AD_____

Award Number: W81XWH-06-1-0590

TITLE: A New Therapeutic Paradigm for Breast Cancer Exploiting Low Dose Estrogen-Induced Apoptosis

PRINCIPAL INVESTIGATOR: Virgil Craig Jordan, OBE, PhD, DSc, FMedSci

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057-0004

REPORT DATE: June 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2013	Annual	19 May 2012- 18 May 2013

4. TITLE AND SUBTITLE
A New Therapeutic Paradigm for Breast Cancer Exploiting Low Dose Estrogen-Induced Apoptosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0590

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Virgil Craig Jordan, OBE, PhD, DSc, FMedSci

E-Mail: vcj2@georgetown.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20057-0004

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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

15. SUBJECT TERMS
Gene expression microarrays, proteomics, c-Src inhibitor PP2, estrogen receptor complex, conformation, estrogen-induced apoptosis, high throughput RNA interference

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU		

Table of Contents

Introduction……5

Body……6

Task 1

Task 1a (*LCCC - Isaacs*)

Work Accomplished……....6

Task 2

Task 2a (*GU - Jordan/Fan)*

Introduction……7

Work Accomplished…….....8

Task 2b (*GU - Jordan/Fan)*

Introduction……...21

Work Accomplished…….....21

Task 2c (*GU- Jordan/Sengupta)*

Introduction……..…….32

Work Accomplished…….…….33

Task 2d (*GU - Jordan/Sengupta/Obiorah)*

Introduction……..…….....39

Work Accomplished…….…….41

Task 2e (*GU- Jordan/Obiorah)*

Introduction……..…….....50

Work Accomplished…….…….51

Task 2f (*GU- Jordan/Obiorah)*

Introduction……..…….....60

Work Accomplished…….…….61

Task 2g (*GU- Jordan/Maximov)*

Introduction……..…….....68

Work Accomplished…….…….69

Task 3

Task 3a (*GU - Riegel/Wellstein*)

Introduction……...76

Work Accomplished…….…….77

Table of Contents continued…..

Task 4
Task 4a (*TGen – Azorsa/Balagurunathan/Cunliffe*)
Introduction……95
Work Accomplished……..95

Key Research Accomplishments..……....96

Reportable Outcomes……...101

Conclusions……..…105

References..……110

Appendix……..……118

INTRODUCTION

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

Figure 1

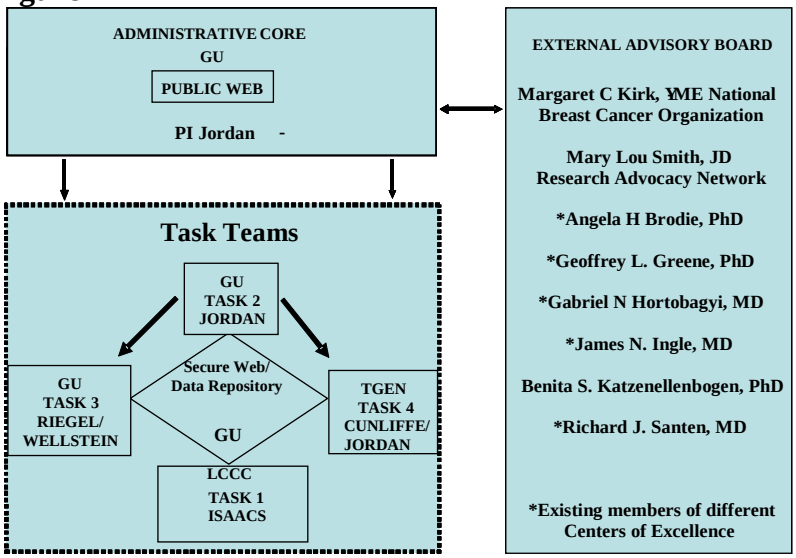


Figure 1. Organization of the COE.

Completion of the Reorganization of the COE

The past year has seen a dramatic increase in our training of new staff that has added to our productivity and guarantees that we will maintain our trajectory to expand knowledge in this important new area of women's health.

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

Body

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

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

Here we report work completed on Task 1a at the LCCC and FCCC sites during year 6 of this COE.

Clinical trial conducted by Claudine Isaacs, MD

DOSE DE-ESCALATION OF ESTROGEN (ESTRACE) TO REVERSE ANTIHORMONE RESISTANCE IN PATIENTS ALREADY EXHAUSTIVELY TREATED WITH ANTIHORMONE THERAPY

Work Accomplished:

Our high dose estrogen protocol is IRB approved, however we have now developed a new strategy to enhance recruitment and export our protocol through a future funding opportunity to a remote site. These opportunities have occurred because of the following changing circumstances. Dr. Alexandra Zimmer is an NCI medical oncology fellow who holds clinics each week at the Georgetown Lombardi Cancer Center with Dr. Isaacs. The fact that a clinical trial comparing high-dose (30mg daily Estrace) versus low dose Estrace (6mg Estrace daily) has now been published (Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A, Jamalabadi-Majidi S, Crowder R, Siegel BA. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 2009;302(7):774-80.) by others based on our laboratory work. The study demonstrates that low-dose estrogen has lower side effects then our current protocol using high-dose estrogen. Georgetown Lombardi Comprehensive Cancer Center has recently entered into negotiations and now concluded an agreement with Hackensack Hospital in New Jersey to create an integrated clinical trial system. Dr. Zimmer and Dr. Isaacs will be exporting our low-dose estrogen protocol to Hackensack to create a baseline study to reproduce the results obtained from the JAMA article above. It is our long-term goal to use the results of this clinical trial to improve clinical responsiveness through combination therapies of low-dose estrogen with selective inhibitors of tumor cell survival. It is our goal to improve response rates from around thirty percent to above fifty percent.

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

Task 2a: Fan and Jordan – To confirm and validate developing pathways of E_2-induced breast cancer cell survival and apoptosis.

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

Modulation of E_2-induced Stress and Apoptosis through c-Src in Long-term Estrogen Deprived Breast Cancer Cells

Introduction

The emergence of antiestrogen resistance in breast cancer is an important clinical phenomenon affecting long-term survival in this disease. Our publications show that physiological concentrations of estrogen (E_2) trigger apoptosis in long-term E_2 deprived breast cancer cells (MCF-7:5C) through inducing stress(3, 4). This new targeted strategy provides novel therapeutic approaches to endocrine resistant breast cancer. A phase II clinical trial reported that E_2 provided a clinical benefit for aromatase inhibitor-resistant advanced breast cancer patients. However, only 30% of patients receive clinical benefit(5). This prompted us to investigate the mechanisms underlying E_2-induced apoptosis to find strategies to increase the therapeutic responsiveness. c-Src is currently of interest, as it is an important adapter protein of ER in breast cancer cells. Here, we found that E_2 elevated c-Src phosphorylation in MCF-7:5C cells and 4-hydroxytamoxifen (4-OHT) blocked this stimulation which suggested that E_2 activated c-Src through ER. E_2 activated the sensors of unfolded protein response (UPR) inositol-requiring protein 1 alpha (IRE1α) and PRK-like endoplasmic reticulum kinase (PERK)/eukaryotic translation initiation factor-2α (eIF2α). The indicator of oxidative stress, heme oxygenase 1 gene (HMOX1), was dramatically up-regulated by E_2. Further examination showed that E_2 significantly increased reactive oxygen species (ROS) production in MCF-7:5C cells. And the energy stress sensor adenosine monophosphate (AMP)-activated protein kinase (AMPK) was activated by E_2. The specific inhibitor of c-Src, PP2, was able to reduce the production of ROS and extrinsic apoptotic signaling pathways induced by E_2, and blocked E_2-induced apoptosis, which was confirmed by knockdown of c-Src with a specific small interfering RNA. All of these data illustrate that c-Src functions as an important transducer in E_2-initiated stress which trigger apoptotic cascades in MCF-7:5C cells. Further examination through transcriptome analysis confirmed that E_2 widely activated apoptosis-related pathways such as oxidative stress and TNF family-related signaling. The c-Src inhibitor, PP2, could abolish the apoptosis-related pathways induced by E_2 which were confirmed by real-time PCR. These data illustrate that c-Src acts as a critical molecule to mediate the downstream signaling of ER (including E_2-induced apoptosis) in MCF-7:5C cells. These data clearly raise a concern regarding the ubiquitous use of c-Src inhibitors to treat patients with advanced aromatase inhibitor-resistant breast cancer, thereby undermining the beneficial effects of E_2-induced apoptosis.

Work Accomplished:

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

It is well documented that E_2 stimulates growth and prevents apoptosis in wild-type breast cancer cells and estrogen-responsive osteoblast cells. In contrast, physiological concentrations of E_2 induce apoptosis in long-term E_2 deprived breast cancer cells(3, 6). c-Src plays a critical role in relaying ER signaling pathways in breast cancer cells. To investigate the function of E_2 and c-Src in long-term E_2-deprived breast cancer cells MCF-7:5C, a specific c-Src tyrosine kinase inhibitor, PP2, was utilized to block phosphorylation of c-Src (Fig. 2A). It also effectively abolished the growth pathways including the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT pathways in MCF-7:5C cells (Fig. 2A). E_2 activated c-Src through ER since 4-hydroxytamoxifen (4-OHT) completely suppressed phosphorylation of c-Src (Fig. 2B). Although our previous finding showed that E_2 initiates apoptosis in MCF-7:5C cells(3), E_2 was able to activate growth pathways in MCF-7:5C cells (Fig. 2C). These actions were blocked by the c-Src inhibitor, PP2 (Fig. 2C). Even though the characteristic E_2-induced apoptosis occurs after 72 hours treatment(3), cell numbers were initially increased by E_2 with a high percentage in S phase (Fig. 2D). All of these results suggested that E_2 caused an imbalance between growth and apoptosis in MCF-7:5C cells.

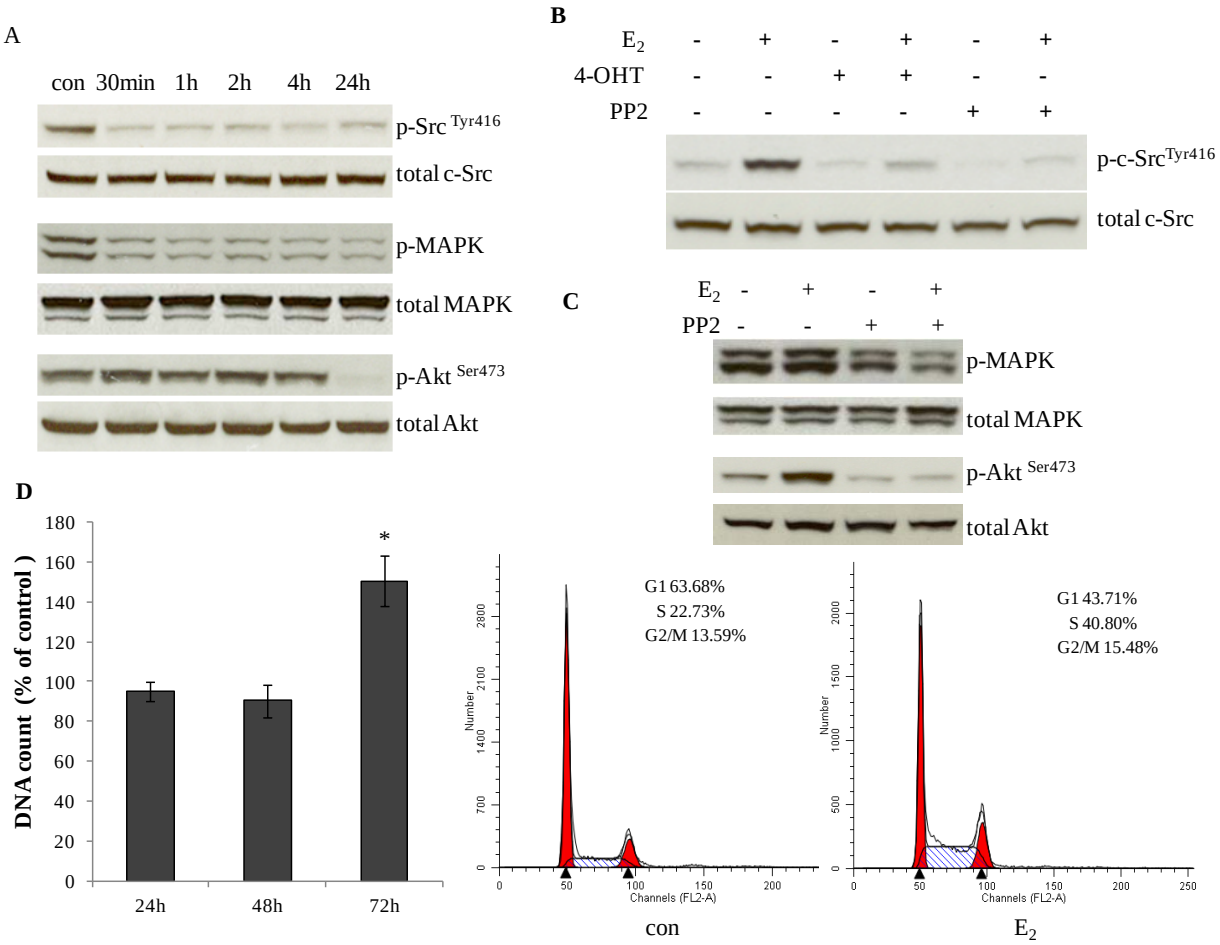


Figure 2. *2A*. The c-Src inhibitor blocked growth pathways in MCF-7:5C cells. MCF-7:5C cells were treated with vehicle (0.1% DMSO) and PP2 (5×10^{-6} mol/L) for different times as indicated. Cell lysates were harvested. Phosphorylated c-Src, MAPK, and Akt were examined by immunoblotting with primary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

MAPK, and Akt were determined for loading controls. *2B*. Estrogen activated c-Src phosphorylation in MCF-7:5C cells. MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9} mol/L), 4-OHT (10^{-6} mol/L), E_2 (10^{-9} mol/L) plus 4-OHT (10^{-6} mol/L), PP2 (5×10^{-6} mol/L), and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) for 48 hours. Cell lysates were harvested. Phosphorylated c-Src was examined by immunoblotting with primary antibody. Immunoblotting for total c-Src was determined for loading control. *2C*. The c-Src inhibitor blocked genomic pathway induced by E_2 in MCF-7:5C cells. MCF-7:5C cells were treated with vehicle (0.1%DMSO), E_2 (10^{-9} mol/L), PP2 (5×10^{-6} mol/L), and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) for 24 hours. Cell lysates were harvested. Phosphorylated MAPK and Akt were examined by immunoblotting with primary antibodies. Immunoblotting for total MAPK and Akt were determined for loading controls. *2D*, E_2 increased S phase of cell cycles in MCF-7:5C cells. MCF-7:5C cells were treated with vehicle and E_2 for different durations. Total DNA was determined using a DNA fluorescence quantitation kit. As a parallel experiment, MCF-7:5C cells were treated with vehicle and E_2 for 72 hours. Cells were fixed for cell cycles analysis. $P<0.05$, * compared with respective control.

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

E_2 was able to activate the non-genomic pathway within minutes (Fig. 3A) which was blocked by the c-Src inhibitor (Fig. 3A). To further investigate the function of the non-genomic pathway in the E_2-induced apoptosis, a synthetic ligand, estrogen dendrimer conjugate (EDC), was used to treat MCF-7:5C cells which is very ineffective in stimulating transcription of endogenous E_2 target genes(7). ER target gene pS2 was selected as a biomarker to measure the dose responsive manner to activate transcription nal activity by EDC (Fig. 3B). In agreement with the result in reference 5, only higher dose of EDC (10^{-6} mol/L) activated pS2 but not in EDC (10^{-8} mol/L) (Fig. 2B). Very similar as E_2, the EDC (10^{-8} mol/L) activated the non-genomic pathway which was blocked by the c-Src inhibitor (Fig. 3C). However, the EDC (10^{-8} mol/L) had no capacity to induce apoptosis (Fig. 3D and 3E). These results indicated that EDC activated the non-genomic pathway but without capacity to activate genomic pathway and did not induce apoptosis in MCF-7:5C cells in certain concentration. All of these data suggest that the non-genomic pathway does not play a critical role in the E_2-induced apoptosis.

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

We have shown that long-term E_2 deprivation increases c-Src activity(8). Therefore, we addressed the question of whether the c-Src inhibitor, PP2, in combination with E_2 would enhance apoptosis in MCF-7:5C cells. Unexpectedly, the c-Src inhibitor blocked apoptosis initiated by E_2 (Fig.4A). To confirm that inhibition of c-Src could block E_2-induced apoptosis, a specific siRNA was utilized to knock down c-Src in MCF-7:5C cells (Fig. 4B), which reduced the percentage of Annexin V binding induced by E_2 (Fig. 3C). Further experiments showed that E_2 disrupted mitochondrial membrane potential ($\Delta\Psi_m$) after 48 hours treatment which was measured by flow cytometry using rhodamine 123 (Rh123) (Fig. 4D). The c-Src inhibitor PP2 and 4-OHT both prevented reduction of Rh123 retention induced by E_2 (Fig. 4D). These data demonstrated that E_2-triggered apoptosis utilize the c-Src tyrosine kinase pathway. All of these findings suggested that the c-Src plays a critical role to mediate E_2-induced apoptosis.

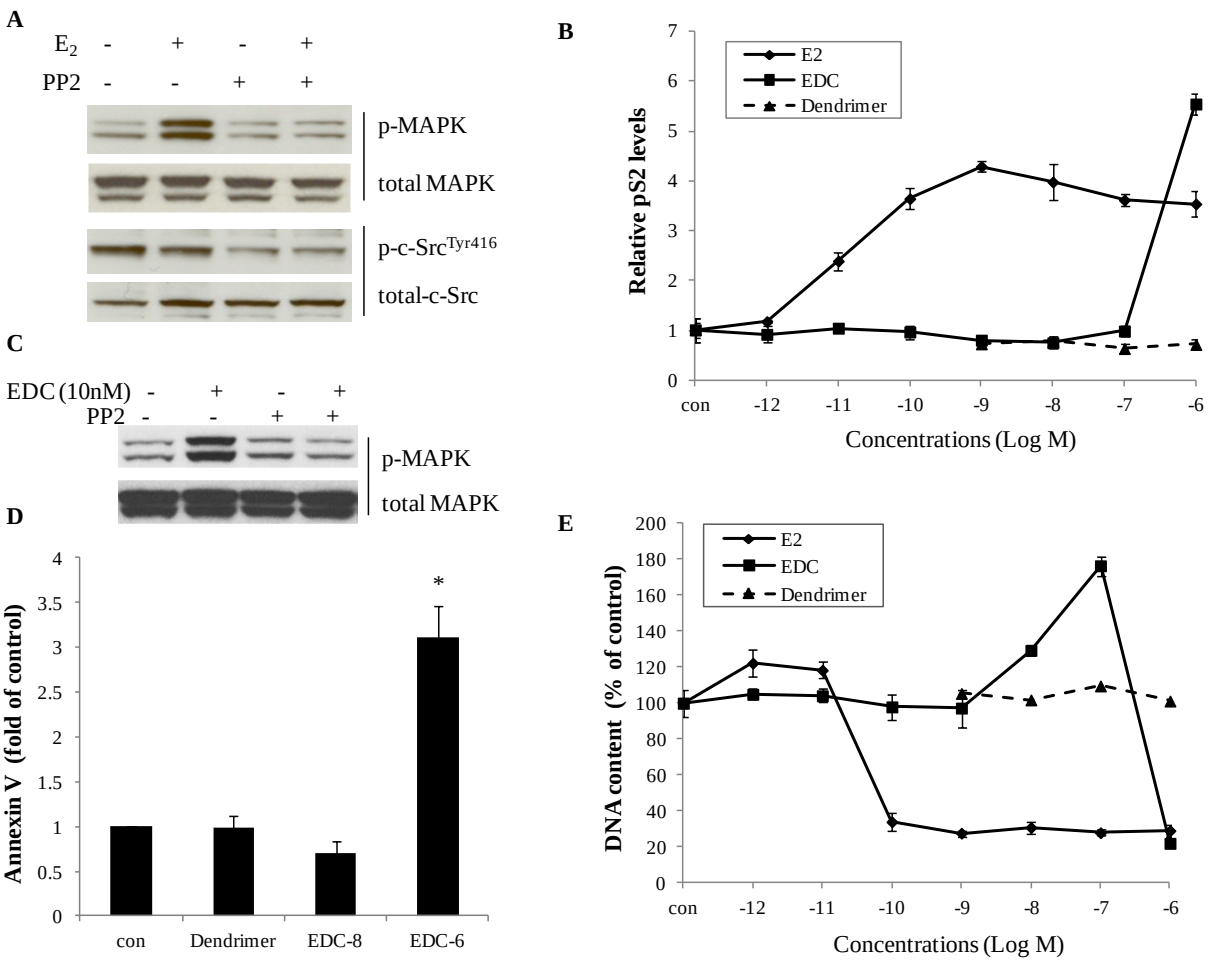


Figure 3. ***3A.*****The c-Src inhibitor blocked non-genomic pathway induced by E_2 in MCF-7:5C cells. MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9} mol/L), PP2 (5×10^{-6} mol/L), and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) for 10 mins. Cell lysates were harvested. Phosphorylated MAPK was examined by immunoblotting with primary antibody. Immunoblotting for total MAPK was determined for loading control. 3*B*. Activation of pS2 by different concentrations of estrogen dendrimer conjugate (EDC). MCF-7:5C cells were treated with vehicle (0.1% MeOH), different concentrations of EDC, E_2, and empty dendrimer as indicated for 8 hours in triplicate. Cells were harvested in TRIzol for real-time PCR. *3C*. The c-Src inhibitor blocked the non-genomic pathway activated by EDC. MCF-7:5C cells were treated with vehicle (0.1% MeOH), EDC (10^{-8} mol/L), PP2 (5×10^{-6} mol/L), EDC (10^{-8} mol/L) plus PP2 (5×10^{-6} mol/L) respectively for 15 minutes and the cell lysates were harvested. Phosphorylated MAPK was examined by immunoblotting with primary antibody. Immunoblotting for total MAPK was used for loading control. *3D.* Detection of apoptosis by different doses of EDC MCF-7:5C cells were treated with vehicle (0.1% MeOH), EDC (10^{-8} mol/L), and EDC (10^{-6} mol/L) for 72 hours. Cells were harvested for the analysis of apoptosis through Annexin V binding assay. *2E*. Cell growth curves after EDC treatment MCF-7:5C cells were treated with vehicle (0.1% MeOH), different concentrations of EDC, E_2, and empty dendrimer as indicated for 7 days in triplicate. Cells were harvested and total DNA was determined using a DNA fluorescence quantitation kit.**

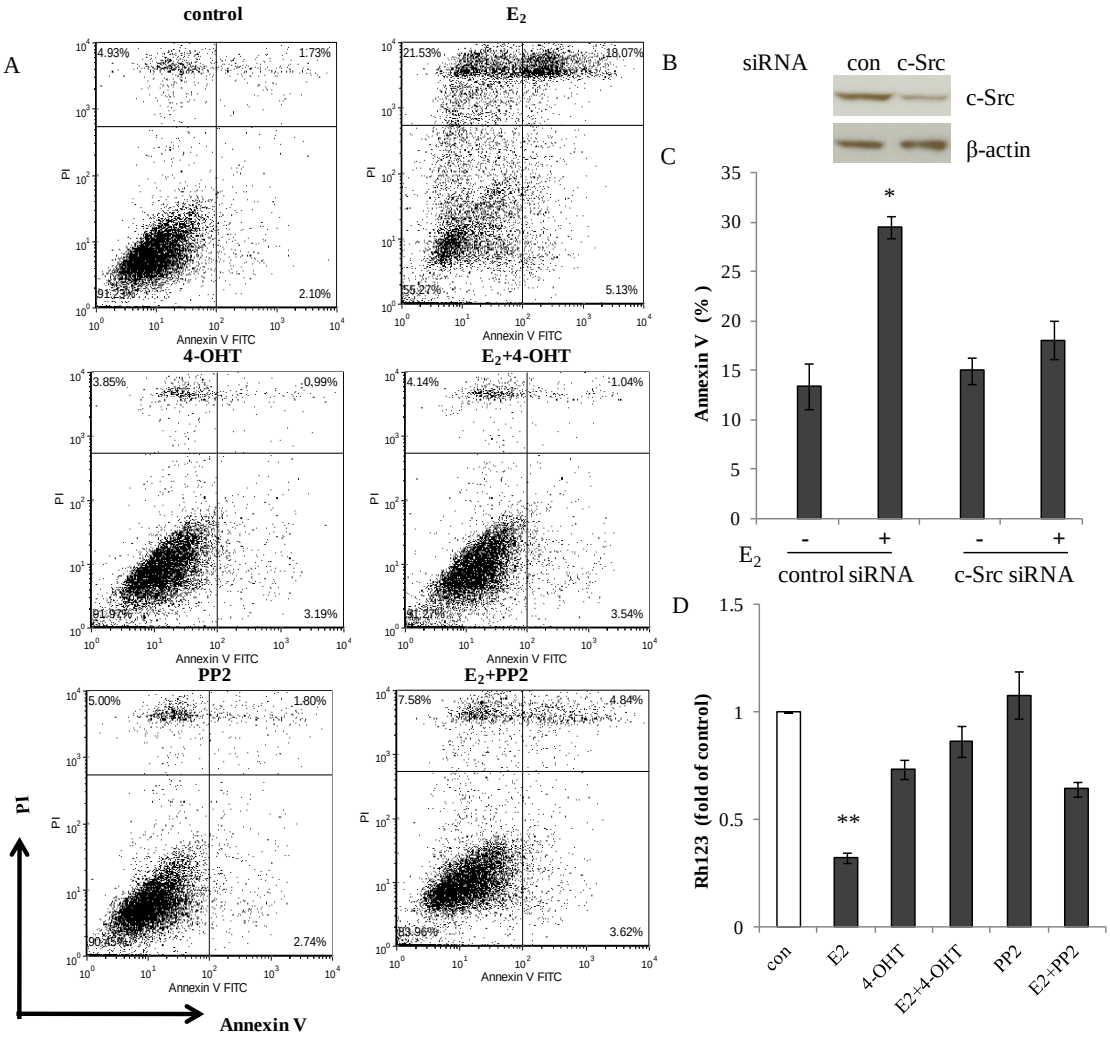


Figure 4. 4*A*. Inhibition of c-Src blocked E_2-induced apoptosis. MCF-7:5C cells were treated with different compounds respectively as above for 72 hours and Annexin V binding assay was used to detect apoptosis. 4*B*. Knockdown of c-Src through interfering RNA MCF-7:5C cells were transfected with siRNA of c-Src for 72 hours using non-target siRNA as control. c-Src was detected by immunoblotting. The β-actin was used for loading control. 4C. Knockdown of c-Src blocked E_2-induced apoptosis. MCF-7:5C cells were transfected with c-Src siRNA and non-target siRNA as above. Then, they were treated with vehicle (0.1% EtOH) and E_2 (10^{-9}mol/L) respectively for 72 hours. Apoptosis was detected through Annexin V binding assay. *P*<0.05, * compared with control. 4D. The c-Src inhibitor blocked the reduction of mitochondrial potential induced by E_2. MCF-7:5C cells were treated with different compounds respectively as above for 48 hours and cells were harvested to detect mitochondrial potential through Rh123. *P*<0.001, ** compared with control.

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

The ER is the initial site for E_2 to induce apoptosis since anti-estrogens ICI 182,780 and 4-OHT completely block apoptosis triggered by E_2 (3). In addition to the mediation of ER growth pathways, c-Src is involved in the process of ligand-activated ER ubiquitylation(9). Therefore, blockade of c-Src tyrosine kinase with PP2 further increased ERα protein and mRNA expression levels in MCF-7:5C cells (Fig. 5A). E_2 activated estrogen response element (ERE) activity which could be blocked by 4-OHT but not by PP2 (Fig. 5B). It was interesting to find that the c-Src inhibitor alone could up-regulate E_2 inducible gene pS2 and was additive with E_2 to elevate pS2 mRNA level (Fig. 5C). Another important ER target gene progesterone receptor (PR) has been regarded as an indicator of a functional ER pathway, since expression

of PR is regulated by E_2. Although the c-Src inhibitor alone did not elevate PR expression, it dramatically synergized with E_2 to up-regulate PR mRNA (Fig. 5D). All of these results demonstrated that blockade of c-Src increased expression of classical ER target genes. It also implied that classical ER pathway might not directly involve in the E_2-induced apoptosis.

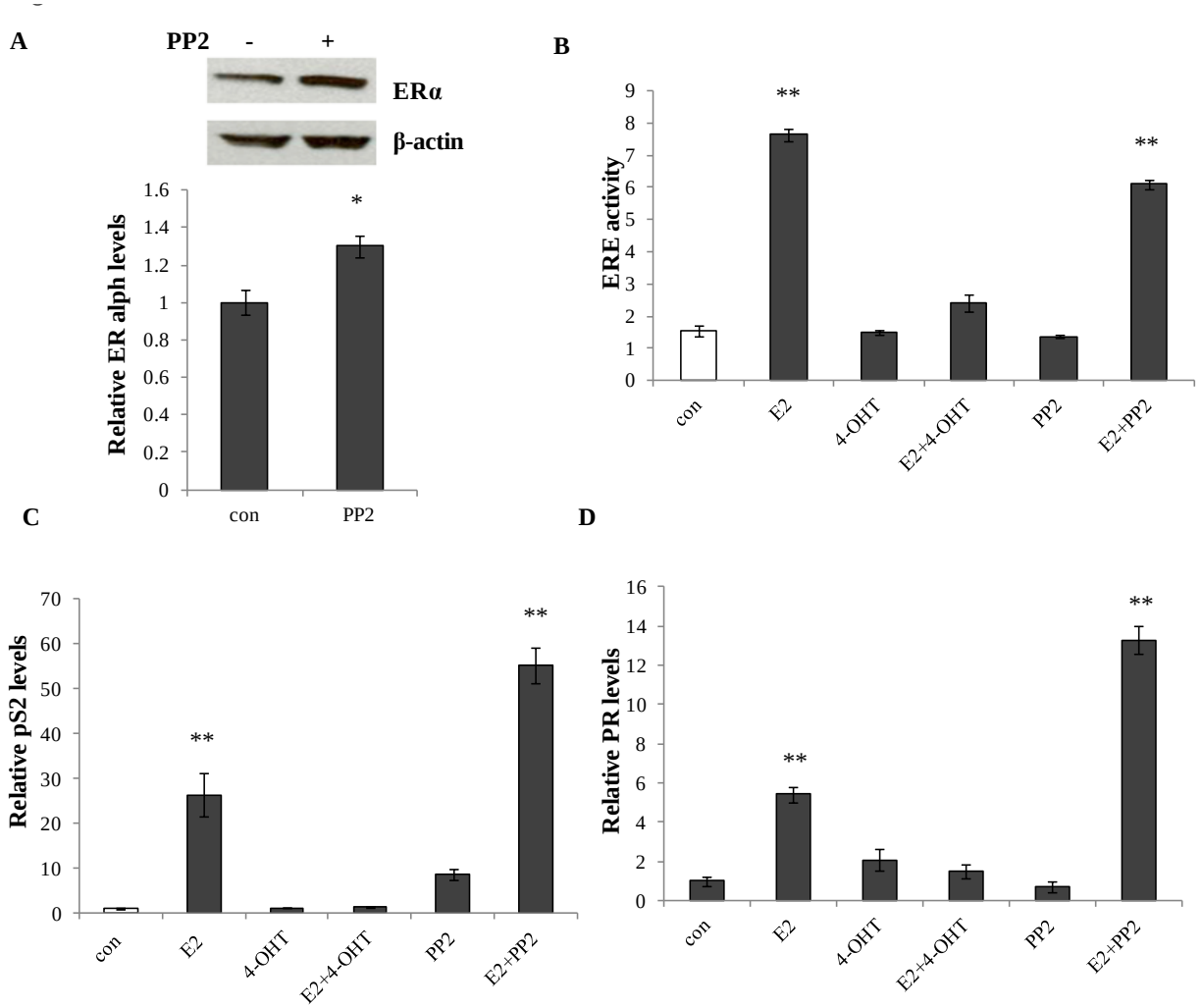


Figure 5. *5A.* The c-Src inhibitor increased levels of ER alpha. MCF-7:5C cells were treated with vehicle (0.1% DMSO) and PP2 (5×10^{-6}mol/L) respectively for 24 hours. ERα protein was detected by immunoblotting. ERα mRNA was quantified with qPCR. *P*<0.05, * compared with control. *5B.* The c-Src inhibitor did not block ERE activity induced by E_2. MCF-7:5C cells were transfected with ERE firefly luciferase plasmid plus renilla luciferase plasmid. Then, cells were treated with different compounds respectively for 24 hours to detect ERE activity. *P*<0.001, ** compared with control. *5C.* The c-Src inhibitor further activated pS2. MCF-7:5C cells were treated with different compounds respectively for 24 hours. The pS2 mRNA was quantified with qPCR. *P*<0.001, ** compared with control. *5D.* The c-Src inhibitor further increased PR levels. MCF-7:5C cells were treated with different compounds respectively for 72 hours. The PR mRNA was quantified with qPCR. *P*<0.001, ** compared with control.

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

To further investigate the mechanisms of the suppression of E_2-induced apoptosis by PP2, RNA-seq analysis was performed to examine the genes regulated by E_2 to trigger apoptosis in MCF-7:5C cells. A wide range of apoptosis-related genes were activated by E_2 (Fig.6A), which were functionally classified into three groups: TP53-related genes (such as TP63, PMAIP1, and CYFIP2), stress-related genes (such as HMOX1, PPP1R15A, ZAK, NUAK2 *etc.*), and inflammatory response-related genes (such as LTB, FAS, TNFRSF21, and CXCR4 *etc.*). Most were stress-related genes (Fig. 6B). Consistent with the biological experiments, 4-OHT and PP2 both blocked apoptosis-related genes induced by E_2 but to a different extent in MCF-7:5C cells (Fig.6A). The majority of these apoptosis-related genes were confirmed by real-time PCR with similar changes noted as in RNA-seq analysis (Fig. 5C). E_2 dramatically increased p63 mRNA levels (Fig. 6C) but did not arrest cells in the G1 phase. In fact, S phase was markedly elevated in MCF-7:5C cells (Fig. 2D). Heme oxygenase 1 (HMOX1) which is active at high concentrations of heme, catalyzes the degradation of heme and is thought to function as an oxidative stress indicator. In breast cancer cells, cytochrome *c* is a major source of heme protein found in the inner membrane of the mitochondrion. E_2 markedly increased HMOX1 in MCF-7:5C cells (Fig. 6C) thereby confirming that E_2 may damage the mitochondria and caused cytochrome *c* release. All of these data suggested that E_2 widely activated intrinsic and extrinsic apoptosis pathways and c-Src was directly involved in mediating apoptosis.

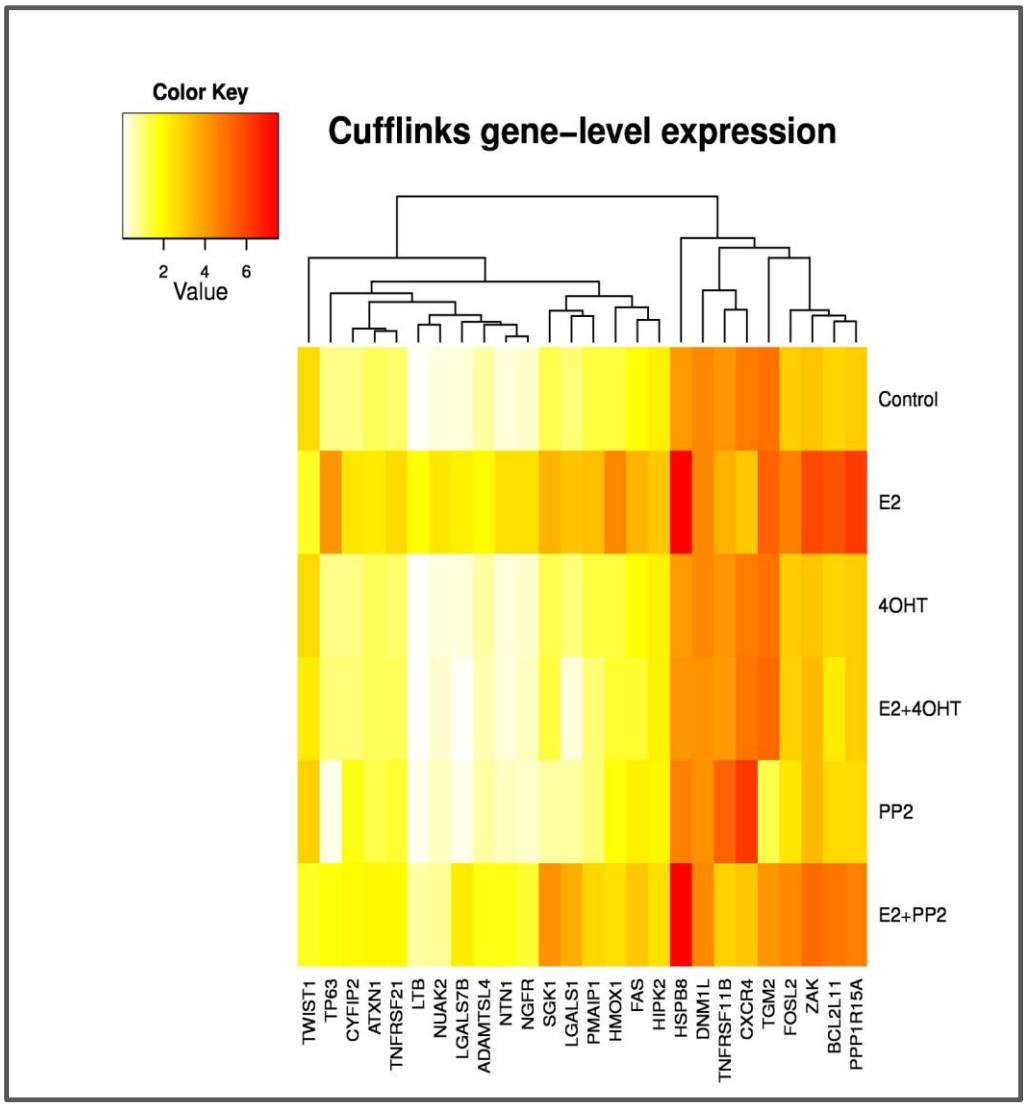


Figure 6A. Estrogen activated apoptosis-related genes in MCF-7:5C cells analyzed through RNA-seq. MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9} mol/L), 4-OHT (10^{-6} mol/L), E_2 (10^{-9} mol/L) plus 4-OHT (10^{-6} mol/L), PP2 (5×10^{-6} mol/L), and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6}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

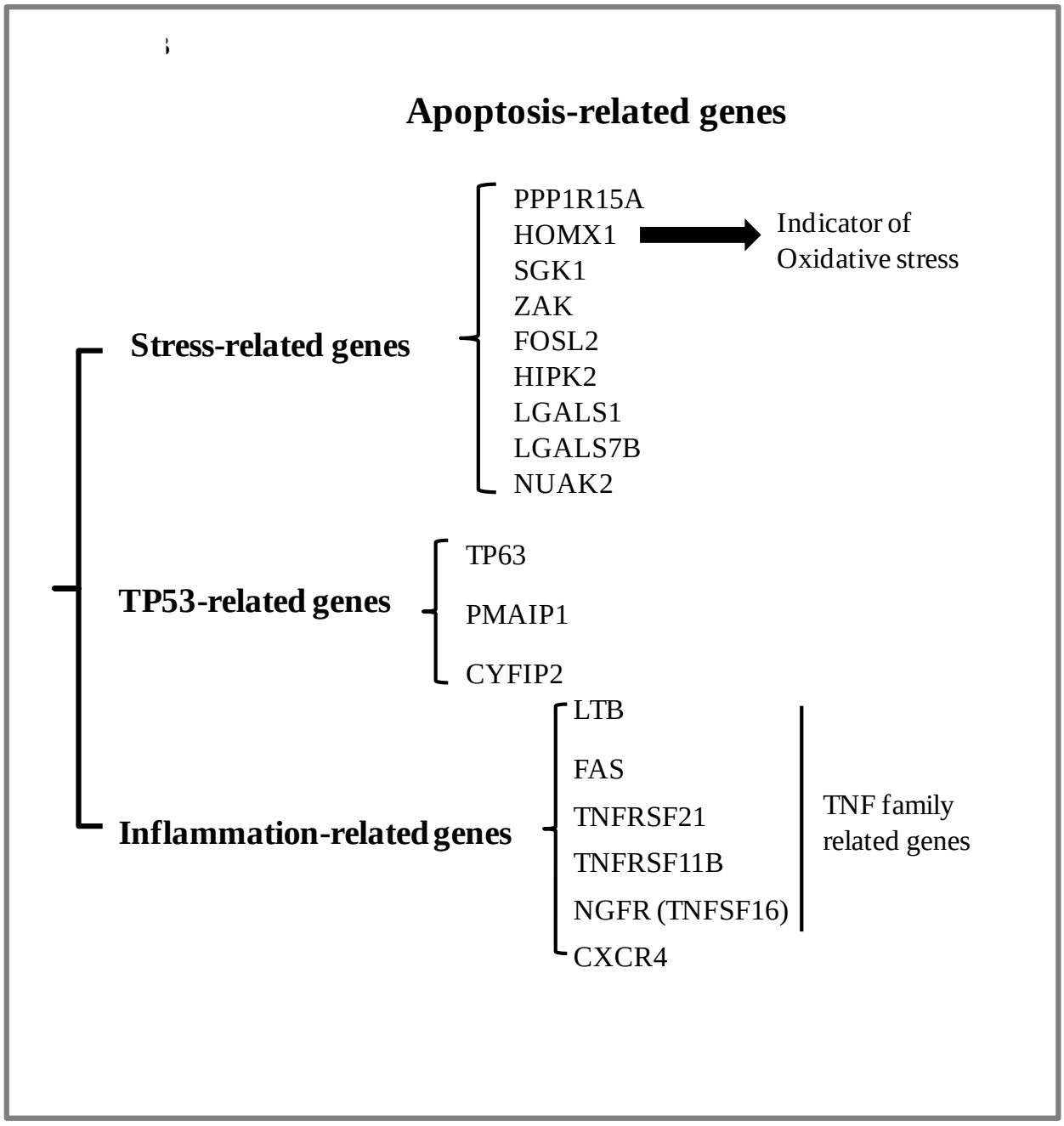


Figure 6B. The apoptosis-related genes selected by RNA-seq were functionally divided into three groups as shown above. Estrogen widely activated apoptosis-related genes to trigger apoptotic cascades in MCF-7:5C cells.

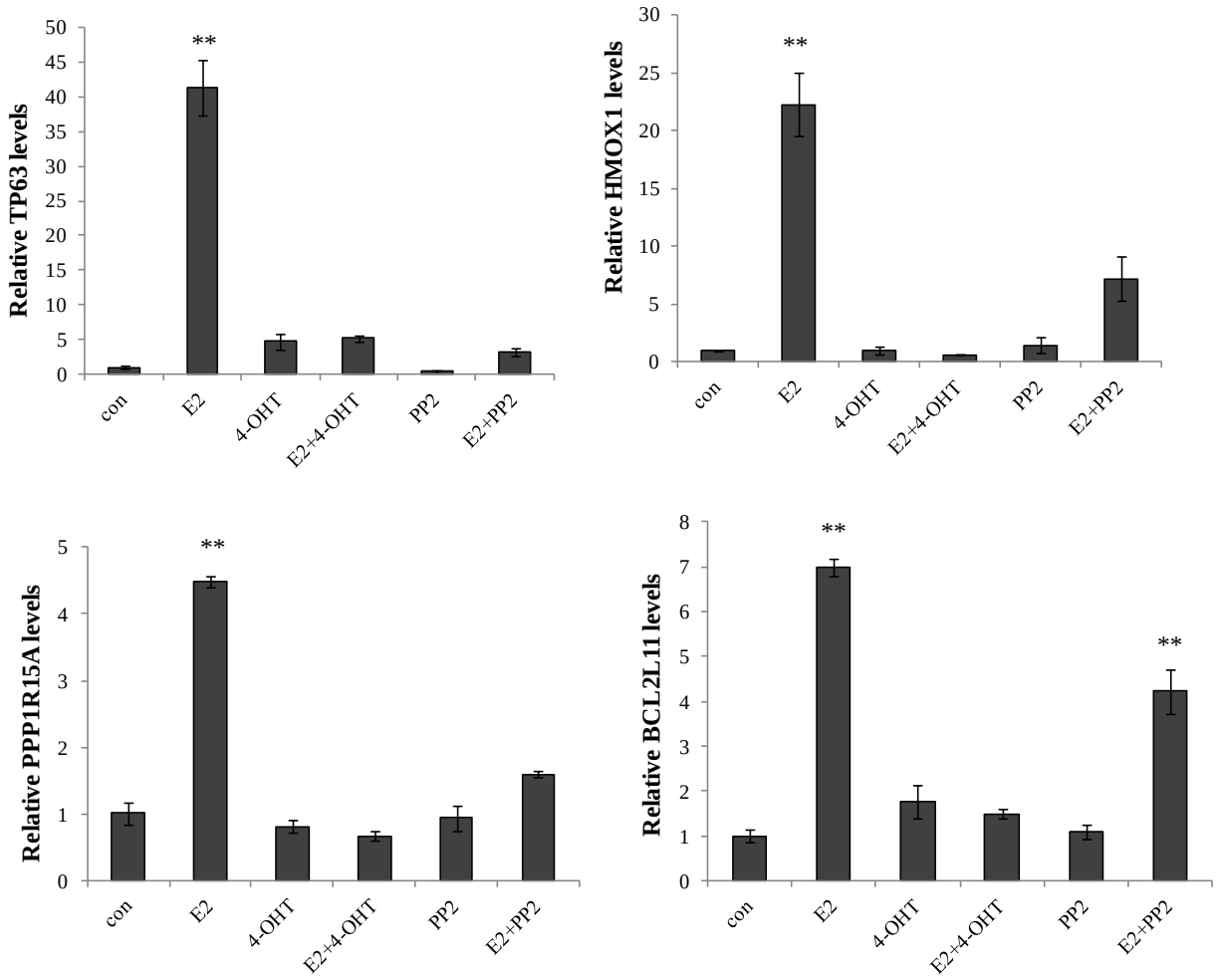


Figure 6C. RNA-seq data were confirmed by real-time PCR. MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9} mol/L), 4-OHT (10^{-6} mol/L), E_2 (10^{-9} mol/L) plus 4-OHT (10^{-6} mol/L), PP2 (5×10^{-6} mol/L), and E_2 (10^{-9} mol/L) plus PP2 (5×10^{-6} mol/L) for 72 hours. Cells were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

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

Reactive oxygen species (ROS) are the product of oxidative stress by mitochondria, whereas an increase in ROS contributes to degenerative changes in mitochondrial function. Under physiological conditions, cellular ROS levels are tightly controlled by low-molecular-weight radical scavengers and by a complex intracellular network of enzymes such as catalases (CAT) and superoxide dismutases (SOD). Under conditions of lethal stress, ROS are considered as key effectors of cell death. Intracellular ROS were detected by CM-H_2DCFDA through flow cytometry (Fig.7A). Detectable ROS appeared after 48 hours of treatment with E_2. The production of ROS reached a peak after 72 hours treatment (Fig.7A and 7B). Blocking ER (by 4-OHT) and c-Src (by PP2) abolished ROS generation induced by E_2 (Fig.7C), indicating that both ER and c-Src were upstream signals of ROS. Free radical scavengers Mn-TBAP, catalase, and sodium formate (SF) which respectively act on superoxide radical (O_2^-), H_2O_2, and hydroxyl radical (OH^-), were utilized to suppress the production of ROS. Our results suggested that H_2O_2 and OH^- were the major sources of ROS induced by E_2. This conclusion was based on the observation that catalase and sodium formate inhibited E_2-induced apoptosis, whereas Mn-TBAP was less effective (Fig. 7D). The RNA-seq

analysis demonstrated that E_2 did not significantly regulate antioxidant enzymes such as catalases (CAT) and superoxide dismutases (SOD) in MCF-7:5C cells (data not shown). Our results suggest that E_2 has the potential to damage mitochondria to cause oxidative stress.

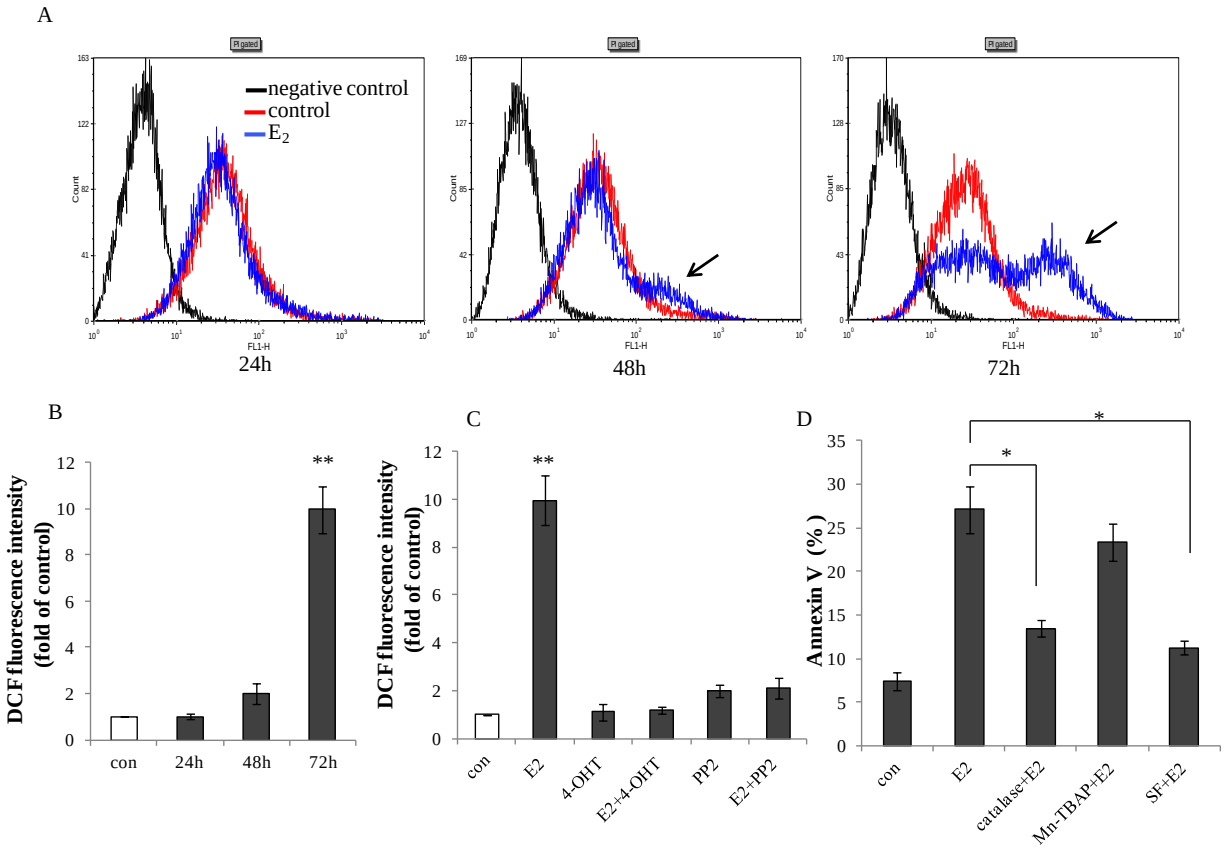


Figure 7. The c-Src inhibitor blocked estrogen-induced oxidative stress in MCF-7:5C cells. *7A*. E_2 increased ROS production in MCF-7:5C cells. MCF-7:5C were treated with vehicle and E_2 for different durations. ROS was detected through flow cytometry with CM-H2DCFDA staining. *7B.* Quantification of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

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

As shown above in Fig. 6A and 6B, E_2 activated TNF family-related genes (such as LTB, FAS, TNFRSF21, and CXCR4 etc.). We confirmed through real-time PCR that members in TNF family members (TNF alpha, LTA, and LTB) were clearly up-regulated by E_2 but blocked by 4-OHT and the c-Src inhibitor in MCF-7:5C cells (Fig. 8A, 8B, and 8C). Of all TNF family members, TNF is the most potent inducer of apoptosis through extrinsic pathway which is mediated by death receptors. We found that low-dose TNF alpha (5ng/ml) could increase the cleavages of caspase 9 and PARP in MCF-7:5C cells (Fig.8D). TNF alpha could significantly inhibit cell growth (Fig.8E). These data demonstrated that E_2-

induced apoptosis in MCF-7:5C cells utilized both intrinsic (mitochondria) and extrinsic (TNF family) pathways.

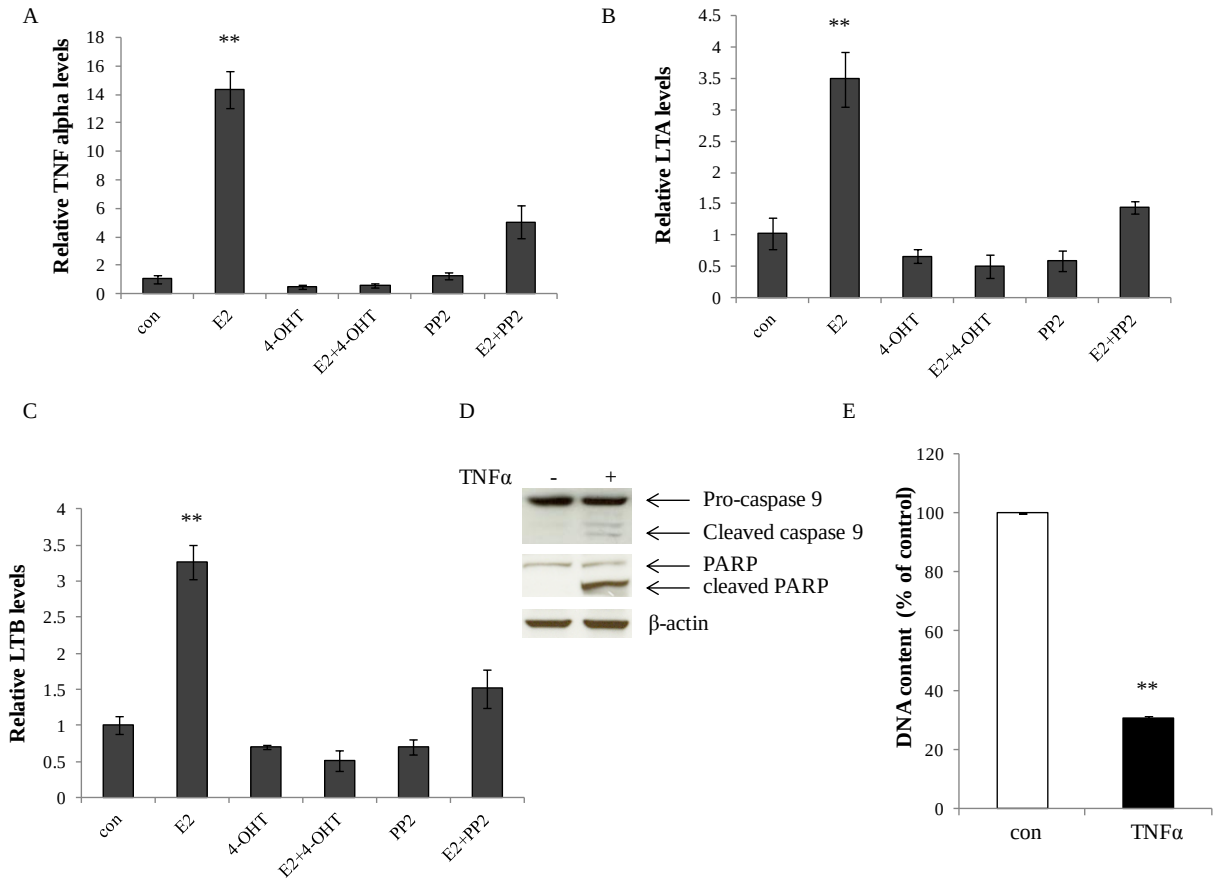


Figure 8. *8A*. The c-Src inhibitor and 4-OHT blocked TNF alpha up-regulated by E_2. MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9}mol/L), 4-OHT (10^{-6}mol/L), E_2 (10^{-9}mol/L) plus 4-OHT (10^{-6}mol/L), PP2 (5×10^{-6} mol/L), and E_2 (10^{-9}mol/L) plus PP2 (5×10^{-6} mol/L) for 72 hours. RNA was isolated with kit (Qiagen) for real-time analysis with specific primers for TNF alpha. *P*<0.001, ** compared with control. *8B*. The c-Src inhibitor and 4-OHT blocked LTA up-regulated by E_2. MCF-7:5C cells were treated with different compounds as above. RNA was isolated with kit (Qiagen) for real-time analysis with specific primers for LTA. *P*<0.001, ** compared with control. 8*C*. The c-Src inhibitor and 4-OHT blocked LTB up-regulated by E_2. MCF-7:5C cells were treated with different compounds as above. RNA was isolated with kit (Qiagen) for real-time analysis with specific primers for LTB. *P*<0.001, ** compared with control. *8D*, TNF alpha increased the cleavages of caspase 9 and PARP. MCF-7:5C cells were treated with vehicle (0.1% DMSO) and TNF alpha (5ng/ml) for 24h. Cell lysates were harvested. Cleavages of caspase 9 and PARP were examined by immunoblotting with primary antibodies. Immunoblotting for β-actin was determined for loading controls. *8E*, TNF alpha inhibited cell growth in MCF-7:5C cells. MCF-7:5C cells were treated with vehicle (H2O) and TNF alpha (5ng/ml) for 7 days. Cells were harvested and DNA content was determined as above. *P*<0.001, ** compared with control.

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

Our previous global gene array data show that E_2 activates genes related to endoplasmic reticulum stress in MCF-7:5C cells(4). To relieve stress, sensors of unfolded protein responses (UPR) are activated as initial responses. In this study, a significant induction of UPR sensors, inositol-requiring protein 1 alpha (IRE1α) and PERK/eukaryotic translation initiation factor-2α (eIF2α), by E_2 occurred after 24 hours of treatment and was further increased by prolonging treatment times in MCF-7:5C cells (Fig. 9A). The antiestrogen 4-OHT completely abolished the response (Fig. 9A). The PERK inhibitor blocked phosphorylation of eIF2α and prevented E_2-induced apoptosis (Fig. 9B and 9C), confirming that endoplasmic reticulum stress was important in the apoptosis initiated by E_2. Phosphorylated eIF2α closely associates with an important cellular energy sensor, adenosine monophosphate (AMP)-activated protein kinase (AMPK), to regulate protein translation and apoptosis. AMPK, which phosphorylates many metabolic enzymes to stimulate catabolic pathways and increases the capacity of cells to produce ATP , was significantly activated after 48 hours treatment with E_2 (Fig. 9D). The c-Src inhibitor, PP2, blocked the phosphorylation of eIF2α but not IRE1α induced by E_2 (Fig. 9E). PP2 also prevented the activation of AMPK after E_2 treatment (Fig. 9F). All of these data indicate that c-Src acts as an important transducer in the protein kinase pathways (eIF2α and AMPK) of stress response (Fig. 9E and 9F) that result in apoptosis.

