.
48. Lewis-Wambi JS, Swaby R, Kim H, Jordan VC. Potential of L-buthionine sulfoximine to enhance the apoptotic action of estradiol to reverse acquired antihormonal resistance in metastatic breast cancer. J Steroid Biochem Mol Biol 2009.
49. Sabnis G, Schayowitz A, Goloubeva O, Macedo L, Brodie A. Trastuzumab reverses letrozole resistance and amplifies the sensitivity of breast cancer cells to estrogen. *Cancer Res* 2009;**69**:1416–28.
50. Medina PJ, Goodin S. Lapatinib: a dual inhibitor of human epidermal growth factor receptor tyrosine kinases. *Clin Ther* 2008;**30**:1426–47.
51. Di Leo A, Gomez HL, Aziz Z, Zvirbule Z, Bines J, Arbushites MC, et al. Phase III, double-blind, randomized study comparing lapatinib plus paclitaxel with placebo plus paclitaxel as first-line treatment for metastatic breast cancer. *J Clin Oncol* 2008;**26**:5544–52.
52. Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev* 2004;**18**: 1926–45.
53. Boulay A, Rudloff J, Ye J, Zumstein-Mecker S, O'Reilly T, Evans DB, et al. Dual inhibition of mTOR and estrogen receptor signaling in vitro induces cell death in models of breast cancer. *Clin Cancer Res* 2005;**11**:5319–28.
54. Awada A, Cardoso F, Fontaine C, Dirix L, De Greve J, Sotiriou C, et al. The oral mTOR inhibitor RAD001 (everolimus) in combination with letrozole in patients with advanced breast cancer: results of a phase I study with pharmacokinetics. *Eur J Cancer* 2008;**44**:84–91.
55. O'Regan RM, Osipo C, Ariazi E, Lee ES, Meeke K, Morris C, et al. Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. *Clin Cancer Res* 2006;**12**:2255–63.
56. Sebolt-Leopold JS. MEK inhibitors: a therapeutic approach to targeting the Ras-MAP kinase pathway in tumors. *Curr Pharm Des* 2004;**10**:1907–14.
57. Morgillo F, Lee HY. Lonafarnib in cancer therapy. *Expert Opin Invest Drugs* 2006; **15**:709–19.
58. Garrofe-Ochoa X, Melero-Fernandez de Mera RM, Fernandez-Gomez FJ, Ribas J, Jordan J, Boix J. BAX and BAK proteins are required for cyclin-dependent kinase inhibitory drugs to cause apoptosis. *Mol Cancer Ther* 2008;**7**:3800–6.

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Estrogen-Induced Apoptosis in Breast Cancer Cells: Translation to Clinical Relevance

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1. Introduction

The first example of hormonal dependency of breast cancer can be dated back as far as 1896, when Dr. G.T. Beatson observed and described the reduction of breast cancer progression in a premenopausal patient after bilateral oophorectomy (Beatson 1896). It was an indication that the ovaries produced something in a woman's body that fueled breast cancer growth. This phenomenon was reconfirmed in a collected series of patients with advanced breast cancer following oophorectomy (Boyd 1900), however there was only a 30% percent response. In 1916 Lathrop and Loeb demonstrated in mice, that ovarian function has an influence on the growth of mammary glands and tumorigenesis, and that castration of immature female mice has delayed the evolution of mammary tumors (Lathrop 1916). However, the chemical control mechanisms of breast cancer progression and the relevance of ovarian function remained uncertain, until the first animal models were introduced to test the effects of oophorectomy and estrogenic properties of different chemical compounds under precise laboratory conditions (Allen 1923). This model allowed the indentification the ovarian hormone, which induced estrus in oophorectomized mice, estrogen.

In subsequent years during the 1930s and 1940s many other compounds, including diethylstilbestrol, and triphenylethylene derivatives would be identified as estrogens utilizing the ovariectomized mouse model (Robson 1937; Dodds 1938). The connection between the beneficial effects of oophorectomy as a treatment for advanced breast cancer provoked questions about the actual role of estrogen and other estrogenic compounds in breast cancer growth. High dose estrogen therapy was the first chemical therapy ("chemotherapy") to treat any cancer successfully. In 1944 Haddow (Haddow 1944) published the results of his clinical trial with the synthetic estrogens triphenylchlorethylene, triphenylmethylethylene, and diethylstilbestrol. He found that 10 out of 22 post-menopausal patients with advanced mammary carcinomas, who were treated with triphenylchlorethylene, had significant regression of tumor growth. Five patients out of 14 who were treated with high dose stilbestrol produced similar responses. The finding that high doses of synthetic estrogens induced regression of tumor growth in some, but not all postmenopausal patients with breast cancer (30% of patients responded to therapy favorably) was similar to the random responsiveness of oophorectomy in premenopausal patients with metastatic breast cancer (Boyd 1900). However, Haddow (Haddow 1944) noted that the first successful use of a chemical therapy to treat breast and prostate cancers

was affiliated with significant systemic side effects, such as nausea, areola pigmentation, uterine bleeding, and edema of the lower extremities. At approximately same time Walpole was investigating the role of diethylstilbestrol and dienestrol in breast cancer (Walpole 1948). He confirmed the results obtained by Haddow that estrogens are effective in the treatment of breast cancer and can be of benefit for patients, but also noticed that older women, and women who received higher doses of estrogens had a better response to hormonal therapy (Walpole 1948; Haddow 1950). However, the mechanisms were again undefined.

The first successful attempt to decipher the biochemistry of estrogens in mammals occurred a decade later. Tritium-labeled hexestrol was found to accumulate in reproductive organs, including mammary glands, in female goats and sheep (Glascock and Hoekstra 1959). This finding was a crucial observation to understand the role of estrogens in processes involving target tissues, such as the mammary gland. Subsequently this research was translated to the clinic with the finding that tritium-labeled hexestrol accumulated at a higher rate in patients that favorably respond to adrenalectomy and oophorectomy, comparing to patients that do not (Folca et al. 1961). This indicated that patients who would accumulate estrogens better in target breast tissue would respond better to surgical castration. However, this technical approach was not pursued further.

During the 1950's Kennedy (Kennedy and Nathanson 1953) systematically investigated the efficacy of synthetic estrogens for the treatment of advanced breast cancer. Kennedy examined a variety of different estrogens, however he found no significant differences and diethylstilbestrol became the standard drug. However, side effects still remained a concern and responses lasted for only about a year in the majority of patients. By the 1960's, the standards for the hormonal treatment of breast cancer were established. Premenopausal women were to be treated with ovarian irradiation therapy or bilateral oophorectomy. However, based on data from the clinical trials, postmenopausal patients with advanced breast cancer were to be treated with high dose of the most potent synthetic estrogenic compound diethylstilboestrol (Kennedy 1965). Overall, one could anticipate that 36 % of patients would respond favorably to high dose estrogen therapy (Kennedy 1965). However, the molecular mechanisms of the anticancer action of estrogen remained elusive. In 1970 Haddow (Haddow 1970) was not enthusiastic about the overall prospects of chemical therapy of breast cancer, he felt that it was important that safer less toxic "estrogens" were developed that might extend therapeutic use. There were clues that deciphering the mysteries of endocrine therapy, such as unknown mechanisms of tumor regression after high-dose estrogen therapy, which could be of major benefit for patient's treatment. Haddow stated: "In spite of the extremely limited practicality of such measure [high dose estrogen], the extraordinary extent of tumor regression observed in perhaps 1% of post-menopausal cases has always been regarded as of major theoretical importance, and it is a matter of some disappointment that so much of the underlying mechanisms continues to elude us". However, as noted previously, high dose estrogen therapy was more successful as a treatment for breast cancer the farther the woman was from the menopause. Estrogen withdrawal somehow played a role in sensitizing tumors to the antitumor actions of estrogen, but this fact was not appreciated at that time. We will return to this concept.

Elwood Jensen predicted the existence of estrogen receptor (ER) in 1962 (Jensen 1962), and the isolation and identification of the ER protein by Toft and Gorski occurred in 1966 (Toft and Gorski 1966). The mediating role of the ER in the estrogen responsiveness of breast

cancer was established, and eventually the ER became the molecular target for targeted therapy and prevention of ER-positive breast cancer (Jensen and Jordan 2003). It was suggested (Lacassagne 1936) in 1936 that a therapeutic agent to block estrogen action would be useful in breast cancer prevention, but there were no clues. Potential candidate antiestrogens were only discovered 20 years later in the late 1950s, but these agents were identified and screened as contraceptive drugs in laboratory animals. MER25 (Lerner et al. 1958), which was first reported as a non-steroidal antiestrogen and subsequently found to be a post-coital contraceptive in animals (Lerner and Jordan 1990). But the drug was too toxic. The first clinically useful compound MRL41 or clomiphene was tested in women; however, it was not a contraceptive, but actually induced ovulation. Nevertheless, clinical trials of clomiphene in the early 1960's did move forward to evaluate its activity in the treatment of breast cancer, but were terminated because of concerns about the drug's potential to cause cataracts (Jordan 2003). In parallel studies stimulated by the initial reports of the non-steroidal antiestrogens, ICI 46,474, the pure trans-isomer of a substituted triphenylethylene, was discovered at Imperial Chemicals Industry (ICI) Pharmaceuticals (now Astra Zeneca) and was described as a postcoital contraceptive in the rat (Harper and Walpole 1967). The Head of the Fertility Control program, Arthur Walpole, earlier in his career was interested in why only some postmenopausal women with metastatic breast cancer respond favorably to high dose estrogen therapy (Walpole 1948). Later Walpole ensured that ICI 46,474 was tested in the clinic and placed on the market as an orphan drug while ICI invested in the scientific research by others in academia to conduct a systematic study of the anticancer actions of tamoxifen and its metabolites (Jordan 2008). This investment reinvented tamoxifen as the first anticancer agent specifically targeted to the ER in the tumor and created the scientific principles to ultimately establish tamoxifen as the "gold standard" for the adjuvant therapy of breast cancer and as the first chemopreventative agent that reduces the incidence of breast cancer in women with elevated risk (Fisher et al. 1999; EBCTCG 2005).

2. Development and clinical application of antihormonal therapy

Since the clinical application of the laboratory principle of targeting the ER with long-term antihormonal therapy (Jordan 2008) to treat breast cancer has become the standard of care, two different approaches to adjuvant antihormonal therapy have been developed in the past 30 years: first, is the blockade of estrogen-stimulated growth (Jensen and Jordan 2003) at the tumor ERs with antiestrogens, and the second one, is the use of aromatase inhibitors to block estrogen biosynthesis in postmenopausal patients (Jordan and Brodie 2007). Tamoxifen was originally referred to as a non-steroidal antiestrogen (Harper and Walpole 1967). However, as more has become known about its molecular pharmacology (Jordan 2001) it has become the pioneering Selective Estrogen Receptor Modulator (SERM). The concept of SERM action was defined by four main pieces of laboratory evidence: 1) ER-positive breast cancer cells inoculated into athymic mice grew into tumors in response to estradiol, but not to tamoxifen (antiestrogenic action), however both estradiol and tamoxifen induced uterine weight increase in mice (estrogen action) (Jordan and Robinson 1987); 2) raloxifene (another non-steroidal antiestrogen), which is less estrogenic in rat uterus, maintained the bone density in ovariectomized rats (estrogen action), as did tamoxifen (Jordan et al. 1987), and prevented mammary carcinogenesis (antiestrogenic action) (Gottardis and Jordan 1987); 3) tamoxifen blocked estradiol-induced growth of ER-positive breast cancer cells in athymic mice

(antiestrogenic action), but induced rapid growth of ER-positive endometrial carcinomas (estrogenic action) (Gottardis et al. 1988); 4) raloxifene was less effective in promoting endometrial cancer growth than tamoxifen (less estrogenic action in uterine tissue) (Gottardis et al. 1990). These laboratory results all translated into clinical practice where it was shown that tamoxifen effectively can reduce the incidence of breast cancer in high-risk pre- and postmenopausal women, however increases the incidence of blood clots and endometrial cancer, which is linked to estrogen-like actions of tamoxifen in these tissues in postmenopausal women, who have a low-estrogen environment (Fisher et al. 1998).

Aromatase inhibitors have an advantage in the therapy of postmenopausal patients over tamoxifen, firstly, because there are fewer side effects, such as blood clots or endometrial cancer, and aromatase inhibitors have a small, but still significant efficacy in increasing disease free survival (Howell et al. 2005). However, most postmenopausal patients worldwide continue treatment with tamoxifen, either for economic reasons or because they were hysterectomized and also have a low risk of developing blood clots (low body mass index and are athletically active). In premenopausal women, long term tamoxifen is the antihormonal therapy of choice for the treatment of ductal carcinoma in situ (DCIS) (Fisher et al. 1999), ER-positive breast cancer treatment (EBCTCG 2005) and the reduction of breast cancer incidence in those premenopausal women at elevated risk (Fisher et al. 1998). It is important to stress that premenopausal women treated with tamoxifen do not have elevations in endometrial cancer and blood clots, thus risk: benefit ratio is in favor of tamoxifen treatment (Gail et al. 1999).

The development of raloxifene from a laboratory concept (Jordan 2007) to a clinically effective drug to prevent both osteoporosis and breast cancer (Cummings et al. 1999; Vogel et al. 2006) has created new opportunities for clinical applications of SERMs. Raloxifene is the result. However, the biggest advantage of raloxifene is that it does not increase the incidence of endometrial cancer (Vogel et al. 2006), which was noted in postmenopausal women taking tamoxifen (Fisher et al. 1998). Raloxifene is used primarily for the prevention of osteoporosis and for the prevention of breast cancer in high risk postmenopausal women.

The current clinical trend for the use of antihormonal therapy for the treatment and prevention of breast cancer is to employ long-term treatment durations. Currently aromatase inhibitors are used for a full 5 years after 5years of tamoxifen (Goss et al. 2005). Though, the clinical application of the SERM concept has proven itself to be successful for the prevention of osteoporosis and 50% of breast cancers (Vogel et al. 2006; Vogel et al. 2010), drug resistance remains an important issue arising from long-term SERM treatment. Studies have shown that after long-term SERM treatment, the pharmacology of the SERMs changes from an inhibitory antiestrogenic state to a stimulatory estrogen-like response (Gottardis and Jordan 1988).

3. Evolution of SERM resistance as deciphered by the laboratory models

Clinical and laboratory studies have identified possible mechanisms for the acquired resistance to SERMs, and tamoxifen. Acquired resistance to SERMs is unique as the tumors are SERM stimulated for growth (Howell et al. 1992). The first laboratory model (Gottardis and Jordan 1988; Gottardis et al. 1988; Gottardis et al. 1990) of transplantable tamoxifen resistant cells demonstrated that 1) tamoxifen or estrogen can cause tumors to grow, 2) tumors require a liganded receptor to grow, 3) an aromatase inhibitors (estrogen deprivation) or a pure antiestrogen that causes ER degradation would be useful second line

agents, 4) there was cross resistance with other SERMs (O'Regan et al. 2002). Currently, numerous model systems exist to study SERM resistance. Some are engineered to increase the likelihood of resistance (Osborne et al. 2003) and others are engineered by transfection of the aromatase gene to study resistance to aromatase inhibitors and compare them with tamoxifen (Brodie et al. 2003). In contrast, others have chosen to develop models naturally through selective pressure either *in vivo* or *in vitro*. The natural selection approach is to either continuously transplant the resulting SERM resistant breast cancer into SERM-treated athymic animals (Wolf and Jordan 1993; Lee et al. 2000) or to employ strategies *in vitro* that use continuous SERM treatment (Herman and Katzenellenbogen 1996; Liu et al. 2003; Park et al. 2005) or long term estrogen deprivation in culture (Song et al. 2001; Lewis et al. 2005). Distinct phases of resistance were elucidated with the use of unique models of tamoxifen-resistant breast cancer developed *in vivo*, in order to better understand the biological consequences of extended antiestrogen treatment on the survival of breast cancer. The model for the treatment phase was developed by injecting ERα-positive MCF-7 cells into athymic mice and supplementing them with post-menopausal doses of estradiol (E2) (86–93 pg/ml) (Robinson and Jordan 1989), which were estradiol-stimulated and tamoxifen (TAM)-inhibited (Figure 1).

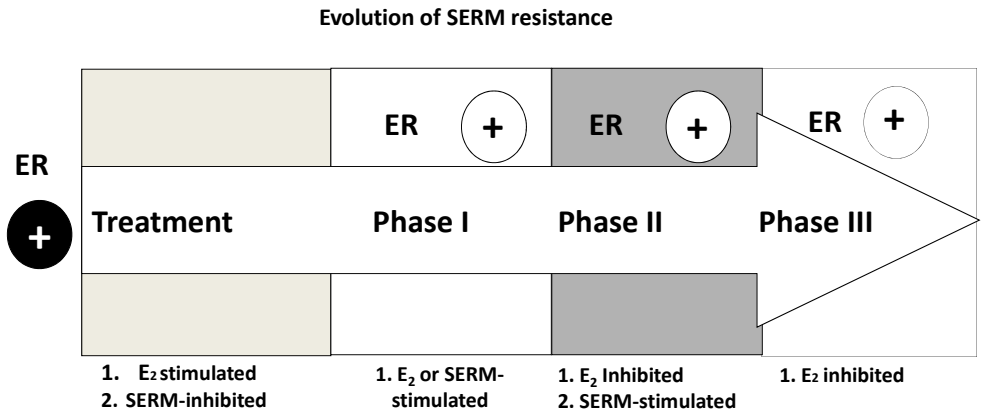

Fig. 1. Evolution of SERM resistance as observed in animal models.

With short term treatment (<2 years) with tamoxifen Phase I TAM-resistant breast tumors developed, which were stimulated to grow by both E2 and tamoxifen (Figure 1) (Gottardis and Jordan 1988; Osborne et al. 1991). The novel model of Phase II resistance to tamoxifen was developed by long-term treatment (>5 years) of breast tumors with tamoxifen (MCF-7TAMLT). These MCF-7TAMLT tumors were stimulated to grow with tamoxifen, but paradoxically were inhibited by estradiol (Figure 1) (Wolf and Jordan 1993; Yao et al. 2000; Osipo et al. 2003). The phase when all known therapies fail and only E2-inhibit the growth is referred to as phase III resistance (Figure 1) (Jordan 2004). Interestingly, during the progression from the treatment phase to Phase III resistance, a cyclic phenomenon was observed where initially estradiol-inhibited growth of Phase II TAM-resistant tumors followed by re-sensitization to estradiol as a growth stimulant (Yao et al. 2000). These new estradiol-stimulated MCF-7 tumors from Phase II tamoxifen-resistant tumors were inhibited by treatment with either TAM or fulvestrant demonstrating complete reversal of drug resistance to tamoxifen (Yao et al. 2000). A similar phenomenon was observed with

raloxifen-resistance (Balaburski et al. 2010). In addition to SERM-resistant tumors, estradiol, at physiologic concentrations, has also been shown to induce apoptosis in long term estrogen deprived (LTED) breast cancer cells *in vitro* and *in vivo*. We noted previously, that in the past, pharmacologic estrogen was employed in therapy of advanced breast cancer that resulted in favorable responses with regression of disease (Haddow 1944). Estrogen therapy yields as high as 40% response rate as first-line treatment in patients with hormonally sensitive breast cancer with metastatic disease (Ingle et al. 1981) and approximately 31% in patients heavily pre-treated with previous endocrine therapies (Lonning et al. 2001). The unique aspect of current laboratory findings is that physiologic estrogen can induce tumor regression in long-term anti-hormone drug resistance (Wolf and Jordan 1993; Yao et al. 2000; Song et al. 2001; Jordan and Ford 2011). But what are the mechanisms?

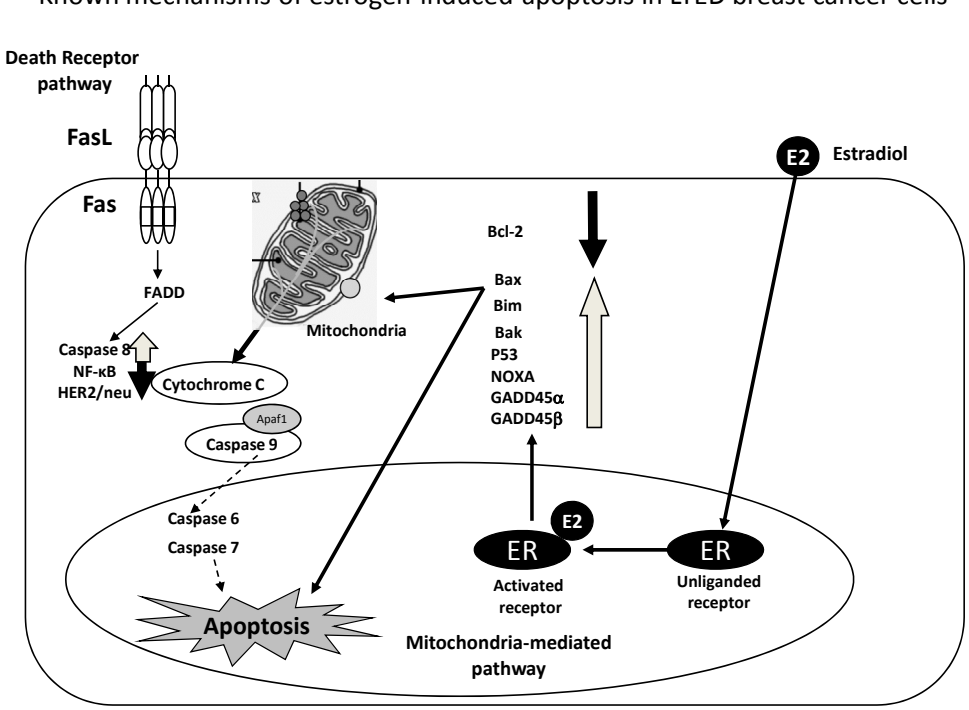

Fig. 2. Mechanisms of estrogen-induced apoptosis in Long-Term Estrogen Deprived (LTED) breast cancer cells. Both FasR/FasL death-signaling and mitochondrial pathways are involved.

4. Mechanism of estrogen-induced apoptosis

To investigate the mechnisms of estradiol-induced apoptosis SERM-stimulated models (Liu et al. 2003; Osipo et al. 2003) or long-term estrogen deprived MCF-7 breast cancer cell lines (Song et al. 2001; Lewis et al. 2005; Lewis et al. 2005) have been interrogated. A link between estradiol-induced apoptosis and activation of the FasR/FasL death-signaling pathway was demonstrated in tamoxifen-stimulated breast cancer tumors by inducing the death receptor

Fas with physiologic levels of estradiol and suppressing the antiapoptotic/prosurvival factors NF-κB and HER2/neu (Osipo et al. 2003; Lewis et al. 2005). A similar finding was reported (Liu et al. 2003) for raloxifene-resistant tumor cells where the growth of raloxifene-resistant MCF-7/Ral cells *in vitro* and *in vivo* was repressed by estradiol via mechanism involving increased Fas expression and decreased NF-κB activity. Furthermore, MCF-7 cells deprived of estrogen for up to 24 months (MCF-7LTED) *in vitro* expressed high levels of Fas compared to the parental MCF-7 cells, which do not express Fas and treatment of the MCF-7/LTED cells with estradiol resulted in a marked increase in Fas ligand (FasL) in these cells (Song et al. 2001). It was also noted that mitochondrial pathway could play a role in mediating estrogen induced apoptosis as the basal expression levels of Bcl-2 were higher in these cells than in the parental MCF-7 cells. Estradiol induced apoptosis occurs in a LTED breast cancer cell line named MCF-7:5C by neutralization of the Bcl-2/Bcl-XL proteins, and upregulation of proapoptotic proteins such as Bax, Bak and Bim, which proves the role of intrinsic mitochondrial pathway (Lewis et al. 2005) (Figure 2).

In MCF-7:5C cells the expression of several pro-apoptotic proteins—including Bax, Bak, Bim, Noxa, Puma, and p53—are markedly increased with estradiol treatment and blockade of Bax and Bim expression using siRNAs almost completely reversed the apoptotic effect of estradiol. Estradiol treatment also led to a loss of mitochondrial potential and a dramatic increase in the release of cytochrome *c* from the mitochondria, which resulted in activation of caspases and cleavage of PARP. Furthermore, overexpression of anti-apoptotic Bcl-x_L was able to protect MCF-7:5C cells from estradiol-induced apoptosis. This particular study was the first to show a link between estradiol-induced cell death and activation of the mitochondrial apoptotic pathway using a breast cancer cell model resistant to estrogen withdrawal (Lewis et al. 2005). Besides the action on the mitohodrial pathway, Bcl-2 overexpression increases cellular glutathione (GSH) level which is associated with increased resistance to chemotherapy-induced apoptosis (Voehringer 1999). GSH is a water-soluble tripeptide composed of glutamine, cysteine, and glycine. It is the most abundant intracellular small molecule thiol present in mammalian cells and it serves as a potent intracellular antioxidant protecting cells from toxins such as free radicals (Schroder et al. 1996; Anderson et al. 1999). Changes in GSH homeostasis have been implicated in the etiology and progression of some diseases and breast cancer (Townsend et al. 2003) and studies have shown that elevated levels of GSH prevent apoptotic cell death whereas depletion of GSH facilitates apoptosis (Anderson et al. 1999). Our laboratory has found evidence which suggests that GSH participates in retarding apoptosis in antihormone-resistant MCF-7:2A human breast cancer cells, which have ~60% elevated levels of GSH compared to wild-type MCF-7 cells and unable to undergo estrogen-induced apoptosis within 1 week unlike MCF-7:5C cells, and that depletion of GSH by 100 μM of L-buthionine sulfoximine (BSO), a potent inhibitor of glutathione biosynthesis, sensitizes these resistant cells to estradiol-induced apoptosis (Lewis-Wambi et al. 2008). However, the question arises as to the actual mechanism of the apoptotic trigger mediated by the ER complex.

5. Structure-function relationship studies for deciphering estrogen-induced apoptosis

The fact that SERMs do not affect the spontaneous growth of MCF-7:5C cells, but can completely block estradiol-induced apoptosis, was an important clue that the shape of the

ER can be modulated to prevent apoptosis. Extensive structure-function relationship studies were initially used to develop a molecular model of estrogen and antiestrogen action (Lieberman et al. 1983; Jordan et al. 1984; Jordan et al. 1986). The hypothetical model presumed the envelopment of a planar estrogen within the ligand-binding domain (LBD) of the ER complex. In contrast, the three-dimensional triphenylethylene binding in the LBD cavity prevents full ER's activation by keeping the LBD open. This structural perturbation of the ER complex is achieved by a correctly positioned bulky side chain on the SERM. This model was enhanced by the subsequent studies to solve the X-ray crystallography of the LBD ER's bound with an estrogen or an antiestrogen (Brzozowski et al. 1997; Shiau et al. 1998). The LBD of ERα is formed by H2-H11 helices and the hairpin β-sheet, while H12, in the agonist bound conformation closes over the LBD cavity filled with E2. E2 is aligned in the cavity by hydrogen bonds at both ends of the ligand, particularly the 3-OH group at the A-ring end of E2. This allows hydrophobic van der Waals contacts along the lipophilic rings of E2, in particular between Phe404 and E2's A-ring, to promote a low energy conformation (Brzozowski et al. 1997). This results in sealing of the ligand-binding cavity by H12, and exposes the AF-2 motif at the surface of the receptor for interaction with coactivators to promote transcriptional transactivation. In contrast, 4-hydroxytamoxifen binds to ER's LBD to block the closure of the cavity by relocating H12 away from the binding pocket, thus preventing coactivator molecules from binding to the appropriate site on the external surface of the complex, which produces an antiestrogenic effect (Shiau et al. 1998). Therefore, it is the external shape of the ERs that is being modulated by the ligand which dictates the binding of coactivator molecules. In other words, the shape of the ligand actually causes the receptor to change shape and programs the ER complex to be able to bind coregulator molecules. However, the simple model of a coregulator controlling the biology of an ER complex is not that simple. The modulation of the estrogen target gene is in fact, regulated by a dynamic process of assembly and destruction of transcription complex at the promoter site of a target gene. After ER is bound to an agonist ligand, its conformation changes allowing coregulator molecules to bind to the complex, for example, SRC-3. SRC-3 is a core coactivator that also attracts other coregulators that do not directly bind to ER, such as p300/CBP histone acetyltransferase, CARM1 methyltransferase, and ubiquitin ligases UbC and UbL. All of these coregulators perform specific subreactions within the protein complex of ER and DNA necessary for transcription of target genes, such as chromatin remodeling through methylation and acetylation modifications, and also direct their enzymatic activity towards adjacent factors, which promote dissociation of the coactivator complex and subsequent ubiquitinilation of select components for proteosomal degradation. As a result, this allows the next cycle of coactivator-receptor-DNA interactions to proceed and the binding and degradation of transcription complexes sustaining the gene transcription (Lonard et al. 2000). However, although AF-2 is deactivated by 4OHTAM, the 4OHTAM:ERα complex has estrogen-like activity (Levenson et al. 1998), whereas raloxifene does not (Levenson et al. 1997). This is believed to be because the side chain of raloxifene shields and neutralizes asp351 to block estrogen action (Levenson and Jordan 1998). In contrast the side chain of tamoxifen is too short. It appears that when helix 12 is not positioned correctly the exposed asp351 can interact with AF-1 to produce estrogen action. This estrogen-like activity can be inhibited by substituting asp351 for glycine an uncharged amino acid (MacGregor Schafer et al. 2000). However, knowledge of the structure of the

4OHTAM: ER LBD complex (Shiau et al. 1998) led to the idea that all estrogens may not be the same in their interactions with ER (Jordan et al. 2001). Previous studies suggest that non-planar TPEs with a bulky phenyl substituent prevents helix-12 from completely sealing the LBD pocket (Jordan et al. 2001). This physical event creates a putative 'anti-estrogen like' configuration within the complex. However, the complex is not anti-estrogenic because Asp351 is exposed to communicate with AF-1 thus causing estrogen-like action. Therefore, there are putative Class I (planar) and Class II (non-planar) estrogens (Jordan et al. 2001). A similar classification and conclusion has been proposed (Gust et al. 2001), but the biological consequences of this classification were unknown until recently.

To further address the hypothesis that the shape of the ER complex can be controlled by the shape of an estrogen, and thereby altering its functional properties, such as induction of apoptosis, a range of hydroxylated TPEs was synthesized (Figure 3) to establish new tools to investigate the relationship of shape with estrogenic activity through the exposure of asp351 (Maximov et al. 2010).

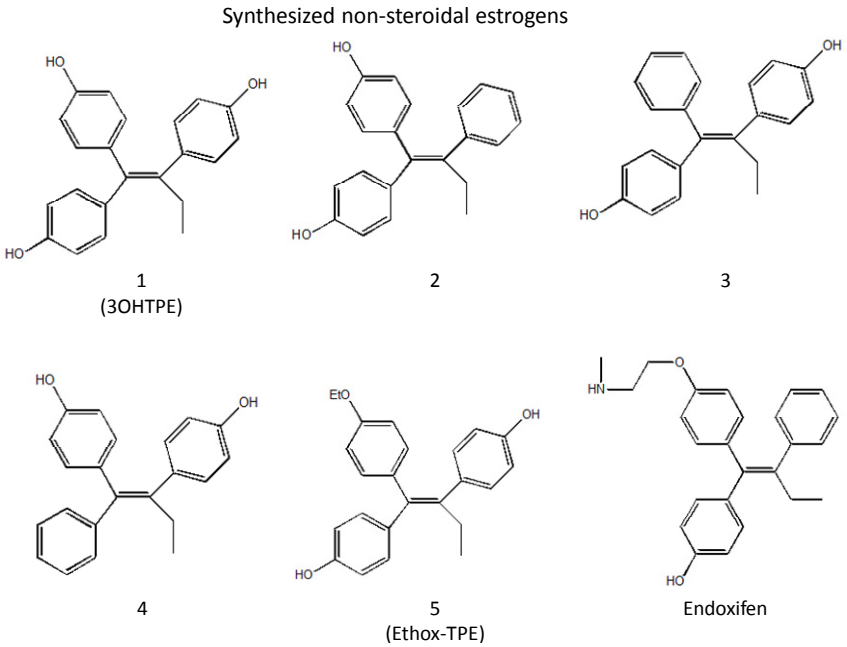

Fig. 3. Synthesized class II non-steroidal estrogens. All estrogens are hydroxylated derivatives of triphenylethylene; 1 – 3-hyrdoxytriphenylethylene (3OHTPE), 2- bisphenoltripenylethylene, 3 – E-dihydroxytriphenylethylene, 4- Z-dihydroxytriphenylethylene, 5- ethoxytripenylethylene, and Endoxifen (a metabolite of the antiestrogenic triphenylethylene tamoxifen with high affinity for the estrogen receprtor).