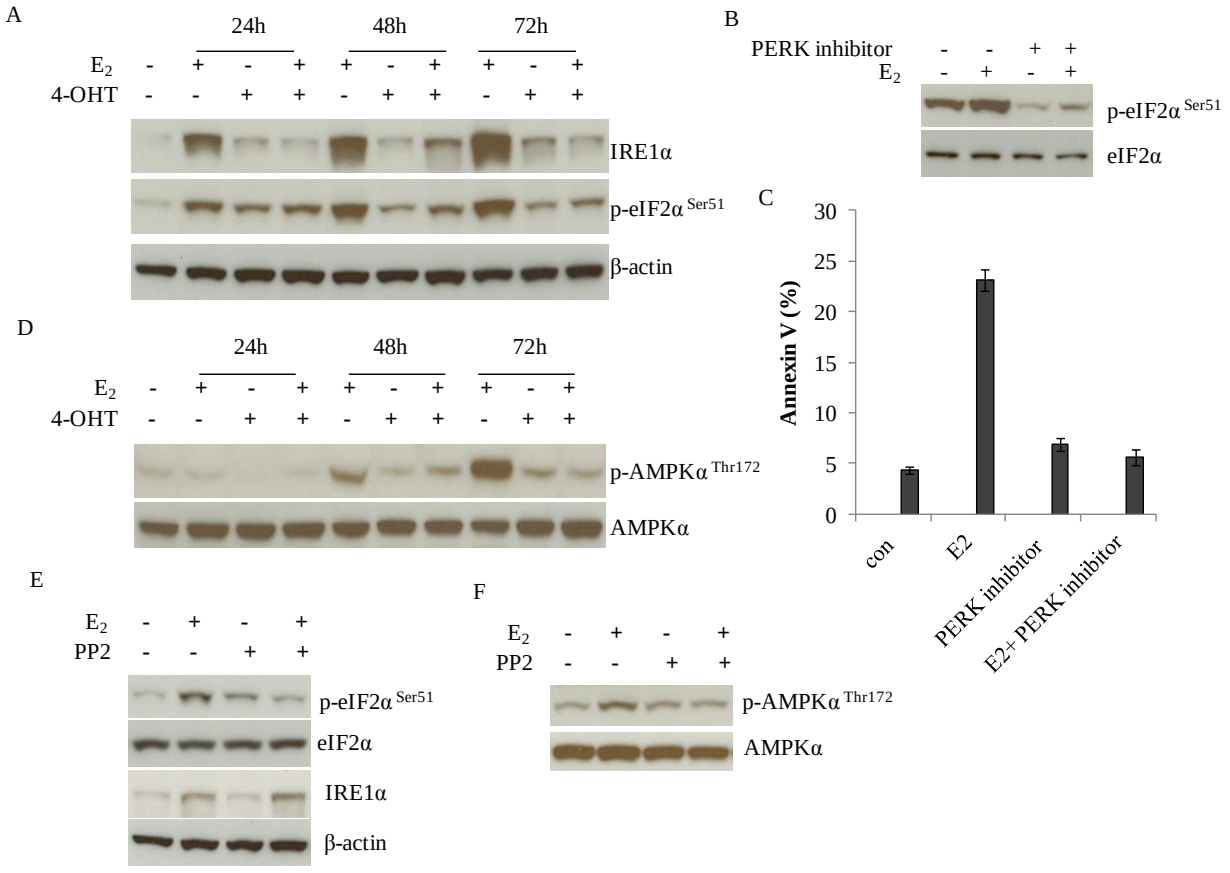


Figure 9. ***9A.*** **E_2 induced endoplasmic reticulum stress in MCF-7:5C cells. MCF-7:5C were treated with E_2 (10^{-9}mol/L) or combined with 4-OHT (10^{-6}mol/L) for different durations. IRE1α and phosphorylated eIF2α were used as indicators of UPR activation.** ***9B.*** **The PERK inhibitor blocked phosphorylation of eIF2α. MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9}mol/L), PERK inhibitor (1×10^{-5}mol/L), E_2 (10^{-9}mol/L) plus PERK inhibitor (1×10^{-5}mol/L) respectively for 24 hours. Phosphorylated eIF2α was examined as the downstream of PERK. Total eIF2α was determined for loading control.** ***9C.*** **The PERK inhibitor blocked E_2-induced apoptosis in MCF-7:5C cells. MCF-7:5C**

cells were treated with E_2 or combined with PERK inhibitor respectively for 72 hours. Apoptosis was detected through Annexin V binding assay. *8D.* E_2 activated energy stress sensor AMPK in MCF-7:5C cells. MCF-7:5C cells were treated with E_2 or combined with 4-OHT as above. Phosphorylated AMPK was examined by immunoblotting. Total AMPK was determined for loading control. *9E.* The c-Src inhibitor blocked phosphorylation of eIF2α but not IRE-1 α. MCF-7:5C cells were treated with E_2 or combined with PP2 for 24 hours. IRE1α and phosphorylated eIF2α were examined by immunoblotting. Total eIF2α and β-actin were determined for loading controls. *9F.* The c-Src inhibitor blocked phosphorylation of AMPK. MCF-7:5C cells were treated with E_2 or combined with PP2 for 48 hours. Phosphorylated AMPK and total AMPK were examined by immunoblotting.

Conclusion

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

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

Task 2b: Fan and Jordan – To investigate the therapeutic effects of the c-Src inhibitor combination with E_2 in long-term E_2-deprived breast cancer cell lines.

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

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

Introduction

Our recent publications demonstrate that physiological concentrations of estrogen (E_2) could induce endoplasmic reticulum stress and oxidative stress(4, 10) which finally resulted in apoptosis in long-term E_2 deprived breast cancer cells, referred as MCF-7:5C cells. And c-Src tyrosine kinase was involved in the process of E_2-induced stresses. To mimic the clinical administration of the c-Src inhibitor, we treated MCF-7:5C cells with different combination for long-term (8 weeks) to further investigate the therapeutic potential of combination the c-Src inhibitor and E_2 on the growth of MCF-7:5C cells. Long-term treatment with PP2 alone or E_2 alone still decreased cell growth with G1 arrest of cell cycles although with different inhibitory rates. In contrast, a combination of PP2 and E_2 actually blocked apoptosis and the resulting cell line (MCF-7:PF) was unique, as they grew vigorously in culture with physiological levels of E_2, which could be blocked by the pure anti-estrogen ICI 182,780**.** In addition to additively up-regulation of endogenous ERα target genes pS2 and progesterone receptor (PR), one of the mechanistic changes was that the c-Src inhibitor could collaborate with E_2 to increase the level of insulin-like growth factor-1 receptor beta (IGF-1Rβ) in MCF-7:PF cells which drove the extracellular signaling pathways. And inhibition of IGF-1Rβ could completely abolish E_2 stimulated growth in MCF-7:PF cells. Furthermore, combination treatment transformed cells with characteristic changes of epithelial-mesenchymal transition (EMT). These data illustrate that caution must be exercised when considering of c-Src inhibitors in clinical trial following the development of acquired resistance to aromatase inhibitors, especially in combination with E_2.

Work Accomplished:

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

We have found that non-receptor tyrosine kinase, c-Src, is activated in long-term E_2 deprived breast cancer cell lines MCF-7:5C and MCF-7:2A and functions as an important transducer to mediate E_2-induced stresses (2, 8). Here, to mimic the clinical administration of the c-Src inhibitor, we long-term (8 weeks) treated MCF-7:5C cells with a specific c-Src inhibitor, PP2, alone or in combination with E_2 to investigate the therapeutic potential in long-term E_2 deprived MCF-7:5C cells. MCF-7:5C cells exhibited a cobblestone-like epithelial phenotype (Fig. 10A). PP2 treated cells appeared smaller and more contracted, with decreased cell spreading (Fig. 10A). And apoptotic impairment could be observed under microscope in E_2 alone treated cells (Fig. 10A). In contrast, combination E_2 and PP2 abolished the growth inhibitory actions by E_2 alone or PP2 alone and the resulting cell line (MCF-7:PF) grew vigorously displaying a spindle-like morphology (Fig. 10A). Further analysis of cell cycles showed that both the c-Src inhibitor and E_2 could clearly arrest cell cycles in G1 phase which was a marker of growth inhibition. However,

combination PP2 and E_2 was unable to arrest cell cycles in G1 (Fig. 10B). These data confirmed that E_2-initiated apoptosis requires c-Src tyrosine kinase pathway(10).

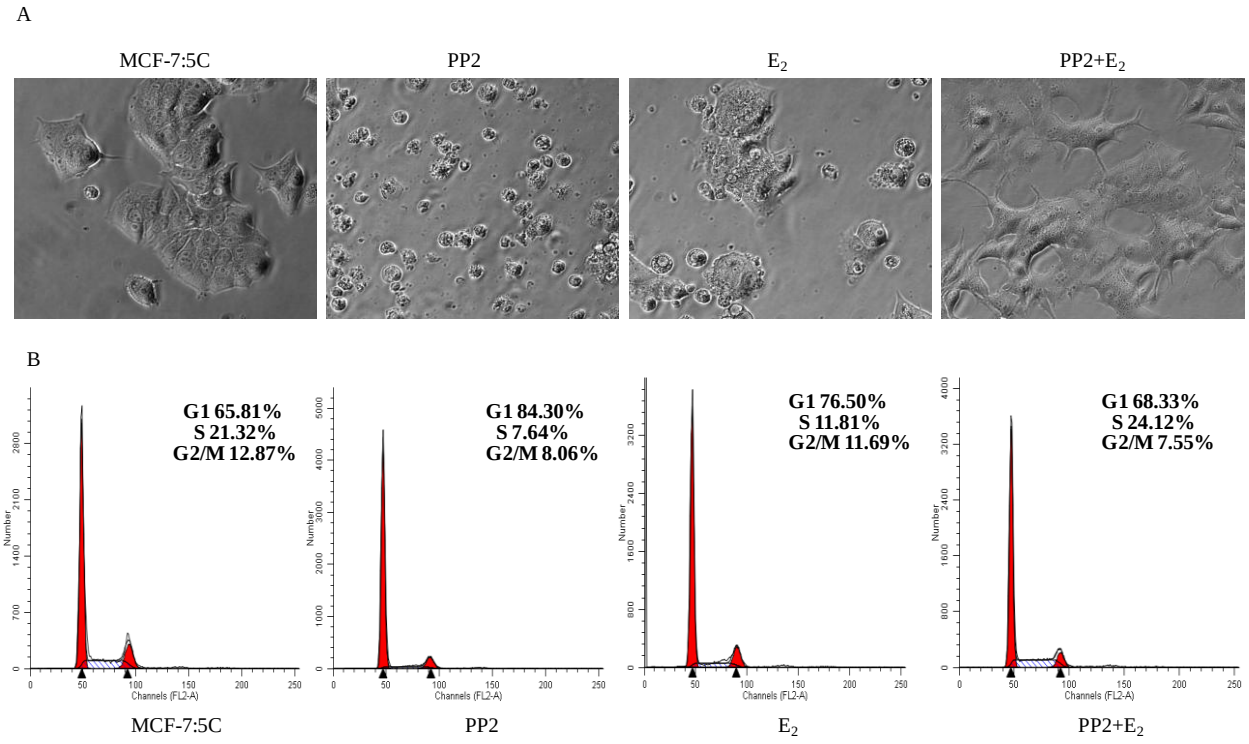


Figure 10. 10*A*. The c-Src inhibitor completely blocked E_2-induced apoptosis after long-term treatment. The morphological changes after 8 weeks treatment with different combination. MCF-7:5C cells were long-term treated with vehicle (0.1% EtOH), PP2 (5×10^{-6} mol/L), E_2 (10^{-9} mol/L), and E_2 (10^{-9}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

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

To further investigate the mechanisms underlying the c-Src inhibitor blocking the apoptosis induced by E_2, the response to E_2 by differently long-term treated cells was first evaluated. Physiological levels of E_2 still caused growth inhibition in MCF-7:5C cells and long-term PP2 treated cells (Fig. 11A). Long-term treatment with E_2 initially caused massive apoptosis, but small fraction of surviving cells subsequently re-grew. Although apoptotic morphology could be observed under microscope at this time point (Fig. 11A), E_2 did not decrease cell number compared with control cells (Fig. 11A). It implied an imbalance between apoptosis and cell growth caused by E_2. In contrast, the resulting cell line (MCF-7:PF) by a combination of PP2 and E_2 grew vigorously in culture with physiological levels of E_2 (Fig. 11A). This stimulation by E_2 could be completely blocked by pure antiestrogen ICI182,780 which demonstrated that proliferation was mediated by ERα (Fig. 11B). Very similarly, the c-Src inhibitor also converted E_2 response from apoptosis to proliferation in another long-term E_2-deprived MCF-7:2A cells, a late-apoptosis cell line (Fig. 11C). It is known that blocking c-Src tyrosine kinase increases ERα expression after 24h treatment(8). This elevated level of ERα was stably expressed after long-term treatment (Fig. 11D). As expected, E_2 alone and MCF-7:PF cells expressed lower levels of ERα due to E_2 down-regulation (Fig. 11D). The ERα mRNA levels were consistent with protein expression (data not shown). There was no change in ERβ

expression after long-term treatment (Fig. 11E). This suggested that MCF-7:PF cells have functional ERα in response to E_2.

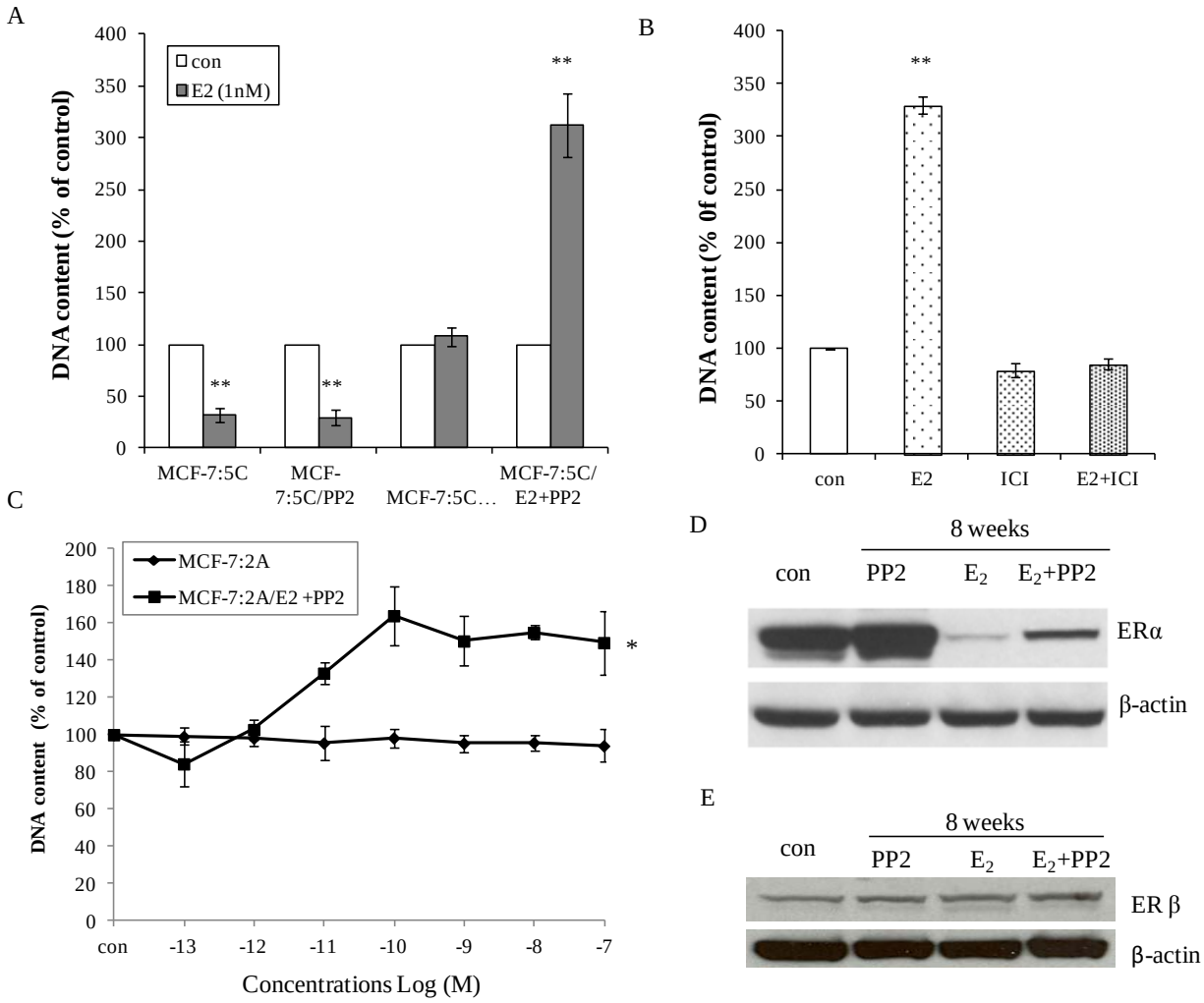


Figure 11. 11*A*. The c-Src inhibitor converted E_2 responses from inducing apoptosis to stimulating growth. Differently treated cells were seeded in 24-well plates in triplicate, respectively. After one day, cells were treated with vehicle (0.1% EtOH) and E_2 (10^{-9} mol/L) respectively. The cells were harvested after 7 days treatment and total DNA was determined using a DNA fluorescence quantitation kit. *P*<0.001, ** compared with control. *11B*. E_2 proliferative effect was blocked by ICI 182,780. MCF-7:PF cells were seeded in 24-well plates in triplicate. After one day, the cells were treated with vehicle (0.1% EtOH), E_2 (10^{-9} mol/L), ICI 182,780 (10^{-6} mol/L), and E_2 (10^{-9} mol/L) plus ICI 182,780 (10^{-6} mol/L) respectively. The cells were harvested as above and total DNA was determined using a DNA fluorescence quantitation kit. *P*<0.001, ** compared with control. *11C*. Inhibition of c-Src converted E_2 from inducing apoptosis to stimulating growth in MCF-7:2A cells. MCF-7:2A cells were long-term (8 weeks) treated with PP2 (5×10^{-6} mol/L) and E_2 (10^{-9} mol/L) as in MCF-7:5C cells. Long-term combination treated cells and MCF-7:2A cells were plated in 24-well plates in triplicate. After one day, cells were treated with different doses of E_2 as indicated. The cells were harvested after 7 days treatment and total DNA was determined using a DNA fluorescence quantitation kit. *P*<0.05, * compared with MCF-7:2A cells. *11D*. Changes of ER alpha after long-term treatment Cell lysates of differently long-term treated cells were harvested. ER alpha was examined by immunoblotting with primary antibody. Immunoblotting for ß-actin was detected for loading control. *11E*. Changes of ER beta after long-term treatment Cell lysates of differently long-term treated cells were harvested. ER beta was examined by immunoblotting with primary antibody. Immunoblotting for ß-actin was detected for loading control.

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

Although ER expression levels were quite different (Fig. 11D), the estrogen response element (ERE) activity was similar among differently treated cells (Fig.12A). It was interesting to find that c-Src inhibitor dramatically elevated E_2 inducible gene pS2 mRNA (Fig. 12B) although the mechanisms were unclear. Moreover, the c-Src inhibitor was additive with E_2 to increase pS2 mRNA in MCF-7:PF cells (Fig. 12B). Another ER target gene, progesterone receptor (PR), was undetectable in MCF-7:5C cells compared with wild type MCF-7 cells (Fig. 12D). However, adding back E_2 in the medium could recover PR expression in E_2 alone treated cells and MCF-7:PF cells (Fig. 12C and 12E). The c-Src inhibitor PP2 alone did not regulate PR expression (Fig. 12E). Nevertheless, it synergized with E_2 to up-regulate PR mRNA although without consistent highest protein expression (Fig. 12C and 12E), which implied existence of a post-translational modification of PR in MCF-7:PF cells(11).

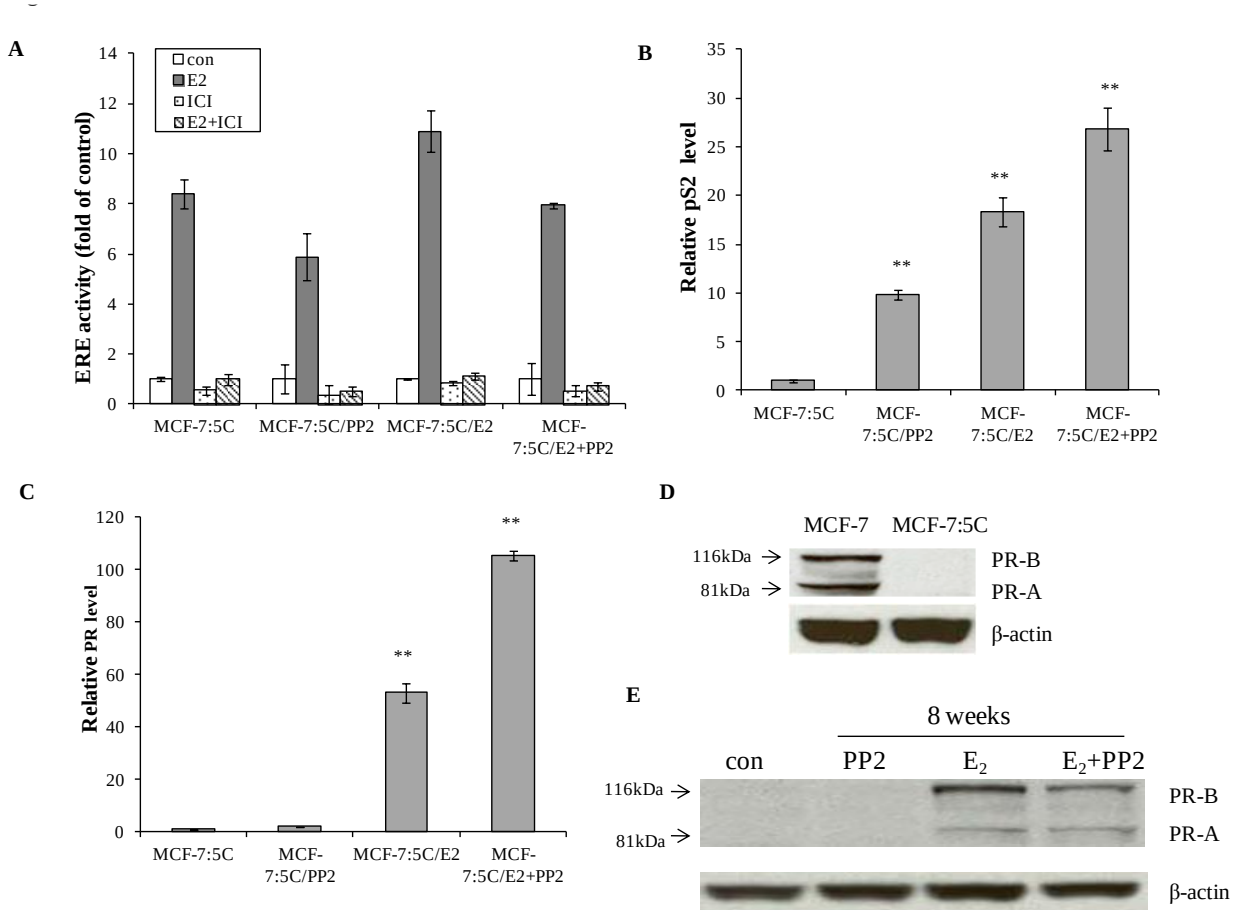


Figure 12. *12A*. ERE activity in different cells MCF-7:5C and differently long-term treated cells were seeded in 24-well plates in triplicate and transfected with ERE firefly luciferase plasmid plus renilla luciferase plasmid as in *Materials and Methods*, respectively. *12B.* The c-Src inhibitor collaborated with E_2 to up-regulate pS2 mRNA. MCF-7:5C and differently long-term treated cells were grown in 6-well plates in triplicate, respectively. The RNA was harvested in TRIzol for real-time PCR analysis. *P*<0.001, ** compared with control. *12C.* The c-Src inhibitor collaborated with E_2 to up-regulate PR mRNA. The RNA of different cells was harvested in TRIzol for real-time PCR analysis. *P*<0.001, ** compared with control. *12D.* PR expression levels were different between wild-type MCF-7 cells and MCF-7:5C cells. Cell lysates were harvested from MCF-7 cells and MCF-7:5C cells. PR was examined by immunoblotting with primary antibody against it. Immunoblotting for ß-actin was detected for loading control. *12E.* PR changes after long-term

treatment. Cell lysates were harvested from differently treated cells. PR was examined by immunoblotting with primary antibody. Immunoblotting for ß-actin was detected for loading control. All the data shown were representative of at least three separate experiments with similar results.

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

As shown in Fig. 12E, E_2 alone treated cells and MCF-7:PF cells had similar levels of PR protein. To further investigate the function of PR, progestin (R5020) was used to examine the activity of progesterone response element (PRE). Interestingly, the progestin uniquely activated PRE activity in MCF-7:PF cells which could be blocked by anti-progestin RU486 (Fig.13A). They also had different cell growth responses to progestin with stimulating growth in MCF-7:PF cells but not in E_2 alone treated cells (Fig. 13B). It has been described that activation of MAPK results in phosphorylation of PR on Ser294, affecting transcriptional function of PR(11). In agreement with this report, we observed that phosphorylated MAPK and PR (Ser294) levels in MCF-7:PF cells were higher than that in E_2 alone treated cells (Fig. 12C). Inhibition of MAPK with U0126 effectively blocked the phosphorylation of PR on Ser294 in MCF-7:PF (Fig. 13D). Although anti-progesterone RU486 blocked PRE activity induced by progestin in MCF-7:PF cells (Fig. 13A), it could not inhibit cell growth activated by progestin whereas itself significantly promoted MCF-7:PF cell growth (data not shown). This estrogenic effects of RU486(12) on MCF-7:PF cells were very similar as on wild-type MCF-7 cells (data not shown). Then, specific siRNA was used to knockdown of PR that effectively inhibited MCF-7:PF cell growth (Fig. 13E). All of these results demonstrated that extracellular signal MAPK may modify PR and affect the transcriptional activity of PR.

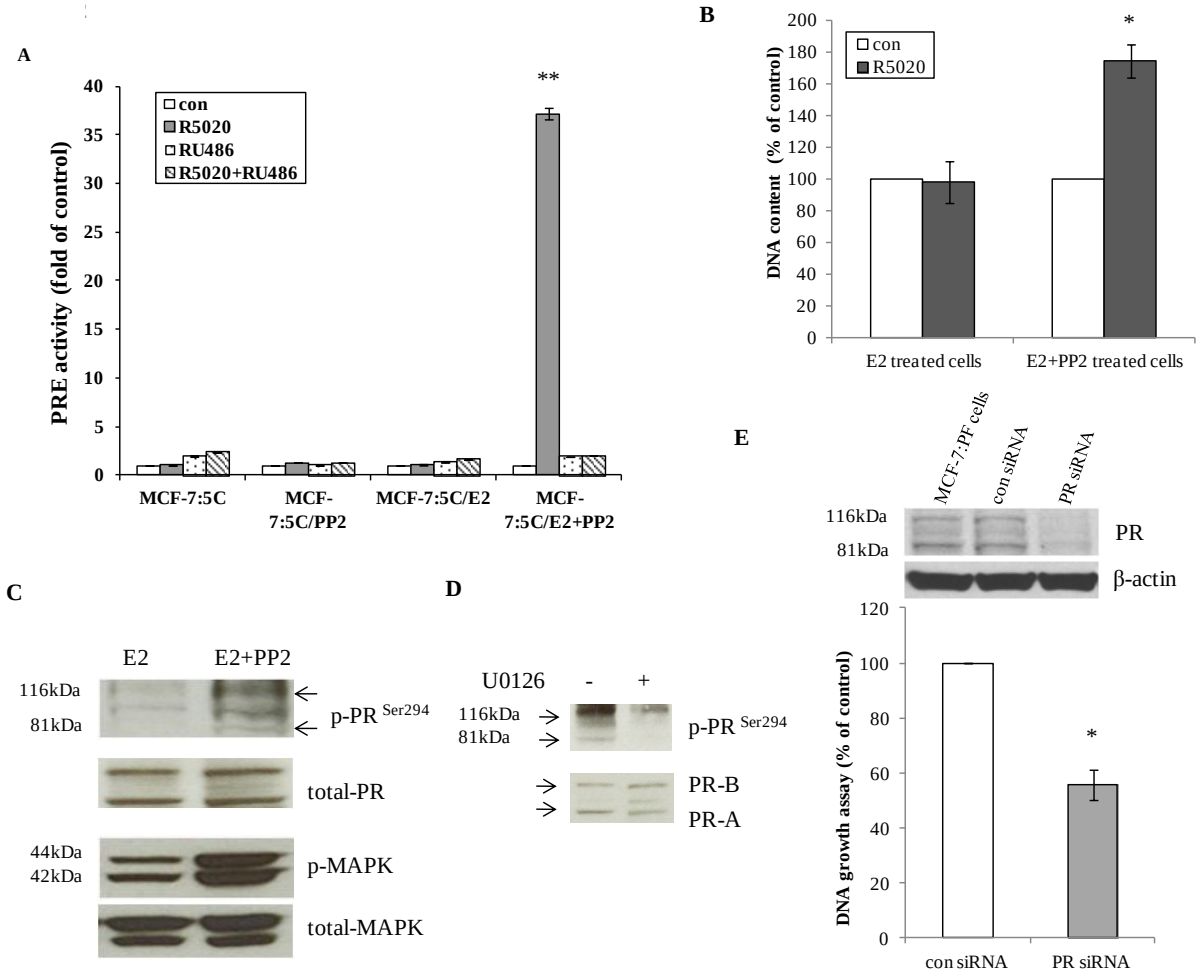


Figure 13. ***13A.*** **The c-Src inhibitor synergized with E_2 to activate PR transcriptional activity. Progestin significantly activated PRE activity in MCF-7:PF cells. Differently long-term treated cells were transfected with PRE firefly luciferase plasmid plus renilla luciferase plasmid as in** ***Materials and Methods*****. The cells were treated with vehicle (0.1% EtOH), progestin (10^{-8}mol/L), RU486 (10^{-6}mol/L), and RU486 (10^{-6}mol/L) plus progestin (10^{-8}mol/L) in triplicate for 24 hours.** ***P*****<0.001, ** compared with control.** ***13B.*** **Different responses to progestin between E_2 alone treated cells and MCF-7:PF cells. E_2 alone treated cells and MCF-7:PF cells were plated in 24-well plates in triplicate. After one day, cells were treated with vehicle (0.1% EtOH) and progestin (10^{-8} mol/L) respectively. Cells were harvested after 7 days treatment and total DNA was determined as above.** ***P*****<0.05, * compared with E_2 alone treated cells.** ***13C.*** **MCF-7:PF cells had higher phosphorylated PR than E_2 alone treated cells. Cell lysates of MCF-7:PF cells and E_2 alone treated cells were harvested. Phosphorylated PR and MAPK were examined by immunoblotting with primary antibodies. Total PR and MAPK were used as loading controls.** ***13D.*** **The PR was phosphorylated by MAPK in MCF-7:PF cells. MCF-7:PF cells were treated with vehicle (0.1% DMSO) and MAPK inhibitor U0126 (10^{-5}mol/L) for 48 hours. Phosphorylated PR was examined by immunoblotting with primary antibody. Total PR was used as loading control.** ***12E.*** **Knockdown of PR by siRNA blocked cell growth. MCF-7:PF cells were transfected with control siRNA and specific PR target siRNA as manufacture's instruction. Cell lysates were harvested after 72 hours to detect PR levels by immunoblotting with primary antibody. Immunoblotting for ß-actin was detected for loading control. As a parallel experiment, cells were harvested after 5 days transfection for DNA growth assay as above.** ***P*****<0.05, * compared with control siRNA.**

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

c-Src mediates the interaction between growth factor receptors and ER in breast cancer(13). These two growth regulatory pathways are tightly linked in ER positive breast cancer(13). Our observations showed that both the c-Src inhibitor and E_2 could increase IGF-1Rβ expression after long-term treatment. Moreover, PP2 and E_2 were additive to elevate IGF-1Rβ in MCF-7:PF cells (Fig. 14A and 14B). To investigate the potential role of IGF-1Rβ in MCF-7:PF cells, a specific inhibitor of IGF-1Rβ, AG1024, was utilized to block receptor tyrosine kinase activity, which effectively abolished MAPK and Akt pathways (Fig. 14C) and inhibited cell growth (Fig. 14D) in MCF-7:PF cells. Importantly, the AG1024 completely abolished E_2 stimulation in a dose dependent manner in MCF-7:PF cells (Fig. 14D). These data indicated that IGF-1Rβ is linked tightly with the ER function in MCF-7:PF cells.

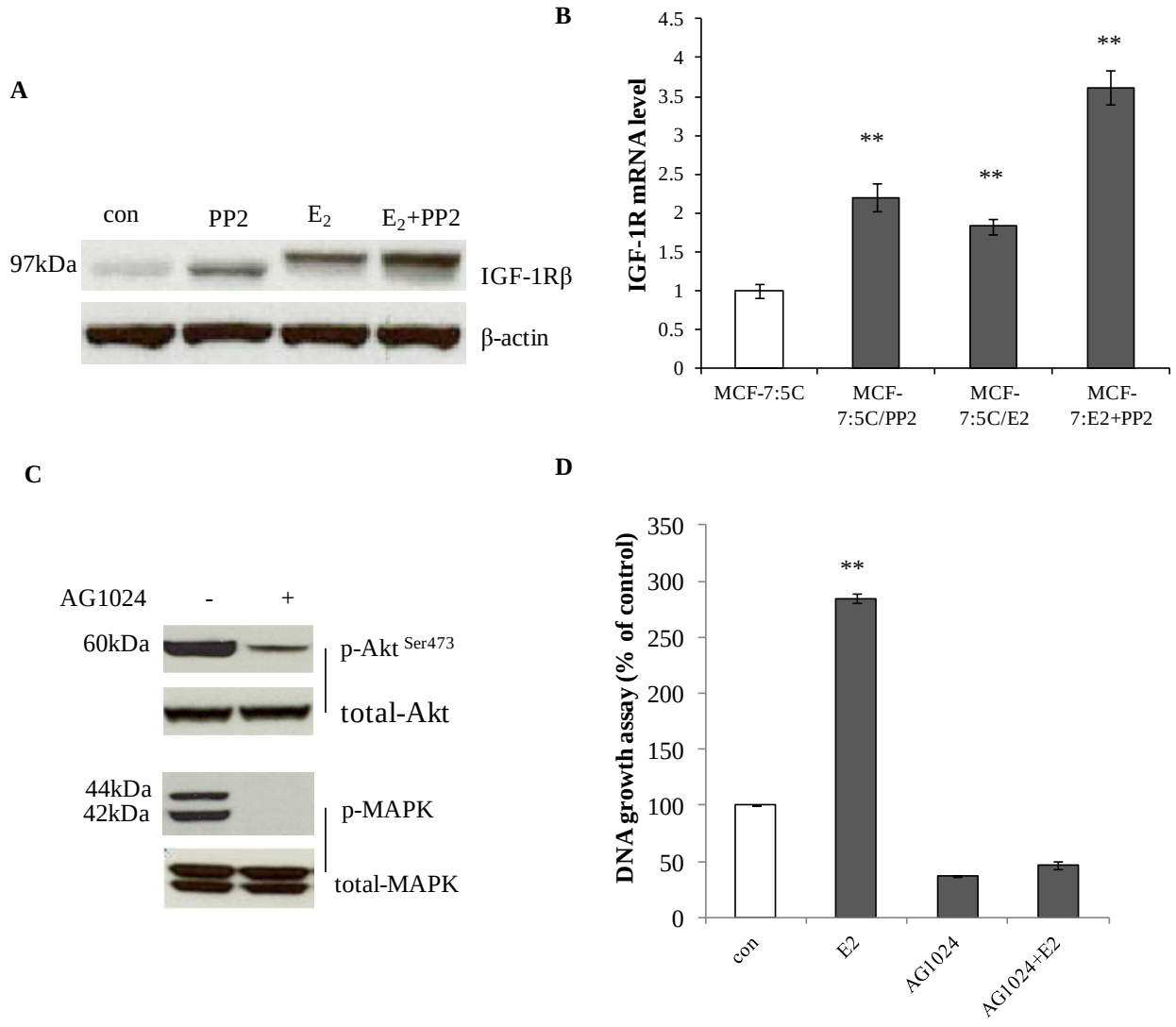


Figure 14. *14A*. The c-Src inhibitor collaborated with E_2 to elevate IGF-1Rβ. Cell lysates of differently long-term treated cells were harvested. IGF-1Rβ was examined by immunoblotting with primary antibody. ß-actin was detected for

loading control. *13B.* IGF-1Rβ mRNA changes were consistent with protein levels. The RNA of differently long-term treated cells was harvested as above. *P*<0.001, ** compared with control. *14C.* Activation of Akt and MAPK pathways by IGF-1Rβ in MCF-7:PF cells. MCF-7:PF cells were treated with vehicle (0.1% DMSO) and AG1024 (10^{-5}mol/L) for 48 hours. Cell lysates were harvested. Phosphorylated Akt and MAPK were determined by immunoblotting with primary antibodies. Total Akt and MAPK were examined for loading controls. *14D.* IGF-1R inhibitor completely blocked E_2 stimulation in MCF-7:PF cells. MCF-7:PF cells were treated with vehicle (0.1% EtOH), E_2 (10^{-9}mol/L), AG1024 (10^{-5}mol/L), and E_2 (10^{-9}mol/L) plus AG1024 (10^{-5}mol/L) for 7 days respectively. The cells were harvested and DNA content was determined as above. *P*<0.001, ** compared with control. All the data shown were representative of at least three separate experiments with similar results.

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

Activation of c-Src kinase has been documented in E-cadherin mediated cell-cell adhesion, which is thought to play an important role in cancer invasion and metastasis(14). Therefore, we sought to examine changes of E-cadherin associated signals after long-term treatment with the c-Src inhibitor in MCF-7:5C cells. Contrary to the effects on wild-type MCF-7 cells(14), PP2 reduced E-cadherin but increased N-cadherin and fibrinogen in MCF-7:5C cells (Fig. 15A), which are characterized features of EMT(14). EMT is regulated by various signal transduction pathways including extracellular signal-regulated kinase (ERK) and Wnt(15). The c-Src inhibitor effectively blocked c-Src phosphorylation in both PP2 alone treated cells and MCF-7:PF cells (Fig. 15B) whereas long-term E_2 treated MCF-7:5C cells still maintained the higher level of phosphorylated c-Src (Fig. 15B). Although the c-Src inhibitor blocks phosphorylated MAPK in the early stage(10), PP2 clearly increased MAPK but continuously blocked Akt after long-term treatment (Fig. 15B). Additionally, inducers of the EMT include several transcription factors such as Snail, Twist, as well as the secreted transforming growth factor beta (TGFβ). In our cell model, the c-Src inhibitor collaborated with E_2 to increase Snail and Twist1 in MCF-7:PF cells (Fig. 15C). Both PP2 alone and E_2 alone increased mRNA levels of TGFβ, but decreased TGFβ in MCF-7:PF cells (Fig. 15D). All of these results suggested that multiple EMT regulators are activated after long-term combination treatment.

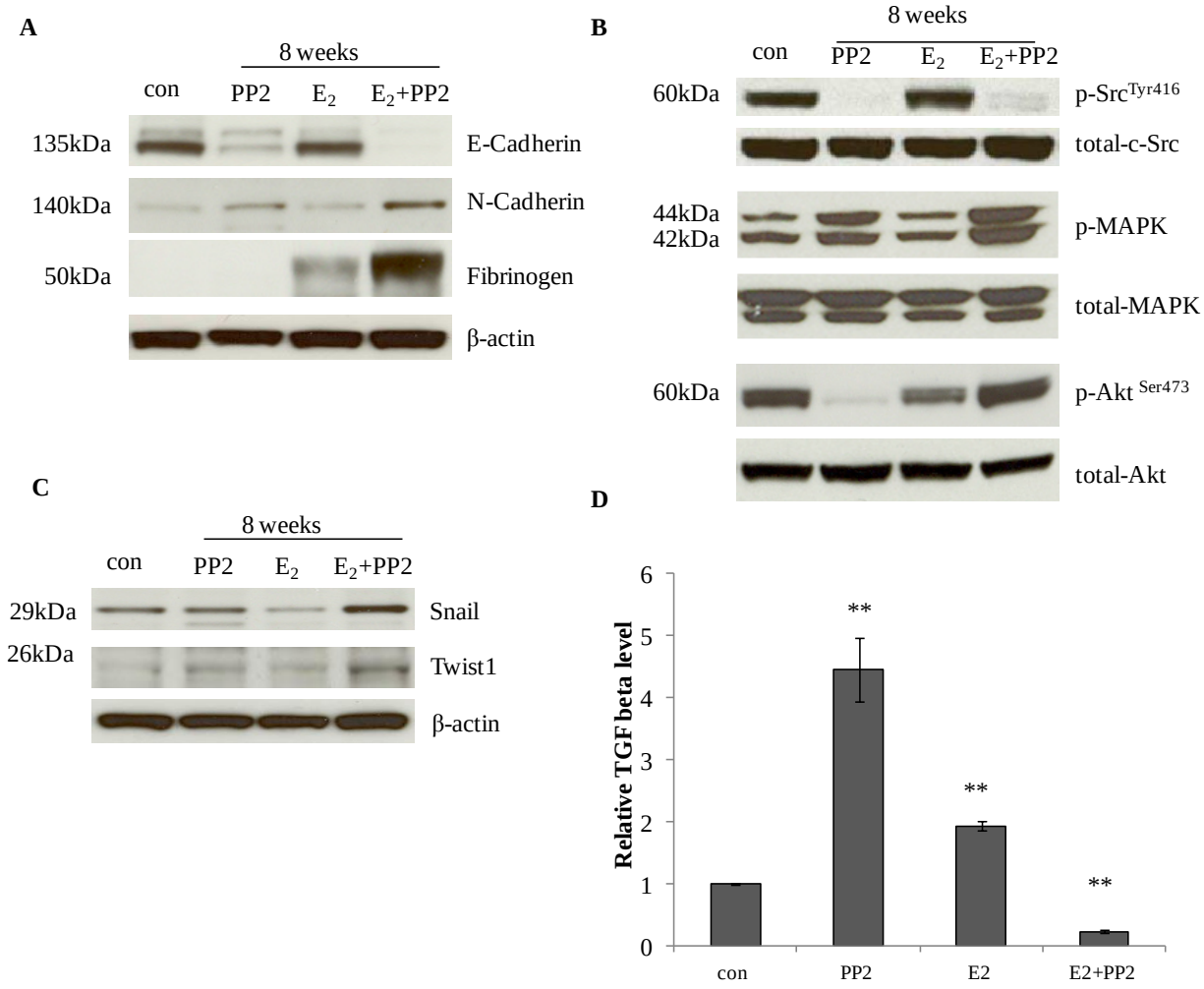


Figure 15. 15*A*. The c-Src inhibitor promoted EMT after combination with E_2 in MCF-7:5C cells. Cell lysates of differently treated cells were harvested. E-cadherin, N-cadherin, and fibrinogen were examined by immunoblotting with primary antibodies. ß-actin was detected for loading control. 15*B*. Signaling pathways changes after long-term combination treatment. Cell lysates of differently treated cells were harvested. Phosphorylated c-Src, MAPK, Akt were examined by immunoblotting with primary antibodies. Total c-Src, MAPK, and Akt were detected for loading controls. 15*C*. Transcription factors Twist1 and Snail were up-regulated. Cell lysates of differently treated cells were harvested. Twist1 and Snail were examined by immunoblotting with primary antibodies. ß-actin was detected for loading control. 15*D*. Regulation of TGFβ after long-term treatment. Differently long-term treated cells were harvested in TRIzol. The mRNA levels were detected through real-time PCR. *P*<0.001, ** compared with control. All the data shown were representative of at least three separate experiments with similar results.

Conclusion

Resistance to aromatase inhibitors is an important clinical problem. We have demonstrated in the laboratory that two long-term E_2-deprived MCF-7 breast cancer cell lines respond to physiological concentrations of E_2 by triggering apoptosis(3, 4). This scientific rationale has clinical relevance(1, 5). However, only 30% of patients receive clinical benefit(5). This prompted us to investigate strategies to increase the therapeutic responsiveness in aromatase inhibitor resistant breast cancer. Oncogene c-Src is activated in E_2-deprived breast cancer cell lines(8). Many observations highlight c-Src as an important therapeutic target to overcome endocrine resistance in breast cancer. We chose an eight-week treatment period in the laboratory to mimic the clinical criteria to evaluate the efficacy of endocrine therapy.

Unexpectedly, the c-Src inhibitor converted E_2 response from inducing apoptosis to stimulating growth in two long-term E_2 deprived breast cancer cell lines. Most importantly, we found that the c-Src inhibitor enhanced the action of E_2 to up-regulate IGF-1Rβ which, in turn, promoted the MCF-7:PF cells to grow. Furthermore, combination treatment enhanced embryonic transcription factors and repressed E-cadherin expression, a characteristic feature of EMT in the generation of invasive tumor cells. In summary, this study suggested that physiological levels of E_2 (probably patient's own E_2) is able to induce apoptosis in long-term E_2-deprived breast cancer. However, administration with a c-Src inhibitor will cause the tumor to grow after aromatase inhibitor resistance, with a variety of signaling networks regulated by the c-Src inhibitor to promote an aggressive phenotype. These data raised a concern regarding the ubiquitous use of c-Src inhibitors in advanced aromatase inhibitor-resistant breast cancer especially combined with E_2.

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

Task 2c: (Sengupta and Jordan) – To elucidate the mechanisms of E_2-independent growth of breast cancer cells mimicking aromatase-inhibitor resistant phenotypes

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

Role of eIF2α-phosphorylation in Mediating Estrogen-induced Apoptosis of Long-term Estrogen deprived MCF7:5C cells

Introduction:

Endocrine therapy is extensively used to treat hormone receptor positive breast cancers. Long-term treatment with either anti-estrogen (tamoxifen) or aromatase inhibitors is associated with resistance in the breast cancer patients(16). Thirty percent patients who have failed multiple endocrine therapies have responded favorably with high or low dose of estrogen treatment with similar efficacy(5, 17). Despite its success in clinic, the precise molecular mechanism of estrogen action in this sub-set of breast cancer patients remains largely unknown. In our lab, we have an estrogen receptor positive cell model, MCF7:5C cells, which have been cultured in estrogen-deprived media for more than a year and undergo estrogen-induced apoptosis *in vitro* as well as in xenograft model in athymic nude mice(3).

Previous studies from our laboratory have indicated that estrogen-induced apoptosis of MCF7:5C cells involves endoplasmic reticulum (ER) stress pathway(4). The precise mechanism by which it activates apoptosis is not known. The ER stress develops because of the load of unfolded proteins in the ER lumen and activates intracellular signaling pathways which are collectively termed the unfolded protein response (UPR)(18). UPR has three distinct pathways of signal transducers, namely ATF6, PERK and IRE1(19). Each of these acts as a sensor for the unfolded protein in the lumen and activates cascades of signaling molecules to protect from excessive protein load and helps in survivalof the cell. However, depending upon the circumstances and the extent of UPR, prolonged ER-stress can induce the cells to undergo apoptosis(20).

This section investigated the role of PERK (protein kinase RNA (PKR)- like ER kinase) mediated signaling of UPR in estrogen mediated apoptosis of MCF7:5C cells (Figure 1). PERK is an ER-resident trans-membrane kinase which oligomerizes and self phosphorylates after sensing ER stress(21). Phosphorylated PERK can in turn phosphorylate the translation initiation factor eIF2α, (Figure 1) which inhibits global mRNA translation. In this way, PERK activation helps in reducing the protein burden in the ER lumen and attempts to alleviate ER stress. Paradoxically, translation of the gene encoding ATF4 (activating transcription factor-4) is favored by the limiting function of eIF2α and its key downstream target CHOP (C/EBP homologous protein)(18). These two key transcription factors induce expression of genes which are important for cellular remediation and apoptosis (Figure 16).

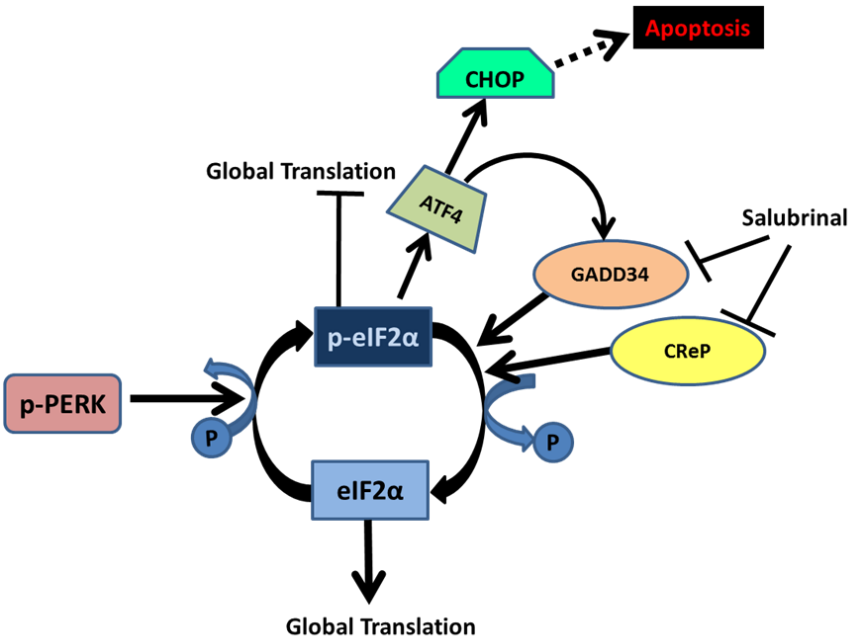


Figure 16.Schematic model of PERK induced unfolded protein response (UPR) through phosphorylation of eIF2α and induction of apoptosis.

Work Accomplished

Estrogen (17β-estradiol) treatment induces phosphorylation of eIF2α and its down-stream targets in MCF7:5C cells

The precisemechanism by which estrogen induces apoptosis in long term estrogen deprived MCF7:5C cell is not completely understood. Our laboratory previously reported involvement of the mitochondrial mediated apoptotic pathway and UPR pathway after estrogen treatment in these cells. Here we reportestrogen-induced phosphorylation of eIF2α in MCF7:5C cells which is activated by PERK- an important branch of UPR. Figure 2 shows time dependent increase in phospho-eIF2α levels after estrogen treatment. As a positive control we used thapsigargin (1μM) a rapid inducer of ER stress and UPR. Since thapsigargin rapidly induces UPR and apoptosis we treated the MCF7:5C cells for 2, 6 and 24 hrs with thapsigargin. On the other hand, estrogen-induced UPR and apoptosis was delayed as a significant increase in phospho eIF2α was evident only after 24 hrs as compared to within 2 hrs after thapsigargin treatment. The cleavage of PARP, a marker of apoptosis, was observed after 48 hrs of estrogen treatment. Interestingly, ATF4 protein, a transcription factor, increases after enhanced phosphorylation of eIF2α. ATF4 can increase the levels of CHOP (also known as DDIT3) which induces apoptosis.

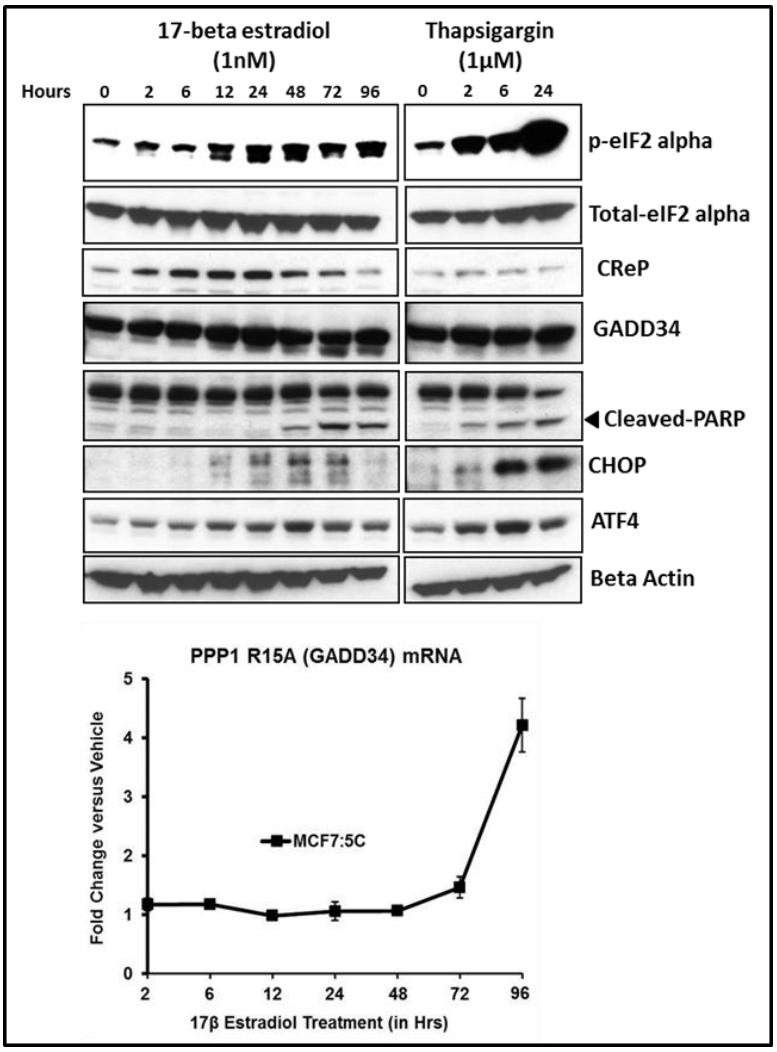


Figure 17 Effect of 17-β estradiol (E2) treatment on phosphorylation of eIF2α and induction of apoptosis.(A) Time dependent effects of E2 on phosphorylation of eIF2α and its down-stream signaling molecules in MCF7:5C cells. Thapsigarin (1µM) was used as a positive control for induction of UPR and apoptosis. MCF7:5C cells were treated with E2 (1nM) or thapsigargin (1µM) for indicated time periods and total protein was extracted. Equal amount of proteins were run on gel and transferred to nitrocellulose membranes by western blotting. The membranes were probed with indicated antibodies. (B) Effect of E2 treatment on the mRNA levels of GADD34 (PPP1 R15A) over 96 hour time period. This data was extracted from the micro-array analysis.

Our results demonstrate that all these factors are involved in the estrogen-induced apoptosis of the MCF7:5C cells.Two enzymes- namely, GADD34 (PPP1 R15A) and CReP are responsible for the de-phosphorylation of eIF2α and help in regain the translation activity of the cell. We checked the regulation of these enzymes in MCF7:5C cells after estrogen treatment and found gradual initial increase in CReP protein levels (upto 24 hrs) but the levels decreased thereafter and reached the levels of vehicle treated cells after 96 hours (Figure 2A). On the other hand, GADD34 (growth arrest and DNA damage-34) protein levels were unchanged throughout the course of estrogen treatment (2-96 hrs). Interestingly, the mRNA levels of GADD34 were induced after 72-96 hours after estrogen treatment (Figure 2B). The GADD34 RNA induction represents a feed-back loop which is a measure for the remediation of ER stress. However, theinduction of mRNA without concurrent increase in the protein levels of GADD34 indicates that the cells are incapacitated to translate the increased mRNA into protein. This may be due to the blockage of global translational machinery of the cells because of high levels of phospho-eIF2α. It is however evident from the figure 4 that neither of the enzymes is able to reduce the phospho-eIF2α levels induced by estrogen in the MCF7:5C cells resulting into a sustained ER stress.

Sustained phosphorylation of eIF2α by salubrinal (SAL) induces apoptosis in MCF7:5C cells.

We tested that if phosphorylation of the eIF2α was sufficient to induce apoptosis of the MCF7:5C

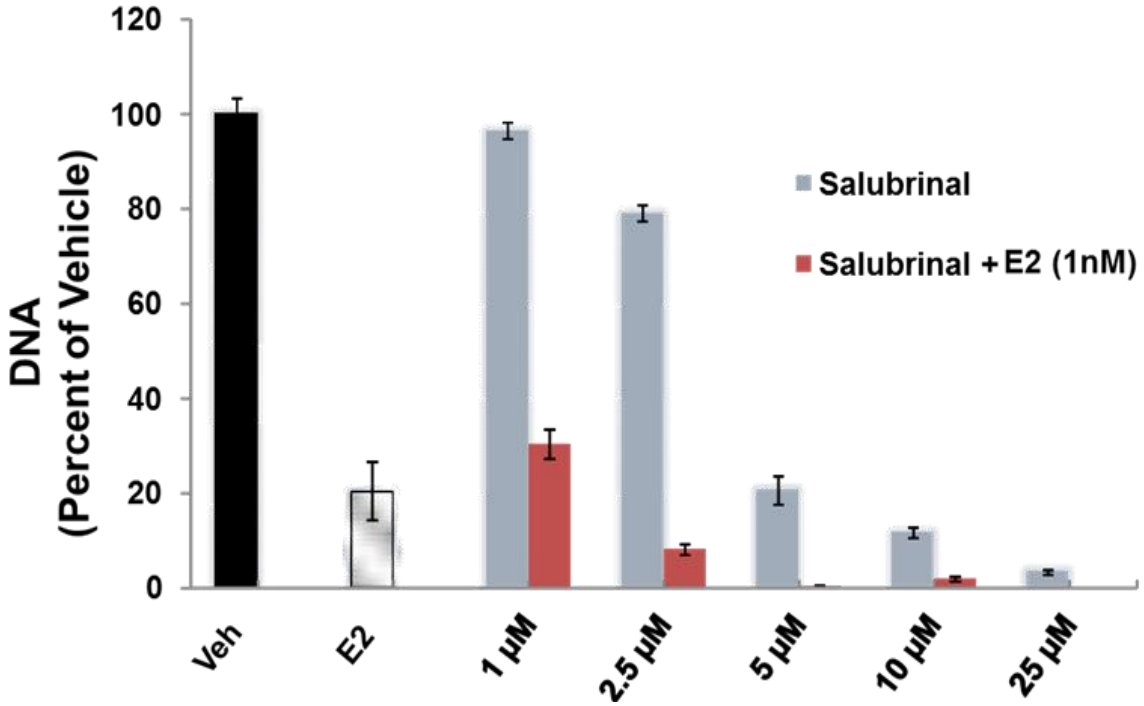


Figure 18 Effect of salubrinal (SAL) in absence or presence of 17-β estradiol (E2; 1nM) treatment on apoptosis in MCF7:5C cells.MCF7:5C cells were treated with vehicle, E2 (1nM), and different concentration of SAL with or without 1nM of E2 over a six day period. Cells were then harvested and Total DNA was assessed as a measure of cell growth/ apoptosis. Data is represented as the percent of DNA of vehicle treatment. All the values are average +/- SD of four replicates.

cells. To induce sustained eIF2α phosphorylation we used the compound salubrinal which is known to inhibit the enzymes GADD34 and CReP (constitutive repressor of eIF2α phosphorylation), which are responsible for de-phosphorylation of eIF2α (Figure 1)(22). Salubrinal was able to induce apoptosis in a dose dependent manner (Figure 18). Five micro-molar of SAL was as efficient as E2 treatment in inducing apoptosis over a period of six day assay. Interestingly, the combination treatment of SAL and E2 was more potent in inducing apoptosis than either of the treatment alone. This suggested that E2 and SAL co-operated in a way which enhanced the apoptotic effect. To further investigate the effect of SAL with or without E2 we determined the levels of phosphorylated eIF2α and its down-stream signaling following the treatment of SAL in presence or absence of E2. SAL treatment alone (2.5µM and 5.0µM) showed

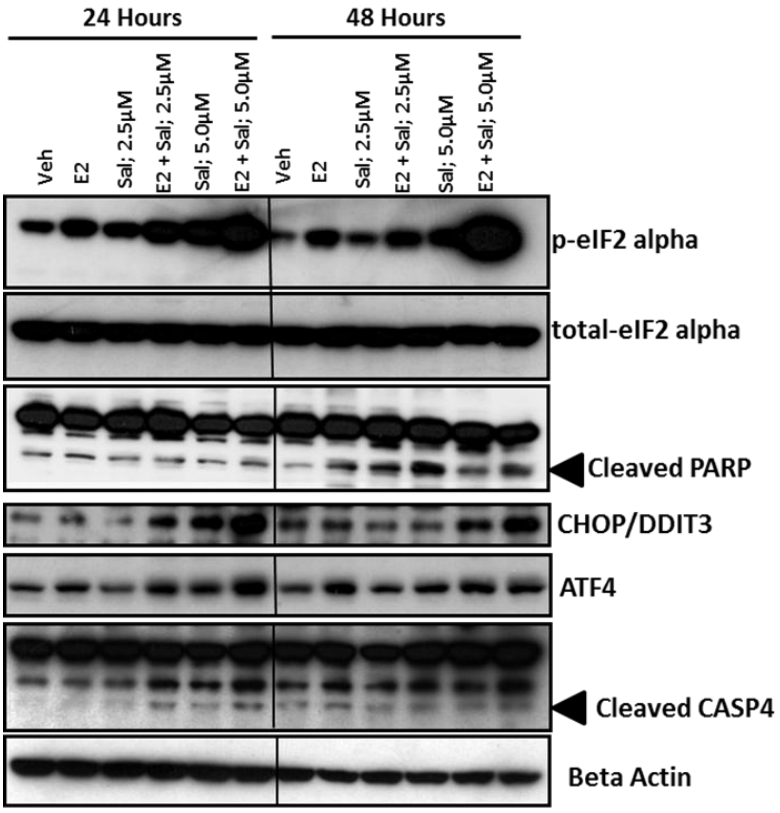


Figure 19 Effect of salubrinal (SAL) in absence or presence of 17-β estradiol (E2; 1nM) treatment on phospho eIF2α and other apoptotic markers in MCF7:5C cells.MCF7:5C cells were treated with vehicle, E2 (1nM), and different concentration of SAL (as indicated) with or without 1nM of E2 for 24 or 48 hrs. Cells were then harvested and total protein was isolated. Equal amount of proteins were run on gel and transferred to nitrocellulose membranes by western blotting. The membranes were then probed with the indicated antibodies.

Phospho-deficient eIF2α prevents apoptotic signaling in MCF7:5C cells.

To further confirm the critical role phosphorylated form of eIF2α (serine 51) we transiently transfected the plasmids harboring a mutation where serine is replaced by alanine at the 51 position, which renders the eIF2α protein as phospho-deficient. We used the wild-type eIF2α plasmid as a control. These plasmids have been previously used by another laboratory. To characterize the plasmids in the MCF7:5C cells we used various amount of plasmids to transfect the MCF7:5C cells using the Fugene HD (Promega, Madison, WI, USA) transfection reagent. After the transfection, we used thapsigargin (1µM) for 4 hours to induce ER stress and eIF2α phosphorylation. The result

(Figure 20) clearly demonstrates that phospho-mutant eIF2α plasmid transfection was able to

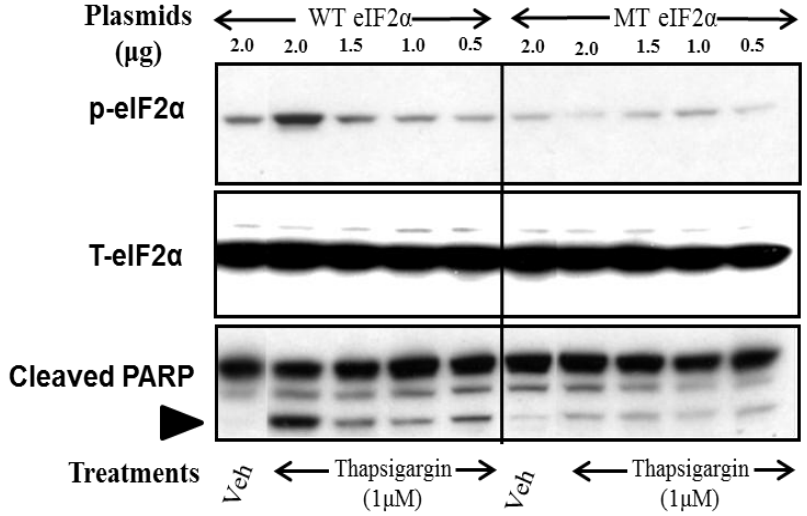


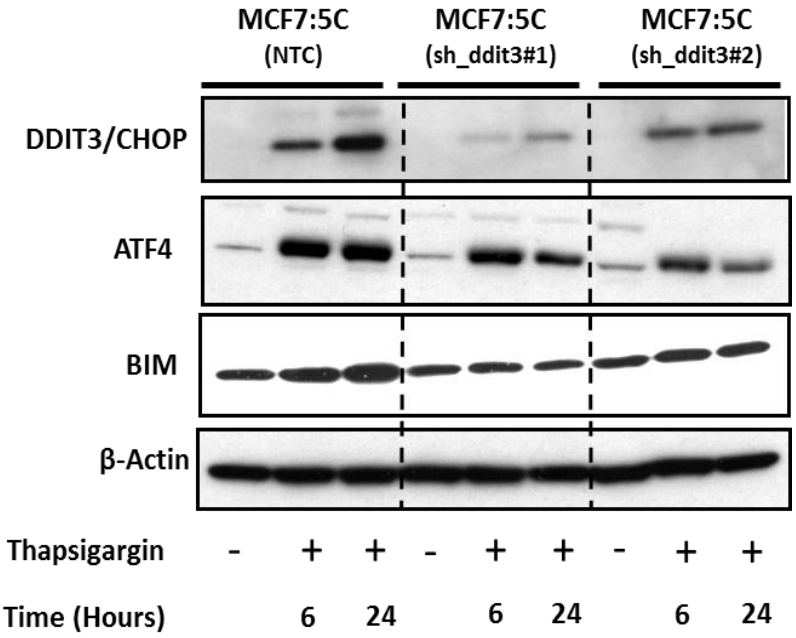
Figure 20. Effect of phospho-mutant eIF2α (serine-51 to alanine; MT eIF2α) transfection on thapsigargin induced UPR and apoptosis in MCF7:5C cells. MCF7:5C cells were transiently transfected with indicated amount of either mutant (MT eIF2α) or wild type (WT eIF2α) plasmids for 48 hrs and treated with thapsigargin (1µM) for 4 hrs and harvested for protein. Equal amount of proteins were run on gel and transferred to nitrocellulose membranes by western blotting. The membranes were then probed with the indicated antibodies.

significantly reduce the eIF2α phosphorylation as compared to wild type eIF2α transfection after 4 hrs of thapsigargin treatment. A sharp reduction in cleavage of PARP (Figure 20) was also noted in the MCF7:5C cells transfected with the mutant plasmid as compared to wild type plasmid. This indicated that phosphorylation of serine 51 of eIF2α was critical in mediating the UPR induced apoptosis in the MCF7:5C cells. Presently, we are conducting the experiments with estrogen treatment under similar conditions to establish that serine 51 phosphorylation of eIF2α is critical for estrogen induced UPR and apoptosis.

Role of C/EBP homologous protein (CHOP)/ DDIT3 in UPR- induced apoptosis in MCF7:5C cells.

Elevated CHOP level is down-stream of ATF4, which in turn is increased following sustained high levels of phosphorylated eIF2α. We therefore, investigated the critical role of CHOP in induction of the ER stress mediated apoptosis in the MCF7:5C cells. For this we first made stably transfected MCF7:5C cells which expressed short hairpin (sh)- RNA to knock-down CHOP levels. Two

Figure 21 Effect of CHOP / DDIT3 knock-down on thapsigargin-induced ER-stress and apoptosis in MCF7:5C cells. MCF7:5C cells were stably transfected using lentivirus containing short hairpin (sh) RNA against ddit3 gene for knock-down of CHOP. Two separate shRNA was used to generate sh_ddit3 #1 and sh_ddit3 #2 respectively. A non-targeting shRNA was used to generate the non-targeting control (NTC) cells for comparison. Thapsigargin (1µM) was used to treat the cells for 6 and 24 hrs. The cells were then harvested for protein. Equal amount of proteins were run on gel and transferred to nitrocellulose membranes by western blotting. The membranes were then probed with the indicated antibodies.



separate shRNA(sh_ddit3 #1 and sh_ddit3 #2) were used to generate two stable cell lines with CHOP depletion. The negative control was generated by stably transfecting MCF7:5C cells using a non-targeting sh RNA (NTC). To characterize the cells we treated it with thapsigargin (1µM) for 4 and 24 hrs which induces ER stress and CHOP protein. As expected CHOPwas drastically induced in NTC cells after 6 and 24 hours of thapsigargin treatment (Figure 21) whereas the MCF7:5C cells stably transfected with ddit3 shRNA(sh_ddit3 #1 and #2) showed very low levels of CHOP protein. The sh_ddit3 #1 cells revealed more effective knock-down of CHOP protein as compared to sh_ddit3#2. We checked the ATF4 levels which is the up-stream factor of CHOP and found that thapsigargin was able to induce ATF4 levels after 6 and 24 hrs in the NTC as well as the CHOP deficient MCF7:5C cells although the level of induction was modestly lower in the latter cell lines. One of most important down-stream target of CHOP is BIM (Bcl2 interacting mediator of cell death) which plays pivotal role in induction of apoptosis after ER stress (23). Previous study from our laboratory has determined that BIM knock-down prevents estrogen-induced apoptosis of MCF7:5C cells(3). Therefore we determinedthe levels of BIM protein in the CHOP deficient MCF7:5C cells after thapsigargin induced ER stress. As expected, in the MCF7:5C cells depleted of CHOP (Figure 6) failed to induce BIM whereas the NTC cells showed a significant increase in BIM levels. These results strongly point towards the facts that CHOP deficient MCF7:5C cells may be resistant to ER-stress induced apoptosis. Further detailed studies are underway to confirm that the same pathway is employed for the estrogen induced ER stress and apoptosis of MCF7:5C cells.

Overall, this section has established that estrogen-induced apoptosis of MCF7:5C cells is mediated by ER-stress and UPR. PERK mediated eIF2α phosphorylation is the key pathway by which it activates the apoptotic signaling. Moreover, pharmacological intervention which increases the phosphorylated status of eIF2α, is sufficient to induce apoptosis in the MCF7:5C cells. We have also studied the downstream effectors of this pathway and determined that ATF4, CHOP and BIM play important roles in implementing the ER-stress mediated apoptosis in these cells.

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

Task 2d: (Sengupta and Jordan) – To confirm and validate developing pathways of E_2-induced breast cancer cell survival and apoptosis.

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

Cyclin Dependent Kinase-9 Mediated Transcriptional De-regulation of cMYC as a Critical Determinant of Aromatase-Inhibitor Resistance in Estrogen Receptor Positive Breast Cancers

Introduction:

Tamoxifen and Aromatase inhibitors which block the estrogen receptor action or estrogen synthesis respectively, are the therapy of choice for the adjuvant treatment of estrogen receptor alpha positive (ERα+) breast cancers (16, 24, 25). Unfortunately, tumor resistance to endocrine therapies, invariably occurs and this represents a major clinical concern for the survivorship of the patients. In case of post-menopausal hormone receptor-positive localized breast cancer patients treated with adjuvant endocrine therapies, 15 % of patients relapsed within 5 year of treatment (26, 27). The majority of hormone receptor positive advanced breast cancer (ABC) patients treated with endocrine therapies report disease progression within 2-3 years of treatment (28-30). Aromatase inhibitors are marginally superior to tamoxifen treatment in ABC but eventually 68% to 75% patients show disease progression within three years (29). It is therefore appropriate to study the underlying molecular mechanisms which contribute to estrogen independence and acquire resistance to identify novel therapeutic targets for the endocrine therapy resistant breast cancers.

Recent clinical studies have found over-expression of the cMYC oncogene and the genes regulated by cMYC as one of the major predictor in the aromatase inhibitor resistant breast cancers (31-33). Besides endocrine resistance, cMYConcoprotein have been found to regulate the expression of "poor-outcome" signature genes responsible for metastasis (34). A meta-analysis reported that amplification of cMYC in breast cancer was significantly associated with risk of relapse and death (35). A recent report has also indicated that gain of cMYC is associated with the progression of invasive ductal carcinoma (IDC) from the ductal carcinoma in situ (DCIS) (36).
Although this information provides a therapeutic opportunity to block the growth of the resistant breast cancer cells by targeting the cMYC, it has not been successful due to lack of a drug-able domain in its 'basic helix-loop-helix' structure (37). Additionally, unacceptable toxicity is associated with cMYC inhibition, as the protein is critically involved in proliferation and regeneration of normal adult tissues (38, 39). Other approaches such as synthetic lethality (40) and modulating chromatin-dependent signal transduction have been used to circumvent direct targeting of cMYC(41).

To determine the relevance and mechanism of the cMYC over-expression in imparting estrogen-independence to the aromatase-resistant breast cancer cells we used the long-term estrogen deprived MCF7 cells, known as MCF7:5C cells (42), which can proliferate in an estrogen-free environment in vitro and are capable of forming xenograft tumors in ovariectomized, athymic mice (3). Paradoxically, these cells undergo apoptosis in response to estrogen treatment *in vitro* as well as *in vivo*(3, 4).

This study dissects the molecular mechanism involved in the transcriptional over-expression of cMYC oncogene in MCF7:5C cells, which imparts estrogen-independence. In addition, we present CDK9 as a potential target for therapeutic intervention which can inhibit the deregulated transcription of cMYC leading to complete inhibition of estrogen-independent proliferation of the aromatase-resistant breast cancer cells.

Work Accomplished

Estrogen-independent growth of long-term estrogen deprived ERα+ breast cancer cells and levels of cMYC

MCF7:5C cells, which are grown in the absence of estrogen for more than a year (42), were used to compare the estrogen-independent growth versus parental MCF7 cells. A comparison of growth of MCF7:5C cells and MCF7 cells in absence of phenol red and estrogen (Figure 22A) shows approximately 5 fold higher proliferation of MCF7:5C cells over six day period. Cell cycle analysis revealed that the increase in growth was due to ~3 fold higher number of "S" phase cells in MCF7:5C cells (Figure 22B). We further investigated the mRNA and protein levels of cMYC in MCF7:5C and parental MCF7 cells. As evident from the figure 1C and 1D, the mRNA and protein levels of cMYC were approximately 3-4 fold higher in MCF7:5C cells as compared to MCF7 cells.

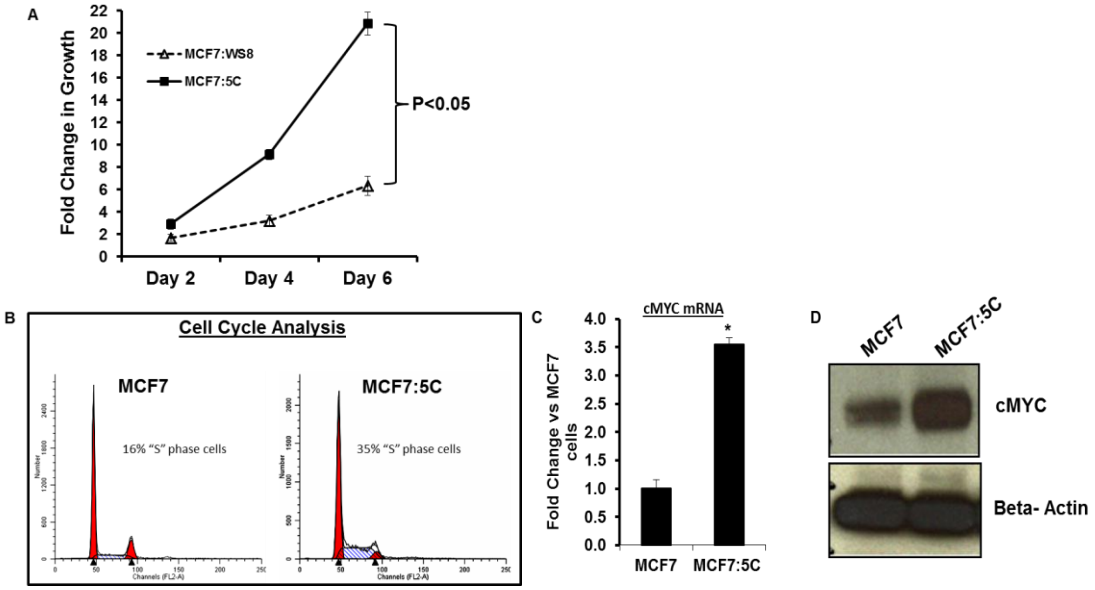


Figure 22: Estrogen independent growth and levels of cMYC mRNA and protein. (A) Estrogen independent growth of MCF7:5C and MCF7 cells over 6 day period. Un-treated cells were grown and total DNA was measured on day 2, 4 and 6 after seeding. The data is represented as fold change in growth. (B) "S" phase cells were assessed by cell cycle analysis of MCF7:5C and MCF7 cells growing in absence of estrogen. (C) cMYC mRNA levels were measured in MCF7:5C cells using RT-PCR. Data is represented as fold change in cMYC mRNA versus MCF7 cells. (D) Western blot of cMYC protein in MCF7 and MCF7:5C cells. Beta actin was used as a control for loading.

Inhibition or depletion of cMYC blocks estrogen-independent proliferation of MCF7:5C cells

We determined the functional role of cMYC over-expression in estrogen-independent growth of long-term estrogen deprived MCF7:5C cells and the parental MCF7 cells. First, we blocked the cMYC action in the cells using a pharmacological inhibitor 10058-F4 which has been shown to specifically inhibit actions of cMYC by blocking its interaction with MAX (43) and stabilize the MYC monomer (44). Treatment with 10058-F4 showed that the compound selectively inhibited the estrogen-independent growth of MCF7:5C cells in a dose-dependent manner as compared to MCF7 cells over a four day period (Figure 23A). Cell cycle analysis confirmed that the decrease in proliferation resulted from a reduction in "S" phase cells in 10058-F4 treated MCF7:5C cells (Figure

23B). The reduction was in MCF7:5C cells was a 60% decrease in ‘S’ phase cells as compared to only a 10% decrease in parental MCF7 cells. We also used the genetic approach to

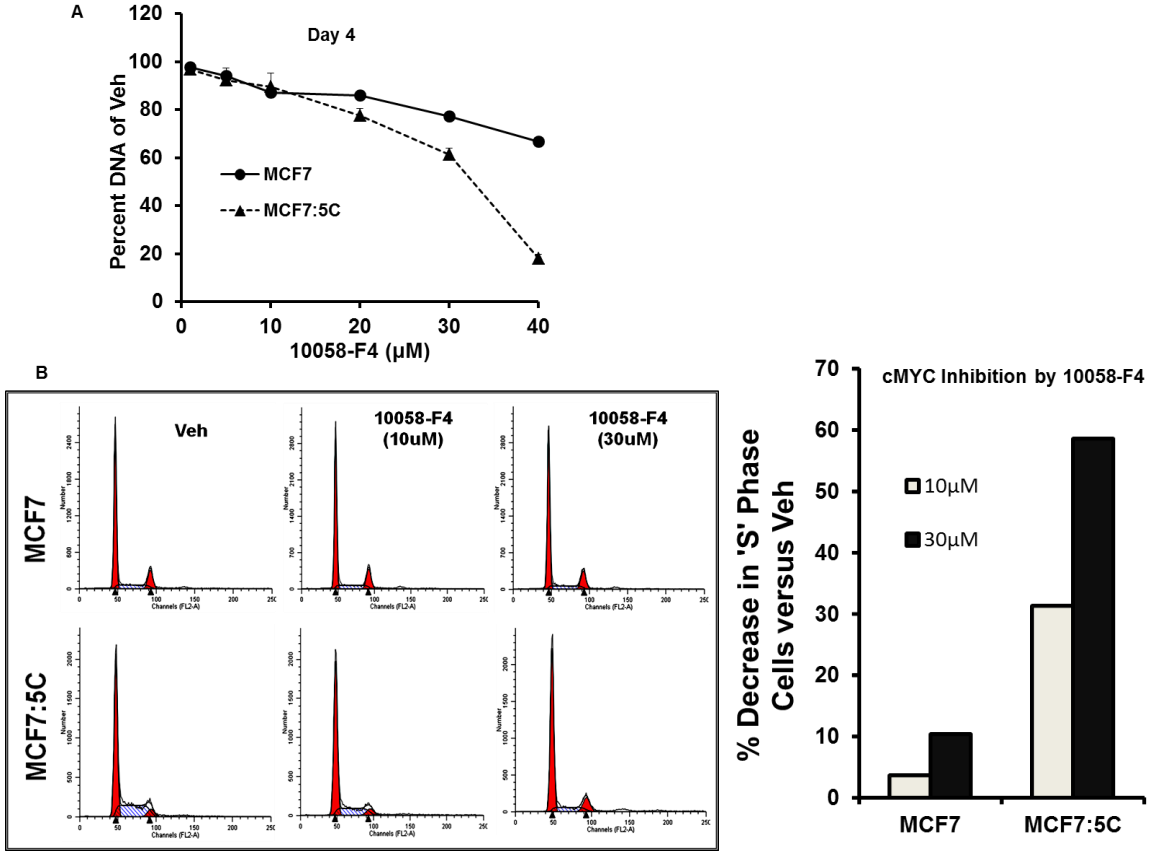


Figure 23: Pharmacological inhibition of cMYC preferentially inhibits estrogen independent growth of MCF7:5C. (A) Total DNA was measured from the MCF7 or MCF7:5C cells after four days of treatment with cMYC inhibitor (10058-F4) with indicated concentration. (B) “S” phase cells were assessed using cell cycle analysis of MCF7 and MCF7:5C cells treated with indicated concentration of cMYC inhibitor. (C) Graphical depiction of decrease in “S” phase cells in MCF7 and MCF7:5C cells after treatment with cMYC inhibitor.

confirm the role of cMYC in MCF7:5C cells, by depleting cMYC levels using short interfering RNA (siRNA). As evident from figure 18A, two different siRNA against cMYC depleted the levels of its protein in MCF7:5C cells. Consistent with the observation of pharmacological intervention, depletion of cMYC led to an inhibition of proliferation and concurrent decrease (50-75%) in number of ‘S’ phase cells (Figure 24B and 24C, respectively).

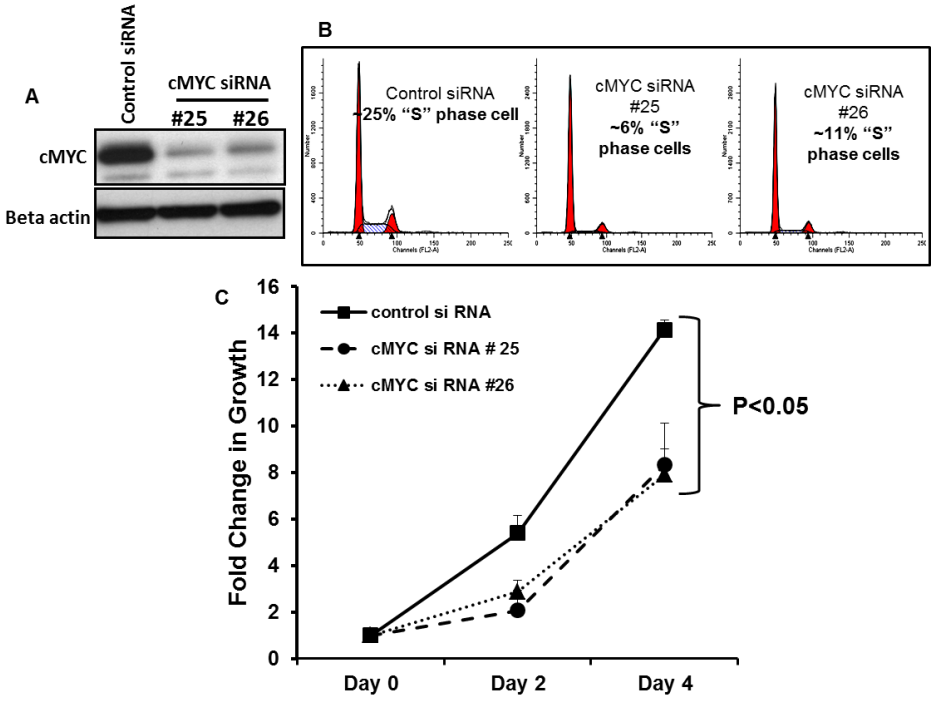


Figure 24: Depletion of cMYC and estrogen independent growth. (A) Knock down of cMYC protein levels after two different siRNA mediated depletion of cMYC. (B) Assessment of "S" phase cells using cell cycle analysis 48 hours after siRNA mediated depletion of cMYC using two different siRNA. (C) Growth of MCF7:5C cells were measured after depletion of cMYC using two separate siRNA and compared with control non-targeting siRNA. Total DNA was assessed after 2 and 4 days of growth and the fold change was calculated after dividing by the DNA content on the day of start of the experiment (Day '0').

cMYC gene expression correlates with relapse free survival (RFS) in endocrine therapy but not chemotherapy treated patients

We used an up-dated on-line tool (www.kmplot.com) which has a combined data set from various annotated breast cancer studies and can be used to study the association of a single gene with patients outcome using various user defined parameters (45). We tested the RFS of the breast cancer patients with high levels of cMYC gene expression. The top 25% percent highest expressing cMYC patients (top quartile) were compared with the rest of the 75%. Kaplan-Meier plots (Figure 25) reveal that

high levels of cMYC expression is associated with poor RFS (P value; 0.0093) in

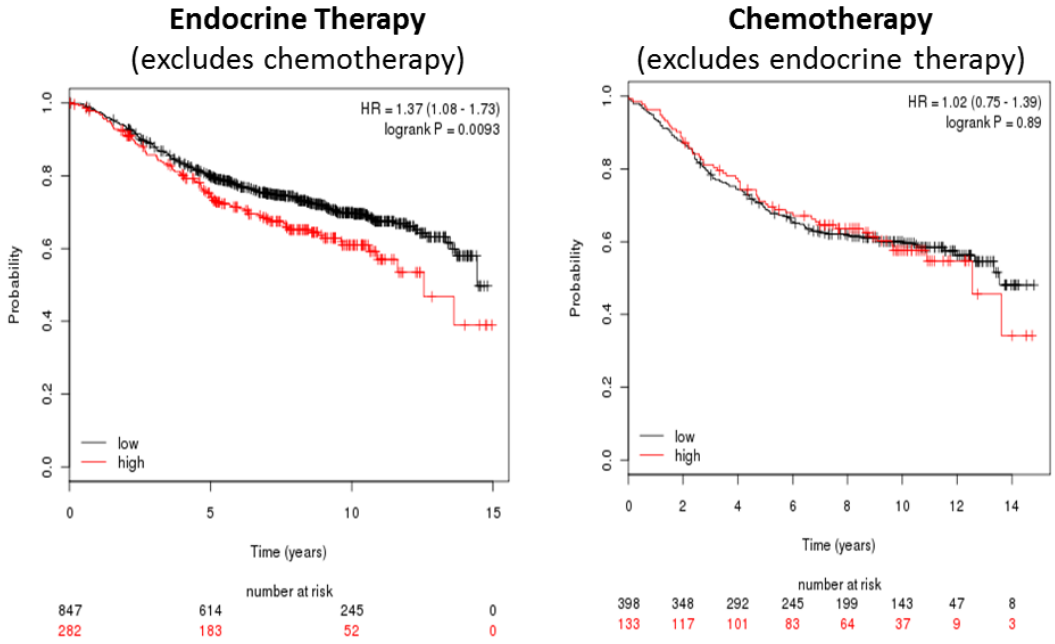


Figure 25: High cMYC expression and relapse free survival (RFS) in ERα+ breast cancer patients. Kaplan meier plots for RFS were generated using the on-line tool (www.kmplot.com) with combined data sets of various breast cancer studies as described in the materials and methods. Top quartile (25%) patients with highest cMYC levels (red line) were compared with rest of the patient population (black line) treated with either endocrine therapy only (left panel) or with chemotherapy only (right panel).

1129 patients treated with endocrine therapy only (Tamoxifen or AIs) whereas this association was not observed in the 531 patients (P value; 0.89) treated with chemotherapy only. This results supported our hypothesis that cMYC over-expression was key factor for the resistance and failure of endocrine therapy.

Stability of cMYC mRNA in MCF7:5C and MCF7 Cells

The steady levels of cMYC mRNA is a function of rate of transcription and its rate of degradation. Therefore to determine the basis of the higher steady state levels of cMYC mRNA in MCF7:5C cells, we performed pulse-chase experiment to study the rate of degradation of cMYC mRNA using the nascent RNA capture kit (Invitrogen; cat # C10365). The cells were pulsed with 5-ethynyl uridine (EU) (a uridine analog) for 24 hrs and thereafter chased with fresh normal media for 60 and 120 minutes. The EU-labeled RNA was isolated and levels of cMYC RNA were measured using RT-PCR. 18s ribosomal RNA was used as an internal control to normalize each sample. The rate of degradation of cMYC mRNA was not significantly different between MCF7:5C cells and its parental control MCF7 cells after 60 and 120 minutes of chase (Figure 26).

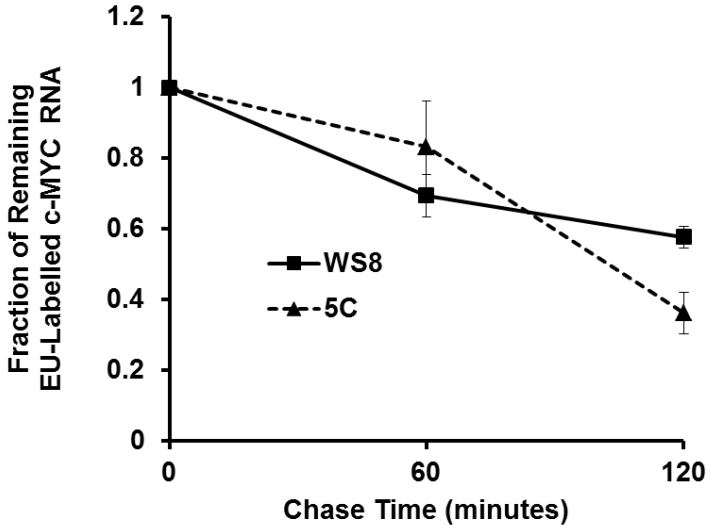


Figure 26: cMYC mRNA stability in MCF7 and MCF7:5C cells. Pulse and chase experiment was performed using 5-ethynyl uridine (uridine analog) to pulse the cells for 24 hours and chased for 60 and 120 minutes with fresh media. The data represents fraction of 5-ethynyl uridine containing cMYC RNA remaining relative to the start of chase time.

To further determine the mechanism of steady-state transcriptional over-expression of the cMYC mRNA in MCF7:5C cells we probed the proximal promoter of the cMYC gene (Figure 27A) in terms of recruitment of phosphorylated serine-5 and phosphorylated serine-2 RNA polymerase II, which is responsible for the initiation and the elongation of the transcription of RNA, respectively. ChIP assay using phospho-specific RNA polymerase II antibodies revealed that in MCF7:5C cells

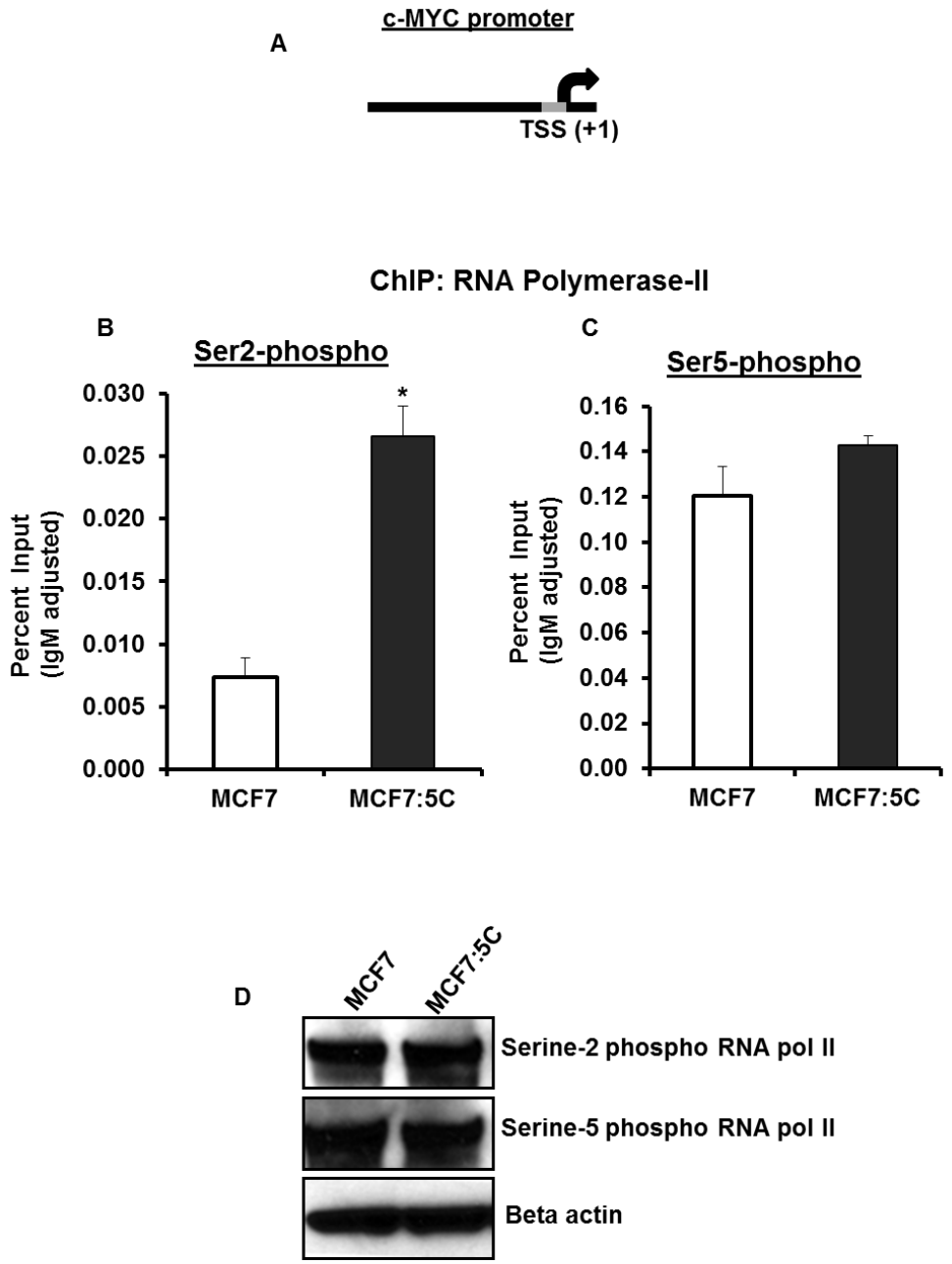


Figure 27 Recruitment of serine-5 and serine-2 -phosphorylated RNA polymerase II at the cMYC promoter. (A) Schematic presentation of cMYC promoter showing the transcription start site (TSS). The grey box represents the region (~150bp upstream of TSS) probed using real-time PCR following ChIP assay. (B) Recruitment of serine-2 phosphorylated RNA polymerase II and (C) serine-5 phosphorylated RNA polymerase II was assessed by ChIP assay followed by real-time PCR in MCF7 and MCF7:5C cells. Values are represented as percent input of the starting chromatin, adjusted for control IgM recruitment for each sample. (* p<.05 versus MCF7 cells) (D) Total protein levels of serine-2 and serine-5 phosphorylated RNA polymerase II in MCF7 and MCF7:5C cells.

the recruitment of serine-2 phosphorylated RNA polymerase II was more than 3 fold higher than parental MCF7 cells (Figure 27B). However, no difference was observed in the recruitment of

serine-5 phosphorylated RNA polymerase II at the cMYC promoter in MCF7:5C and MCF7 cells (Figure 27C). We further ascertained that the total levels of phosphorylated serine-2 or serine-5 RNA polymerase was not different in MCF7:5C cells as compared to MCF7 cells (Figure 27D).

Levels of cyclin dependent kinase 9 (CDK9) in MCF7:5C and MCF7 cells and its role in estrogen-independent growth

CDK9 is a major kinase which is responsible for the phosphorylation of serine-2 RNA polymerase II but not serine-5 RNA polymerase II (46, 47) and the elongation of RNA transcripts (48). We therefore examined the total and the phosphorylated CDK9 levels in MCF7:5C and MCF7 cells.

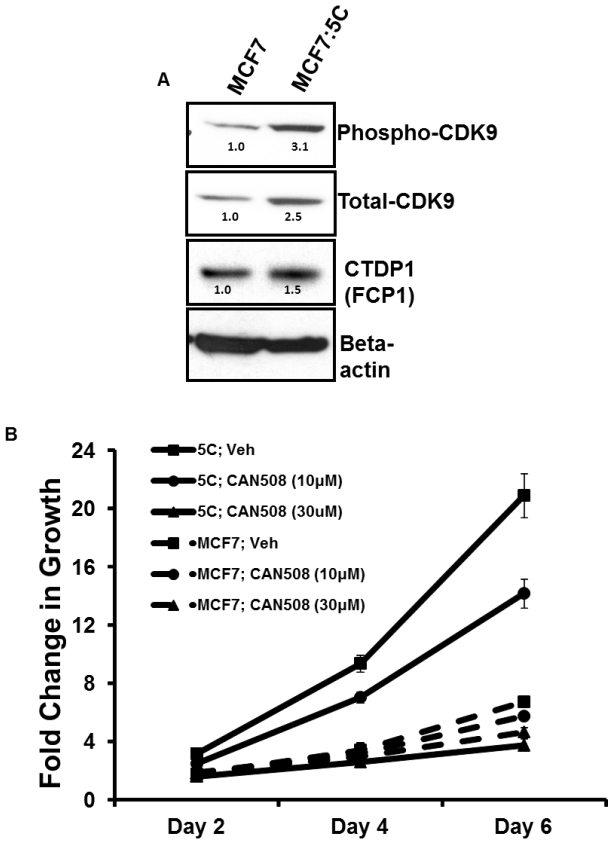


Figure 28: Total CDK9 levels and effect of its inhibition on estrogen-independent growth. (A) Protein levels of phospho and total CDK9 and CTDP1 was assessed using western blotting in MCF7 and MCF7:5C cells. The numbers above each band correspond to the fold change in protein levels versus MCF7 cells adjusted for beta actin levels for each sample. (B) Total DNA was measured to assess the growth of MCF7 and MCF7:5C cells after 2, 4 and 6 days of treatment with indicated doses of the CDK9 inhibitor, CAN508.

The total as well as the phosphorylated CDK9 levels was elevated in MCF7:5C cells by 2.5 and 3.1 fold respectively (Figure 28A). We also observed a slight increase in the levels of CTDP/ FCP1 protein in MCF7:5C cells, which is known to dephosphorylate CDK9 (46) (Figure 28A). Interestingly, FCP1 has also been reported to stimulate transcription elongation (49). Next, we used specific potent, competitive inhibitor of CDK9, known as CAN 508 (50) to study the role of CDK9 in estrogen-independent growth of MCF7:5C cells and compared it with the parental MCF7 cells. CAN 508 treatment completely block the growth of the MCF7:5C cells in a selective and dose dependent manner (Figure 28B). By contrast, minimal growth inhibitory effects were observed in case of parental MCF7 cells (Figure 28C).

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

Inhibition of CDK9 in MCF7:5C cells by using 100 μM CAN 508, resulted in approximately 60% decrease in cMYC mRNA within one hour of treatment (Figure 29A). This was followed by

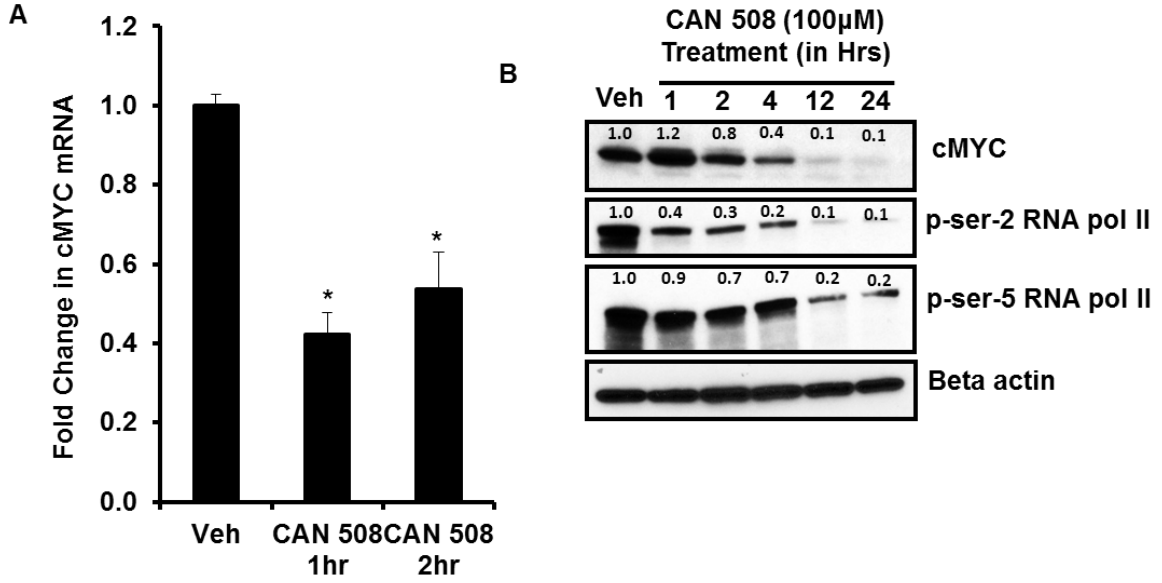


Figure 29: CDK9 inhibition reduces cMYC mRNA and protein. (A) Levels of cMYC mRNA was measured by quantitative RT-PCR in MCF7:5C cells after one and two hrs of CDK9 inhibition by 100μM of CAN508.(* p<.05 versus vehicle (Veh) treatment.(B) Protein levels of cMYC, phospho-serine-2 and serine-5 RNA polymerase II after inhibition of CDK9 by 100μM of CAN508 for indicated time points. The numbers above each band correspond to the fold change in protein levels versus vehicle (Veh) treatment adjusted for beta actin levels for each sample.

dependent decline in cMYC protein levels (Figure 29B). Concomitant inhibition of serine-2 phosphorylated RNA polymerase II CTD was also observed within an hour of treatment (Figure 14B) indicating its role in cMYC transcription. As evident from figure 29B, serine-5 phosphorylation of RNA polymerase II CTD was not much altered within 4 hours of CDK9 inhibition. Although later time points showed marked reduction in serine-5 phosphorylation, along with serine-2 phosphorylation which was most likely due to secondary effects.

In this section, we have delineated the transcriptional mechanism of cMYC over-expression, following long-term estrogen deprivation of hormone responsive ERα+ breast cancer cells, and propose a model (Figure 30) which mediates the estrogen independent proliferation of these cells. We suggest that there will be a potential clinical benefit by using CDK9 inhibitors in the treatment of endocrine therapy resistant breast cancers.

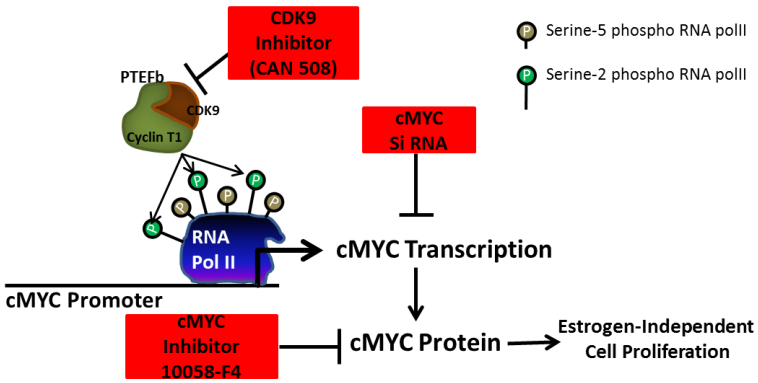


Figure 30: Proposed model of cMYC transcriptional regulation in MCF7:5C cells. The cartoon depicts our findings on the CDK9 mediated cMYC transcriptional regulation and its role in estrogen-independent growth of the MCF7:5C cells.

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

Task 2e :Obiorah and Jordan – To determine the trigger point for estradiol induced apoptosis and explore differential gene expression in comparison to cytotoxic chemotherapy induced apoptosis.

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

DELAYED TRIGGERING OF ESTROGEN INDUCED APOPTOSIS THAT CONTRASTS WITH RAPID PACLITAXEL INDUCED BREAST CANCER CELL DEATH

INTRODUCTION

Endocrine therapy remains the standard of care in the treatment of estrogen receptor (ER) positive breast cancer(51). Tamoxifen inhibits estradiol (E_2)-induced tumor growth; but continuous tamoxifen treatment of nude mice with transplantable ER positive tumors results in tumor growth with either E2 or tamoxifen within a year (52, 53). Retransplantation of ER positive tamoxifen resistant tumors demonstrated growth that occurred with either physiologic E_2 or tamoxifen (53, 54). After 5 years of retransplantation and tamoxifen treatment, these serially transplanted tamoxifen stimulated tumors grew in response to tamoxifen but paradoxically rapidly regressed with physiologic E_2 treatment (55). Development of acquired resistance to long term (5 years) antihormonal therapy in breast cancer causes a reconfiguration of the tumor cells that now makes them vulnerable to physiologic estrogen induced apoptosis. We have previously shown that MCF7 breast cancer cells that are resistant to long term estrogen withdrawal also undergoes apoptosis in response to E_2(56, 57). Clinical trials have evaluated this concept for patients with advanced breast cancer who have acquired resistant to antihormone therapy. A clinical study (58)found that high dose diethylstilbestrol induced an objective response in 30 percent of postmenopausal breast cancer patients who had previous exhaustive antihormone therapy. Ellis and colleagues (59)showed that postmenopausal women with aromatase inhibitors resistant metastatic breast cancer, had a 29% clinical benefit with low dose estrogen (6mg daily) but the same clinical benefit but more side effects with high dose estrogen (30mg daily). Additional clinical evidence for the antitumor action of low dose estrogen comes from the Women Health Initiative (WHI) trial (60) which compared conjugated equine estrogen (CEE) therapy with placebo in hysterectomised postmenopausal women. The original report (61)noted a paradoxical decrease in incidence of breast cancer compared to combination CEE and progestin (62, 63)and this observation was subsequently supported by results from the Million Women Study(64). In neither clinical study (61, 64)was a molecular mechanism offered to explain the apparent anomaly that CEE alone does not induce a profound significant increase in breast cancer risk. However reanalysis of the mature data from the WHI CEE alone study (65) now demonstrates a persistent and sustained decrease in the incidence and mortality of breast cancer in women who received estrogen alone therapy. We recently reported that constituents of CEE cause apoptosis in long term estrogen deprived MCF7 cells (66). Given that these laboratory observations translate to clinical benefit for patients, it is appropriate to investigate the molecular events that precede the induction of apoptosis by E_2.

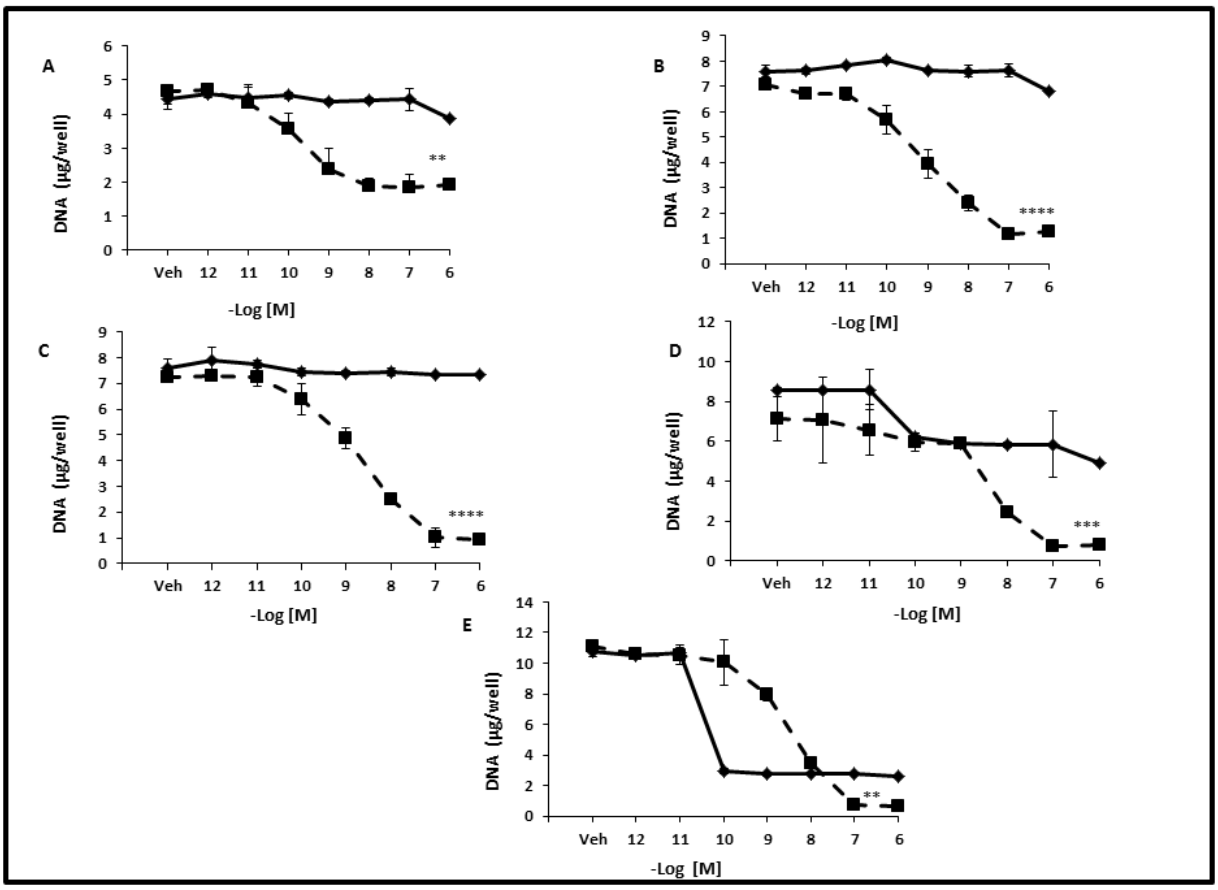
Cancer chemotherapy induces rapid death of neoplastic cells(67, 68), but estrogen induced apoptosis, in contrast, is a delayed event. We (69) recently identified the total gene activation sequence that occurs over a 7 day period during E_2 induced apoptosis using long term estrogen deprived estrogen responsive breast cancer cells. Endoplasmic reticulum stress (ERS) is induced by E_2, which activates unfolded protein response leading to upregulation of mitochondrial proapoptotic genes. Involvement of the extrinsic pathway in E_2 induced apoptosis have been implicated but its exact role is not clearly defined(70, 71). However, nothing is known on the effect of cytotoxic chemotherapy in the MCF7:5C cells. Paclitaxel, a member of the drug family, the taxanes is a mitotic spindle inhibitor that prevents destabilization of microtubules(72, 73). Taxanes are used extensively as part of combination therapy in metastatic breast cancer (74, 75)and are the gold standard in the adjuvant therapy of early breast cancer where they decrease risk of cancer recurrence and mortality(76, 77).

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

WORK ACCOMPLISHED

Antiproliferative effects of estradiol and paclitaxel in MCF7:5C cells

Long term estrogen deprived MCF7 cells grow independently of estrogens and undergo apoptosis in the presence of E_2(56). We sought to compare the antiproliferative activity between paclitaxel and E_2 in the MCF7:5C cell line and explore their potential to induce apoptosis. Paclitaxel induced rapid inhibition of growth in a concentration dependent manner with maximum inhibition at 0.1μM. Fifty percent growth inhibition was achieved by 24h (Fig. 31A), which increased to almost 100% after 48h of treatment (Fig. 31B). In contrast, E_2 achieved maximal growth inhibition at 0.1nM, and did not quantitatively prevent cell proliferation until after 72hrs (Fig. 31C). Twenty five percent of growth inhibition occurred at 96h with E_2 treatment (Fig.31D) and this increased to 80% at the 120h time point (Fig.31E).



Determination of the critical trigger point of estradiol induced apoptosis

Figure 31 Effect of E2 and Paclitaxel on the growth characteristics and apoptosis in the MCF-7:5C Cells. MCF-7:5C cells were seeded in 24 well plates and treated with E2 and paclitaxel over a range of doses and cells were harvested after (A)24H (B) 48h (C) 72h (D) 96h and (E) 120h. Data points shown are the average of 3 replicates +/- SD. [p<0.02. *** p<0.003, ****p<0.001]**

Determination of the critical trigger point of estradiol induced apoptosis

Although E_2 treatment induces apoptosis of MCF7:5C cells in a concentration dependent manner by the end of seven days treatment, the cells are unresponsive to the antiestrogen, 4OHT. Rather 4OHT blocks E_2 mediated apoptosis. To further investigate the delayed response to E_2 mediated apoptosis and determine the critical trigger point for E_2 induced apoptosis, we used 4OHT to block and rescue the cells from the apoptotic effect of E_2. In this way, we established when the cells are committed to cell death. MCF7:5C cells were treated with 1 nM of E_2 and subsequently 1µM of 4OHT was used to block the apoptotic effects of E2 at the indicated time points over a range of 96h after the addition of E_2. Cells were then all collected for DNA assay on day 7. Apoptosis triggered by E_2 was competitively inhibited and rescued for up to 24hrs, and thereafter, it lost the ability to rescue cells committed to E_2 induced apoptosis (Fig. 32). At 36 hrs, the cells are committed to apoptosis despite the antiestrogenic action of 4OHT.These data suggest that the critical trigger for the commitment of the cell to the induction of apoptosis by E_2 lies at this time point.

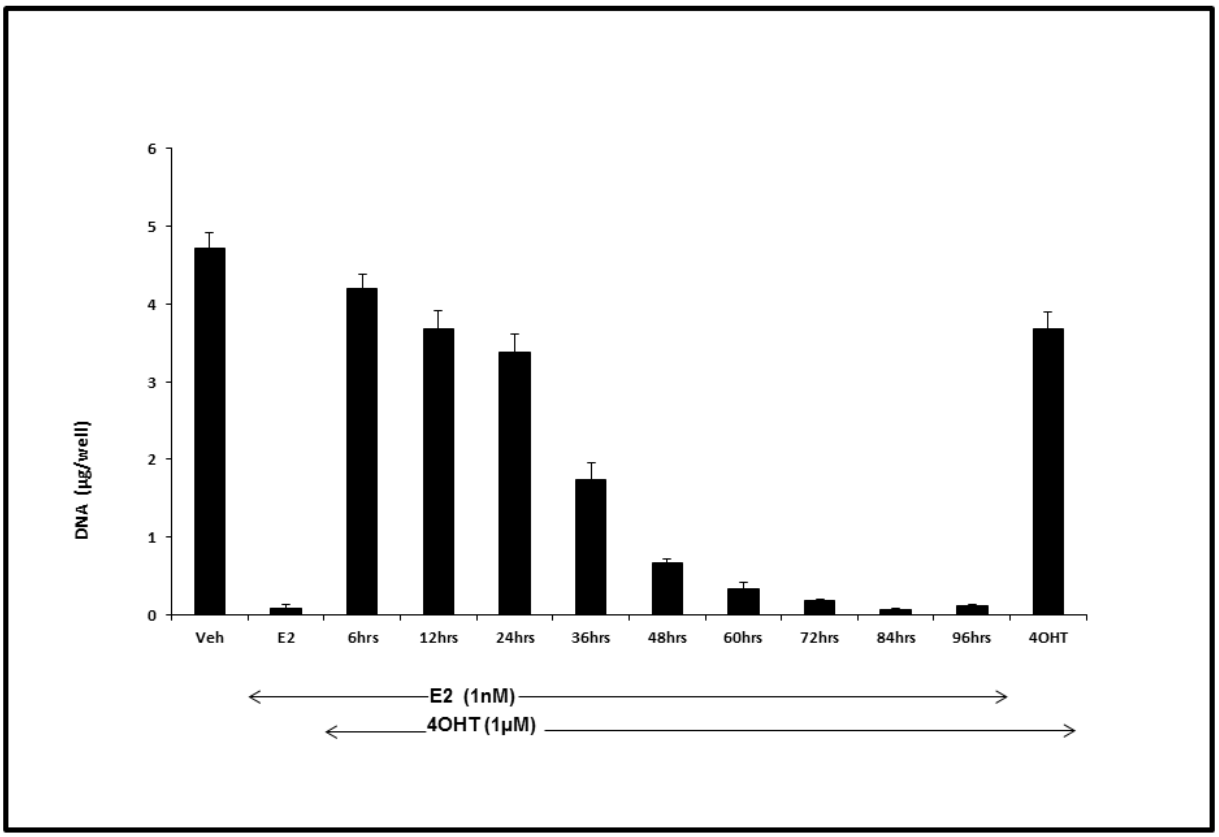


Figure 32. Deciphering the trigger point for E_2 induced apoptosis. Cells were treated with E_2 (1nM) alone and 1µM 4OHT was added and used to block and reverse E_2 action at 6h, 12h,24h,36h, 48h, 60h,72h,84h,and 96h. The cells were harvested after 7 days of treatment. The extent of apoptosis was determined by measuring the DNA content of the remaining cells in each well. The experiment was done in triplicates and the data represent the mean of three independent experiments with 95% confidence intervals. The trigger point for E_2 mediated apoptosis was elucidated at the time when the apoptotic effects of E2 could not be blocked by 4OHT.

Differential gene expression of E2 mediated apoptosis at the critical trigger point

To identify genes associated with E2-induced apoptosis with a particular focus on the critical trigger time point, differential regulation of apoptotic gene expression in response to E2 was interrogated in the MCF7:5C cells. Cells were treated with 1nM E_2or without E_2 (control), 1µM 4OHT and E_2 in combination with 4OHT over a 48 h time course consisting of 3 time points(24,36 and 48h).Gene expression was measured using customized RT-PCR profiler assay kits that include 384-well plates to profile expression of 370 apoptosis related human genes (see Methods). The PCR arrays performs gene expression analysis with real-time PCR sensitivity and the multi-gene profiling capability of a microarray. Analyses for significantly regulated genes are described in the Materials and Methods. At 24h, as expected significant evidence of apoptotic gene induction is not apparent, rather proapoptotic genes such as BAD,BCL2L10 and Caspases 1, 9 and 10 are differentially downregulated by E_2 (STable 1). TNF related genes, TNFRSF8 and TNFSF14 are induced by both E_2 and 4OHT and they do not play a definitive role in the TNF mediated apoptosis but rather are involved in the T cell response. Interestingly, at 36hrs (Fig. 33A), which represents the trigger point for apoptosis, E_2 induces proinflammatory genes such as CEBPB, CEBPG, and DAPK1 and

endoplasmic reticulum stress (ERS) genes; DDIT3 and ERN 1. BCL2L11(BIM), an important member of the mitochondrial pathway and an apoptosis activator is also upregulated by E2, suggesting an early involvement of the intrinsic pathway. Following 48hrs of E_2 treatment (Fig. 33B), the gene expression expands to involve the TNF death receptor genes FAS, TNFRSF21 and TNF and continued increased expression of ERS and proinflammmatory genes. In addition p53 expression is increased at 48hrs. PMAIP 1 (also known as NOXA), a Bcl-2 homology (BH3) only family NOXA, a p53 regulated gene is also upregulated by E_2. 4OHT acted as an antiestrogen and was able to block all effects of E_2. The identified apoptosis related genes are listed in STable 1-3.