We compared and contrasted the estrogen-like properties of the hydroxylated TPEs to promote proliferation in the ERα-positive human breast cancer cell line MCF-7:WS8 cells (Figure 4A), which are hypersensitive to the proliferative actions of E2. Compounds were compared with the tamoxifen metabolites 4-OHT and endoxifen. Results show that our

MCF-7:WS8 human breast cancer cells were exquisitely sensitive to E2 which produced a concentration-dependent increase in growth, and all of the TPE's were potent agonists with the ability to stimulate MCF-7:WS8 breast cancer cell growth, however, their agonist potency was less compared to E2. The metabolites, 4-OHT and endoxifen, had no significant agonist effect in MCF-7:WS8 cells, however, these compounds at 1 μM were able to completely inhibit estradiol-stimulated MCF-7:WS8 breast cancer cell growth, thus confirming their role as antiestrogens (data not shown). To determine the ability of the test TPEs to activate the ER, MCF-7:WS8 cells were transiently transfected with an ERE-luciferase reporter gene encoding the firefly reporter gene with 5 consecutive Estrogen Responsive Elements (EREs) under the control of a TATA promoter. The binding of ligand-activated ER complex at the EREs in the promoter of the luciferase gene activates transcription. The measurement of the luciferase expression levels permits a determination of agonist activity of the TPE:ER complex. All the phenolic TPEs were estrogenic and induced the increase of ERE-luciferase activity, but were less potent compared to E2. To confirm and advance the hypothesis that the shape of the estrogen ER complex was different for planar and nonplanar (TPE –like) estrogens, series of tested phenolic TPEs were evaluated in the ER-negative breast cancer cell line T47D:C42 (Pink et al. 1996) which was transiently transfected with an ERE luciferase plasmid and either the wild-type ER or the D351G mutant ER plasmids. Previously it was found that the mutant D351G ER completely suppressed estrogen-like properties of 4-OHT at an endogenous TGFα target gene(MacGregor Schafer et al. 2000). We established that in the presence of the wild-type ER all of the tested TPE compounds were potent agonists with the ability to significantly enhance ERE luciferase activity (Figure 4C). In contrast, when the D351G mutant ER gene was transfected with the ERE luciferase reporter only the planar E_2 was estrogenic whereas the TPEs did not activate the ERE reporter gene (Figure 4D). These results confirm the importance of Asp351 in ER activation by TPE ligands to trigger estrogen action. To further confirm the hypothesis, the best "fits" of the tested TPEs and endoxifen, obtained from docking simulations ran against the antagonist conformation of the ER, were superimposed on the experimental agonist conformation of the ER. Overall the TPEs are unlikely to be accommodated in the agonist conformation of the ER due to the sterical clashes between "Leu crown", mostly Leu525 and Leu540, helix 12 and ligands, indicating, that these ligands most likely bind to ER's conformation more closely related with the antagonist form. X-ray crystallography of ER-4OHTAM and ER-Raloxifene complexes, demonstrating that the presence of the alkyaminoethoxy sidechain of 4OHTAM is crucial for the ER to gain an antagonistic conformation by displacing the H12 of the receptor by 4OHTAM's bulky sidechain, thus preventing the binding of the coactivators (Shiau et al. 1998). The absence of the alkyaminoethoxy sidechain on the tested TPEs does not allow these compounds to act as antiestrogens, like 4-OHT or endoxifen, which posseses the alkyaminoethoxy sidechain (Shiau et al. 1998). However, the fact that these TPEs were able to significantly induce growth and ERE activation in MCF-7:WS8 cells demonstrated that they are still full agonists, despite the changes in biological potencies of the tested TPEs, due to repositioning of the hydroxyl groups and addition of the ethoxy group. Thus cell growth is a very sensitive property of the ligand:ER complex and can occur minimally with an AF-1 function alone in the case of TPEs but also with the possibility for interacting with a perturbated LBD. 4OHT does not stimulate growth so possibly a corepressor binds in the case of a SERM:ER complex. An interesting aspect of the study (Maximov et al. 2010) is the importance of Asp351 in activation of the ER thereby acting as a molecular test for the presumed structure

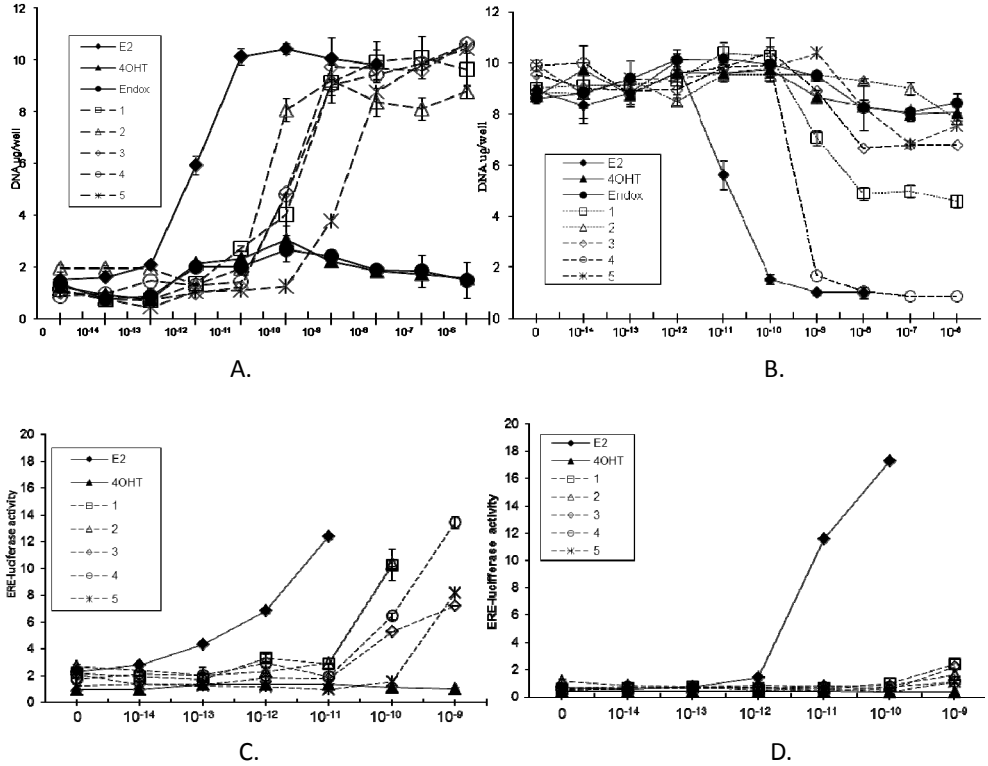

Fig. 4. A: Agonist activity in MCF-7:WS8 cells of synthesized TPEs and E2 and anti-estrogens 4-OHT and Endoxifen; B: E2 induces apoptosis in long-term estrogen deprived MCF-7:5C cells and synthesized TPEs are unable to act as full agonists resembling more anti-estrogens 4-OHT and Endoxifen; C: E2 and all TPEs are able to increase the activity of luciferase in T47D:C4:2 cells transiently transfected with wild-type ER DNA construct; D: E2 is the only agonist in D351G ER mutant T47D:C4:2 cells, as TPEs are unable to increase the luciferase activity in cells expressing the mutant form of ER, indicating the importance of Asp351 of the ER for activation with non-planar TPEs.

of the TPE:ER complex. Based on the X-ray crystallography of the ER in complex with 4OHTAM (Shiau et al. 1998) and raloxifene (Brzozowski et al. 1997), it was determined that the basic side chains of these antiestrogens are in proximity of Asp351 in the ER. It was hypothesized that this interaction with raloxifene actually neutralizes and shields Asp351 preventing it from interacting with ligand-independent activating function 1 (AF-1). In contrast, 4OHTAM possesses some estrogenic activity, because the side chain is too short (Shiau et al. 1998). Substitution of Asp351 with Glycine which is a non-charged aminoacid, leads to loss of estrogenic activity of the ER bound with 4OHTAM (MacGregor Schafer et al. 2000; Levenson et al. 2001). Results from ERE luciferase assays in T47:C4:2 cells transiently transfed with wild type and D351G mutant ER expression plasmids demonstrated that wild type ER was activated by all of the tested TPEs, however substitution of Asp351 by Gly prevented the increase of ERE luciferase activity by all TPEs and only planar E2, which does

not interact with Asp351 at all, or exposes it on the surface of the complex, was able to activate ERE in D351G ER transfected cells. This confirms and expands the classification of estrogens, where planar estrogens such as E2 are classified as class I and all TPE-related estrogens are classified as class II estrogens based on the mechanism of activation of the ER (Jordan et al. 2001).

Further we tested the hypothesis that, the shape of the ER complex with either planar estrogens (Class I) or angular estrogens (Class II), can modulate the apoptotic actions of estrogen through the shape of the resulting complex. In this study MCF-7:5C cells were employed to investigate the actions of 4-OHT and our model TPEs on estradiol-induced apoptosis. As estrogen-induced apoptosis can be reversed in a concentration related manner by the nonsteroidal antiestrogen 4-OHT, paradoxically, all tested TPEs were able to reverse the apoptotic effect of estradiol in MCF-7:5C cells, at the same time the tested TPEs alone were not able to induce apoptosis in these cells significantly (Figure 4B). However, the tested TPEs have still retained their ability to induce ERE-luciferase activity in MCF-7:5C cells, indicating that these compounds are still agonists of the ER in these cells, but biologically acted as antagonists. Besides differences in biological effects of TPEs in MCF-7 cells and MCF-7:5C cells, biochemical effects of tested TPEs on ER complex similar to those with 4-OHT were studied. 4-OHT is known to retard the destruction of the 4-OHT ER complex (Pink and Jordan 1996; Wijayaratne and McDonnell 2001). Similarly, the TPEs do not facilitate the rapid destruction of the TPE:ER complex, as it was shown via Western blotting that the TPE:ER levels are analogous to 4-OHT:ER levels rather than estradiol ER-like, where ER is rapidly degraded. As it was noted previously, ER degradation plays a crucial role in estrogen-mediated gene expression. It was previously shown that ER protein degradation is proteosome mediated (Lonard et al. 2000; Reid et al. 2003), and ER coactivator SRC3/AIB1 links the transcriptional activity of the receptor and its proteosome degradation (Shao et al. 2004). Our results indicate that the transcriptional activity of ER, based on qRT-PCR results, is similar on the pS2 gene in both MCF-7:WS8 cells and MCF-7:5C cells with the tested TPE compounds, and based on our ChIP assay results for evaluating the ER's recruitment on the pS2 gene promoter, the E2:ER complex has robust binding in the promoter region and SRC-3 is detected presumably bound to the ER complex, however, 4-OHT:ER complexes only have modest binding of ERα and virtually no SRC-3 in the promoter region, at the same time, the TPEs permit some binding of the TPE:ER complexes in the promoter region but there are lower levels of SRC-3 and a reduced ability to stimulate PS2 mRNA synthesis (Figure 5).

We believe that the changed conformation of the TPE:ER complex, prevents the complete closure of H12 over the ligand-binding cavity and thus does not allow co-activators to bind to the incompletely open AF-2 motif on the ER's surface. Indeed, LeClercq's group (Bourgoin-Voillard et al. 2010) have recently confirmed and extended our molecular classifications of estrogens, with a larger series of compounds and have also shown that an angular TPE does not cause the destruction of the ER complex in a manner analogous to estradiol when MCF-7 cells are examined by immunohistochemistry for the ER, and that the putative Class II estrogens that do not permit the appropriate sealing of the LBD with helix 12 do not efficiently bind co-activators, therefore our respective studies are in agreement.

In summary, the proposed hypothesis that the TPE-ER complex significantly changes the shape of the ER to adopt a conformation that mimics that adopted by 4-OHT when it binds to the ER. A co-activator now has difficulty in binding to the TPE-ER complex

appropriately, but whereas this does affect cell replication, it dramatically impairs the events that must be triggered to cause apoptosis. Future studies will confirm or refute our hypothesis based upon the known intrinsic activity of mutant ERs and their capacity to investigate estrogen-target genes.

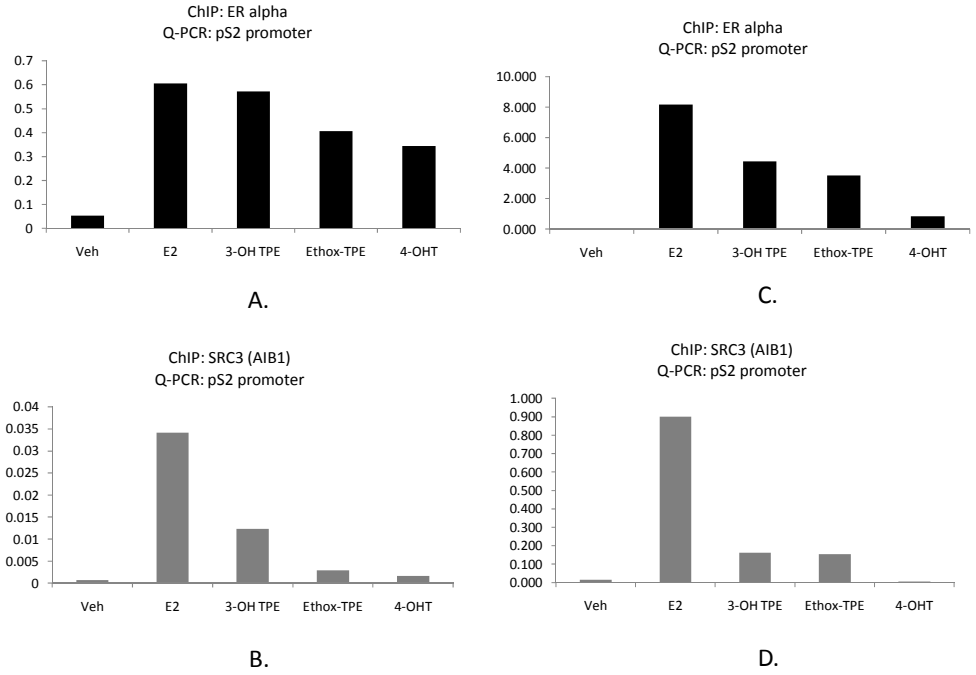

Fig. 5. A&B: ChIP analysis performed in MCF-7:WS8 cells with pS2 promoter region was pulled down via anti-ERα antibody (A) and anti-SRC3/AIB1 antibody (B); C&D: ChIP analysis performed in MCF-7:5C cells with pS2 promoter region pulled down via anti-ERα antibody (C) and anti-SRC3/AIB1 antibody (D). All results indicate that in both cell lines tested TPEs and E2 recruit ERα complex to the pS2 promoter region, but interestingly, class II estrogens are unable to co-recruit sufficient amount of SRC-3 co-activator, unlike E2.

6. Relevance to current clinical research

Laboratory studies show that low concentrations of estrogen can cause apoptotic death of breast tumor cells, following estrogen deprivation with antihormonal treatment. This has translated very well into the clinic, and recent clinical trials have demonstrated that low-dose estrogen treatment can effectively be utilized after the formation of resistance to antihormonal treatment. Ellis and colleagues (Ellis et al. 2009) have shown, that a daily dose of 6 mg of estradiol could stop the growth of tumors or even cause them to shrink in about 25% of women with metastatic breast cancer that had developed resistance to antihormonal therapy. At the same time, these results correlate with earlier results obtained by Loenning and coworkers (Lonning et al. 2001), who have studied the efficacy of high dose of DES on the responsiveness of metastatic breast cancer following exhaustive antihormonal treatment

with tamoxifen, aromatase inhibitors and etc. 4 out of 32 patients had complete responses (Lonning et al. 2001) and 1 patient after 5 year treatment with DES had no recurrence for a following 6 years (Lonning 2009). The question at that moment remains whether estrogen at physiologic concentrations can be efficient as antitumor agent in estrogen-deprived breast tumors. As mentioned previously, Ellis and coworkers have demonstrated that an equivalent clinical benefit for high (30 mg daily) and low (6 mg daily) dose of estradiol in metastatic breast cancer patients who had failed aromatase inhibitor therapy, which is long-term estrogen deprivation. Overall, the results demonstrate that low dose estrogen therapy has fewer systemic sideffects, but the same efficacy as a treatment for long-term antihormone resistant breast cancer as high dode estrogen therapy. This can be seen as "replacement with" physiologic estrogen to premenopausal levels. The benefit-risk ratio is in favor of low-dose estrogen therapy. These results correlate well with results from WHI trial of estrogen-replacement therapy (ERT) in hysterectomized postmemopausal women (LaCroix et al. 2011). The WHI results show a sustained reduction in the incidence of breast cancer in postmenopausal women up to 5 years after the intervention with conjugated equine estrogens for 5 years prior. It was demonstrated that the group of patients receiving conjugated equine estrogens had incidence of breast cancer 0.27% in comparison to the control group of patients the incidence was 0.35%. The idea that woman's own estrogen can act as an antitumor agent after estrogen-deprivation to prevent metastization and tumor growth (Wolf and Jordan 1993) has lead to incorporation into the Study of Letrozole Extension (SOLE) trial. This trial is addressing the question whether regular drug holydays can decrease recurrence of breast cancer by physiologic estrogen after deprivation with aromatase inhibitor letrozole. Subsequent trials may have to use ERT for a few weeks to trigger apoptosis.

7. Conclusion

Taken together, the demonstrations of the apoptotic actions of estrogen as a potential anticancer agent in postmenopausal breast cancer patients, now provides a rationale to further explore and decipher mechanisms of estrogen-induced apoptosis. There is a possibility that future studies on the molecular mechanism of estrogen-induced apoptosis will help to indentify new more safer and specific agents for breast cancer therapy.

8. Acknowledgments

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9. References

Allen, E., Doisy, E.A. (1923). An ovarian hormone: Preliminary reports on its localization extraction and partial purification and action in test animals. *JAMA* 81: 810-821.

Anderson, C. P., J. M. Tsai, et al. (1999). Depletion of glutathione by buthionine sulfoxine is cytotoxic for human neuroblastoma cell lines via apoptosis. *Exp Cell Res* 246(1): 183-192.

Balaburski, G. M., R. C. Dardes, et al. (2010). Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. *Int J Oncol* 37(2): 387-398.

Beatson, G. T. (1896). On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. *Lancet* 2: 162-165.

Bourgoin-Voillard, S., D. Gallo, et al. (2010). Capacity of type I and II ligands to confer to estrogen receptor alpha an appropriate conformation for the recruitment of coactivators containing a LxxLL motif-Relationship with the regulation of receptor level and ERE-dependent transcription in MCF-7 cells. *Biochem Pharmacol* 79(5): 746-757.

Boyd, S. (1900). On Oophorectomy in cancer of the breast. *British Medical Journal* 2: 134-141.

Brodie, A. H., D. Jelovac, et al. (2003). The intratumoral aromatase model: studies with aromatase inhibitors and antiestrogens. *J Steroid Biochem Mol Biol* 86(3-5): 283-288.

Brzozowski, A. M., A. C. Pike, et al. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 389(6652): 753-758.

Cummings, S. R., S. Eckert, et al. (1999). The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* 281(23): 2189-2197.

Dodds, E. C., Goldberg, L., Lawson, W., Robinson, R. (1938). Estrogenic activity of certain synthetic compounds. *Nature* 141: 247-248.

EBCTCG (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 365(9472): 1687-1717.

Ellis, M. J., F. Gao, et al. (2009). Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *JAMA* 302(7): 774-780.

Fisher, B., J. P. Costantino, et al. (1998). Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 90(18): 1371-1388.

Fisher, B., J. Dignam, et al. (1999). Tamoxifen in treatment of intraductal breast cancer: National Surgical Adjuvant Breast and Bowel Project B-24 randomised controlled trial. *Lancet* 353(9169): 1993-2000.

Folca, P. J., R. F. Glascock, et al. (1961). Studies with tritium-labelled hexoestrol in advanced breast cancer. Comparison of tissue accumulation of hexoestrol with response to bilateral adrenalectomy and oophorectomy. *Lancet* 2(7206): 796-798.

Gail, M. H., J. P. Costantino, et al. (1999). Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J Natl Cancer Inst* 91(21): 1829-1846.

Glascock, R. F. and W. G. Hoekstra (1959). Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep. *Biochem J* 72: 673-682.

Goss, P. E., J. N. Ingle, et al. (2005). Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17. *J Natl Cancer Inst* 97(17): 1262-1271.

Gottardis, M. M. and V. C. Jordan (1987). Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* 47(15): 4020-4024.

Gottardis, M. M. and V. C. Jordan (1988). Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 48(18): 5183-5187.

Gottardis, M. M., M. E. Ricchio, et al. (1990). Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. *Cancer Res* 50(11): 3189-3192.

Gottardis, M. M., S. P. Robinson, et al. (1988). Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 48(4): 812-815.

Gust, R., R. Keilitz, et al. (2001). Investigations of new lead structures for the design of selective estrogen receptor modulators. *J Med Chem* 44(12): 1963-1970.

Haddow, A. (1950). The chemotherapy of cancer. *Br Med J* 2(4691): 1271-1272.

Haddow, A. (1970). David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer* 26(4): 737-754.

Haddow, A., Watkinson, J.M., Paterson, E. (1944). Influence of synthetic oestrogens upon advanced malignant disease. *British Medical Journal*: 393-398.

Harper, M. J. and A. L. Walpole (1967). A new derivative of triphenylethylene: effect on implantation and mode of action in rats. *J Reprod Fertil* 13(1): 101-119.

Herman, M. E. and B. S. Katzenellenbogen (1996). Response-specific antiestrogen resistance in a newly characterized MCF-7 human breast cancer cell line resulting from long-term exposure to trans-hydroxytamoxifen. *J Steroid Biochem Mol Biol* 59(2): 121-134.

Howell, A., J. Cuzick, et al. (2005). Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* 365(9453): 60-62.

Howell, A., D. J. Dodwell, et al. (1992). Response after withdrawal of tamoxifen and progestogens in advanced breast cancer. *Ann Oncol* 3(8): 611-617.

Ingle, J. N., D. L. Ahmann, et al. (1981). Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *N Engl J Med* 304(1): 16-21.

Jensen, E. V., Jacobson, H.I. (1962). Basic guidelines to the mechanism of estrogen action. *Recent Progr. Hormone Res.* 18: 387-414.

Jensen, E. V. and V. C. Jordan (2003). The estrogen receptor: a model for molecular medicine. *Clin Cancer Res* 9(6): 1980-1989.

Jordan, V. C. (2001). Selective estrogen receptor modulation: a personal perspective. *Cancer Res* 61(15): 5683-5687.

Jordan, V. C. (2003). Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2(3): 205-213.

Jordan, V. C. (2004). Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 5(3): 207-213.

Jordan, V. C. (2007). SERMs: meeting the promise of multifunctional medicines. *J Natl Cancer Inst* 99(5): 350-356.

Jordan, V. C. (2008). Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 44(1): 30-38.

Jordan, V. C. and A. M. Brodie (2007). Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* 72(1): 7-25.

Jordan, V. C. and L. G. Ford (2011). Paradoxical Clinical Effect of Estrogen on Breast Cancer Risk: A "New" Biology of Estrogen-induced Apoptosis. *Cancer Prev Res (Phila)* 4(5): 633-637.

Jordan, V. C., R. Koch, et al. (1986). Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. *Br J Pharmacol* 87(1): 217-223.

Jordan, V. C., M. E. Lieberman, et al. (1984). Structural requirements for the pharmacological activity of nonsteroidal antiestrogens in vitro. *Mol Pharmacol* 26(2): 272-278.

Jordan, V. C., E. Phelps, et al. (1987). Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 10(1): 31-35.

Jordan, V. C. and S. P. Robinson (1987). Species-specific pharmacology of antiestrogens: role of metabolism. *Fed Proc* 46(5): 1870-1874.

Jordan, V. C., J. M. Schafer, et al. (2001). Molecular classification of estrogens. *Cancer Res* 61(18): 6619-6623.

Kennedy, B. J. (1965). Hormone therapy for advanced breast cancer. *Cancer* 18(12): 1551-1557.

Kennedy, B. J. (1965). Systemic effects of androgenic and estrogenic hormones in advanced breast cancer. *J Am Geriatr Soc* 13: 230-235.

Kennedy, B. J. and I. T. Nathanson (1953). Effects of intensive sex steroid hormone therapy in advanced breast cancer. *J Am Med Assoc* 152(12): 1135-1141.

Lacassagne, A. (1936). Hormonal pathogenesis of adenocarcinoma of the breast. *Am J Cancer* 27: 217-225.

LaCroix, A. Z., R. T. Chlebowski, et al. (2011). Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. *JAMA* 305(13): 1305-1314.

Lathrop, A. E. C., Loeb, L. (1916). Further Investigations on the Origin of Tumors in Mice. *J Cancer Res* 1(1): 1-19.

Lee, E. S., J. M. Schafer, et al. (2000). Cross-resistance of triphenylethylene-type antiestrogens but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 6(12): 4893-4899.

Lerner, L. J., F. J. Holthaus, Jr., et al. (1958). A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenylethanol. *Endocrinology* 63(3): 295-318.

Lerner, L. J. and V. C. Jordan (1990). Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. *Cancer Res* 50(14): 4177-4189.

Levenson, A. S., W. H. Catherino, et al. (1997). Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. *J Steroid Biochem Mol Biol* 60(5-6): 261-268.

Levenson, A. S. and V. C. Jordan (1998). The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. *Cancer Res* 58(9): 1872-1875.

Levenson, A. S., J. I. MacGregor Schafer, et al. (2001). Control of the estrogen-like actions of the tamoxifen-estrogen receptor complex by the surface amino acid at position 351. *J Steroid Biochem Mol Biol* 76(1-5): 61-70.

Levenson, A. S., D. A. Tonetti, et al. (1998). The oestrogen-like effect of 4-hydroxytamoxifen on induction of transforming growth factor alpha mRNA in MDA-MB-231 breast cancer cells stably expressing the oestrogen receptor. *Br J Cancer* 77(11): 1812-1819.

Lewis-Wambi, J. S., H. R. Kim, et al. (2008). Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* 10(6): R104.

Lewis, J. S., K. Meeke, et al. (2005). Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 97(23): 1746-1759.

Lewis, J. S., C. Osipo, et al. (2005). Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 94(1-3): 131-141.

Lieberman, M. E., J. Gorski, et al. (1983). An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. *J Biol Chem* 258(8): 4741-4745.

Liu, H., E. S. Lee, et al. (2003). Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. *J Natl Cancer Inst* 95(21): 1586-1597.

Lonard, D. M., Z. Nawaz, et al. (2000). The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. *Mol Cell* 5(6): 939-948.

Lonning, P. E. (2009). Additive endocrine therapy for advanced breast cancer - back to the future. *Acta Oncol* 48(8): 1092-1101.

Lonning, P. E., P. D. Taylor, et al. (2001). High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 67(2): 111-116.

MacGregor Schafer, J., H. Liu, et al. (2000). Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. *Cancer Res* 60(18): 5097-5105.

Maximov, P. Y., C. B. Myers, et al. (2010). Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. *J Med Chem* 53(8): 3273-3283.

O'Regan, R. M., C. Gajdos, et al. (2002). Effects of raloxifene after tamoxifen on breast and endometrial tumor growth in athymic mice. *J Natl Cancer Inst* 94(4): 274-283.

Osborne, C. K., V. Bardou, et al. (2003). Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst* 95(5): 353-361.

Osborne, C. K., E. Coronado, et al. (1991). Acquired tamoxifen resistance: correlation with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxytamoxifen. *J Natl Cancer Inst* 83(20): 1477-1482.

Osipo, C., C. Gajdos, et al. (2003). Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* 95(21): 1597-1608.

Park, W. C., H. Liu, et al. (2005). Deregulation of estrogen induced telomerase activity in tamoxifen-resistant breast cancer cells. *Int J Oncol* 27(5): 1459-1466.

Pink, J. J., M. M. Bilimoria, et al. (1996). Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. *Br J Cancer* 74(8): 1227-1236.

Pink, J. J. and V. C. Jordan (1996). Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 56(10): 2321-2330.

Reid, G., M. R. Hubner, et al. (2003). Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. *Mol Cell* 11(3): 695-707.

Robinson, S. P. and V. C. Jordan (1989). Antiestrogenic action of toremifene on hormone-dependent, -independent, and heterogeneous breast tumor growth in the athymic mouse. *Cancer Res* 49(7): 1758-1762.

Robson, J. M., Schonberg, A. (1937). Estrous reactions, including mating, produced by triphenyl ethylene. *Nature* 140: 196.

Schroder, C. P., A. K. Godwin, et al. (1996). Glutathione and drug resistance. *Cancer Invest* 14(2): 158-168.

Shao, W., E. K. Keeton, et al. (2004). Coactivator AIB1 links estrogen receptor transcriptional activity and stability. *Proc Natl Acad Sci U S A* 101(32): 11599-11604.

Shiau, A. K., D. Barstad, et al. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 95(7): 927-937.

Song, R. X., G. Mor, et al. (2001). Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *J Natl Cancer Inst* 93(22): 1714-1723.

Toft, D. and J. Gorski (1966). A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *Proc Natl Acad Sci U S A* 55(6): 1574-1581.

Townsend, D. M., K. D. Tew, et al. (2003). The importance of glutathione in human disease. *Biomed Pharmacother* 57(3-4): 145-155.

Voehringer, D. W. (1999). BCL-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity. *Free Radic Biol Med* 27(9-10): 945-950.

Vogel, V. G., J. P. Costantino, et al. (2006). Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* 295(23): 2727-2741.

Vogel, V. G., J. P. Costantino, et al. (2010). Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. *Cancer Prev Res (Phila)* 3(6): 696-706.

Walpole, A. L., Patterson, E. (1948). Synthetic oestrogens in mammary cancer. *Lancet* 2(2583): 783-786.

Wijayaratne, A. L. and D. P. McDonnell (2001). The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. *J Biol Chem* 276(38): 35684-35692.

Wolf, D. M. and V. C. Jordan (1993). A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res* 127: 23-33.

Yao, K., E. S. Lee, et al. (2000). Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 6(5): 2028-2036.

Early Breast Cancer

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From Screening to Multidisciplinary Management

Third Edition

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5 Endocrine prevention of breast cancer

Russell E. McDaniel and V. Craig Jordan

INTRODUCTION

The idea of prevention of breast cancer is not new, but significant practical progress has been made, through translational research, to make the idea feasible in some women. It is now possible to reduce the incidence of breast cancer through the inhibition of estrogen action.

Professor Antoine Lacassagne (1) stated a vision for the prevention of breast cancer at the annual meeting of the American Association of Cancer Research in Boston in 1936.

> "*If one accepts the consideration of adenocarcinoma of the breast as the consequence of a special hereditary sensibility to the proliferative actions of oestrone, one is led to imagine a therapeutic preventative for subjects predisposed by their heredity to this cancer. It would consist – perhaps in the very near future when the knowledge and use of hormones will be better understood – in the suitable use of a hormone antagonistic or excretory, to prevent the stagnation of oestrone in the ducts of the breast.*"

But no agent that was "antagonistic to prevent the stagnation of oestrone in the breast" was available for clinical trial until tamoxifen (2,3). Tamoxifen (Fig. 5.1) became the "antiestrogen" of choice because (*i*) there was a large body of basic biological evidence that this was a valid hypothesis to test; (*ii*) tamoxifen was noted to reduce the incidence of contralateral breast cancer when used as an adjuvant therapy to treat micrometastases from the ipsilateral primary tumor and most importantly (*iii*) there was a huge and expanding clinical experience with tamoxifen as a long-term treatment for node-positive and node-negative breast cancer. The latter point was important as the majority of patients with estrogen receptor (ER)-positive node-negative breast cancers are cured by surgery (plus radiation) alone. So five years of adjuvant tamoxifen was essentially being used in the majority of "well women" (4,5).

In this chapter, the changing fashions in endocrine chemoprevention are described. These have occurred because of significant advances in our understanding of the pharmacology of the drug group the "nonsteroidal antiestrogens" (6) that underwent a metamorphosis in the mid 1980s (7) to become the new drug group, the selective estrogen receptor modulators (SERMs) (8,9). This laboratory work on SERM action and the finding that antihormone resistance in breast cancer is not static but evolves (10,11) ultimately led to discovery (rediscovery?) of a new biology of estrogen action—estrogen-induced apoptosis (12). Remarkably, this conversation between the laboratory and the clinical research community now provides a fascinating insight into a paradoxical clinical finding in the Women's Health Initiative (WHI) trial of conjugated equine estrogen (CEE) alone in hysterectomized postmenopausal women in their late 60s. Since dogma dictates that estradiol is the survival signal that fuels breast cancer cell replication, the WHI trial unexpectedly noted a significant decrease in the incidence of breast cancer during CEE treatment and for the six years after treatment stops (cumulative annualized incidence of 151 invasive breast cancers with CEE treatment as opposed to 199 invasive breast cancers with placebo) (13). These data might provide a starting point for consideration of estrogen-induced apoptosis as a chemoprevention strategy in the future.

THE LINK BETWEEN ESTROGEN AND BREAST CANCER

The topic has been reviewed (14) in the referred research literature so only essential facts will be considered here. The link between estrogen action for breast cancer growth, the tumor prior to treatment, ER, and five years of adjuvant tamoxifen therapy to block tumor growth is compelling and proven in randomized clinical trials (15). The findings can be simply summarized: breast tumors that are ER negative do not respond to tamoxifen treatment, tamoxifen dramatically reduces recurrence and mortality during 5 years of treatment for patients with ER-positive breast cancer, and this is maintained for at least 15 years following completion of therapy. Tamoxifen reduces the incidence of contralateral breast cancer by 50% and this is sustained but tamoxifen also increases the incidence of endometrial cancer in postmenopausal women (and mortality). The negative actions of adjuvant tamoxifen, such as deaths from endometrial cancer or thromboembolic disease, do not affect the overall benefit of treatment (15) but do impact on the use of tamoxifen for chemoprevention. Profound target-site-specific actions of tamoxifen on the uterus in the recent overview (15) recapitulate and confirm findings from translational research with tamoxifen completed in the 1980s (16,17). There is thus recognition of a small but significant increase in the incidence of endometrial cancer in postmenopausal women treated with tamoxifen. This finding eventually resulted in the paradigm shift away from tamoxifen to new opportunities but this advances our story too quickly. In the 1980s, tamoxifen was the only medicine available for testing therapeutic and chemopreventive strategies with SERMs in the 1990s. The clinical community advanced with a responsibility to weigh risks and benefits in clinical trials to ensure the safety and long-term health of women at risk for breast cancer.

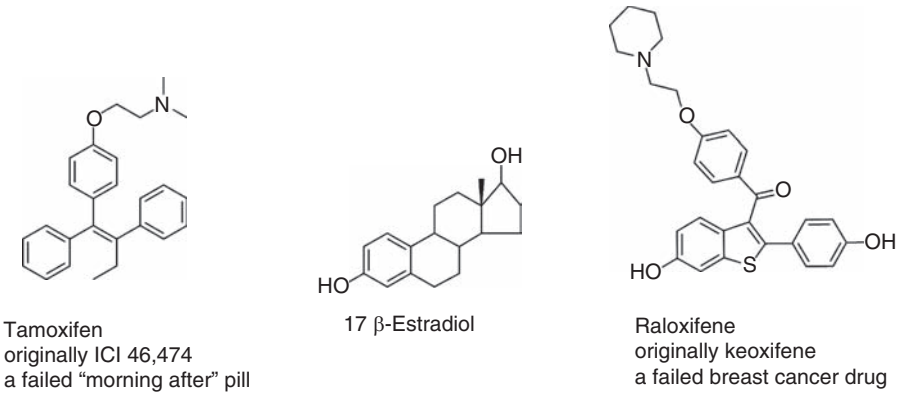

Figure 5.1 A comparison of the structures of the potent steroidal estrogen 17β-estradiol and the nonsteroidal antiestrogens (now called SERMs) tamoxifen and raloxifene. Tamoxifen and raloxifene are both approved in the United States (US) for the reduction of risk for breast cancer in high risk pre- and postmenopausal women or postmenopausal women alone respectively.

The treatment trials' database and translational research were essential to address the hypothesis that tamoxifen, a nonsteroidal antiestrogen, could effectively block the genesis and growth of ER-positive breast cancer but would be ineffective against the growth of ER-negative disease. Nevertheless in the 1980s, estrogen was also considered to be an essential component of women's health, maintaining bone density and preventing coronary heart disease. Thus, if tamoxifen, an antiestrogen, prevented the development and growth of ER-positive breast cancer in half a dozen high-risk women per year per 1000 (18), hundreds of other women in the selected population might subsequently develop osteoporosis and coronary heart disease. The intervention with tamoxifen would be detrimental to public health. The good news was that tamoxifen was not an antiestrogen everywhere; it was the lead compound of a class of drug that selectively modulated ER target tissues around the body. This discovery ultimately facilitated the development of a new strategy for the utilization of SERMs as chemopreventives in breast cancer.

SERM ACTION IN THE LABORATORY

The original work to investigate the target site pharmacology of tamoxifen in the laboratory was to provide a database with which to predict clinical outcomes and safety for future chemoprevention trials. Historically in the 1960s, there was general interest in the chance finding that nonsteroidal antiestrogens lowered circulating cholesterol. Unfortunately, severe toxicological findings were an issue for some compounds because of their ability to increase the level of circulatory desmosterol, which was associated with cataract formation. This toxicity made a search for safer antiestrogens imperative (2). The discovery of ICI 46474 (Fig. 5.1), the pure trans isomer of the substituted triphenylethylene that was to become tamoxifen, was notable because there was a low conversion to desmosterol though circulating cholesterol was lowered profoundly in rats (19). Indeed the first patent application for tamoxifen in the United Kingdom (UK) in 1965 stated the following (2):

> "*The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolaemic activity.*"

However, the patent was denied in the United States (US)and the statements concerning breast cancer had to be removed initially as the claim was considered to be "fantastic" and without experimental evidence. The patent for tamoxifen in the US was finally awarded in 1986 just at the time that the National Cancer Institute recommended adjuvant tamoxifen as the standard of care for patients with ER-positive breast cancer (20).

Parenthetically, all studies conducted in the senior author's laboratory during the 1970s and 1980s on the application of tamoxifen for the treatment and prevention of breast cancer in the US and UK were at a time of no patent protection in the US. No other company exploited the findings as no one cared because it was unlikely to be a successful therapeutic strategy!

During the 1980s, the Wisconsin Tamoxifen study followed up the question of tamoxifen treatment lowering circulating cholesterol in postmenopausal patients (21,22) and noted a decrease in low density lipoprotein cholesterol but no effect on high density lipoprotein cholesterol. There was certainly some initial enthusiasm that there would be a significant decrease in coronary heart disease but despite some encouraging reports (23–25) no consistent decrease in coronary events has been noted in the Oxford Overview Analysis for tamoxifen treatment.

Tamoxifen maintains bone density in ovariectomized rats (26–28) and this counterintuitive laboratory result for an "antiestrogen" formed the scientific basis for the Wisconsin Tamoxifen Study. The clinical study was a placebo controlled double blind trial to establish the actions of two years of tamoxifen on bone density in postmenopausal patients with node-negative breast cancer (at the time of recruitment, these patients were several years post diagnosis and surgery and no adjuvant treatment was the standard of care). Tamoxifen significantly improved bone density compared with placebo treatment (29).

Thus tamoxifen was estrogen-like, lowering circulating cholesterol and estrogen-like, maintaining bone density; so tamoxifen might provide benefit for women enrolled in a chemoprevention trial. The anticancer actions of tamoxifen were well established and supported by the inhibition of mammary carcinogenesis in rat (30,31) and mouse (32) models. But an

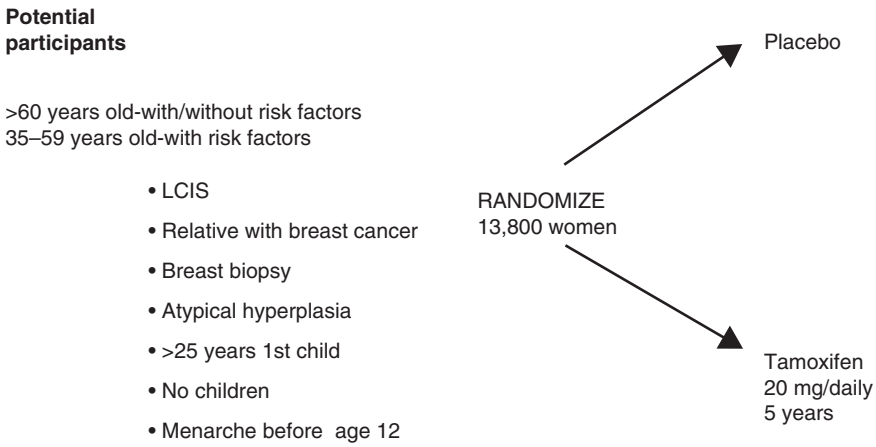

Figure 5.2 The risk requirements for recruitment to the National Surgical Breast and Bowel Project (NSABP)/National Cancer Institute (NCI) study P-1 to determine the worth of tamoxifen for preventing breast cancer in high-risk pre- and postmenopausal women (38).

increase in the incidence of endometrial cancer was a predictable concern, based on earlier work (16,17) before major clinical trials of chemoprevention in breast cancer started. Also the finding that tamoxifen was a hepatocarcinogen in specific rat strains (33) was of significance from a toxicology point of view and for safety reasons in any chemopreventive trial. However, no evidence either at that time (34,35) or subsequently has emerged which demonstrates hepatocarcinogenesis in humans with the use of tamoxifen.

The first pilot chemoprevention study was initiated by Trevor Powles at the Royal Marsden Hospital in the early 1980s (36). This study grew over the years of accrual and interestingly showed benefit at 20 years for those women taking tamoxifen for eight years following recruitment (37). However, the pivotal chemoprevention study was the Fisher P-1 study (Fig. 5.2) conducted by the National Surgical Advent Breast and Bowel Project (NSABP) (38). This landmark study was an adequately powered prospective, placebo controlled trial primarily used by the Food and Drug Administration (FDA) as evidence to approve tamoxifen for the reduction of risk of breast cancer in pre- and postmenopausal women at high risk for the disease.

There are significant benefits for women at risk for breast cancer nested within the results of the P-1 prevention trial during treatment with tamoxifen. There were fewer fractures but this was not significant overall. Tamoxifen reduces ER-positive invasive breast cancer incidence by 50% and the same is true for ductal carcinoma *in situ* (DCIS) (38). Benefits from breast chemoprevention last for years following cessation of treatment (39) and this has been confirmed by others (40). This is clearly a consistent long-term "antitumor action" of tamoxifen which is imprinted following therapy and is analogous to the sustained antitumor effect of tamoxifen following adjuvant treatment (15,41). We will comment further on the new concept of "imprinting" in the section "SERM Summary".

Despite extensive testing, tamoxifen is seen as presenting the well woman with significant risks such as endometrial cancer and blood clots (although only in postmenopausal women) (Fig. 5.3) (38). There is also the nagging concern about rat hepatocarcinoma. Tamoxifen has a human carcinogen black box designation in the US. With all these uncertainties, clearly another strategy for chemoprevention was necessary for an appropriate science-based advance in public health. This was obvious (7) even before the NSABP trial had been launched in the early 1990s (38) but tamoxifen was the only medicine available with sufficient clinical trial experience to move forward into chemoprevention. Nevertheless, the recognition of SERMs in the laboratory (7) also catalyzed a change in the development of another nonsteroidal antiestrogen, keoxifene (Fig. 5.1). Keoxifene was initially investigated in the 1980s as a competitor for tamoxifen as a breast cancer drug, but failed to advance in development and was abandoned in clinical trials (42). Surprisingly, keoxifene also maintained bone density in rats similar to tamoxifen but was significantly less uterotrophic than the latter (26,43) which would translate to a reduced risk of endometrial cancer in all subsequent clinical trials. The name was changed from keoxifene to raloxifene (Fig. 5.1).

Keoxifene prevented mammary cancer in rats but because of poor pharmacokinetics and rapid excretion keoxifene does not have the sustained actions of tamoxifen (31) and continuous therapy was necessary. Thus the scene was set for a move away from a broad therapeutic strategy with tamoxifen administered to high-risk populations where a few ER-positive invasive breast cancers can be prevented but most women are exposed to side effects with no benefit to balance the risks. In response, a "roadmap" was created based on laboratory science and the emerging clinical trial data that would significantly advance women's health.

USE OF SERMs TO PREVENT MULTIPLE DISEASES IN WOMEN

A plan to prevent breast cancer as a public health initiative was initially described at the First International Chemoprevention meeting in New York in 1987 (44). It was reasonable simply to state the proposal, published from the 1987 meeting (44) and subsequently to refine and present again at the annual meeting of the American Association for Cancer Research in San Francisco in 1989 (7). "*The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused on the identification of a population of high-risk women to test "chemopreventive" agents. But, are resources being used less than optimally? An alternative would be to seize on the*

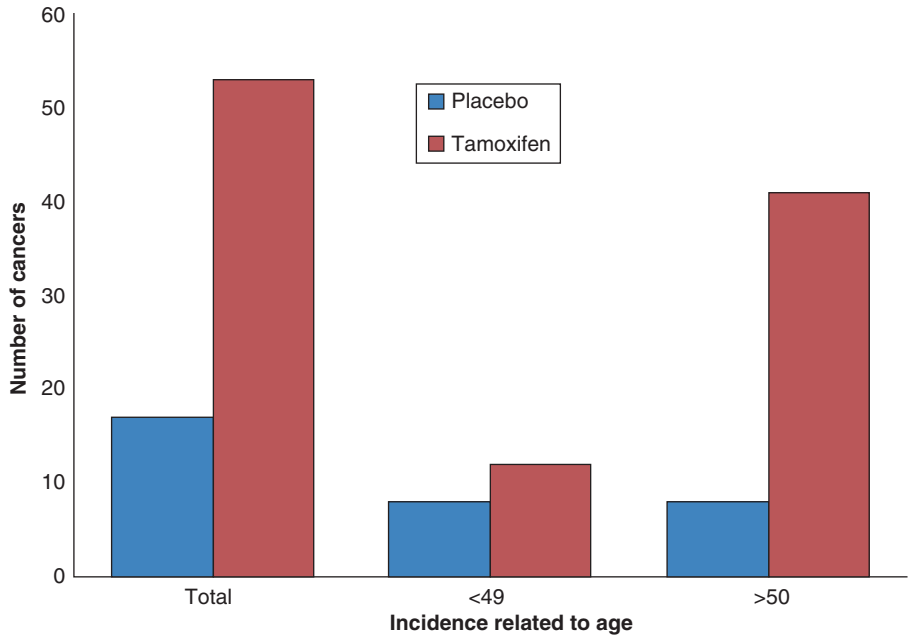

Figure 5.3 The total and age-related incidence of endometrial cancer in the NSABP/NCI P-1 chemoprevention trial (39). Premenopausal women have no increased risk of developing endometrial cancer during or following five years of tamoxifen treatment.

developing clues provided by an extensive clinical investigation of available antioestrogens. Could analogues be developed to treat osteoporosis or even retard the development of atherosclerosis? If this proved to be true, then a majority of women in general would be treated for these conditions as soon as menopause occurred. Should the agent also retain anti-breast tumour actions, then it might be expected to act as a chemosuppressive on all developing breast cancers if these have an evolution from hormone-dependent disease to hormone-independent disease. A bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century (44)." The vision of the concept was refined and focused by 1990 (7). "*We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids, so apparently, derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high-risk group to prevent breast cancer.*" This concept is exactly what has been translated to clinical practice (45,46): use a SERM (raloxifene) to treat osteoporosis and reduce the incidence of breast cancer as a beneficial side effect (45–47).

THE SERMS SURFACE IN CLINICAL PRACTICE

Raloxifene is the pioneering SERM approved for the prevention of osteoporosis around the world. The pivotal registration trial was the Multiple Outcomes of Raloxifene Evaluation (MORE) trial. Raloxifene reduced spine fractures by 50% compared with placebo (47). A separate analysis of breast cancer incidence demonstrated a 76% decrease in the incidence of invasive breast cancer (Fig. 5.4) over the three-year evaluation. There was no increase in endometrial cancer but DCIS remained unaffected (45). A long running trial, Raloxifene Use for the Heart (RUTH), to examine whether coronary heart events could be reduced in high-risk populations, did not show any benefit for raloxifene (48). Looked at another way, it showed little harm, but coronary heart disease (CHD) in a high-risk population was unaffected.

The use of estrogen-like medicines to treat and prevent osteoporosis in the postmenopausal woman demands a long-term therapy—perhaps an indefinite therapy. The extension trial to MORE was Continuing Outcomes Relevant to Evista (CORE) (46). An evaluation of both breast cancer and endometrial cancer in the CORE trial confirmed a sustained efficacy to prevent the development of breast cancer over the nine years of raloxifene treatment (Fig. 5.5) and this effect was entirely expressed in the prevention of ER-positive disease with no effect on the development of ER-negative disease.

Not unexpectedly, the promising data from the MORE trial (45) would propel raloxifene into a head to head study comparing tamoxifen with raloxifene for chemoprevention in high-risk postmenopausal women (Fig. 5.6). The Study of Tamoxifen and Raloxifene (STAR) trial illustrates several important lessons; however the dramatic decrease in invasive breast cancer noted in the MORE trial (raloxifene reducing the risk of ER-positive breast cancer by 90% and a 76% reduction of any newly diagnosed invasive breast cancer) (45) was not noted in the STAR trial with raloxifene. There was no difference between the incidence of breast cancer during treatment with tamoxifen or raloxifene (49) notwithstanding the

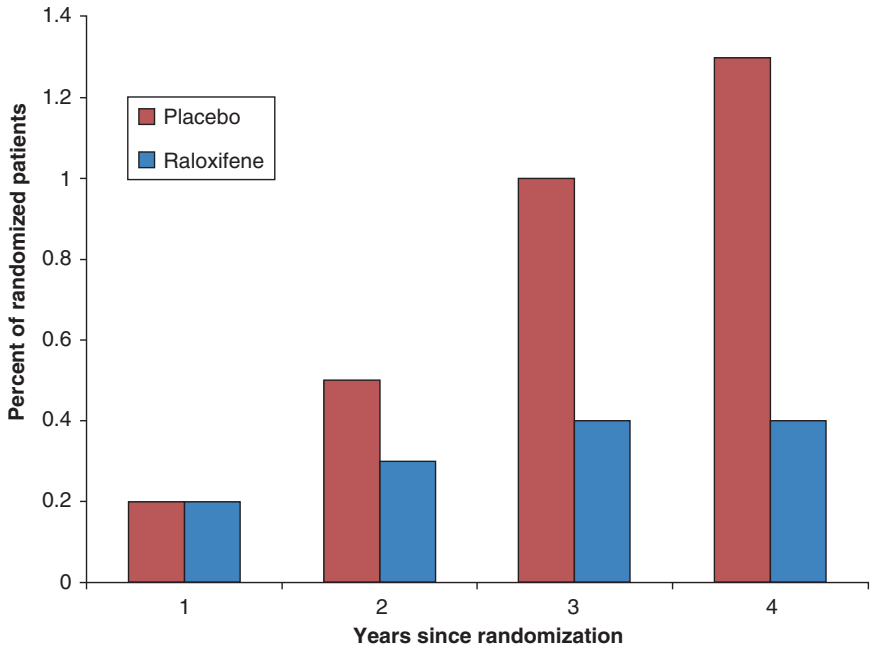

Figure 5.4 The annual accumulative incidence breast cancers represented as a percent of affected randomized patients in the Multiple Outcomes of Raloxifene Evaluation (MORE) trial that randomized women with an increased risk for osteoporotic fractures to placebo (2576 women) or raloxifene (5129 women) (45).

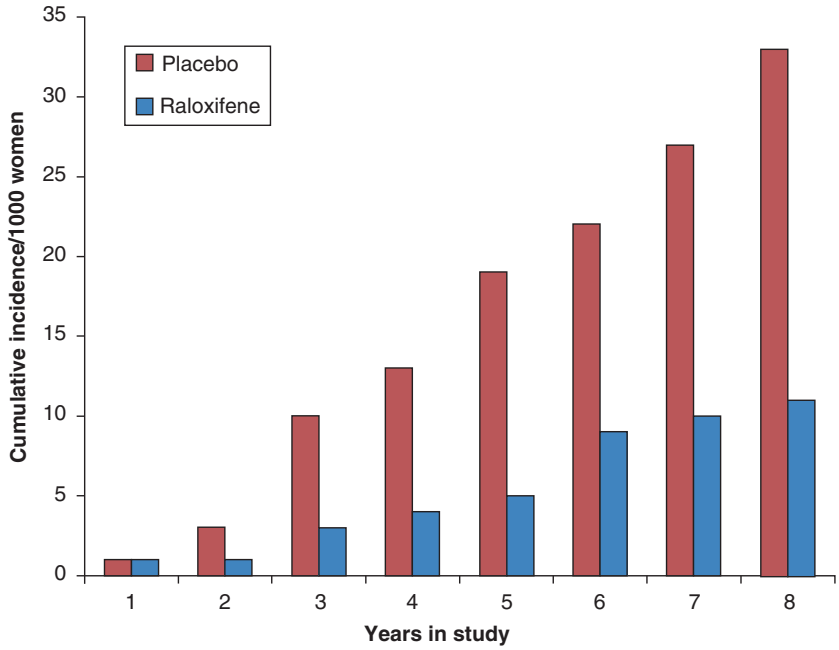

Figure 5.5 The cumulative incidence of invasive breast cancer for the combined MORE and Continuing Outcomes Relevant to Evista (CORE) studies. Shown are patients at high risk for osteoporotic fractures receiving either placebo or raloxifene (60 mg daily) (46).

presumed 50% decrease based on the results from the P-1 trial (38,39). Raloxifene had a very low proliferative effect on the uterine epithelium when compared with tamoxifen and this translated to fewer hysterectomies in the raloxifene-treated women (49). Additionally, there were fewer thrombotic events with raloxifene and fewer operations for cataracts (see earlier concerns with the triphenyl ethylene based nonsteroidal antiestrogens (2)). Overall raloxifene seems to be equivalent to tamoxifen as a chemopreventive for invasive breast cancer but is less effective than tamoxifen at controlling the development of DCIS. Nevertheless, raloxifene confers greater safety.

However, the importance of long-term follow-up for clinical trials is illustrated by the STAR trial. A re-evaluation of the STAR trial three years after stopping five years of treatment showed that although tamoxifen retained its "imprinting" as an antitumor agent raloxifene did not. Raloxifene was only 78% as effective at reducing primary breast cancer incidence as tamoxifen. These clinical data reflect the superiority

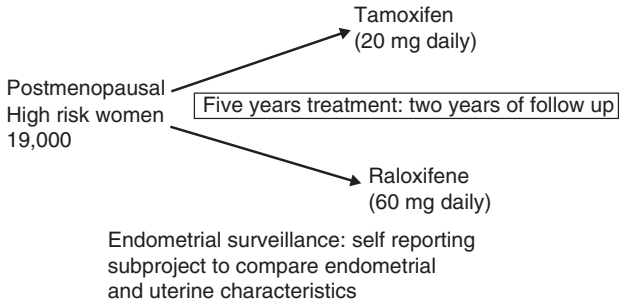

Figure 5.6 The design of the Study of Tamoxifen and Raloxifene (STAR). The STAR trial for postmenopausal women at an elevated risk for breast cancer had fewer serious side effects when taking raloxifene but a similar reduction in the incidence of breast cancer (49). However, after stopping the SERM treatment, the antitumor action of raloxifene was not maintained (50); so a continuous treatment with raloxifene was recommended (as this is the approval for the treatment and prevention of osteoporosis).

of tamoxifen in preclinical studies (31,50) and based on the raloxifene extension study, this agent (46) may need to be given indefinitely to prevent both osteoporosis and breast cancer.

INNOVATIONS IN SERM DEVELOPMENT

The story of the initial discovery and clinical application of the SERMs, tamoxifen and raloxifene, can be partially attributed to the play of chance; the right people were in the right place at the right time and willing to seize an opportunity that ultimately resulted in progress in medicine and pharmaceutical profits. The profits are necessary to permit progress in medicine. This is not a new idea and was stated as being essential by Professor Paul Ehrlich in the late 19th century for the successful development of what was the first chemical therapy for any disease (51). The anti-syphilitic Salvarsan™ (606; Hoechst) was discovered through systematic organic synthesis and testing of hundreds of compounds in appropriate animal models of human disease. But syphilis would not have been conquered if the pharmaceutical company Hoechst had not developed the drug. Without successful drug development, there would be no medicines. This fact is critical to the next part of the SERM story.

There has been considerable innovation by pharmaceutical chemists to refine the selectivity of SERMs and advance in the creation of the ideal SERM. The goal is illustrated in Figure 5.7. Numerous compounds have been synthesized and tested in preclinical studies but it is not our intention to survey progress in the laboratory here. This progress has been documented elsewhere (52,53). Rather, four SERMs are selected for consideration: ospemifene, arzoxifene, bazedoxifene, and lasofoxifene (Fig. 5.8). The reason for the selection of these four is that significant progress has been made in completed clinical trials of these drugs.

Ospemifene

Ospemifene (FC-1271a) is a new SERM that has shown estrogen-like effects in bone marrow (54), enhancing osteoblast formation *in vitro* by a mechanism unlike that of raloxifene. Ospemifene, Z-2-(4-(4-chloro-1,2-diphenyl-but-1-enyl)phenoxy) ethanol) is a metabolite of toremifene (55). Ospemifene also has estrogenic activity in the vaginal epithelium, though not in the endometrium, suggesting its application as a treatment for vaginal dryness associated with menopause (56,57). Ospemifene has been shown to inhibit the growth of the ER-positive MCF-7 cells in culture (56).

Preclinical studies *in vivo* have shown ospemifene to prevent bone loss and increase bone strength in ovariectomized rats and to have a benefit in lowering serum cholesterol levels (58).

Phase I, II, and III clinical trials have been carried out with ospemifene (55) with no significant toxicity evident. Phase II trials (56,59) and a phase III trial (60) indicate that ospemifene is effective for treating vulvar and vaginal atrophy in postmenopausal women. Ospemifene's estrogen-like activity on the vagina improved symptoms of vaginal dryness, unlike raloxifene (61).

Arzoxifene

Arzoxifene (LY353381) is a potent SERM that was evaluated by Eli Lilly and Company (62). This SERM binds to the estrogen receptor alpha with higher affinity than raloxifene (62–64). It was found to have antagonistic effects on the uterus while being 30–100 times more potent than raloxifene in the prevention of body weight, bone, and serum cholesterol changes secondary to ovariectomization of rats (65). Furthermore, arzoxifene and its metabolite, demethylated arzoxifene, have been shown to not have a proliferative effect on endometrial tissue while protecting the bone.

In clinical trials, arzoxifene has shown promise for treatment of osteoporosis. In a phase III trial (66), arzoxifene treatment of postmenopausal osteoporotic women increased spine and hip bone density. Other trials have suggested that arzoxifene was effective against vertebral fractures but not nonvertebral fractures.

In spite of arzoxifene's encouraging preclinical and early clinical findings, arzoxifene is not on the market and is not being developed. Arzoxifene has some adverse effects in common with all SERMs such as hot flashes, increased risk of venous thromboembolic events, and cramps. In addition, a phase III breast cancer clinical trial was stopped because "*Arzoxifene was statistically significantly inferior to tamoxifen with regard to progression-free survival and other time-to-event parameters, although tumor response was comparable between the treatments*" (67). Arzoxifene has not been developed further.

Bazedoxifene

Bazedoxifene, a SERM for the treatment and prevention of osteoporosis in postmenopausal women (as well as, in combination with conjugated equine estrogens, for treatment of menopausal symptoms (68)), is currently approved for use in the European Union (EU) and it is under review by the US' Food and Drug Administration. This SERM, developed from a collaborative effort between Wyeth Pharmaceuticals and Ligand Pharmaceuticals, has a binding affinity for ERα about 10-fold lower than 17β-estradiol (69,70). Preclinical studies on bazedoxifene have been two-tiered: those studying bazedoxifene alone as treatment and as a preventative agent for osteoporosis and those of bazedoxifene in combination with conjugated estrogens. Bazedoxifene alone shows its efficacy in maintaining

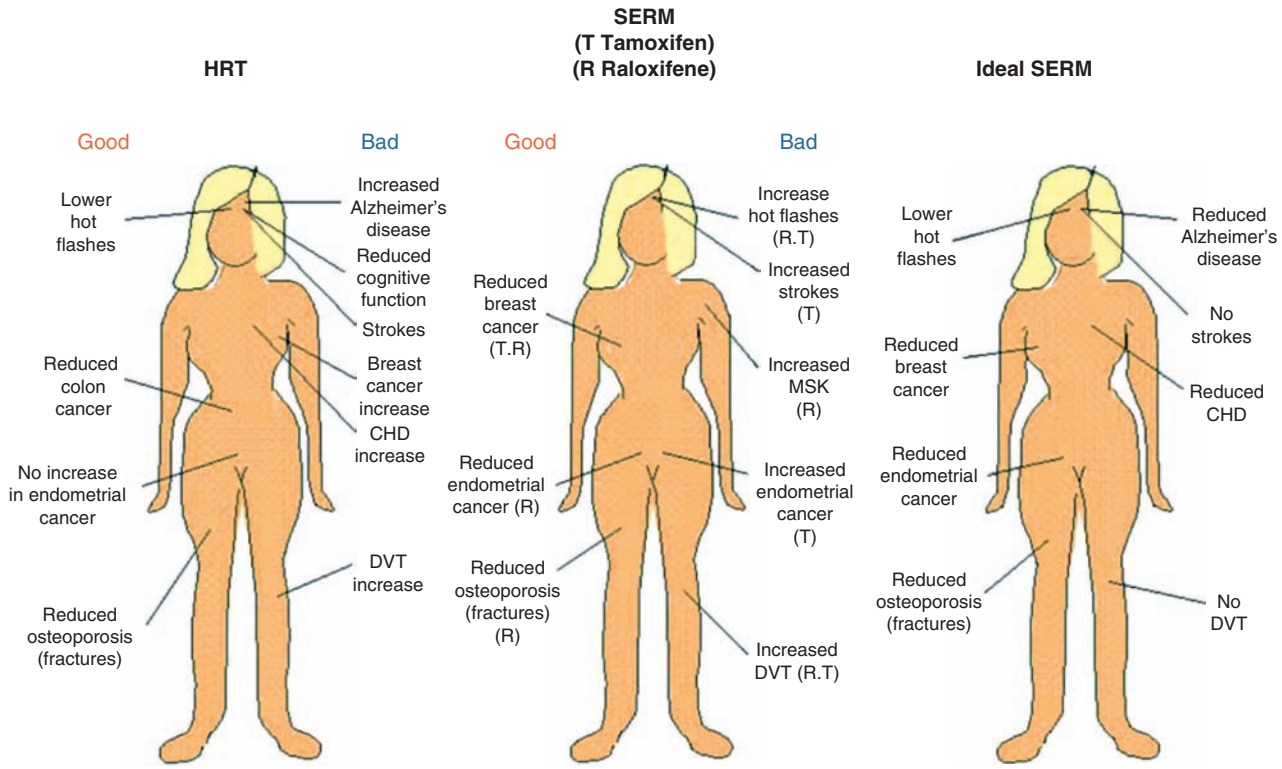

Figure 5.7 A comparison of the good and bad aspects of hormone replacement therapy (HRT) and current selective estrogen receptor modulators (SERMs) tested in postmenopausal women. On the right is the ideal SERM of the future. *Source*: From Ref. (95).

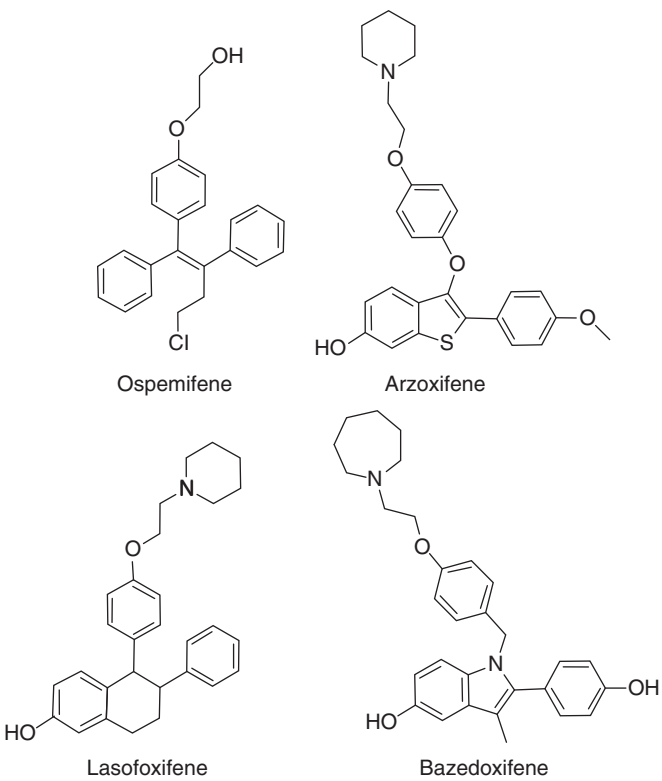

Figure 5.8 The structures of SERMs that have completed clinical testing over the last decade. Arzoxifene has not been pursued for clinical use and ospemifene is targeted for an application for vaginal atrophy. Lasofoxifene is the newest SERM thus far to attain the pharmacological profile of an ideal SERM (Fig. 5.7). Bazedoxifene is targeted for treatment and prevention for osteoporosis, or with conjugated equine oestrogen as an estrogen replacement therapy for hot flashes.

bone mass in doses as low as 0.1 mg/kg/day in ovariectomized rats (69,70). This bone preservation is comparable to that of raloxifene and lasofoxifene (71,72).

Combination studies have been carried out on bazedoxifene given with a mixture of the 10 principal conjugated estrogens (CEs) in Premarin® (Pfizer Pharmaceuticals; New York, NY). Bazedoxifene (3.0 mg/kg) was given in tandem with the CE. Bazedoxifene antagonized CE-induced dose-dependent increase in uterine weight to control levels (73).

Bazedoxifene has gone through several phase III clinical trials. It has been shown to reduce bone turnover and to prevent bone loss without undue endometrial, ovarian, and breast risks (74,75). Another phase III study showed that bazedoxifene reduced the incidence of vertebral fractures as compared with placebo (76). Among high-risk women for breast cancer, bazedoxifene significantly lowered the risk of nonvertebral fracture relative to both placebo and raloxifene (77). Bazedoxifene is considered to be well tolerated; serious adverse events and discontinuations are similar to those of a placebo group (77). Any increased risks of venous thromboembolism are similar to raloxifene and lasofoxifene (78,79). Bazedoxifene is considered safe regarding osteoporosis treatment and prevention (77) but cannot be considered a chemopreventive agent for breast cancer (76,77).

Lasofoxifene

Lasofoxifene is a SERM which binds with high affinity to the ER that is approved for the treatment of osteoporosis in the UK and EU but not currently in the US (80). Animal model studies of

lasofoxifene have shown it to inhibit osteoclastogenesis, prevent bone loss, and reduce bone turnover (72,81).

Phase II and phase III clinical studies have confirmed improvements in bone mineral density (BMD). In one phase II study (82) one year's treatment with lasofoxifene showed significant improvement regarding lumbar spine BMD as compared with calcium and vitamin D. In another study, lasofoxifene acted positively by increasing BMD comparably to CEE (83).

Three phase III clinical studies have been carried out on lasofoxifene: The Postmenopausal Evaluation and Risk-Reduction with Lasofoxifene (PEARL) study, The Osteoporosis Prevention and Lipid Lowering (OPAL) study, and the Comparison of Raloxifene and Lasofoxifene (CORAL) study. The PEARL study found that both lumbar spine and femoral neck BMDs were increased after three years' treatment. Lasofoxifene also significantly reduced the risk of ER-positive breast cancer as compared with placebo (84–86). The OPAL trial tested three doses of lasofoxifene against placebo. All doses showed improved lumbar spine and hip BMD as compared with placebo (87). All doses also showed reduced serum levels of C-terminal telopeptide of type 1 collagen, serum osteocalcin, and no increase in breast density or pain (88). The CORAL study found that lasofoxifene maintained BMD in the lumbar spine better than raloxifene, with no difference in hip BMD; lasofoxifene also lowered total cholesterol more than raloxifene (89).

Lasofoxifene is a major advance toward improved potency and side effect profile. Lasofoxifene is 100 times more potent than raloxifene, but unlike the latter, lasofoxifene reduces the risk of coronary heart disease and strokes. Like raloxifene, lasofoxifene reduces the incidence of fractures and ER-positive breast cancers with no increase in the risk of endometrial cancer (79,86).

SERM SUMMARY

The practical application of SERMs for the chemoprevention of breast cancer has only resulted from the research philosophy first espoused by Professor Paul Ehrlich to achieve successful outcomes in experimental therapeutics, that is, the four Gs (in German): *Gluck* (luck), *Geduld* (patience), *Geshick* (skill), and *Geld* (money) (51). The discoveries with both tamoxifen and raloxifene, in the same laboratory, were not predictable. Some would say lucky. But with patience and skill over decades and the investment of money from philanthropy and the pharmaceutical industry to develop the new concepts further and "sell" the idea to physicians, millions of women are alive and millions more continue to benefit. The approved drugs, tamoxifen and raloxifene, are safe and effective if used in the correct manner for the right patients: tamoxifen for five years in high-risk premenopausal women (or the postmenopausal woman without a uterus) or raloxifene indefinitely in high-risk postmenopausal women. This therapeutic intervention will reduce the incidence of breast cancer in select populations. By contrast, 40 years ago there was nothing.