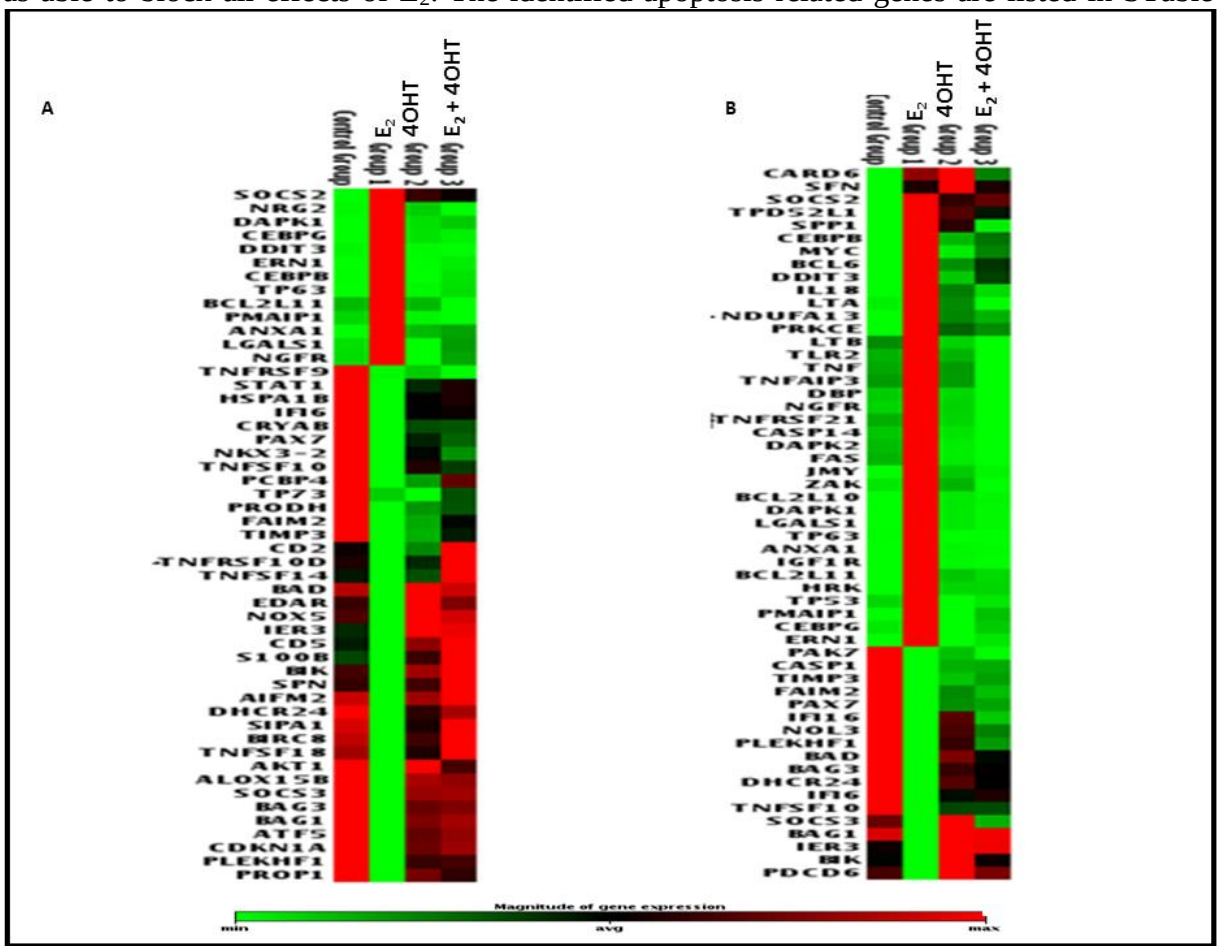
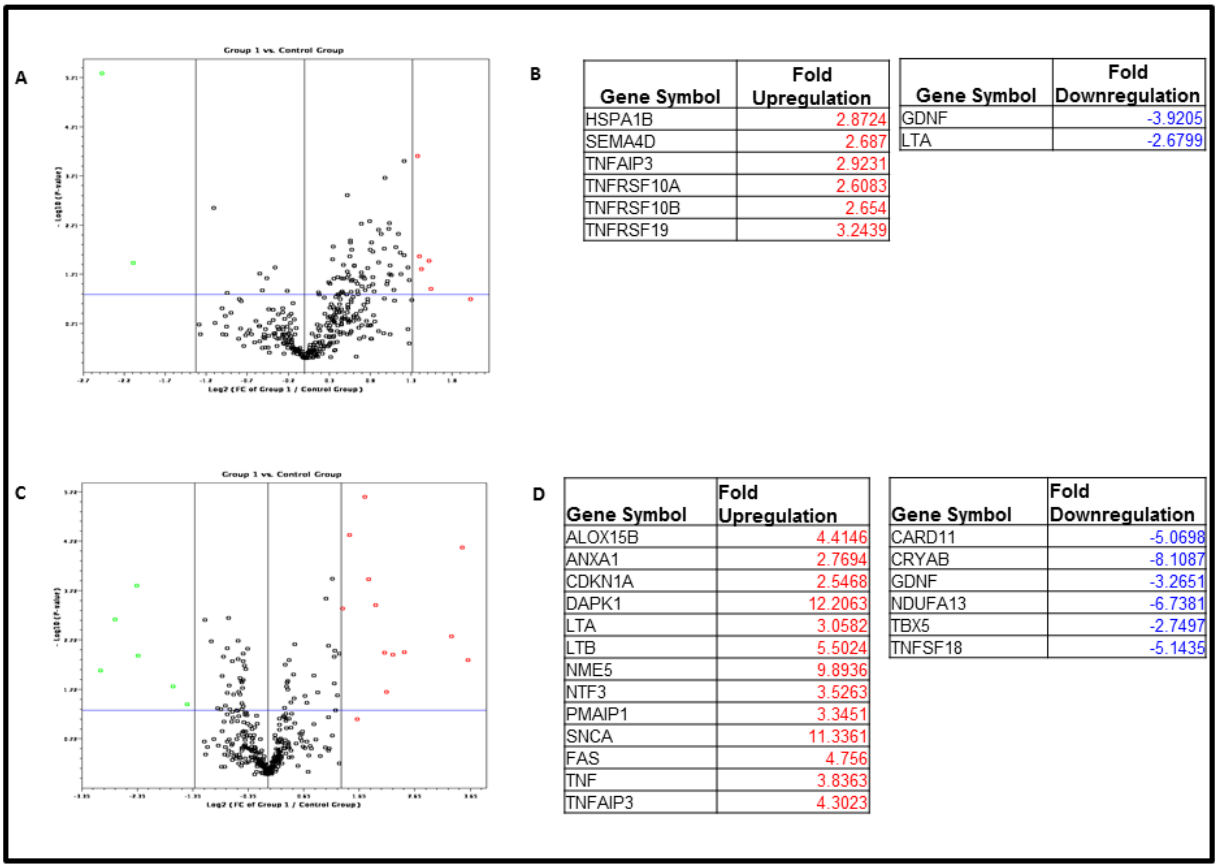


Figure 33. Heat map of E_2 mediated apoptotic genes which are differentially expressed by 36h and 48 h of treatment. Cells were parsed into groups of 3 replicates per treatment per time point, and then treated with either 0.1%ethanol (control), 1nM E2, 1 µM 4OHT in the presence or absence of E2 over a period of 48 h. Total RNA was extracted and reverse transcribed as described in materials and methods. Samples were loaded onto customized PCR array plates with primers for indicated apoptotic genes. Gene expression values were obtained and analyzed in comparison to the controls at (A) 36h and (B) 48h. The maximum expressed level of any given gene is represented by red color and minimum levels are presented as green color. Control group and group 1, 2, 3, 4 are the re-presentation of the vehicle, E_2 , 4OHT, and combination therapy of E_2 and 4OHT respectively.

Paclitaxel induces apoptosis in MCF7:5C cells through a death receptor mediated pathway.

Paclitaxel induces apoptosis rapidly in MCF7:5C cells (Fig. 31). We further investigated expressed genes activated by paclitaxel that may define a molecular mechanism. MCF7:5C cells were treated with 1µM paclitaxel at indicated time points and samples were quality controlled for gene expression using PCR array. Paclitaxel selectively activated the TNF family of apoptotic related genes. After an initial 12hrs of treatment (Fig. 34 a-b), paclitaxel stimulated TNFRSF10A (TNF receptor superfamily, member 10a) and TNFRSF10B (TNF receptor superfamily, member 10b) which are known to be activated by the ligand TNF-related apoptosis inducing ligand (TNFSF10/TRAIL), and causes death through the extramitochondrial pathway. TNFRSF19 (TNF receptor superfamily, member 19) induces apoptosis in a caspase-independent manner. In addition, TNF proapoptotic genes, including FAS, TNF and other TNF proinflammatory genes; LTA, LTB and TNFAIP3 are activated by 24h of treatment with paclitaxel (Fig. 4c-d). Paclitaxel further induces NOXA and CDKN1A (p21) which is known to inhibit the activity of cyclin-CDK2 or -CDK4 complexes at the G1 phase. Although these two p53 regulated genes were upregulated by paclitaxel, p53 induction was not observed at 24h. Unlike E_2, which increases BIM and TNF mRNA levels(Fig.35a-b), paclitaxel was only able to induce TNF expression (Fig.35c-d). These results highlight the differences in apoptotic pathways between the two treatments.



Gene Symbol	Fold Upregulation
HSPA1B	2.8724
SEMA4D	2.687
TNFAIP3	2.9231
TNFRSF10A	2.6083
TNFRSF10B	2.654
TNFRSF19	3.2439

Gene Symbol	Fold Downregulation
GDNF	-3.9205
LTA	-2.6799

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

Gene Symbol	Fold Downregulation
CARD11	-5.0698
CRYAB	-8.1087
GDNF	-3.2651
NDUFA13	-6.7381
TBX5	-2.7497
TNFSF18	-5.1435

Figure 34.Determination of apoptotic genes induced by a cytotoxic chemotherapy in MCF7:5C cells. MCF7: 5C cells were treated with either 0.1%ethanol (control), or 1uM paclitaxel for 12h, and 24 h. Gene expression values were obtained and analyzed in comparison to the controls and volcano plots were generated at 12h of treatment (A) and the expressed genes listed (B). Similarly, gene expression levels are analyzed after 24h of paclitaxel treatment (C) and genes are listed in (D).The genes selected were at least 2.5 fold over-expressed or under-expressed as compared to vehicle at p value of 0.05. Genesupregulated are represented in pink and dowregulated genes are represented in green.

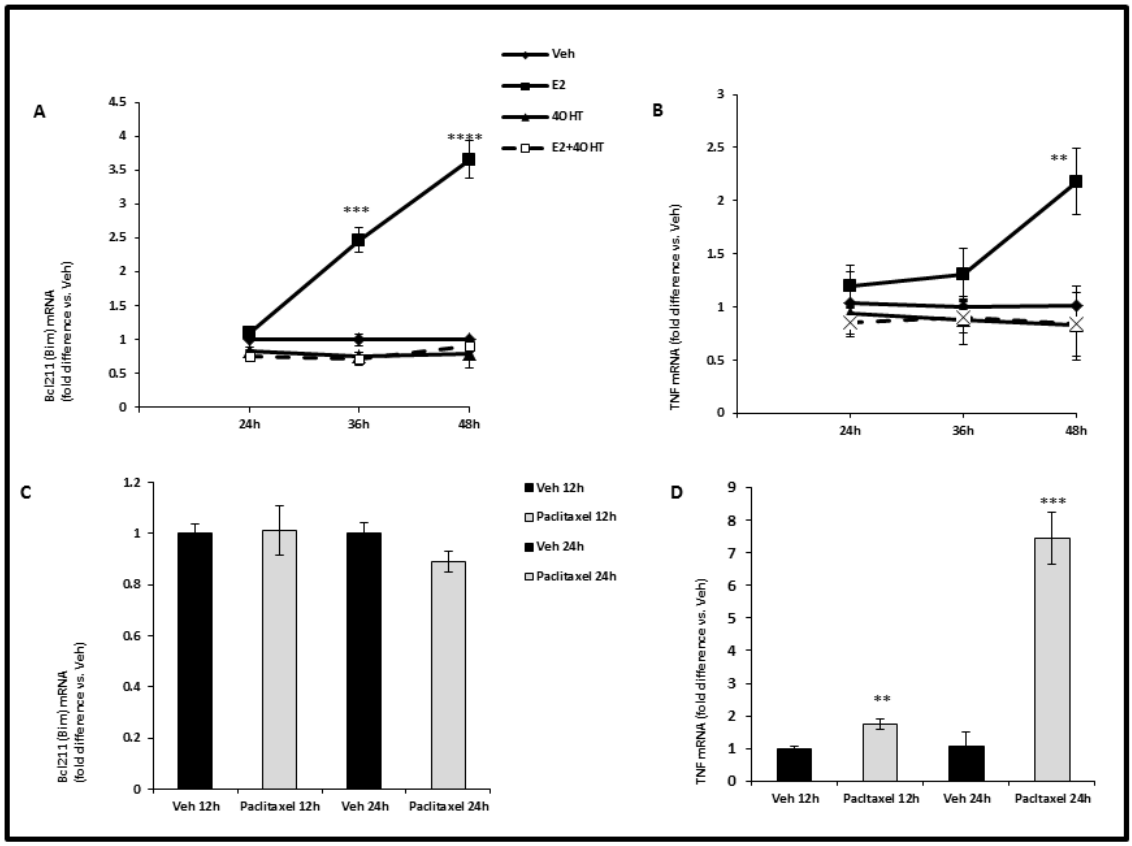


Figure 35. E2 Activates both Mitochondrial and Extrinsic Pathway of Apoptosis, while Paclitaxel Activates only the Extrinsic Pathway.
MCF7:5C cells were treated with Vehicle (Veh), 1 nM E2, 1µM or combination treatment of E_2 and 4OHT for 24, 36 and 48 h. Total RNA was reverse transcribed and assessed for (A) BIM and (B) TNF gene expression. Induction of (C) BIM and (D) TNF mRNA was determined in MCF7:5C cells treated either Veh or 1µM paclitaxel for 12h and 24h using RT-PCR. PCR data values are presented as fold difference versus vehicle treated cells ± SEM. [p<0.02,*** p<0.0003,****p<0.0001]**

Differential effect of paclitaxel in induction of G2 blockade in comparison to E_2.

Paclitaxel prevents progression of mitosis and activates the mitotic checkpoint, paving a path for apoptosis. To elucidate whether the apoptotic effects of paclitaxel in comparison to E_2 were mediated through cell cycle arrest, we performed cell cycle analysis in MCF7:5C cells treated with either vehicle control (Veh), 1nME_2 or 1µM paclitaxel for 12hrs, 24hrs, and 48hrs using flow cytometry. Our results reveal that paclitaxel treatment causes accumulation of cells in G_2/M phase with a concomitant reduction in the number of cells in G_1 phase and S phase (Fig. 5) Cell cycle arrest in G_2/M phase was about 3-fold higher compared with control and E_2 at 12h and rapidly increased to 7-fold by 48h. In contrast, a G_1 or G_2 blockade was not observed with E_2treatment, rather an increase in S phase consistently occurred at all time points. Based on these observations, we hypothesize that the apoptotic effects of paclitaxel in MCF7:5C cells results from a perturbation

in the cell cycle check points, whereas E_2 induces cell proliferation finally resulting in apoptosis.

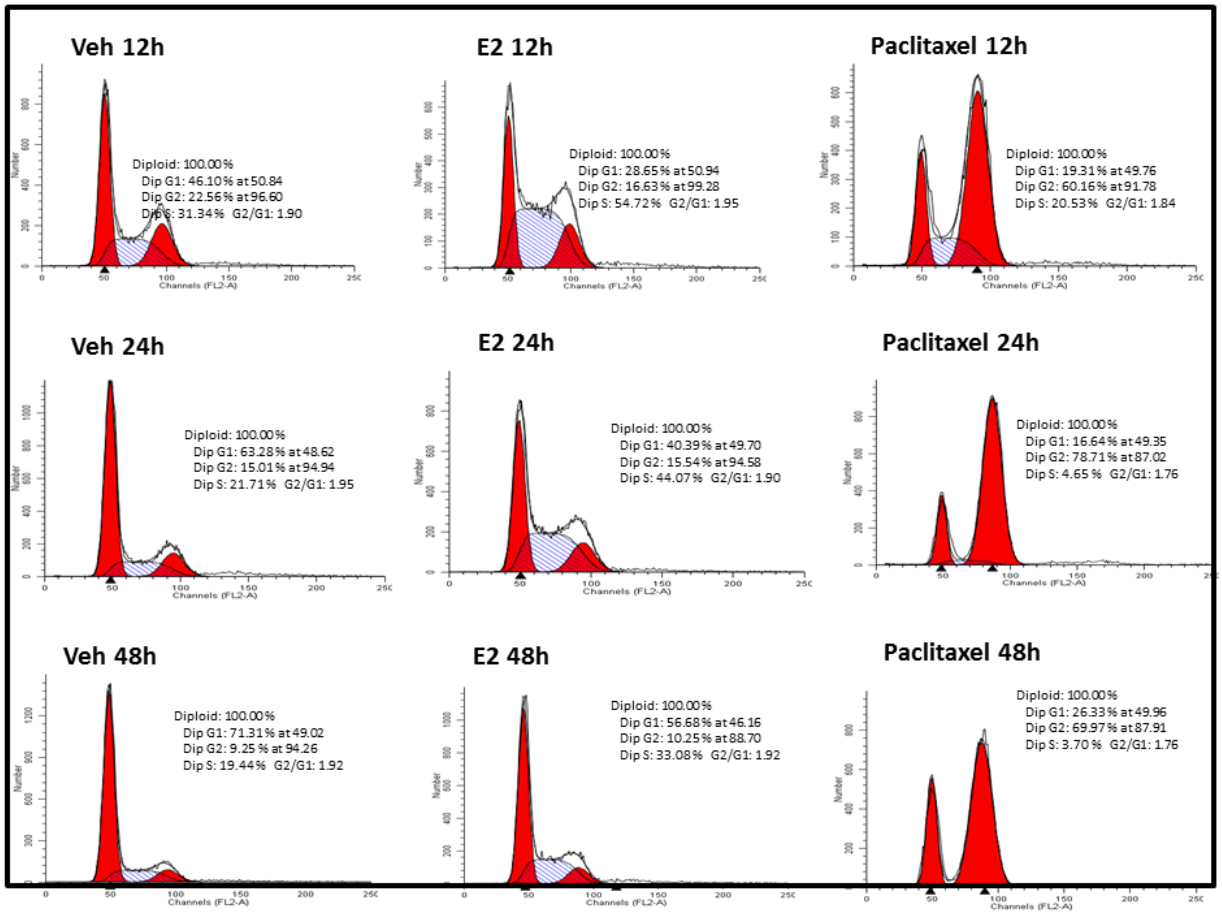


Figure 36. Cell cycle analysis of the effects of E2 and paclitaxel in the MCF7:5C cells
Representative cell cycle **profiles of MCF7:5C** cells **treated with either 0.1%ethanol (vehicle), E_2 (1nM) or paclitaxel (1µM) for 12h,24h and 48 h.. FL2-A represents the intensity of propidium iodide, and the *y* axis represents the** cell **number.**

DISCUSSION

The molecular sequence of events resulting in either E_2 induced apoptosis and paclitaxel induced apoptosis is completely different in antihormone resistant MCF7 breast cancer cells.. Paclitaxel rapidly inhibits the growth of MCF7:5C cells and achieves maximum growth inhibition by 48hrs. In contrast, 80% growth inhibition occurs with E_2 after 5 days of treatment, showing a delayed process for the induction of apoptosis. Based on our observations, we further investigated the slow apoptotic response of E_2 to determine the exact duration of E_2 exposure necessary to trigger apoptosis. Using 4OHT to block and rescue E_2 induced commitment to an apoptotic response, we observed that the trigger for apoptosis occurs after 24hrs and the cells become committed to apoptosis by 36hrs. There is activation by E_2 of ERS genes; DDIT3 and ERN1 and proinflammatory genes such as CEBPB, CEBPG, and DAPK1 at 36 hrs. Activation of the mitochondrial pathway was indicated by increased expression of BCL2L11,BIM, which continued to be upregulated at 48hrs. Involvement of the extrinsic pathway was evidenced by induction of FAS, TNFRSF21 and TNF and TNFAIP3 at 48hrs. The TNF family of genes are a group of cytokines that are involved in a number of processes including apoptosis(78, 79) and inflammation(80). The increased involvement of ERS and inflammatory genes in E2 induced apoptosis, is not surprising because both pathways are known

to intersect(81, 82). Multiple genes induced by E_2 are NF-κB responsive which is a major regulator of inflammatory response(83, 84). Upregulation of these genes such as BIM, CEBPB/G, TNF, TNFAIP3,LTA and LTB provide a potential mechanism for E_2 to target a variety of inflammatory and apoptotic genes.

We have previously reported that MCF7:5C cells respond to E_2 by suppressing ERα signaling leading to activation of ERS and inflammatory stress(69). As a consequence, unfolded proteins accumulate and prolonged ERS leads to activation of BCL2L11/BIM and BAX. The importance of BIM and Bax have previously been noted and verified by selective increased expression of both proteins by E_2(56). Depletion of BIM and BAX using siRNA blocked E_2 mediated apoptosis. Involvement of the death receptor signaling (extrinsic) pathway in E_2 induced apoptosis has been also observed. Osipo et al(71) showed that E_2 induced regression of tamoxifen stimulated breast cancer tumors, by activating the death receptor Fas and inhibiting the antiapoptotic/prosurvival factors NF-kB and HER2/neu**.** In addition, the growth of raloxifene resistant MCF7 cells *in vitro* and *in vivo* was inhibited by E_2 by increasing Fas expression and reduced NF-kB activity (85). However unlike the present study, none of the previous studies investigated a time course of the intrinsic and extrinsic pathway in the MCF7:5C cells in estrogen induced apoptosis. To our knowledge, this study is the first to show a sequential activation of the intrinsic and extrinsic pathways in E_2 mediated apoptosis.

Paclitaxel a mitotic spindle inhibitor and a cytotoxic chemotherapy extensively used in the treatment of breast cancer was used as a comparator to E_2 to demonstrate differences in the expression of apoptosis related genes. Paclitaxel selectively induces the TRAIL/TNFRSF10A/B pathway as well as the orphan TNF receptor, TNFRSF19 at 12hrs. Increased expression of TNF family members is evident at 24hrs including FAS, TNF and other TNF proinflammatory genes LTA,LTB and TNFAIP3 with inhibition of the cell cycle at G1 checkpoint by p21. Upregulation of NOXA along with p21 indicates an involvement of p53 but p53 levels did not reach statistical significance. On the other hand, paclitaxel kills the MCF-7 cells through the intrinsic pathway of apoptosis by displacement of BIM from the BIM/BCL2 complex(86). Knockdown of BIM with siRNA significantly impairs the ability of paclitaxel to cause apoptosis in the MCF-7 cell line and the extrinsic pathway was not required for apoptosis in the MCF7 breast cancer cells(86, 87). Furthermore, another study(88) showed that BIM was not required for paclitaxel mediated apoptosis in MCF-7 cells and these apparent discrepancies could be due to differences in that exist from cell lines obtained from different sources. However, long term deprivation of estrogen from the MCF7 cells may have induced changes in the microenvironment that may be responsible for the taxane to act immediately via the extrinsic apoptosis pathway in the MCF7:5C cells. Flow cytometry studies show that E_2causes both proliferation and apoptosis of the MCF7:5C cells indicating that before the trigger for apoptosis occurs, the cells grow in response to E_2. Because cells continue to divide early, the antiproliferative activity of E_2do not become evident until after 4 days of treatment. In contrast, Paclitaxel causes an immediate G2 blockade by 12hrs which may explain the rapid induction of apoptosis.

In conclusion, E_2 induces ERS which leads to an initial activation of the mitochondrial apoptotic pathway and a later recruitment of the TNF apoptotic pathway, whereas, paclitaxel induces a G2/M blockade and rapidly induces apoptosis through the TNF pathway of apoptosis. The novel delayed aspect of E2 induced apoptosis in antihormone resistant breast cancer creates a new dimension in our dimension in our opportunities to apply the knowledge for this targeted therapy of clinical significance(59, 66, 89). E_2 induces ERS which leads to an initial activation of the mitochondrial apoptotic pathway and a later activation of the TNF apoptotic pathway. Whereas,

paclitaxel induces a G2/M blockade and rapidly induces apoptosis through the TNF pathway of apoptosis.

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

Task 2f: Obiorah and Jordan – To develop laboratory models that illustrate the new biology of estrogen induced apoptosis or growth to explain the effects of estrogen replacement therapy.

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

The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality.

Introduction

Despite the extensive progress made in the management of breast cancer, it still remains the most common cause of cancer and the 2nd leading cause of cancer death in women in the United States. An estimated(90) 230,480 new cases of invasive breast cancer was projected to occur in 2011, as well as an estimated 57,650 cases of breast carcinoma in situ. In addition, approximately 39,520 women were expected to die from breast cancer in 2011(90).Multiple risk factors has been established for breast cancer and estrogen is a key growth stimulus in the development and progression of the disease. George Beatson(91) provided the first medical evidence of the estrogen dependency of breast cancer in 1896. The conclusion that a woman's ovaries provided the fuel that maintained breast cancer was based on the observation of remission of advanced breast tumors in a premenopausal patient that underwent bilateral oophorectomy. Stanley Boyd(92) surveyed all known cases in 1900 and concluded a 30% responsive rate, a figure that has stood the test of time for the response rate of breast cancer to any anti-hormone therapy. Animal models provided further evidence on the role of estrogens in breast cancer growth. Lanthrop and Loeb(93) observed in 1916, a decrease in the occurrence of mammary carcinomas in castrated immature female mice. Estrogen, an ovarian hormone, was subsequently extracted and purified and induced vaginal cornification in ovarectomised mice(94). This advance led to the elucidation of the biological properties of synthetic estrogens using ovariectomised mice, therefore establishing a connection between mitogenic potential of estrogens and breast cancer. The strategy of targeting the estrogen receptor (ER) has led to the discovery of endocrine therapies which function to either block estrogen action by using selective estrogen receptor modulators (SERMS) or depriving the ER of estrogens by using aromatase inhibitors(95). Antihormonal therapies remain the gold standard of care in the treatment and prevention of ER positive breast cancer(96).

WORK ACCOMPLISHED

Conjugated equine estrogens

Extensive progress in the production of estrogen preparations for commercial use was made by scientists at Wyeth pharmaceuticals (then Ayerst) Canada who extracted conjugated estrogens from pregnant horse's urine(97). In 1942, Food and Drug Administration (FDA) approval(98) was obtained in the United States for the clinical use of conjugated equine estrogens (premarin)for the treatment of menopausal symptoms and related conditions. There was an initial worldwide acceptance of CEE in the 1960s, however increased risks of developing endometrial cancer led to a decline in prescriptions for postmenopausal women(99, 100). A new generation of interest in the use estrogen therapy in the treatment of osteoporosis in the 1980s led to clinical studies of women receiving either estrogen alone or estrogen plus progestin therapies. Women on estrogen and progestin treatment had lower incidence of endometrial cancer(101, 102) indicating that progestin blocked the proliferative effect of estrogens on the endometrial lining. As a result, CEE was approved for the treatment and prevention of osteoporosis; women with an intact uterus were given progestin in addition to the estrogens. CEE is made up of conjugated estrogens and the tablet consists of at least 10 estrogens (fig. 37) which include estrone (59.2%), equilin (26.9%),17α-dihydroequilin (16.3%) , 17α-estradiol (4.32%) , 17β-dihydroequilin (1.76%), 17α-dihydroequilenin (1.76%), 17β-dihydroequilenin (3.36%), equilenin (2.4%), 17β-estradiol (0.8%), and $\Delta^{8,9}$-dehydroestrone (4.16%). Generic synthetic versions of CEE are not currently approved by the FDA based on inadequacies noted on their active ingredients, bioequivalence, safety and effectiveness(103).

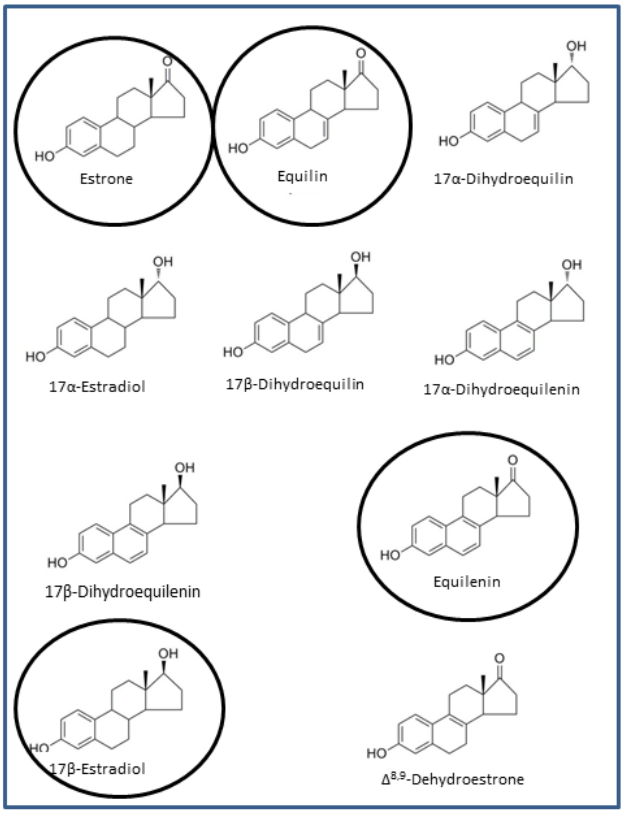


Figure 37 Structures of the estrogenic constituents of premarin.Estradiol, equilin, estrone and equilenin were used in our experimental studies.

Effect of Conjugated Equine Estrogens on breast cancer cells

Long term concentrations of estrogen deprived MCF-7 breast cancer cells undergo apoptosis upon treatment with physiologic estradiol(3). Based on the preliminary results of the WHI CEE study, we decided to elucidate the biological properties of the main estrogens in CEE in two different models of breast cancer cells. Estrogens have been shown to regulate the growth of ER positive MCF-7 breast cancer cells. To study the biological activity of the actual estrogens namely equilin, estrone and equilenin, we tested their ability to induce proliferation in MCF7:WS8 cells which contain ER and have retained estrogen responsiveness for a sustained period of continuous cell culture(104). MCF-7 cells were grown in estrogen free media for 3 days and treated with various concentrations of the equilin, estrone and equilenin and their effects were compared to E_2 (fig 38A). All the three estrogens were able to induce cell growth of MCF-7 cells in a dose dependent manner to the maximum level as E_2. Equilin and estrone induced cell proliferation with maximum stimulation occurring at 0.1nM, whereas equilenin reached maximal stimulation at 1nM as compared to 0.01nM for E_2. Next we investigated the growth properties of the equilin, estrone and equilenin in long term estrogen deprived MCF7:5C cells in comparison to E_2. Fig 38B shows that equilin, estrone and equilenin drastically inhibited the growth of the MCF7:5C cells at comparable concentrations to E_2. Maximum growth inhibition was achieved with E_2 at 0.1M, while equilin and estrone reach

maximum growth inhibition at 1nM and equilenin at 10nM after 7 days of treatment. To determine if the observed estrogen induced growth inhibition of the MCF7:5C cells was due to apoptosis, MCF7:5C cell were either the control, E_2, equilin, estrone or equilenin for 72 hours and the level of apoptosis was measured using annexin V staining. E_2, equilin, estrone and equilenin all show increased apoptotic staining compared to the control treated cells (Fig 39). The ability of the conjugated estrogens to inhibit the growth and induce apoptosis in the MCF7:5C cells and not the parental MCF7 cells suggest that these biological properties are dependent on the duration of deprivation of estrogen in the breast cancer cells.

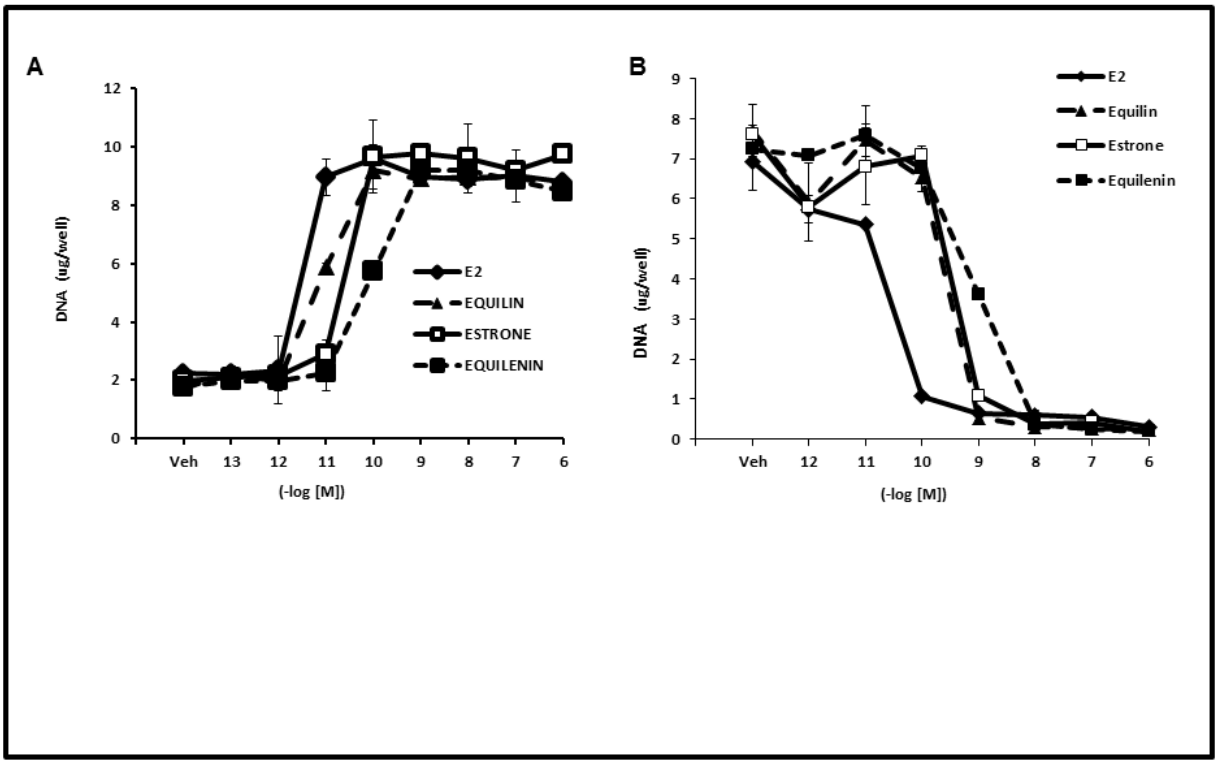


Figure 38.Cell proliferation assay analysis of the biological properties of active steroids in CEE in breast cancer cells. (A) MCF7 cells were grown in E_2 stripped media for 3 days and treated for 7 days with various concentrations of E_2, equilin, estrone and equilenin and compared to the Veh (control). (B) Equilin, estrone and equilenin drastically inhibited the growth of MCF7:5C cells in a similar manner as E_2. The experiments were completed in triplicates and performed as previously described (105)

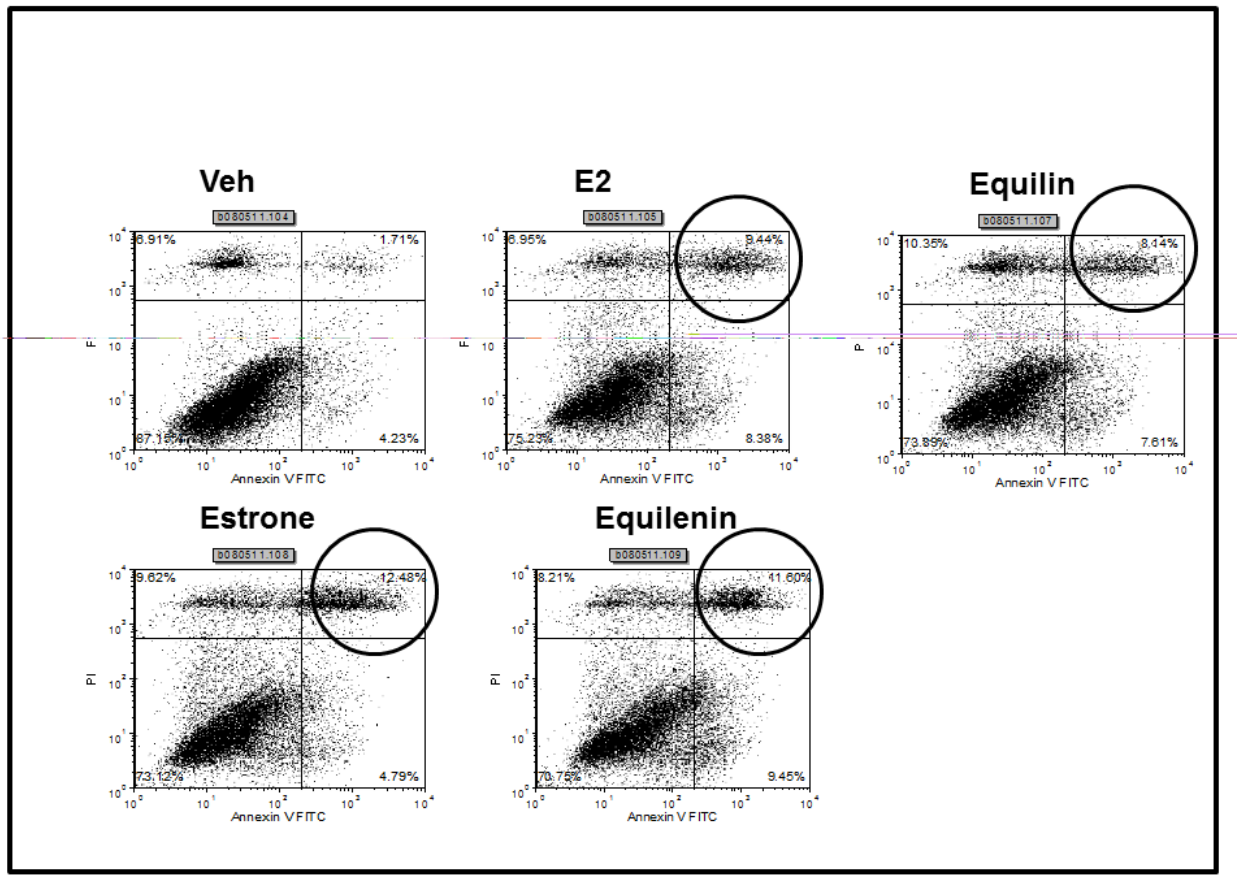


Figure 39. Effects of estradiol and active estrogens in CEE on apoptosis in MCF7:5C cells. MCF7:5C cells were seeded in 100mm plates and treated with Veh (control), 1nM E_2, 1nM Equilin, 1nM estrone and 1uM equilenin for 72 hours and cells were stained with FITC-annexin V and propidium iodide and analyzed by flow cytometry and performed as previously described(105). The upper right box of vehicle treated (Veh) cells have low apoptotic cells (1.71%), whereas all for estrogens, this fraction is increased (circled upper right hand box).

Molecular mechanisms of estrogen induced apoptosis

To decipher the precise series of events that precede estrogen induced apoptosis, differential gene expression in response to E2 was interrogated using affymetrix based microarray analysis(4). Specific genes were identified for MCF7:5C which indicate that E_2 induced endoplasmic reticulum stress (ERS) and inflammatory stress responses that lead to apoptosis. Identified ERS genes indicated that E_2 inhibited protein folding leading to accumulation of unfolded proteins and widespread inhibition of protein translation with subsequent induction of cell death. In response to severe ERS, Bcl-2 interacting mediator of cell death (Bim; BCL211) was induced. Further evidence of the involvement of the mitochondrial pathway in E_2 induced apoptosis was reported by Lewis and colleagues(105) who showed increased expression of several proapoptotic proteins including, Bax, Bak, Bim, Noxa, Puma and p53 in E2 treated MCF7:5C cells. Reversal of the apoptotic effect of E_2 in these cells was observed with blockade of Bax and Bim expression using short interfering RNAs (siRNAs). Involvement of the Fas/Fasl death signaling (extrinsic) pathway in the apoptotic effect of E_2 has been investigated. Osipo et al(106) demonstrated that E_2 induced regression of tamoxifen

stimulated breast cancer tumors, by activating the death receptor Fas and suppressing the antiapoptotic/prosurvival factors NF-kB and HER2/neu**.** Similarly,the growth of raloxifene resistant MCF7 cells *in vitro* and *in vivo* was attenuated by E_2 by increasing Fas expression and reduced NF-kB activity(107). Studies are currently ongoing to determine the sequence of events that occur before E_2 induces apoptosis in the MCF7:5C cells.

The resolution of the crystal structure provided insight into the activation of the ER by E_2 and silencing by antiestrogens(108, 109) and is providing the insight into the "trigger" mechanism for the ER complex. The shape the ligands make with the ER is imperative to their ability to induce apoptosis in the MCF7:5C cells. E_2 is sealed within the hydrophobic pocket of the ligand binding domain of the ER by helix 12 and coactivators bind leading to activation of apoptotic genes. On the other hand, 4-hydroxytamoxifen (4OHT) pushes back helix 12, prevents coactivator binding and this may responsible for its ability to block estrogen induced apoptosis in the MCF7:5C cells. Knockdown of coactivator AIB1/SRC3 in MCF7:5C cells led to the loss of apoptosis inducing effect of E_2 suggesting that AIB1is a significant control hub E_2 in induced apoptosis in these breast cancer cells(110). Structure function studies show that the shape of the estrogen(111) can modulate the shape of the estrogen-ER complex to induce apoptosis(112). Hydroxylated triphenylethylenes (TPE) which are structurally similar to 4OHT and have estrogenic properties in MCF7 cells, have been shown to block E_2 induced apoptosis(113). The antiestrogenic shape they make with the ER may be responsible for the delayed apoptotic effect of the TPEs in the MCF7:5C cells. These pharmacologic studies are currently under investigation and will be the focus of further reports.

Discussion

Before the clinical use of antiestrogen therapy, high dose estrogens were effective in the induction of tumor regression in metastatic breast cancer(114, 115). In more recent times, estrogen therapy show significant clinical benefit in postmenopausal women who have undergone extensive anti-hormone treatment(5). Development of tamoxifen stimulated tumors in athymic mice following a five year treatment with tamoxifen suggest that the development of anti-hormone resistance over years of treatment reconfigures the survival mechanism of breast cancer so that estrogen is no longer a potent mitogen that stimulates cell proliferation but rather becomes a death signal. Preclinical data clearly show that long term estrogen deprivation of ER positive MCF-7 breast cancers and subsequent treatment of the cells with E_2 causes apoptosis of these cells. Creation of an estrogen deprived environment either by withdrawal of estrogen treatment(116) or by exhaustive anti-hormone therapy increases sensitivity of breast tumors to estrogen therapy which subsequently induces tumor regression. Similarly CEE alone reduces the incidence of breast cancer in hysterectomised postmenopausal women. This protective effect is not observed in women who received addition progesterone therapy, suggesting that the progestin may play a potential role in the increase in breast cancer seen in postmenopausal women who received combined hormonal therapy.
To explain the aforementioned clinical data, laboratory studies show that estrogens in the CEE were able to cause proliferation of MCF7 cells after growing these cells in an estrogen free medium for 3 days. This cell population is adapted to an environment rich in estrogen, so naturally all the cells grow with a "resupply" of natural steroidal estrogens. However, these same estrogens induce apoptosis to a similar extent as E_2 in MCF-7 cells that have been deprived of estrogen treatment for many years. The ability of estrogen therapy to treat or prevent tumors is related to the menopausal status of a woman and how long they have been physiologically deprived of estrogen. In the Stoll data(116) (Table 1), the rate of remission of advanced breast cancer was significantly less in women who were less than 5 years postmenopausal(9%), and there was a 35% remission rate in women who

were more than 5 years postmenopausal. It is important to stress that majority of the women in the WHI CEE trial were above 60 years and the mean age at screening was 63.6 years. Here, the overall result was a reduction in breast cancer and mortality. There is a need for an "estrogen holiday" before starting estrogen therapy. Induction of menopause in a woman gradually deprives the cells of estrogen. However immediate treatment with estrogens may cause growth of nascent ER positive breast tumors which may increase breast cancer risk (fig 40A). The cells vulnerable to death with estrogens in CEE, have been selected because estrogen deprivation at menopause causes estrogen dependent nascent breast cancers to die, but all do not die. Remaining cells that survive learn to grow without estrogen (fig 40B). These cells will continue to grow to produce breast cancer unless exogenous estrogens induces apoptotic death. Therefore 5 years of CEE treatment immediately after menopause will cause sustained continuing growth of ER positive tumor cells. Because nascent ER+ tumor cells have been estrogen deprived in women who are 5 to 10 years postmenopausal, 5 years of CEE therapy induces massive apoptotic cell death and subsequent tumor cell death and an enhanced patient survival.

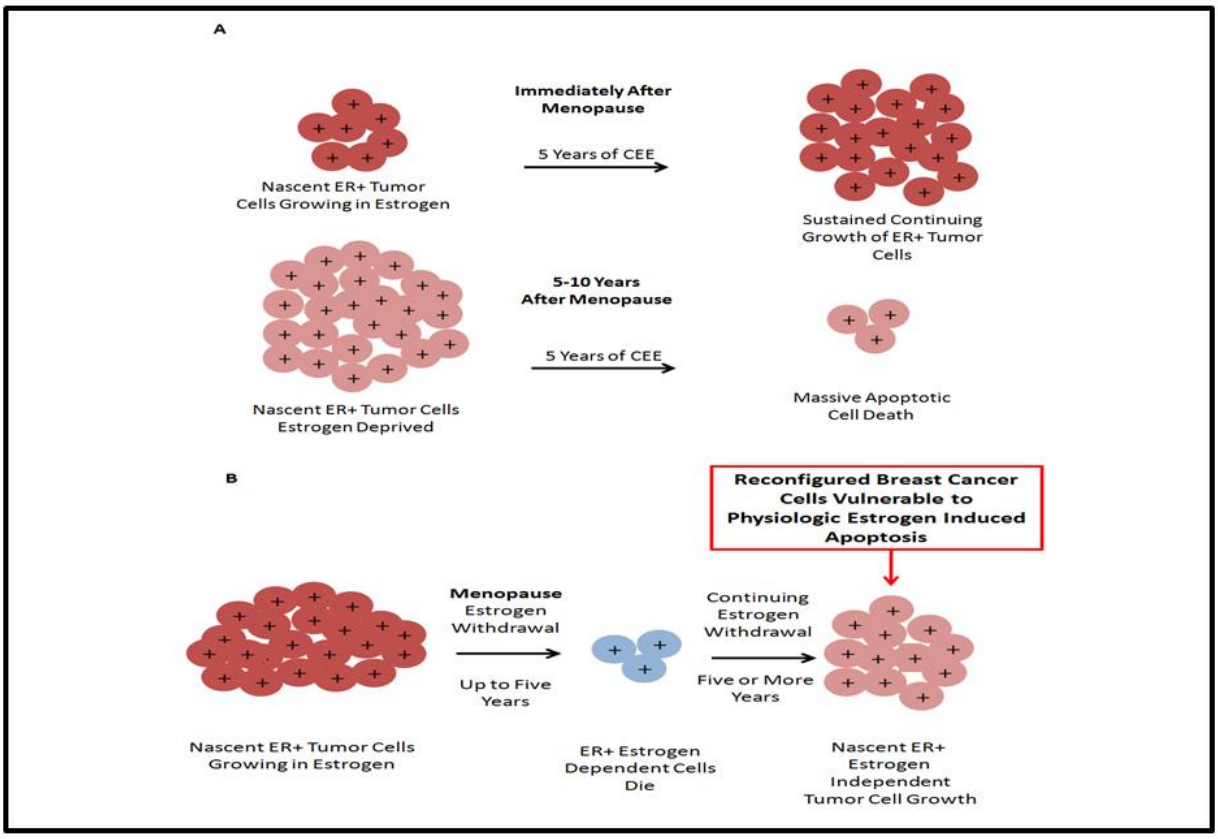


Figure 40. The success of estrogen replacement therapy is dependent on menopausal status of a woman. A. Treatment of women immediately after menopause with CEE results in sustained growth of nascent ER positive tumors, whereas treatment 5. years after menopause causes apoptotic cell death. B. Estrogen withdrawal in postmenopausal women causes ER positive dependent cells to die but some cells continue to grow independent of estrogen

Conclusion

High dose estrogen treatment is effective in causing tumor regression in metastatic breast cancer. The mechanism for this treatment was a paradox and unknown for 60 years but is now being deciphered(4). Objective tumor remission was seen in women over 5 years postmenopausal(114, 115). Estrogen replacement therapy administered to women in their late 60s causes a sustained decrease in breast cancer incidence and a decrease in mortality(1). The question was, why? Long term estrogen deprivation (LTED) for ER positive breast cancer cells is the key. We have created LTED breast cancer cell lines and for the first time, described the mechanism of estrogen-induced apoptosis. This new biology of estrogen induced apoptosis and can be now used to explain the effects of ET in reducing breast cancer incidence and mortality for women in the 60s.

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

Task 2g: (Jordan/Maximov): To elucidate the structure-function relashionship of estrogenic fixed ring triphenylethylene derivatives on estrogen-induced apoptosis in estrogen-independent breast cancer cell lines.

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

Structure-function relationships of fixed ring derivatives of estrogenic isomers of triphenylethylene on estrogen-induced apoptosis in estrogen-independent human breast cancer cell line.

Introduction

Estrogen-induced apoptosis is a relatively new phenomenon observed in antiestrogen and hormone independent breast cancers(117). Antihormone treatment of breast cancer consequently changes breast cancer cells and produces resistance to therapy. Paradoxically woman's own physiological estrogen is able at this point to induce breast cancer regression(5, 17). To decipher the mechanisms of estrogen-induced apoptosis and to discover potentially safer and more specific agents to induce apoptosis one must study the structure-function relationship of such compounds at the estrogen receptor (ER) to decipher a trigger for apoptosis in hormone-independent breast cancer cell line. One such breast cancer cell lines was developed by our group using selective cloning of MCF-7 cellsunder long-term estrogen deprivation conditions. This cell line is called MCF-7:5C(42). The cells are grown in estrogen-deprived conditions in culture media with supplementation of charcoal-stripped serum. The cell line grows independently from estrogen and is resistant to antiestrogens, however addition of estradiol (E2) induces apoptosis. To further study the mechanisms of this phenomenon, structure-functional studies were undertaken using estrogenic triphenylethylene derivatives (113, 118). It was shown that the shape of the receptor does matter for estrogen-induced apoptosis, however later studies shown that MCF-7/5C require more time to induce apoptosis with triphenylethylenes.This study is the structure-functional investigation of estrogen-induced apoptosis using fixed ring isomerically stable derivatives of an ethoxy-derivative of triphenylethylene, that was previously shown to be estrogenic in breast cancer cells (112). The results of this study will help to understand the relationship between the structure of the ligand and the conformation of the ER to induce apoptosis.

Work Accomplished

The isomers of the tested compounds have a distinctly different estrogenic profile

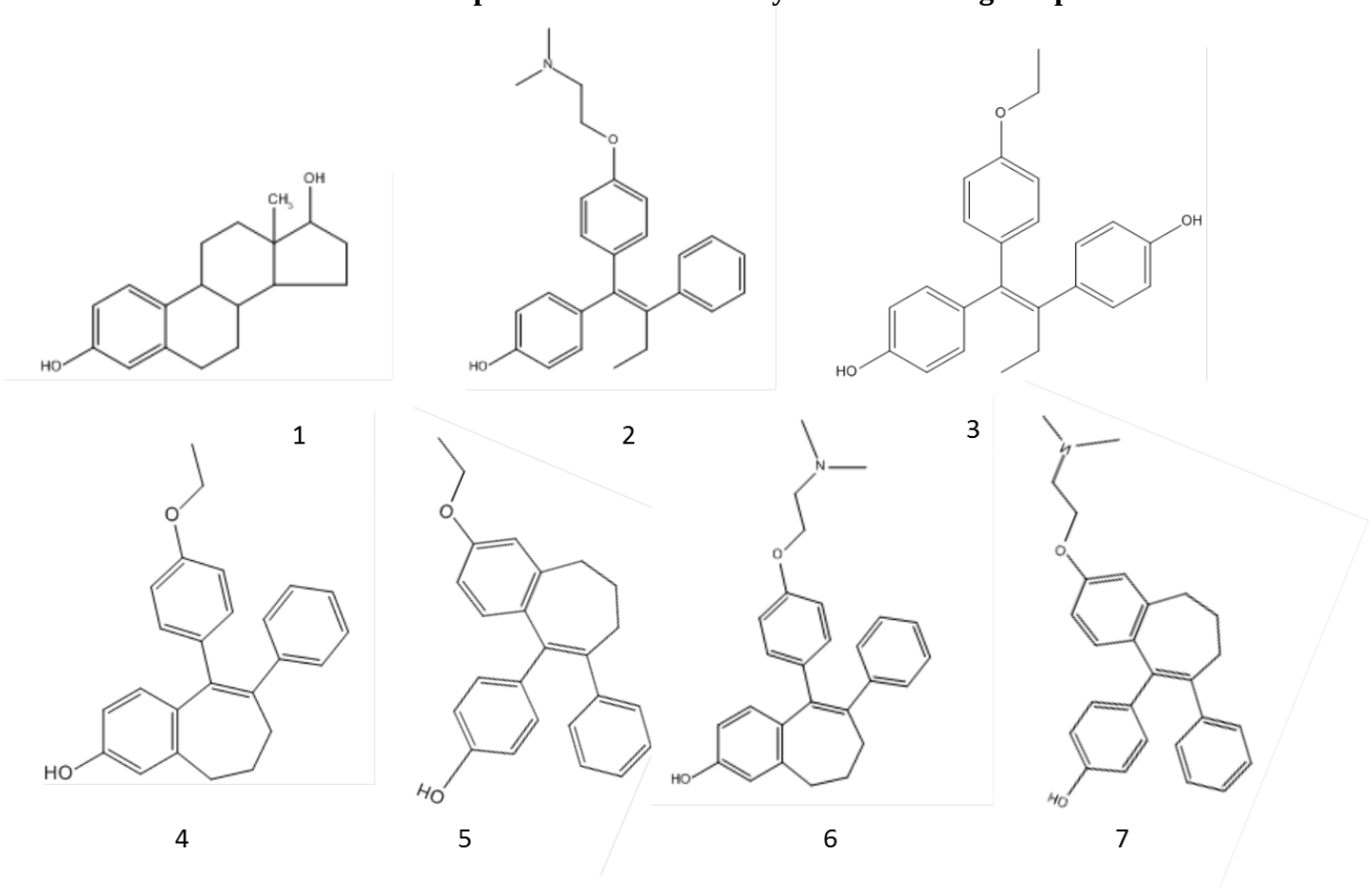


Figure 41. The tested Estrogen Receptor (ER) ligands. 1. 17β-estradiol (E2), 2. 4-hydroxytamoxifen (4OHT), 3.Ethoxytriphenylethylene (ethoxyTPE), 4. Trans-4-hydroxy fixed ring ethoxyTPE (TFREthoxTPE), 5. Cis-4-hydroxy fixed ring ethoxyTPE (CFREthoxTPE), 6. Trans fixed ring 4OHT (TFR4OHT), 7.Cis fixed ring 4OHT (CFR4OHT).

Above mentioned results (112) published earlier, with an ethoxy derivative of triphenylethylene, actually represent the action of a 50/50 mixture of cis- and trans-isomers. For studying the structure-functional relationship of the separate isomers we have synthesized isomerically stable fixed-ring derivatives of the same compound. The isomerically stable derivatives of triphenylethylenes were used previously to study the structure-functional relationships of antiestrogenisity of tamoxifen's isomers (119). The compounds were synthesized at Fox Chase Cancer Center's Organic Synthesis Facility, Philadelphia, PA. The compounds were tested for their estrogenic/antiestrognic properties in MCF-7:WS8wild phenotype breast cancer cell line. At this time, the synthesis of the cis-isomer of the fixed-ring ethoxy compound is in its final steps. Besides the trans-isomer of the fixed ring ethoxy-compound, we have compared the properties of the later with fixed ring cis and trans isomers of 4-hydroxytamoxifen (4OHT), which we have also synthesized, to elucidate the role of the ligand structure further. Also we have used the previously described ethoxy derivative of triphenylethylene (ethoxyTPE) and regular 4OHT for comparison. The tested compounds are shown in figure 41.

Isomers of the tested compounds have different action with regards to estrogen-induced apoptosis in MCF-7/5C cells.

The effects of the tested compound were studied using a DNA-based cell proliferation assay. To study the estrogenic properties of the tested compounds, the compounds were dissolved via serial dilutions in RPMI1640 culture media supplemented with charcoal stripped serum, and no phenol-red. The cells were estrogen starved in the same media for 3 days before the assay. The treatment was carried out for 7 days.

The proliferation assay in MCF-7/WS8 cells after treatment with serial dilutions of tested componds (Fig.42) show that the TFREthoxyTPE is an agonist and is equivalent to the EthoxyTPE compound. In comparison 4OHT and TFR4OHT in equimolar concentrations do not show any signs of estrogenicity. Interestingly CFR4OHT compound is a partial agonist at higher concentrations, which could be explained by its lower binding affinity to the ER. These results indicate a different biology of the isomers of the same compound (FR4OHT), probably due to difference in conformation of the ligand-binding domain (LBD) of the ER.

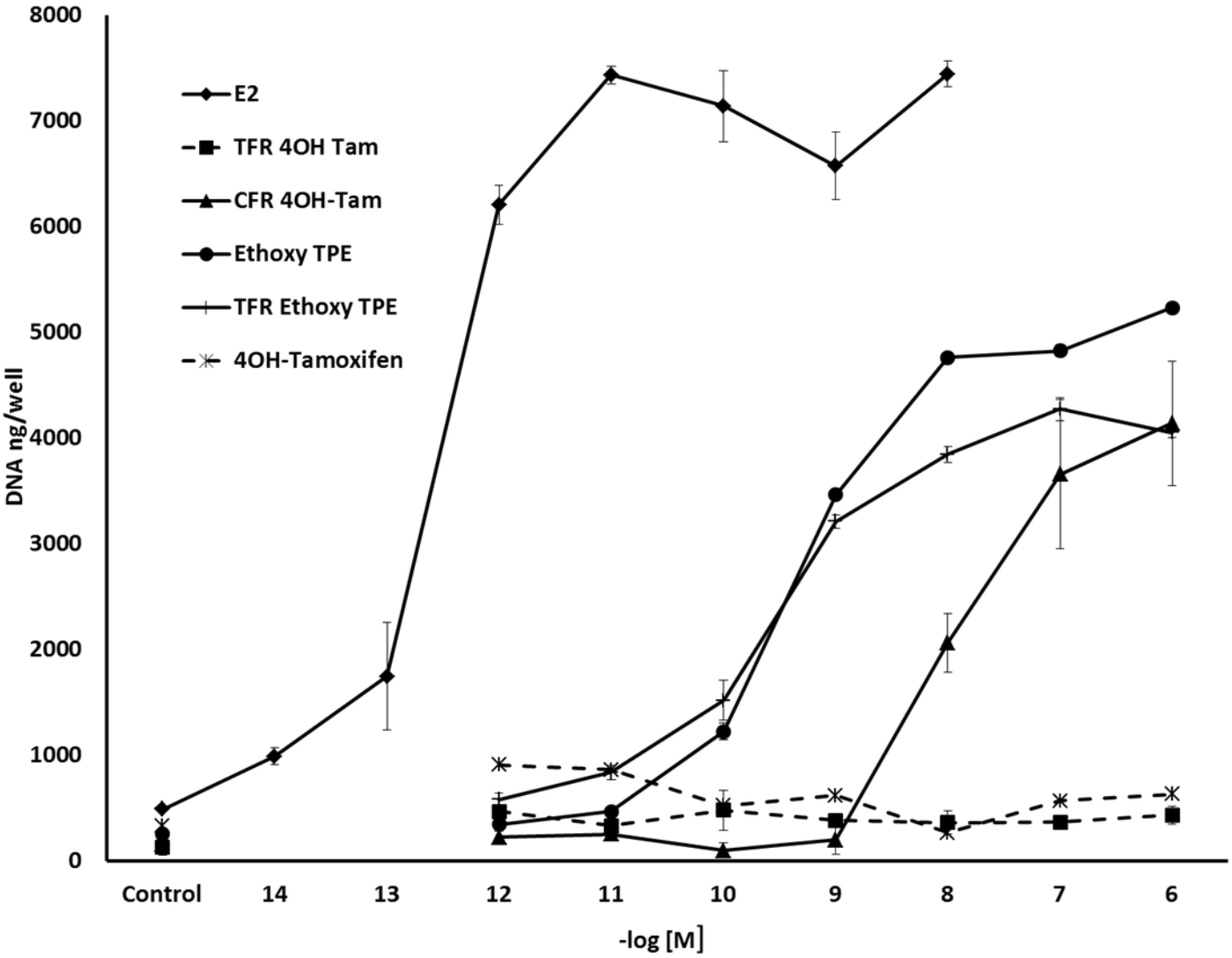


Figure 42. Results of the proliferation assay in MCF-7/WS8 cells showing estrogenic properties of the tested compounds. The cis-isomer of the fixed ring 4OHT is a partial agonist inducing cell proliferation at higher concentrations. However the EthoxyTPE and TFREthoxyTPE that have a shorter side chain are also partial agonists, but due to higher binding affinity to the ER induce cell proliferation at lower EC50.

We anticipate that the tests with CFREthoxyTPE will similarly demonstrate estrogenic properties in these cells and thus confirm this hypothesis. This compound is currently being synthesized.

Similar treatments in MCF-7/5C cells showed that only E2 was able to induce apoptosis at a 1 week timepoint. The only compounds that was able to reduce proliferation was EthoxyTPE with 28% in proliferation reduction rate (Fig.43).

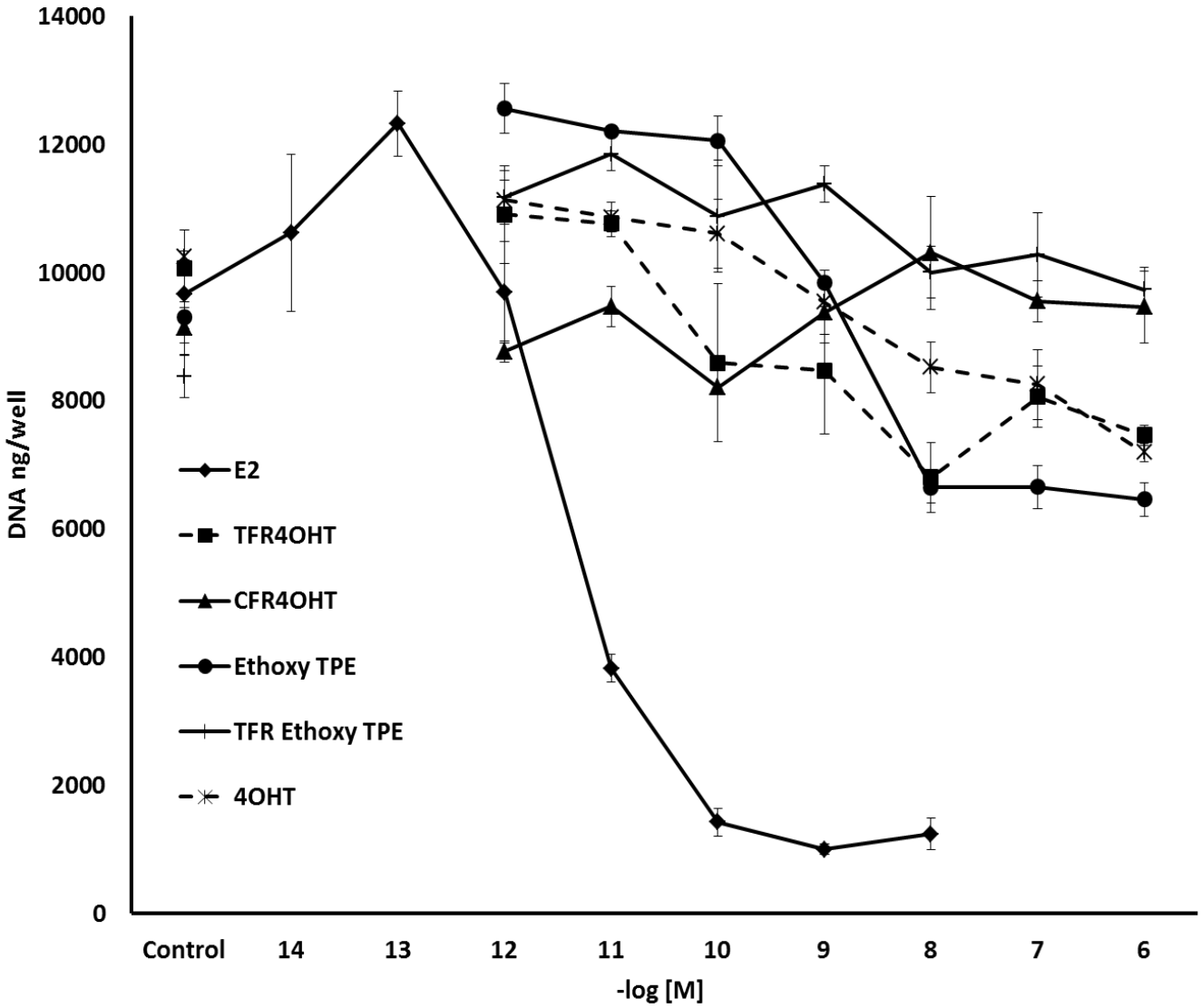


Figure 43. Proliferation in hormone-independent breast cancer cell line MCF-7:5C. E2 is able to induce apoptosis in these cells within a week virtually killing all the cells at 0,1nM. However all other tested compounds appear to be inactive during 1 week treatment. The compounds that have shown estrogenic properties in MCF-7/WS8 cells are inactive, except EthoxyTPE has some minor estrogenic properties at higher concntrations.

All of the tested compounds were able to inhibit the 1nM E2-induced apoptosis in combination treatments, except Ethoxy TPE (Fig.44).

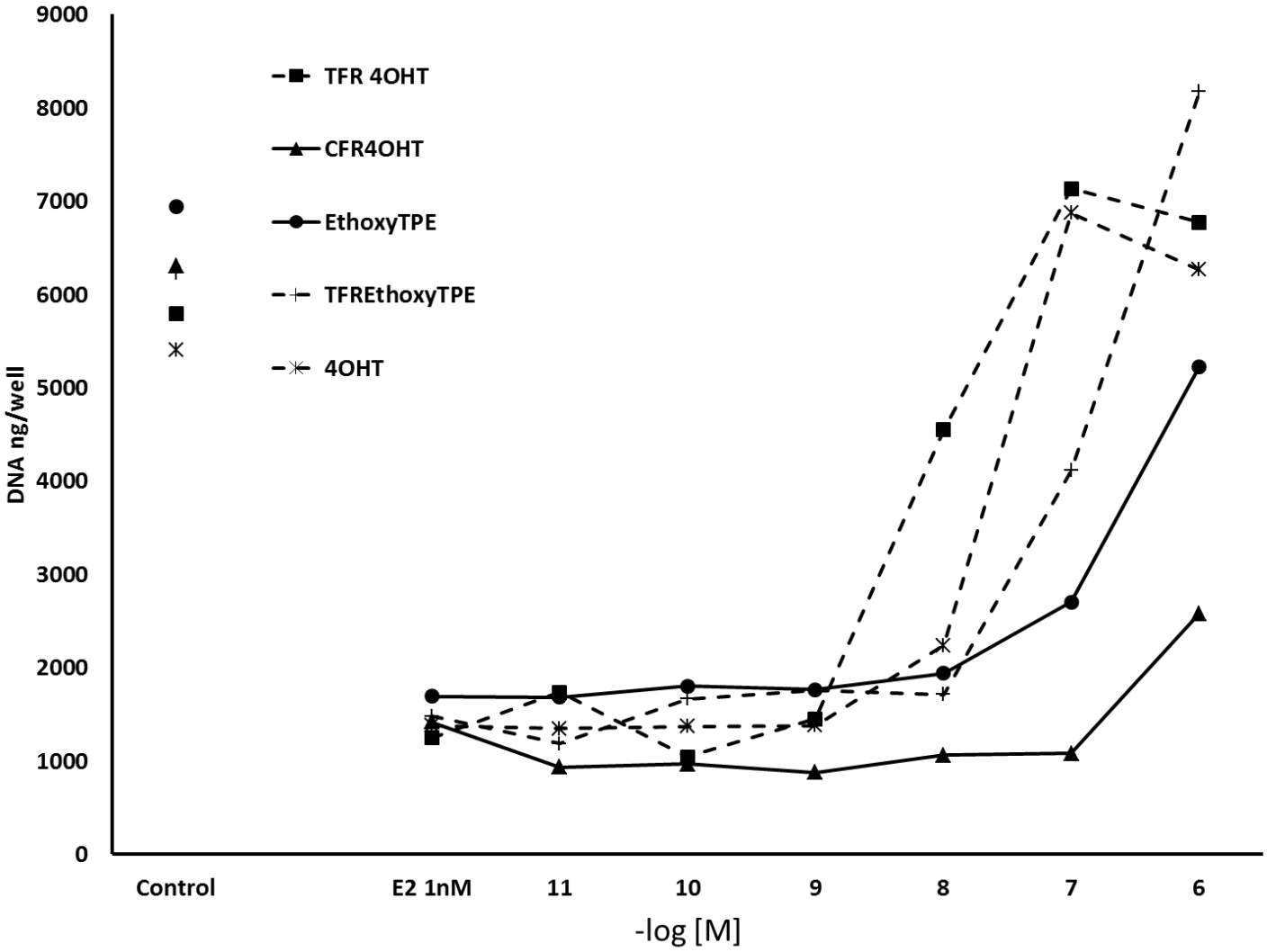


Figure 44. Blockade of apoptosis in MCF-7/5C cells by increasing concentrations of test compounds incubated with 1nM E2. The tested compounds were diluted in media containing 1nM E2, and the effects were compared with E2 alone and vehicle control. The results show that all of the compounds possess antiestrogenic properties. The least potent compound is CFR4OHT, probably due to its low binding affinity for the ER, whereas 4OHT and TFR4OHT are equivalent, and TFREthoxyTPE though shows antiestrogenic properties in this cell line it has a higher IC50 than 4OHT, the EthoxyTPE compound has only partial antiestrogenic properties in this cell line as it is a partial agonist.

Interestingly, CFR4OHT compound was able to only weakly inhibit the estrogen-induced apoptosis. All treatments were performed following 1 week treatment. The most potent antiestrogens were 4OHT and TFR4OHT with similar IC50. This is explained by those compounds have a bulky side chain and similarly prevent the closure of Helix 12 of the LBD of the ER to produce an antiestrogenic effect.

Compounds change their biology in MCF-7/5C cell after 2 weeks of treatment.

A new biological effect emerged by treatment of MCF-7/5C cells with tested compounds for 2 weeks. The compounds that had shown low antiestrogenic properties in MCF-7/5C cells in the first week of treatment and no estrogenic effect alone are now killing cells by the second week (Fig.45).

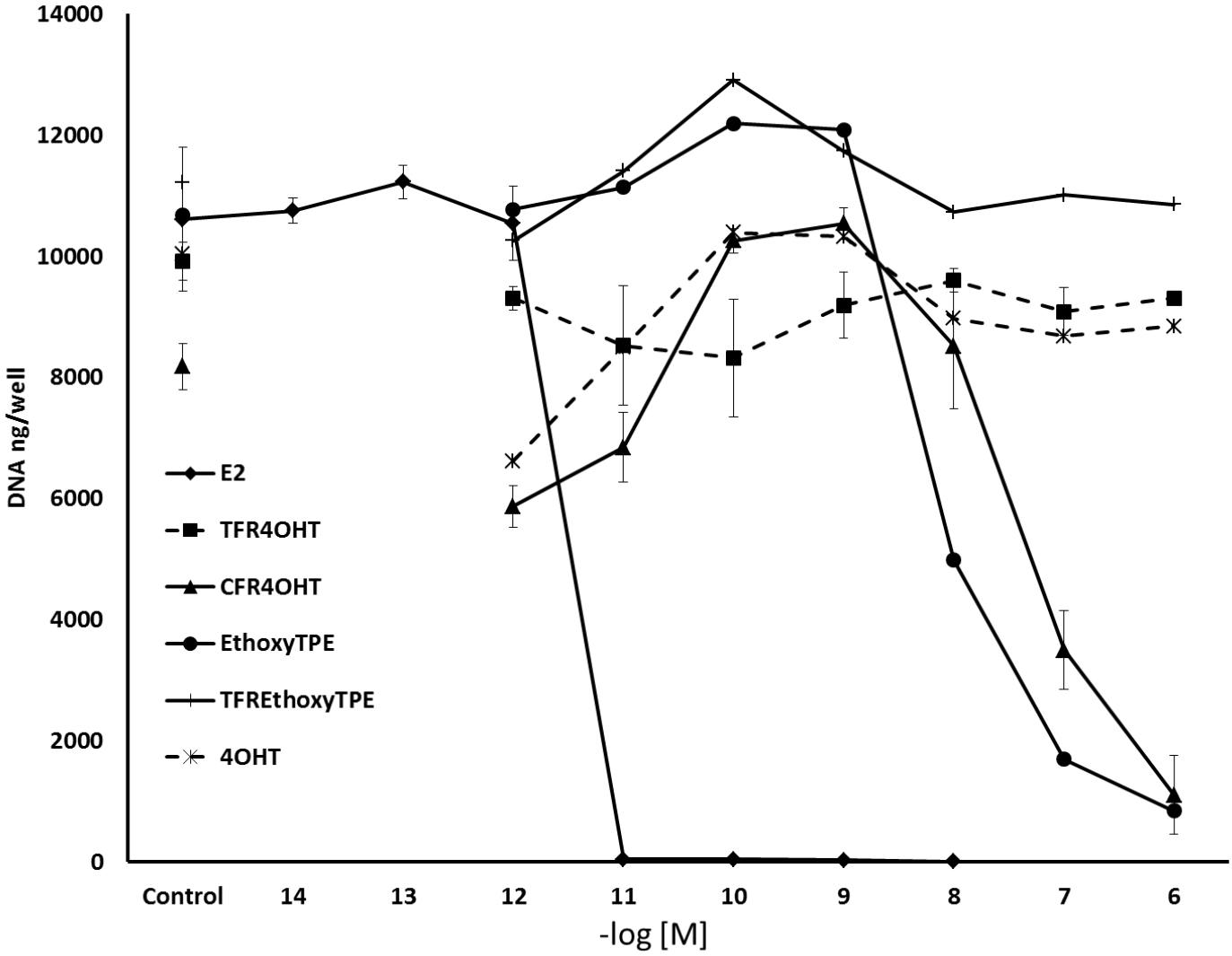


Figure 45 Proliferation assay in MCF-7:5C cells after two weeks of treatment with tested compounds. The results of two week treatment show that E2 is the most potent estrogen in MCF-7:5C cells and now kills the cells completely at a minimal concentration of 0.01 nM. CFR4OHT and EthoxyTPE which were estrogenic in MCF-7/WS8 cells are now killing the 5C cells after 2 weeks. However TFREthoxy compound still does not induce apoptosis.

Compounds that have demonstrated estrogenic properties in proliferation assays in MCF-7/WS8 cells CFR4OHT and EthoxyTPE are killing in the MCF-7/5C cells. Interestingly, TFREthoxy compound that showed estrogenic properties equivalent to EthoxyTPE is not inducing apoptosis in MCF-7/5C cells after two weeks of treatment at any concentration. This could be explained by the ethoxy group on the second phenyl ring of the TFREthoxyTPE and EthoxyTPE compound being short enough to induce growth in MCF-7/WS8 when compared to TFR4OHT or 4OHT, which have a longer bulkier side chain and push out the helix 12 far enough to block the activation of the ER. But this conformation induced in the ER complex with TFREthoxyTPE is insufficient to activate the ER complex to induce apopt osis. Nevertheless, the cis- isomers of the compounds are binding to the ER in such a fashion that they are able to activate the receptor to induce both growth and apoptosis. We propose that the presence of the cis-isomeric contaminant in the EthoxyTPE compound is actually producing the apoptotic effect, similar to the CFR4OHT, however the only way to prove

that would to perform further experiments with CFREthoxyTPE compound which is currently synthesized.

Conclusions

The tested compounds with various structures were evaluated for their estrogenic/antiestrogenic properties in human breast cancer cell lines to induce growth or estrogen-induced apoptosis. The compounds properties were evaluated using a DNA cell proliferation assay. The results of the assays demonstrate that there is a different biology of the isomers of the same antiestrogen FR4OHT and that the conformation of the ER that is dictated by the structure of the ligand is crucial in inducing apoptosis in hormone independent breast cancer cells. Further experiments are needed to confirm the hypothesis.

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

Introduction:

From our previously published experiments on the characterization of the MCF7:5C cell culture *in vitro* we identified ER and AIB1 interacting proteins ((82, 110); Kirilyuk et al. COE report 2012).We were particularly interested in AIB1 interacting proteins that had known transcriptional repressor function and that were differentially expressed between MCF-7 and MCF-7:5C. We hypothesized that repressing AIB1 function would allow changes in the apoptotic responses in cells since we have previously published that AIB1 is rate limiting for estrogen induced proliferation and inhibition of apoptosis in parental MCF7 cells. Loss of AIB1 function would predict switch to a pro-apoptotic effect of AIB1. To investigate potential repressors of AIB1 function we examined all AIB1 interacting proteins identified by Mass spectrometry in the interaction lists previously published under this COE(82, 110), We determined that a known co-repressor Fox G1 was of interest since it bound to AIB1 differentially from MCF7 vs MCF7:5C. In the past year we have investigated the effects of Fox G1 on apoptosis and regulation of AIB1 gene expression and those findings are described below and published recently(120). Highlights of this work and manuscript are included and quoted below.

WORK ACCOMPLISHED

Background:

Amplified in breast cancer 1 (AIB1, ACTR, RAC3, SRC3, NCOA3 and p/CIP**)** belongs to the p160 family of steroid receptor coactivators and is found to be frequently amplified in multiple human cancers(121). Similar to the other p160 coactivators, AIB1 can associate with hormone-bound nuclear receptors, and potentiate transcriptional activation by enhancing transcriptional complex assembly and through local chromatin remodeling(122-124). AIB1 is an oncogene and has been strongly implicated in the development of hormone-responsive and nonresponsive cancers(125, 126) by coactivating not only nuclear receptors but also nonreceptor transcription factors such as E2F1, nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and PEA3(127-130). In mouse models, AIB1 overexpression results in the development of mammary hyperplasia and tumorigenesis(131). The overexpression of AIB1 has been observed in 30-60% of human breast tumors and a strong correlation exists between high levels of AIB1 and high HER2 levels, larger tumor size, higher tumor grade, increased cancer reoccurrence and worse prognosis(132).

AIB1 expression can be controlled at multiple levels. AIB1 protein levels are regulated by a number of proteasomal degradation pathways(133-135). In terms of AIB1 mRNA, we have previously reported that all-*trans* retinoic acid, antiestrogens and tamoxifen, and TGF-β can upregulate AIB1 transcripts, whereas estrogen can suppress AIB1 gene expression(136). In addition, a recent study demonstrates that transcription of the AIB1 gene is controlled by regulatory sequences within the bp -250 to +350 region of its promoter which enable AIB1 to auto-regulate and enhance the expression of its own gene(137, 138). In these studies, an Sp1-binding site down-stream of exon 1 was described within the -250/+350 region that also recruited E2F1. This enables AIB1 to complex with E2F1, and this Sp1-associated transcription complex significantly increases the coactivation of the AIB1 gene(138).

AIB1 is also known to directly bind to other coactivators such as histone acetyltransferase p300/CBP, p300/CBP-associated cofactor p/CAF, and arginine methyltransferase CARM 1, and enhances transcriptional activation by bringing these potent cofactors capable of modifying chromatin organization to the target gene promoter(123, 139, 140). The ability to interact with a wide range of transcriptional cofactors allows AIB1 to act as a potent coactivator(132). In contrast, only a few transcriptional corepressors that interact with the SRC family proteins are known(141, 142). Therefore, we conducted broad screens of AIB1-interacting proteins using mass spectrometry (MS) to detect low abundance AIB1 binding partners that may potentially suppress AIB1 function and negatively regulate the AIB1 gene expression. We focused on AIB1-interacting proteins that segregated under the category of "transcriptional repressors," and here we demonstrate that the winged-helix, DNA-binding transcriptional corepressor FoxG1 (also known as brain factor 1, BF1) which we identified as an AIB1 interacting protein, can downregulate AIB1 promoter activity and suppress both AIB1 transcript and protein expression in MCF-7 cells. FoxG1 belongs to the forkhead-box family of transcriptional regulators, and is a protein mainly expressed in the brain and testis in human(143, 144). FoxG1 controls the development of the telencephalon and cerebral corticogenesis(143) and is shown to interact with global transcriptional corepressors and histone deacetylases to potentiate transcriptional repression(145). FoxG1 can also directly interact with androgen receptor (AR) and suppress AR-mediated transactivation(144). While FoxG1 knockout

mice develop cerebral hypoplasia and die at birth, humans with FoxG1 haploinsufficiency show severe mental retardation and microcephaly(143, 146, 147). The prominent developmental phenotype associated with FoxG1 pathology has focused most investigations of FoxG1 function on the brain and neurogenesis, therefore not much is known about the role of FoxG1 in cancer and the molecular mechanism underlying FoxG1 function.

Our data indicate that FoxG1 is directly recruited to the AIB1 promoter. We report a mechanism by which FoxG1 overexpression compromises the integrity of an Sp-1-associated activating transcriptional complex. This complex is required for the upregulation of AIB1 gene expression, and FoxG1 reduces its recruitment by disassembly and detachment of the activating complex from the AIB1 promoter. We also show that FoxG1 downregulates AIB1 expression which leads to apoptosis in human breast cancer cells.

Results obtained:

AIB1 interacts with the transcriptional corepressor FoxG1

To identify proteins that interact with AIB1, we performed AIB1-specific immunoprecipitations (IP) of lysates from HEK293T cells transfected with a FLAG-tagged AIB1 construct. After IP with a FLAG antibody, we isolated FLAG-associated immunocomplexes by denaturing gel electrophoresis, followed by Coomassie Blue staining. Twelve visible bands were excised, and extracted proteins were subjected to MS analysis (described in(110). FoxG1 was identified as a candidate AIB1-interacting protein through our MS evaluation. It was of interest for further investigation since it is known to function in certain contexts as a transcriptional repressor(145) and could potentially regulate AIB1 function and gene expression. To verify AIB1 interaction with FoxG1, we performed coimmunoprecipitation (co-IP) experiments. FLAG-AIB1 was expressed by transient transfection together with HA-FoxG1 in HEK293T cells and HA-FoxG1 was detected in the immunoprecipitates of FLAG-AIB1 from whole cell lysates (Fig. 46A). We also confirmed the interaction of endogenous AIB1 and FoxG1 in MCF-7 breast cancer cells which harbor the 20q AIB1 gene amplicon and express high levels of AIB1 protein and a detectable amount of FoxG1 (Fig. 46B). These IP results confirm the MS data and demonstrate that FoxG1 is present in complexes that co-immunoprecipitate with AIB1.

FoxG1 is predominantly expressed in the brain and its non-neuronal expression in normal tissues is low(143, 144). The expression of FoxG1 in human cancer has not been widely reported. In a comparison of normal breast cells and breast cancer cell lines, we found that the mRNA expression level of FoxG1 was significantly lower in breast cancer cell lines, irrespective of their estrogen receptor (ER) status, as compared to FoxG1 expression in the normal human mammary epithelial cells (HMEC) (Fig. 46C). These data suggest a loss of FoxG1 expression from normal to cancerous transition (Fig. 46C) and indicate that reduced FoxG1 expression might have prognostic significance in human breast cancer. Our reanalysis of published microarray data (www.oncomine.org) from human breast cancer clinical samples of four independent studies (Zhao et al.(148) , Turashvili et al. (149), Richardson et al.(150), and The Cancer Genome Atlas - Invasive Breast Carcinoma Gene Expression Data/TCGA) further supported this hypothesis and showed a significant ($p<0.0001$) negative correlation between AIB1 and FoxG1 mRNA expression over 714 samples, where higher expression of FoxG1 coincided with lower expression of AIB1 and vice versa (Fig. 46D).

In addition, we used unbiased gene expression data compiled by Kaplan-Meier (KM) Plotter (http://kmplot.com) (45) from a series of suitable studies that allow for an analysis of clinical outcomes correlated with a single gene expression. We saw that higher FoxG1 mRNA levels, in

2241 breast cancer samples, correlated with increased relapse-free survival rate (Fig. 46E) In contrast, in the same data set, elevated AIB1 transcript levels correlated with reduced relapse-free survival (RFS) (Fig. 46E), which is consistent with previous reports on AIB1 prognostic significance in human breast cancer (reviewed in(132)).

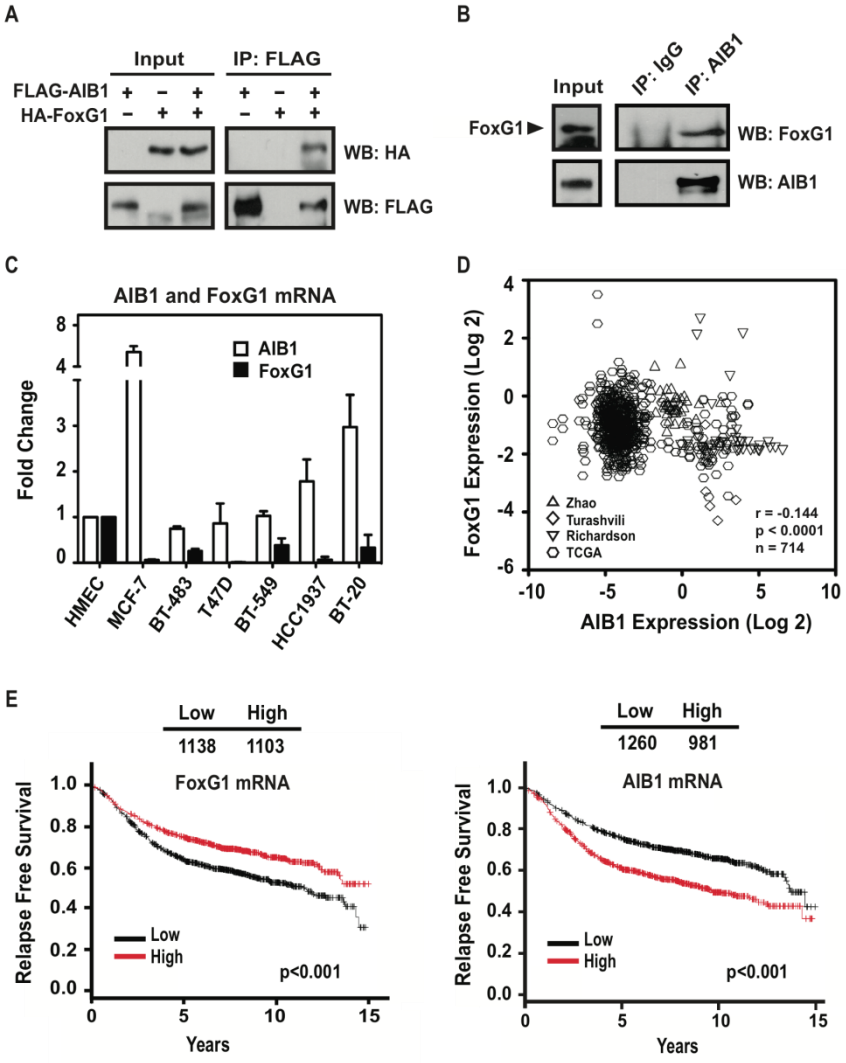


Figure 46. AIB1 and FoxG1 interact in mammalian cells.
A, AIB1 interacts with FoxG1 in HEK293T cells. FLAG-AIB1 was cotransfected with HA-FoxG1 constructs. 48 hours post transient transfection, whole cell lysates were collected and used for immunoprecipitation (IP) and western blot (WB) analysis with anti-FLAG and anti-HA antibodies as indicated. B, Interaction of endogenous AIB1 with FoxG1 in MCF-7 breast cancer cells. Nuclear lysates were prepared from MCF-7 cells and immunoprecipitated with an AIB1 antibody or control IgG. FoxG1 protein associated with AIB1 in the IP was detected by WB as indicated. C, AIB1 and FoxG1 mRNA expression levels in breast cancer cell lines. Total RNA was harvested from breast cancer cell lines to determine the relative gene expression for AIB1 and FoxG1. D, FoxG1 and AIB1 mRNA expression are inversely correlated. Data from Zhao et al. (148), Turashvili et al.(149) , Richardson et al.(150) , and TCGA (The Cancer Genome Atlas - Invasive Breast Carcinoma Gene Expression Data) were analyzed using Oncomine (www.oncomine.org). Higher expression of FoxG1 coincides with lower expression of AIB1 and vice versa. E, Analysis of the levels of AIB1 and FoxG1 mRNA on a gene expression microarray of breast cancer samples from patients with known relapse-free survival (RFS) times provided by

Kaplan-Meier Plotter (http://www.kmplot.com) (KM analysis parameters are described in Material and Methods) (45).

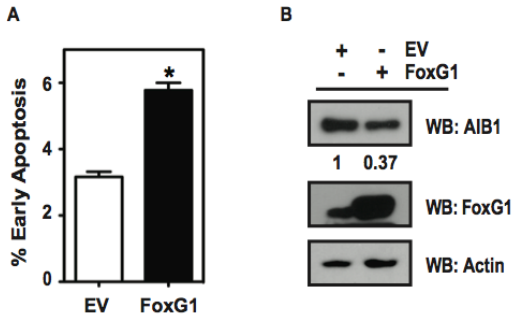
FoxG1 induces apoptosis in MCF-7 cells

The expression pattern for AIB1 and FoxG1 in MCF-7 cells was of interest because these cells significantly overexpress AIB1 and show low levels of FoxG1 mRNA expression compared to HMEC (Fig. 46C). We therefore chose to study the phenotypic effect of FoxG1 overexpression on MCF-7 cells. Twenty-four hours after expression vector transfection, overexpressing FoxG1 led to cell detachment from culture dishes, and the induction of apoptosis as determined by annexin V staining (Fig. 47A, upper panel). The early and total apoptosis average indices for duplicate samples of 14.5 and 28.64 % in FoxG1-expressing cells were significantly elevated compared to MCF-7 cells transfected with the control empty vector (EV) with average apoptosis indices of 1.68 and 9.4 %, respectively (Fig. 47A, lower panel). Increased expression of FoxG1 in MCF-7 cells was correlated with the apoptotic response.

Previous studies reported increased incidence of apoptosis in MCF-7 cells when AIB1 expression is downregulated by small-interfering RNA (siRNA)-directed gene silencing(151) . Thus, since AIB1 and FoxG1 form a complex (Fig. 46A and B), we conjectured that a portion of the FoxG1-induced apoptotic effect might be mediated through changes in AIB1 expression. Consistent with this notion, FoxG1 overexpression caused a twofold decrease in both AIB1 mRNA and protein expression levels (Fig. 47B). We also observed a similar effect of FoxG1 on AIB1 expression in the metastatic MDA-MB-231 cells. In these cells, FoxG1 overexpression caused a 3% to 6% increase in early apoptosis and a near 60% reduction in the levels of AIB1 protein (Supplemental Fig. 1A and B). This indicates that FoxG1 can have a negative regulatory effect on AIB1 expression also in other different subtypes of breast cancer cells that are estrogen receptor (ER) negative.

We next asked whether the FoxG1 induction of apoptosis was mediated directly by the loss of AIB1 by determining whether exogenously expressed AIB1 was sufficient to rescue these cells from FoxG1-induced apoptosis. Our analysis revealed that AIB1 co-expression with FoxG1 allowed an approximately 50% rescue from apoptosis compared to cells transfected with only FoxG1 (Fig. 47C, right panel; compare 2nd bar with the 4th bar). Thus, our phenotypic studies argue that a significant portion of the FoxG1 induction of apoptosis in MCF-7 cells is mediated through down-regulation of AIB1 expression.

Supplemental Figure 1



<u>Supplemental Fig. 1</u>. FoxG1 induces apoptosis and down-regulates AIB1 protein expression in MDA-MB-231 breast cancer cells. A, MDA-MB-231 cells were transfected with either an empty vector (EV) control or FoxG1 constructs. 24 hours after transfection, cells were subjected to Annexin V apoptosis analysis and cells undergoing early apoptosis were quantified. The mean ± SEM values were obtained from duplicate samples from each transfection condition. *, P<0.05 relative to EV. Statistical analysis was done by Student's t test. B, Analysis of endogenous AIB1 protein expression in MDA-MB-231 cells overexpressing FoxG1. Cells were transfected with EV or FoxG1 as in (A), whole cell lysates were then collected to determine the relative protein levels for AIB by WB with antibodies as indicated

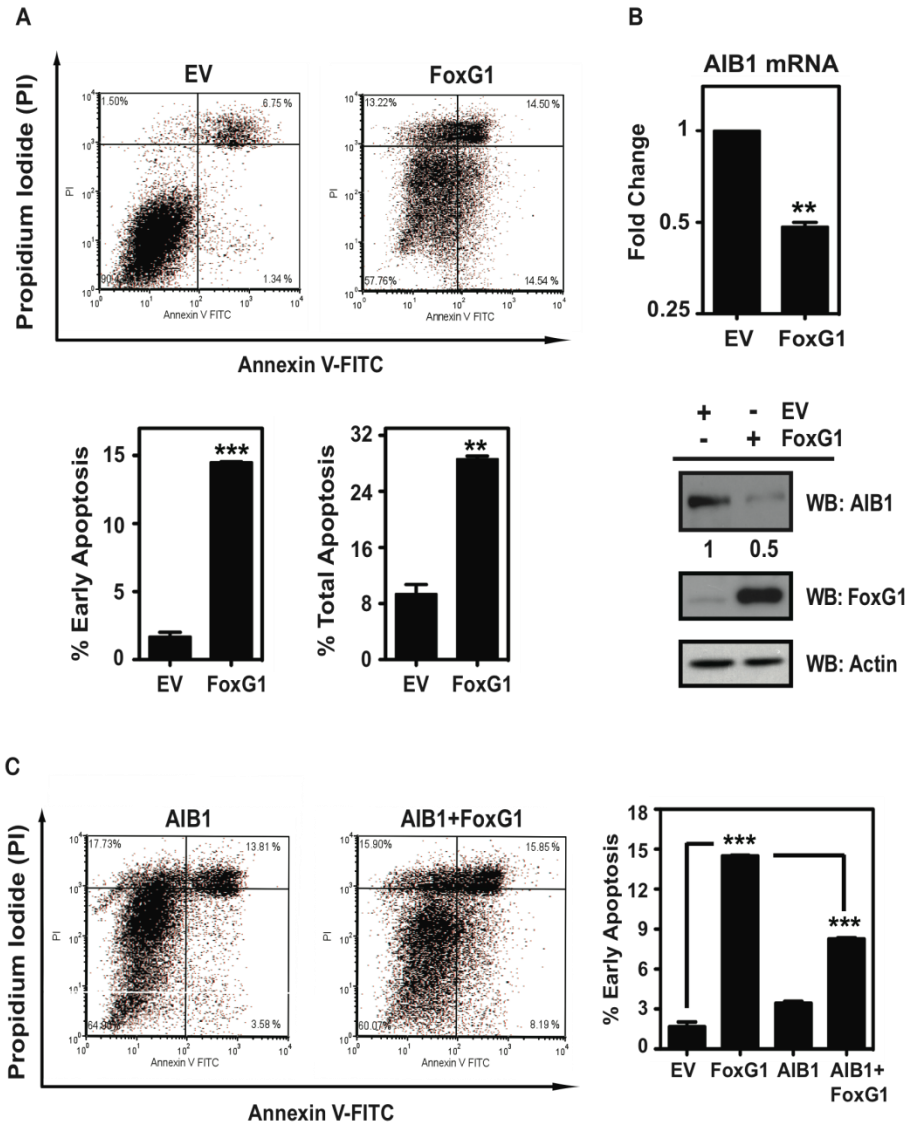


Figure 47. FoxG1 induces apoptosis and down-regulates AIB1 expression in MCF-7 cells. A, MCF-7 cells were transfected with either an empty vector (EV) control or FoxG1 constructs. 24 hours after transfection, cells were subjected to Annexin V apoptosis analysis. The percentages of cells in early- and late apoptosis are represented by bottom right- and top right quadrants of the FACS analysis, respectively. Percent total apoptosis was the total percentage of cells in both early- and late apoptosis. The mean ± SEM values were obtained from duplicate samples from each transfection condition. ***, P<0.001; **, P<0.01 relative to EV. Statistical analysis was done by Student's t test. B, Analysis of endogenous AIB1 expression in MCF-7 cells overexpressing FoxG1. Cells were transfected with EV or FoxG1 as in (A). Total RNA and whole cell lysates were collected to determine the relative levels of mRNA and protein for AIB1. Cells transfected with EV were arbitrarily set at 1 and cells expressing FoxG1 were analyzed in reference to it. Student's t test. **, P<0.01 relative to EV. Relative protein levels were determined by WB with antibodies as indicated. C, AIB1 rescues MCF-7 cells from FoxG1-induced apoptosis. MCF-7 cells were transfected separately with expression vectors for either EV control, FoxG1, AIB1, or AIB1 and FoxG1 together. Cells were assessed for apoptosis as in (A). ***, P<0.001, EV vs. FoxG1; or FoxG1 vs. AIB1+FoxG1. One-way ANOVA with Bonferroni post test.

FoxG1 represses AIB1 promoter activity

Our data demonstrate that FoxG1 overexpression represses the levels of AIB1 transcript (Fig. 47B), thus, we hypothesized that FoxG1 could directly regulate the AIB1 promoter. Previous studies have identified a region in the AIB1 gene promoter responsible for the positive auto-regulation of transcription through the recruitment of an activating transcriptional protein complex involving AIB1, E2F1, and Sp1 (138). This critical positive regulatory sequence covers a -250 to +350 span up and downstream of the transcription start site of the AIB1 gene. An intronic Sp1 binding site, marked by a GC box at bp +150/+160 is required for the AIB1 promoter activation by E2F1 and AIB1. Moreover, the Sp1 binding sequence serves as a docking site for the recruitment of the AIB1-E2F1 complex, through which AIB1 can act as a coactivator on its own promoter, establishing a positive auto-regulatory loop of AIB1 gene expression (Fig. 48A) (138).

To determine whether FoxG1 impacted AIB1 promoter activity we cotransfected MCF-7 cells with an E2F1 expression vector in the presence or absence of FoxG1 and a wild-type (WT) AIB1 promoter-luciferase reporter containing the intact positive regulatory sequence of the AIB1 gene promoter (Fig. 48B i). E2F1 significantly enhanced the AIB1 promoter reporter activity, as shown previously (Fig. 48B ii) (138), whereas the addition of FoxG1 suppressed E2F1-induced AIB1 promoter activation back to the basal level, indicating an inhibitory role for FoxG1 in AIB1 gene expression (Fig. 48B ii).

Because the intronic GC-rich, Sp1 binding sequence is essential for the recruitment of Sp1, AIB1 and E2F1 (138), we next tested whether FoxG1 is also recruited to the AIB1 gene promoter through this element. We performed chromatin immunoprecipitation (ChIP) assays using HEK293T cells transfected with either the WT luciferase reporter AIB1(-250/+350) or the same reporter with a deleted Sp1 site - AIB1(-250/+350)-Sp1-del (Fig. 48B i). PCR primers with a forward primer positioned on exon 1 of the AIB1 gene and a reverse primer in the luciferase sequence were used to specifically detect and distinguish the transfected luciferase vectors from the endogenous AIB1 promoter (Fig. 48B i, red arrows). Our ChIP analysis showed that FoxG1 is associated with the WT AIB1(-250/+350) reporter but not the mutant reporter AIB1(-250/+350)-Sp1-del (Fig. 48B iii). Consistent with published literature (138), our data also demonstrated that AIB1, E2F1 and Sp1 bind to the WT AIB1(-250/+350) reporter, whereas the Sp1 site deletion abolished recruitment of these proteins (Fig. 48B iii). These results indicate that the Sp1 binding site is not only required for Sp1, E2F1 and AIB1 binding to the AIB1 promoter, but it is also critical for the recruitment of FoxG1.

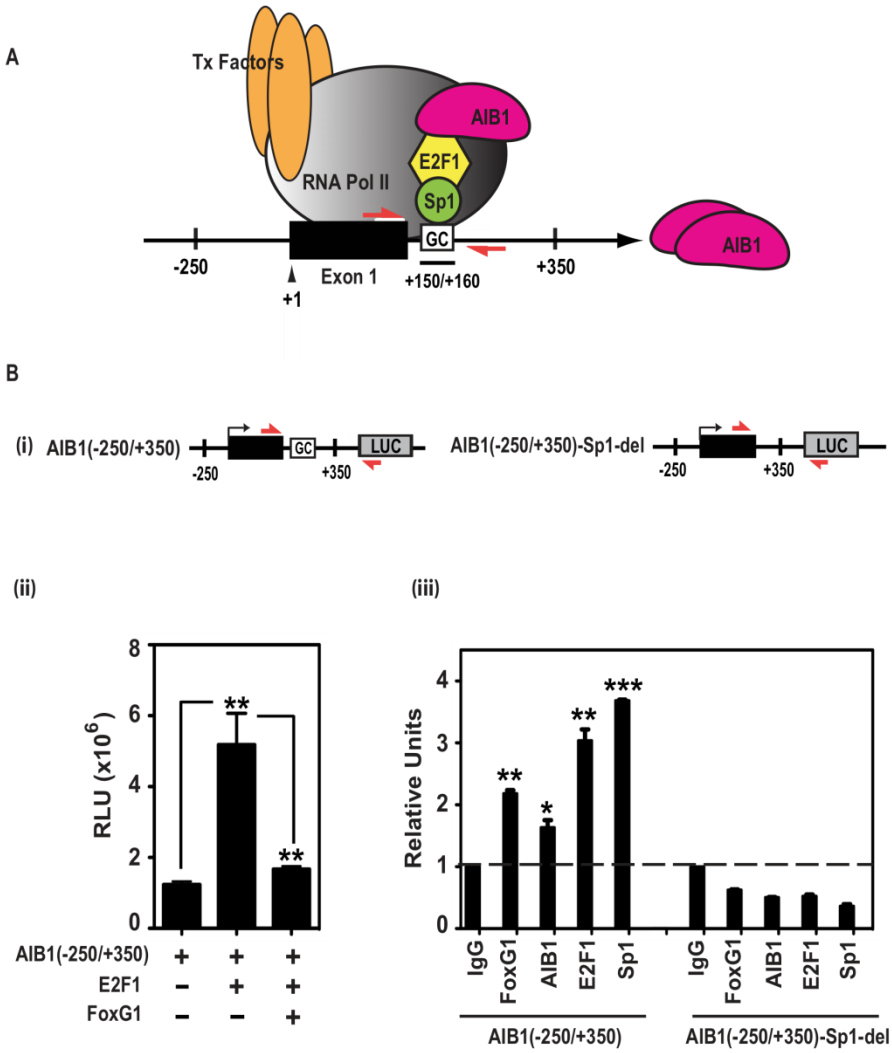


Figure 48. FoxG1 represses AIB1 gene promoter activity. _A, Model of the AIB1 gene promoter. Model showing an activating transcriptional complex consisting of AIB1, E2F1 and Sp1, anchored to DNA through a Sp1-binding site, the GC box, which is down stream of exon 1 (black box) in the -250 to +350 base pair region of the AIB1 promoter. The red arrows represent the locations and orientations of the AIB1 promoter-specific primers. B, panel i, AIB1 wild type (WT) and mutant Sp1 site-deleted promoter luciferase reporters. The red arrows are primers that specifically detect these reporters. Panel ii, FoxG1 represses the activity of the AIB1 promoter reporter. MCF-7 cells were transfected with WT AIB1(-250/+350) reporter alone, or together with E2F1 in the presence or absence of FoxG1. A representative graph is shown from two independent experiments and data were analyzed by one-way ANOVA with Bonferroni post test. **, P<0.01 when E2F1 is compared to promoter alone; or FoxG1 and E2F1 together relative to E2F1. Panel iii, Protein association to the Sp1 binding site in the transfected AIB1 gene promoter. HEK293T cells were transfected with either the WT AIB1 reporter or the mutant reporter where the Sp1 binding sequence is deleted. Cells were processed for ChIP 6 hours post transfection. Recruitment of FoxG1, AIB1, E2F1 and Sp1 to both the WT- and mutant reporters was assessed with a pair of primers that specifically detect the transfected reporter DNA (panel i, red arrows). The IgG-ChIP was arbitrarily set as 1 and all the samples were analyzed and plotted in reference to IgG. Data represent two independent experiments and were analyzed by Student's t test. ***, P<0.001; **, P<0.01; *, P<0.05 compared with IgG control.

FoxG1 forms a complex with AIB1 and E2F1 on the endogenous AIB1 gene promoter

We next performed ChIP assays to investigate whether FoxG1 is directly recruited to the -250/+350 region of the endogenous AIB1 gene promoter in MCF-7 cells. Using a pair of AIB1 promoter-specific primers where the forward primer is positioned on exon 1 of the AIB1 gene and the reverse primer is situated down-stream of the Sp1 binding sequence (Fig. 48A, red arrows), we found that an antibody specific to FoxG1, but not the IgG control, successfully immunoprecipitated endogenous FoxG1 on the AIB1 promoter (Fig. 49A). As a negative control we demonstrated no specific FoxG1 binding relative to the IgG control to a region in the coding sequence of exon 4 of the AIB1 gene (Fig. 49A). We also show that FoxG1 is not recruited specifically to the non-target albumin promoter (Fig. 49A). Together, these ChIP data support FoxG1 specific recruitment to the AIB1 promoter.

AIB1 has been shown to directly interact with E2F1 and the AIB1-E2F1 complex is essential for E2F1-regulated gene transcription(127). Individual binding of AIB1 and E2F1 to the -250/+350 region of the endogenous AIB1 gene promoter is well established (138), and our goal was to determine whether AIB1 and E2F1 are recruited to this region as a complex. We performed reciprocal ChIP-reChIP (reChIP) assays to address this question. Cross-linked and sonicated chromatin was prepared from MCF-7 cells, incubated and immunoprecipitated first with either AIB1 or E2F1 antibodies. The AIB1- or E2F1 ChIP, followed by "release" of the protein-enriched chromatin, was subjected to a subsequent ChIP with antibodies specific to either E2F1 or AIB1, respectively. As a control, isotype IgG was used for the first round of ChIP followed by E2F1- or AIB1 reChIP. The two-step reciprocal reChIP successfully precipitated the endogenous AIB1 promoter, indicating that AIB1 and E2F1 form a protein complex at the -250/+350 region of the AIB1 promoter (Fig. 49B).

We next assayed for endogenous FoxG1 participation in the AIB1-E2F1 complex by reciprocal reChIP assays. We found that the endogenous AIB1 promoter was precipitated from FoxG1/AIB1 and FoxG1/E2F1 reChIP immunoprecipitates, as well as AIB1/FoxG1 and E2F1/FoxG1 reChIP immunoprecipitates (Fig. 49C and D). These data suggest simultaneous chromatin co-occupancy of the three proteins, and indicate that there is basal level recruitment of endogenous AIB1, E2F1 and FoxG1 to the -250/+350 regulatory sequence of the AIB1 promoter.

We have confirmed an interaction between AIB1 and FoxG1 (Fig. 46A and B), and shown that the two proteins are recruited to the endogenous AIB1 gene promoter as a complex (Fig. 49C). To examine whether FoxG1 occupancy at the endogenous AIB1 promoter is dependent on the co-recruitment of AIB1, we infected MCF-7 cells with lentiviral vectors expressing shRNAs targeting AIB1 (shRNA-AIB1) or control scrambled shRNAs (shRNA-Control). Depletion of AIB1 protein by shRNA silencing led to a threefold decrease in AIB1 occupancy at the endogenous AIB1 gene promoter as well as a twofold reduction in the recruitment of FoxG1 to the promoter (Fig. 49E). This decrease in FoxG1 occupancy at the AIB1 promoter was not due to a reduction in FoxG1 expression since its protein levels remained unchanged while the levels of AIB1 protein were significantly reduced (Fig. 49E, Western Blot). Together our data indicate that the presence of AIB1 is required for the co-recruitment of FoxG1 to the AIB1 gene promoter.

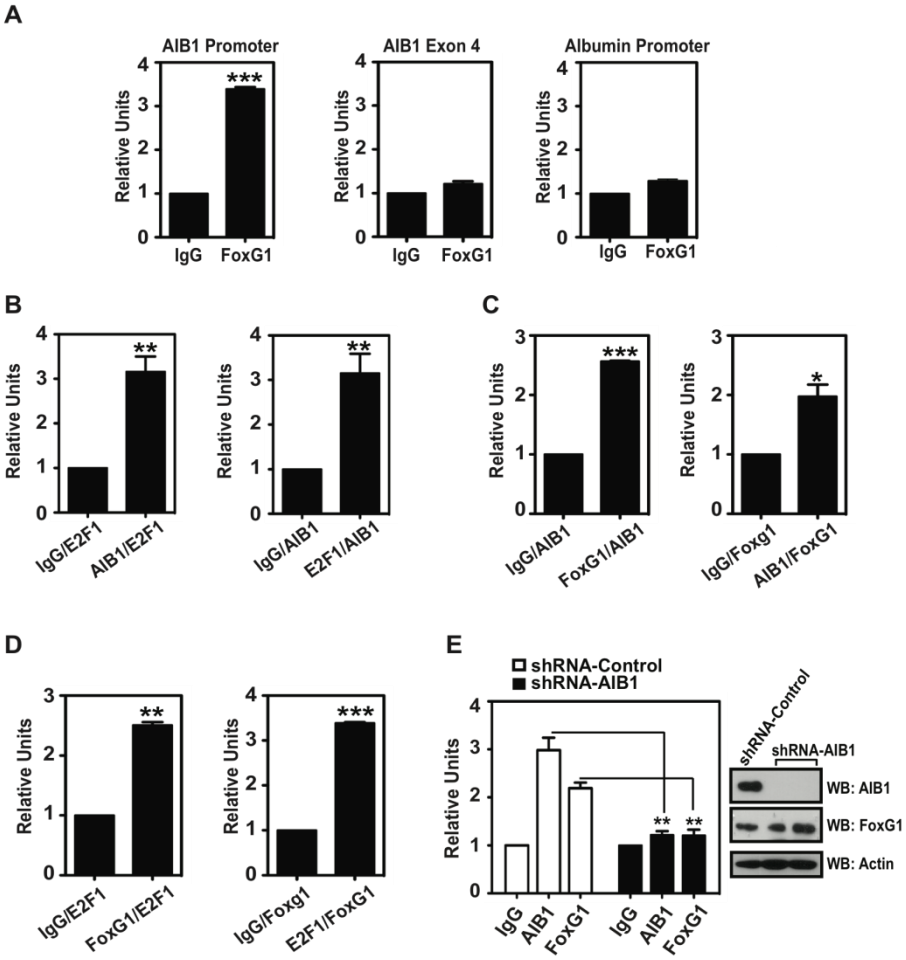


Figure 49. FoxG1 forms a complex with AIB1 and E2F1 on the AIB1 gene promoter. A, FoxG1 is recruited to the endogenous AIB1 promoter. ChIP assays were performed in MCF-7 cells, where endogenous FoxG1-DNA complex was immunoprecipitated with anti-FoxG1 antibody or isotype IgG. Protein-enriched DNA was analyzed by RT-PCR using AIB1 promoter-specific primers (Fig. 3A, red arrows) or primers that will either amplify a region in exon 4 of the AIB1 gene or the albumin promoter. ChIP results were analyzed by Student's t test, where ***, P<0.001 relative to IgG. B to D Two-step ChIP-reChIP assays were performed in MCF-7 cells, and all DNA samples were subjected to two rounds of ChIPs. Sonicated chromatin was immunoprecipitated first with (B) AIB1 or E2F1-, (C) FoxG1 or AIB1-, or (D) FoxG1 or E2F1 antibodies, followed by reChIP with antibodies specific to (B) E2F1 or AIB1, (C) AIB1 or FoxG1, or (D) E2F1 or FoxG1. As a negative control, isotype IgG was used for the first-round ChIPs followed by reChIP of the respective second-round antibodies. The endogenous AIB1 promoter bound to each immunocomplex as indicated in the figure was analyzed by RT-PCR using the AIB1 promoter-specific primers. Student's t test. ***, P<0.001; **, P<0.01; *, P<0.05 when compared with each respective IgG-reChIP control. E, FoxG1 recruitment to the endogenous AIB1 promoter is dependent on AIB1. Endogenous AIB1 was depleted by infection of MCF-7 cells with lentiviral vectors expressing shRNAs targeting a distinct sequence in AIB1 (shRNA-AIB1) or control scrambled shRNA (shRNA-Control). 96 hours post lentiviral infection, cells were subjected to ChIP analyses where protein-DNA complexes were immunoprecipitated with antibodies against either AIB1 or FoxG1, or an isotype IgG. Student's t test. **, P<0.01; shRNA-Control vs. shRNA-AIB1. The amount of AIB1 protein knocked down 96 hours post infection was assessed by Western blot with antibodies as indicated.

FoxG1 compromises the integrity of the activating complex on the AIB1 gene promoter

We next performed ChIP assays to investigate the effect of overexpressing FoxG1 (at levels that suppress AIB1 mRNA expression and cause apoptosis as shown in Fig. 47A and B) on the transcription complex present on the endogenous AIB1 promoter at bp +150/+160 (Fig. 48A). ChIP assays were performed from MCF-7 cells that had been transfected with either an EV control or a FoxG1-expression vector. We demonstrate that increased expression of FoxG1 resulted in >50% decline in the promoter-binding activities of AIB1, E2F1, p300 and RNA polymerase II (Pol II), although there was no significant change in Sp1 recruitment at the promoter level (Fig. 50A). The loss of promoter binding was not due to FoxG1-induced changes in gene expression since protein levels of these factors were either unchanged or increased (e.g. E2F1) when FoxG1 was overexpressed (Fig. 50B). Interestingly, we did not observe increased chromatin occupancy of FoxG1 when it was overexpressed (Fig. 50A) suggesting that once cellular expression of FoxG1 is above a threshold level, it can promote rapid disassembly of the Sp1-associated complex without affecting the direct binding of Sp1 to the +150/+160 AIB1 promoter binding element. To test this, we performed reciprocal reChIP experiments to assess the integrity of the transcriptional protein complex with increased expression of FoxG1. We show that FoxG1 overexpression in MCF-7 cells caused significant reduction in the recruitment of protein complexes comprising Sp1 and E2F1, AIB1 and FoxG1 respectively, or E2F1 and Sp1, AIB1 and FoxG1 respectively (Fig. 50C and D). Most interestingly, we observed that overexpressing FoxG1 led to a near three- to twelvefold decrease (depending on the orientation of the reChIP) in the co-recruitment of E2F1 complexed with Sp1 to the AIB1 promoter (Fig. 50C and D; compare “Sp1/E2F1” and “E2F1/Sp1” between EV, white bar and FoxG1, black bar).

The recruitment of p300 to the +150/+160 Sp1-associated complex has not been described previously and overexpression of FoxG1 also reduces its association with the complex at the AIB1 promoter (Fig. 5A), without a reduction in p300 protein expression (Fig. 50B). The binding of p300 to AIB1 is known to promote and stabilize transcriptional complex formation, and exert a positive effect on gene transcription(123, 128, 152). Therefore, we wanted to determine whether overexpressing FoxG1 had any effect on the recruitment of the AIB1-p300 complex. We assessed co-occupancy of AIB1 and p300 at the AIB1 promoter by reciprocal reChIP, and discovered a five- to eightfold reduction in the recruitment of AIB1-p300 complex to the AIB1 promoter in MCF-7 cells transfected with FoxG1 as compared to control (Fig. 50E).

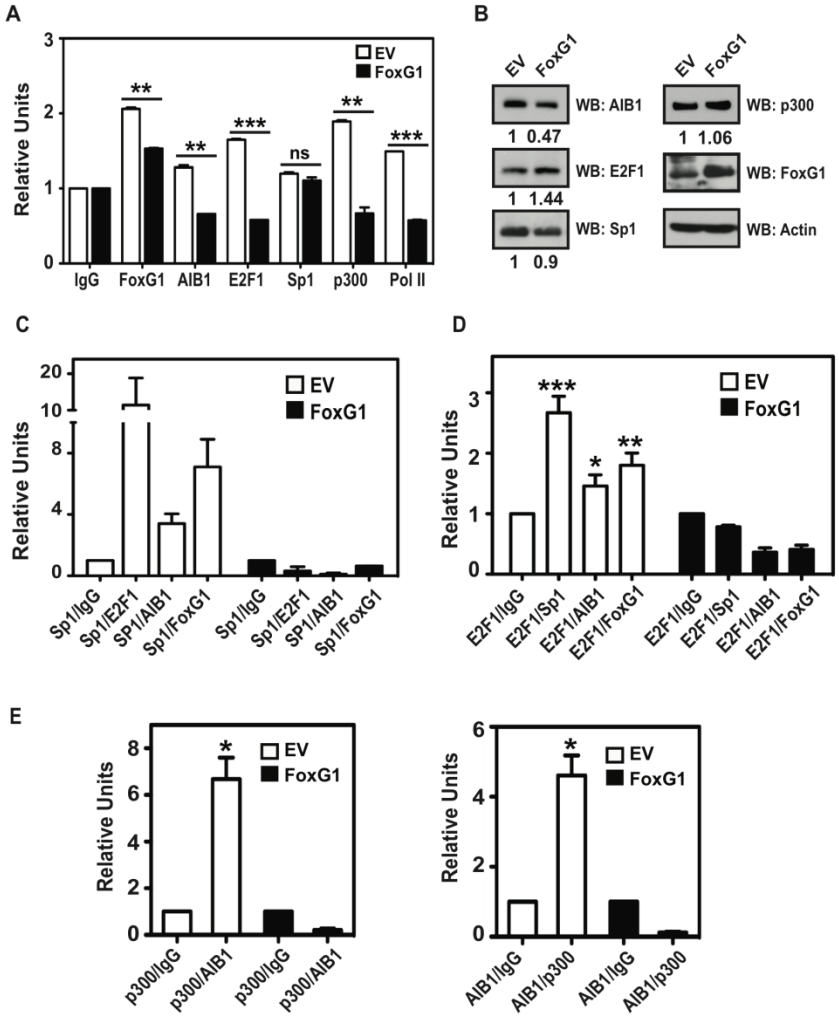


Figure 50. FoxG1 destabilizes the Sp1-assoicated transcription complex on the AIB1 gene promoter. A, FoxG1 overexpression leads to decreased recruitment of the members of the transcriptional complex to the endogenous AIB1 promoter. ChIP assays were performed in MCF-7 cells transfected with EV or FoxG1 vectors, by enriching protein-bound endogenous AIB1 promoter with antibodies as indicated. Student's t test, where ***, P<0.001; **, P<0.01 were FoxG1-expressing cells (black bars) relative to EV (white bars). B, Relative protein levels after FoxG1 transfection in MCF-7 cells are shown by WB and probed with antibodies as indicated. C and D, Overexpressing FoxG1 compromises the integrity of the transcription complex. The immunocomplexes associated with the AIB1 promoter were assessed by ChIP-reChIP experiments, where chromatin was immunoprecipitated sequentially first with an anti-Sp1 antibody, followed by reChIP with antibodies specific to either E2F1, AIB1, or FoxG1; or first with an anti-E2F1 antibody, followed by reChIP with antibodies specific to either Sp1, AIB1, or FoxG1. The Sp1-ChIP and E2F1-ChIP were also followed by a reChIP of IgG as a negative control. ***, P<0.001; **, P<0.01; *, P<0.05 relative to E2F1/IgG. Student's t test. E, Overexpressing FoxG1 causes reduction in p300-AIB1 co-occupancy at the AIB1 promoter. MCF-7 cells were transfected with EV or FoxG1 as in (A), and harvested for reChIP experiments by performing reciprocal and sequential ChIPs using antibodies specific to p300, followed by AIB1, or to AIB1, followed by p300. The AIB1 promoter-specific primers were used to assess the relative occupancy of the AIB1-p300 complex at the endogenous AIB1 promoter. *, P<0.05 relative to p300/IgG or AIB1/IgG. Student's t test.