Unfortunately to advance further, it is unclear whether the new SERMs have the "uniqueness" to supersede raloxifene as the SERM of choice to prevent both osteoporosis and significantly reduce the risk of breast cancer. Arzoxifene can be viewed as a "long-acting raloxifene" but following completion of clinical trials the decision was made not to seek FDA approval. In contrast, bazedoxifene for osteoporosis or bazedoxifene plus CEE to treat menopausal symptoms appears to hold merit for the postmenopausal women with a uterus. Bazedoxifene is approved in several countries. Ospemifene could have a "niche" application to ameliorate vaginal dryness, but an application to prevent breast cancer, like toremifene before, is unlikely without major clinical trials for chemoprevention, osteoporosis, or other indications. Lasofoxifene is approved in the EU but the drug has not been launched. The FDA has not approved lasofoxifene. It is all about *Geld* and the fear of financial failure by the pharmaceutical industry. The same was true for tamoxifen and raloxifene. Now the market may be overcrowded but there have been advances. The ideal SERM is illustrated in Figure 5.7 with the goal to achieve an improvement on raloxifene, the failed breast cancer drug. Raloxifene is seen to be a safe advance over tamoxifen as there is no endometrial cancer incidence and no rat hepatocarcinogenicity with the former. If we only focus on SERMs that have successfully moved to approval for osteoporosis (or hot flashes in the case of bazedoxifene) it is clear that lasofoxifene has solved additional issues by reducing strokes and reducing CHD. Significant progress has been made. The innovation of using CEE with bazedoxifene to protect the uterus (and breasts in early menopausal women) may yet prove to be useful as estrogen replacement therapy in younger postmenopausal women.

So if SERMs are currently optimal for the foreseeable future what about "no estrogen" at all. The aromatase inhibitors (AIs) have been rigorously tested in clinical trials of treatment and there is a recent trial of letrozole versus placebo in high-risk women that has shown promise for future consideration (91). However, despite claims about low incidence of side effects such as bone loss, joint pain, and vaginal dryness (with the attendant sexual issues), it would be hard to believe that the side effects of the many could ever outweigh the benefits of the few. If large populations are to benefit from AIs, issues of increased risk of CHD will again demand rigorous monitoring (91). Good quality of life is essential for any chemopreventive strategy. This was the basis some 60 years ago, for the introduction of estrogen replacement therapy/hormone replacement therapy (HRT) to improve quality of life for the many. Unfortunately, estrogen and HRT have a bad reputation relating to growth of breast cancer for the few (92). Nevertheless, there has been a recent surprise and once again science is poised to propel innovation forward and make progress.

The surprise was counterintuitive in the estrogen alone trial of the WHI (93). The finding that administration of CEE to postmenopausal hysterectomized women in their late 60s reduced the incidence of breast cancer for up to five years after stopping CEE (13) demands explanation. Clues as to the mechanisms for these paradoxical antitumor effects of low dose estrogen administration to women in their late 60s come from work of the mechanisms of antihormone resistance during long-term therapy (12). Two decades of laboratory study of the consequences of long-term SERM therapy demonstrated an evolution of types of resistance culminating in the discovery of a new biology of estrogen-induced apoptosis (94). It appears that five years of adjuvant antihormone therapy for breast cancer accelerates a process of breast cancer cell survival

that is similar to what occurs over the 20 years with long-term estrogen deprivation following the menopause. Physiologically estrogen deprivation after the menopause needs decades to change the cell sensitivity from estrogen being a survival signal in breast cancer to an apoptotic trigger. In contrast it takes less than a decade to achieve the same effect on breast cancer with antihormone therapy. The WHI results and the associated laboratory evidence now pose a provocative dilemma in the era of "individualized" medicine. The application of low dose (physiological) estrogen-induced apoptosis has already moved successfully from the laboratory to clinical trial (95), and is being tested as a "purge strategy" for long-term AI adjuvant therapy with three-month drug holidays annually in the Study of Letrozole Extension (SOLE) trial (96). Maybe the era of individualized chemoprevention is soon to dawn as we piece together all the advances being made in cancer research and treatment. This era will deploy new knowledge of genetics, lifestyle, detection, and molecular medicine for the right preventive for the right women. If we can understand the mechanism of estrogen-induced apoptosis (97) as currently applied to second-line treatment after SERMs or AIs and use the knowledge to alternate or "purge" nascent breast cancer cells resistant to SERMs used as long-term preventatives with CEE for a few months, this new approach may be added to the armamentarium available to physicians as inexpensive but effective.

ACKNOWLEDGEMENTS

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REFERENCES

1. Lacassagne A. Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer 1936; 27: 217–25.
2. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2003; 2: 205–13.
3. Jordan VC. Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 2006; 147: S269–76.
4. Report from the Breast Cancer Trials Committee, Scottish Cancer Trials Office (MRC), Edinburgh. Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Lancet 1987; 2: 171–5.
5. Fisher B, Costantino J, Redmond C, et al. A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. N Engl J Med 1989; 320: 479–84.
6. Jordan VC. Biochemical pharmacology of antiestrogen action. Pharmacol Rev 1984; 36: 245–76.
7. Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 1990; 50: 4177–89.
8. Jordan VC. The science of selective estrogen receptor modulators: concept to clinical practice. Clin Cancer Res 2006; 12: 5010–13.
9. Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nat Rev Cancer 2007; 7: 46–53.
10. Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 1993; 127: 23–33.
11. Yao K, Lee ES, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000; 6: 2028–36.
12. Jordan VC, Ford LG. Paradoxical clinical effect of estrogen on breast cancer risk: a "new" biology of estrogen-induced apoptosis. Cancer Prev Res (Phila) 2011; 4: 633–7.
13. LaCroix AZ, Chlebowski RT, Manson JE, et al. Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. JAMA 2011; 305: 1305–14.
14. Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 2009; 69: 1243–54.
15. EBCTG. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 2011; 378: 771–84.
16. Gottardis MM, Robinson SP, Satyaswaroop PG, et al. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 1988; 48: 812–15.
17. Fornander T, Rutqvist LE, Cedermark B, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1989; 1: 117–20.
18. Gail MH, Brinton LA, Byar DP, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. J Natl Cancer Inst 1989; 81: 1879–86.
19. Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 1967; 13: 101–19.
20. Consensus Conference. Adjuvant chemotherapy for breast cancer. JAMA 1985; 254: 3461–3.
21. Love RR, Newcomb PA, Wiebe DA, et al. Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 1990; 82: 1327–32.
22. Love RR, Wiebe DA, Newcomb PA, et al. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann Intern Med 1991; 115: 860–4.
23. Nordenskjöld B, Rosell J, Rutqvist LE, et al. Coronary heart disease mortality after 5 years of adjuvant tamoxifen therapy: results from a randomzed trial. J Natl Cancer Inst 2005; 97: 1609–10.
24. Hackshaw A, Roughton M, Forsyth S, et al. Long-term benefits of 5 years of tamoxifen: 10-year follow-up of a large randomized trial in women at least 50 years of age with early breast cancer. J Clin Oncol 2011; 29: 1657–63.
25. McDonald CC, Stewart HJ. Fatal myocardial infarction in the Scottish adjuvant tamoxifen trial. The Scottish Breast Cancer Committee. BMJ 1991; 303: 435–7.
26. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987; 10: 31–5.
27. Turner RT, Wakley GK, Hannon KS, et al. Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. J Bone Miner Res 1987; 2: 449–56.
28. Turner RT, Wakley GK, Hannon KS, et al. Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. Endocrinology 1988; 122: 1146–50.
29. Love RR, Mazess RB, Barden HS, et al. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 1992; 326: 852–6.
30. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 1976; 12: 419–24.
31. Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987; 47: 4020–4.
32. Jordan VC, Lababidi MK, Langan-Fahey S. Suppression of mouse mammary tumorigenesis by long-term tamoxifen therapy. J Natl Cancer Inst 1991; 83: 492–6.
33. Greaves P, Goonetilleke R, Nunn G, et al. Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. Cancer Res 1993; 53: 3919–24.
34. Jordan VC. What if tamoxifen (ICI 46,474) had been found to produce rat liver tumors in. 1973? A personal perspective. Ann Oncol 1995; 6: 29–34.
35. Jordan VC, Morrow M. Should clinicians be concerned about the carcinogenic potential of tamoxifen? Eur J Cancer 1994; 30A: 1714–21.

36. Powles TJ, Hardy JR, Ashley SE, et al. A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. Br J Cancer 1989; 60: 126–31.
37. Powles TJ, Ashley S, Tidy A, et al. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. J Natl Cancer Inst 2007; 99: 283–90.
38. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: report of the national surgical adjuvant breast and bowel project P-1 Study. J Natl Cancer Inst 1998; 90: 1371–88.
39. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for the prevention of breast cancer: current status of the national surgical adjuvant breast and bowel project P-1 study. J Natl Cancer Inst 2005; 97: 1652–62.
40. Cuzick J, Forbes JF, Sestak I, et al. Long-term results of tamoxifen prophylaxis for breast cancer–96-month follow-up of the randomized IBIS-I trial. J Natl Cancer Inst 2007; 99: 272–82.
41. (EBCTCG) EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005; 365: 1687–717.
42. Lewis JS, Jordan VC. Case histories: raloxifene. In: Taylor J, Triggle D, eds. Comprehensive Medicinal Chemistry II. Volume 8 Oxford, UK: Elsevier Limited, 2006: 103–21.
43. Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 1983; 32: 1031–6.
44. Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 1988; 6: 589–95.
45. Cummings SR, Eckert S, Krueger KA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA 1999; 281: 2189–97.
46. Martino S, Cauley JA, Barrett-Connor E, et al. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 2004; 96: 1751–61.
47. Ettinger B, Black DM, Mitlak BH, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 1999; 282: 637–45.
48. Barrett-Connor E, Mosca L, Collins P, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 2006; 355: 125–37.
49. Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 2006; 295: 2727–41.
50. Vogel VG, Costantino JP, Wickerham DL, et al. Update of the national surgical adjuvant breast and bowel project Study of Tamoxifen and Raloxifene (STAR) P-2 trial: preventing breast cancer. Cancer Prev Res (Phila) 2010; 3: 696–706.
51. Baumler E. Paul Ehrlich: Scientist for Life. New York: Holmes & Meier, 1984: 288.
52. Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. J Med Chem 2003; 46: 1081–111.
53. Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 1. Receptor interactions. J Med Chem 2003; 46: 883–908.
54. Qu Q, Harkonen PL, Vaananen HK. Comparative effects of estrogen and antiestrogens on differentiation of osteoblasts in mouse bone marrow culture. J Cell Biochem 1999; 73: 500–7.
55. Gennari L, Merlotti D, Valleggi F, et al. Ospemifene use in postmenopausal women. Expert Opin Investig Drugs 2009; 18: 839–49.
56. Taras TL, Wurz GT, DeGregorio MW. In vitro and in vivo biologic effects of ospemifene (FC-1271a) in breast cancer. J Steroid Biochem Mol Biol 2001; 77: 271–9.
57. Voipio SK, Komi J, Halonen K, et al. Effects of ospemifene (FC-1271a) on uterine endometrium, vaginal maturation index, and hormonal status in healthy postmenopausal women. Maturitas 2002; 43: 207–14.
58. Qu Q, Zheng H, Dahllund J, et al. Selective estrogenic effects of a novel triphenylethylene compound, FC1271a, on bone, cholesterol level, and reproductive tissues in intact and ovariectomized rats. Endocrinology 2000; 141: 809–20.
59. Rutanen EM, Heikkinen J, Halonen K, et al. Effects of ospemifene, a novel SERM, on hormones, genital tract, climacteric symptoms, and quality of life in postmenopausal women: a double-blind,randomized trial. Menopause 2003; 10: 433–9.
60. Bachmann GA, Komi JO, Group OS. Ospemifene effectively treats vulvovaginal atrophy in postmenopausal women: results from a pivotal phase 3 study. Menopause 2010; 17: 480–6.
61. Komi J, Lankinen KS, Harkonen P, et al. Effects of ospemifene and raloxifene on hormonal status, lipids, genital tract, and tolerability in postmenopausal women. Menopause 2005; 12: 202–9.
62. Palkowitz AD, Glasebrook AL, Thrasher KJ, et al. Discovery and synthesis of [6-hydroxy-3-[4-[2-(1-piperidinyl)ethoxy]phenoxy]-2-(4-hydroxyphenyl)] b enzo[b]thiophene: a novel, highly potent, selective estrogen receptor modulator. J Med Chem 1997; 40: 1407–16.
63. Bryant HU, Glasebrook AL, Yang NN, et al. An estrogen receptor basis for raloxifene action in bone. J Steroid Biochem Mol Biol 1999; 69: 37–44.
64. Grese TA, Cho S, Finley DR, et al. Structure-activity relationships of selective estrogen receptor modulators: modifications to the 2-arylbenzothiophene core of raloxifene. J Med Chem 1997; 40:146–67.
65. Sato M, Turner CH, Wang T, et al. LY353381.HCl: a novel raloxifene analog with improved SERM potency and efficacy in vivo. J Pharmacol Exp Ther 1998; 287: 1–7.
66. Kendler DL, Palacios S, Cox DA, et al. Arzoxifene versus raloxifene: effect on bone and safety parameters in postmenopausal women with osteoporosis. Osteoporos Int 2011; 23: 1091–101.
67. Deshmane V, Krishnamurthy S, Melemed AS, et al. Phase III double-blind trial of arzoxifene compared with tamoxifen for locally advanced or metastatic breast cancer. J Clin Oncol 2007; 25: 4967–73.
68. Gruber C, Gruber D. Bazedoxifene (Wyeth). Curr Opin Investig Drugs 2004; 5: 1086–93.
69. Miller CP, Collini MD, Tran BD, et al. Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. J Med Chem 2001; 44: 1654–7.
70. Komm BS, Kharode YP, Bodine PV, et al. Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity. Endocrinology 2005; 146: 3999–4008.
71. Black LJ, Sato M, Rowley ER, et al. Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 1994; 93: 63–9.
72. Ke HZ, Paralkar VM, Grasser WA, et al. Effects of CP-336,156, a new, nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. Endocrinology 1998; 139: 2068–76.
73. Kharode Y, Bodine PV, Miller CP, et al. The pairing of a selective estrogen receptor modulator, bazedoxifene, with conjugated estrogens as a new paradigm for the treatment of menopausal symptoms and osteoporosis prevention. Endocrinology 2008; 149: 6084–91.
74. Miller PD, Chines AA, Christiansen C, et al. Effects of bazedoxifene on BMD and bone turnover in postmenopausal women: 2-yr results of a randomized, double-blind, placebo-, and active-controlled study. J Bone Miner Res 2008; 23: 525–35.
75. Pinkerton JV, Archer DF, Utian WH, et al. Bazedoxifene effects on the reproductive tract in postmenopausal women at risk for osteoporosis. Menopause 2009; 16: 1102–8.
76. Silverman SL, Christiansen C, Genant HK, et al. Efficacy of bazedoxifene in reducing new vertebral fracture risk in postmenopausal women with osteoporosis: results from a 3-year, randomized, placebo-, and active-controlled clinical trial. J Bone Miner Res 2008; 23: 1923–34.
77. de Villiers TJ, Chines AA, Palacios S, et al. Safety and tolerability of bazedoxifene in postmenopausal women with osteoporosis: results of a 5-year, randomized, placebo-controlled phase 3 trial. Osteoporos Int 2011; 22: 567–76.
78. Grady D, Ettinger B, Moscarelli E, et al. Safety and adverse effects associated with raloxifene: multiple outcomes of raloxifene evaluation. Obstet Gynecol 2004; 104: 837–44.
79. Cummings SR, Ensrud K, Delmas PD, et al. Lasofoxifene in postmenopausal women with osteoporosis. N Engl J Med 2010; 362: 686–96.
80. UK Medicines Information New Drugs Online. New Drugs Online Report for lasofoxifene. Availabile from http://www.ukmi.nhs.uk/applications/ndo/record_view_open.asp?newDrugID=3184 Af] [Cited 2011, 29 September].

81. Maeda T, Ke HZ, Simmons H, et al. Lasofoxifene, a next generation estrogen receptor modulator: preclinical studies. Clin Calcium 2004; 14: 85–93.
82. Moffett A, Ettinger M, Bolognese M, et al. Lasofoxifene, a next generation SERM, is effective in preventing loss of BMD and reducing LDL-C in postmenopausal women. J Bone Min Res 2004; 19: S96.
83. Lee A, Radecki D, Wolter K. Lasofoxifene Phase II and Phase III clinical trial design and strategy. J Bone Min Res 2005; 20: M384.
84. Eastell R, Reid DM, Vukicevic S, et al. The effects of lasofoxifene on bone turnover markers: the PEARL trial. J Bone Min Res 2008; 23: 1287.
85. Cummings SR, Ensrud K, Delmas PD, et al. Lasofoxifene in postmenopausal women with osteoporosis. N Engl J Med 2010; 362: 686–96.
86. LaCroix AZ, Powles T, Osborne CK, et al. Breast cancer incidence in the randomized PEARL trial of lasofoxifene in postmenopausal osteoporotic women. J Natl Cancer Inst 2010; 102: 1706–15.
87. McClung M SE, Cummings S, et al. Lasofoxifene increased BMD of the spine and hip and decreased bone turnover markers in postmenopausal women with low or normal BMD. J Bone Min Res 2005; 20: S97.
88. Davidson M, Moffett A, Welty F, et al. Extraskeletal effects of lasofoxifene on postmenopausal women. J Bone Min Res 2005; 20: S173.
89. McClung MR, Siris E, Cummings S, et al. Prevention of bone loss in postmenopausal women treated with lasofoxifene compared with raloxifene. Menopause 2006; 13: 377–86.
90. Goss PE, Ingle JN, Ales-Martinez JE, et al. Exemestane for breast-cancer prevention in postmenopausal women. N Engl J Med 2011; 364: 2381–91.
91. Cuppone F, Bria E, Verma S, et al. Do adjuvant aromatase inhibitors increase the cardiovascular risk in postmenopausal women with early breast cancer? Meta-analysis of randomized trials. Cancer 2008; 112: 260–7.
92. Chlebowski RT, Kuller LH, Prentice RL, et al. Breast cancer after use of estrogen plus progestin in postmenopausal women. N Engl J Med 2009; 360: 573–87.
93. The Women's Health Initiative Steering Committee. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative Randomized Control Trial. JAMA 2004; 291: 1701–12.
94. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. Cancer Cell 2004; 5: 207–13.
95. Ellis MJ, Gao F, Dehdashti F, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 2009; 302: 774–80.
96. Jordan VC, Ford LG. Paradoxical clinical effect of estrogen on breast cancer risk: a "new" biology of estrogen-induced apoptosis. Cancer Prev Res (Phila) 2011; 4: 633–7.
97. Ariazi EA, Brailoiu E, Yerrum S, et al. The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. Cancer Res 2010; 70: 1184–94.

6 The biological basis for breast cancer screening and its relevance to treatment

John R. Benson

INTRODUCTION

The principles of screening were enunciated by the World Health Organization (WHO) in 1968 (1) and formed the basis for breast screening programs designed to detect disease before it becomes clinically apparent. The aims of screening are to prevent, delay, or reduce the clinical impact of a target disease. Included among the preconditions for effective population screening was the assumption that the natural history of the disease in question should be "well understood" with a recognizable early stage for which treatment outcome would be enhanced compared with that of later stages. Thus there should be a preclinical phase with a consistent abnormality that is easily detectable with affordable, non-invasive methods. It is implicit that screening programs cannot yield any survival advantage when no effective treatments exist, and conversely no benefit will be apparent in terms of reduction of mortality if the treatments for screen-detected and symptomatic cancers are equally efficacious. Thus the benefits of screening derive from early treatment rather than early detection *per se*. A further consideration with screening programs is the prevalence of a disease which will determine the cost-effectiveness within a particular healthcare system (2). It has also been argued that implementation of a large scale screening program, such as for breast cancer, can only be justified when the cancer in question has serious consequences in terms of mortality and morbidity (3).

Randomized trials of breast screening have now confirmed the efficacy of screening in women over 50 years of age where reductions of breast cancer mortality of between 25 and 30% are attainable (4–7). The World Health Organization in 2002 upheld these conclusions for screening mammography within this age group of women (8). There are clearly trans-Atlantic differences both in philosophy and practice in terms of breast cancer screening. Most of the key trials have been carried out in countries outside the United States and a majority of them are based on European populations. Interpretation of these trial data has varied among experts around the world and indeed within individual countries. Some of this controversy is related to the vagaries of statistical processing and manipulation while other aspects of contention are based on issues of cost-effectiveness and perceived value for money. For example, in 1993 the American Cancer Society and the European Society of Mastology met in New York and Paris respectively to review results of screening trials; they each arrived at opposite conclusions regarding screening of younger women between 40 and 49 years of age (9).

CLINICAL TRIALS OF BREAST CANCER SCREENING

Within the international breast community, it is generally accepted that clinical trials to date support the conclusion that mammographic screening reduces the risk of death from breast cancer in women aged between 50 and 69 years (mortality reduction approximately 20%) (10). Individual trials and limited meta-analyses have reported mortality reductions of up to 30% for postmenopausal women (11). Some of the trials include postmenopausal women up to the age of 75 years, but there are no data assessing the value of screening in women beyond this age (12). Moreover, these trials involved two- or three-yearly mammography and thus the recommendations for biennial screening mammography for women aged 50–74 years seem reasonable and justified on the basis of published data alone (as opposed to patient demand or any emotive/intuitive influences) (13). It must be remembered that false positivity is a potential downside to screening and older patients are at a higher risk from general anesthesia performed for "diagnostic excision biopsies" resulting from indeterminate imaging and percutaneous needle biopsy results.

For women between 40 and 50 years of age, there is evidence for effectiveness of screening in terms of mortality reduction, but it takes rather longer (12–14 years) for that benefit to emerge (14). Moreover, the magnitude of the mortality reduction is only about 15% compared with greater than 20% for women more than 50 years of age. Furthermore, many experts have argued that the "delayed benefit" of screening mammography in younger women can be attributed to screening these women after the age of 50 years! Previous claims for fair evidence that mammographic screening every one to two years significantly reduced the mortality for women aged 40 years and more did not take into account of the potential harms and downside of screening in this age group. Over the past few years, both professionals and the public have become more aware of these concerns about screening (false-positive results, increased anxiety, unnecessary visits, further imaging and biopsy, and false reassurance) and the risk/benefit/(cost) ratio has been shifted such that now some organizations within the United States have recommended against routine screening mammography in women aged 40–49 years in the absence of any known genetic mutation or chest wall irradiation (13). It is essential that women are fully informed and aware of both the benefits and harms of screening, particularly in the younger age group where mortality reductions are more modest. There are issues of overdiagnosis and detection of disease which would not have progressed in a woman's lifetime and which

might have led to a surgical recommendation for mastectomy with or without immediate breast reconstruction (which can be associated with complications and long-term adverse sequelae on quality of life).

There is no evidence as yet from clinical trials that breast self-examination is beneficial (15). Once again, though this might seem intuitively beneficial in terms of early cancer detection, overall there is potential to cause harm from false-positive prompts and increased referral to breast specialists for assessment. Many women have a degree of breast lumpiness and it is not surprising that they may find an area which they deem suspicious or worrying!

Likewise, there are currently no data to show any additional benefit from clinical breast examination (CBE) over screening mammography for women either under or over the age of 50 years. Some have suggested that CBE should be carried out at the same time as screening mammography as some cancers are radiologically occult but clinically palpable. Indeed within the Health Insurance Plan trial carried out in the 1960s, a significant proportion of cancers (45%) were detected with this mode of screening. However, methods of mammography have improved enormously since then and in the Edinburgh trial, only 6% of cancers were detected with CBE alone (16).

There is still a powerful patient–consumer-led demand for screening mammography in younger women in the United States, but hopefully a better informed public will lead to attrition of this demand. Health policy must be based on evidence and not emotion, and physicians should abide by these recommendations but respect an individual woman's values and personal perspectives. However, it is incumbent upon them to provide full information for their patients and correct any misguided preconceptions and biases arising from ignorance or misinformation. Some women will insist on screening mammography within the 40–49 year age group despite the most robust reassurance and information on potential harms; it cannot be denied that there is any mortality reduction from screening in this age group. The challenge is to convey the concept of risk–benefit ratio and convince most women that overall the disadvantages outweigh the benefits for most women in this age group. Discretion must be exercised and occasionally biennial mammography offered to these women.

Despite the apparent success of screening as judged by the endpoint of population mortality, it could be argued that the aforementioned screening criteria have not been fully satisfied in relation to breast cancer. For this reason, the underlying mechanism and extent by which advancing the time of diagnosis improves overall survival of a screened population remains unclear and some of the controversy relating to screening trials may emanate from limitations based on disease biology and the natural history of breast cancer. The latter is a heterogeneous disease, in terms both of variation among different tumors and of cellular composition within any individual tumor. This biological heterogeneity confers a variable natural history upon breast cancer, and may ultimately undermine and limit the potential impact not only of screening programs, but also treatment schedules for breast cancer. Conservative estimates reveal a broad range of individual tumor growth rates (17–19), with stochastic models being most applicable to breast cancer growth (20). This limits the breadth of predictions about the growth of mammary tumors and may hinder the design and planning of optimum schedules for breast cancer screening, where there are often "trade-offs" between the generalizability of screening outcomes and the validity of individual trials (7).

BIOLOGICAL MODELS OF BREAST CANCER

The essence of breast cancer screening is to detect malignant lesions at an earlier stage in their natural developmental history for which instigation of appropriate therapies will lead to mortality reduction. Detection of a lesion at a smaller size *per se* will not necessarily impact on mortality; diagnosis of a disease at an earlier chronological stage will only translate into improved outcomes if effective therapies can be instituted at this time. Otherwise, increased survival will be ascribed to "lead time" bias, and individuals merely acquire advance knowledge of their disease, with date of death and mortality remaining unchanged.

For screening to produce a genuine improvement in survival and reduction of cause-specific mortality, there must be an event in the natural history of the disease beyond which prognosis is adversely affected, and for which there is a threshold effect reflected in the size of a detectable lesion. If a lesion progressively increased in size without any such concomitant "event," then it would be of no consequence whether this was excised at size x or x+1 provided that excision was complete.

There are two events which may occur in the natural history and progression of malignant breast lesions which could account for the efficacy of screening and provide a biological rationale for early detection strategies; (*i*) early dissemination and (*ii*) phenotypic progression. Should one or both of these events occur at some stage in neoplastic development which is dependent upon tumor size, then earlier detection and intervention may pre-empt the formation of micrometastases and/or a more biologically aggressive primary tumor and so lead to improved prognosis (21). Stochastic models of tumor growth imply that such an event could occur relatively suddenly during tumor development due to a random growth "spurt" (20). Concepts of orderly progression may be deceptive; tumors can disseminate prior to reaching thresholds of either mammographic detection or clinical presentation (19,22). Screening aims to treat asymptomatic individuals at an early time point in this neoplastic continuum which represents a "window of opportunity" for treatment and can potentially reduce mortality by mechanisms outlined earlier.

There are two dominant paradigms of breast cancer biology which have governed the management of breast cancer over the past century: the Halstedian paradigm and the paradigm of biological predeterminism. Although the latter has become pre-eminent during the past two decades, both are relevant to the philosophy of breast screening and indeed an intermediate paradigm may be most appropriate contemporaneously (Fig. 6.1) (23).

Halstedian Paradigm

Virchow proposed a centrifugal theory for dissemination of breast cancer in which a tumor was considered to initially invade local tissues and to subsequently spread in a progressive,

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CHAPTER 7

Selective Estrogen Receptor Modulators as Treatments and Preventives of Breast Cancer

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Abstract: Estrogen is important in human health and diseases. Estrogen binds to estrogen receptors (ER), alpha and beta which subsequently function as transcription factors. Selective estrogen receptor modulators (SERMs) are synthetic molecules which bind to ER and can modulate its transcriptional capabilities in different ways in diverse estrogen target tissues. Tamoxifen, the first SERM, is extensively used for targeted therapy of ER positive breast cancers and is also approved as the first chemo-preventive agent for lowering breast cancer incidence in high risk women. The strategy for the therapeutic and preventive applications of tamoxifen was initially demonstrated in the laboratory which laid the foundation for its success in the clinic. Unfortunately, use of tamoxifen is associated with *de novo* and acquired resistance and some undesirable side effects. The molecular study of the resistance provides an opportunity to understand the mechanism of SERM action which may further help in designing new and improved SERMs. Clinical studies demonstrate that another SERM, raloxifene, used to treat post-menopausal osteoporosis, is also as efficient as tamoxifen in preventing breast cancers with fewer side effects. Overall, these findings provide opportunities for SERMs as a new class of drugs which not only can be used for therapeutic and preventive purposes of breast cancers but also for other disease states. The goal is to create new SERMs with a better therapeutic profile and fewer side effects.

Keywords: Breast cancer, osteoporosis, estrogen receptor, tamoxifen, raloxifene, SERMs, endocrine therapy, drug resistance.

1. INTRODUCTION

Breast cancer incidences and death rates have dropped significantly during recent years, which is associated strongly with improvement in early detection methods

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and decrease of menopausal hormone replacement therapy (HRT) [1, 2]. HRT, in the form of estrogen alone or estrogen plus progesterone, had been widely used since the 1960s until recent years, to treat conditions associated with aging as well as unpleasant menopausal symptoms. HRT was also known to protect post-menopausal women from osteoporosis and also thought to protect women from heart disease and Alzheimer's disease. However, the Women's Health Initiative (WHI) study indicated that taking estrogen with or without progesterone for 5 or more years placed the women at higher risk of breast cancer, Alzheimer's disease, heart disease, blood clot and stroke, although HRT is effective to reduce the risks of osteoporosis and colon cancer [3, 4]. The Million Women Study (MWS) conducted in the UK also showed that women taking HRT were more likely to develop breast cancer [5], endometrial cancer [6] and ovarian cancer [7]. In the US, the use of estrogen-plus-progestin HRT has dropped almost 50% when the WHI announced their findings in 2002, and this was followed by a sharp 7% decrease of new breast cancers in 2003 [2]. Although the decrease of HRT uses is not the sole reason leading to less breast cancer incidences, much effort has been focused on finding more effective and safer compounds to replace HRT which not only relieve menopausal symptoms but also prevent and treat hormone-responsive cancers. One most promising approach is to use selective estrogen receptor modulators (SERMs).

SERMs are synthetic compounds that bind to estrogen receptors alpha and/or beta (ERα and/or ERβ) and exert estrogenic or antiestrogenic activities in a tissue/cell-specific manner. The first SERM that has been used successfully in the clinic to prevent and treat breast cancer is tamoxifen, a failed postcoital contraceptive that evolved into the "gold standard" for breast cancer treatment [8, 9]. Tamoxifen is estimated to have saved the lives of over 400,000 women with breast cancer [8]. The second generation SERM, raloxifene (formally called keoxifene), failed as a treatment for breast cancer but is effective against osteoporosis and prevents breast cancer at the same time. Raloxifene is as effective as tamoxifen to reduce invasive breast cancer risks without an increase in the risk of endometrial cancer observed with tamoxifen [10]. Indeed a recent study suggests that raloxifene might even be effective in preventing endometrial cancer [11]. These findings have acted as a catalyst for the search of new SERMs which are estrogen-like in

bones and circulating lipids but antiestrogenic in women's reproductive organs and therefore are anti-cancer agents. However, there are problems associated with the current SERMs such as drug resistance and side effects. For example, both tamoxifen and raloxifene increase both hot flashes and blood clots [12].

Besides SERMs, other endocrine therapies target the ER indirectly to prevent and treat breast cancer. Aromatase inhibitors (AIs) that block the synthesis of estrogen from androgen in peripheral tissues have been extensively studied and show efficacy equivalent or superior to tamoxifen to treat postmenopausal breast cancer [13]. Since the mechanism for AIs to treat ER-positive breast cancer is to deplete estrogen in postmenopausal patients, they do not increase risks of endometrium cancer or blood clot and may be a better choice for postmenopausal breast cancer patients than tamoxifen. However, AIs are not effective in premenopausal women with actively functioning ovaries because AIs do not inhibit ovarian estrogen production. In addition, AIs lack the estrogenic protective function for cardiovascular diseases or osteoporosis. As a result, the side effects of AIs are mostly consistent with estrogen deprivation. AIs are associated with a greater incidence of bone loss and musculoskeletal symptoms, and probably higher risk of cardiovascular disease suggested by adjuvant trials comparing AIs and tamoxifen [14]. However, AIs are associated with a lower incidence of gynecological symptoms, thromboembolic diseases and hot flashes compared to tamoxifen in adjuvant setting [14]. Another strategy is to use selective estrogen receptor down-regulators (SERDs), such as fulvestrant, that cause degradation of ERs. Fulvestrant has been approved to treat advanced breast cancer after tamoxifen failure, and a recent phase III trial indicated that fulvestrant and AI exemestane were equally effective with a similar safety profile [15].

Resistance is a common problem associated with endocrine therapy therefore alternative treatment strategies without cross-resistance are necessary. Compared to the pure anti-estrogenic actions like AIs or fulvestrant, an ideal SERM with beneficial estrogenic effects has great potential for breast cancer prevention and treatment, especially in postmenopausal women as they often suffer from unpleasant symptoms resulting from lower estrogen. A perfect SERM would reduce the risk of breast cancer, ovarian cancer and uterine cancer, as well as strengthen the bone, prevent coronary heart disease, strokes and Alzheimer's

disease, and relieve menopausal discomfort like hot flashes and vaginal atrophy [12].

The complicated outcome of SERMs action cannot just be explained by turning on or off the ERs and their downstream genes. Although much new knowledge is being developed, we are still evolving in our understanding of the detailed mechanism of SERMs and their interaction with the ERs. In the past decade, another group of protein factors, nuclear receptor coregulators, have been identified that are essential for modulating the functions of SERMs and ERs. In this article, we will review the evolving understanding of the molecular mechanisms of SERMs action in the context of other signal transduction pathways and nuclear receptor coregulators, as well as the problems associated with the application of SERMs as a treatment or preventative for breast cancer. Finally, the new SERMs with potential as new agents to treat or prevent breast cancer will be described.