FoxG1 disrupts AIB1's coactivator function

We next tested if the effect of FoxG1 on AIB1-containing transcription complexes was limited to the Sp1 binding site in the AIB1 gene promoter or if other promoter elements known to involve AIB1 were also affected. AIB1 has previously been shown to coactivate NF-κB and AP-1(128, 130). Therefore we examined the impact of FoxG1 on promoters containing these transcription factor binding sites. As reported previously (153), AIB1 overexpression induced transcription from all these reporters (Fig. 51A to C) but concomitant FoxG1 overexpression caused a significant reduction in the AIB1-induced transcription of the AP-1 and NF-κB promoters (Fig. 51A and B). We also observed a near-complete reversal of AIB1 coactivation on the estrogen-responsive promoter (ERE) reporter in the presence of FoxG1 and estrogen (Fig. 51C). Interestingly, FoxG1 expression caused a slight decrease in AIB1 coactivation on the promoter-reporters in the empty vector transfected cells (Fig. 51A to C). HEK293T cells express endogenous AIB1 protein, which may coactivate the reporters and affect transcription from the empty vector control. Thus, FoxG1 overexpression should dampen basal AIB1 coactivating activity. To assess the impact of FoxG1 expression on endogenous genes in MCF-7 cells we used quantitative RT-PCR to generate a mRNA expression profile of 168 genes that are known to participate in or respond to ER or NF-κB signaling. In addition, five housekeeping genes were included as a loading control (ACTB, B2M, GAPDH, HPRT1, RPLP0). Using a cutoff of gene expression changes of >1.5 fold and P<0.01, we found that in the NF-κB gene set, 30 genes were upregulated and 14 were downregulated (40 were unchanged); in the ER responsive gene set, 8 genes were upregulated and 42 genes were downregulated (34 were unchanged) after overexpression of FoxG1. In supplemental table 1 we detailed some of the notable genes involved in breast cancer pathogenesis that were downregulated by FoxG1 from the NF-κB and ER gene array sets. However, the repressive effect of FoxG1 on AIB coactivated gene promoters was not universal since we found in parallel experiments that FoxG1 overexpression had no impact on endogenous E2F1-regulated genes such as CDK2, CDC25a, MCM7, E2F1 and CDC6 in MCF-7 cells (Fig. 51D). These data indicate that FoxG1 is important not only for the control of AIB1 promoter, but also for some AIB1 regulated steroid-dependent and -independent transcription, although the impact of FoxG1 is dependent on the promoter context.

Supplemental Table 1

NF-κB Responsive	
Gene (acronym)	Regulation (fold, p-value)
CCL2	+ 18.3 **
CCL5	+ 5.3 **
CSF1	+ 2.1 **
CSF2	+ 1.7 **
CSF3	- 12.1 **
ICAM1	+ 4.9 **
IFNG	- 3.0 **
IL8	+ 1.1 ns
LTA	+ 1.1 ns
TNF	+ 4.0 **

ER Responsive	
Gene (acronym)	Regulation (fold, p-value)
AIB1	- 2.0 **
BRCA1	- 2.4 **
EBAG9	- 1.6 **
ESR1	- 2.2 **
HER2	- 1.6 **
IRS1	- 2.1 **
PGR	- 1.8 **
PS2=TFFI	- 1.9 **
XB1	- 2.0 **

Supplemental Table 1. The impact of FoxG1 expression on endogenous genes in MCF-7 breast cancer cells. MCF-7 cells were transfected with an EV control or FoxG1 expressing vectors. Total RNA was collected 24 hours post transfection. Quantitative real time PCR was performed to assess the mRNA expression of 168 genes that are known to participate in or respond to ER or NF-κB signaling (SA-Biosciences Array). All gene expressions were corrected for loading controls (5 housekeeping genes). The mean and 99% confidence interval were calculated for all 168 genes. A cutoff of gene expression changes of >1.5 fold was used to assess up- or downregulation of gene expression. **, P<0.01 relative to EV; ns = not significant.

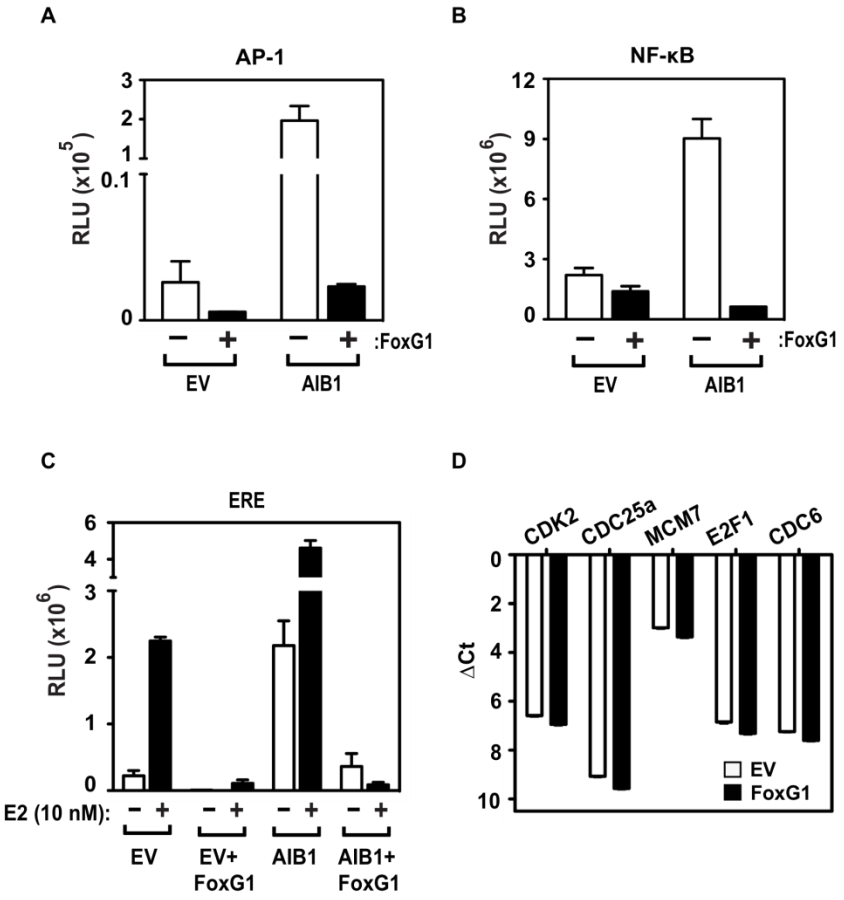


Figure 51. FoxG1 disrupts AIB1's coactivator function.
A and B, FoxG1's effect on steroid-independent promoters. HEK293T cells were transfected with AIB1 expression constructs as indicated with either (A) a multimerized AP-1 reporter or (B) a multimerized NF-κB reporter, in the presence or absence of FoxG1. c-fos and c-jun expression vectors were also cotransfected with the AP-1 reporter. 24 hours after transfection, cells were lysed to measure luciferase activity. C, FoxG1's effect on estrogen-stimulated transcription. AIB1 was cotransfected with ERα and estrogen-responsive promoter reporter (ERE) constructs into hormone-stripped HEK293T cells, with or without cotransfection of FoxG1. Cells were treated with ethanol (-) or 10 nM estradiol (E2) (+) for 24 hours and analyzed for reporter activity. Results are expressed as changes in the level of activation compared with EV-transfected cells. D, FoxG1 has no effect on E2F1-regulated gene expression. MCF-7 cells were transfected with EV or FoxG1 and total RNA was harvested from cells to determine the relative gene expression for CDK2, CDC25A, MCM7, E2F1, and CDC6. The Ct values were normalized to actin expression as control.

Conclusions

The control of the overall levels and activity of the nuclear receptor coactivator AIB1 in a cell occurs at multiple levels including control of AIB1 levels of gene transcription(137, 138), control of AIB1 mRNA stability and degradation (with e.g. *miRNA-17-5p* (154)) , control of protein modification including phosphorylation (153, 155, 156), acetylation (123) and sumoylation (157) and control of proteasomal degradation of AIB1 protein (133, 134, 158). In the current study we have determined that FoxG1 can control levels of AIB1 mRNA by directly influencing the transcription of the AIB1 gene. Based on our data we propose a model (Fig. 52) whereby the Sp1 site at bp +150/+160 of the AIB1 gene promoter is directly repressed by increasing levels of FoxG1. AIB1 can complex with E2F1 and together regulate the activity of its own promoter (137, 138). E2F1 can regulate AIB1 promoter activity by interacting with Sp1 bound at bp +150/+160 which, via direct binding to DNA, appears to anchor the E2F1-AIB1 coactivating complex to the AIB1 gene promoter (Fig. 52) (138).

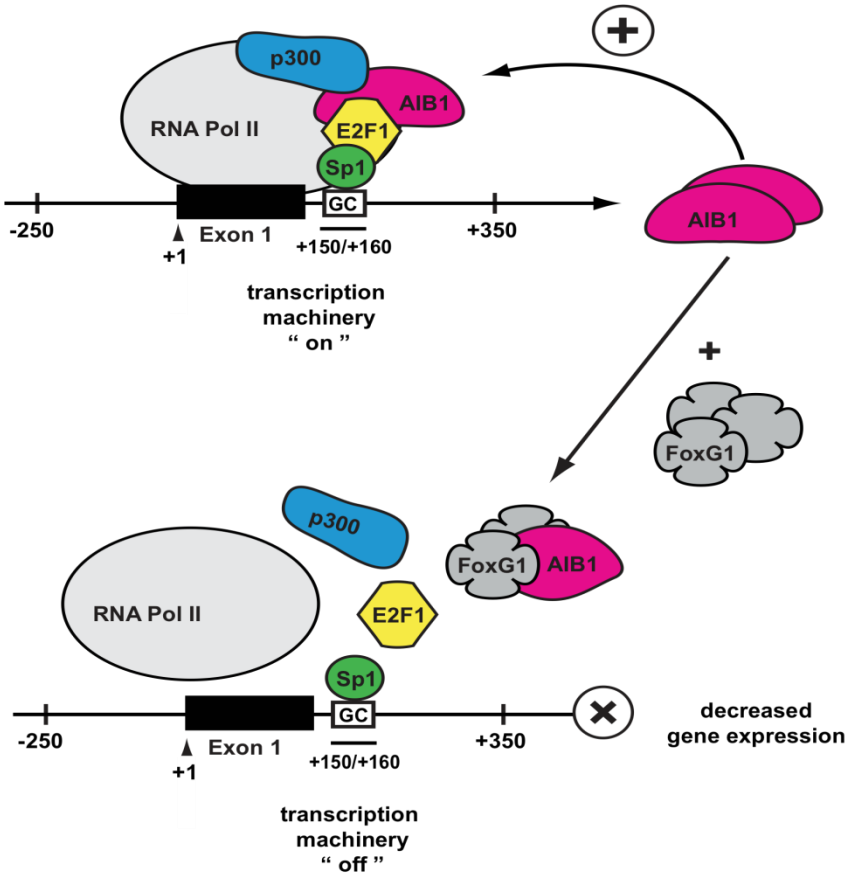


Figure 52. A proposed model for the role of FoxG1 in regulating AIB1 gene expression.
FoxG1 binds to, and reduces AIB1 binding to the components of the activating transcription complex that is required for the upregulation of AIB1 gene expression. In the presence of increased FoxG1 levels, the activating complex disassembles and disassociates from the AIB1 promoter, leading to reduced AIB1 gene transcription.

This allows AIB1 to coactivate and enhance the transcriptional activity of its own promoter. Our data show a reduction in the recruitment of the "anchorage complex", E2F1-Sp1, as well as the

essential “coactivating complex”, E2F1-AIB1 to the AIB1 promoter when FoxG1 is overexpressed (Fig. 50C and D). Our data also indicate that p300 is recruited as part of the activating complex necessary for high levels of AIB1 gene transcription (Fig. 50A and 52). CBP/p300 can bind AIB1 directly, promote stable formation of the transcription complex and has strong histone acetylase activity necessary for altering local chromatin structure and activating transcription (123, 152). We show that overexpressing FoxG1 led to a dramatic reduction in the recruitment of p300-AIB1 complex to the AIB1 promoter (Fig. 50E). Our data indicate that as FoxG1 levels rise in the cell, the Sp1-associated transcription complex is disrupted, causing E2F1, AIB1 and p300 to dissociate from Sp1, thus reducing AIB1 gene transcription. Interestingly, Hsia et al. have shown that E2F family proteins together with AAA+ nuclear coregulator cancer associated (ANCCAs) proteins are recruited to the AIB1 gene promoter by binding to multiple noncanonical E2F binding sequences within the AIB1 first exon and intron regions, and are able to directly control AIB1 expression in breast cancer cells (159). However, we believe that the inhibition of AIB1 gene transcription by FoxG1 requires no other elements of the AIB1 gene promoter since deletion of the Sp1 binding sequence alone effectively prevents recruitment of FoxG1, AIB1, E2F1, and Sp1 to the AIB1 promoter reporter (Fig. 48B iii).

Previous studies have shown that FoxG1 can cause transcriptional repression by binding DNA directly (160) and nucleating a repressosome by recruiting histone deacetylase 1 (HDAC1) and TLE family proteins (145). However, a search (http://www.ncbi.nlm.nih.gov/nuccore) of the region flanking the Sp1 site in the AIB1 gene promoter using AIB1 genomic DNA (GenBank accession no. AL353777) shows no match for the consensus FoxG1 binding sequence, AATGTAAACA, which is evolutionarily conserved and commonly shared by avian, rat and human FoxG1 gene (161). Furthermore, the interaction of AIB1 with FoxG1 in cell lysates occurs in the absence of tethering DNA. This suggests that in the context of the Sp1 binding site in the AIB1 promoter, FoxG1 inhibits transcription by disrupting an activating transcription complex bearing histone acetylase activity, rather than by forming a *de novo* repressosome after direct DNA-binding to the AIB1 gene promoter. Also consistent with this paradigm of FoxG1-induced repression mechanism is that, in our ChIP assays in cells overexpressing FoxG1, we did not observe increased FoxG1 binding to the AIB1 promoter in the vicinity of the Sp1 binding site. In fact, as exogenous FoxG1 levels increase in the cell, the amount of FoxG1 present in the Sp1-associated transcription complex decreases along with the loss of AIB1 and E2F1. This suggests that at higher concentrations of FoxG1, there is an increase in its access or affinity for AIB1 binding, possibly though dimerization, and this in turn would accelerate degeneration and disassembly of the activating transcription complex. Similar models of repression have been seen with Foxp1, a transcriptional repressor of the forkhead protein family which has been shown to be tumor suppressive in several types of cancers (162). Foxp1 can homo- and heterodimerize with Foxp2 and Foxp4, and dimerization is required for interacting with other transcription cofactors and for executing transcriptional repression (163).

Our data also suggest that FoxG1 may fall under the category of “short-range repressors,” which generally act within 100 bp of, or bind adjacently to a transcriptional activator, causing inhibition through “quenching” (164-166). Short-range repressors may also directly interact with an activating cofactor and interfere with its activity or block its access to the basal transcriptional machinery (167, 168). It is been demonstrated that several members of the short-range repressors mediate transcriptional repression in a repressor concentration-dependent manner, in which higher repressor protein levels (as compared to low levels) are sufficient to switch a gene from an active to an inactive state (168-170). Therefore, it is possible that activation of the AIB1 gene promoter occurs when FoxG1 protein levels are lowered or lost in a cell. Since high AIB1 expression can lead to uncontrolled cell proliferation and tumorigenesis (131), it is possible that cells employ FoxG1 to

control and dampen AIB1 transcription. FoxG1 thus may serve as a short-range repressor for the AIB1 promoter, as we have shown that FoxG1 binding to AIB1 leads to the detachment of the critical activating protein complexes from the AIB1 gene promoter which subsequently causes the disintegration of the Sp1-associated positive regulatory transcription complex (Fig 50C and D). In this sense, FoxG1 acts like a tumor suppresser and this would be consistent with the loss of FoxG1 expression as cells evolve from normal to cancerous (Fig. 46C).

A question that arises from the proposed model in Fig. 52 is: How does FoxG1 cause destabilization of the Sp1-associated transcription complex? One possibility is that through binding, FoxG1 induces a conformational change in AIB1 which leads to reduced affinity between AIB1 and the other members of the Sp1 transcription complex. Future studies will be directed to examine the domains of AIB1 and FoxG1 responsible for complex formation and repression of gene transcription. Another intriguing observation from our study is that, despite the disruptive effect FoxG1 exerts on AIB1-mediated coactivation, we found that this is not limited to, or exclusive to the Sp1 regulatory sequence. In fact, we observed dramatic reductions in AIB1 coactivation at NF-κB and AP-1 regulatory elements (Fig. 51A and B). Although not all AIB1-associated promoter elements are influenced by FoxG1, since the transcription of a number of E2F1-driven genes was unaffected by increasing amounts of FoxG1 in the cell. This implies that FoxG1 repression of a gene promoter activity is context-specific for AIB1 in a transcription complex. A recent genome-wide location analysis of AIB1 chromatin affinity sites in 17β-estradiol (E2) -treated MCF-7 cells demonstrated a significant overlap of AIB1 with FoxA1 binding sites in the breast cancer cell DNA (171). FoxA1 is another member of the forkhead family and a determining factor for estrogen receptor function and endocrine response (172). It would be interesting to investigate the portion of AIB1 genomic binding sites that are also engaged by FoxG1, and to determine whether such a population represent a subset of FoxG1- regulated genes.

Reintroducing AIB1 into FoxG1-induced apoptotic MCF-7 cells was only able to partially restore viability in these cells (Fig. 47C). This could indicate that FoxG1 induction of apoptosis also involves genes that are not directly regulated by AIB1. However, complete replenishment of endogenous AIB1 levels is difficult to achieve after knockdown and also the temporal response of different AIB1 regulated genes can be variable. Global ChIP assays have revealed that AIB1 is widely distributed in the genome (171) and our data have shown that FoxG1 downregulates AIB1 coactivation of AP-1 and NF-κB transcription (Fig. 51A and B). AP-1 is known to promote the expression of genes involved in cell cycle progression (173), and NF-κB-dependent gene transcription is crucial for pro-proliferation and anti-apoptosis signals (174). Thus reduction in AIB1 levels by FoxG1 repression likely has effects on multiple AIB1-regulated pathways which result in apoptosis. Of note is that FoxG1 is also known to antagonize TGF-β signaling by binding to, and blocking, the action of SMAD-3/4 proteins, both of which are major signal-transducers of TGF-β (175). Interestingly, AIB1 is one of a number of TGF-β responsive genes in A549 human lung carcinoma cells (176) and TGF-β can significantly upregulate AIB1 gene transcription in MCF-7 cells (136). Thus FoxG1 inhibition of TGF-β signaling might also be involved in the FoxG1-mediated apoptosis in MCF-7 cells.

The overexpression of the oncogene AIB1 is associated with worse disease outcome in multiple types of tumors (177). However, loss of AIB1 can also be pro-oncogenic in certain contexts, such as in B-cell lymphoma (178). Similarly, although FoxG1 can interact with AR directly *in vitro* and acts as a corepressor to both AR- and PR-mediated transactivation (144), FoxG1 is also shown to be upregulated in ovarian cancer (179), and its gene amplification is associated with the development of bladder cancer and medulloblastoma (180, 181). This suggests that FoxG1 can be both pro- or anti-oncogenic, depending on the cellular environment. Our analysis of the

microarray data generated from breast cancer populations indicates that lower levels of FoxG1 segregate with worse clinical outcome (Fig. 46E). Since estrogen is reported to suppress cellular AIB1 expression in MCF-7 cells (136), it is possible that ER status may contribute to the better prognosis associated with higher FoxG1 levels in patients. However, the risk of relapse with low FoxG1 was not significantly different in the ER-positive and -negative patient populations analyzed in Fig. 46E (data not shown). Overall our observations in the present study suggest that FoxG1 can act like a tumor suppressor in breast cancer, and downregulation of FoxG1 function could represent an important mechanism to drive AIB1-dependent survival and growth. Mimicking FoxG1 binding to AIB1 with small molecule inhibitors is therefore a possible therapeutic approach in AIB1 overexpressing cancers.

Future Directions:

We are currently determining whether loss or gain of FoxG1 causes a change in the proliferative or apoptotic response of MCF7 or MCF7:5C cells. We are testing different FoxG1 shRNA constructs to determine which of these will produce efficient knock down and will then utilize the most effective shRNAs for inducible knockdown experiments. In parallel, we are also trying to determine if gain or loss of FoxG1 expression is due to epigenetic changes in the FoxG1 gene promoter or changes in expression of miRNAs that target FoxG1.

TASK 4: (FCCC/Ariazi; TGen/Cunliffe; Jordan/GU) – To analyze E_2-induced survival and apoptotic pathways using gene arrays and siRNAs.

Task 4a: (Cunliffe,Azorsa, Balagurunathan) - Interrogate pathways of endocrine resistance using high throughput RNA interference (HT-RNAi)

There are two current projects that are ongoing now at the discovery and writing stage based upon the databases created at TGen with two specific objectives.

1 CGH of Cell lines

Dr. Heather Cunliff earlier completed the CGH for MCF-7:WS8 (estrogen deprived), MCF-7:5C and MCF-7:2A (two estrogen deprived clones that grow spontaneously). Results for these studies were presented as an oral presentation by Dr. Cunliff in 2007 at the Era of Hope Meeting in Baltimore. Dr. Cunliff has now completed the Agilent gene array for MCF-7:2A and MCF-7:5C hybridized against MCF-7:WS8 (estrogen deprived) RNAs. These data will be amalgamated with the prior CGH data and are currently being prepared for publication.

2 Pharmacology of the MCF-7:PF Cell Line (see Task 2b)

We have discovered that long-term treatment of MCF-7:5C cells with physiologic estrogen and the c-Src inhibitor, PP2, results in the inhibition of estrogen-induced apoptosis causes the evolution of a new cell population that is ER positive, PgR positive, and now grows in response to a panel of SERMs. This new observation is currently being developed and interrogated in the laboratory, as this is the first time that there has been any report of a SERM stimulated cell in culture. As a first step, Dr. Heather Cunliff has worked with Dr. Ping Fan to created an Agilent gene array database mapping the pathways for estrogen stimulated growth or SERM stimulated growth. It is clear that the complex survival networks can rapidly produce new clones of cells to subvert antihormone therapy and reverse apoptosis into cell growth.

KEY RESEARCH ACCOMPISHMENTS

Task 1 (LCCC, Isaacs)

- The creation of a new low-dose estrogen protocol to treat women who have been exhaustively treated with antihormone therapy.
- The consolidation of the Georgetown Lombardi Comprehensive Cancer Center vision of clinical trials testing with Hackensack Hospital in New Jersey.

Task 2a (GU - Jordan/Fan)

- Estrogen (E_2) widely activated apoptosis-related genes detected by RNA-sequence analysis in MCF-7:5C cells. Majority of these genes were related with stress.
- We further found that E_2 activated sensors of unfolded protein response (UPR) after 24 hours treatment in MCF-7:5C cells. It indicated that E_2 caused extra unfolded protein accumulating in the endoplasmic reticulum.
- E_2 caused mitochondrial dysfunction through disrupting mitochondrial membrane integrity.
- E_2 activated energy stress sensor AMPK after 48 hours treatment in MCF-7:5C cells which demonstrated the function of mitochondria was damaged after E_2 treatment.
- E_2 activated oxidative stress indicator HMOX1 and increased the production of reactive oxygen species (ROS).
- The scavengers of free radicals prevented E_2-induced apoptosis, confirming that oxidative stress played an important role in apoptosis.
- E_2 activated TNF family members (TNFα, LTA, and LTB), suggesting that E_2 activated extrinsic apoptotic pathways in MCF-7:5C cells.
- Inhibition of c-Src tyrosine kinase partially blocked some kinase activities such as eIF2α and AMPK involved in the process of stress.
- The c-Src inhibitor, PP2, reduced the production of ROS induced by E_2.
- The c-Src inhibitor effectively blocked the extrinsic pathways induced by E_2.
- The c-Src inhibitor blocked apoptosis induced by E_2, confirming by the specific siRNA to knock down c-Src.
- We confirmed that E_2-triggered apoptosis might be utilizing c-Src tyrosine kinase as an important signaling pathway. c-Src tyrosine kinase acted as an important signal transducer in the process of stress induced by E_2.
- c-Src was involved in the non-genomic and genomic pathways activated by E_2 in MCF-7:5C cells. We excluded that the non-genomic pathway was involved in triggering apoptosis by E_2.
- Our data implied that classical ERE regulated transcriptional pathways were not required by the E_2-induced apoptosis. We are currently investigating the functions of non-classical transcription factors such as AP-1 family members in regulation of stress responses and causing apoptosis.
- These data provided an important therapeutic rationale for the patient selection in clinical trials with c-Src inhibitors in aromatase inhibitor resistant breast cancer.

Task 2b (GU - Jordan/Fan)

- Based on the findings in the **Task 2a** section, we treated MCF-7:5C cells long-term (8 weeks) with different combinations to further investigate the therapeutic potential of the combination of the c-Src inhibitor PP2 and E_2 on the growth of MCF-7:5C cells.
- Long-term treatment with PP2 alone or E_2 alone still decreased cell growth with G1 arrest of cell cycles.
- In contrast, a combination of PP2 and E_2 blocked apoptosis and the resulting cell line (MCF-7:PF) was unique, as they grew vigorously in culture with physiological levels of E_2.
- E2-stimulated growth could be blocked by the pure antiestrogen ICI 182,780, implying existence of functional estrogen receptor (ER).
- Progesterone receptor (PR) was strictly regulated by E_2 which made E_2 alone treated cells and MCF-7:PF cells have higher levels of PR protein.
- However, the progestin uniquely activated PRE activity in MCF-7:PF cells which could be blocked by anti-progestin RU486. They also had different cell growth responses, progestin stimulated growth in MCF-7:PF cells, but not in E_2 alone treated cells.
- The c-Src inhibitor synergized with E_2 to elevate transcriptional activity of in MCF-7:PF cells.
- The c-Src inhibitor continuously inhibited Akt pathway but transiently blocked MAPK in MCF-7:5C cells. The increased MAPK phosphorylated PR on Ser294 site, affecting the transcriptional activity of PR.
- The c-Src inhibitor collaborated with E_2 to increase the level of insulin-like growth factor-1 receptor beta (IGF-1Rβ). Blockade of IGF-1Rβ completely abolished E_2-stimulated growth in MCF-7:PF cells.
- Furthermore, combination treatment up-regulated transcription factors Twist1 and Snail and repressed E-cadherin expression which made MCF-7:PF cells display a characteristic phenotype of epithelial-mesenchymal transition (EMT).
- These data illustrate that caution must be exercised when considering c-Src inhibitors in clinical trials following the development of acquired resistance to aromatase inhibitors, especially in the presence of the patient's own estrogen.

Task 2c (GU- Jordan/Sengupta)

- Estrogen induced apoptosis of MCF7:5C cells is mediated by ER-stress and unfolded protein response which uses the PERK pathway.Estrogen treatment increases phosphorylation of the translational initiation factor, eIF2α after 12 hours remains elevated.
- The phosphorylation of eIF2α, at serine 51, is critical for activation of the down-stream factors, ATF4 and CHOP for inducing apoptosis by apoptosis.
- The RNA levels of GADD34, which de-phosphorylates eIF2α, was up-regulated after 72 hours of estrogen treatment but the protein levels remained unchanged throughout the course of treatment.

- Salubrinal, a compound which inhibits de-phosphorylation of eIF2α at serine 51, was able to mimic estrogen in inducing apoptosis in the MCF7:5C cells suggesting eIF2α phosphorylation is a key event for apoptosis.
- Over-expression of the phospho-mutant eIF2α in the MCF7:5C cells was able to decrease the levels of phospho-eIF2α and inhibit the thapsigargin induced apoptosis.
- Knock-down of CHOP (a down-stream target of PERK pathway) also resisted the ER-stress induced apoptosis most likely by blocking the up-regulation of the pro-apoptotic BIM protein.

Task 2d (GU - Jordan/Sengupta/Obiorah)

- Basal expression of cMYC transcripts and protein level is 3-4 fold higher in the AI-resistant ER+ breast cancer cell model, MCF7:5C cells, as compared to parental counterpart MCF7 cells.
- Pharmacological inhibition of cMYCusing 10058-F4, inhibited the estrogen independent growth by reducing the 'S' phase cellsof MCF7:5C cells.
- Knock-down of cMYC gene in MCF7:5C cells inhibited the estrogen independent growth of the cells by reducing the 'S' phase cells.
- High cMYC expression correlated with poor relapse free survival in patients treated with endocrine therapy but not chemotherapy.
- High levels of serine-2-phosphorylated RNA polymerase II (a marker of elongation of RNA synthesis) was recruited at the cMYC promoter in MCF7:5C cells, as compared to parental MCF7 cells, is most likely responsible for the higher levels of cMYC transcripts.
- High levels of phosphorylated-CDK9 were found in MCF7:5C cells which is known for the phosphorylation of serine-2 residue of RNA polymerase II.
- Pharmacological inhibition of CDK9 not only blocked the estrogen-independent growth of the MCF7:5C cells but also inhibited the transcription of cMYC gene and the protein levels.

Task 2e (GU- Jordan/Obiorah)

- E2-induced apoptosis occurs as a delayed event in MCF7:5C cells in contrast to the generally accepted norm.
- Paclitaxel, a cytotoxic chemotherapy, rapidly induces apoptosis in the same cell line by
- 24 hrs, while E2 begins this process after 72 hrs using a cell proliferation assay.
- E2 induces ERS and inflammatory stress genes as well as apoptotic genes that induce
- both the intrinsic apoptosis pathway at 36 hrs and the extramitochondrial pathway at 48hrs.
- Given the above results, it is proposed that E2-induced apoptosis involves a number of multifactorial events that may explain thedelayed apoptosis that is observed in the MCF7:5C cells.
- Paclitaxel selectively induces the TRAIL/TNFRSF10A/B pathway initially which expand
- to involve more death receptors with inhibition of the cell cycle at G1 checkpoint by p21.
- Cell cycle analysis show that paclitaxel causes rapid reduction of the S phase as well as G2/M blockade by 12 h of treatment. By contrast, E_2 causes an initial proliferation, then apoptosis of the MCF7:5C cells.

Task 2f (GU- Jordan/Obiorah)

- ERS genes indicated that E_2 inhibited protein folding leading to accumulation of unfolded proteins and widespread inhibition of protein translation with subsequent induction of cell death.
- In response to severe ERS, Bcl-2 interacting mediator of cell death (Bim; BCL211) was induced.

Task 2g (GU- Jordan/Maximov)

- The cis and trans isomers of the tested compounds have different estrogenic properties in wild type breast cancer cells.
- The cis and trans isomers of the tested compounds have a different ability to induce estrogen-induced apoptosis and with a different timecourse (delayed apoptosis), when compared to estradiol in antiestrogen resistant breast cancer cells.

Task 3a (GU - Riegel/Wellstein)

- The oncogene nuclear receptor coactivator amplified in breast cancer 1 (AIB1) is a transcriptional coactivator that is overexpressed in various types of human cancers. However, the molecular mechanisms controlling AIB1 expression in breast cancer remain unclear.
- We identified a novel interacting protein of AIB1, forkhead-box protein G1 (FoxG1), which is an evolutionarily conserved forkhead-box transcriptional corepressor.
- We show that FoxG1 expression is low in breast cancer cell lines, and that low levels of FoxG1 are correlated with a worse prognosis in breast cancer.
- We demonstrate that transient overexpression of FoxG1 can suppress endogenous levels of AIB1 mRNA and protein in MCF-7 breast cancer cells. Exogenously expressed FoxG1 in MCF-7 cells also leads to apoptosis that can be rescued in part by AIB1 overexpression.
- Using chromatin immunoprecipitation (ChIP), we determined that FoxG1 is recruited to a region of the AIB1 gene promoter previously characterized to be responsible for AIB1-induced, positive auto-regulation of transcription through the recruitment of an activating, multiprotein complex, involving AIB1, E2F1 and Sp1. Increased FoxG1 expression significantly reduces the recruitment of AIB1, E2F1 and p300 to this region of the endogenous AIB1 gene promoter.
- Our data imply that FoxG1 can function as a pro-apoptotic factor in breast cancer in part through suppression of AIB1 coactivator transcription complex formation, thereby reducing the expression of the AIB1 oncogene

Task 4a (TGen – Azorsa/Balagurunathan/Cunliffe)

- The original CGH for our three cell lines our now in the process of being analyzed and melded with Agilent gene arrays of RNA from MCF-7:5C and MCF-7:2A hybridized against MCF-7:WS8 (estrogen deprived).
- The unique MCF-7:PF cell line, that is SERM stimulated for growth is being evaluated using Agilent arrays to determine the mechanism of estrogen versus SERM stimulated growth.

REPORTABLE OUTCOMES

Publications

1. Korch C., Spillman, M.A., Jackson, T.A., Jacobsen, B.M., Murphy S.K., Lessey, B.A., Jordan, V.C. and Bradford, A.P. (2012). DNA profiling analysis of endometrial and ovarian cell lines reveals misidentification, redundancy and contamination. Gynecologic Oncology 127:241-248.

2. Sengupta, S. S., Obiorah, I. O., Maximov, P. Y., Curpan, R., and Jordan, V. C. (2013) Molecular Mechanism of Action of Bisphenol and Bisphenol-A Mediated by Estrogen Receptor alpha in Growth and Apoptosis of Breast Cancer Cells. British Journal of Pharmacology 169:167-78.

3. Obiorah, I. and Jordan, V. C. 2013. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. North American Menopause Society/Pfizer-Wulf H. Utian Endowed Lecture. Menopause 20:372-382.

4. Maximov, P.Y., Lee, T.M., and Jordan, V.C. (2013). The discovery and development of Selective Estrogen Receptor Modulators (SERMs) for clinical practice. Current Clinical Pharmacology 8:135-155

5. Jordan, V.C. (2013). Estrogen Action, SERMs and Women's Health. Imperial College Press, London.

6. Segala G., de Medina P., Iuliano L., Zerbinati C., Paillasse M. R., Noguer E., Dalenc F., Payre B., Jordan V. C., Record M., Silvente-Poirot S., Poirot M (2013).5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of tamoxifen in breast cancer cells. Biochemical Pharmacology (epub ahead of print).

7. Li J. V., Chien C. D., Garee J. P., Xu J., Wellstein A., Riegel A. T. (2013) Transcriptional repression of AIB1 by FoxG1 leads to apoptosis in breast cancer cells. Molecular Endocrinology (epub ahead of print)

8. Fan P., Griffith O. L., Agboke F., Anur P., McDaniel R. E., Creswell K., Kim S. H., Katzenellenbogen J. A., Gray J. W., Jordan V. C. (2013) c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. Cancer Research (epub ahead of print).

9. McDaniel, R.E., Maximov, P.Y., and Jordan, V.C. (2013) Estrogen-mediated mechanisms to control the growth and apoptosis of breast cancer cells: a translational research success story. In: Vitamins and Hormones (ed. Gerald Litwack) Elsevier (*in press*).

10. Maximov, P.Y., McDaniel, R. E., and Jordan, V.C. (2013). Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basal, Switzerland (*in press*).

Abstracts

1. *Abstract # 5642 was published in the 2013 Proceedings of the 104th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 6-10 2013.*
   Transcriptional deregulation of cMYC as a critical determinant of estrogen independence and aromatase inhibitor resistance in breast cancers.
   Surojeet Sengupta, Michael C. Biarnes, V. Craig Jordan.
2. *Abstract # 2939 was published in the 2013 Proceedings of the 104th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 6-10 2013.*
   Novel molecular mechanism for estradiol-induced apoptosis that contrasts with cytotoxic chemotherapy-induced apoptosis in breast cancer.
   Ifeyinwa Obiorah, Surojeet Sengupta, V. Craig Jordan
3. *Abstract # 828 was published in the 2013 Proceedings of the 104th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 6-10 2013.*
   Mechanisms underlying differential response to estrogen-induced apoptosis in long-term estrogen-deprived breast cancer cell lines.
   Elizabeth E. Sweeney, Ping Fan, V. Craig Jordan.
4. *Abstract # 830 was published in the 2013 Proceedings of the 104th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 6-10 2013.*
   Transcriptional modulation of estrogen-induced apoptosis through activation of c-Fos/c-Jun in long-term estrogen deprived breast cancer cells.
   Ping Fan, Fadeke Agboke, Obi L. Grifith, Russell E. McDaniel, V. Craig Jordan.
5. *Abstract # 4409 was published in the 2013 Proceedings of the 104th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 6-10 2013.*
   The characterization of isomerically stable fixed ring derivatives of tamoxifen metabolites 4-hydroxytamoxifen and 4-hydroxy-N-desmethyltamoxifen (endoxifen) in vitro.
   Philipp Y. Maximov, Cynthia Myers, Daphne J. Fernandes, V. Craig Jordan.
6. Abstract # P2-13-09. Was presented at the San Antonio Breast Cancer Symposium, December 4-8, 2012 and was published in Cancer Research 2012;72(24 Suppl).
   Pharmacological impact of endoxifen in a laboratory simulation of tamoxifen therapy in postmenopausal breast cancer patients
   PY Maximov, RE McDaniel, P Bhatta, H Brauch, and VC Jordan

Presentations

2012:

1. Jordan VC "Scientific Survival Suggestions after Four Decades of Discovery." University of Liverpool, Liverpool, UK, July 25 2012.
2. Jordan VC "The Science of SERMs." University of Arkansas Medical School, Little Rock, AK, September 13, 2012.
3. Jordan VC "Scientific Survival Suggestions after Four Decades of Discovery." National Center for Toxicological Research Food and Drug Administration, Jefferson, AK, September 14, 2012
4. Jordan VC "The treatment and prevention of breast cancer by selective estrogen receptor modulation- something from nothing." Robert M. Hearin Distinguished Lecture University of Mississippi Medical Center. Jackson, MS, September 27, 2012
5. Jordan VC "Chemoprevention of Breast Cancer: Lessons Learned." 14TH Annual Lynn Sage Breast Cancer Symposium, Chicago IL, October 5, 2012.
6. Jordan VC "The Pfizer-Wulf H. Utian Endowed Lecture:The Paradox of Estradiol-Induced Breast Cancer Cell Growth and Apoptosis." North American Menopause Society, Orlando, FL, October 6, 2012.
7. Jordan VC "The Paradox of Estradiol-Induced Breast Cancer Cell Growth and Apoptosis: a Change in Clinical Practice?." Georgetown University - Wednesday Research Update Seminar Series. Washington, DC, October 17, 2012.
8. Jordan VC "The New Biology of Estrogen Induced Apoptosis for the Treatment and Prevention of Breast Cancer." Robert H. Lurie Comprehensive Cancer Center Northwestern University. Chicago, IL, November 2, 2012.
9. Jordan VC "Scientific Survival Suggestions to Graduate Students and Postdoctoral Fellows." Robert H. Lurie Comprehensive Cancer Center Northwestern University. Chicago, IL, November 2, 2012.
10. Jordan VC "Scientific Survival Suggestions to Medical and Surgical Fellows." Robert H. Lurie Comprehensive Cancer Center Northwestern University. Chicago, IL, November 3, 2012.
11. Jordan VC "THE SCIENCE OF SERMs: FOUR DECADES OF DISCOVERY Something from Nothing." Southern California Clinical and Translational Science Institute. USC Health Sciences Campus, November 7, 2012.
12. Jordan VC "Improving The Response Rate to Endocrine Therapy." 19th Annual Multi-Disciplinary Cancer Symposium New Perspectives on Breast Cancer 2012Washington Adventist Hospital (11-2012). Bethesda, MD, November 9, 2012.

2013

1. Jordan VC "Molecular Mechanism of Estrogen-Induced Apoptosis in Breast Cancer:Paradox to Paradigm" Georgetown University - Wednesday Research Update Seminar Series. Washington, DC, October 17, 2012.

2. Jordan VC "Four Decades of Discovery for the Treatment and Prevention of Breast Cancer: The SERM Story The 38th Sir Edward Mellanby Memorial Oration". CSIR-CDRI
3. Lucknow, India, February 11, 2013.
4. Jordan VC "The Origins of the New Biology of Estrogen-Induced Apoptosis in Breast Cancer Research, Treatment, and Prevention Inaugural Dr. Gary D. Kruh Memorial Lecture" University of Illinois, Chicago, IL, May 9, 2013.

Awards and Honorary Memberships

1. Fellow of the City and guilds Institute, 2013 (UK)
2. Fellow of the American Association for Cancer Research Academy, 2013

Appointments

1. Russell E. McDaniel
   September 3, 2012, Laboratory Manager
2. Daphne Fernandes
   October 1, 2012, Research Assistant I

CONCLUSIONS

It is important to stress at the outset of this section, that there is growing momentum within the clinical community that our work is an important new dimension in women‟s health.

This is illustrated by three, well-defined facts:

1) Our focus on the applicability of our laboratory results through the use of low dose estrogen for the treatment of metastatic breast cancer following antihormone drug resistance is now a general topic of discussion. Our work has been pivotal for the publication of others; the concept we proposed from the laboratory data enhances the treatment of women with breast cancer(59, 182).

2) Our concepts form the basis of a major clinical trial in Europe and around the world, described as the Study of Letrozole Extension (SOLE)(183). The strategy for the study is to examine whether continuous long term antihormone therapy is better or worse for the adjuvant treatment of ER-positive breast cancer than therapy that has three months per year drug holidays, where the women‟s own estrogen can destroy the antihormone resistant breast cancer cells before drug resistance disease gets a hold.

3) The recent published findings of the Women‟s Health Initiative (WHI) of estrogen replacement therapy in hysterectomized postmenopausal women showed a reduction in the incidence of breast cancer that in fact continues for five years after estrogen therapy stops(1). We are providing all of the scientific knowledge database to explain this apparently paradoxical finding (estrogen replacement reduces the risk of breast cancer!). We obviously take very seriously, the fact that we are the pioneering group scientifically in this area and through the investment of the DOD CoE grant via their visionary peer reviewed system, we have been given the responsibility to decipher the mechanisms involved in this new biology of estrogen-induced apoptosis in breast cancer.

It is clear from the aforementioned three broad applications in clinical medicine that we have an opportunity to revolutionize women‟s health through the prudent application of remaining resources awarded through our CoE grant to be used in our no cost extension. I will systematically create an executive summary conclusion for each of our ongoing Tasks.

Our high dose estrogen protocol is IRB approved, however we have now developed a new strategy to enhance recruitment and export our protocol through a future funding opportunity to a remote site. These opportunities have occurred because of the following changing circumstances. Dr. Alexandra Zimmer is an NCI medical oncology fellow who holds clinics each week at the Georgetown Lombardi Cancer Center with Dr. Isaacs. The fact that a clinical trial comparing high-dose (30mg daily Estrace) versus low dose Estrace (6mg Estrace daily) has now been published (Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A, Jamalabadi-Majidi S, Crowder R, Siegel BA. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 2009;302(7):774-80.) by others based on our laboratory work. The study demonstrates that low-dose estrogen has lower side effects then our current protocol using high-dose estrogen. Georgetown Lombardi Comprehensive Cancer Center has recently entered into negotiations and now concluded an agreement with Hackensack Hospital in New Jersey to create an integrated clinical trial system. Dr. Zimmer and Dr. Isaacs will be exporting our low-dose estrogen protocol to Hackensack to create a baseline study to reproduce the results obtained from the JAMA article above. It is our long-term goal to use the results of this clinical trial to improve clinical responsiveness through combination therapies of low-dose estrogen with selective inhibitors of tumor cell survival. It is our goal to improve response rates from around thirty percent to above fifty percent (**Task 1a**).

Our major accomplishment to date on the grant has been to create a map of the life and death of breast cancer cells in response to physiological estrogen. This is a new unique dataset that is invaluable, but the complexity of our dataset is currently a challenge to the best brains in bioinformatics in the world, with whom we are currently collaborating. It is important to realize that our visionary approach proposed the equivalent of creating a movie, of the life and death of breast cancer cells through gene activation and suppression, but every other research group in the world is studying only single photographs of cells and tumors at a single point in time. Nevertheless, it is our accomplishment that has been enhanced by considerable bioinformatics input and the development of new computer modeling systems to analyze gene dosing activation against time for the growth and death of human breast cancer cells in response to estrogen. We have taken all of our enormous gene array data against time (96 hours) andprovided it to Dr. Joe Gray at the University of Oregon. He will be working with us over the next year of the grant to create an unique pathway analysis "movie". This has never been done before. With our database, we have already identified the sequence of events for estrogen-induced apoptosis in our endocrine resistant breast cancer cells. Estrogen induces a stress response and activates inflammatory genes. This discovery now allows us to interrogate this mechanism of inflammation-mediated cell death through its modulation with anti-inflammatory agents such as glucocorticoids. The other major finding from our database is a description of the caspase cascade that provokes cell death and destruction following estrogen-induced apoptosis. We have precisely defined and identified caspase 4 as the trigger caspase in the initiation of estrogen-induced apoptosis. However, we now seek to build upon our database and use molecular pharmacology to define and refine the input signal through the estrogen receptor that modulates estrogen-induced apoptosis. We are addressing the issue of what are the basic estrogen-ER related events that trigger estrogen induced apoptosis?

Our publications demonstrate that physiological concentrations of estrogen (E_2) induce endoplasmic reticulum and oxidative stress which finally result in apoptosis in E_2-deprived breast cancer cells, MCF-7:5C. c-Src is involved in the process of E_2-induced stress. To mimic the clinical administration of c-Src inhibitors, we treated cells with either E_2, a c-Src inhibitor PP2, or the combination for 8 weeks to further explore the apoptotic potential of the c-Src inhibitor and E_2 on MCF-7:5C cells. Protein levels of receptors and signaling pathways were examined by immunoblotting. Expression of mRNA was detected through real-time PCR. Cell cycles were analyzed by flow cytometry. Long-term treatment with PP2 alone or E_2 alone decreased cell growth. In contrast, a combination of PP2 and E_2 blocked apoptosis and the resulting cell line (MCF-7:PF) was unique, as they grew vigorously in culture with physiological levels of E_2, which could be blocked by the pure antiestrogen ICI182,780. One major change was that PP2 collaborated with E_2 to increase the level of insulin-like growth factor-1 receptor beta (IGF-1Rβ). Blockade of IGF-1Rβ completely abolished E_2-stimulated growth in MCF-7:PF cells. Furthermore, combination treatment up-regulated transcription factors Twist1 and Snail and repressed E-cadherin expression which made MCF-7:PF cells display a characteristic phenotype of epithelial-mesenchymal transition (EMT). These data illustrate the role of the c-Src inhibitor to block E_2-induced apoptosis and enhance E_2-stimulated growth. Caution must be exercised when considering c-Src inhibitors in clinical trials following the development of acquired resistance to aromatase inhibitors, especially in the presence of the patient's own estrogen (**Task 2a**).

We have shown that estrogen (E_2) induces apoptosis in long-term E_2 deprived breast cancer cells (MCF-7:5C) through stress responses, but the molecular mechanism underlying E_2-induced stress remains to be elucidated. Here, we report that the oncogene c-Src acts as an important adapter

protein of estrogen receptor (ER) involved in stress responses induced by E_2 in MCF-7:5C cells. E_2 elevated c-Src phosphorylation in MCF-7:5C cells and 4-hydroxytamoxifen (4-OHT) blocked this stimulation which suggested that E_2 activated c-Src through ER. E_2 activated the sensors of unfolded protein response (UPR) inositol-requiring protein 1 alpha (IRE1α) and PRK-like endoplasmic reticulum kinase (PERK)/eukaryotic translation initiation factor-2α (eIF2α). The indicator of oxidative stress, heme oxygenase 1 gene (HMOX1), was dramatically up-regulated by E_2. Further examination showed that E_2 significantly increased reactive oxygen species (ROS) production in MCF-7:5C cells. And the energy stress sensor adenosine monophosphate (AMP)-activated protein kinase (AMPK) was activated by E_2. The specific inhibitor of c-Src, PP2, was able to abolish the phosphorylation of eIF2α and AMPK and reduced the production of ROS induced by E_2. Therefore, PP2 blocked E_2-induced apoptosis, which was confirmed by knockdown of c-Src with a specific small interferon RNA. All of these data illustrate that c-Src functions as a critical transducer in E_2-initiated endoplasmic reticulum stress and oxidative stress which trigger apoptotic cascades in MCF-7:5C cells. This study provides an important rationale for further exploration of the stress responses in endocrine resistant breast cancer to improve clinical benefit (**Task 2b**).

Overall, we have shown that estrogen-induced apoptosis of MCF7:5C cells is mediated by ER-stress and UPR. PERK mediated eIF2α phosphorylation is the key pathway by which it activates the apoptotic signaling. Moreover, pharmacological intervention which increases the phosphorylated status of eIF2α, is sufficient to induce apoptosis in the MCF7:5C cells. We have also studied the downstream effectors of this pathway and determined that ATF4, CHOP and BIM play important roles in implementing the ER-stress mediated apoptosis in these cells (**Task 2c**)

Development of resistance to existing endocrine-therapies in estrogen receptor alpha positive (ERα +) breast cancers is the major obstacle for maintaining efficacy of targeted therapy. Recent clinical studies have indicated over-expression of cMYC oncogene is associated with aromatase inhibitor (AI) resistant breast cancers. To understand the mechanisms involved in acquiring resistance we investigated the significance of cMYC over-expression in an endocrine-therapy resistant breast cancer cell model, MCF7:5C cells, which have been cultured long-term in estrogen-deprived media. Compared to the parental counterpart MCF7 cells, cMYC mRNA and protein was 3 fold over-expressed in MCF7:5C cell, which was found to be driving the estrogen-independent growth of these cells. Further investigation suggested transcriptional de-regulation of cMYC gene was responsible for its over-expression in the MCF7:5C cells. Chromatin immuno-precipitation assay revealed markedly higher recruitment of phosphorylated serine-2 carboxy-terminal domain (CTD) of RNA polymerase-II at the proximal promoter of cMYC gene in MCF7:5C cells as compared to its parental cells. The level of phospho-CDK9, a factor responsible for phosphorylation of serine-2 of RNA polymerase II CTD, was found to be elevated in MCF7:5C cells. Pharmacological inhibition of CDK9 not only reduced the transcripts and the protein levels of cMYC in MCF7:5C cells but also selectively inhibited its growth. This study describes the molecular events involved in the transcriptional over-expression of cMYC gene in AI-resistant breast cancer cells and identifies CDK9 as a potential novel drug target for therapeutic intervention in endocrine-resistant breast cancers (**Task 2d**).

Estrogen receptor alpha (ERα) binds to different ligand which can function as complete / partial estrogen-agonist or antagonist. This depends on the chemical structure of the ligands which modulates the transcriptional activity of the estrogen-responsive genes by altering the conformation of the liganded-ERα complex. This study determined the molecular mechanism of estrogen-agonistic / antagonistic action of structurally similar ligands, bisphenol (BP) and bisphenol-A (BPA) on cell proliferation and apoptosis of ERα+ve breast cancer cells. DNA was measured to assess the proliferation and apoptosis of breast cancer cells. RT- PCR and ChIP assays were performed to

quantify the transcripts of TFF1 gene and recruitment of ERα and SRC3 at the promoter of TFF1 gene, respectively. Molecular docking was used to delineate the binding modes of BP and BPA with the ERα. PCR-based arrays were used study the regulation of the apoptotic genes. BP and BPA induced the proliferation of breast cancer cells, however, unlike BPA, BP failed to induce apoptosis. BPA consistently acted as an agonist in our studies but BP exhibited mixed agonistic/antagonistic properties. Molecular docking revealed agonistic and antagonistic mode of binding for BPA and BP respectively. BPA treatment resembled E2 treatment in terms of PCR based regulation of apoptotic genes whereas BP was similar to 4OHT treatment. The chemical structure of ERα ligand determines the agonistic or antagonistic biological responses by the virtue of their binding mode, conformation of the liganded-ERα complex and the context of the cellular function.

The administration of high dose synthetic estrogens was the first successful chemical therapy used in the treatment of metastatic breast cancer in postmenopausal women and this approach became the standard of care in postmenopausal women with metastatic breast cancer between 1950s and the end of the 1970s. The most recent analysis of the Women's Health Initiative estrogen alone trial in hysterectomised women revealed a persistent significant decrease in the incidence of breast cancer as well as breast cancer mortality. Although estrogens are known to induce proliferation of breast cancer cells, we have shown that physiologic concentrations induce apoptosis in long term estrogen deprived breast cancer cells. We have developed laboratory models that illustrate the new biology of estrogen induced apoptosis or growth to explain the effects of estrogen replacement therapy. The key to the success of estrogen therapy lies in a sufficient period of withdrawal of physiological estrogens (5-10years) and the subsequent regrowth of nascent breast tumor cells that survive under estrogen deprived conditions. These nascent tumors are now vulnerable to estrogen induced apoptosis.

The clinical basis for the use of physiologic estrogen treatment of metastatic anti hormone resistant breast tumors is based on laboratory studies which show that resistance to long- term antihormone breast cancer therapy evolves over 5 years. Taxanes have been used extensively in the treatment of early and advanced breast cancer and play an active role in the survival of breast cancer patients. We have interrogated the sequence of events that involve the apoptotic signaling pathway induced by estradiol (E_2) in comparison to paclitaxel. Cell culture studies show estrogen induced apoptosis to be a slow process, while paclitaxel rapidly inhibits the growth and induces death of long term estrogen deprived MCF7 cells (MCF7:5C). Using 4-hydroxytamoxifen to block E_2 induced apoptosis at different times, we established that the cellular commitment for E_2 triggered apoptosis occur only after 24 h. Activation of the intrinsic pathway was observed by 36h of E_2 treatment with subsequent induction of the extrinsic apoptotic pathway by 48h. Apoptosis was induced by paclitaxel exclusively through the extramitochodrial pathway and caused rapid G2/M blockade by 12 h of treatment. By contrast, E_2 causes an initial proliferation, then apoptosis of the MCF7:5C cells. These data indicate that E_2 induced apoptosis involves a novel, multidynamic process that is distractedly different from that of a classic cytotoxic chemotherapeutic drug used in breast cancer (**Task 2e**).

We have created LTED breast cancer cell lines and for the first time, described the mechanism of estrogen-induced apoptosis. This new biology of estrogen induced apoptosis and can be now used to explain the effects of ET in reducing breast cancer incidence and mortality for women in the 60s (**Task 2f**).

The cis and trans isomers of the tested metabolites of tamoxifen have different estrogenic properties in wild type breast cancer cells.The cis and trans isomers of the tested compounds have a different ability to induce estrogen-induced apoptosis and with a different timecourse (delayed apoptosis), when compared to estradiol in antiestrogen resistant breast cancer cells. The tested compounds with various structures were evaluated for their estrogenic/antiestrogenic properties in

human breast cancer cell lines to induce growth or estrogen-induced apoptosis. The compounds properties were evaluated using a DNA cell proliferation assay. The results of the assays demonstrate that there is a different biology of the isomers of the same antiestrogen FR4OHT and that the conformation of the ER that is dictated by the structure of the ligand is crucial in inducing apoptosis in hormone independent breast cancer cells. Further experiments are needed to confirm the hypothesis (**Task 2g**).

The oncogene nuclear receptor coactivator amplified in breast cancer 1 (AIB1) is a transcriptional coactivator that is overexpressed in various types of human cancers. However, the molecular mechanisms controlling AIB1 expression in the majority of cancers remain unclear. In this study,we identified a novel interacting protein of AIB1, forkhead-box protein G1 (FoxG1), which is an evolutionarily conserved forkhead-box transcriptional corepressor. We show that FoxG1 expressionis low in breast cancer cell lines, and that low levels of FoxG1 are correlated with a worse prognosis in breast cancer. We also demonstrate that transient overexpression of FoxG1 can suppress endogenous levels of AIB1 mRNA and protein in MCF-7 breast cancer cells. Exogenously expressed FoxG1 in MCF-7 cells also leads to apoptosis that can be rescued in part by AIB1 overexpression. Using chromatin immunoprecipitation (ChIP), we determined that FoxG1 is recruited to a region of the AIB1 gene promoter previously characterized to be responsible for AIB1-induced, positive auto-regulation of transcription through the recruitment of an activating, multiprotein complex, involving AIB1, E2F1 and Sp1. Increased FoxG1 expression significantly reduces the recruitment of AIB1, E2F1 and p300 to this region of the endogenous AIB1 gene promoter. Our data imply that FoxG1 can function as a pro-apoptotic factor in part through suppression of AIB1 coactivator transcription complex formation, thereby reducing the expression of the AIB1 oncogene (**Task 3a**).

Dr. Heather Cunliff earlier completed the CGH for MCF-7:WS8 (estrogen deprived), MCF-7:5C and MCF-7:2A (two estrogen deprived clones that grow spontaneously). Results for these studies were presented as an oral presentation by Dr. Cunliff in 2007 at the Era of Hope Meeting in Baltimore. Dr. Cunliff has now completed the Agilent gene array for MCF-7:2A and MCF-7:5C hybridized against MCF-7:WS8 (estrogen deprived) RNAs. These data will be amalgamated with the prior CGH data and are currently being prepared for publication.

We have discovered that long-term treatment of MCF-7:5C cells with physiologic estrogen and the c-Src inhibitor, PP2, results in the inhibition of estrogen-induced apoptosis causes the evolution of a new cell population that is ER positive, PgR positive, and now grows in response to a panel of SERMs. This new observation is currently being developed and interrogated in the laboratory, as this is the first time that there has been any report of a SERM stimulated cell in culture. As a first step, Dr. Heather Cunliff has worked with Dr. Ping Fan to created an Agilent gene array database mapping the pathways for estrogen stimulated growth or SERM stimulated growth. It is clear that the complex survival networks can rapidly produce new clones of cells to subvert antihormone therapy and reverse apoptosis into cell growth (**Task 4a**).

Our future plans for the no cost extension will complete our original plan for deciphering the molecular mechanism (mechanisms) of estrogen-induced apoptosis. Our unique team has built on our strengths and we are now poised to interrogate the models and move rapidly towards ublication. We are the leaders in this area. It is important to stress that our data was used to obtain grants from other sources (SU2C, Susan G. Komen For The Cure). This enhances our capacity for interaction with the best breast cancer research scientists in the world.

REFERENCES

1. Anderson GL, Chlebowski RT, Aragaki AK, Kuller LH, Manson JE, Gass M, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. Lancet Oncol. 2012 May;13(5):476-86.
2. Chlebowski RT, Anderson GL. Changing concepts: Menopausal hormone therapy and breast cancer. J Natl Cancer Inst. 2012 Apr 4;104(7):517-27.
3. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst. 2005 Dec 7;97(23):1746-59.
4. Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, et al. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci U S A. 2011 Nov 22;108(47):18879-86.
5. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA. 2009 Aug 19;302(7):774-80.
6. Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst. 2001 Nov 21;93(22):1714-23.
7. Harrington WR, Kim SH, Funk CC, Madak-Erdogan Z, Schiff R, Katzenellenbogen JA, et al. Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. Mol Endocrinol. 2006 Mar;20(3):491-502.
8. Fan P, McDaniel RE, Kim HR, Clagett D, Haddad B, Jordan VC. Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines. Eur J Cancer. 2012 Dec;48(18):3488-98.
9. Chu I, Arnaout A, Loiseau S, Sun J, Seth A, McMahon C, et al. Src promotes estrogen-dependent estrogen receptor alpha proteolysis in human breast cancer. J Clin Invest. 2007 Aug;117(8):2205-15.
10. Ping Fan OG, Fadeke Abgoke, Pavana Anur, Xiojun Zou, Russell McDaniel, Karen Creswell, Sung Hoon Kim, John Katzenellenbogen, Joe Gray, and V. Craig Jordan. c-Src Modulates Estrogen-Induced Stress and Apoptosis in Estrogen-Deprived Breast Cancer Cells. Cancer Research. 2013;In Press.
11. Lange CA, Shen T, Horwitz KB. Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1032-7.
12. Jeng MH, Langan-Fahey SM, Jordan VC. Estrogenic actions of RU486 in hormone-responsive MCF-7 human breast cancer cells. Endocrinology. 1993 Jun;132(6):2622-30.
13. Fan P, Wang J, Santen RJ, Yue W. Long-term treatment with tamoxifen facilitates translocation of estrogen receptor alpha out of the nucleus and enhances its interaction with EGFR in MCF-7 breast cancer cells. Cancer Res. 2007 Feb 1;67(3):1352-60.
14. Nam JS, Ino Y, Sakamoto M, Hirohashi S. Src family kinase inhibitor PP2 restores the E-cadherin/catenin cell adhesion system in human cancer cells and reduces cancer metastasis. Clin Cancer Res. 2002 Jul;8(7):2430-6.
15. Moustakas A, Heldin CH. Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci. 2007 Oct;98(10):1512-20.
16. Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol. 2007 Dec 20;25(36):5815-24.
17. Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jorgensen LM, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Res Treat. 2001 May;67(2):111-6.
18. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. Science. 2011 Nov 25;334(6059):1081-6.
19. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol. 2007 Jul;8(7):519-29.
20. Kim I, Xu W, Reed JC. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. Nat Rev Drug Discov. 2008 Dec;7(12):1013-30.
21. Wek RC, Jiang HY, Anthony TG. Coping with stress: eIF2 kinases and translational control. Biochem Soc Trans. 2006 Feb;34(Pt 1):7-11.
22. Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D, et al. A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. Science. 2005 Feb 11;307(5711):935-9.

23. Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND, et al. ER stress triggers apoptosis by activating BH3-only protein Bim. Cell. 2007 Jun 29;129(7):1337-49.
24. Sengupta S, Jordan VC. Selective estrogen modulators as an anticancer tool: mechanisms of efficiency and resistance. Adv Exp Med Biol. 2008;630:206-19.
25. Obiorah I, Jordan VC. Progress in endocrine approaches to the treatment and prevention of breast cancer. Maturitas. 2011 Dec;70(4):315-21.
26. Baum M, Budzar AU, Cuzick J, Forbes J, Houghton JH, Klijn JG, et al. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. Lancet. 2002 Jun 22;359(9324):2131-9.
27. Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, Forbes JF, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet. 2005 Jan 1-7;365(9453):60-2.
28. Chia S, Gradishar W, Mauriac L, Bines J, Amant F, Federico M, et al. Double-blind, randomized placebo controlled trial of fulvestrant compared with exemestane after prior nonsteroidal aromatase inhibitor therapy in postmenopausal women with hormone receptor-positive, advanced breast cancer: results from EFECT. J Clin Oncol. 2008 Apr 1;26(10):1664-70.
29. Nabholtz JM, Buzdar A, Pollak M, Harwin W, Burton G, Mangalik A, et al. Anastrozole is superior to tamoxifen as first-line therapy for advanced breast cancer in postmenopausal women: results of a North American multicenter randomized trial. Arimidex Study Group. J Clin Oncol. 2000 Nov 15;18(22):3758-67.
30. Baselga J, Campone M, Piccart M, Burris HA, 3rd, Rugo HS, Sahmoud T, et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med. 2012 Feb 9;366(6):520-9.
31. McNeil CM, Sergio CM, Anderson LR, Inman CK, Eggleton SA, Murphy NC, et al. c-Myc overexpression and endocrine resistance in breast cancer. J Steroid Biochem Mol Biol. 2006 Dec;102(1-5):147-55.
32. Ellis MJ, Ding L, Shen D, Luo J, Suman VJ, Wallis JW, et al. Whole-genome analysis informs breast cancer response to aromatase inhibition. Nature. 2012 Jun 21;486(7403):353-60.
33. Miller TW, Balko JM, Ghazoui Z, Dunbier A, Anderson H, Dowsett M, et al. A gene expression signature from human breast cancer cells with acquired hormone independence identifies MYC as a mediator of antiestrogen resistance. Clin Cancer Res. 2011 Apr 1;17(7):2024-34.
34. Wolfer A, Wittner BS, Irimia D, Flavin RJ, Lupien M, Gunawardane RN, et al. MYC regulation of a "poor-prognosis" metastatic cancer cell state. Proc Natl Acad Sci U S A. 2010 Feb 23;107(8):3698-703.
35. Deming SL, Nass SJ, Dickson RB, Trock BJ. C-myc amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. Br J Cancer. 2000 Dec;83(12):1688-95.
36. Heselmeyer-Haddad K, Berroa Garcia LY, Bradley A, Ortiz-Melendez C, Lee WJ, Christensen R, et al. Single-Cell Genetic Analysis of Ductal Carcinoma in Situ and Invasive Breast Cancer Reveals Enormous Tumor Heterogeneity yet Conserved Genomic Imbalances and Gain of MYC during Progression. Am J Pathol. 2012 Nov;181(5):1807-22.
37. Darnell JE, Jr. Transcription factors as targets for cancer therapy. Nat Rev Cancer. 2002 Oct;2(10):740-9.
38. Soucek L, Whitfield J, Martins CP, Finch AJ, Murphy DJ, Sodir NM, et al. Modelling Myc inhibition as a cancer therapy. Nature. 2008 Oct 2;455(7213):679-83.
39. Trumpp A, Refaeli Y, Oskarsson T, Gasser S, Murphy M, Martin GR, et al. c-Myc regulates mammalian body size by controlling cell number but not cell size. Nature. 2001 Dec 13;414(6865):768-73.
40. Toyoshima M, Howie HL, Imakura M, Walsh RM, Annis JE, Chang AN, et al. Functional genomics identifies therapeutic targets for MYC-driven cancer. Proc Natl Acad Sci U S A. 2012 Jun 12;109(24):9545-50.
41. Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell. 2011 Sep 16;146(6):904-17.
42. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol. 1992 Dec;90(1):77-86.
43. Yin X, Giap C, Lazo JS, Prochownik EV. Low molecular weight inhibitors of Myc-Max interaction and function. Oncogene. 2003 Sep 18;22(40):6151-9.
44. Follis AV, Hammoudeh DI, Wang H, Prochownik EV, Metallo SJ. Structural rationale for the coupled binding and unfolding of the c-Myc oncoprotein by small molecules. Chem Biol. 2008 Nov 24;15(11):1149-55.
45. Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat. 2010 Oct;123(3):725-31.
46. Saunders A, Core LJ, Lis JT. Breaking barriers to transcription elongation. Nat Rev Mol Cell Biol. 2006 Aug;7(8):557-67.
47. Shim EY, Walker AK, Shi Y, Blackwell TK. CDK-9/cyclin T (P-TEFb) is required in two postinitiation pathways for transcription in the C. elegans embryo. Genes Dev. 2002 Aug 15;16(16):2135-46.

48. Larochelle S, Amat R, Glover-Cutter K, Sanso M, Zhang C, Allen JJ, et al. Cyclin-dependent kinase control of the initiation-to-elongation switch of RNA polymerase II. Nat Struct Mol Biol. 2012 Nov;19(11):1108-15.
49. Sims RJ, 3rd, Belotserkovskaya R, Reinberg D. Elongation by RNA polymerase II: the short and long of it. Genes Dev. 2004 Oct 15;18(20):2437-68.
50. Krystof V, Cankar P, Frysova I, Slouka J, Kontopidis G, Dzubak P, et al. 4-arylazo-3,5-diamino-1H-pyrazole CDK inhibitors: SAR study, crystal structure in complex with CDK2, selectivity, and cellular effects. J Med Chem. 2006 Nov 2;49(22):6500-9.
51. Jordan VC. A Century of Deciphering the Control Mechanisms of Sex Steroid Action in Breast and Prostate Cancer: The Origins of Targeted Therapy and Chemoprevention. *Cancer Res*. 2009 February 15, 2009;**69**(4):1243-54.
52. Osborne CK, Coronado EB, Robinson J. Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. Eur J Cancer Clin Oncol. 1987;**23**(8):1189-96.
53. Gottardis MM, Jordan V. Development of Tamoxifen-stimulated Growth of MCF-7 Tumors in Athymic Mice after Long-Term Antiestrogen Administration. Cancer Res. 1988 September 15, 1988;**48**(18):5183-7.
54. Gottardis MM, Jiang S-Y, Jeng M-H, Jordan VC. Inhibition of Tamoxifen-stimulated Growth of an MCF-7 Tumor Variant in Athymic Mice by Novel Steroidal Antiestrogens. Cancer Res. 1989 August 1, 1989;**49**(15):4090-3.
55. Yao K, Lee E, Bentrem D, England G, Schafer J, O'Regan R, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res. 2000 May 1, 2000;**6**(5):2028-36.
56. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, et al. Intrinsic Mechanism of Estradiol-Induced Apoptosis in Breast Cancer Cells Resistant to Estrogen Deprivation. J Natl Cancer Inst. 2005 7 December 2005;**97**(23):1746-59.
57. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol*. 2005;**94**(1–3):131-41.
58. Lønning P, Taylor P, Anker G, Iddon J, Wie L. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. Breast Cancer Research and Treatment. 2001 2001/05/01;**67**(2):111-6.
59. Ellis M, Gao F, Dehdashti F, Jeffe D, Marcom P, Carey L, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor–positive, aromatase inhibitor–resistant advanced breast cancer: A phase 2 randomized study. JAMA. 2009;**302**(7):774-80.
60. WHI. Design of the Women's Health Initiative Clinical Trial and Observational Study. Controlled Clin Trials. 1998;**19**:61-109.
61. Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, et al. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. JAMA. 2004 Apr 14;291(14):1701-12.
62. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA. 2002;**288**(3):321-33.
63. Chlebowski R, Manson J, Anderson G, Cauley J, Aragaki A, Stefanick M, et al. Estrogen Plus Progestin and Breast Cancer Incidence and Mortality in the Women's Health Initiative Observational Study. J Natl Cancer Inst. 2013 April 17, 2013;**105**(8):526-35.
64. Beral V, Reeves G, Bull D, Green J. Breast Cancer Risk in Relation to the Interval Between Menopause and Starting Hormone Therapy. J Natl Cancer Inst. 2011;**103**(4):296-305.
65. Anderson GL, Chlebowski RT, Aragaki AK, Kuller LH, Manson JE, Gass M, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *Lancet Oncol*. 2012;**13**(5):476-86.
66. Obiorah I, Jordan VC. 2012 NAMS/PFIZER- Wulf H. utian endowed lecture. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. Menopause. 2013;**20**(4):372-82.
67. Kaufmann SH, Earnshaw WC. Induction of Apoptosis by Cancer Chemotherapy. Exp Cell Res. 2000;**256**(1):42-9.
68. Makin G, Dive C. Apoptosis and cancer chemotherapy. Trends Cell Biol. 2001;**11**(11):22-6.
69. Ariazi E, Cunliffe H, Lewis-Wambi JS, Slifker M, Willis A, Ramos P, et al. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci USA. 2011 November 22, 2011;**108**(47):18879-86.
70. Song R, Mor G, Naftolin F, McPherson R, Song J, Zhang Z, et al. Effect of Long-Term Estrogen Deprivation on Apoptotic Responses of Breast Cancer Cells to 17β-Estradiol. J Natl Cancer Inst. 2001 November 21, 2001;**93**(22):1714-23.
71. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical Action of Fulvestrant in Estradiol-Induced Regression of Tamoxifen-Stimulated Breast Cancer. *J Natl Cancer Inst*. 2003 November 5, 2003;**95**(21):1597-608.

72. Jordan M, Toso R, Thrower D, Wilson L. Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. Proc Natl Acad Sci USA. 1993 October 15, 1993;**90**(20):9552-6.
73. Yvon AC, Wadsworth P, Jordan MA, Toso RJ, Thrower D, Wilson L. Taxol suppresses dynamics of individual microtubules in living human tumor cells. Mol Biol Cell. 1999 April 1, 1999;**10**(4):947-59.
74. Kelly C, Green M, Broglio K, Thomas E, Brewster A. Phase III Trial Evaluating Weekly Paclitaxel Versus Docetaxel in Combination With Capecitabine in Operable Breast Cancer. J Clin Oncol. 2012 March 20, 2012;**30**(9):930-5.
75. Robert N, Saleh M, Paul D, Generali D, Gressot L, Copur M, et al. Sunitinib plus paclitaxel versus bevacizumab plus paclitaxel for first-line treatment of patients with advanced breast cancer: a phase III, randomized, open-label trial. Clin Breast Cancer. 2011;**11**(2):82-92.
76. Gines J, Sabater E, Martorell C, Grau M, Monroy M, Casado M. Efficacy of taxanes as adjuvant treatment of breast cancer: a review and meta-analysis of randomised clinical trials. Clin Transl Oncol. 2011;**13**(7):485-98.
77. Ward S, Simpson E, Davis S, Hind D, Rees A, Wilkinson A. Taxanes for the adjuvant treatment of early breast cancer: systematic review and economic evaluation. Health Technol Assess. 2007;**11**(40):1-144.
78. Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell. 2003;**114**(2):181-90.
79. Thorburn A. Death receptor-induced cell killing. Cell Signal. 2004;**16**(2):139-44.
80. De Paepe B, Creus KK, De Bleecker JL. The tumor necrosis factor superfamily of cytokines in the inflammatory myopathies: potential targets for therapy. Clin Dev Immunol. 2012;**369432**(10):23.
81. Zhang K, Kaufmann R. From endoplasmic-reticulum stress to the inflammatory response. Nature. 2008;**454**:455-62.
82. Hu P, Han Z, Couvillon A, Kaufman R, Exton J. Autocrine tumor necrosis factor a links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1α-mediated NF-κB activation and down-regulation of TRAF2 expression. Mol Cell Biol. 2006;**26**:3071–84.
83. Baldwin A. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol. 1996;**14**:649-83.
84. Dobrovolskaia MA, Kozlov, S V. Inflammation and cancer: when NF-kappaB amalgamates the perilous partnership. Curr Cancer Drug Targets. 2005;**5**(5):325-44.
85. Liu H, Lee E-S, Gajdos C, Pearce S, Chen B, Osipo C, et al. Apoptotic Action of 17β-Estradiol in Raloxifene-Resistant MCF-7 Cells In Vitro and In Vivo. J Natl Cancer Inst. 2003 November 5, 2003;**95**(21):1586-97.
86. Kutul O, Lethai, A. . Displacement of Bim by BmF and Puma rather than increase in Bim level mediates paclitaxel induced apoptosis in breast cancer cells. Cell Death Differ. 2010;**17(10)**:1624-35.
87. Ajabnoor GM, Crook T, Coley H. Paclitaxel resistance is associated with switch from apoptotic to autophagic cell death in MCF-7 breast cancer cells. Cell death dis. 2012 **3(1)**:e260.
88. Czernick M, Rieger A, Goping I. Bim is reversibly phosphorylated but plays a limited role in paclitaxel cytotoxicity of breast cancer cell lines. . *Biochem biophys res commun* 2009;**379:1**:145-50.
89. Anderson GL, Chlebowski RT, Aragaki AK, Kuller LH, Manson JE, Gass M, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *Lancet Oncol*. 2012;**13**(5):476-86.
90. American Cancer Society. Breast Cancer Facts & Figures 2011-2012. Atlanta: American Cancer Society Inc.; 2011.
91. Beatson G. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. Lancet. 1896;2:101 - 11.
92. Boyd S. On oophorectomy in cancer of the breast. BMJ. 1900;2:1161-7.
93. Lathrop AEC, Loeb L. Further Investigations on the Origin of Tumors in Mice: III. On the Part Played by Internal Secretion in the Spontaneous Development of Tumors. J Cancer Res. 1916 January 1, 1916;1(1):1-19.
94. Allen E, Doisy E. An ovarian hormone: Preliminary report on its localization, extraction and partial purification, and action in test animals. JAMA. 1923;81(10):819-21.
95. Jordan VC, Brodie AMH. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids. 2007;72(1):7-25.
96. Obiorah I, Jordan VC. Progress in endocrine approaches to the treatment and prevention of breast cancer. Maturitas. 2011;70(4):315-21.
97. Watkins E. The Estrogen Elixer: A History of Hormone Replacement Therapy in America. Baltimore: The Johns Hopkins Universtiy Press; 2007.
98. Research UFaDACfDEa. FDA: FDA Background on Conjugated Estrogens.2005.
99. Smith D.C. PR, Thompson D.J, Herrmann W.L. Association of exogenous estrogen and endometrial carcinoma. N Engl J Med. 1975;293:1164-7.

100. Ziel H.K. FWD. Increased risk of endometrial cancinoma among users of conjugated estrogens. N Engl J Med. 1975;293:1167-70.
101. Greenblatt R, Gambrell, RD, Stoddard, LD. The protective role of progesterone in the prevention of endometrial cancer. Pathol Res Pract. 1982 Aug;174(3):297-318.
102. Gambrell R. The prevention of endometrial cancer in postmenopausal women with progestogens. Maturitas. 1978 Sep;1(2):107-12.
103. Research UFaDACfDEa. FDA: Conjugated estrogens - Letter from Dr. Janet Woodstock1997.
104. Soule HD, Vazquez J, Long A, et al. A Human Cell Line From a Pleural Effusion Derived From a Breast Carcinoma. J Natl Cancer Inst. 1973 November 1, 1973;51(5):1409-16.
105. Lewis J, Meeke K, Osipo C, Ross E, Kidawi N, Li T, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst. 2005;97:1746 - 59.
106. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst. 2003 Nov 5;95(21):1597-608.
107. Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, et al. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst. 2003 Nov 5;95(21):1586-97.
108. Brzozowski AM, Pike ACW, Dauter Z, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature. [10.1038/39645]. 1997;389(6652):753-8.
109. Shiau AK, Barstad D, Loria P, et al. The Structural Basis of Estrogen Receptor/Coactivator Recognition and the Antagonism of This Interaction by Tamoxifen. Cell. 1998;95(7):927-37.
110. Hu ZZ, Kagan BL, Ariazi EA, Rosenthal DS, Zhang L, Li JV, et al. Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. PLoS One. 2011;6(6):e20410.
111. Jordan VC, Schafer JM, Levenson AS, Liu H, Pease KM, Simons LA, et al. Molecular classification of estrogens. Cancer Res. 2001 Sep 15;61(18):6619-23.
112. Maximov PY, Myers CB, Curpan RF, Lewis-Wambi JS, Jordan VC. Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. J Med Chem. 2010 Apr 22;53(8):3273-83.
113. Maximov P, Sengupta S, Lewis-Wambi JS, Kim HR, Curpan RF, Jordan VC. The Conformation of the Estrogen Receptor Directs Estrogen-Induced Apoptosis in Breast Cancer: A Hypothesis. Horm Mol Biol Clin Investig. 2011 Mar 1;5(1):27-34.
114. Stoll B. Palliation by castration or by hormone administration. In Stoll B, ed. *Breast Cancer Management Early and Late.*
. London, UK: William Herman Medical Books Ltd; 1977.
115. Haddow A, Watkinson J.M., Patterson E. Influence of synthetic oestrogens upon advanced malignant disease. BMJ. 1944;2:393-98.
116. Palliation by castration and by hormone administration. In: Stoll B, editor. Breast Cancer Management-Early and Late: William Heinemann Medical Books, Chicago; 1977. p. 133-46.
117. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol. 2005 Feb;94(1-3):131-41.
118. Sengupta S, Obiorah I, Maximov P, Curpan R, Jordan V. Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. Br J Pharmacol. 2013 May;169(1):167-78.
119. Murphy CS, Parker CJ, McCague R, Jordan VC. Structure-activity relationships of nonisomerizable derivatives of tamoxifen: importance of hydroxyl group and side chain positioning for biological activity. Mol Pharmacol. 1991 Mar;39(3):421-8.
120. Li JV, Chien CD, Garee JP, Xu J, Wellstein A, Riegel AT. Transcriptional repression of AIB1 by FoxG1 leads to apoptosis in breast cancer cells. Mol Endocrinol. 2013 May 9.
121. Xu J, Wu RC, O'Malley BW. Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer. 2009 Sep;9(9):615-30.
122. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science. 1997 Aug 15;277(5328):965-8.
123. Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, et al. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell. 1997 Aug 8;90(3):569-80.
124. Suen CS, Berrodin TJ, Mastroeni R, Cheskis BJ, Lyttle CR, Frail DE. A transcriptional coactivator, steroid receptor coactivator-3, selectively augments steroid receptor transcriptional activity. J Biol Chem. 1998 Oct 16;273(42):27645-53.
125. Xu J, Li Q. Review of the in vivo functions of the p160 steroid receptor coactivator family. Mol Endocrinol. 2003 Sep;17(9):1681-92.

126. Hsia EY, Zou JX, Chen HW. The roles and action mechanisms of p160/SRC coactivators and the ANCCA coregulator in cancer. Prog Mol Biol Transl Sci. 2009;87:261-98.
127. Louie MC, Zou JX, Rabinovich A, Chen HW. ACTR/AIB1 functions as an E2F1 coactivator to promote breast cancer cell proliferation and antiestrogen resistance. Mol Cell Biol. 2004 Jun;24(12):5157-71.
128. Yan J, Yu CT, Ozen M, Ittmann M, Tsai SY, Tsai MJ. Steroid receptor coactivator-3 and activator protein-1 coordinately regulate the transcription of components of the insulin-like growth factor/AKT signaling pathway. Cancer Res. 2006 Nov 15;66(22):11039-46.
129. Qin L, Liao L, Redmond A, Young L, Yuan Y, Chen H, et al. The AIB1 oncogene promotes breast cancer metastasis by activation of PEA3-mediated matrix metalloproteinase 2 (MMP2) and MMP9 expression. Mol Cell Biol. 2008 Oct;28(19):5937-50.
130. Werbajh S, Nojek I, Lanz R, Costas MA. RAC-3 is a NF-kappa B coactivator. FEBS Lett. 2000 Nov 24;485(2-3):195-9.
131. Torres-Arzayus MI, Font de Mora J, Yuan J, Vazquez F, Bronson R, Rue M, et al. High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. Cancer Cell. 2004 Sep;6(3):263-74.
132. Lahusen T, Henke RT, Kagan BL, Wellstein A, Riegel AT. The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer. Breast Cancer Res Treat. 2009 Jul;116(2):225-37.
133. Mani A, Oh AS, Bowden ET, Lahusen T, Lorick KL, Weissman AM, et al. E6AP mediates regulated proteasomal degradation of the nuclear receptor coactivator amplified in breast cancer 1 in immortalized cells. Cancer Res. 2006 Sep 1;66(17):8680-6.
134. Wu RC, Feng Q, Lonard DM, O'Malley BW. SRC-3 coactivator functional lifetime is regulated by a phospho-dependent ubiquitin time clock. Cell. 2007 Jun 15;129(6):1125-40.
135. Li C, Ao J, Fu J, Lee DF, Xu J, Lonard D, et al. Tumor-suppressor role for the SPOP ubiquitin ligase in signal-dependent proteolysis of the oncogenic co-activator SRC-3/AIB1. Oncogene. 2011 Oct 20;30(42):4350-64.
136. Lauritsen KJ, List HJ, Reiter R, Wellstein A, Riegel AT. A role for TGF-beta in estrogen and retinoid mediated regulation of the nuclear receptor coactivator AIB1 in MCF-7 breast cancer cells. Oncogene. 2002 Oct 17;21(47):7147-55.
137. Louie MC, Revenko AS, Zou JX, Yao J, Chen HW. Direct control of cell cycle gene expression by proto-oncogene product ACTR, and its autoregulation underlies its transforming activity. Mol Cell Biol. 2006 May;26(10):3810-23.
138. Mussi P, Yu C, O'Malley BW, Xu J. Stimulation of steroid receptor coactivator-3 (SRC-3) gene overexpression by a positive regulatory loop of E2F1 and SRC-3. Mol Endocrinol. 2006 Dec;20(12):3105-19.
139. Teyssier C, Chen D, Stallcup MR. Requirement for multiple domains of the protein arginine methyltransferase CARM1 in its transcriptional coactivator function. J Biol Chem. 2002 Nov 29;277(48):46066-72.
140. Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell. 2000 Dec 8;103(6):843-52.
141. Li X, Kimbrel EA, Kenan DJ, McDonnell DP. Direct interactions between corepressors and coactivators permit the integration of nuclear receptor-mediated repression and activation. Mol Endocrinol. 2002 Jul;16(7):1482-91.
142. Karmakar S, Gao T, Pace MC, Oesterreich S, Smith CL. Cooperative activation of cyclin D1 and progesterone receptor gene expression by the SRC-3 coactivator and SMRT corepressor. Mol Endocrinol. 2010 Jun;24(6):1187-202.
143. Xuan S, Baptista CA, Balas G, Tao W, Soares VC, Lai E. Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. Neuron. 1995 Jun;14(6):1141-52.
144. Obendorf M, Meyer R, Henning K, Mitev YA, Schroder J, Patchev VK, et al. FoxG1, a member of the forkhead family, is a corepressor of the androgen receptor. J Steroid Biochem Mol Biol. 2007 May;104(3-5):195-207.
145. Yao J, Lai E, Stifani S. The winged-helix protein brain factor 1 interacts with groucho and hes proteins to repress transcription. Mol Cell Biol. 2001 Mar;21(6):1962-72.
146. Hanashima C, Li SC, Shen L, Lai E, Fishell G. Foxg1 suppresses early cortical cell fate. Science. 2004 Jan 2;303(5654):56-9.
147. Shoichet SA, Kunde SA, Viertel P, Schell-Apacik C, von Voss H, Tommerup N, et al. Haploinsufficiency of novel FOXG1B variants in a patient with severe mental retardation, brain malformations and microcephaly. Hum Genet. 2005 Oct;117(6):536-44.
148. Zhao H, Langerod A, Ji Y, Nowels KW, Nesland JM, Tibshirani R, et al. Different gene expression patterns in invasive lobular and ductal carcinomas of the breast. Mol Biol Cell. 2004 Jun;15(6):2523-36.
149. Turashvili G, Bouchal J, Baumforth K, Wei W, Dziechciarkova M, Ehrmann J, et al. Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis. BMC Cancer. 2007;7:55.

150. Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, et al. X chromosomal abnormalities in basal-like human breast cancer. Cancer Cell. 2006 Feb;9(2):121-32.
151. Oh A, List HJ, Reiter R, Mani A, Zhang Y, Gehan E, et al. The nuclear receptor coactivator AIB1 mediates insulin-like growth factor I-induced phenotypic changes in human breast cancer cells. Cancer Res. 2004 Nov 15;64(22):8299-308.
152. Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, et al. The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature. 1997 Jun 12;387(6634):677-84.
153. Oh AS, Lahusen JT, Chien CD, Fereshteh MP, Zhang X, Dakshanamurthy S, et al. Tyrosine phosphorylation of the nuclear receptor coactivator AIB1/SRC-3 is enhanced by Abl kinase and is required for its activity in cancer cells. Mol Cell Biol. 2008 Nov;28(21):6580-93.
154. Hossain A, Kuo MT, Saunders GF. Mir-17-5p regulates breast cancer cell proliferation by inhibiting translation of AIB1 mRNA. Mol Cell Biol. 2006 Nov;26(21):8191-201.
155. Wu RC, Qin J, Yi P, Wong J, Tsai SY, Tsai MJ, et al. Selective phosphorylations of the SRC-3/AIB1 coactivator integrate genomic reponses to multiple cellular signaling pathways. Mol Cell. 2004 Sep 24;15(6):937-49.
156. Font de Mora J, Brown M. AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. Mol Cell Biol. 2000 Jul;20(14):5041-7.
157. Wu H, Sun L, Zhang Y, Chen Y, Shi B, Li R, et al. Coordinated regulation of AIB1 transcriptional activity by sumoylation and phosphorylation. J Biol Chem. 2006 Aug 4;281(31):21848-56.
158. Naeem H, Cheng D, Zhao Q, Underhill C, Tini M, Bedford MT, et al. The activity and stability of the transcriptional coactivator p/CIP/SRC-3 are regulated by CARM1-dependent methylation. Mol Cell Biol. 2007 Jan;27(1):120-34.
159. Hsia EY, Kalashnikova EV, Revenko AS, Zou JX, Borowsky AD, Chen HW. Deregulated E2F and the AAA+ coregulator ANCCA drive proto-oncogene ACTR/AIB1 overexpression in breast cancer. Mol Cancer Res. 2010 Feb;8(2):183-93.
160. Li J, Thurm H, Chang HW, Iacovoni JS, Vogt PK. Oncogenic transformation induced by the Qin protein is correlated with transcriptional repression. Proc Natl Acad Sci U S A. 1997 Sep 30;94(20):10885-8.
161. Li J, Chang HW, Lai E, Parker EJ, Vogt PK. The oncogene qin codes for a transcriptional repressor. Cancer Res. 1995 Dec 1;55(23):5540-4.
162. Banham AH, Beasley N, Campo E, Fernandez PL, Fidler C, Gatter K, et al. The FOXP1 winged helix transcription factor is a novel candidate tumor suppressor gene on chromosome 3p. Cancer Res. 2001 Dec 15;61(24):8820-9.
163. Li S, Weidenfeld J, Morrisey EE. Transcriptional and DNA binding activity of the Foxp1/2/4 family is modulated by heterotypic and homotypic protein interactions. Mol Cell Biol. 2004 Jan;24(2):809-22.
164. Gray S, Levine M. Short-range transcriptional repressors mediate both quenching and direct repression within complex loci in Drosophila. Genes Dev. 1996 Mar 15;10(6):700-10.
165. Arnosti DN, Gray S, Barolo S, Zhou J, Levine M. The gap protein knirps mediates both quenching and direct repression in the Drosophila embryo. EMBO J. 1996 Jul 15;15(14):3659-66.
166. Barolo S, Levine M. hairy mediates dominant repression in the Drosophila embryo. EMBO J. 1997 May 15;16(10):2883-91.
167. Li C, Manley JL. Even-skipped represses transcription by binding TATA binding protein and blocking the TFIID-TATA box interaction. Mol Cell Biol. 1998 Jul;18(7):3771-81.
168. Sauer F, Jackle H. Concentration-dependent transcriptional activation or repression by Kruppel from a single binding site. Nature. 1991 Oct 10;353(6344):563-6.
169. Kosman D, Small S. Concentration-dependent patterning by an ectopic expression domain of the Drosophila gap gene knirps. Development. 1997 Apr;124(7):1343-54.
170. Hewitt GF, Strunk BS, Margulies C, Priputin T, Wang XD, Amey R, et al. Transcriptional repression by the Drosophila giant protein: cis element positioning provides an alternative means of interpreting an effector gradient. Development. 1999 Mar;126(6):1201-10.
171. Lanz RB, Bulynko Y, Malovannaya A, Labhart P, Wang L, Li W, et al. Global characterization of transcriptional impact of the SRC-3 coregulator. Mol Endocrinol. 2010 Apr;24(4):859-72.
172. Hurtado A, Holmes KA, Ross-Innes CS, Schmidt D, Carroll JS. FOXA1 is a key determinant of estrogen receptor function and endocrine response. Nat Genet. 2011 Jan;43(1):27-33.
173. Liu Y, Lu C, Shen Q, Munoz-Medellin D, Kim H, Brown PH. AP-1 blockade in breast cancer cells causes cell cycle arrest by suppressing G1 cyclin expression and reducing cyclin-dependent kinase activity. Oncogene. 2004 Oct 28;23(50):8238-46.

174. Biswas DK, Shi Q, Baily S, Strickland I, Ghosh S, Pardee AB, et al. NF-kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. Proc Natl Acad Sci U S A. 2004 Jul 6;101(27):10137-42.
175. Seoane J, Le HV, Shen L, Anderson SA, Massague J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell. 2004 Apr 16;117(2):211-23.
176. Akiyama N, Matsuo Y, Sai H, Noda M, Kizaka-Kondoh S. Identification of a series of transforming growth factor beta-responsive genes by retrovirus-mediated gene trap screening. Mol Cell Biol. 2000 May;20(9):3266-73.
177. Ma G, Ren Y, Wang K, He J. SRC-3 has a role in cancer other than as a nuclear receptor coactivator. Int J Biol Sci. 2011;7(5):664-72.
178. Coste A, Antal MC, Chan S, Kastner P, Mark M, O'Malley BW, et al. Absence of the steroid receptor coactivator-3 induces B-cell lymphoma. EMBO J. 2006 Jun 7;25(11):2453-64.
179. Chan DW, Liu VW, To RM, Chiu PM, Lee WY, Yao KM, et al. Overexpression of FOXG1 contributes to TGF-beta resistance through inhibition of p21WAF1/CIP1 expression in ovarian cancer. Br J Cancer. 2009 Oct 20;101(8):1433-43.
180. Kim TH, Jo SW, Lee YS, Kim YJ, Lee SC, Kim WJ, et al. Forkhead box O-class 1 and forkhead box G1 as prognostic markers for bladder cancer. J Korean Med Sci. 2009 Jun;24(3):468-73.
181. Adesina AM, Nguyen Y, Mehta V, Takei H, Stangeby P, Crabtree S, et al. FOXG1 dysregulation is a frequent event in medulloblastoma. J Neurooncol. 2007 Nov;85(2):111-22.
182. Munster PN, Carpenter JT. Estradiol in breast cancer treatment: reviving the past. JAMA. 2009 Aug 19;302(7):797-8.
183. Jordan VC, Ford LG. Paradoxical clinical effect of estrogen on breast cancer risk: a "new" biology of estrogen-induced apoptosis. Cancer Prev Res (Phila). 2011 May;4(5):633-7.

APPENDIX

1. Korch C., Spillman, M.A., Jackson, T.A., Jacobsen, B.M., Murphy S.K., Lessey, B.A., Jordan, V.C. and Bradford, A.P. (2012). DNA profiling analysis of endometrial and ovarian cell lines reveals misidentification, redundancy and contamination. Gynecologic Oncology 127:241-248.
2. Sengupta, S. S., Obiorah, I. O., Maximov, P. Y., Curpan, R., and Jordan, V. C. (2013) Molecular Mechanism of Action of Bisphenol and Bisphenol-A Mediated by Estrogen Receptor alpha in Growth and Apoptosis of Breast Cancer Cells. British Journal of Pharmacology 169:167-78.
3. Obiorah, I. and Jordan, V. C. 2013. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. North American Menopause Society/Pfizer-Wulf H. Utian Endowed Lecture. Menopause 20:372-382.
4. Maximov, P.Y., Lee, T.M., and Jordan, V.C. (2013). The discovery and development of Selective Estrogen Receptor Modulators (SERMs) for clinical practice. Current Clinical Pharmacology 8:135-155
5. Jordan, V.C. (2013). Estrogen Action, SERMs and Women's Health. Imperial College Press, London.
6. Segala G., de Medina P., Iuliano L., Zerbinati C., Paillasse M. R., Noguer E., Dalenc F., Payre B., Jordan V. C., Record M., Silvente-Poirot S., Poirot M (2013).5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of tamoxifen in breast cancer cells. Biochemical Pharmacology (epub ahead of print).
7. Li J. V., Chien C. D., Garee J. P., Xu J., Wellstein A., Riegel A. T. (2013) Transcriptional repression of AIB1 by FoxG1 leads to apoptosis in breast cancer cells. Molecular Endocrinology (epub ahead of print)
8. Fan P., Griffith O. L., Agboke F., Anur P., McDaniel R. E., Creswell K., Kim S. H., Katzenellenbogen J. A., Gray J. W., Jordan V. C. (2013) c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. Cancer Research (epub ahead of print).
9. McDaniel, R.E., Maximov, P.Y., and Jordan, V.C. (2013) Estrogen-mediated mechanisms to control the growth and apoptosis of breast cancer cells: a translational research success story. In: Vitamins and Hormones (ed. Gerald Litwack) Elsevier (*in press*).
10. Maximov, P.Y., McDaniel, R. E., and Jordan, V.C. (2013). Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Springer Basel AG, Basal, Switzerland (*in press*).

Gynecologic Oncology 127 (2012) 241–248

Contents lists available at SciVerse ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

DNA profiling analysis of endometrial and ovarian cell lines reveals misidentification, redundancy and contamination

Christopher Korch [a,1], Monique A. Spillman [b,1], Twila A. Jackson [b,1], Britta M. Jacobsen [c], Susan K. Murphy [d], Bruce A. Lessey [e], V. Craig Jordan [f], Andrew P. Bradford [b,*]

[a] Department of Medicine, Division of Medical Oncology, University of Colorado Anschutz Medical Campus, Aurora, CO 80045, USA
[b] Department of Obstetrics & Gynecology, University of Colorado Anschutz Medical Campus, Aurora, CO 80045, USA
[c] Department of Medicine, Division of Endocrinology, University of Colorado Anschutz Medical Campus, Aurora, CO 80045, USA
[d] Department of Obstetrics & Gynecology, Duke University Medical Center, Durham, NC 27708, USA
[e] Division of Reproductive Endocrinology and Infertility, Greenville Hospital System, Greenville, SC 29605, USA
[f] Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC 20057, USA

ARTICLE INFO

Article history:
Received 7 May 2012
Accepted 11 June 2012
Available online 16 June 2012

Keywords:
STR profiling
Endometrial
Ovarian
Cell lines
Authenticity

ABSTRACT

Objectives. Cell lines derived from human ovarian and endometrial cancers, and their immortalized non-malignant counterparts, are critical tools to investigate and characterize molecular mechanisms underlying gynecologic tumorigenesis, and facilitate development of novel therapeutics. To determine the extent of misidentification, contamination and redundancy, with evident consequences for the validity of research based upon these models, we undertook a systematic analysis and cataloging of endometrial and ovarian cell lines.

Methods. Profiling of cell lines by analysis of DNA microsatellite short tandem repeats (STR), p53 nucleotide polymorphisms and microsatellite instability was performed.

Results. Fifty-one ovarian cancer lines were profiled with ten found to be redundant and five (A2008, OV2008, C13, SK-OV-4 and SK-OV-6) identified as cervical cancer cells. Ten endometrial cell lines were analyzed, with RL-92, HEC-1A, HEC-1B, HEC-50, KLE, and AN3CA all exhibiting unique, uncontaminated STR profiles. Multiple variants of Ishikawa and ECC-1 endometrial cancer cell lines were genotyped and analyzed by sequencing of mutations in the p53 gene. The profile of ECC-1 cells did not match the EnCa-101 tumor, from which it was reportedly derived, and all ECC-1 isolates were genotyped as Ishikawa cells, MCF-7 breast cancer cells, or a combination thereof. Two normal, immortalized endometrial epithelial cell lines, HES cells and the hTERT-EEC line, were identified as HeLa cervical carcinoma and MCF-7 breast cancer cells, respectively.

Conclusions. Results demonstrate significant misidentification, duplication, and loss of integrity of endometrial and ovarian cancer cell lines. Authentication by STR DNA profiling is a simple and economical method to verify and validate studies undertaken with these models.

© 2012 Elsevier Inc. All rights reserved.

Introduction

Cell lines, immortalized from normal human tissues or derived from tumors, are widely used models to address molecular mechanisms underlying the physiology and pathology of the female reproductive tract, and to evaluate novel therapeutics or preventive strategies [1–3]. Verification of the provenance and integrity of such cell lines is clearly of paramount importance, but historically, has rarely been undertaken by investigators. The problem of cross-contamination, identified and characterized by examination of isozyme patterns, karyotyping, and cytogenetics, dates back to the establishment of the prototypical HeLa cell line in culture in 1951 and remains a significant concern [4–7]. Over one-third (18–50%) of cell lines may be mixtures, misidentified or intra-species contaminants [2,8–15]. Furthermore, there are many examples of redundancy among reportedly unique cell lines, and instances of contamination during original derivations, such that the intended novel cell line was never established [5,10,16–19]. Thus, it is evident that authentication of cell line origins and integrity is crucial to validate results and conclusions obtained using these model systems.

Short tandem repeat (STR) profiling or 'DNA fingerprinting' identifies variants in tetranucleotide microsatellite loci on multiple human chromosomes and is the accepted international standard for genetic analysis of cell lines for authentication by comparison to established STR databases [20–24].

A comprehensive analysis of cell lines commonly used in the study of ovarian and endometrial cancer had not been undertaken, particularly with respect to those cell lines not obtained from established cell repositories. We used STR profiling, sequencing of p53 mutations, and

* Corresponding author at: University of Colorado, Anschutz Medical Campus, Department of Obstetrics & Gynecology, MS 8613, 12700 E. 19th Avenue, Aurora, CO 80045, USA. Fax: +1 303 724 3512.
E-mail address: Andy.Bradford@ucdenver.edu (A.P. Bradford).
[1] Joint first authors.

0090-8258/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygyno.2012.06.017

human papilloma virus screening to examine cell lines of purported ovarian and endometrial origins. We observed examples of cross-contamination, misidentification of lines and/or tissue of origin, and redundancy among established cancer cells, and found evidence that immortalized normal endometrial epithelial cell lines are genetically identical to previously established cervical and breast cancer cells. We provide reference DNA profiles for women's cancer cell lines that are not currently in public cell banks and extend the number of loci for profiles currently available through central repositories.

Materials and methods

DNA isolation and STR profiling

Cell lines were grown in appropriate specific standard media. Genomic DNA was isolated from 0.5 to 5×10^6 cells using a Zymo Research ZR genomic DNA II kit and quantified by gel electrophoresis and ethidium bromide staining by comparison to a DNA mass ladder. Multiplex PCR amplified products were generated using 1–2 ng of genomic DNA with an Applied Biosystems Identifiler kit and ABI 3730 capillary sequencer as described [2,18]. STR loci were analyzed with Gene Mapper 4.0. Profiles were compared to published reports [22,25], consolidated (ATCC, DSMZ, JCRB and RIKEN) databases, and an in-house database, using a custom search algorithm designed to facilitate comparison of cell lines with related profiles and identify individual cell lines in a mixture (C. Korch and J. West, Vanderbilt University, unpublished). STR profiles of the ovarian and endometrial cancer cells analyzed in this study are available online at http://DNAsequencingcore.UCDenver.edu.

TP53 sequence analysis and microsatellite instability assays

PCR amplification was used to generate overlapping products spanning the Variable Number Tandem Repeat (VNTR; a pentanucleotide repeat of A_4T) in intron 1, through the protein encoding exons 2–11, including intervening introns 2–8 and 10 [26]. Sequencing primers and p53 gene structure are shown in Fig. S1. DNAs were screened for microsatellite instability [27] using Promega MSI analysis system version 1.2 according to the manufacturers' protocol.

HPV testing

Aliquots of cells were placed into ThinPrep (Hologic) solution. DNA was isolated and tested in the University of Colorado Hospital Clinical Laboratory using the hybrid capture PCR, Digene HC2 High Risk HPV test (Qiagen).

Ovarian and endometrial cell lines

We obtained cell lines from multiple institutions in the United States, Europe and Japan, including, where possible, the originating laboratories. Multiple independent samples of the earliest available passages from each institution were analyzed and, if available, profiles of each individual cell line were compared from several sources. Ovarian cancer cell lines are listed in Table S1. Ishikawa cells were obtained from Dr. K.K. Leslie (University of Iowa), Dr. B.A. Lessey (Greenville Hospital System, SC), Dr. M. Brown (Dana Farber Cancer Institute, Harvard University) and Drs. H. Philpott and P. Thraves (European Collection of Cell Cultures, ECACC). ECC-1 cells were from Drs. B.A. Lessey, M. Brown and V.C. Jordan (Lombardi Comprehensive Cancer Center, Georgetown University). EnCa-101 tumors were provided by Drs. V.C. Jordan and G. Balburski (Fox Chase Cancer Center). HES cells were from Dr. D. Kniss (Ohio State University) and hTERT-EEC cells from Dr. T. Klonisch (University of Manitoba, Canada). KLE and HEC-50 cells were from Dr. K.K. Leslie. RL-95-2, HEC-1A, HEC-1B and AN3CA cells were from the American Type Culture Collection (ATCC, Manassas, VA).

Results

Analysis of endometrial cancer cell lines

Endometrial carcinomas are derived from glandular epithelium and are typically divided into two subtypes based on clinical, histological and molecular characteristics [28–30]. Cell lines derived from type I (Ishikawa, ECC-1 and RL-95-2) and type II (HEC-1, HEC-50, KLE and AN3CA) tumors have been widely used as models to investigate molecular genetics and mechanisms underlying their development, progression and response to therapeutics [31–35].

HEC-1B cells, the first to be derived from a human endometrial carcinoma [32,36,37], exhibited a unique profile (Table S3). HEC-1A, derived from the same patient, cells are predominantly diploid, while the HEC-1B line is tetraploid [38,39]. HEC-50 cells [38,40], also have a unique profile consistent with that on file with the Japanese Collection of Research Bioresources (JCRB: 1145).

Similarly, KLE (CRL-1622) and AN3CA (HTB-111) cells, originating from peritoneal and lymph node metastases, respectively [34,41,42], and RL-95-2 cells (CRL-1671) derived from a moderately differentiated (Grade 2) endometrial adenosquamous carcinoma [35], all have STR profiles consistent with those reported by the ATCC (Table S3).

Ishikawa cells were established from the epithelial component of a moderately differentiated, stage 2, endometrial adenocarcinoma [43,44]. At least three variants of Ishikawa cells, the original line, 3-H-4 and 3-H-12, differing in their reported degree of differentiation, relative expression of estrogen (ER) and progesterone (PR) receptors, growth and colony formation rates, were distributed to investigators [45].

We profiled multiple isolates of the original Ishikawa cells and 3-H-12 variants obtained from a number of laboratories as detailed in the Materials and methods section. Samples with unique profiles, which may represent the 3-H-4 variant based upon their date of origin are designated '3-H-4'. The results are summarized in Table 1.

Overall the Ishikawa cell lines exhibit very similar profiles, indicative of their origin from the same patient. Identical alleles were present at several loci (CSF1PO, D5S818, D16S539, D21S11, THO1 and TPOX). Others reflect loss or gain of alleles (D8S1179, D13S317 and FGA) or alterations in the number of repeats (D2S1338, D3S1358, D19S433 and vWA). At the D7S820 locus, the original Ishikawa isolate exhibits 8.3- and 11-repeat alleles, while subsequent sublines display 9- or 10-repeats. The D18S51 locus was found to be highly polymorphic in most Ishikawa lines.

Minor differences in the number of repeats at certain loci are consistent with the known microsatellite instability (MSI) of these lines, due to mutations in mismatch repair systems [46–48], and suggest that these variants arose by genetic drift between different clonal isolates over hundreds of cell passages. Accordingly, all Ishikawa cell lines exhibited high variability/instability at microsatellite loci (Table S2). Defective mismatch repair also underlies allelic variation in AN3CA cells (Table S3) [49]. In contrast, EnCa-101 tumors and MCF-7 cells were MSI stable.

We also profiled a variant of Ishikawa cells lacking ER [50]. Previous reports implied that these cells, also known as Ishikawa B, were derived from a different patient [51,52]. The STR profile of ER-negative Ishikawa cells exhibits minor variations from other Ishikawa sublines (Table 1), but overlap at the majority of loci indicates a common origin.

A second type 1, ER and PR positive cell line, ECC-1, was established from a grade 2, well-differentiated, endometrial carcinoma adenocarcinoma [42,53,54]. The line was derived by passage of the tumor, designated EnCa-101, in nude mice and subsequent isolation of PR positive cells from an epithelial monolayer culture [42,55]. ECC-1 cells were described as a well-differentiated, steroid responsive line with a phenotype characteristic of luminal surface epithelium, distinct from Ishikawa cells, which expressed markers of glandular endometrial epithelium [33].

Table 1
Summary of STR profiles of Ishikawa and ECC-1 endometrial cancer cells and EnCa-101 tumor.

Cell line	Amelogenin	CSF1PO	D2S1338	D3S1358	D5S818	D7S820	D8S1179	D13S317	D16S539	D18S51	D19S433	D21S11	FGA	THO1	TPOX	vWA
Ishikawa original	X	11, 12	18, 20	17, 18	10, 11	8.3, 11	12, 16	9, 12	9	14, (19) 20, 21 polymorphic	12.2, 14	28	21	9, 10	8	14, 18
Ishikawa '3-H-4'	X	11, 12	19, 20	16, 17	10, 11	9, 10	12, 16	9, 12	9	13, 21, 22	12.2, 14	28	21, 22	9	8	14, 17
Ishikawa 3-H-12	X	11, 12, (13)	19, 20	16, 17	10, 11	9, 10	12, 13, 16	9, 12, 13	9	12, 19, 20	13.2, 14	28	20, 21	9, 10	8	14, 17
Ishikawa 3-H-12	X	11, 12	20	16, 17, (18)	10, 11, (12)	9, 10	12, (13), 16	9, 12	9, (10)	13, 20	12.2, 14, (15)	28	21	9, 10 or 11	8	14, 17 or 18
Ishikawa ER -ve	X	11, 13	20	16, 17	10, 11	9, 10	12, 13, 16	9, 12	8, 9	13, 19	12.2, 14	28	20	9,10	8	14, 17
Ishikawa, ECACC, this report	X	11,12, (13)	20	15, 17	10, 11, 12	9, 10	12, 16	9, 12, 13	9	13, 19, (14, 20)	12.2, 14	28	21	9, 10	8	14, 17
Ishikawa, ECACC	X	11,12	NT	NT	10, 11	9, 10	NT	9, 12	9	NT	NT	NT	NT	9, 10	8	14, 17
ECC-1	X	11, 12	20	16, 17	10, 11	9, 10	13, 16	9, 12,	9	12, 19	12.2, 14 or 15	28	21	9, 10	8	14, 17
ECC-1 ATCC CRL-2923	X	11, 12	NT	NT	10, 11	9, 10	NT	9, 12	9	NT	NT	NT	NT	9, 10	8	14, 17
EnCa-101	X	13, 14	23, 27	15, 21	14, 15	11.3, 12	18, 21	10, 13	12, 13	16	13.2, 14 or 15	27, 30	21	9, 9.3	8	18, 23

Number of STRs at each of 16 surveyed loci. Numbers after decimal point indicate number of bases in an incomplete STR. Commas separate allele calls for multiple peaks. Alleles in parenthesis indicate low amplitude peaks suggesting only a minor fraction of the cells in the population carry that allele. ECACC: DNA profile from European Collection of Cell Cultures; ATCC: DNA profile from American Type Culture Collection. NT: locus not tested. X: only the amelogenin allele on the X chromosome was detected.

Upon STR and MSI analyses, ECC-1 samples exhibited DNA profiles essentially identical to Ishikawa 3-H-12 cells (Tables 1 and S2). In addition, the ATCC profile for ECC-1 also closely matched that of earlier Ishikawa cells on file with the European Collection of Cell Cultures (ECACC). Other 'ECC-1' cell lines were found to be identical to MCF-7 breast cancer cells or consist of a mixture of Ishikawa and MCF-7 cells (not shown). Unfortunately, following the death of Dr. Satyaswaroop, records and cell lines from his laboratory were lost or destroyed (Zaino, R. and Lessey, B., personal communication). Thus, we could not obtain reference samples of the original ECC-1 line or EnCa-101 tumor from which it was purportedly derived. However, the EnCa-101 tumor has been continuously maintained in mice [56] and we obtained and analyzed 3 independent samples. Profiling of these tumors showed minor variations, but results indicated that they were derived from the same human patient. In contrast, the unique EnCa-101 profiles did not match ECC-1, Ishikawa or MCF-7 cell lines (Table 1). These data are inconsistent with the reported origins of ECC-1 cells and suggest that the original line has been lost. Our results show that currently available ECC-1 cells are Ishikawa cells, MCF-7 breast cancer cells, or a mixture of both.

Sequencing of p53 mutations in endometrial cancer cells

To confirm the apparent equivalence of Ishikawa and ECC-1 cells, we screened for p53 mutations by PCR amplification and sequencing of the Variable Number Tandem Repeat (VNTR) region in intron 1, and the protein encoding exons and introns (Fig. S1). Table 2 lists the observed p53 mutations and SNPs compared to the reference/normal sequence.

In agreement with previous reports [31,57], Ishikawa original and 3-H-12 cells harbor a Met 246 Val mutation in exon 7. These two lines are also homozygous in the VNTR region with 8 repeats of A_4T, heterozygous in exon 4 for the Asp 49 Val mutation (nucleotide G12069S), and heterozygous in intron 10 for deletion of the seventh T in a heptanucleotide repeat (17822delT). The original Ishikawa sample has two additional heterozygous mutations, 12724insA (intron 4) and 13764delA (intron 6), which are not present in the 3-H-12 line (Table 2).

Possible '3-H-4' sublines have a similar profile, but lack the intronic 12724insA and 13764delA mutations of poly A stretches, present in the original Ishikawa lines (Table 2). An additional heterozygous mutation in intron 4 (G12299K (G + T)) was detected in some Ishikawa 3-H-12 sublines. Interestingly, consistent with their closely matched STR profiles, the ER-negative Ishikawa cells, despite their purported distinct origin, exhibit TP53 mutations identical to Ishikawa 3-H-12 and '3-H-4' (not shown). TP53 mutations unique to the original Ishikawa lines are insertions or deletions in homopolymer A or T stretches, which are consistent with microsatellite instability due to mutations in the mismatch repair system [46].

In agreement with their identical STR profiles, ECC-1 cells show the same TP53 mutations as Ishikawa 3-H-12 lines, further evidence that ECC-1 cells are misidentified Ishikawa cells. In contrast, EnCa-101 tumors have completely different TP53 mutations from the Ishikawa and ECC-1 lines (Table 2), again demonstrating that ECC-1 cells are not derived from the EnCa-101 tumor. 'ECC-1' cells shown to be contaminated with or identical to MCF-7 cells were not subjected to TP53 analysis.

Finally, our data suggest that only one copy of the p53 gene is expressed in Ishikawa cells. In the genomic DNA, both the A14063R (A + G) and G12069S (G + C) positions are heterozygous. However, only the 14063G mutation is present in the cDNA sequence [31,57], suggesting that the G12069C mutation is in the unexpressed copy of the gene.

Analysis of normal endometrial epithelial cells

Immortalized, non-transformed endometrial epithelial cells are a potentially valuable resource to investigate normal uterine physiology and tumorigenesis. We profiled two such lines, human endometrial (HES) cells [58] and hTERT-EEC [59], obtained from their developers, which have been extensively used as models of normal endometrium. Neither cell line was authenticated as they exhibited DNA profiles corresponding to HeLa and MCF-7 cancer cells, respectively.

HES cells were established, in 1989, from a primary culture of benign proliferative endometrium, which apparently underwent spontaneous transformation after serial passage [58,60]. Profiling of these cells

Table 2
Summary of TP53 mutations and single nucleotide polymorphisms (SNPs).

TP53 reference sequence	Ishikawa original	Ishikawa '3-H-4'	Ishikawa 3-H-12	Ishikawa 3-H-12	ECC-1	EnCa-101 tumor
Intron 1: VNTR A_4T repeats	Homozygous 8 repeats	Homozygous 8 repeats	Homozygous 8 repeats	Homozygous 8 repeats	Homozygous 8 repeats	Heterozygous 7 and 9 repeats
Exon 4: G12069 Asp 49	Heterozygous G12069S Asp49His	Heterozygous G12069S Asp49His	Heterozygous G12069S Asp49His	Heterozygous G12069S Asp49His	Heterozygous G12069S Asp49His	
Intron 4: G12299			Heterozygous G12299K			
Intron 4: Poly A_7 12718–12724	Heterozygous 12724insA Poly A_7/A_8					
Intron 5: G12786						Homozygous G12786T SNP
Intron 5: C13253						Heterozygous C13253Y SNP
Intron 6: G13642						Heterozygous G13462K SNP
Intron 6: Poly A_9 13756–13764	Heterozygous 13764delA Poly A_9/A_8					
Exon 7: A14063 Met246	Heterozygous A14063R Met246Val	Heterozygous A14063R Met246Val	Heterozygous A14063R Met246Val	Heterozygous A14063R Met246Val	Heterozygous A14063R Met246Val	
Intron 10: Poly T_7 17816–17822	Heterozygous 17822delT Poly T_7/T_6	Heterozygous 17822delT Poly T_7/T_6	Heterozygous 17822delT Poly T_7/T_6	Heterozygous 17822delT Poly T_7/T_6	Heterozygous 17822delT Poly T_7/T_6	Homozygous/Hemizygous Poly T_7

Tumor protein p53 (TP53) genomic DNA, from multiple independent samples of each cell line, was sequenced as described in the Materials and methods section. The normal reference normal is GenBank HSP53G, a.k.a. X54156, which is used by the International Agency for Research on Cancer IARC (http://www-p53.iarc.fr). A blank cell in the table indicates the DNA sequence that matches the reference/normal sequence. VNTR: Variable Number Tandem Repeat. Symbols — K: G and T; R: A and G; S:G and C; Y:C and T; del: nucleotide deletion; ins: nucleotide insertion.

(Table 3) indicated that they are identical at all loci to HeLa cervical carcinoma cells, specifically the HeLaS3 variant. HES cells are also identical to WISH cells, a cell line originally described as derived from human amnion [61] but subsequently also identified as HeLa [7,62,63]. These results were independently confirmed by the STR fragment analysis facility at Johns Hopkins University (D. Kniss, Ohio State University; personal communication).

hTERT-EECs were isolated from normal proliferative phase endometrial epithelium and immortalized by stable transfection with the catalytic subunit of human telomerase (hTERT) [59]. Replicate STR

Table 3
Summary of STR profiles of normal immortalized endometrial epithelial cells.

Cell line	Amelogenin	CSF1PO	D2S1338	D3S1358	D5S818	D7S820	D8S1179	D13S317	D16S539	D18S51	D19S433	D21S11	FGA	THO1	TPOX	vWA
hTERT-EEC-B37	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
hTERT-EEC-15	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
hTERT-EEC-17	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
hTERT-EEC-38	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
hTERT-EEC-49	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
MCF-7 (HTB-22) this report	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
MCF-7 NCI-60	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15
MCF-7 ATCC (HTB-22)	X	10	NT	NT	11, 12	8, 9	NT	11	11, 12	NT	NT	NT	NT	6	9, 12	14, 15
HES	X	9, 10	17	15, 18	11, 12	8, 12	12, 13	13.3	9, 10	16	13, 14	27, 28	21	7	8, 12	16, 18
HeLa this report	X	9, 10	17	15, 18	11, 12	8, 12	12, 13	12, 13.3	9, 10	16	13, 14	27, 28	18, 21	7	8, 12	16, 18
HeLa ATCC (CCL-2)	X	9, 10	NT	NT	11, 12	8, 12	NT	12, 13.3	9, 10	NT	NT	NT	NT	7	8, 12	16, 18
HeLaS3 ATCC (CCL-2.2)	X	9, 10	NT	NT	11, 12	8, 12	NT	13.3	9, 10	NT	NT	NT	NT	7	8, 12	16, 18
WISH ATCC (CCL-25)	X	9, 10	NT	NT	11, 12	8, 12	NT	13.3	9, 10	NT	NT	NT	NT	7	8, 12	16, 18

Number of STRs at each of 16 surveyed loci. Numbers after decimal point indicate number of bases in an incomplete STR. Commas separate allele calls for multiple peaks. NT: not tested. Numbers following hTERT-ECC indicate clones. Samples were analyzed in duplicate independent reactions. MCF-7 breast cancer cells reference STR profiles from ATCC (HTB-22) and NCI-60 panel [25]. HeLa and WISH reference profiles from ATCC database. X: only the amelogenin allele on the X chromosome was detected.

profiling of the earliest available passages of multiple clonal lines indicated all isolates of hTERT-EEC cells to be genetically identical to MCF-7 breast cancer cells (Table 3). As for HES cells, this was not attributable to contamination as no other profiles were detected in the samples.

Analysis of ovarian cancer cell lines

We obtained and genotyped fifty-one ovarian cancer cell lines (Table S1), many of which are not available from public repositories. Two of the lines (IGROV1 and OVCAR-10) gave mixed genotypes indicating cross-contamination and were excluded from further analysis. The mixed genotype for IGROV1 was confirmed in multiple isolates including those obtained directly from the National Cancer Institute.

Several purported 'ovarian cancer' lines were genotypically identical to other known, non-ovarian, cancer cells: BG-1[64] was identified as MCF-7 breast cancer cells, and CH1, CH1cisR, and 222 as the teratocarcinoma line PA1. C13, A2008 and OV2008 were identical to the ME-180 (ATCC: HTB-33) cervical cancer cell line, and confirmed to be HPV positive (Table 4). The genotypically distinct 2008 cell line [65], obtained directly from the originating laboratory of Dr. Peter Disaia [66], was HPV negative. Finally, SK-OV-4 and SK-OV-6 lines matched HPV-negative C-33A (HTB-31) cervical cancer cells (Table 4).

Two 'normal ovarian' cell lines, NOSE06 and NOSE07, were genotyped as the ovarian cancer line DOV-13. Similarly, Caov-2 was identical to the earlier NIH:OVCAR-2 line (Table S4) and some samples of COLO-720E were found to be COLO-704 (not shown). Ovary1847 cells were genotyped as NIH:OVCAR-8.

The remaining ovarian cancer cell lines exhibited unique, uncontaminated genotypes and are listed with their STR profiles in Table S4.

We noted disparate genotypes for several cell lines with similar names; 2008 cells are distinct from A2008 and OV2008, and 167 differs from OV167 cells. In contrast, the TOV-112D cell line is identical to TOV-21D, which appears to have arisen via transposition of numbers and letters in the name. Some isolates of TOV-112D were misidentified and matched TOV-21G cells.

The heterogeneity of ovarian tumor cells in ascitic fluid has previously lead to the establishment of several cell lines with different phenotypic

Table 4
STR profiles of cervical and other cancer cell lines misclassified as ovarian.