2. MECHANISM OF ESTROGEN ACTION

2.1. Structure and Function of ER

The existence of estrogen binding protein was first predicted by Elwood Jensen and colleagues in early 1960's [16]. The first ER cDNA, now known as ERα, was later cloned in the mid-1980's [17, 18]. In 1996, an additional ER was cloned from rat prostate [19] and designated as ERβ. The action of estrogen in cells is therefore almost entirely mediated by these two related but distinct subtype of estrogen receptors, ERα and ERβ. Both receptors function as ligand activated transcription factors which can bind the cognate DNA sequences known as estrogen responsive elements (ERE), and activate transcription. The ER proteins can be structurally subdivided into six domains on the basis of the functions controlled by the region, as shown in Fig. (**1**). The A/B domain contains one of the two transcriptional activation functions (AFs), designated as AF1 which is involved in estrogen-independent activation of transcription. Another activation function domain, AF2, is located in the E domain which also harbors the ligand binding domain (LBD), and is involved in estrogen/ ligand dependent activation

[20, 21]. The ERβ has 97% homology in the DBD and 61% homology in the LBD with ERα suggesting differential ligand binding capability of ERs [21].

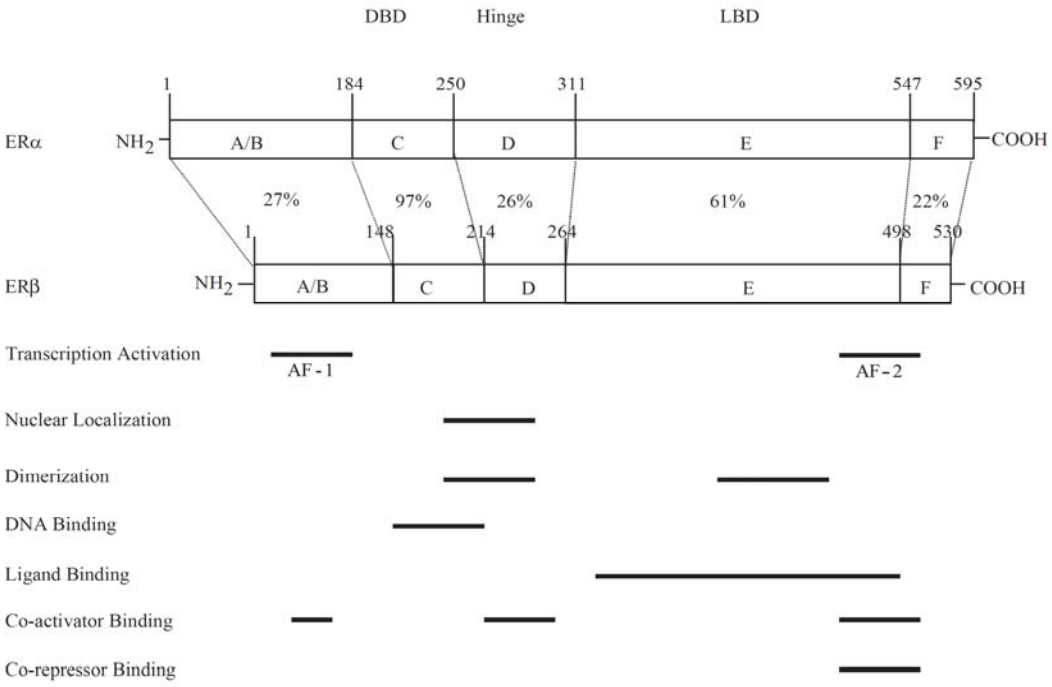


Fig. (1). Schematic comparison of human ER-α and ER-β structure. The structural domains are shown, and the percentage of amino acid identity shared by the two ERs is indicated for each domain. The horizontal bars highlight areas of different functions.

SERMs, the molecules which can bind to ERα and/or β and can either stimulate estrogen-like actions (agonist) or oppose estrogen actions (antagonist) in various estrogen target tissues and cells. This pharmacologic knowledge advanced studies to decipher the details of the molecular mechanism of estrogen action in different cell and tissue types.

The structural studies of a SERM complexed with the LBD of ERα and ERβ reveal that re-orientation of the AF2 helix (helix 12) after the binding of the SERM to the hydrophobic pocket of the LBD [22, 23]. The interaction of amino acid Asp351 of ERα with the alkylaminoethoxyphenyl side chain of tamoxifen or raloxifene is crucial to prevent the recruitment of coactivators to the SERM-receptor complex surface [22, 23]. Using different mutants of ERα for the amino

acid Asp351, it was shown that shielding and neutralization of Asp351 by the side chain of raloxifene is critical in defining the antiestrogenicity of this SERM. Furthermore, it has been shown that changing the Asp351 from aspartate to glycine (D351G) abolishes the estrogen-agonist activity of the tamoxifen-ER complex, while retaining its antagonistic property. The AF2 region of the agonist-bound receptor is particularly important for the interactions of steroid receptor coactivators (SRCs 1-3) *via* the interacting amino acid motif LxxLL, known as nuclear receptor interacting domain (NRID). It is important to note that the affinity of ERs for these NRIDs of SRCs is highly dependent upon the ER subtype, α and β, and ligand bound to the ER [24-26]. Recruitment of these co-activator(s) is also responsible for facilitating the activation of estrogen responsive genes by modifying the chromatin structure and activating the transcriptional machinery. Additionally, SERMs may also show differential AF1 activity mediated by co-repressor binding. Using ERE-reporter constructs, it has been shown that the AF1 domain of ERα is actively involved in agonist-induced gene expression whereas the AF1 domain of ERβ is involved very weakly [27].

Estrogen can also modulate the expression of genes by another mechanism in which the receptor complex can interact with other transcription factors such as activating protein 1 (AP1) or stimulating protein 1 (Sp1) through a process known as a tethering mechanism. Intriguing differences are observed in the mechanism of action between ERα and ERβ through an AP1 site. In the presence of estrogen, ERα induces AP1 driven reporter activity but ERβ has no effect [28]. The raloxifene bound ERβ complex can induce transcriptional activity through the AP1 site but the activity through ERα bound to raloxifene is negligible.

ERs also act in a non-genomic manner initiated from the cell membrane. These actions are very fast (seconds to minutes) and occur without RNA or protein synthesis. They often mobilize second messenger molecules such as Ca^{2+} and cAMP, and are associated with protein kinase cascades such as PI3K/Akt and MAPK [29, 30]. Several explanations have been offered to explain these effects. There could be a subpopulation of nuclear ERs associate with the plasma membrane, either through posttranslational modification such as palmitoylation or mediated by scaffold proteins such as caveolin-1 and MNAR, since ERs do not have a transmembrane domain [29, 30]. Another membrane bound protein, G

protein coupled receptor GPR30, was identified in recent years that mediates non-genomic actions of estrogen [31, 32]. The cellular localization of GPR30 is still controversial. Some evidence suggests it is at plasma membrane [33, 34] and other evidence suggests it is in the endoplasmic reticulum [32]. GPR30 binds to 17β-estradiol, tamoxifen and fulvestrant with high affinity [34] and is associated with breast cancer metastasis and transactivation of the epidermal growth factor receptor (EGFR) [35].

2.2. Co-Regulators

The co-regulators are protein molecules which can physically interact with the liganded or un-liganded ERs and modulate the transcription of the genes. The transcriptional activation or repression of the responsive genes is a combinatorial function of ligand-receptor interaction, recognition of cognate DNA sequence and recruitment of specific co-regulators onto the promoter of the gene. The assembly of the whole transcriptional complex is also dependent upon the affinity of the above mentioned individual components among themselves and their relative concentrations in the cell. Co-regulators play defining roles in the final tissue outcome in terms of transcriptional activation or repression mediated by estrogen or SERMs. The co-regulators can be broadly classified on the basis of their function, as co-activators which promote the activation of the transcriptional process, or co-repressors which are associated with repression of transcription of genes (Fig. **2**).

2.2.1. Co-Activators

Presently, around 200 co-activators are known, which are associated with 48 nuclear receptors [36]. The family of p160 proteins known as steroid receptor co-activators (SRCs) have been studied extensively. The relative abundance of SRC1 in uterine cells is responsible for the agonistic activity of tamoxifen, whereas in breast cancer cells, with low SRC1 levels, tamoxifen acts as an estrogen antagonist [37]. However, raloxifene, another related SERM, does not recruit SRC-1 even in the uterine cells [37], suggesting that the interaction with specific ligand which elicits a unique conformation of the receptor is critical for the

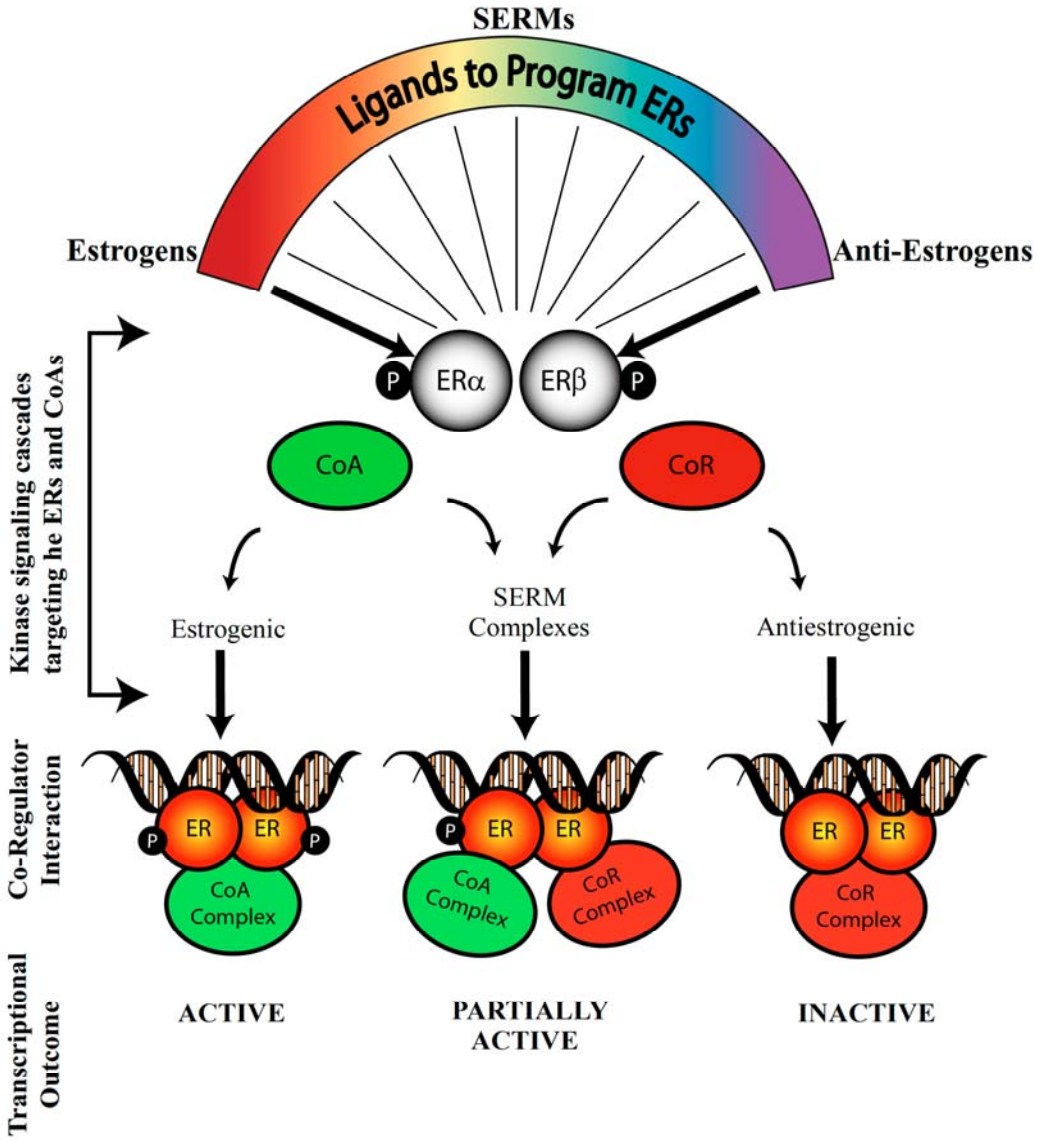


Fig. (2). Schematic representation of different liganded-ER complexes interacting with co-regulators and consequent transcriptional activities. ERs that bind to estrogenic ligands interact with co-activators (CoA) and activate transcription. Anti-estrogen liganded-ER complexes interact with co-repressors (CoR) and inactivate transcription of responsive genes. Selective estrogen receptor modulators (SERMs) bind to ERs and interact with either co-activator or co-repressor complexes eliciting partial transcriptional activity depending upon the cellular context.

interaction of co-regulators. These observations further provide an explanation for the earlier studies, where tamoxifen have been reported to induce growth of

endometrial cancer cells but not of breast cancer cells in athymic mice [38] and also that estrogen agonistic properties of raloxifene is less in endometrial cancer cells [39]. These finding also translate very well to clinical experience [40, 41]. In addition, the SERMs can enhance the stability of the co-activators (SRC1 and SRC3) and thereby influence the transcriptional capability of other nuclear receptors [42]. Post-translational modifications of the co-activators, including but not limited to phosphorylation, methylation, ubiquitylation, sumoylation and acetylation, can also regulate the gene activation by influencing the ability of the co-activators to interact with ER and other components of the transcriptional complex [33, 35, 36]. The understanding of structure-function relationship of ligands at the ER has formed the basis of designing effective new SERMs with fewer side effects.

2.2.2. Co-Repressors

Co-repressors are functional counterparts of co-activators, which are associated with transcriptionally inactive promoters and help repress the expression of genes [43]. Fewer co-repressors have been reported compared to the co-activators. In the case of ER, the co-repressors are known to interact with the un-liganded and/or antagonist bound receptor. The two most extensively studied co-repressors in connection with ER are Nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). The ER bound to raloxifene or 4-hydroxytamoxifen (a potent antagonist metabolite of tamoxifen) is known to recruit NCoR and SMRT to the promoters of estrogen responsive genes and repress transcription [37, 44, 45]. It has been shown that inhibition of NCoR or SMRT with monoclonal antibodies can enhance the agonistic property of 4-hydroxytamoxifen [46]. Moreover, using fibroblasts from NCoR null mice, 4-hydroxytamoxifen was shown to be a relatively potent ERα agonist [47]. The critical role of NCoR and SMRT in 4-hydroxytamoxifen-induced arrest of cell proliferation of ERα positive breast cancer cells is confirmed because 4-hydroxytamoxifen-stimulated cell cycle progression now occurs in NCoR-and-SMRT-deficient breast cancer cells [48]. However not all estrogen responsive genes are activated by 4-hydroxytamoxifen in NCoR and SMRT deficient cells, clearly indicating that additional molecules are important in SERM-induced repression of estrogen responsive genes. Indeed, there are several other co-

repressor proteins known for ER. Metastasis associated protein 1 (MTA1) is a corepressor found to mediate the ER transcriptional repression [49]. Another corepressor, known as repressor of estrogen action (REA) potentiates the inhibitory effects of anti-estrogens including 4-hydroxytamoxifen. Additionally, REA interacts with ER and competes with the co-activator SRC1 for binding to the estrogen bound ER [50, 51]. This again emphasizes the fact that the relative levels of co-regulators may be important in deciding the outcome of the SERM action. The proteasomal regulation of NCoR is another factor which may influence the SERM action. Degradation of NCoR occurs through the 26S proteasome, which is mediated by seven in absentia homologue 2 (Siah2) [52]. Interestingly, estrogen mediated upregulation of Siah2 in ER positive breast cancer cells has been implicated in the proteasomal degradation of NCoR, and subsequent de-repression of NCoR regulated genes [53].

In addition to acting as a "transcriptional adapter" between the receptors and the transcriptional machinery, the coregulator itself or its complex possess various enzymatic activities such as acetylation, phosphorylation, methylation or de-acetylation by which they are able to modify the local chromatin structure thereby making the local environment conducive for gene expression or repression. Intrinsic histone acetyl transferase activity was found to be associated with co-activator SRC1 which helps in the activation of transcriptional expression [54]. In contrast, the 4-hydroxytamoxifen bound ER complex which recruits the co-repressors NCoR and SMRT is associated with histone de-acetylases and other chromatin modifying enzymes [37, 55]. The deacetylase activity promotes transcriptional repression [37, 55]. Interestingly, another enzyme in the co-activator complex, CARM1 (coactivator associated arginine methyltransferase 1) has recently been implicated in modifying the coactivator itself and inducing the degradation of the complex [56]. This suggests the ability of the enzymes in the complex to modify other proteins in its own complex apart from a role in the modification of chromatin.

With this background of the molecular biology of SERM action, it is now appropriate to describe our evolving understanding about drug resistance. This is important not only because tumor drug resistance is the consequence of long term

SERM administration, but also because new knowledge will aid patients with the development of novel treatment strategies for SERM-resistant breast cancer.

3. DRUG RESISTANCE TO SERMs

There are three types of resistance to SERMs based on the mechanism: metabolic resistance, intrinsic resistance and acquired resistance [57].

3.1. Metabolic Resistance

Metabolic resistance to tamoxifen is mostly related to CYP2D6, an enzyme product that metabolizes tamoxifen into its active forms 4-hydroxytamoxifen and endoxifen [58]. This has been extensively reviewed recently and will only be briefly mentioned here [13, 59]. CYP2D6 is genetically polymorphic and 5-8% of Caucasian subjects are CYP2D6 “poor metabolizers” thus are less likely to benefit from tamoxifen treatment, although it has been shown that these women tolerate tamoxifen better and tend to remain on the drug for longer [59]. The genotype of CYP2D6 has been shown in some clinical trials to be directly related to the outcome of tamoxifen use, however, the results are not always consistent. Eight studies indicated that CYP2D6 “poor metabolizer” genotypes have worse outcome of breast cancer patients who received tamoxifen but two studies contradicted this conclusion [60]. In addition to the genotype of CYP2D6, it is important to consider that other drugs may interact with the enzyme system and block the metabolic activation of tamoxifen. Unfortunately, selective serotonin reuptake inhibitors (SSRIs) that are used to relieve the menopausal side effects of tamoxifen are also metabolized by CYP2D6 and block the metabolic activation of tamoxifen. The proper choice of SSRI may be important so as not to impair tamoxifen metabolism. The SSRI of choice is venlafaxine that has only a low affinity for the CYP2D6 enzyme [61]. Although these emerging data about CYP2D6 genotypes and the drug interaction between tamoxifen and SSRIs are important, it is perhaps too early to use CYP2D6 status to routinely choose between tamoxifen and aromatase inhibitors to treat postmenopausal women with breast cancer. At present, an international consortium is evaluating the overall CYP2D6 status of completed clinical trials with tamoxifen to assemble a large

scale retrospective analysis of the worth of genotyping. The aim is to answer the question of whether "poor metabolizers" should avoid tamoxifen use.

3.2. Intrinsic Resistance

Approximately 30% ER-positive breast cancer patients do not respond to tamoxifen [62]. This type of resistance is referred to as "*de novo*" resistance or intrinsic resistance. Clinical studies showed that only 40% patients with ER-positive, progesterone receptor (PR)-negative breast cancers are responsive to anti-estrogen treatment (tamoxifen or endocrine ablation) compared to 80% responsive rate in ER-and-PR-positive patients [58, 59]. Historically, the status of PR has been regarded as an indicator of a functional ER pathway, since expression of PR is regulated by estrogen. On the other hand, recent evidence suggested that the absence of PR is associated with excessive growth factor signaling such as overexpression of HER2 [63, 64], which has been known to impair estrogen induction of PR and reduce the effectiveness of tamoxifen treatment for breast cancer [65]. However, the negative association between PR and HER2 seems more evident in older women (> 45 yrs) [66] and it remains controversial that PR-status could be used for clinical decision on choosing between tamoxifen or AIs [67].

Growth factor signaling, especially through epidermal growth factor (EGF) pathway, has been studied extensively in the past two decades and linked to SERM resistance. This has been recently reviewed [68] and will only be briefly summarized here. EGF binds to ErbB family of cell surface receptors that include four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4). Overexpression of HER2 has been clinically linked to less response to endocrine therapies and worse prognosis [69-71], so has the overexpression of EGFR [72]. Different ErbB family members can form heterodimers and activate multiple signaling pathways including PI3K/Akt and MAPK. The major molecular mechanisms leading to SERM resistance can be summarized as follows: 1. Activation of downstream kinase cascade results in the phosphorylation of ER at key residues (Ser106/107, 118, 167, 305 and Thr 311) which activates transcription in a ligand-independent manner. Phosphorylation may change the binding of ER with ligands, DNA and

coregulators, which may ultimately alter the activity of SERMs [73]. For example, phosphorylation of ER at Ser167 by Akt and Ser118 by the MAPK pathway both cause ligand-independent activation [74-76]. A recent study showed that phosphorylation of ER at Ser305 altered the orientation between the C-terminus of ER and SRC-1 that led to the recruitment of ER transcription coactivators and RNA polymerase II even in the presence of tamoxifen [77]. 2. Phosphorylation of ER co-regulators is equally important as the phosphorylation of ER itself, since phosphorylated co-activators have increased activity in the presence of SERMs [78-80]. Phosphorylation of co-repressors such as SMRT is associated with the co-repressor's nuclear export and impaired transcriptional suppressing function [81]. 3. Other than enhancing the transcriptional activity of the ER by phosphorylation, overexpression of EGFR or HER2 increases the non-genomic actions of ER, and SERMs may now act as estrogen agonists *via* the membrane effects of ER [82, 83]. In addition to the EGF signal pathway, the insulin-like growth factor (IGF) signal pathway is also involved in tamoxifen resistance [84]. It can activate PI3K/Akt pathway [71] and turn on genes that are otherwise activated by estrogen [85, 86].

Dysregulation of ER co-regulators is another major contributor to intrinsic SERM resistance. Overexpression of both AIB1 (SRC-3, ACTR, p/CIP, RAC3, TRAM-1) and HER2 have been shown to convert tamoxifen into an estrogen agonist in breast cancer cells [78]. Elevated AIB1 was found to associate with tamoxifen resistance, DNA-nondiploidy, high S-phase fraction and HER2 amplification in samples from clinical study [87]. Although a study indicated that high expression of AIB1 was not associated with relapse during tamoxifen treatment [88], AIB1 was shown to associate with tamoxifen resistance in breast cancers that overexpressed ErbB family proteins [88, 89]. AIB1 might be a predictor marker for tamoxifen ineffectiveness in ER-positive, HER2-positive and PR-negative breast cancer. On the other hand, low expression of ER co-repressor NcoR is associated with shorter relapse-free survival in breast cancer patients who only received tamoxifen after surgery [90]. Based on the emerging importance of co-regulators and tamoxifen resistance, one novel approach to overcome tamoxifen resistance is by the use of disulfide benzamide (DIBA) to disrupt the zinc finger in the ERα DNA binding domain. The approach facilitates ERα dissociation from

coactivator AIB1 and concomitant association of corepressor NcoR without changing the phosphorylation of HER2, MAPK, Akt or AIB1 [91].

Another group of regulators associated with tamoxifen resistance are microRNAs (miRNA). These are naturally occurring single-stranded RNAs with the length of 21-23 nucleotides that do not code for proteins. They regulate gene expression mainly by inducing target mRNA degradation or inhibiting translation (protein synthesis). Dysregulation of miRNAs is associated with many cancers including breast cancer [92, 93]. Two recent studies show that miRNA-221/222 are upregulated in tamoxifen-resistant breast cancer cells and primary tumors, and they may contribute to tamoxifen resistance by down-regulating p27Kip1 or ERα [94, 95].

3.3. Acquired Resistance

Breast cancer patients who initially respond to tamoxifen later develop “acquired resistance” that is characterized by tamoxifen stimulated growth. This can be replicated in the laboratory with MCF-7 xenograft tumors implanted in ovariectomized athymic mice. Tamoxifen initially inhibits estrogen stimulated tumor growth but eventually some tumors start to grow during tamoxifen therapy [96]. These tumors now grow in response to either estrogen or tamoxifen and stop growing with no treatment or during treatment with fulvestrant [96]. The laboratory model is consistent with the clinic observation that aromatase inhibitor or fulvestrant are equally effective after the failure of tamoxifen treatment [97, 98]. It therefore appears that ER remains fully functional in the laboratory model of acquired tamoxifen resistance. In clinical studies, only 17-28% patients with acquired tamoxifen resistance have a loss of ER function [99, 100], and it is more likely that acquired resistance is associated with the stimulation of other growth/survival pathways [101]. For example, activated mammalian target of rapamycin (mTOR, downstream of PI3K/Akt and MAPK pathway) and c-Src (downstream of EGFR/HER2) were observed in breast cancer cells and mTOR and c-Src inhibitors can restore tamoxifen sensitivity in these cells, respectively [102, 103]. Several genes involved in cell proliferation and survival have altered expression level in breast cancer cells with acquired tamoxifen resistance. Examples of genes which down regulation is associated with acquired tamoxifen

resistance include cyclin-dependent kinase inhibitors p21Cip [104] and p27Kip [105]. Examples of genes which upregulation is associated with tamoxifen acquired resistance include cyclin-dependent kinase 10 (CDK10) [106] and anti-apoptotic protein survivin [107].

Laboratory observation showed that acquired tamoxifen-resistant breast cancer cells/tumors respond differently to estrogen, and three phases of tamoxifen-resistance have been described, which seems to depend on the length of tamoxifen exposure [12]. Tumors with phase I resistance are stimulated by estrogen and tamoxifen but inhibited by AIs and fulvestrant; tumors with phase II resistance are stimulated by tamoxifen but are inhibited by estrogen due to apoptosis; tumors with phase III resistance (automatous growth) grow in a hormone-independent manner that is not responsive to either AIs or fulvestrant or SERMs, but estrogen still exerts apoptotic actions on those tumors [12]. The laboratory models suggest a new treatment strategy, in which limited duration, low-dose estrogen can be used to purge phase II- or phase III-resistant breast cancer cells so that the tumors will be responsive to antiestrogen therapy again. Phase II clinical study is ongoing to test this treatment plan [108].

Most studies on SERM-resistance are related to tamoxifen and little is known about raloxifene resistance. Based on a few studies on raloxifene resistance using cell culture and animal models, raloxifene-resistant tumors are likely to have similar properties as tamoxifen-resistant ones [109]. Raloxifene-resistant MCF7 cells generated by long-term exposure to raloxifene *in vitro* are also resistant to tamoxifen *in vitro* and *in vivo*. They exhibit phase II SERM-resistance as estradiol treatment causes tumor regression by inducing G2/M cell cycle arrest and apoptosis [110]. Another raloxifene-resistant breast tumor model generated by exposing MCF7 breast tumors to raloxifene *in vivo* exhibits phase I SERM-resistance whose growth is stimulated by tamoxifen, raloxifene and estrogen [109]. Interestingly, protein levels of EGFR and HER2 are also increased in this phase I raloxifene-resistant tumor model, which suggests raloxifene-resistant tumors share similar molecular mechanisms as tamoxifen-resistant ones [109].

Overall, the classifications of different forms of antihormonal drug resistance can be used as a basis to evaluate the pharmacology of new SERMs. The goal is to

improve on tamoxifen, the pioneer that over the past 30 years found ubiquitous long term applications in the treatment and prevention of breast cancer.

4. NEW SERMs

The discovery of the first antibiotic penicillin initiated a search for further antibiotics to delay drug resistance and to target specific diseases. Similarly, the successful clinical application of tamoxifen in medicine has resulted in the investigation of numerous related molecules to develop the "ideal SERM". However, it has been challenging to find a SERM that is superior to tamoxifen, which retains or extends its benefit to treat and prevent breast cancer but with fewer side effects. Tamoxifen maintains bone density in animals [111] and humans [112] so SERMs are being developed to treat osteoporosis, but the potential to prevent breast cancer and uterine cancer will also increase their clinical value and commercial success. The core structures of SERMs are diverse, including triphenylethylene, benzothiophene, chromene (benzopyran), naphthalene, indole and steroid, but each of the newer SERM is really a mimic of tamoxifen, raloxifene or estradiol. The development of dozens of SERMs have been discontinued due to ineffectiveness for human disease or severe side effects, but several new SERMs are under active investigations with great potential in breast cancer treatment and/or prevention, alone or in combination with other type of drugs. In addition, since the identification of ERβ in 1996 [19], ER subtype selective SERMs have been developed which could potentially be used as breast cancer preventives. Thus this area of medicinal chemistry remains an important topic of interest as new ER regulated targets emerge. We will review the current status of several agents that are either approved or in the process of drug development (summarized in Table **1**).

4.1. Tamoxifen-Like SERMs

4.1.1. Toremifene (Fareston)

Toremifene (**2**) is a chlorinated tamoxifen analogue which has been approved in the US and several other countries for the treatment of metastatic breast cancer.

Table 1. Current Status of New SERMs

Drug Name	Category (Structure)	Effects	Preclinical Results	Clinical Status
Toremifene	Tamoxifen-like	Breast cancer treatment Heart protection Mastalgia treatment Prostate cancer prevention Relieve side effects of androgen deprivation therapy	Fewer genotoxic effects than tamoxifen [113], bone effects similar to tamoxifen [119]	FDA approved for metastatic breast cancer Phase II trial (65 women) better than tamoxifen regulating lipid metabolism [121, 122] Phase II trials (62 and 195 women) effective [126, 127] Phase II trial (514 men) decreases prostate cancer incidence [128] Phase III trial (1,389 men) improves lipid profiles [130] Phase III trial (1,392 men) increases bone mineral density [129]
Ospemifene	Tamoxifen-like	Vaginal atrophy treatment Osteoporosis treatment Breast cancer prevention	Estrogenic effects on vaginal epithelium that is not observed with tamoxifen or raloxifene [134-136] Inhibits tumor growth in animal models as effective as tamoxifen [140, 141]	Phase III trial (826 women) relieves vaginal dryness Phase II trial (118 women): Comparable to or slightly better than raloxifene [139] Phase III trial planned (detail not available) Not available
GW5638 (DPC974) & GW7604	Tamoxifen-like	Breast cancer treatment (2^{nd} line therapy)	Works as a SERM and as a SERD [148], effective in tamoxifen-resistant tumors [144, 145]; functions as an ER agonist in bone and cardiovascular system but an antagonist in breast and endometrium [142]	Phase I trial (9 patients who failed first-line hormone therapy) low toxicity [ASCO meeting 2002, abstract 452]
Arzoxifene (LY353381)	Raloxifene-like	Breast cancer treatment Breast cancer prevention	Antiestrogenic in breast and endometrium, estrogenic in bone and lipids [150] Effective to prevent ER-positive and ER-negative mammary tumors especially in combination with LG100268 [140, 155]	Phase III trial (200 patients) inferior to tamoxifen [154] Phase I trials (50 and 76 women) low toxicity and favorable biomarker profile [156]

(Table 1) contd…..

Drug Name	Category (Structure)	Effects	Preclinical Results	Clinical Status
Lasofoxifene (CP-336156, Fablyn)	Raloxifene-like	Osteoporosis treatment and prevention Vaginal atrophy treatment Breast cancer treatment and prevention Heart disease prevention	Higher potency than tamoxifen and raloxifene [158]; higher oral bioavailability than raloxifene [160] Effects similar to tamoxifen to prevent and treat NMU-induced mammary tumor in rats [163]	Phase III trial (1,907 women) significantly increases bone mineral density compared to placebo, no endometrial effects, no association with thromboembolic disorder [159] Phase III trial to compare with raloxifene (CORAL trial, details not available) Phase III trial (445 patients) improves vaginal atrophy compared to placebo Phase III trial (PEARL trial with 8,556 women), reduces ER-positive breast cancer incidence compared to placebo; slightly decreases major coronary disease risk; reduces vertebral and non-vertebral fractures; increases risks of venous thromboembolic events but not stroke; no endometrial effects [SABCS 2008, abstract 11]
Pipendoxifene (ERA-923)	Raloxifene-like	Breast cancer treatment	Inhibits tamoxifen-sensitive and -resistant tumors in mice and rats no uterotrophic activities compared to raloxifene [167]	Phase II trial to treat tamoxifen-refractory breast cancer in postmenopausal women (details not available)
Bazedoxifene (TSE-424 WAY-140424)	Raloxifene-like	Osteoporosis treatment and prevention Breast cancer prevention	Increases bone density with little uterine or vasomotor effects Inhibits estrogen-stimulated breast cancer cells growth [169]	Phase III trial (7,492 women) reduces vertebral and non-vertebral fracture incidences, while raloxifene is not effective against non-vertebral fracture [171] Phase III trial (497 women) reduces endometrial thickness, unique property among known SERMs [170] Not available
Acolbifene (EM-652, SCH57068) & EM-800 (SCH57050)	Raloxifene-like	Breast cancer treatment (2^{nd} line therapy) Breast cancer treatment (1st line therapy) Breast cancer prevention	Highest affinity for ER, inhibit growth of multiple breast cancer cells *in vitro* and *in vivo* [180]	Phase III trial, less effective than anastrozole to treat tamoxifen-resistance breast cancer, study halted [182] Phase III trial planned [182] Phase II trial (started in February, 2009) for premenopausal women

(Table 1) contd…..

Drug Name	Category (Structure)	Effects	Preclinical Results	Clinical Status
CHF4227	Raloxifene-like	Breast cancer and osteoporosis prevention	Prevents DMBA-induced mammary tumors and preserves bone mass in rats [184, 185];	Phase I trials (24 and 56 women) beneficial on bone markers and lipid metabolism; no effects on endometrium; not causing hot flashes
SP500263	Raloxifene-like	Breast cancer and osteoporosis treatment	Inhibits breast cancer cell growth *in vitro* and *in vivo* without stimulating uterine weight gain [188, 189], protects bone *in vitro* [190]	Not available
HMR3339	Steroidal	Osteoporosis and cardiovascular disease prevention	Better than raloxifene to protect cancellous bones [191]	Phase II trials (96 and 118 and 94 women) better than raloxifene at improving some beneficial cardiovascular markers [192-194]
PSK3471	Steroidal	Osteoporosis and breast cancer prevention and treatment	Prevents bone loss *in vivo*, inhibits growth of breast cancer cells *in vivo* [197]	Not available

Its structure is shown in Fig. (**3**). Toremifene is as effective as tamoxifen in the treatment of ER-positive breast cancer but with the potential of fewer genotoxic effects, since it does not produce DNA adducts in rat liver and human endometrium [113]. The mechanism for the reduced genotoxicity of toremifene can be explained as follows: Tamoxifen-DNA adducts are primarily formed *via* sulfonation of the α-hydroxylated tamoxifen metabolites, but the α-hydroxy metabolites of toremifene is poorly esterified or sulfonated, and even sulfonated α-hydroxy toremifene, α-sulfoxytoremifene, reacts poorly with DNA [114, 115]. However, there are some reports to show toremifene induces DNA damages and hepatocarcinogenesis in rats [116, 117].

The effects of toremifene and tamoxifen on bones are similar [118], as are the endometrial effects. However, a recent safety evaluation demonstrates that secondary endometrial cancer incidence is lower with toremifene than with tamoxifen and is similar to that with raloxifene [119]. Nevertheless, toremifene stimulates the growth of human endometrial cancer implanted in athymic mice in the same way as tamoxifen [120]. The positive effects of toremifene on lipid

profiles are superior to tamoxifen's. Toremifene lowers the low-density lipoprotein (LDL) cholesterol to a level similar to that seen with tamoxifen, but unlike tamoxifen, toremifene slightly increases high-density lipoprotein (HDL) cholesterol and lowers triglycerides in the serum [121, 122].

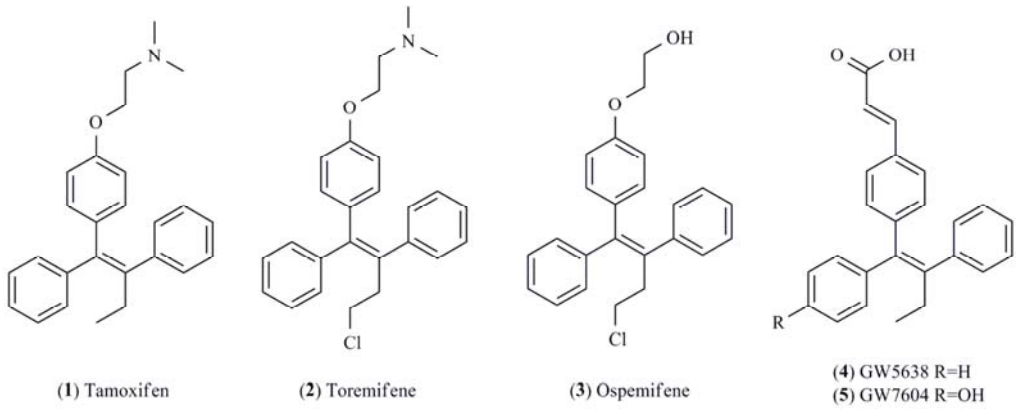


Fig. (3). SERMs with a structure mimicking tamoxifen containing a triphenylethylene core.

Cross-resistance with tamoxifen is an important issue to consider when using toremifene for recurrent breast cancer because the majority of patients have received or failed adjuvant tamoxifen. Toremifene is completely cross-resistant with tamoxifen in human breast tumors implanted in athymic mice [123], as well as in breast cancer patients [124, 125]. Therefore, toremifene would not be effective as a second-line endocrine therapy after tamoxifen failure and may offer no therapeutic advantages over tamoxifen as an adjuvant therapy.

In recent years, toremifene has been developed to treat other estrogen-related diseases. Toremifene is effective to treat mastalgia in some small phase II trials [126, 127], and is also effective at decreasing prostate cancer incidences in a high-risk population [128]. In addition, a recent multicenter randomized phase III trial showed that toremifene increased bone density and improved lipid profile in men receiving androgen deprivation therapy for prostate cancer [129, 130].

4.1.2. Ospemifene (Deaminohydroxytoremifene, FC-1271a)

Ospemifene (**3**), or deaminohydroxytoremifene, is a metabolite of toremifene (Fig. **3**). Like toremifene, ospemifene is generally well tolerated and has a

favorable safety profile. It does not induce DNA adducts in mice [131], rats [132] and monkey [133]. Ospemifene exerts a very weak estrogenic effect on endometrial histology, like raloxifene and decreases cholesterol [134]. However, unlike tamoxifen or raloxifene, ospemifene has significant estrogenic effects on vaginal epithelium [134-136] and is being developed for postmenopausal vaginal atrophy, a chronic condition experienced by about 40% postmenopausal women. Ospemifene is being evaluated in a phase III trial that has already recruited 826 women. Early results suggested that a 12-week course of ospemifene treatment significantly relieves symptoms of dryness in the vagina.

Ospemifene has showed promise in the prevention and treatment of osteoporosis. Cell culture studies indicated that ospemifene inhibits osteoclast formation and bone resorption and protects osteoblast-derived cells from apoptosis [137, 138]. In a recent phase II trial to compare effects of ospemifene and raloxifene on biochemical markers of bone turnover in postmenopausal women, ospemifene showed similar effects as raloxifene in regulating most of the bone markers examined, and at the 90-mg dose, ospemifene increased procollagen type I N propeptide (PINP) more than raloxifene [139]. Ospemifene is currently in phase III development for the treatment of postmenopausal osteoporosis.

Studies based on animal models suggest ospemifene might be effective in breast cancer prevention. Ospemifene prevented dimethylbenzanthracene (DMBA)-induced mammary tumors in female Sencar mice as effectively as tamoxifen, while raloxifene was not effective [140]. In a transplantable mouse model of ductal carcinoma *in situ* (DCIS), ospemifene had inhibitory effects equivalent to tamoxifen in terms of tumor growth and progression [141]. Nevertheless, the chemoprevention effects of ospemifene in breast cancer need to be further studied and substantiated by clinical trials.

4.1.3. GW5638 (DPC974) and GW7604

GW5638 (**4**) is a derivative of tamoxifen with an acrylate side chain in place of the dimethylaminoethoxy side chain in tamoxifen. GW7604 (**5**) is the 4-hydroxy version of GW5638, analogous to the major metabolite of tamoxifen, 4-hydroxytamoxifen. Their structures are shown in Fig. (**3**). GW5638 functions as a

full ER agonist in bone and the cardiovascular system but as an antagonist in breast and endometrial system in rodent models [142].

Although the structures of tamoxifen and GW5638/GW7604 are similar, GW5638/GW7604 acts with a mechanism different from tamoxifen/4-hydroxy tamoxifen as suggested by the following evidence: 1). GW7604 acts as an antagonist in MDA-MB-231 cells transfected with wild-type ERα, but 4-hydroxytamoxifen acts as an agonist [143]; 2). Phage display experiments indicate that GW7604 bound ERα or ERβ is associated with different peptides from 4-hydroxytamoxifen, raloxifene or fulvestrant bound ERs [144]; 3). GW5638 inhibits the growth of tamoxifen-resistant breast tumor xenograft [144, 145]; 4). The crystal structure of the ERα LBD bound by GW5638 shows that GW5638 induces a distinct conformation of H12 in the ERα AF2 region, which increased exposure of hydrophobic residues and results in ERα destabilization in MCF7 cells [146].

GW5638 and GW7604 are also classified as selective estrogen receptor down-regulators (SERDs) because they induce ERα degradation, a property observed with the pure antiestrogen fulvestrant which was approved for the treatment of metastatic breast cancer [147]. However, a recent report [148] indicates that GW5638 induces ERα degradation through a different mechanism from fulvestrant and another SERD RU58,668, as the protein/protein interaction surface on ER required for fulvestrant-induced degradation is not necessary for GW5638-induced degradation. The fact that GW5638 has a unique mechanism to antagonize estrogen function and induces ER degradation in breast cancer cells makes it a possible second line therapy after tamoxifen failure and as an alternative to fulvestrant. Currently, GW5638 is under clinical development under the name DPC974 [148].

4.2. Raloxifene-Like SERMs

4.2.1. Arzoxifene (LY353381)

Arzoxifene (**7**) is a derivative of raloxifene with the ketone group replaced by an ether group and the hydroxy group is replaced by a methoxy group (Fig. **4**). These

modifications have improved the pharmokinetic properties [149]. Arzoxifene has antiestrogenic effects on breast and endometrium but pro-estrogenic effects on bone and lipids [150]. Arzoxifene is cross-resistant in some but not all tamoxifen-stimulated breast tumor xenografts [151]. Phase II clinical trials indicate that arzoxifene is effective to treat tamoxifen-sensitive or tamoxifen-refractory patients with advanced or metastatic breast cancer [152] and patients with recurrent or advanced endometrial cancer [153] with minimal toxicity. However, a phase III trial showed arzoxifene was inferior to tamoxifen to treat patients with locally advanced and metastatic breast cancer [154]. The main role of arzoxifene may reside in its chemoprevention potential since it is more potent than raloxifene in pre-clinical studies [149].

The breast cancer chemoprevention property of arzoxifene has been studied with animal models and small short-term clinical trials. Arzoxifene effectively prevented nitrosomethylurea (NMU)-induced mammary tumor in rats [140] and induced apoptosis of breast cancer cells in rodent models especially when used in combination with rexinoid LG100268, a selective ligand for the retinoid X receptors (RXR) [155]. In two phase I clinical trials of women with newly diagnosed ductal carcinoma *in situ* or T1/T2 invasive cancer, arzoxifene did not demonstrate a significant reduction of tumor cell proliferation compared to placebo in 2-6 weeks treatment [156]. However, there were some favorable findings, such as a decrease of serum insulin like growth factor I (IGF-1) *vs* IGF binding protein 3 (IGFBP3) ratio and an increase of sex hormone binding globulin [156]. Another interesting aspect of the pharmacology of arzoxifene is that it might have chemopreventive properties for ER-negative breast cancer when used in combination with LG100268. A recent study showed that both SERMs arzoxifene and acolbifene alone prevent ER-negative mammary tumor in a mouse model and the effect is synergized with LG100268 [155]. Although the SERMs by themselves are not functional in the treatment of established tumors, together with LG100268 they inhibit proliferation and induce apoptosis in the ER-negative mammary tumors [155]. The mechanism how SERMs prevent tumorigenesis of ER-negative breast tissue is unknown, but the results suggest that arzoxifene has the potential for further clinical development as a chemoprevention drug of both ER-positive and negative breast cancer, especially in combination with rexinoids.

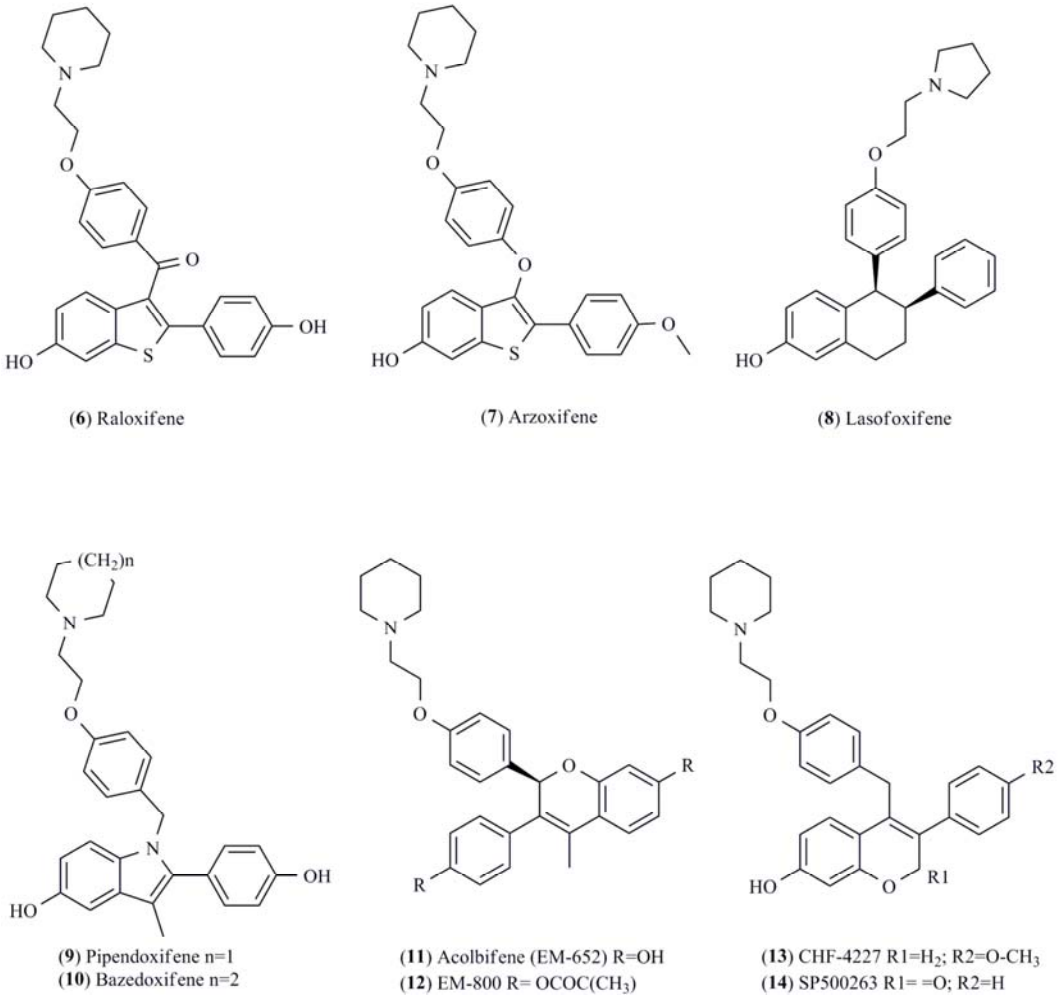


Fig. (4). SERMs with a structure mimicking raloxifene.

4.2.2. Lasofoxifene (CP-336156, Fablyn)

Lasofoxifene (**8**) has a naphthalene core structure, which is different from all the other SERMs discussed in this article (Fig. **4**). However, the crystal structure shows that lasofoxifene fits into the ERα LBD pocket in a similar manner as other ligands [157]. In addition, lasofoxifene-bound ERα LBD has similar conformational features as other SERM-bound ERα LBDs, such as tamoxifen or raloxifene, in which H12 in the "antagonist-bound" conformation and occludes the coactivator binding surface [157]. Lasofoxifene has a high affinity for ER with

an IC_{50} of 1.5 nM, which is comparable to 17β-estradiol and higher than tamoxifen and raloxifene [158]. It preserves bone density and lowers serum cholesterol, and also has chemopreventive and chemotherapeutic effects in rat mammary tumor models without any uterine hypertrophic effects [159]. Lasofoxifene is currently undergoing an extensive clinical evaluation for the prevention and treatment of osteoporosis [159]. One advantage of lasofoxifene over raloxifene is its increased oral bioavailability due to the nonpolar naphthalene structure that makes it a poor substrate for intestinal wall glucuronidation [160]. In addition to its effects on bone, lasofoxifene significantly improves symptoms of vaginal atrophy [161] and a recently completed phase III trial indicated that lasofoxifene decreased vaginal pH and improved the vaginal-cell maturation index in osteoporotic postmenopausal women. These effects may be due to the increased vaginal ERβ and androgen receptor protein levels [162]. Lasofoxifene acts as a chemopreventive and treatment in the NMU-induced rat mammary tumor model. The results are similar to the comparator drug tamoxifen [163]. Phase III trials are currently ongoing to evaluate its ability to prevent breast cancer and cardiovascular diseases in postmenopausal women [164].

4.2.3. Pipendoxifene (ERA-923)

Pipendoxifene (**9**) has an indole core structure (Fig. **4**). It was designed by adding an alkylaminoethoxyphenyl side chain to zindoxifene (D-16726), a 2-phenylindol based SERM which failed as a treatment for breast cancer [165]. Pipendoxifene, also named ERA-923, mimics the structure of raloxifene and is devoid of uterotrophic activities in immature rats and ovariectomized mice compared to raloxifene [166]. It inhibits the growth of tamoxifen-sensitive and -resistant tumors in rats and mice [167] and is under phase II clinical development for the treatment of tamoxifen-resistant metastatic breast cancer. In a recent study, a combination of pipendoxifene and temsirolimus, which is a mammalian target of rapamycin (mTOR) inhibitor, synergistically inhibited growth of MCF-7 cells and xenograft models even at suboptimal doses, primarily by causing G1 cell cycle arrest [168]. This suggested that combination of a SERM and an mTOR inhibitor might be of clinic value as breast cancer treatments.

4.2.4. Bazedoxifene (TSE-424, WAY-140424)

Bazedoxifene (**10**) is another indole SERM, designed and synthesized at the same time as pipendoxifene with a slight structural difference, as shown in Fig. (**4**) [166]. This SERM is being actively developed to treat osteoporosis with the potential to prevent breast cancer. Bazedoxifene binds to ERα and ERβ with an affinity lower than raloxifene but is less selective for ERα [169]. It inhibits estrogen-mediated proliferation of breast cancer MCF7 cells and increases bone density with little uterine or vasomotor effects in rat models [169]. A Phase III trial with 497 healthy postmenopausal women showed that 6-month bazedoxifene treatment decreases endometrium thickness and uterine bleeding, suggesting antagonistic effects of bazedoxifene in endometrium [170]. Bazedoxifene is currently under review by the Food and Drug Administration (FDA) for the prevention and treatment of postmenopausal osteoporosis. The completed 3-year phase III trial which enrolled 7,492 postmenopausal women with moderate to severe osteoporosis showed bazedoxifene significantly reduced the incidences of vertebral and non-vertebral fracture compared to placebo, while raloxifene was not effective against non-vertebral fracture [171]. No safety concerns related to breast or endometrium were observed, however, a statistical insignificant increase of venous thromboembolic events was observed with groups treated with either bazedoxifene or raloxifene in the same study [172]. Based on studies using rodent models, combination of bazedoxifene and conjugated estrogens exerted positive vasomotor, lipid, and skeletal responses with minimal uterine stimulation [173]. This suggested that pairing SERMs and estrogen might be effective in the treatment of menopausal symptoms and prevention of osteoporosis. However, further studies are needed to examine the effectiveness of bazedoxifene in breast cancer prevention.

4.2.5. Acolbifene (EM-652, SCH57068) and EM-800 (SCH57050)

Acolbifene (EM-652) (**11**) and its orally active prodrug EM-800 (**12**) have a chromene core structure (Fig. **4**). They were initially misclassified as pure antiestrogens and their side chain was depicted by analogy with the pure antiestrogen fulvestrant [174]. However, the structure of acolbifene is actually similar to that of raloxifene, and unlike fulvestrant, the antiestrogenic side chain

of acolbifene does not mask the mutant ER amino acid D351Y to produce an estrogenic action [175]. In addition, acolbifene and EM-800 act as antiestrogens in mammary and uterine tissues, but have estrogenic effects to prevent bone loss and have a favored function in the regulation of lipid metabolism by lowering plasma cholesterol and triglyceride in rodent models [176, 177]. Therefore, acolbifene and EM-800 should be classified as SERMs.

Acolbifene has the highest ER-binding affinity among all known compounds [178]. Preclinical studies indicated that acolbifene and EM-800 were more potent than tamoxifen, idoxifene, raloxifene, GW-5638, toremifene and droloxifene to inhibit the growth of breast cancer cell lines MCF-7, ZR-75-1, MCF-7 and T47D as well as ZR-75-1 xenograft in mice models [179, 180]. Interestingly, acolbifene caused disappearance of 65% ZR-75-1 xenograft in ovariectomized nude mice, while other SERMs tested (tamoxifen, toremifene, raloxifene, droloxifene, idoxifene and GW 5638) only decreased the tumor growth rate stimulated by estrone [180]. Acolbifene was evaluated as a second line therapy for tamoxifen-refractory breast cancers, since it was regarded as a pure anti-estrogen. In a small clinical trial involved 43 postmenopausal or ovariectomized women with breast cancer who had received tamoxifen for over a year but relapsed, the objective response to EM-800 was 12% with 1 complete response and 4 partial responses [181]. In a phase III trial to compare acolbifene with the aromatase inhibitor anastrozole in breast cancer patients who did not respond to tamoxifen, acolbifene did not show superior antitumor activity to anastrozole and the study was halted [182]. However, acolbifene and EM-800 may be more suitable as first line therapy and a phase III trial for untreated metastatic breast cancer patients is planned [182].

Recent studies indicate that acolbifene might be used in combination with other drugs. Acolbifene synergizes with rexinoid LG100268 in the prevention and treatment of mice with ER-negative mammary tumor [155]. It also synergizes with dehydroepiandrosterone (DHEA), which is a naturally produced prohormone for androgen and estrogen, in the prevention of dimethylbenzanthracene (DMBA)-induced mammary tumors in the rats [183]. A phase III trials of acolbifene plus DHEA for vaginal atrophy and uterine safety has been planned.

4.2.6. CHF4227

CHF4227 (**13**) is a SERM with a chromene (benzopyran) core structure, as shown in Fig. (**4**). Compared with raloxifene, CHF4227 binds to ERα and ERβ with higher affinity and inhibits the uterotropic action of 17alpha-ethynyl estradiol with more potency [184]. CHF4227 significantly prevents the development of DMBA-induced mammary tumors in rats [184]. It preserves bone mass without affecting uterine weight and decreases serum cholesterol and fat mass in ovariectomized rats [185]. A recent phase I study showed CHF4227 is well-tolerated, as 28 days of treatment has a positive effect on the serum lipid profile and bone markers without any negative effects on the endometrium or the fibrinolytic system. Additionally, CHF4227 does not cause vaginal bleeding or hot flashes [186]. These results suggest that CHF4227 is safe and worthy of further clinical development for osteoporosis and the chemoprevention of breast cancer.

4.2.7. SP500263

SP500263 (**14**) was discovered in a screen to identify ER agonist in bone cells [187]. It has a chromene core structure and binds to both ERα and ERβ with high affinity similar to raloxifene's (Fig. **4**) [187]. SP500263 inhibits the growth of breast cancer MCF7 cells and xenografts in nude mice, and does not stimulate uterine weight gain in immature rats or ovariectomized adult rats [188, 189]. SP500263 also blocks osteoclastogenesis in human bone cell model [190]. These preclinical results suggest that SP500263 has potential for the treatment of both breast cancer and osteoporosis. However, clinical value of this drug has yet to be determined.

4.3. Steroidal SERMs

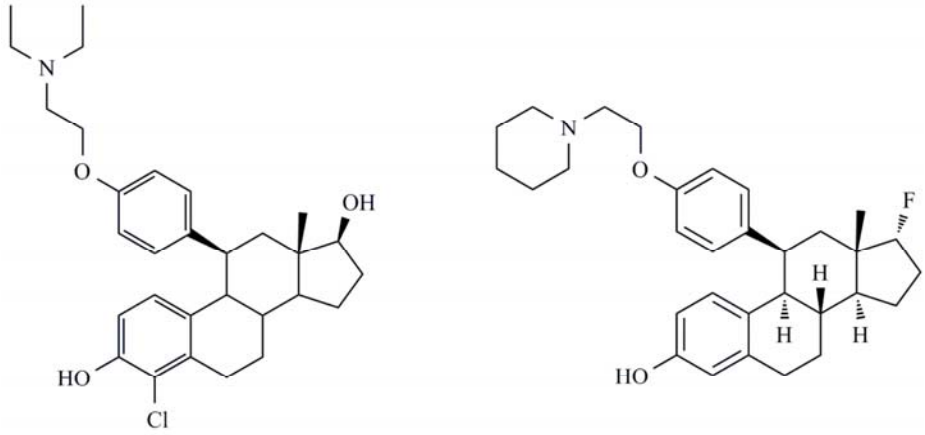
4.3.1. HMR3339

All of the SERMs described to this point are non-steroidal. Recently, steroidal SERMs have been described (Fig. **5**). In rats, HMP3339 (**15**) not only increases bone mineral density but also restores the mechanical strength at multiple sites even after ovariectomy, and it affects both cortical and cancellous bones, while

raloxifene was effective only at cancellous sites [191]. HMR3339 has entered clinical investigation for the prevention of osteoporosis and cardiovascular diseases. In a series of small phase II trials with healthy postmenopausal women, HMR3339 was found to reduce total cholesterol, LDL cholesterol, C-reactive protein (CRP, a pro-inflammatory cytokine and a cardiovascular disease risk factor), asymmetric dimethylarginine (AMDA, a nitric oxide synthase inhibitor) and homocysteine [192-194]. Elevation of AMDA or homocysteine is linked to a high incidence of cardiovascular disease but raloxifene treatment does not reduce the level of either AMDA or homocysteine [192]. HMR3339 reduces concentrations of procarboxypeptidase U (pro-CpU, an inhibitor of fibrinolysis), antithrombin and fibrinogen to a degree similar to raloxifene and shows beneficial effects on some markers of fibrinolysis [195, 196]. Therefore, HMR3339 has potential to prevent cardiovascular diseases and possibly also osteoporosis. However, whether or not there is potential as a cancer preventive has not been determined.

4.3.2. PSK3471

PSK3471 (**16**) is a newly developed SERM with a structure similar to HMR3339 (Fig. **5**). It was reported to prevent gonadectomy-induced bone loss in male and female mice, and antagonize estradiol-stimulated MCF-7 cell proliferation [197].



(**15**) HMR3339 (**16**) PSK3471

Fig. (5). Steroidal SERMs.

4.4. ER Subtype Selective SERMs

ERα and ERβ have a different tissue distribution and have overlapping but distinct biological functions [198]. Unlike ERα, ERβ expression is not routinely examined in the clinic and its function in breast cancer remains unclear. ERβ expression is found in both normal and breast cancer specimens but does not correlate with ERα expression [199]. It seems that ERβ functions differently if it is expressed alone or co-expressed with ERα in breast cancers. In ERα-positive breast tumors, ERβ often antagonizes the pro-proliferation actions of ERα [200, 201] and its expression is associated with better response to endocrine therapy and a favorable clinical outcome in most cases [202]. Thus ERβ seems to function as a tumor suppressor. However in ERα-negative breast tumors, several studies indicated that the expression of ERβ correlates with proliferation markers such as Ki67 and cyclin A [202, 203], which suggested that ERβ might stimulate cancer growth. In the latter situation, ERβ could serve as an endocrine therapy target in those patients who would otherwise be regarded as ER-negative and have limited choice but chemotherapy. The presence of ERβ in ERα-negative breast cancers may partly explain why some "ER-negative" patients respond to SERMs. The reason that ERβ functions differently in the absence or presence of ERα might be due to the different activities between the ERα/β heterodimer and ERα or ERβ homodimers.

A new direction to consider is the estrogen related receptor (ERR) [204, 205]. There is emerging evidence that ERRα is critical for the growth of ER-negative breast-cancer MDA-MD-231 xenografts in mice [206], as ERRα appears to be involved in angiogenesis by inducing the expression of vascular endothelial growth factor (VEGF) [207, 208]. Novel therapeutic agents targeted to ERRα would be valuable to treat breast cancer.

Several ER-subtype selective SERMs have been reported, although it is difficult to design subtype selective ligands given the fact that only two amino acids are different in the ligand binding pocket between ERα and ERβ (despite that they have 61% amino acid identity in LBD). All the SERMs discussed previously were designed against ERα and have low subtype selection in terms of binding affinity. In contrast to the focus on ERα and breast cancer, most of ER subtype selective SERMs are developed for diseases other than breast cancer. In animal models,

ERβ-selective agonists ERB041 and diaryl-propionitrile (DPN) have been shown to have anti-inflammatory properties and antidepressant-like effects, respectively [209, 210]. An ERβ agonist, 8-vinylestra-1,3,5 (10)-triene-3,17β-diol, stimulates ovarian follicular development in hypophysectomized rats and gonadotropin-releasing hormone a tagonist-treated mice [211], thus this drug could be used to enhance fertility [198]. A few ERβ agonists are being developed for clinical applications in Alzheimer's disease and rheumatoid arthritis [212]. For breast cancer prevention and treatment, it is conceivable that ERβ agonist might have potential for ERα-and-β-positive tumors, especially in combination of an ERα selective antagonist, since the preclinical studies indicate a protective role of ERβ. However, this strategy poses a difficult pharmacologic issue of tissue pharmacodynamics. Nevertheless, a couple of ERβ modulators have been shown with positive effects to treat advanced postmenopausal breast cancer, which will be discussed below.

4.4.1. Discovery of New Properties of SERMs

The original discovery of SERMs provided both opportunities and challenges. The challenge is the control of hot flashes as this is a quality of life issue. No matter how good a medicine is at preventing a few spontaneous breast cancers in a large population, or controlling the increase in fractures noted with osteoporosis, if a woman does not accept the side effects of persistent hot flashes and stops taking the medicine- there is no advance. Numerous pharmaceutical companies are addressing the issue of hot flush control to create a more acceptable SERM.

Dainippon pharmaceuticals has employed spiro[indene-1,1'-indane]-5,5'-diol as a SERM core to design new SERM with all the traditional SERM effects on preserving bone, reducing circulating cholesterol with minimum effects on the breast and endometrium but preventing hot flashes. This is done assessing ovariectomized rat tail skin temperature [213]. They report a useful lead compound (Fig. **6**) that merits further investigation.

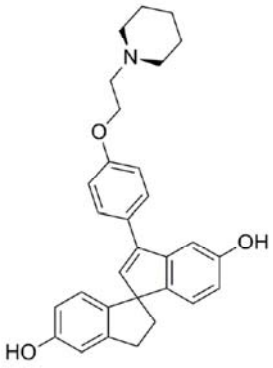
Eli Lilly has studied the structure activity relationships of oxachrysene derivative (Fig. **6**) to find a compound that binds to ERα and β, blocks the growth of MCF-7 breast cancer cells, Ishikawa endometrial cancer cells and is a powerful antagonist

of estrogen-stimulated increases in rat uterine weight. In ovariectomized rats, bone density is maintained and there is a decrease in circulating cholesterol levels. Most importantly the compound was able to control temperature in a morphine dependent rat model [214].

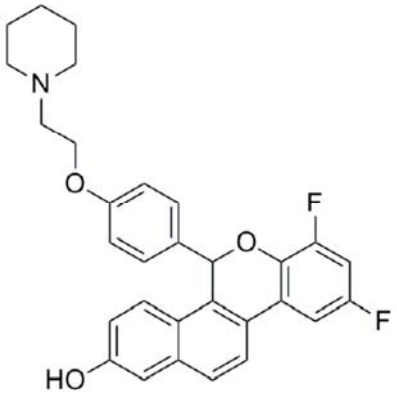
Johnson and Johnson has a long standing program to address the issue of the control of hot flashes in novel SERMs. They originally reported that a chromene derived bisbenzopyran (Fig. **6**) had all the appropriate characteristics of a SERM plus alleviation of hot flashes (changes in the tail skin temperature during morphine withdrawal in the rat) and the alleviation of vaginal dryness [215]. Subsequently, the same group reported improved biology for a chromene derived selective ER modulator (Fig. **6**) [216] as an improved "back-up" to their original phase 2 development of the chromene based molecule.

Finally two additional innovations deserve mention. Eli Lilly has described a SERM targeted to premenopausal women for the treatment of leiomyomas [217]. Their compounds are naphthalene derivatives but their compound of interest LY2066948 (Fig. **7**) contains a methyl sulphone moiety that permits potent anti-uterotrophic activity but ensures the compound does not cross the blood brain barrier. They demonstrate that substitution of the methyl sulphone for fluorine (Fig. **7**) that passage of the blood brain barrier occurs resulting in 25 fold higher circulating estrogen. Thus LY2066948 could be a useful agent to stop the growth benign leiomyomas in premenopausal women.

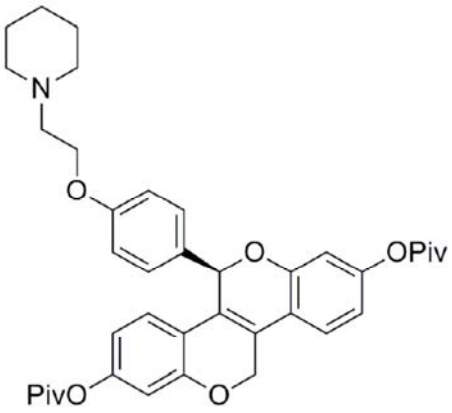
Alzheimer's disease is a devastating neurodegenerative condition affecting 4.5 million people in the United States. Estrogen is known to stop the degeneration of neurons but current preparations have additional toxicological issue. Interestingly, there was a chance finding that the pure antiestrogen fulvestrant can mimic estrogen to stop neuronal degeneration [218] (Fig. **8**).



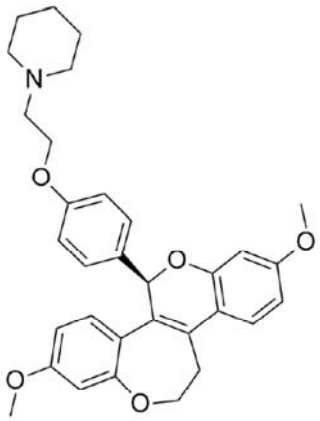
(**17**) Dainippon (+ enantiomer)



(**18**) Eli Lilly ((R) –(+) enantiomer)



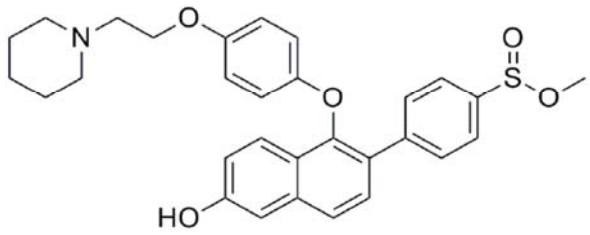
(**19**) Johnson and Johnson
(R enantiomer; original clinical candidate)



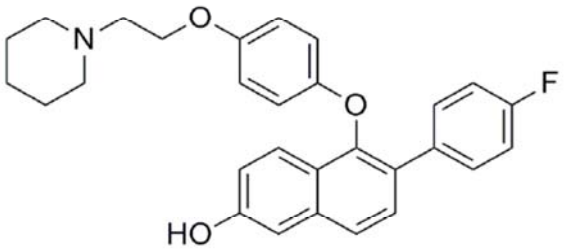
(**20**) Johnson and Johnson
(R enantiomer; back up)

Fig. (6). Ligands developed by various pharmaceutical companies for the treatment of 'hot flashes' in postmenopausal women.

Unfortunately, fulvestrant does not cross the blood brain barrier. A swap of the similar side chain of Vitamin E to the 7α position of fulvestrant demonstrates that it may be possible to create a "brain specific" estrogen [219] (Fig. **8**).



Eli Lilly (LY 2066948)



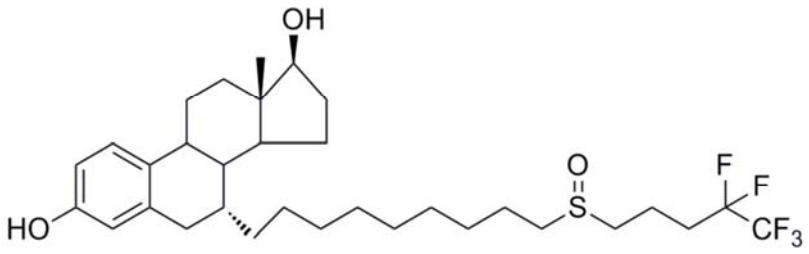
Eli Lilly (crosses blood brain barrier)

Fig (7). Chemical structure of SERM (LY 2066948) which could be used to treat leiyomyomas in premenopausal women. The molecule does not cross the blood brain barrier because of the methyl sulphone group. Exchange for fluorine allows passage of the blood brain barrier.

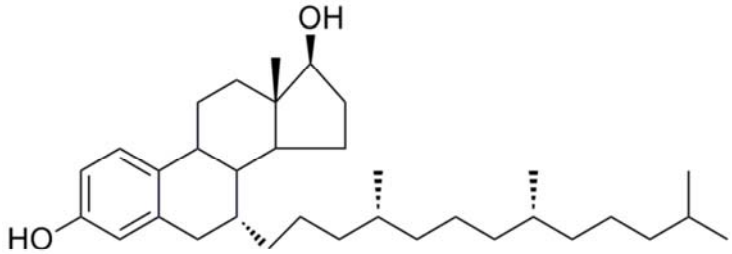
5. CONCLUDING REMARKS

Endocrine therapy targeting to ERα has been very successful in the treatment and prevention of breast cancer [220, 221]. It is very effective and less toxic compared to combinational cytotoxic chemotherapy that was the only option 30 years ago. In the ensuing period, multiple strategies have been developed to antagonize estrogen action. Most experience has accumulated with the competitive inhibitor of estrogen action tamoxifen, but targeting aromatase to deplete estrogen with AIs in postmenopausal patients or to induce ER degradation with SERDs have been valuable innovations in therapies. The goal for treatment is to create a "no-estrogen environment". However, SERMs that maintain the beneficial effects of estrogen but antagonize the harmful effects of estrogen have great potential in the

prevention of multiple diseases in common. It is clear that many new SERMs are being developed that could provide better choices for patients in the future.



(**21**) Fulvestrant



(**22**) Hybrid of 17β-estradiol and Vitamin E

Fig (8). Chemical structures of fulvestrant, and the hybrid molecule of 17β-estradiol and vitamin E which is capable of crossing the blood brain barrier and can function as a brain specific estrogen.

To overcome the unwanted side effects and problems with drug resistance, combination therapy might be another important direction in addition to the development of new SERMs. For example, combination of SERM acolbifene and DHEA could be protective against breast cancer and osteoporosis with beneficial effects to stimulate vaginal maturation and decrease skin dryness [182]. As traditional HRT is less acceptable to regulatory authorities because of the increased risk of breast cancer, a combination of HRT and a SERM may be a reasonable idea to relieve unpleasant menopausal effects while decrease breast cancer risks. With regards to avoiding drug resistance, combining a SERM and an inhibitor targeting significant survival signal transduction pathway is under active evaluation. By way of example, a combination of tamoxifen and inhibitors of the HER2 signal transduction pathway may prevent acquired tamoxifen resistance

[222]. Similarly, SERM pipendoxifene and mTOR inhibitor temsirolimus synergistically inhibits the proliferation of MCF7 breast cancer cells and xenograft at suboptimal concentrations [168]. Additionally, combinations of a SERM (arzoxifene or acolbifene) and a rexinoid LG100268 are effective to prevent and treat ER-negative mammary tumors in animal models [155]. The potential combination seems endless but the marriage of molecular biology and medicine holds great promise for advances in targeted therapeutics based on the SERM model.

In summary, it is clear that the original idea of targeting specific hormone receptor with selective medicine has proven its worth by advancing medicine with the SERMs tamoxifen and raloxifene. Now there are a whole range of new SERMs poised for clinical applications. But this is not the end of the story. Novel selective modulators of all members of the nuclear receptor superfamily are under investigation addressing the treatment or prevention of diseases never before considered possible [57, 221].

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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DISCLOSURE

This e-Book chapter is an updated version of the review article entitled "Potential of Selective Estrogen Receptor Modulators as Treatments and Preventives of Breast Cancer" published in *Anti-Cancer Agents in Medicinal Chemistry*, **2009**, *9*(5), 481-499.

REFERENCES

[1] Berry DA.; Cronin KA.; Plevritis SK.; Fryback DG.; Clarke L.; Zelen M.; Mandelblatt JS.; Yakovlev AY.; Habbema JD.; Feuer EJ. Effect of screening and adjuvant therapy on mortality from breast cancer. *N Engl J Med*. **2005**, 353(17):1784-92.

[2] Ravdin PM.; Cronin KA.; Howlader N.; Berg CD.; Chlebowski RT.; Feuer EJ.; Edwards BK.; Berry DA. The Decrease in Breast-Cancer Incidence in 2003 in the United States. *N Engl J Med*. **2007**, 356(16):1670-4.

[3] Writing Group for the Women's Health Initiative I. Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women: Principal Results From the Women's Health Initiative Randomized Controlled Trial. *JAMA*. **2002**, 288(3):321-33.

[4] The Women's Health Initiative Steering C. Effects of Conjugated Equine Estrogen in Postmenopausal Women With Hysterectomy: The Women's Health Initiative Randomized Controlled Trial. *JAMA*. **2004**, 291(14):1701-12.

[5] Beral V. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet*. **2003**, 362(9382):419-27.

[6] Beral V.; Bull D.; Reeves G. Endometrial cancer and hormone-replacement therapy in the Million Women Study. *Lancet*. **2005**, 365(9470):1543-51.

[7] Beral V.; Bull D.; Green J.; Reeves G. Ovarian cancer and hormone replacement therapy in the Million Women Study. *Lancet*. **2007**, 369(9574):1703-10.

[8] Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov*. **2003**, 2(3):205-13.

[9] Veronesi U.; Boyle P.; Goldhirsch A.; Orecchia R.; Viale G. Breast cancer. *Lancet*. **2005**, 365(9472):1727-41.

[10] Cummings SR.; Eckert S.; Krueger KA.; Grady D.; Powles TJ.; Cauley JA.; Norton L.; Nickelsen T.; Bjarnason NH.; Morrow M.; Lippman ME.; Black D.; Glusman JE.; Costa A.; Jordan VC. The Effect of Raloxifene on Risk of Breast Cancer in Postmenopausal Women: Results From the MORE Randomized Trial. *JAMA*. **1999**, 281(23):2189-97.

[11] DeMichele A.; Troxel AB.; Berlin JA.; Weber AL.; Bunin GR.; Turzo E.; Schinnar R.; Burgh D.; Berlin M.; Rubin SC.; Rebbeck TR.; Strom BL. Impact of Raloxifene or Tamoxifen Use on Endometrial Cancer Risk: A Population-Based Case-Control Study. *J Clin Oncol*. **2008**, 26(25):4151-9.

[12] Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell*. **2004**, 5(3):207-13.

[13] Jordan VC.; Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids*. **2007**, 72(1):7-25.

[14] Perez EA. Safety profiles of tamoxifen and the aromatase inhibitors in adjuvant therapy of hormone-responsive early breast cancer. *Ann Oncol*. **2007**, 18(suppl_8):viii26-35.

[15] Chia S.; Gradishar W.; Mauriac L.; Bines J.; Amant F.; Federico M.; Fein L.; Romieu G.; Buzdar A.; Robertson JF.; Brufsky A.; Possinger K.; Rennie P.; Sapunar F.; Lowe E.; Piccart M. Double-blind, randomized placebo controlled trial of fulvestrant compared with exemestane after prior nonsteroidal aromatase inhibitor therapy in postmenopausal women with hormone receptor-positive, advanced breast cancer: results from EFECT. *J Clin Oncol*. **2008**, 26(10):1664-70.

[16] Jensen EV.; Jacobson HI. Basic guides to the mechanism of estrogen action. *Recent Prog Horm Res*. **1962**, 18:387-414.

[17] Green S.; Walter P.; Kumar V.; Krust A.; Bornert JM.; Argos P.; Chambon P. Human oestrogen receptor cDNA: sequence, expression and homology to v- erb-A. *Nature*. **1986**, 320(6058):134-9.

[18] Greene GL.; Gilna P.; Waterfield M.; Baker A.; Hort Y.; Shine J. Sequence and expression of human estrogen receptor complementary DNA. *Science*. **1986**, 231(4742):1150-4.

[19] Kuiper GG.; Enmark E.; Pelto-Huikko M.; Nilsson S.; Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A*. **1996**, 93(12):5925-30.

[20] Tsai MJ.; O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem*. **1994**, 63:451-86.

[21] MacGregor JI.; Jordan VC. Basic guide to the mechanisms of antiestrogen action. *Pharmacol Rev*. **1998**, 50(2):151-96.

[22] Brzozowski AM.; Pike AC.; Dauter Z.; Hubbard RE.; Bonn T.; Engstrom O.; Ohman L.; Greene GL.; Gustafsson JA.; Carlquist M. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*. **1997**, 389(6652):753-8.

[23] Pike AC.; Brzozowski AM.; Hubbard RE.; Bonn T.; Thorsell AG.; Engstrom O.; Ljunggren J.; Gustafsson JA.; Carlquist M. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *Embo J*. **1999**, 18(17):4608-18.

[24] Bramlett KS.; Wu Y.; Burris TP. Ligands specify coactivator nuclear receptor (NR) box affinity for estrogen receptor subtypes. *Mol Endocrinol*. **2001**, 15(6):909-22.

[25] Wong CW.; Komm B.; Cheskis BJ. Structure-function evaluation of ER alpha and beta interplay with SRC family coactivators. ER selective ligands. *Biochemistry*. **2001**, 40(23):6756-65.

[26] Warnmark A.; Almlof T.; Leers J.; Gustafsson JA.; Treuter E. Differential recruitment of the mammalian mediator subunit TRAP220 by estrogen receptors ERalpha and ERbeta. *J Biol Chem*. **2001**, 276(26):23397-404.

[27] Hall JM.; McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology*. **1999**, 140(12):5566-78.

[28] Paech K.; Webb P.; Kuiper GG.; Nilsson S.; Gustafsson J.; Kushner PJ.; Scanlan TS. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites [see comments]. *Science*. **1997**, 277(5331):1508-10.

[29] Bjornstrom L.; Sjoberg M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol Endocrinol*. **2005**, 19(4):833-42.

[30] Manavathi B.; Kumar R. Steering estrogen signals from the plasma membrane to the nucleus: two sides of the coin. *J Cell Physiol.* **2006**, 207(3):594-604.
[31] Filardo EJ.; Quinn JA.; Frackelton AR, Jr..; Bland KI. Estrogen action *via* the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endocrinol.* **2002**, 16(1):70-84.
[32] Revankar CM.; Cimino DF.; Sklar LA.; Arterburn JB.; Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science.* **2005**, 307(5715):1625-30.
[33] Funakoshi T.; Yanai A.; Shinoda K.; Kawano MM.; Mizukami Y. G protein-coupled receptor 30 is an estrogen receptor in the plasma membrane. *Biochem Biophys Res Commun*. **2006**, 346(3):904-10.
[34] Thomas P.; Pang Y.; Filardo EJ.; Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology.* **2005**, 146(2):624-32.
[35] Filardo EJ.; Quinn JA.; Sabo E. Association of the membrane estrogen receptor, GPR30, with breast tumor metastasis and transactivation of the epidermal growth factor receptor. *Steroids.* **2008**, 73(9-10):870-3.
[36] Lonard DM.; O'Malley BW. The expanding cosmos of nuclear receptor coactivators. *Cell*. **2006**, 125(3):411-4.
[37] Shang Y.; Brown M. Molecular Determinants for the Tissue Specificity of SERMs. *Science*. **2002**, 295(5564):2465-8.
[38] Gottardis MM.; Robinson SP.; Satyaswaroop PG.; Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res.* **1988**, 48(4):812-5.
[39] Gottardis MM.; Ricchio ME.; Satyaswaroop PG.; Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. *Cancer Res.* **1990**, 50(11):3189-92.
[40] Vogel VG.; Costantino JP.; Wickerham DL.; Cronin WM.; Cecchini RS.; Atkins JN.; Bevers TB.; Fehrenbacher L.; Pajon ER, Jr..; Wade JL, 3rd.; Robidoux A.; Margolese RG.; James J.; Lippman SM.; Runowicz CD.; Ganz PA.; Reis SE.; McCaskill-Stevens W.; Ford LG.; Jordan VC.; Wolmark N. Effects of tamoxifen *vs* raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *Jama*. **2006**, 295(23):2727-41.
[41] Vogel VG.; Costantino JP.; Wickerham DL.; Cronin WM.; Cecchini RS.; Atkins JN.; Bevers TB.; Fehrenbacher L.; Pajon ER.; Wade JL, 3rd.; Robidoux A.; Margolese RG.; James J.; Runowicz CD.; Ganz PA.; Reis SE.; McCaskill-Stevens W.; Ford LG.; Jordan VC.; Wolmark N. Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. *Cancer Prev Res (Phila).* **2010**, 3(6):696-706.
[42] Lonard DM.; Tsai SY.; O'Malley BW. Selective estrogen receptor modulators 4-hydroxytamoxifen and raloxifene impact the stability and function of SRC-1 and SRC-3 coactivator proteins. *Mol Cell Biol*. **2004**, 24(1):14-24.
[43] McKenna NJ.; Lanz RB.; O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev*. **1999**, 20(3):321-44.
[44] Shang Y.; Hu X.; DiRenzo J.; Lazar MA.; Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell*. **2000**, 103(6):843-52.

[45] Smith CL.; Nawaz Z.; O'Malley BW. Coactivator and Corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. *Mol Endocrinol*. **1997**, 11:657-66.

[46] Lavinsky RM.; Jepsen K.; Heinzel T.; Torchia J.; Mullen TM.; Schiff R.; Del-Rio AL.; Ricote M.; Ngo S.; Gemsch J.; Hilsenbeck SG.; Osborne CK.; Glass CK.; Rosenfeld MG.; Rose DW. Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc Natl Acad Sci U S A.* **1998**, 95(6):2920-5.

[47] Jepsen K.; Hermanson O.; Onami TM.; Gleiberman AS.; Lunyak V.; McEvilly RJ.; Kurokawa R.; Kumar V.; Liu F.; Seto E.; Hedrick SM.; Mandel G.; Glass CK.; Rose DW.; Rosenfeld MG. Combinatorial roles of the nuclear receptor corepressor in transcription and development. *Cell*. **2000**, 102(6):753-63.

[48] Keeton EK.; Brown M. Cell cycle progression stimulated by tamoxifen-bound estrogen receptor-alpha and promoter-specific effects in breast cancer cells deficient in N-CoR and SMRT. *Mol Endocrinol*. **2005**, 19(6):1543-54.

[49] Mazumdar A.; Wang RA.; Mishra SK.; Adam L.; Bagheri-Yarmand R.; Mandal M.; Vadlamudi RK.; Kumar R. Transcriptional repression of oestrogen receptor by metastasis-associated protein 1 corepressor. *Nat Cell Biol.* **2001**, 3(1):30-7.

[50] Montano MM.; Ekena K.; Delage-Mourroux R.; Chang W.; Martini P.; Katzenellenbogen BS. An estrogen receptor-selective coregulator that potentiates the effectiveness of antiestrogens and represses the activity of estrogens. *Proc Natl Acad Sci U S A.* **1999**, 96(12):6947-52.

[51] Delage-Mourroux R.; Martini PG.; Choi I.; Kraichely DM.; Hoeksema J.; Katzenellenbogen BS. Analysis of estrogen receptor interaction with a repressor of estrogen receptor activity (REA) and the regulation of estrogen receptor transcriptional activity by REA. *J Biol Chem*. **2000**, 275(46):35848-56.

[52] Zhang J.; Guenther MG.; Carthew RW.; Lazar MA. Proteasomal regulation of nuclear receptor corepressor-mediated repression. *Genes Dev*. **1998**, 12(12):1775-80.

[53] Frasor J.; Danes JM.; Funk CC.; Katzenellenbogen BS. Estrogen down-regulation of the corepressor N-CoR: mechanism and implications for estrogen derepression of N-CoR-regulated genes. *Proc Natl Acad Sci U S A*. **2005**, 102(37):13153-7.

[54] Spencer TE.; Jenster G.; Burcin MM.; Allis CD.; Zhou J.; Mizzen CA.; McKenna NJ.; Onate SA.; Tsai SY.; Tsai MJ.; O'Malley BW. Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature*. **1997**, 389(6647):194-8.

[55] Liu XF.; Bagchi MK. Recruitment of distinct chromatin-modifying complexes by tamoxifen-complexed estrogen receptor at natural target gene promoters *in vivo*. *J Biol Chem*. **2004**, 279(15):15050-8.

[56] Feng Q.; Yi P.; Wong J.; O'Malley BW. Signaling within a coactivator complex: methylation of SRC-3/AIB1 is a molecular switch for complex disassembly. *Mol Cell Biol*. **2006**, 26(21):7846-57.

[57] Jordan VC.; O'Malley BW. Selective Estrogen-Receptor Modulators and Antihormonal Resistance in Breast Cancer. *J Clin Oncol.* **2007**, 25(36):5815-24.

[58] Jin Y.; Desta Z.; Stearns V.; Ward B.; Ho H.; Lee KH.; Skaar T.; Storniolo AM.; Li L.; Araba A.; Blanchard R.; Nguyen A.; Ullmer L.; Hayden J.; Lemler S.; Weinshilboum RM.; Rae JM.; Hayes DF.; Flockhart DA. CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. *J Natl Cancer Inst*. **2005**, 97(1):30-9.

[59] Weinshilboum R. Pharmacogenomics of endocrine therapy in breast cancer. *Adv Exp Med Biol*. **2008**, 630:220-31.

[60] Flockhart D. CYP2D6 genotyping and the pharmacogenetics of tamoxifen. *Clin Adv Hematol Oncol*. **2008**, 6(7):493-4.

[61] Borges S.; Desta Z.; Li L.; Skaar TC.; Ward BA.; Nguyen A.; Jin Y.; Storniolo AM.; Nikoloff DM.; Wu L.; Hillman G.; Hayes DF.; Stearns V.; Flockhart DA. Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. *Clin Pharmacol Ther*. **2006**, 80(1):61-74.

[62] Harvell DM.; Richer JK.; Singh M.; Spoelstra N.; Finlayson C.; Borges VF.; Elias AD.; Horwitz KB. Estrogen regulated gene expression in response to neoadjuvant endocrine therapy of breast cancers: tamoxifen agonist effects dominate in the presence of an aromatase inhibitor. *Breast Cancer Res Treat.* **2008**.

[63] Arpino G.; Weiss H.; Lee AV.; Schiff R.; De Placido S.; Osborne CK.; Elledge RM. Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance. *J Natl Cancer Inst.* **2005**, 97(17):1254-61.

[64] Kim H-J.; Cui X.; Hilsenbeck SG.; Lee AV. Progesterone Receptor Loss Correlates with Human Epidermal Growth Factor Receptor 2 Overexpression in Estrogen Receptor-Positive Breast Cancer. *Clin Cancer Res.* **2006**, 12(3):1013s-8.

[65] Osborne CK.; Schiff R.; Arpino G.; Lee AS.; Hilsenbeck VG. Endocrine responsiveness: understanding how progesterone receptor can be used to select endocrine therapy. *Breast.* **2005**, 14(6):458-65.

[66] Huang HJ.; Neven P.; Drijkoningen M.; Paridaens R.; Wildiers H.; Van Limbergen E.; Berteloot P.; Amant F.; Christiaens MR.; Vergote I. Association between HER-2/neu and the progesterone receptor in oestrogen-dependent breast cancer is age-related. *Breast Cancer Res Treat.* **2005**, 91(1):81-7.

[67] Jordan VC.; Gelber RD. Problems With the Progesterone Receptor in Practice? *J Clin Oncol.* **2007**, 25(15):1957-9.

[68] Arpino G.; Wiechmann L.; Osborne CK.; Schiff R. Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. *Endocr Rev.* **2008**, 29(2):217-33.

[69] Slamon DJ.; Clark GM.; Wong SG.; Levin WJ.; Ullrich A.; McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. **1987**, 235(4785):177-82.

[70] Konecny G.; Pauletti G.; Pegram M.; Untch M.; Dandekar S.; Aguilar Z.; Wilson C.; Rong HM.; Bauerfeind I.; Felber M.; Wang HJ.; Beryt M.; Seshadri R.; Hepp H.; Slamon DJ. Quantitative association between HER-2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. *J Natl Cancer Inst.* **2003**, 95(2):142-53.

[71] De Laurentiis M.; Arpino G.; Massarelli E.; Ruggiero A.; Carlomagno C.; Ciardiello F.; Tortora G.; D'Agostino D.; Caputo F.; Cancello G.; Montagna E.; Malorni L.; Zinno L.; Lauria R.; Bianco AR.; De Placido S. A meta-analysis on the interaction between HER-2 expression and response to endocrine treatment in advanced breast cancer. *Clin Cancer Res*. **2005**, 11(13):4741-8.

[72] Tsutsui S.; Ohno S.; Murakami S.; Hachitanda Y.; Oda S. Prognostic value of epidermal growth factor receptor (EGFR) and its relationship to the estrogen receptor status in 1029 patients with breast cancer. *Breast Cancer Res Treat.* **2002**, 71(1):67-75.

[73] Likhite VS.; Stossi F.; Kim K.; Katzenellenbogen BS.; Katzenellenbogen JA. Kinase-Specific Phosphorylation of the Estrogen Receptor Changes Receptor Interactions with Ligand, Deoxyribonucleic Acid, and Coregulators Associated with Alterations in Estrogen and Tamoxifen Activity. *Mol Endocrinol*. **2006**, 20(12):3120-32.

[74] Bunone G.; Briand PA.; Miksicek RJ.; Picard D. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *Embo J*. **1996**, 15(9):2174-83.

[75] Campbell RA.; Bhat-Nakshatri P.; Patel NM.; Constantinidou D.; Ali S.; Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. *J Biol Chem*. **2001**, 276(13):9817-24.

[76] Kato S.; Endoh H.; Masuhiro Y.; Kitamoto T.; Uchiyama S.; Sasaki H.; Masushige S.; Gotoh Y.; Nishida E.; Kawashima H.; *et al*. Activation of the estrogen receptor through phosphorylation by mitogen- activated protein kinase. *Science*. **1995**, 270(5241):1491-4.

[77] Zwart W.; Griekspoor A.; Berno V.; Lakeman K.; Jalink K.; Mancini M.; Neefjes J.; Michalides R. PKA-induced resistance to tamoxifen is associated with an altered orientation of ERalpha towards co-activator SRC-1. *Embo J*. **2007**, 26(15):3534-44.

[78] Font de Mora J.; Brown M. AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Mol Cell Biol*. **2000**, 20(14):5041-7.

[79] Lopez GN.; Turck CW.; Schaufele F.; Stallcup MR.; Kushner PJ. Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. *J Biol Chem*. **2001**, 276(25):22177-82.

[80] Wu RC.; Qin J.; Hashimoto Y.; Wong J.; Xu J.; Tsai SY.; Tsai MJ.; O'Malley BW. Regulation of SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) Coactivator activity by I kappa B kinase. *Mol Cell Biol*. **2002**, 22(10):3549-61.

[81] Hong SH.; Privalsky ML. The SMRT corepressor is regulated by a MEK-1 kinase pathway: inhibition of corepressor function is associated with SMRT phosphorylation and nuclear export. *Mol Cell Biol*. **2000**, 20(17):6612-25.

[82] Schiff R.; Massarweh SA.; Shou J.; Bharwani L.; Mohsin SK.; Osborne CK. Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. *Clin Cancer Res*. **2004**, 10(1 Pt 2):331S-6S.

[83] Shou J.; Massarweh S.; Osborne CK.; Wakeling AE.; Ali S.; Weiss H.; Schiff R. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J Natl Cancer Inst*. **2004**, 96(12):926-35.

[84] Nicholson RI.; Hutcheson IR.; Knowlden JM.; Jones HE.; Harper ME.; Jordan N.; Hiscox SE.; Barrow D.; Gee JMW. Nonendocrine Pathways and Endocrine Resistance: Observations with Antiestrogens and Signal Transduction Inhibitors in Combination. *Clin Cancer Res*. **2004**, 10(1):346S-54.

[85] Creighton CJ.; Casa A.; Lazard Z.; Huang S.; Tsimelzon A.; Hilsenbeck SG.; Osborne CK.; Lee AV. Insulin-like growth factor-I activates gene transcription programs strongly associated with poor breast cancer prognosis. *J Clin Oncol*. **2008**, 26(25):4078-85.

[86] Lee AV.; Weng CN.; Jackson JG.; Yee D. Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. *J Endocrinol*. **1997**, 152(1):39-47.

[87] Dihge L.; Bendahl PO.; Grabau D.; Isola J.; Lovgren K.; Ryden L.; Ferno M. Epidermal growth factor receptor (EGFR) and the estrogen receptor modulator amplified in breast cancer (AIB1) for predicting clinical outcome after adjuvant tamoxifen in breast cancer. *Breast Cancer Res Treat*. **2008**, 109(2):255-62.

[88] Kirkegaard T.; McGlynn LM.; Campbell FM.; Muller S.; Tovey SM.; Dunne B.; Nielsen KV.; Cooke TG.; Bartlett JM. Amplified in breast cancer 1 in human epidermal growth factor receptor - positive tumors of tamoxifen-treated breast cancer patients. *Clin Cancer Res*. **2007**, 13(5):1405-11.

[89] Osborne CK.; Bardou V.; Hopp TA.; Chamness GC.; Hilsenbeck SG.; Fuqua SA.; Wong J.; Allred DC.; Clark GM.; Schiff R. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst.* **2003**, 95(5):353-61.

[90] Girault I.; Lerebours F.; Amarir S.; Tozlu S.; Tubiana-Hulin M.; Lidereau R.; Bieche I. Expression Analysis of Estrogen Receptor {alpha} Coregulators in Breast Carcinoma: Evidence That NCOR1 Expression Is Predictive of the Response to Tamoxifen. *Clin Cancer Res*. **2003**, 9(4):1259-66.

[91] Wang LH.; Yang XY.; Zhang X.; An P.; Kim HJ.; Huang J.; Clarke R.; Osborne CK.; Inman JK.; Appella E.; Farrar WL. Disruption of estrogen receptor DNA-binding domain and related intramolecular communication restores tamoxifen sensitivity in resistant breast cancer. *Cancer Cell*. **2006**, 10(6):487-99.

[92] Iorio MV.; Ferracin M.; Liu CG.; Veronese A.; Spizzo R.; Sabbioni S.; Magri E.; Pedriali M.; Fabbri M.; Campiglio M.; Menard S.; Palazzo JP.; Rosenberg A.; Musiani P.; Volinia S.; Nenci I.; Calin GA.; Querzoli P.; Negrini M.; Croce CM. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res*. **2005**, 65(16):7065-70.

[93] Lu J.; Getz G.; Miska EA.; Alvarez-Saavedra E.; Lamb J.; Peck D.; Sweet-Cordero A.; Ebert BL.; Mak RH.; Ferrando AA.; Downing JR.; Jacks T.; Horvitz HR.; Golub TR. MicroRNA expression profiles classify human cancers. *Nature*. **2005**, 435(7043):834-8.

[94] Miller TE.; Ghoshal K.; Ramaswamy B.; Roy S.; Datta J.; Shapiro CL.; Jacob S.; Majumder S. MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27(Kip1). *J Biol Chem*. **2008**:M804612200.

[95] Zhao J-J.; Lin J.; Yang H.; Kong W.; He L.; Ma X.; Coppola D.; Cheng JQ. MicroRNA-221/222 negatively regulates ERalpha and associates with tamoxifen resistance in breast cancer. *J Biol Chem*. **2008**:M806041200.

[96] Gottardis MM.; Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res*. **1988**, 48(18):5183-7.

[97] Osborne CK.; Pippen J.; Jones SE.; Parker LM.; Ellis M.; Come S.; Gertler SZ.; May JT.; Burton G.; Dimery I.; Webster A.; Morris C.; Elledge R.; Buzdar A. Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant *versus* anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. *J Clin Oncol*. **2002**, 20(16):3386-95.

[98] Howell A.; Robertson JF.; Quaresma Albano J.; Aschermannova A.; Mauriac L.; Kleeberg UR.; Vergote I.; Erikstein B.; Webster A.; Morris C. Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. *J Clin Oncol.* **2002**, 20(16):3396-403.

[99] Johnston SR.; Saccani-Jotti G.; Smith IE.; Salter J.; Newby J.; Coppen M.; Ebbs SR.; Dowsett M. Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. *Cancer Res*. **1995**, 55(15):3331-8.

[100] Gutierrez MC.; Detre S.; Johnston S.; Mohsin SK.; Shou J.; Allred DC.; Schiff R.; Osborne CK.; Dowsett M. Molecular changes in tamoxifen-resistant breast cancer: relationship

between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. *J Clin Oncol*. **2005**, 23(11):2469-76.

[101] Normanno N.; Di Maio M.; De Maio E.; De Luca A.; de Matteis A.; Giordano A.; Perrone F. Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. *Endocr Relat Cancer*. **2005**, 12(4):721-47.

[102] deGraffenried LA.; Friedrichs WE.; Russell DH.; Donzis EJ.; Middleton AK.; Silva JM.; Roth RA.; Hidalgo M. Inhibition of mTOR activity restores tamoxifen response in breast cancer cells with aberrant Akt Activity. *Clin Cancer Res*. **2004**, 10(23):8059-67.

[103] Chu I.; Sun J.; Arnaout A.; Kahn H.; Hanna W.; Narod S.; Sun P.; Tan CK.; Hengst L.; Slingerland J. p27 phosphorylation by Src regulates inhibition of cyclin E-Cdk2. *Cell*. **2007**, 128(2):281-94.

[104] Abukhdeir AM.; Vitolo MI.; Argani P.; De Marzo AM.; Karakas B.; Konishi H.; Gustin JP.; Lauring J.; Garay JP.; Pendleton C.; Konishi Y.; Blair BG.; Brenner K.; Garrett-Mayer E.; Carraway H.; Bachman KE.; Park BH. Tamoxifen-stimulated growth of breast cancer due to p21 loss. *Proceedings of the National Academy of Sciences*. **2008**:0710887105.

[105] Yuan Y.; Qin L.; Liu D.; Wu R-C.; Mussi P.; Zhou S.; Songyang Z.; Xu J. Genetic Screening Reveals an Essential Role of p27kip1 in Restriction of Breast Cancer Progression. *Cancer Res*. **2007**, 67(17):8032-42.

[106] Iorns E.; Turner NC.; Elliott R.; Syed N.; Garrone O.; Gasco M.; Tutt ANJ.; Crook T.; Lord CJ.; Ashworth A. Identification of CDK10 as an Important Determinant of Resistance to Endocrine Therapy for Breast Cancer. *Cancer Cell*. **2008**, 13(2):91-104.

[107] Moriai R.; Tsuji N.; Moriai M.; Kobayashi D.; Watanabe N. Survivin plays as a resistant factor against tamoxifen-induced apoptosis in human breast cancer cells. *Breast Cancer Res Treat*. **2008**.

[108] Jordan VC.; Lewis JS.; Osipo C.; Cheng D. The apoptotic action of estrogen following exhaustive antihormonal therapy: a new clinical treatment strategy. *Breast*. **2005**, 14(6):624-30.

[109] O'Regan RM.; Osipo C.; Ariazi E.; Lee ES.; Meeke K.; Morris C.; Bertucci A.; Sarker MA.; Grigg R.; Jordan VC. Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. *Clin Cancer Res*. **2006**, 12(7 Pt 1):2255-63.

[110] Liu H.; Lee ES.; Gajdos C.; Pearce ST.; Chen B.; Osipo C.; Loweth J.; McKian K.; De Los Reyes A.; Wing L.; Jordan VC. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*. *J Natl Cancer Inst*. **2003**, 95(21):1586-97.

[111] Jordan VC.; Phelps E.; Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat*. **1987**, 10(1):31-5.

[112] Love RR.; Mazess RB.; Barden HS.; Epstein S.; Newcomb PA.; Jordan VC.; Carbone PP.; DeMets DL. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med*. **1992**, 326(13):852-6.

[113] Umemoto A.; Lin CX.; Ueyama Y.; Komaki K.; Santosh Laxmi YR.; Shibutani S. Absence of DNA adduct in the leukocytes from breast cancer patients treated with toremifene. *Chem Res Toxicol*. **2006**, 19(3):421-5.

[114] da Costa GG.; Pereira PC.; Churchwell MI.; Beland FA.; Marques MM. DNA adduct formation in the livers of female Sprague-Dawley rats treated with toremifene or alpha-hydroxytoremifene. *Chem Res Toxicol*. **2007**, 20(2):300-10.

[115] Shibutani S.; Ravindernath A.; Terashima I.; Suzuki N.; Laxmi YR.; Kanno Y.; Suzuki M.; Apak TI.; Sheng JJ.; Duffel MW. Mechanism of lower genotoxicity of toremifene compared with tamoxifen. *Cancer Res*. **2001**, 61(10):3925-31.

[116] Dragan VP.; Vaughan J.; Jordan VC.; Pitot HC. Comparison of the effects of tamoxifen and toremifene on liver and kidney tumor promotion in female rats. *Carcinogenesis*. **1995**, 16(11):2733-41.

[117] Sargent LM.; Dragan YP.; Sattler C.; Bahnub N.; Sattler G.; Martin P.; Cisneros A.; Mann J.; Thorgeirsson S.; Jordan VC.; Pitot HC. Induction of hepatic aneuploidy *in vivo* by tamoxifen, toremifene and idoxifene in female Sprague-Dawley rats. *Carcinogenesis*. **1996**, 17(5):1051-6.

[118] Erkkola R.; Mattila L.; Powles T.; Heikkinen J.; Toivola B.; Korhonen P.; Mustonen M. Bone mineral density and lipid changes during 5 years of follow-up in a study of prevention of breast cancer with toremifene in healthy, high-risk pre- and post-menopausal women. *Breast Cancer Res Treat.* **2005**, 93(3):277-87.

[119] Harvey HA.; Kimura M.; Hajba A. Toremifene: an evaluation of its safety profile. *Breast.* **2006**, 15(2):142-57.

[120] O'Regan RM.; Cisneros A.; England GM.; MacGregor JI.; Muenzner HD.; Assikis VJ.; Bilimoria MM.; Piette M.; Dragan YP.; Pitot HC.; Chatterton R.; Jordan VC. Effects of the antiestrogens tamoxifen, toremifene, and ICI 182,780 on endometrial cancer growth. *J Natl Cancer Inst.* **1998**, 90(20):1552-8.

[121] Kusama M.; Kaise H.; Nakayama S.; Ota D.; Misaka T.; Aoki T. Crossover trial for lipid abnormality in postmenopausal breast cancer patients during selective estrogen receptor modulators (SERMs) administrations. *Breast Cancer Res Treat.* **2004**, 88(1):9-16.

[122] Kusama M.; Miyauchi K.; Aoyama H.; Sano M.; Kimura M.; Mitsuyama S.; Komaki K.; Doihara H. Effects of toremifene (TOR) and tamoxifen (TAM) on serum lipids in postmenopausal patients with breast cancer. *Breast Cancer Res Treat*. **2004**, 88(1):1-8.

[123] Lee ES.; Schafer JM.; Yao K.; England G.; O'Regan RM.; De Los Reyes A.; Jordan VC. Cross-resistance of triphenylethylene-type antiestrogens but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res.* **2000**, 6(12):4893-9.

[124] Stenbygaard LE.; Herrstedt J.; Thomsen JF.; Svendsen KR.; Engelholm SA.; Dombernowsky P. Toremifene and tamoxifen in advanced breast cancer--a double-blind cross-over trial. *Breast Cancer Res Treat.* **1993**, 25(1):57-63.

[125] Vogel CL.; Shemano I.; Schoenfelder J.; Gams RA.; Green MR. Multicenter phase II efficacy trial of toremifene in tamoxifen-refractory patients with advanced breast cancer. *J Clin Oncol.* **1993**, 11(2):345-50.

[126] Gong C.; Song E.; Jia W.; Qin L.; Guo J.; Jia H.; Hu X.; Su F. A double-blind randomized controlled trial of toremifen therapy for mastalgia. *Arch Surg*. **2006**, 141(1):43-7.

[127] Oksa S.; Luukkaala T.; Maenpaa J. Toremifene for premenstrual mastalgia: a randomised, placebo-controlled crossover study. *Bjog*. **2006**, 113(6):713-8.

[128] Price D.; Stein B.; Sieber P.; Tutrone R.; Bailen J.; Goluboff E.; Burzon D.; Bostwick D.; Steiner M. Toremifene for the prevention of prostate cancer in men with high grade prostatic intraepithelial neoplasia: results of a double-blind, placebo controlled, phase IIB clinical trial. *J Urol*. **2006**, 176(3):965-70; discussion 70-1.

[129] Smith MR.; Malkowicz SB.; Chu F.; Forrest J.; Price D.; Sieber P.; Barnette KG.; Rodriguez D.; Steiner MS. Toremifene increases bone mineral density in men receiving

androgen deprivation therapy for prostate cancer: interim analysis of a multicenter phase 3 clinical study. *J Urol.* **2008**, 179(1):152-5.

[130] Smith MR.; Malkowicz SB.; Chu F.; Forrest J.; Sieber P.; Barnette KG.; Rodriquez D.; Steiner MS. Toremifene improves lipid profiles in men receiving androgen-deprivation therapy for prostate cancer: interim analysis of a multicenter phase III study. *J Clin Oncol.* **2008**, 26(11):1824-9.

[131] Hellmann-Blumberg U.; Taras TL.; Wurz GT.; DeGregorio MW. Genotoxic effects of the novel mixed antiestrogen FC-1271a in comparison to tamoxifen and toremifene. *Breast Cancer Res Treat.* **2000**, 60(1):63-70.

[132] Qu Q.; Zheng H.; Dahllund J.; Laine A.; Cockcroft N.; Peng Z.; Koskinen M.; Hemminki K.; Kangas L.; Vaananen K.; Harkonen P. Selective estrogenic effects of a novel triphenylethylene compound, FC1271a, on bone, cholesterol level, and reproductive tissues in intact and ovariectomized rats. *Endocrinology*. **2000**, 141(2):809-20.

[133] Wurz GT.; Hellmann-Blumberg U.; DeGregorio MW. Pharmacologic effects of ospemifene in rhesus macaques: a pilot study. *Basic Clin Pharmacol Toxicol*. **2008**, 102(6):552-8.

[134] Komi J.; Lankinen KS.; Harkonen P.; DeGregorio MW.; Voipio S.; Kivinen S.; Tuimala R.; Vihtamaki T.; Vihko K.; Ylikorkala O.; Erkkola R. Effects of ospemifene and raloxifene on hormonal status, lipids, genital tract, and tolerability in postmenopausal women. *Menopause*. **2005**, 12(2):202-9.

[135] Rutanen EM.; Heikkinen J.; Halonen K.; Komi J.; Lammintausta R.; Ylikorkala O. Effects of ospemifene, a novel SERM, on hormones, genital tract, climacteric symptoms, and quality of life in postmenopausal women: a double-blind, randomized trial. *Menopause*. **2003**, 10(5):433-9.

[136] Voipio SK.; Komi J.; Kangas L.; Halonen K.; DeGregorio MW.; Erkkola RU. Effects of ospemifene (FC-1271a) on uterine endometrium, vaginal maturation index, and hormonal status in healthy postmenopausal women. *Maturitas*. **2002**, 43(3):207-14.

[137] Kallio A.; Guo T.; Lamminen E.; Seppanen J.; Kangas L.; Vaananen HK.; Harkonen P. Estrogen and the selective estrogen receptor modulator (SERM) protection against cell death in estrogen receptor alpha and beta expressing U2OS cells. *Mol Cell Endocrinol.* **2008**.

[138] Michael H.; Harkonen PL.; Kangas L.; Vaananen HK.; Hentunen TA. Differential effects of selective oestrogen receptor modulators (SERMs) tamoxifen, ospemifene and raloxifene on human osteoclasts *in vitro*. *Br J Pharmacol*. **2007**, 151(3):384-95.

[139] Komi J.; Lankinen KS.; DeGregorio M.; Heikkinen J.; Saarikoski S.; Tuppurainen M.; Halonen K.; Lammintausta R.; Vaananen K.; Ylikorkala O.; Erkkola R. Effects of ospemifene and raloxifene on biochemical markers of bone turnover in postmenopausal women. *J Bone Miner Metab*. **2006**, 24(4):314-8.

[140] Wurz GT.; Read KC.; Marchisano-Karpman C.; Gregg JP.; Beckett LA.; Yu Q.; Degregorio MW. Ospemifene inhibits the growth of dimethylbenzanthracene-induced mammary tumors in Sencar mice. *J Steroid Biochem Mol Biol.* **2005**, 97(3):230-40.

[141] Namba R.; Young LJ.; Maglione JE.; McGoldrick ET.; Liu S.; Wurz GT.; DeGregorio MW.; Borowsky AD.; MacLeod CL.; Cardiff RD.; Gregg JP. Selective estrogen receptor modulators inhibit growth and progression of premalignant lesions in a mouse model of ductal carcinoma *in situ*. *Breast Cancer Res*. **2005**, 7(6):R881-9.

[142] Willson TM.; Norris JD.; Wagner BL.; Asplin I.; Baer P.; Brown HR.; Jones SA.; Henke B.; Sauls H.; Wolfe S.; Morris DC.; McDonnell DP. Dissection of the molecular mechanism of action of GW5638, a novel estrogen receptor ligand, provides insights into the role of estrogen receptor in bone. *Endocrinology*. **1997**, 138(9):3901-11.

[143] Bentrem D.; Dardes R.; Liu H.; MacGregor-Schafer J.; Zapf J.; Jordan V. Molecular mechanism of action at estrogen receptor alpha of a new clinically relevant antiestrogen (GW7604) related to tamoxifen. *Endocrinology*. **2001**, 142(2):838-46.

[144] Connor CE.; Norris JD.; Broadwater G.; Willson TM.; Gottardis MM.; Dewhirst MW.; McDonnell DP. Circumventing tamoxifen resistance in breast cancers using antiestrogens that induce unique conformational changes in the estrogen receptor. *Cancer Res.* **2001**, 61(7):2917-22.

[145] Dardes RC.; O'Regan RM.; Gajdos C.; Robinson SP.; Bentrem D.; De Los Reyes A.; Jordan VC. Effects of a new clinically relevant antiestrogen (GW5638) related to tamoxifen on breast and endometrial cancer growth *in vivo*. *Clin Cancer Res.* **2002**, 8(6):1995-2001.

[146] Wu YL.; Yang X.; Ren Z.; McDonnell DP.; Norris JD.; Willson TM.; Greene GL. Structural basis for an unexpected mode of SERM-mediated ER antagonism. *Mol Cell.* **2005**, 18(4):413-24.

[147] Fan M.; Rickert EL.; Chen L.; Aftab SA.; Nephew KP.; Weatherman RV. Characterization of molecular and structural determinants of selective estrogen receptor downregulators. *Breast Cancer Res Treat.* **2007**, 103(1):37-44.

[148] Wittmann BM.; Sherk A.; McDonnell DP. Definition of functionally important mechanistic differences among selective estrogen receptor down-regulators. *Cancer Res.* **2007**, 67(19):9549-60.

[149] Sporn MB. Arzoxifene: a promising new selective estrogen receptor modulator for clinical chemoprevention of breast cancer. *Clin Cancer Res.* **2004**, 10(16):5313-5.

[150] Munster PN. Arzoxifene: the development and clinical outcome of an ideal SERM. *Expert Opin Investig Drugs*. **2006**, 15(3):317-26.

[151] Schafer JM.; Lee ES.; Dardes RC.; Bentrem D.; O'Regan RM.; De Los Reyes A.; Jordan VC. Analysis of cross-resistance of the selective estrogen receptor modulators arzoxifene (LY353381) and LY117018 in tamoxifen-stimulated breast cancer xenografts. *Clin Cancer Res*. **2001**, 7(8):2505-12.

[152] Buzdar A.; O'Shaughnessy JA.; Booser DJ.; Pippen JE, Jr..; Jones SE.; Munster PN.; Peterson P.; Melemed AS.; Winer E.; Hudis C. Phase II, randomized, double-blind study of two dose levels of arzoxifene in patients with locally advanced or metastatic breast cancer. *J Clin Oncol.* **2003**, 21(6):1007-14.

[153] McMeekin DS.; Gordon A.; Fowler J.; Melemed A.; Buller R.; Burke T.; Bloss J.; Sabbatini P. A phase II trial of arzoxifene, a selective estrogen response modulator, in patients with recurrent or advanced endometrial cancer. *Gynecol Oncol.* **2003**, 90(1):64-9.

[154] Deshmane V.; Krishnamurthy S.; Melemed AS.; Peterson P.; Buzdar AU. Phase III Double-Blind Trial of Arzoxifene Compared With Tamoxifen for Locally Advanced or Metastatic Breast Cancer. *J Clin Oncol*. **2007**, 25(31):4967-73.

[155] Liby K.; Rendi M.; Suh N.; Royce DB.; Risingsong R.; Williams CR.; Lamph W.; Labrie F.; Krajewski S.; Xu X.; Kim H.; Brown P.; Sporn MB. The combination of the rexinoid, LG100268, and a selective estrogen receptor modulator, either arzoxifene or acolbifene, synergizes in the prevention and treatment of mammary tumors in an estrogen receptor-negative model of breast cancer. *Clin Cancer Res.* **2006**, 12(19):5902-9.

[156] Fabian CJ.; Kimler BF.; Anderson J.; Tawfik OW.; Mayo MS.; Burak WE, Jr..; O'Shaughnessy JA.; Albain KS.; Hyams DM.; Budd GT.; Ganz PA.; Sauter ER.; Beenken SW.; Grizzle WE.; Fruehauf JP.; Arneson DW.; Bacus JW.; Lagios MD.; Johnson KA.; Browne D. Breast cancer chemoprevention phase I evaluation of biomarker modulation by arzoxifene, a third generation selective estrogen receptor modulator. *Clin Cancer Res.* **2004**, 10(16):5403-17.

[157] Vajdos FF.; Hoth LR.; Geoghegan KF.; Simons SP.; LeMotte PK.; Danley DE.; Ammirati MJ.; Pandit J. The 2.0 A crystal structure of the ERalpha ligand-binding domain complexed with lasofoxifene. *Protein Sci.* **2007**, 16(5):897-905.

[158] Ke HZ.; Paralkar VM.; Grasser WA.; Crawford DT.; Qi H.; Simmons HA.; Pirie CM.; Chidsey-Frink KL.; Owen TA.; Smock SL.; Chen HK.; Jee WS.; Cameron KO.; Rosati RL.; Brown TA.; Dasilva-Jardine P.; Thompson DD. Effects of CP-336,156, a new, nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. *Endocrinology*. **1998**, 139(4):2068-76.

[159] Gennari L. Lasofoxifene: a new type of selective estrogen receptor modulator for the treatment of osteoporosis. *Drugs Today (Barc)*. **2006**, 42(6):355-67.

[160] Rosati RL.; Da Silva Jardine P.; Cameron KO.; Thompson DD.; Ke HZ.; Toler SM.; Brown TA.; Pan LC.; Ebbinghaus CF.; Reinhold AR.; Elliott NC.; Newhouse BN.; Tjoa CM.; Sweetnam PM.; Cole MJ.; Arriola MW.; Gauthier JW.; Crawford DT.; Nickerson DF.; Pirie CM.; Qi H.; Simmons HA.; Tkalcevic GT. Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryltetrahydronaphthalene. *J Med Chem.* **1998**, 41(16):2928-31.

[161] Gass M.; Kagan R.; Simon J.; Spino C.; Barcomb L. Objective Measures of Vaginal Atrophy Are Improved With Lasofoxifene, A Next-Generation Selective Estrogen Receptor Modulator. *Obstetrics & Gynecology*. **2005**, 105(4):71S.

[162] Wang XN.; Simmons HA.; Salatto CT.; Cosgrove PG.; Thompson DD. Lasofoxifene enhances vaginal mucus formation without causing hypertrophy and increases estrogen receptor beta and androgen receptor in rats. *Menopause*. **2006**, 13(4):609-20.

[163] Cohen LA.; Pittman B.; Wang CX.; Aliaga C.; Yu L.; Moyer JD. LAS, a novel selective estrogen receptor modulator with chemopreventive and therapeutic activity in the N-nitroso-N-methylurea-induced rat mammary tumor model. *Cancer Res.* **2001**, 61(24):8683-8.

[164] Vogelvang TE.; van der Mooren MJ.; Mijatovic V.; Kenemans P. Emerging selective estrogen receptor modulators: special focus on effects on coronary heart disease in postmenopausal women. *Drugs*. **2006**, 66(2):191-221.

[165] Stein RC.; Dowsett M.; Cunningham DC.; Davenport J.; Ford HT.; Gazet JC.; von Angerer E.; Coombes RC. Phase I/II study of the anti-oestrogen zindoxifene (D16726) in the treatment of advanced breast cancer. A Cancer Research Campaign Phase I/II Clinical Trials Committee study. *Br J Cancer*. **1990**, 61(3):451-3.

[166] Miller CP.; Collini MD.; Tran BD.; Harris HA.; Kharode YP.; Marzolf JT.; Moran RA.; Henderson RA.; Bender RH.; Unwalla RJ.; Greenberger LM.; Yardley JP.; Abou-Gharbia MA.; Lyttle CR.; Komm BS. Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. *J Med Chem*. **2001**, 44(11):1654-7.

[167] Greenberger LM.; Annable T.; Collins KI.; Komm BS.; Lyttle CR.; Miller CP.; Satyaswaroop PG.; Zhang Y.; Frost P. A new antiestrogen, 2-(4-hydroxy-phenyl)-3-methyl-1-[4-(2-piperidin-1-yl-ethoxy)-benzyl]-1H-in dol-5-ol hydrochloride (ERA-923),

inhibits the growth of tamoxifen-sensitive and -resistant tumors and is devoid of uterotropic effects in mice and rats. *Clin Cancer Res*. **2001**, 7(10):3166-77.

[168] Sadler TM.; Gavriil M.; Annable T.; Frost P.; Greenberger LM.; Zhang Y. Combination therapy for treating breast cancer using antiestrogen, ERA-923, and the mammalian target of rapamycin inhibitor, temsirolimus. *Endocr Relat Cancer*. **2006**, 13(3):863-73.

[169] Komm BS.; Kharode YP.; Bodine PV.; Harris HA.; Miller CP.; Lyttle CR. Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity. *Endocrinology*. **2005**, 146(9):3999-4008.

[170] Ronkin S.; Northington R.; Baracat E.; Nunes MG.; Archer DF.; Constantine G.; Pickar JH. Endometrial effects of bazedoxifene acetate, a novel selective estrogen receptor modulator, in postmenopausal women. *Obstet Gynecol*. **2005**, 105(6):1397-404.

[171] Silverman SL.; Christiansen C.; Genant HK.; Vukicevic S.; Zanchetta JR.; de Villiers TJ.; Constantine GD.; Chines AA. Efficacy of Bazedoxifene in Reducing New Vertebral Fracture Risk in Postmenopausal Women With Osteoporosis: Results From a 3-Year, Randomized, Placebo- and Active-Controlled Clinical Trial*. *J Bone Miner Res*. **2008**.

[172] Bazedoxifene: Bazedoxifene Acetate, TSE 424, TSE-424, WAY 140424. *Drugs R D*. **2008**, 9(3):191-6.

[173] Kharode Y.; Bodine PVN.; Miller CP.; Lyttle CR.; Komm BS. The Pairing of a Selective Estrogen Receptor Modulator, Bazedoxifene, With Conjugated Estrogens as a New Paradigm for the Treatment of Menopausal Symptoms and Osteoporosis Prevention. *Endocrinology*. **2008**:en.2008-0817.

[174] Tremblay A.; Tremblay GB.; Labrie C.; Labrie F.; Giguere V. EM-800, a novel antiestrogen, acts as a pure antagonist of the transcriptional functions of estrogen receptors alpha and beta. *Endocrinology*. **1998**, 139(1):111-8.

[175] Schafer JI.; Liu H.; Tonetti DA.; Jordan VC. The interaction of raloxifene and the active metabolite of the antiestrogen EM-800 (SC 5705) with the human estrogen receptor. *Cancer Res*. **1999**, 59(17):4308-13.

[176] Martel C.; Picard S.; Richard V.; Belanger A.; Labrie C.; Labrie F. Prevention of bone loss by EM-800 and raloxifene in the ovariectomized rat. *J Steroid Biochem Mol Biol*. **2000**, 74(1-2):45-56.

[177] Lemieux C.; Gelinas Y.; Lalonde J.; Labrie F.; Richard D.; Deshaies Y. Hypocholesterolemic action of the selective estrogen receptor modulator acolbifene in intact and ovariectomized rats with diet-induced hypercholesterolemia. *Metabolism*. **2006**, 55(5):605-13.

[178] Martel C.; Provencher L.; Li X.; St Pierre A.; Leblanc G.; Gauthier S.; Merand Y.; Labrie F. Binding characteristics of novel nonsteroidal antiestrogens to the rat uterine estrogen receptors. *J Steroid Biochem Mol Biol*. **1998**, 64(3-4):199-205.

[179] Simard J.; Labrie C.; Belanger A.; Gauthier S.; Singh SM.; Merand Y.; Labrie F. Characterization of the effects of the novel non-steroidal antiestrogen EM-800 on basal and estrogen-induced proliferation of T-47D, ZR-75-1 and MCF-7 human breast cancer cells *in vitro*. *Int J Cancer*. **1997**, 73(1):104-12.

[180] Gutman M.; Couillard S.; Roy J.; Labrie F.; Candas B.; Labrie C. Comparison of the effects of EM-652 (SCH57068), tamoxifen, toremifene, droloxifene, idoxifene, GW-5638 and raloxifene on the growth of human ZR-75-1 breast tumors in nude mice. *Int J Cancer*. **2002**, 99(2):273-8.

[181] Labrie F.; Champagne P.; Labrie C.; Roy J.; Laverdiere J.; Provencher L.; Potvin M.; Drolet Y.; Pollak M.; Panasci L.; L'Esperance B.; Dufresne J.; Latreille J.; Robert J.; Samson B.; Jolivet J.; Yelle L.; Cusan L.; Diamond P.; Candas B. Activity and Safety of the Antiestrogen EM-800, the Orally Active Precursor of Acolbifene, in Tamoxifen-Resistant Breast Cancer. *J Clin Oncol*. **2004**, 22(5):864-71.

[182] Labrie F. Future perspectives of selective estrogen receptor modulators used alone and in combination with DHEA. *Endocr Relat Cancer*. **2006**, 13(2):335-55.

[183] Luo S.; Sourla A.; Labrie C.; Belanger A.; Labrie F. Combined effects of dehydroepiandrosterone and EM-800 on bone mass, serum lipids, and the development of dimethylbenz(A)anthracene-induced mammary carcinoma in the rat. *Endocrinology*. **1997**, 138(10):4435-44.

[184] Galbiati E.; Caruso PL.; Amari G.; Armani E.; Ghirardi S.; Delcanale M.; Civelli M. Pharmacological actions of a novel, potent, tissue-selective benzopyran estrogen. *J Pharmacol Exp Ther*. **2002**, 303(1):196-203.

[185] Armamento-Villareal R.; Sheikh S.; Nawaz A.; Napoli N.; Mueller C.; Halstead LR.; Brodt MD.; Silva MJ.; Galbiati E.; Caruso PL.; Civelli M.; Civitelli R. A new selective estrogen receptor modulator, CHF 4227.01, preserves bone mass and microarchitecture in ovariectomized rats. *J Bone Miner Res*. **2005**, 20(12):2178-88.

[186] Civelli M.; Preti AP.; Cenacchi V.; Rondelli I.; Guastalla D.; Tarral A.; Dostert P.; Guillevic Y.; Homery MC. Single and multiple ascending dose studies of a novel tissue-selective oestrogen receptor modulator, CHF 4227, in healthy postmenopausal women. *Br J Clin Pharmacol*. **2007**, 64(3):304-16.

[187] Brady H.; Doubleday M.; Gayo-Fung LM.; Hickman M.; Khammungkhune S.; Kois A.; Lipps S.; Pierce S.; Richard N.; Shevlin G.; Sutherland MK.; Anderson DW.; Bhagwat SS.; Stein B. Differential response of estrogen receptors alpha and beta to SP500263, a novel potent selective estrogen receptor modulator. *Mol Pharmacol*. **2002**, 61(3):562-8.

[188] Brady H.; Desai S.; Gayo-Fung LM.; Khammungkhune S.; McKie JA.; O'Leary E.; Pascasio L.; Sutherland MK.; Anderson DW.; Bhagwat SS.; Stein B. Effects of SP500263, a novel, potent antiestrogen, on breast cancer cells and in xenograft models. *Cancer Res*. **2002**, 62(5):1439-42.

[189] Sutherland MK.; Brady H.; Gayo-Fung LM.; Leisten J.; Lipps SG.; McKie JA.; O'Leary E.; Patnaik N.; Anderson DW.; Bhagwat SS.; Stein B. Effects of SP500263, a novel selective estrogen receptor modulator, on bone, uterus, and serum cholesterol in the ovariectomized rat. *Calcif Tissue Int*. **2003**, 72(6):710-6.

[190] Kung Sutherland MS.; Lipps SG.; Patnaik N.; Gayo-Fung LM.; Khammungkune S.; Xie W.; Brady HA.; Barbosa MS.; Anderson DW.; Stein B. SP500263, a novel SERM, blocks osteoclastogenesis in a human bone cell model: role of IL-6 and GM-CSF. *Cytokine*. **2003**, 23(1-2):1-14.

[191] Ammann P.; Bourrin S.; Brunner F.; Meyer JM.; Clement-Lacroix P.; Baron R.; Gaillard M.; Rizzoli R. A new selective estrogen receptor modulator HMR-3339 fully corrects bone alterations induced by ovariectomy in adult rats. *Bone*. **2004**, 35(1):153-61.

[192] Vogelvang TE.; Mijatovic V.; Kenemans P.; Teerlink T.; van der Mooren MJ. HMR 3339, a novel selective estrogen receptor modulator, reduces total cholesterol, low-density lipoprotein cholesterol, and homocysteine in healthy postmenopausal women. *Fertil Steril*. **2004**, 82(6):1540-9.

[193] Vogelvang TE.; Mijatovic V.; Kenemans P.; Schalkwijk CG.; van der Mooren MJ. Effect of HMR 3339, a novel selective estrogen receptor modulator, on C-reactive protein levels in healthy postmenopausal women. *Am J Cardiol.* **2004**, 94(9):1205-8.

[194] Verhoeven MO.; Teerlink T.; Kenemans P.; Vogelvang TE.; van der Mooren MJ. Effects on asymmetric dimethylarginine of HMR 3339, a novel selective estrogen receptor modulator: a 12-week, randomized, placebo-controlled, double-blind, dose-ranging study in healthy postmenopausal women. *Menopause.* **2007**, 14(2):235-42.

[195] Vogelvang TE.; Leurs JR.; Mijatovic V.; Willemse J.; van der Mooren MJ. HMR 3339, a novel selective estrogen receptor modulator, reduces concentrations of procarboxypeptidase U, an inhibitor of fibrinolysis. A randomized, placebo-controlled study in postmenopausal women. *J Thromb Haemost.* **2005**, 3(5):1090-2.

[196] Vogelvang TE.; Mijatovic V.; Kenemans P.; Emeis JJ.; Heijst JA.; van der Mooren MJ. The effects of 12 weeks of HMR 3339, a novel selective estrogen receptor modulator, on markers of coagulation and fibrinolysis: a randomized, placebo-controlled, double-blind, dose-ranging study in healthy postmenopausal women. *Am J Obstet Gynecol.* **2005**, 193(4):1384-94.

[197] Windahl SH.; Galien R.; Chiusaroli R.; Clement-Lacroix P.; Morvan F.; Lepescheux L.; Nique F.; Horne WC.; Resche-Rigon M.; Baron R. Bone protection by estrens occurs through non-tissue-selective activation of the androgen receptor. *J Clin Invest.* **2006**, 116(9):2500-9.

[198] Zhao C.; Dahlman-Wright K.; Gustafsson JA. Estrogen receptor beta: an overview and update. *Nucl Recept Signal*. **2008**, 6:e003.

[199] Skliris GP.; Leygue E.; Watson PH.; Murphy LC. Estrogen receptor alpha negative breast cancer patients: estrogen receptor beta as a therapeutic target. *J Steroid Biochem Mol Biol*. **2008**, 109(1-2):1-10.

[200] Chang EC.; Frasor J.; Komm B.; Katzenellenbogen BS. Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. *Endocrinology*. **2006**, 147(10):4831-42.

[201] Lin CY.; Strom A.; Li Kong S.; Kietz S.; Thomsen JS.; Tee JB.; Vega VB.; Miller LD.; Smeds J.; Bergh J.; Gustafsson JA.; Liu ET. Inhibitory effects of estrogen receptor beta on specific hormone-responsive gene expression and association with disease outcome in primary breast cancer. *Breast Cancer Res.* **2007**, 9(2):R25.

[202] Murphy LC.; Watson PH. Is oestrogen receptor-beta a predictor of endocrine therapy responsiveness in human breast cancer? *Endocr Relat Cancer*. **2006**, 13(2):327-34.

[203] Rosa FE.; Caldeira JR.; Felipes J.; Bertonha FB.; Quevedo FC.; Domingues MA.; Moraes Neto FA.; Rogatto SR. Evaluation of estrogen receptor alpha and beta and progesterone receptor expression and correlation with clinicopathologic factors and proliferative marker Ki-67 in breast cancers. *Hum Pathol.* **2008**, 39(5):720-30.

[204] Ariazi EA.; Jordan VC. Estrogen-related receptors as emerging targets in cancer and metabolic disorders. *Curr Top Med Chem.* **2006**, 6(3):203-15.

[205] Stein RA.; McDonnell DP. Estrogen-related receptor {alpha} as a therapeutic target in cancer. *Endocr Relat Cancer.* **2006**, 13(Supplement_1):S25-32.

[206] Stein RA.; Chang C-y.; Kazmin DA.; Way J.; Schroeder T.; Wergin M.; Dewhirst MW.; McDonnell DP. Estrogen-Related Receptor {alpha} Is Critical for the Growth of Estrogen Receptor-Negative Breast Cancer. *Cancer Res.* **2008**, 68(21):8805-12.

[207] Arany Z.; Foo SY.; Ma Y.; Ruas JL.; Bommi-Reddy A.; Girnun G.; Cooper M.; Laznik D.; Chinsomboon J.; Rangwala SM.; Baek KH.; Rosenzweig A.; Spiegelman BM. HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. *Nature*. **2008**, 451(7181):1008-12.

[208] Ao A.; Wang H.; Kamarajugadda S.; Lu J. Involvement of estrogen-related receptors in transcriptional response to hypoxia and growth of solid tumors. *Proc Natl Acad Sci U S A*. **2008**, 105(22):7821-6.

[209] Leventhal L.; Brandt MR.; Cummons TA.; Piesla MJ.; Rogers KE.; Harris HA. An estrogen receptor-beta agonist is active in models of inflammatory and chemical-induced pain. *Eur J Pharmacol*. **2006**, 553(1-3):146-8.

[210] Meyers MJ.; Sun J.; Carlson KE.; Marriner GA.; Katzenellenbogen BS.; Katzenellenbogen JA. Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *J Med Chem*. **2001**, 44(24):4230-51.

[211] Hegele-Hartung C.; Siebel P.; Peters O.; Kosemund D.; Muller G.; Hillisch A.; Walter A.; Kraetzschmar J.; Fritzemeier KH. Impact of isotype-selective estrogen receptor agonists on ovarian function. *Proc Natl Acad Sci U S A*. **2004**, 101(14):5129-34.

[212] Barker S. Non-steroidal anti-estrogens in the treatment of breast cancer. *Curr Opin Investig Drugs*. **2006**, 7(12):1085-91.

[213] Watanabe N.; Ikeno A.; Minato H.; Nakagawa H.; Kohayakawa C.; Tsuji J. Discovery and preclinical characterization of (+)-3-[4-(1- piperidinoethoxy)phenyl]spiro[indene- 1,1'-indane]-5,5'-diol hydrochloride: a promising nonsteroidal estrogen receptor agonist for hot flush. *J Med Chem*. **2003**, 46(19):3961-4.

[214] Wallace OB.; Lauwers KS.; Dodge JA.; May SA.; Calvin JR.; Hinklin R.; Bryant HU.; Shetler PK.; Adrian MD.; Geiser AG.; Sato M.; Burris TP. A selective estrogen receptor modulator for the treatment of hot flushes. *J Med Chem*. **2006**, 49(3):843-6.

[215] Jain N.; Kanojia RM.; Xu J.; Jian-Zhong G.; Pacia E.; Lai MT.; Du F.; Musto A.; Allan G.; Hahn D.; Lundeen S.; Sui Z. Novel chromene-derived selective estrogen receptor modulators useful for alleviating hot flushes and vaginal dryness. *J Med Chem*. **2006**, 49(11):3056-9.

[216] Jain N.; Xu J.; Kanojia RM.; Du F.; Jian-Zhong G.; Pacia E.; Lai MT.; Musto A.; Allan G.; Reuman M.; Li X.; Hahn D.; Cousineau M.; Peng S.; Ritchie D.; Russell R.; Lundeen S.; Sui Z. Identification and structure-activity relationships of chromene-derived selective estrogen receptor modulators for treatment of postmenopausal symptoms. *J Med Chem*. **2009**, 52(23):7544-69.

[217] Hummel CW.; Geiser AG.; Bryant HU.; Cohen IR.; Dally RD.; Fong KC.; Frank SA.; Hinklin R.; Jones SA.; Lewis G.; McCann DJ.; Rudmann DG.; Shepherd TA.; Tian H.; Wallace OB.; Wang M.; Wang Y.; Dodge JA. A selective estrogen receptor modulator designed for the treatment of uterine leiomyoma with unique tissue specificity for uterus and ovaries in rats. *J Med Chem*. **2005**, 48(22):6772-5.

[218] Zhao L.; O'Neill K.; Brinton RD. Estrogenic agonist activity of ICI 182,780 (Faslodex) in hippocampal neurons: implications for basic science understanding of estrogen signaling and development of estrogen modulators with a dual therapeutic profile. *J Pharmacol Exp Ther*. **2006**, 319(3):1124-32.

[219] Zhao L.; Jin C.; Mao Z.; Gopinathan MB.; Rehder K.; Brinton RD. Design, synthesis, and estrogenic activity of a novel estrogen receptor modulator--a hybrid structure of 17beta-estradiol and vitamin E in hippocampal neurons. *J Med Chem*. **2007**, 50(18):4471-81.

[220] Jensen EV.; Jordan VC. The estrogen receptor: a model for molecular medicine. *Clin Cancer Res*. **2003**, 9(6):1980-9.

[221] Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer*. **2008**, 44(1):30-8.

[222] Massarweh S.; Osborne CK.; Creighton CJ.; Qin L.; Tsimelzon A.; Huang S.; Weiss H.; Rimawi M.; Schiff R. Tamoxifen Resistance in Breast Tumors Is Driven by Growth Factor Receptor Signaling with Repression of Classic Estrogen Receptor Genomic Function. *Cancer Res*. **2008**, 68(3):826-33.