Cell line	Amelogenin	CSF1PO	D2S1338	D3S1358	D5S818	D7S820	D8S1179	D13S317	D16S539	D18S51	D19S433	D21S11	FGA	THO1	TPOX	vWA	HPV
A2008	X	11	18	16	12	9, 10	14	11, 12	12, 13	12	13, 15.2	30, 31	23	8, 9.3	8, 10	15, 17	+
C13	X	11	18	16	12	9	14	11, 12	12, 13	12	15.2	30, 31	23	8, 9.3	8, 10	15, 17	NT
ME-180	X	11	18	16	12	9, 10	14	11, 12	12, 13	12	13, 15.2	30, 31	23	8, 9.3	8, 10	15, 17	NT
OV2008	X	11	18	16	12	9, 10	14	11, 12	12, 13	12	13, 15.2	30, 31	23	8, 9.3	8, 10	15, 17	+
ME-180 ATCC (HTB-33)	X	11	NT	NT	12	9, 10	NT	11, 13	12, 13	NT	NT	NT	NT	8, 9.3	8, 10	15, 17	+
SKOV4	X	12	23, 25	16	11, 12	10	10, 14	13	13, 14	15, (17), 18	11, 13	29, 31, 32	21, 26	7, 8	9	18, 20 (19)	NT
SKOV6	X	12	23, 25	16	11, 12	10	10, 14	13	13, 14	15, (17),18	11, 13	29, 30, 31, 32	21, 26	7, 8	9	18, 20	NT
C-33 A	X	12	23, 25	16	11, 12	10	10, 14	13	13, 14	15, (17), 18	11, 13, 14	29, 30, 31	21, 26	7, 8	9	18, 20	NT
C-33 A ATCC (HTB-31)	X	12	NT	NT	11, 12	10	NT	13	13, 14	NT	NT	NT	NT	7, 8	9	18, 20	−
BG-1	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 24, 25	6	9, 12	14, 15	NT
MCF-7 NCI-60	X	10	21, 23	16	11, 12	8, 9	10, 14	11	11, 12	14	13, 14	30	23, 25	6	9, 12	14, 15	NT
MCF-7 ATCC (HTB-22)	X	10	NT	NT	11, 12	8, 9	NT	11	11, 12	NT	NT	NT	NT	6	9, 12	14, 15	NT
CH1	X	9, 12, 13	24	15	11	9	14, 15	9, 10	9, 12	15, 18	13	29, 31.2	24	7, 9	11	15, 17	NT
CH1-cisR	X	9, 13	24	15	11	9	14, 15	9, 10	9, 12	15, 18	13	29, 31.2	24	7, 9	11	15, 17	NT
222	X	9, 13	24	15	11	9	14, 15	9, 10	9, 12	15, 18	13	29, 31.2	24	7, 9	11	15, 17	NT
PA-1 JCRB (9061)	X	9, 12	NT	NT	11	9	NT	9, 10	9, 12	NT	NT	NT	NT	7, 9	11	15, 17	NT
NOSE06	X	8, 10	20, 24	14, 16	11	10	14	11	10, 13	12, 16	13, 14	32.2, 33.2	21, 24	6, 9.3	6, 8	19	NT
NOSE07	X	8, 10	20, 24	14, 16	11	10	14	11	10, 13	12, 16	13, 14	32.2, 33.2	21, 24	6, 9.3	6, 8	19	NT
DOV-13	X	8, 10	20, 24	14, 16	11	10	14	11	10, 13	12, 16	13, 14	32.2, 33.2	21, 24	6, 9.3	6, 8	19	NT

Number of STRs at each of 16 surveyed loci. Numbers after decimal point indicate number of bases in an incomplete STR. Commas separate allele calls for multiple peaks. Alleles in parentheses indicate low amplitude peaks suggesting only a minor fraction of the cells in the population carry that allele. NT: allele not tested. ATCC is a reference DNA profile from the American Type Culture Collection. HPV: human papilloma virus status (+: positive; −: negative). MCF-7 breast cancer cells reference STR profiles from ATCC (HTB-22) and NCI-60 panel. X: only the amelogenin allele on the X chromosome was detected.

characteristics [67]. We profiled very early passages of OV429 and OV433 [68,69] and found identical genotypes, indicative of either a common patient origin or early cross-contamination (Table S4). Of historical note, OV433 was the cell line used originally to select for reactivity to the OC125 monoclonal antibody to the ovarian tumor marker CA125.

The cluster of PEO1/PEO4/PEO6 cells is known to originate from the same patient [70], and genotype accordingly. Similarly, HEY/HEYA8/HEYC2 cells [71] are derived from the same original line, and share identical genotypes (Table S4).

Chemotherapy resistant derivatives mirror parental cell line genotypes

We tested five original and cisplatin-resistant paired cell lines and all five parent and derivative combinations were confirmed by genotyping. However, as shown earlier (Table 4), the OV2008/C13 cells are cervical, not ovarian cancer cells and the CH1/CH1cisR lines [72] are PA1 teratocarcinoma cells. Table S5 shows STR profiles of the matched cisplatin-sensitive/-resistant ovarian cancer cell lines. The 41M/41McisR, TYKnu/TYKnucisR and A2780/A2780cisR pairs each have unique profiles. The paired lines demonstrate some genetic instability, consistent with cisplatin-induced MSI [73]. Cisplatin-resistant A2780 cells have lost alleles at the D3S1358, FGA, D8S1179. D5S818, D7S820, CSF1PO, and D2S1338 loci, and gained an allele at the D18S51 locus. The 41M/41McisR pair is more stable, with the cisplatin-resistant line differing only at the vWA locus. The original derivation of the 41M cisplatin-resistant lines lists three isolates (41McisR2, 41McisR4 and 41McisR6), which differed in their IC_{50} [74]. The subline profiled herein is unknown, as the identifying number has been lost.

Discussion

Gynecologic cancer research is critically dependent on the use of cell culture models, to investigate molecular mechanisms underlying the development and progression of tumors, to design and test novel therapeutic strategies, and to identify potential diagnostic or prognostic markers. In this report, we profiled the most widely used endometrial and ovarian cell lines and discovered several examples of misidentification, redundancy and cross-contamination.

Genotyping and HPV testing of ovarian cancer cell lines identified eight (BG-1 [64], CH1/CH1cisR [72], 222 [75], C13 [76], A2008 [77,78], OV2008, SKOV-4 and SKOV-6 [79]) as previously existing, breast cancer, teratocarcinoma or cervical cancer cell lines. In addition, two 'normal ovarian' cell lines, NOSE06 and NOSE07 [80], were genotyped as the ovarian cancer line DOV-13 [81]. We also highlight the possibility for confusion of several ovarian cancer cell lines with similar names, but distinct genotypes; e.g. 167 and OV167, 2008 and A2008/OV2008.

We profiled a number of variants of Ishikawa endometrial cancer cells. Results are consistent with a common origin for these sublines, with variations and polymorphisms in some STR loci attributable to genetic instability, mismatch repair defects, and high passage number [75–77]. Analyses of mutations in the p53 gene (TP53) are consistent with previous reports [31,57] and provide additional genetic markers to perhaps distinguish the original, 3-H-4 and 3-H-12 Ishikawa lines. Furthermore, STR profiling, TP53 sequencing, and MSI analysis confirm that currently available isolates of ECC-1 cells are not authentic but are identical to Ishikawa cells, specifically the 3-H-12 line. This conclusion is reinforced by evidence that the EnCa-101 tumor, from which the original EEC-1 line was purportedly derived [42,55], is genetically distinct from both Ishikawa and ECC-1 cells. We also observed several ECC-1 isolates to be misidentified MCF-7 cells or a cross-contaminated mixture of Ishikawa and MCF-7 lines.

ECC-1 cells were initially characterized as distinct from Ishikawa lines based on differential expression of cytokeratin 13 and osteopontin [33]. However, both markers were present in the two lines, which otherwise showed identical patterns of expression of steroid hormone receptors and their coactivators [33]. The karyotypes of Ishikawa and ECC-1 cells also exhibit some apparent differences [31,33], but chromosomal number and structural rearrangements in both lines were complex with high intercellular variability [31,33]. Comparative cytogenetic analysis found that, given the evident heterogeneity and differential capabilities of the techniques used (FISH or SKY) to detect abnormalities in small chromosomal segments, the karyotypic similarity was likely underestimated, and is consistent with the two lines sharing a common origin.

Thus, we conclude that the original ECC-1 cell line has been lost, although the persistence of the EnCa-101 tumor [56] provides an opportunity for its re-derivation. ECC-1 cells have been extensively used as models of ER positive, type 1, endometrial cancers. Since Ishikawa cells are also representative of such endometrioid tumors, our evidence that the two lines are identical may not significantly impact conclusions drawn from these studies, beyond the use of two redundant cell lines. However, the possible misidentification of MCF-7 breast cancer cells as ECC-1, or cross contamination with the former, should be considered in interpreting results using ECC-1 cells.

We identified the normal endometrial epithelial cell line (HES) as HeLa cervical carcinoma cells. HES cells have been used as a model of benign endometrial epithelium to study mucosal immunity [82], implantation [83,84], decidualization [85] and endometriosis [86], and have served as 'normal' controls for novel chemotherapeutics [87,88] and analysis of signaling pathways in the endometrium [89–93]. Similarly, the telomerase immortalized endometrial epithelial cell line, hTERT-EEC [59], was an exact genotypic match to MCF-7 breast cancer cells. hTERT-EEC has been proposed as model to study steroids in normal endometrial physiology, including, endometriosis and implantation [59,94,95]. Clearly, conclusions derived from studies utilizing HES cells (HeLa) or hTERT-EEC (MCF-7) should be interpreted with caution, in the light of evidence that they are neither normal nor endometrial in origin.

Cell line authentication is essential for their meaningful use in research. We recommend that cell lines be quarantined and authenticated by DNA profiling prior to use, and periodically evaluated by STR genotype, to check for cross-contamination and validate construction of stably transfected, genetically modified or clonally selected variants. Derivation of novel cell lines should be accompanied, where possible, by STR profiles of the patient germ line, tumor or tissue, and cell line DNA. We also suggest the use of histological or phenotypic markers to verify the tissue of origin, since STR profiling cannot provide this information resulting in debate as to the tissue type of some cancer cell lines [2,96].

The origins and mechanisms of cell line contamination, including poor tissue culture technique, inadequate quality control, clerical and labeling errors, and aerosol transfer of cells, have been reviewed previously [63] and, despite best laboratory practices, are probably unavoidable. Accordingly, even among cell lines that exhibited unique profiles, we found examples, from all sources, of individual aliquots that were misidentified or contaminated, indicating a widespread and pervasive problem. STR profiling is a simple, widely available and relatively inexpensive method to document and authenticate cell lines, and has been recommended as an internationally accepted standard for human cells [22,63,97,98]. Despite repeated calls for journals to require DNA profiling of cells for publication, this practice has not been widely adopted [63,99]. Complacency and denial of the existence and extent of the problem with validation and authenticity of cell lines, while prevalent [7,24,63,99], are antithetical to the conduct of responsible research in gynecologic oncology.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygyno.2012.06.017.

Conflict of interest statement

No conflict of interest.

Acknowledgments

We are grateful to the investigators who generously provided cell lines for analysis. STR profiling and sequence analysis were carried out in the University of Colorado Cancer Center (UCCC) DNA Sequencing and Analyses Core. We thank Dr. Marileila Varella Garcia of the UCCC Molecular Pathology Shared Resource Cytogenetics Core (NCI-P30CA046934) for karyotype comparison and analysis and Jerry Haney for MSI assays. This work was funded in part by NCI CA125427, NICHD HD067721, Lombardi Comprehensive Cancer Center support grant NIH P30 CA051008, Department of Defense grant W81XWH-11-1-0469, Cancer League of Colorado, Ovarian Cancer Research Fund, Foundation for Women's Cancer, Adelson Family Cancer Foundation and University of Colorado Department of Obstetrics and Gynecology.

References

[1] Burdall SE, Hanby AM, Lansdown MR, Speirs V. Breast cancer cell lines: friend or foe? Breast Cancer Res 2003;5:89–95.
[2] Schweppe RE, Klopper JP, Korch C, Pugazhenthi U, Benezra M, Knauf JA, Fagin JA, Marlow LA, Copland JA, Smallridge RC, Haugen BR. Deoxyribonucleic acid profiling analysis of 40 human thyroid cancer cell lines reveals cross-contamination resulting in cell line redundancy and misidentification. J Clin Endocrinol Metab 2008;93:4331–41.
[3] Vollmer G. Endometrial cancer: experimental models useful for studies on molecular aspects of endometrial cancer and carcinogenesis. Endocr Relat Cancer 2003;10:23–42.
[4] Lucey BP, Nelson-Rees WA, Hutchins GM. Henrietta Lacks, HeLa cells, and cell culture contamination. Arch Pathol Lab Med 2009;133:1463–7.
[5] MacLeod RA, Dirks WG, Matsuo Y, Kaufmann M, Milch H, Drexler HG. Widespread intraspecies cross-contamination of human tumor cell lines arising at source. Int J Cancer 1999;83:555–63.
[6] McCormick D. Sleeper cell. Biotechniques 2008;45:11.
[7] Masters JR. HeLa cells 50 years on: the good, the bad and the ugly. Nat Rev Cancer 2002;2:315–9.
[8] Identity crisis. Nature 2009;457:935–6.
[9] Chatterjee R. Cell biology. When 60 lines don't add up. Science 2007;315:929.
[10] Chatterjee R. Cell biology. Cases of mistaken identity. Science 2007;315:928–31.
[11] Freshney RI. Authentication of cell lines: ignore at your peril! Expert Rev Anticancer Ther 2008;8:311–4.
[12] Hughes P, Marshall D, Reid Y, Parkes H, Gelber C. The costs of using unauthenticated, over-passaged cell lines: how much more data do we need? Biotechniques 2007;43:575 [577–8, 581–2 passim.].
[13] Nelson-Rees WA, Daniels DW, Flandermeyer RR. Cross-contamination of cells in culture. Science 1981;212:446–52.
[14] Rae JM, Creighton CJ, Meck JM, Haddad BR, Johnson MD. MDA-MB-435 cells are derived from M14 melanoma cells—a loss for breast cancer, but a boon for melanoma research. Breast Cancer Res Treat 2007;104:13–9.
[15] Capes-Davis A, Theodosopoulos G, Atkin I, Drexler HG, Kohara A, MacLeod RA, Masters JR, Nakamura Y, Reid YA, Reddel RR, Freshney RI. Check your cultures! A list of cross-contaminated or misidentified cell lines. Int J Cancer 2010;127:1–8.
[16] Lacroix M. Persistent use of "false" cell lines. Int J Cancer 2008;122:1–4.
[17] van Bokhoven A, Varella-Garcia M, Korch C, Hessels D, Miller GJ. Widely used prostate carcinoma cell lines share common origins. Prostate 2001;47:36–51.
[18] van Bokhoven A, Varella-Garcia M, Korch C, Johannes WU, Smith EE, Miller HL, Nordeen SK, Miller GJ, Lucia MS. Molecular characterization of human prostate carcinoma cell lines. Prostate 2003;57:205–25.
[19] van Bokhoven A, Varella-Garcia M, Korch C, Miller GJ. TSU-Pr1 and JCA-1 cells are derivatives of T24 bladder carcinoma cells and are not of prostatic origin. Cancer Res 2001;61:6340–4.
[20] Dirks WG, Drexler HG. Online verification of human cell line identity by STR DNA typing. Methods Mol Biol 2011;731:45–55.
[21] Dirks WG, Faehnrich S, Estella IA, Drexler HG. Short tandem repeat DNA typing provides an international reference standard for authentication of human cell lines. ALTEX 2005;22:103–9.
[22] Masters JR, Thomson JA, Daly-Burns B, Reid YA, Dirks WG, Packer P, Toji LH, Ohno T, Tanabe H, Arlett CF, Kelland LR, Harrison M, Virmani A, Ward TH, Ayres KL, Debenham PG. Short tandem repeat profiling provides an international reference standard for human cell lines. Proc Natl Acad Sci U S A 2001;98:8012–7.
[23] Parson W, Kirchebner R, Muhlmann R, Renner K, Kofler A, Schmidt S, Kofler R. Cancer cell line identification by short tandem repeat profiling: power and limitations. FASEB J 2005;19:434–6.
[24] Cell line misidentification. the beginning of the end. Nat Rev Cancer 2010;10:441–8.
[25] Lorenzi PL, Reinhold WC, Varma S, Hutchinson AA, Pommier Y, Chanock SJ, Weinstein JN. DNA fingerprinting of the NCI-60 cell line panel. Mol Cancer Ther 2009;8:713–24.
[26] Mouchawar J, Korch C, Byers T, Pitts TM, Li E, McCredie MR, Giles GG, Hopper JL, Southey MC. Population-based estimate of the contribution of TP53 mutations to subgroups of early-onset breast cancer: Australian Breast Cancer Family Study. Cancer Res 2010;70:4795–800.
[27] Gologan A, Sepulveda AR. Microsatellite instability and DNA mismatch repair deficiency testing in hereditary and sporadic gastrointestinal cancers. Clin Lab Med 2005;25:179–96.
[28] Hecht JL, Mutter GL. Molecular and pathologic aspects of endometrial carcinogenesis. J Clin Oncol 2006;24:4783–91.
[29] Lax SF. Molecular genetic pathways in various types of endometrial carcinoma: from a phenotypical to a molecular-based classification. Virchows Arch 2004;444:213–23.
[30] Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E, Vergote I. Endometrial cancer. Lancet 2005;366:491–505.
[31] Albitar L, Pickett G, Morgan M, Davies S, Leslie KK. Models representing type I and type II human endometrial cancers: Ishikawa H and Hec50co cells. Gynecol Oncol 2007;106:52–64.
[32] Kuramoto H, Hamano M, Imai M. HEC-1 cells. Hum Cell 2002;15:81–95.
[33] Mo B, Vendrov AE, Palomino WA, DuPont BR, Apparao KB, Lessey BA. ECC-1 cells: a well-differentiated steroid-responsive endometrial cell line with characteristics of luminal epithelium. Biol Reprod 2006;75:387–94.
[34] Richardson GS, Dickersin GR, Atkins L, MacLaughlin DT, Raam S, Merk LP, Bradley FM. KLE: a cell line with defective estrogen receptor derived from undifferentiated endometrial cancer. Gynecol Oncol 1984;17:213–30.
[35] Way DL, Grosso DS, Davis JR, Surwit EA, Christian CD. Characterization of a new human endometrial carcinoma (RL95-2) established in tissue culture. In Vitro 1983;19:147–58.
[36] Kuramoto H, Tamura S, Notake Y. Establishment of a cell line of human endometrial adenocarcinoma in vitro. Am J Obstet Gynecol 1972;114:1012–9.
[37] Kuramoto H. Studies of the growth and cytogenetic properties of human endometrial adenocarcinoma in culture and its development into an established line. Acta Obstet Gynaecol Jpn 1972;19:47–58.
[38] Kuramoto H, Nishida M, Morisawa T, Hamano M, Hata H, Kato Y, Ohno E, Iida T. Establishment and characterization of human endometrial cancer cell lines. Ann N Y Acad Sci 1991;622:402–21.
[39] Satyaswaroop PG, Fleming H, Bressler RS, Gurpide E. Human endometrial cancer cell cultures for hormonal studies. Cancer Res 1978;38:4367–75.
[40] Kuramoto H, Suzuki K. Effects of progesterone on the growth kinetics and the morphology of a human endometrial cancer cell-line. Acta Obstet Gynaecol Jpn 1976;23:123–32.
[41] Dawe CJ, Banfield WG, Morgan WD, Slatick MS, Curth HO. Growth in continuous culture, and in hamsters, of cells from a neoplasm associated with acanthosis nigricans. J Natl Cancer Inst 1964;33:441–56.
[42] Satyaswaroop PG. Endometrial cancer. In: Masters JRW, Palsson B, editors. Human cell culture. Kluwer Academic Publishers; 1999. p. 71–8.
[43] Nishida M, Kasahara K, Kaneko M, Iwasaki H, Hayashi K. Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors. Nihon Sanka Fujinka Gakkai Zasshi 1985;37:1103–11.
[44] Nishida M, Kasahara K, Oki A, Satoh T, Arai Y, Kubo T. Establishment of eighteen clones of Ishikawa cells. Hum Cell 1996;9:109–16.
[45] Nishida M. The Ishikawa cells from birth to the present. Hum Cell 2002;15:104–17.
[46] Lewis KA, Mullany S, Thomas B, Chien J, Loewen R, Shridhar V, Cliby WA. Heterozygous ATR mutations in mismatch repair-deficient cancer cells have functional significance. Cancer Res 2005;65:7091–5.
[47] Inoue M. Current molecular aspects of the carcinogenesis of the uterine endometrium. Int J Gynecol Cancer 2001;11:339–48.
[48] Risinger JI, Berchuck A, Kohler MF, Watson P, Lynch HT, Boyd J. Genetic instability of microsatellites in endometrial carcinoma. Cancer Res 1993;53:5100–3.
[49] Umar A, Boyer JC, Thomas DC, Nguyen DC, Risinger JI, Boyd J, Ionov Y, Perucho M, Kunkel TA. Defective mismatch repair in extracts of colorectal and endometrial cancer cell lines exhibiting microsatellite instability. J Biol Chem 1994;269:14367–70.
[50] Ignar-Trowbridge DM, Teng CT, Ross KA, Parker MG, Korach KS, McLachlan JA. Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. Mol Endocrinol 1993;7:992–8.
[51] Collins F, MacPherson S, Brown P, Bombail V, Williams AR, Anderson RA, Jabbour HN, Saunders PT. Expression of oestrogen receptors, ERalpha, ERbeta, and ERbeta variants, in endometrial cancers and evidence that prostaglandin F may play a role in regulating expression of ERalpha. BMC Cancer 2009;9:330.
[52] Wenger SL, Senft JR, Sargent LM, Bamezai R, Bairwa N, Grant SG. Comparison of established cell lines at different passages by karyotype and comparative genomic hybridization. Biosci Rep 2004;24:631–9.
[53] Clarke CL, Feil PD, Satyaswaroop PG. Progesterone receptor regulation by 17 beta-estradiol in human endometrial carcinoma grown in nude mice. Endocrinology 1987;121:1642–8.
[54] Satyaswaroop PG, Sivarajah A, Zaino RJ. Hormonal control of growth of human endometrial carcinoma in nude mouse model. In: Brescani F, King RG, Lippman ME, editors. Progress in cancer research and therapy. New York: Raven Press; 1988. p. 430–55.
[55] Satyaswaroop PG, Tabibzadeh SS. Extracellular matrix and the patterns of differentiation of human endometrial carcinomas in vitro and in vivo. Cancer Res 1991;51:5661–6.
[56] Patel RR, Sengupta S, Kim HR, Klein-Szanto AJ, Pyle JR, Zhu F, Li T, Ross EA, Oseni S, Fargnoli J, Jordan VC. Experimental treatment of oestrogen receptor (ER) positive breast cancer with tamoxifen and brivanib alaninate, a VEGFR-2/FGFR-1 kinase inhibitor: a potential clinical application of angiogenesis inhibitors. Eur J Cancer 2010;46:1537–53.

[57] Yaginuma Y, Westphal H. Analysis of the p53 gene in human uterine carcinoma cell lines. Cancer Res 1991;51:6506–9.
[58] Desai NN, Kennard EA, Kniss DA, Friedman CI. Novel human endometrial cell line promotes blastocyst development. Fertil Steril 1994;61:760–6.
[59] Hombach-Klonisch S, Kehlen A, Fowler PA, Huppertz B, Jugert JF, Bischoff G, Schluter E, Buchmann J, Klonisch T. Regulation of functional steroid receptors and ligand-induced responses in telomerase-immortalized human endometrial epithelial cells. J Mol Endocrinol 2005;34:517–34.
[60] Kniss DA, Zimmerman PD, Garver CL, Fertel RH. Interleukin-1 receptor antagonist blocks interleukin-1-induced expression of cyclooxygenase-2 in endometrium. Am J Obstet Gynecol 1997;177:559–67.
[61] Hayflick L. The establishment of a line (WISH) of human amnion cells in continuous cultivation. Exp Cell Res 1961;23:14–20.
[62] Kniss DA, Xie Y, Li Y, Kumar S, Linton EA, Cohen P, Fan-Havard P, Redman CW, Sargent IL. ED(27) trophoblast-like cells isolated from first-trimester chorionic villi are genetically identical to HeLa cells yet exhibit a distinct phenotype. Placenta 2002;23:32–43.
[63] Masters JR. False cell lines: the problem and a solution. Cytotechnology 2002;39: 69–74.
[64] Geisinger KR, Kute TE, Pettenati MJ, Welander CE, Dennard Y, Collins LA, Berens ME. Characterization of a human ovarian carcinoma cell line with estrogen and progesterone receptors. Cancer 1989;63:280–8.
[65] DiSaia PJ, Sinkovics JG, Rutledge FN, Smith JP. Cell-mediated immunity to human malignant cells. A brief review and further studies with two gynecologic tumors. Am J Obstet Gynecol 1972;114:979–89.
[66] Orth K, Hung J, Gazdar A, Bowcock A, Mathis JM, Sambrook J. Genetic instability in human ovarian cancer cell lines. Proc Natl Acad Sci U S A 1994;91:9495–9.
[67] Woods LK, Morgan RT, Quinn LA, Moore GE, Semple TU, Stedman KE. Comparison of four new cell lines from patients with adenocarcinoma of the ovary. Cancer Res 1979;39:4449–59.
[68] Bast Jr RC, Feeney M, Lazarus H, Nadler LM, Colvin RB, Knapp RC. Reactivity of a monoclonal antibody with human ovarian carcinoma. J Clin Invest 1981;68:1331–7.
[69] Berkowitz R, Kabawat S, Lazarus H, Colvin R, Knapp R, Bast Jr RC. Comparison of a rabbit heteroantiserum and a murine monoclonal antibody raised against a human epithelial ovarian carcinoma cell line. Am J Obstet Gynecol 1983;146:607–12.
[70] Langdon SP, Lawrie SS, Hay FG, Hawkes MM, McDonald A, Hayward IP, Schol DJ, Hilgers J, Leonard RC, Smyth JF. Characterization and properties of nine human ovarian adenocarcinoma cell lines. Cancer Res 1988;48:6166–72.
[71] Buick RN, Pullano R, Trent JM. Comparative properties of five human ovarian adenocarcinoma cell lines. Cancer Res 1985;45:3668–76.
[72] Hills CA, Kelland LR, Abel G, Siracky J, Wilson AP, Harrap KR. Biological properties of ten human ovarian carcinoma cell lines: calibration in vitro against four platinum complexes. Br J Cancer 1989;59:527–34.
[73] Watanabe Y, Koi M, Hemmi H, Hoshai H, Noda K. A change in microsatellite instability caused by cisplatin-based chemotherapy of ovarian cancer. Br J Cancer 2001;85:1064–9.
[74] Loh SY, Mistry P, Kelland LR, Abel G, Harrap KR. Reduced drug accumulation as a major mechanism of acquired resistance to cisplatin in a human ovarian carcinoma cell line: circumvention studies using novel platinum (II) and (IV) ammine/amine complexes. Br J Cancer 1992;66:1109–15.
[75] Nio Y, Zighelboim J, Berek JS, Bonavida B. Sensitivity of fresh and cultured ovarian tumor cells to tumor necrosis factor, interferon-alpha 2, and OK-432. Cancer Immunol Immunother 1988;27:246–54.
[76] Andrews PA, Murphy MP, Howell SB. Differential potentiation of alkylating and platinating agent cytotoxicity in human ovarian carcinoma cells by glutathione depletion. Cancer Res 1985;45:6250–3.
[77] Eva A, Robbins KC, Andersen PR, Srinivasan A, Tronick SR, Reddy EP, Ellmore NW, Galen AT, Lautenberger JA, Papas TS, Westin EH, Wong-Staal F, Gallo RC, Aaronson SA. Cellular genes analogous to retroviral onc genes are transcribed in human tumour cells. Nature 1982;295:116–9.
[78] Ozols RF, Hogan WM, Young RC. Direct cloning of human ovarian cancer in soft agar: clinical limitations and pharmacologic applications. Recent Results Cancer Res 1984;94:41–50.
[79] Yuan Y, Kim WH, Han HS, Lee JH, Park HS, Chung JK, Kang SB, Park JG. Establishment and characterization of human ovarian carcinoma cell lines. Gynecol Oncol 1997;66:378–87.
[80] Berchuck A, Rodriguez G, Olt G, Whitaker R, Boente MP, Arrick BA, Clarke-Pearson DL, Bast Jr RC. Regulation of growth of normal ovarian epithelial cells and ovarian cancer cell lines by transforming growth factor-beta. Am J Obstet Gynecol 1992;166:676–84.
[81] Young TN, Rodriguez GC, Moser TL, Bast Jr RC, Pizzo SV, Stack MS. Coordinate expression of urinary-type plasminogen activator and its receptor accompanies malignant transformation of the ovarian surface epithelium. Am J Obstet Gynecol 1994;170:1285–96.
[82] King AE, Fleming DC, Critchley HO, Kelly RW. Regulation of natural antibiotic expression by inflammatory mediators and mimics of infection in human endometrial epithelial cells. Mol Hum Reprod 2002;8:341–9.
[83] Banerjee P, Sapru K, Strakova Z, Fazleabas AT. Chorionic gonadotropin regulates prostaglandin E synthase via a phosphatidylinositol 3-kinase-extracellular regulatory kinase pathway in a human endometrial epithelial cell line: implications for endometrial responses for embryo implantation. Endocrinology 2009;150:4326–37.
[84] Wang P, Julian JA, Carson DD. The MUC1 HMFG1 glycoform is a precursor to the 214D4 glycoform in the human uterine epithelial cell line, HES. Biol Reprod 2008;78:290–8.
[85] Menkhorst E, Salamonsen L, Robb L, Dimitriadis E. IL11 antagonist inhibits uterine stromal differentiation, causing pregnancy failure in mice. Biol Reprod 2009;80: 920–7.
[86] Bukulmez O, Hardy DB, Carr BR, Auchus RJ, Toloubeydokhti T, Word RA, Mendelson CR. Androstenedione up-regulation of endometrial aromatase expression via local conversion to estrogen: potential relevance to the pathogenesis of endometriosis. J Clin Endocrinol Metab 2008;93:3471–7.
[87] Cho JH, Lee JG, Yang YI, Kim JH, Ahn JH, Baek NI, Lee KT, Choi JH. Eupatilin, a dietary flavonoid, induces G2/M cell cycle arrest in human endometrial cancer cells. Food Chem Toxicol 2011;49:1737–44.
[88] Kim JH, Yang YI, Lee KT, Park HJ, Choi JH. Costunolide induces apoptosis in human endometriotic cells through inhibition of the prosurvival Akt and nuclear factor kappa B signaling pathway. Biol Pharm Bull 2011;34:580–5.
[89] Khorram O, Han G, Magee T. Cigarette smoke inhibits endometrial epithelial cell proliferation through a nitric oxide-mediated pathway. Fertil Steril 2010;93:257–63.
[90] Carino C, Olawaiye AB, Cherfils S, Serikawa T, Lynch MP, Rueda BR, Gonzalez RR. Leptin regulation of proangiogenic molecules in benign and cancerous endometrial cells. Int J Cancer 2008;123:2782–90.
[91] Jabbour HN, Boddy SC. Prostaglandin E2 induces proliferation of glandular epithelial cells of the human endometrium via extracellular regulated kinase 1/2-mediated pathway. J Clin Endocrinol Metab 2003;88:4481–7.
[92] Luo X, Xu J, Chegini N. Gonadotropin releasing hormone analogue (GnRHa) alters the expression and activation of Smad in human endometrial epithelial and stromal cells. Reprod Biol Endocrinol 2003;1:125.
[93] Luo X, Xu J, Chegini N. The expression of Smads in human endometrium and regulation and induction in endometrial epithelial and stromal cells by transforming growth factor-beta. J Clin Endocrinol Metab 2003;88:4967–76.
[94] Aboussahoud W, Aflatoonian R, Bruce C, Elliott S, Ward J, Newton S, Hombach-Klonisch S, Klonisch T, Fazeli A. Expression and function of Toll-like receptors in human endometrial epithelial cell lines. J Reprod Immunol 2010;84:41–51.
[95] Willing C, Peich M, Danescu A, Kehlen A, Fowler PA, Hombach-Klonisch S. Estrogen-independent actions of environmentally relevant AhR-agonists in human endometrial epithelial cells. Mol Hum Reprod 2011;17:115–26.
[96] Chambers AF. MDA-MB-435 and M14 cell lines: identical but not M14 melanoma? Cancer Res 2009;69:5292–3.
[97] O'Brien SJ. Cell culture forensics. Proc Natl Acad Sci U S A 2001;98:7656–8.
[98] Barallon R, Bauer SR, Butler J, Capes-Davis A, Dirks WG, Elmore E, Furtado M, Kline MC, Kohara A, Los GV, MacLeod RA, Masters JR, Nardone M, Nardone RM, Nims RW, Price PJ, Reid YA, Shewale J, Sykes G, Steuer AF, Storts DR, Thomson J, Taraporewala Z, Alston-Roberts C, Kerrigan L. Recommendation of short tandem repeat profiling for authenticating human cell lines, stem cells, and tissues. In Vitro Cell Dev Biol Anim 2010;46: 727–32.
[99] Katsnelson A. Biologists tackle cells' identity crisis. Nature 2010;465:537.

BJP British Journal of Pharmacology

DOI:10.1111/bph.12122
www.brjpharmacol.org

RESEARCH PAPER

Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells

S Sengupta[1]*, I Obiorah[1]*, PY Maximov[1], R Curpan[2] and VC Jordan[1]

[1]*Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, USA, and* [2]*Institute of Chemistry, Romanian Academy, Timisoara, Romania*

Correspondence
V Craig Jordan, Georgetown University Medical Center, 3970 Reservoir Rd NW, Washington, DC 20057, USA. E-mail: vcj2@georgetown.edu

*These authors contributed equally to this work.

Keywords
oestrogen; oestrogen receptor; breast cancer; apoptosis; triphenylethylenes; antioestrogens; bisphenol and bisphenol A

Received
14 September 2012
Revised
21 December 2012
Accepted
8 January 2013

BACKGROUND AND PURPOSE

Oestrogen receptor alpha (ERα) binds to different ligand which can function as complete/partial oestrogen-agonist or antagonist. This depends on the chemical structure of the ligands which modulates the transcriptional activity of the oestrogen-responsive genes by altering the conformation of the liganded-ERα complex. This study determined the molecular mechanism of oestrogen-agonistic/antagonistic action of structurally similar ligands, bisphenol (BP) and bisphenol A (BPA) on cell proliferation and apoptosis of ERα + ve breast cancer cells.

EXPERIMENTAL APPROACH

DNA was measured to assess the proliferation and apoptosis of breast cancer cells. RT-PCR and ChIP assays were performed to quantify the transcripts of *TFF1* gene and recruitment of ERα and SRC3 at the promoter of *TFF1* gene respectively. Molecular docking was used to delineate the binding modes of BP and BPA with the ERα. PCR-based arrays were used to study the regulation of the apoptotic genes.

KEY RESULTS

BP and BPA induced the proliferation of breast cancer cells; however, unlike BPA, BP failed to induce apoptosis. BPA consistently acted as an agonist in our studies but BP exhibited mixed agonistic/antagonistic properties. Molecular docking revealed agonistic and antagonistic mode of binding for BPA and BP respectively. BPA treatment resembled E2 treatment in terms of PCR-based regulation of apoptotic genes whereas BP was similar to 4OHT treatment.

CONCLUSIONS AND IMPLICATIONS

The chemical structure of ERα ligand determines the agonistic or antagonistic biological responses by the virtue of their binding mode, conformation of the liganded-ERα complex and the context of the cellular function.

Abbreviations

4OHT, 4-hydroxy tamoxifen; BP, bisphenol; BPA, bisphenol A; ChIP, chromatin-immunoprecipitation assay; DES, diethylstilbestrol; E2, 17β-oestradiol; ERα, oestrogen receptor alpha; LBD, ligand binding domain; RAL, raloxifene; RT-PCR, real time PCR; SRC3, steroid coactivator 3; *TFF1*, *trefoil factor 1*

© 2013 The Authors
British Journal of Pharmacology © 2013 The British Pharmacological Society

Introduction

Oestrogen receptor alpha (ERα) mediates its action in cells and tissues by binding to its cognate ligands and function as a 'ligand-activated' transcription factor (Jordan and O'Malley, 2007). Apart from its natural ligands, many different compounds can bind to ERα and thus can function as its ligand (Sengupta and Jordan, 2008). However, depending upon the chemical structures of these ligands, they can either function as a complete/partial oestrogen- agonist or antagonist. Broadly, the oestrogenic compounds can be classified as class I and class II depending upon their planar or non-planar chemical structures respectively (Jordan *et al.*, 2001). Different ligands bind to the same core of the ligand binding domain (LBD) of ERα protein but can evoke distinct three-dimensional conformation of the liganded-ERα complex which can either interact with the coactivators or the corepressors (collectively known as coregulators) at the promoters of oestrogen-responsive genes (Jordan and O'Malley, 2007). Consequently, this complex modulates the transcriptional activity of the various oestrogen-responsive genes and eventually determines the outcome of the ERα-dependent physiological responses of a particular cell or tissue type. The molecular basis of this differential recruitment of the coregulators has been attributed to the ability of the liganded-ERα to reorient the helix 12 (H12) of the LBD in such a manner that the complex can interact with the coactivators at the structural interface formed by H3, H4 and H5 helices; when ERα is bound to an agonist [17β-oestradiol (E2) or diethylstilbestrol (DES)] (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998), but this interaction is completely blocked when the ERα is bound to antagonists, such as 4-hydroxy-tamoxifen (4OHT) (Brzozowski *et al.*, 1997) or raloxifene (RAL) (Shiau *et al.*, 1998). Interestingly, when ERα is liganded with an antagonist, such as 4OHT, an active metabolite of tamoxifen, which is extensively used in treatment and prevention of breast cancers (Jordan, 1993), it can now interact with the corepressors and can inhibit the transcriptional activity from the oestrogen-responsive genes (Metivier *et al.*, 2002; Shang and Brown, 2002; Liu and Bagchi, 2004). Besides the interaction of coregulators with the liganded ERα, the levels of coactiavtors and corepressors in a given cell can also determine the physiological responses to different ligands of ERα (Shang and Brown, 2002).

Earlier studies from our laboratory have identified that the amino acid aspartate at 351 (which is in the H3) of the ERα LBD is critically important for maintaining the integrity of antioestrogenic activity of keoxifene (RAL) and 4OHT (Levenson *et al.*, 1997; 1998). Earlier, the mutation of ERα encoding amino acid 351 which substituted the aspartate to tyrosine amino acid was detected in one of the xenograft tumours stimulated by tamoxifen in the athymic mice (Wolf and Jordan, 1994). Further investigations have revealed that changing the amino acid aspartate 351 of the ERα to glycine (D351G) abolishes the oestrogenic effect of 4OHT but does not affect oestradiol action on *TGFα* gene activation in the ER negative breast cancer cells stably transfected with either wild type ERα or D351G mutated ERα (MacGregor Schafer *et al.*, 2000). Using these models, oestrogens were classified as either type I, which have the planar structures or type II, which have the angular or non-planar structures (Jordan *et al.*, 2001; Bentrem *et al.*, 2003). A recent confirmatory study evaluated the ability of several type I and II liganded ERα to associate with the specific peptide motif 'LXXLL' which coactivators use to interact with the ERα (Bourgoin-Voillard *et al.*, 2010).

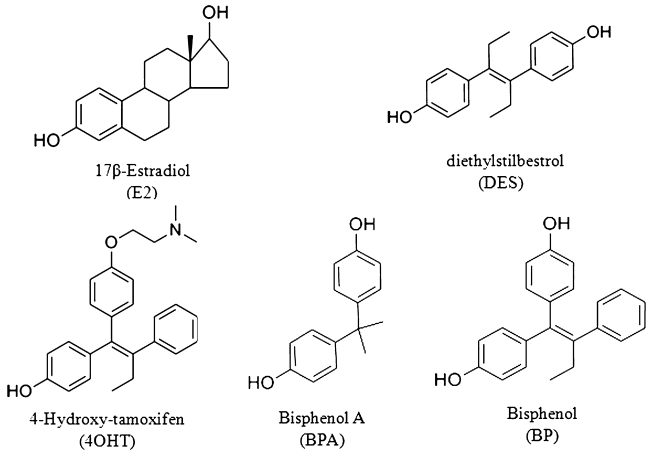


Figure 1

Chemical structures of 17β-oestradiol (E2), Diethylstilbestrol (DES), 4-Hydroxy tamoxifen (4OHT), Bisphenol (BP) and Bisphenol A (BPA).

A previous study (Maximov *et al.*, 2011) from our laboratory indicated that the conformation of the ERα complex can govern the oestrogen-induced apoptosis in the MCF7 : 5C breast cancer cells. The present study dissects the ERα mediated effect of two structurally similar oestrogenic ligands, namely, bisphenol (BP) and bisphenol A (BPA) (Figure 1), on two critical physiological responses, that is growth and apoptosis in the breast cancer cells. BP is structurally related to 4OHT with E2-like agonistic properties, whereas BPA has been characterized as an endocrine disruptor with weak oestrogenic properties. Using various investigative tools, this study underscore the fact that minor difference in the shape of the ERα-liganded complex has profound modulation on oestrogen-induced apoptosis but not on oestrogen-induced replication of breast cancer cells.

Materials and methods

Cell culture and reagents

Cell culture media were purchased from Invitrogen Inc. (Grand Island, NY, USA) and fetal calf serum (FCS) was obtained from HyClone Laboratories (Logan, UT, USA). Compounds E2, 4OHT and BPA were obtained from Sigma-Aldrich (St. Louis, MO, USA). BP was synthesized and the details of the synthesis have been reported previously (Maximov *et al.*, 2010). The ER positive breast cancer cells MCF-7 : WS8 (hereafter mentioned as MCF7) and oestrogen-deprived MCF7 : 5C were derived from MCF7 cells obtained from the Dr. Dean Edwards, San Antonio, TX, USA as reported previously (Jiang *et al.*, 1992). MCF7 cells were maintained in RPMI media supplemented with 10% FCS, 6 ng·mL^{-1} bovine insulin and penicillin and streptomycin. MCF7 : 5C cells were maintained in phenol red-free RPMI media containing 10% char-

coal dextran treated FCS, 6 ng·mL^{-1} bovine insulin and penicillin and streptomycin. Three to four days prior to harvesting the MCF7, cells were cultivated in phenol red-free media containing 10% charcoal dextran treated FCS. The cells were treated with indicated compounds (with media changes every 48 h) for the specified time and were subsequently harvested for growth assay. MDA-MB-231 cells stably transfected with wild type ERα (MC2) or D351G ERα (JM6) were grown in minimal essential medium without phenol red in the presence of 5% charcoal dextran treated calf serum, glutamine, bovine insulin, penicillin, streptomycin, nonessential amino acids and 500 μg·mL^{-1} G418 as described previously (MacGregor Schafer *et al.*, 2000). All the experiments were repeated at least three times, in triplicate to confirm the results.

Cell growth assay

The cell growth was monitored by measuring the total DNA content per well in 24 well plates. Fifteen thousand cells were plated per well and treatment with indicated concentrations of compounds was started after 24 h, in triplicate. Media containing the specific treatments were changed every 48 h. On day 6 (144 h post treatment), the cells were harvested and total DNA was assessed using a fluorescent DNA quantitation kit (Cat # 170–2480; Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. Briefly, the cells were harvested using hypotonic buffer solution and were subsequently sonicated. The DNA content was estimated using a fluorescent dye (Hoechst 33258) provided in the kit.

RNA isolation and real-time PCR (RT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNAeasy kit according to the manufacturer's instructions. RT-PCR was performed as previously described (Sengupta *et al.*, 2010). Briefly, high capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA, USA) was used to generate cDNA was using 1 ug of total RNA in a total volume of 20 uL. The cDNA was subsequently diluted to 500 uL and RT-PCR was performed using ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). In each well, 20 uL reaction volume included 10 uL SYBR green PCR master mix (Applied Biosystems), 125 nM each of forward and reverse primers and 5 uL of diluted cDNA. The change in expression of transcripts was determined as described previously and used the ribosomal protein 36B4 mRNA as the internal control (Sengupta *et al.*, 2010).

Chromatin-immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously (Maximov *et al.*, 2011). Briefly, cells were treated with indicated compounds for 45 min and cross-linked using 1.25% paraformaldehyde for 15 min and subsequently stopped cross-linking with 2 M glycine. Cells were collected, followed by nuclei isolation by centrifugation. Isolated nuclei were resuspended in SDS-lysis buffer followed by sonication and centrifugation at 14 000× *g* for 20 min at 4°C. The supernatant were diluted 1 : 10 with ChIP dilution buffer. Normal rabbit IgG and Magna ChIP protein A magnetic bead (Upstate Cell Signaling Solutions, Temecula CA, USA) were used to immunoclear the supernatant followed by immunoprecipitation with antibodies against ERα (1:1 mixture of cat# sc-543 and sc-7207; Santa Cruz Biotechnology Inc., Dallas, TX, USA) and steroid receptor coactivator-3 (SRC3) (cat# 13066; Santa Cruz Biotechnology, Inc.). Immunocomplexes were pulled down using protein A magnetic beads and a magnet. The beads bound to immunocomplexes were washed using different buffers as described previously (Maximov *et al.*, 2011). Precipitates were finally extracted twice using freshly made 1% SDS and 0.1 M $NaHCO_3$ followed by de-crosslinking. The DNA fragments were purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA). RT-PCR was performed using 2 μL isolated DNA, using primers specific for *PS2* promoter (Maximov *et al.*, 2011). The data are presented as percent input of starting chromatin input after subtracting the percent input pull down of the negative control (normal rabbit IgG).

Molecular modelling

A commonly used method to evaluate the docking method efficiency is to dock the cocrystallized ligand to its native experimental structure. The expected outcome would be a docking solution, pose, which recapitulates the binding mode of the ligand in the binding site of the experimental structure. For this reason, 3D-conformations of E2, DES and 4OHT were generated, optimized with MMFF94 force field and then subjected to preparation for docking using the LigPrep utility. The same protocol was followed for BPA and BP. Protein Preparation Workflow (Schrödinger, LLC, New York, NY, 2011) was employed to prepare the proteins for molecular docking. The residues well known to be important for biological activity D351 and E353 were kept charged in all three receptors, the free rotation of hydroxyl group for T347 was allowed and H524 residue was protonated at the epsilon nitrogen atom in the complexes 1GWR and 3ERT based on the available literature data. In the case of 3ERD complex, two structures were prepared for docking runs having H524 protonated at epsilon (3ERD_ε) and delta (3ERD_δ) nitrogen.

The best docking poses were selected based on the composite score, Emodel, which accounts not only for the binding affinity but also for the energetic terms, such as ligand strain energy and interaction energy. When E2, DES and 4OHT were docked to their native structures the top ranked docking solutions have a ligand RMSD of 0.353 for E2, 0.416 for DES docked to 3ERD_ε and 0.372 when docked to 3ERD_δ and 0.629 for 4OHT.

Real time profiler assay for apoptosis

RT-PCR profiler assay kits for apoptosis was used from a commercial vendor which uses 384 well plates to profile the expression of 370 apoptosis related human genes (Qiagen; SABiosciences Corp, Fredrick, MD, USA; Cat#330231 PAHS-3012E). All the procedures were followed as per the manufacturer's instructions. Briefly, MCF7 : 5C cells were treated with E2 (10^{-9} M) for 24, 48 and 72 h or with indicated compounds (in triplicate) for 48 h and total RNA was isolated using the method mentioned earlier. Two micrograms of total RNA was reverse transcribed and RT-PCR was performed using ABI 7900HT. The fold change was calculated by ΔΔCt method and volcano plots were generated using the web based tool, RT^2 profile PCR array data analysis version 3.5 (Qiagen; SABiosciences Corp.).

Statistics

Statistical significance of our data was assessed using the Student's *t*-test wherever relevant. A *P*-value of <0.05 was considered as statistically significant.

Results

Differential effect of BP and BPA in inducing apoptosis in MCF7 : 5C cells but not growth in MCF7 cells

BP (Figure 1) a triphenylethylene (TPE) is a known partial oestrogenic ligand which can induce growth of the ERα positive breast cancer cells (Maximov *et al.*, 2010) and can also partially initiate prolactin synthesis from primary culture of cells from immature rat pituitary glands (Jordan and Lieberman, 1984). Another compound with similar chemical structure, BPA (Figure 1) is also a well-characterized but weak oestrogenic ligand (Routledge *et al.*, 2000). Here, we evaluated the ability of these two oestrogenic compounds to induce growth and apoptosis in MCF7 and MCF7 : 5C cells, respectively as both these responses are dependent on oestrogen-agonistic action. As expected, BP as well as BPA was able to induce the concentration dependent growth in the MCF7 cells (Figure 2A). BPA was less potent compared to BP as maximal growth was achieved by BP at 10^{-9} M concentra-

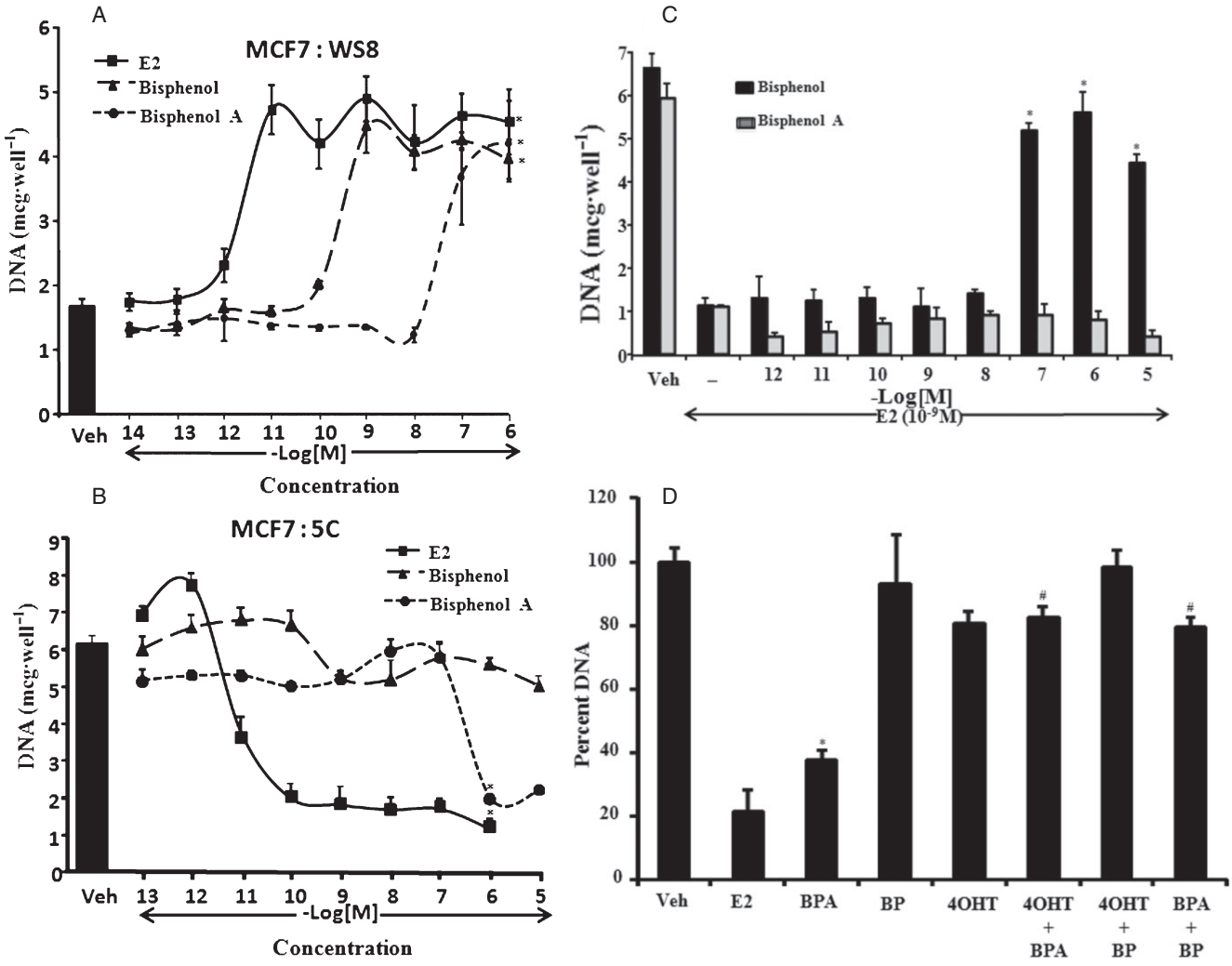


Figure 2

Differential effect of bisphenol (BP) and bisphenol A (BPA) on growth and apoptosis of ERα positive breast cancer cells. (A) Dose-dependent effects of BP, BPA and (oestradiol) E2 on growth of MCF7 cells treated for 6 days as indicated. The black bar denotes the level of DNA in vehicle treated cells over a 6-day period. The growth is measured as amount of DNA present in each well. (*P < 0.05 vs. vehicle treatment) (B) Dose-dependent effect of BP, BPA and E2 on apoptosis of MCF7 : 5C cells treated for 6 days as indicated. The black bar denotes the level of DNA in vehicle treated cells over a 6-day period. The growth is measured as amount of DNA present in each well. (*P < 0.05 vs. vehicle treatment) (C) Dose dependent effect of BP and BPA on E2 (1 nM)-induced apoptosis in MCF7 : 5C cells, treated over a six day period. The growth is measured as amount of DNA present in each well. (*P < 0.05 vs. 1 nM E2 treatment) (D) Effect of BP (10^{-6} M) and 4OHT (10^{-6} M) on BPA (10^{-6} M) induced apoptosis in MCF7 : 5C cells over 6-day period. (*P < 0.05 vs. vehicle treatment; $^{\#}P$ < 0.05 vs. BPA treatment) The data are presented as percent of growth considering the vehicle treated cells as 100 percent. Each value is average of at least three replicates ±SD.

tion as compared to 10^{-6} M for BPA. By comparison, E2 induced maximal growth at 10^{-11} M concentration in the MCF7 cells. In the case of MCF7 : 5C cells, which undergo apoptosis with E2 treatment (Lewis *et al.*, 2005; Ariazi *et al.*, 2011), a marked contrast was observed between BP and BPA in the induction of apoptosis. BPA was able to induce apoptosis to the same extent as E2 in these cells at a higher (10^{-6} M) concentration (Figure 2B) as compared to E2 which achieved maximal effect at 10^{-10} M. However, BP failed to induce apoptosis even at 10^{-5} M concentration (Figure 2B). We further investigated that if BP was actually binding to the ERα in the MCF7 : 5C cells by treating these cells with BP in combination with 10^{-9} M of E2. BP was able to block the effect of E2 in the MCF7 : 5C cells (Figure 2C and Supporting Information Figure S3) in a concentration dependent manner indicating that the effect of BP was through the ERα, thus inhibiting the E2 action. On the other hand, BPA was not able to block the effect E2 action (Figure 2C). In addition, we also show that the oestrogenic effect of BPA (10^{-6} M) in inducing apoptosis in MCFF7 : 5C cells was completely blocked by BP (10^{-6} M) as well as 10^{-6} M of 4OHT (Figure 2D).

Regulation of oestrogen-responsive gene trefoil factor 1(TFF1 or PS2) by BP and BPA

We next investigated the transcriptional regulation of a well-characterized oestrogen-regulated gene, *TFF1* (*PS2*) (Metivier *et al.*, 2003) by BP and BPA and compared it with E2 and 4OHT. MCF7 cells were treated for 4 h with the 0.1% ethanol (veh), E2 (10^{-9} M), 4OHT (10^{-6} M), BP (10^{-6} M and 10^{-5} M) or BPA (10^{-6} M and 10^{-5} M) and the transcripts levels of *PS2* gene were measured using RT-PCR. Two different concentrations (10^{-6} M and 10^{-5} M) were used for BP and BPA, because BPA is a weak oestrogen and we wanted to evaluate the concentration dependent regulation of these compounds. As expected, *PS2* mRNA was up-regulated around fivefold by E2 (10^{-9} M) compared to vehicle treatment and 4OHT (10^{-6} M) which completely failed to induce the levels of *PS2* mRNA (Figure 3A). On the other hand, BP treatment at 10^{-6} M concentration moderately (~2 fold) up-regulated the *PS2* mRNA levels and higher concentration (10^{-5} M) of BP failed to further increase the levels of *PS2* (Figure 3A). Conversely, cells treated with BPA exhibited concentration dependent increase in up-regulation of the *PS2* mRNA and the magnitude of up-regulation with high concentration (10^{-5} M) of BP was equivalent to the E2-mediated up-regulation of *PS2* mRNA (Figure 3A).

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

To understand the differences in the molecular mechanism of the transcriptional activation of *PS2* gene *in vivo* by BP and BPA in comparison to E2 and 4OHT treatment, we performed ChIP assay to evaluate the recruitment of ERα and SRC3 at the promoter region of *TFF1* (*PS2*) gene (Figure 3B) which has a well-characterized functional oestrogen-responsive element (ERE) (Metivier *et al.*, 2002). MCF7 cells were treated with either 0.1% ethanol (veh), E2 (10^{-9} M), 4OHT (10^{-6} M), BP (10^{-6} M or 10^{-5} M) or BPA (10^{-6} M or 10^{-5} M) for 45 min and thereafter harvested for ChIP assay. The results (Figure 3C) reveal that both concentrations of BPA (10^{-6} M and 10^{-5} M) recruited ERα to the *PS2* promoter with ERE in a concentration-dependent manner which was equivalent to results obtained with E2 treatment. In contrast, BP did not show a concentration-related effect and the levels of ERα plateaued at 50% of either E2 or BPA (Figure 3C). Recruitment of the coactivator, SRC3 (AIB1), which plays a key role in transcriptional activation of several oestrogen-regulated genes, including *PS2* gene (Shao *et al.*, 2004; Labhart *et al.*, 2005), followed the similar pattern as the ERα (Figure 3D). BPA treatment at both the concentrations (10^{-6} M or 10^{-5} M) recruited SRC3 in a concentration-dependent manner to become equivalent to levels observed with E2 treatment whereas BP treatment (both concentration) plateaued at 50% of E2 or BPA recruitment levels (Figure 3D). As expected, 4OHT treatment did not recruit SRC3 and was comparable to vehicle treatment. The ChIP data correlates very well with the observed pattern of transcriptional activation of *PS2* gene (Figure 3A) under same treatment conditions.

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

Previous studies from our laboratory have established an *in vitro* system to evaluate and differentiate the conformation of liganded ERα induced by planar and non-planar ligands (Jordan *et al.*, 2001). Activation of *TGFα* gene in MDA : MB 231 cells stably transfected with wt ERα (MC2 cells) or mutant ERα (JM6 cells, D351G; which has the aspartate substituted with glycine at amino acid 351), is used as a marker to distinguish the ERα interactions between planar and non-planar oestrogen ligands (Jordan *et al.*, 2001). We treated the MC2 and JM6 cells with increasing concentrations of BP and BPA and measured the *TGFα* induction in these cells. E2 was used as a positive control. In MC2 cells, (wt ERα), all the tested ligands induced *TGFα* transcripts level to similar levels (Figure 4A). Induction of *TGFα* by BPA was observed at higher concentrations whereas BP and E2 had similar effects (Figure 4A). On the other hand, in JM6 cells (mutant; D351G ERα), BP failed to induce *TGFα* transcription even at higher concentrations (Figure 4B), whereas E2 and BPA treatment induced TGFα (Figure 4B), although the maximal induction with BPA was observed at higher concentration (10^{-5} M) which was less than 50% of E2 treatment. We further confirmed that E2-induced *TGFα* stimulation in JM6 cells was completely blocked by BP and 4OHT in a dose-dependent manner; whereas co-treatment of BPA in presence of E2 failed to inhibit it (Figure 4C).

Molecular docking of BP and BPA to the LBD of ERα

To determine the binding mode of BPA and BP to ERα, the ligands were docked to the agonist and antagonist conformations of the receptor. The experimental structure, 3ERT, was selected from protein database for the antagonist conformation of ERα (Figure 5A) containing 4OHT, while for the agonist conformation, two experimental structures were selected, namely the receptor cocrystallized with E2, 1GWR (Figure 5B) and DES, 3ERD (Figure 5C) respectively.

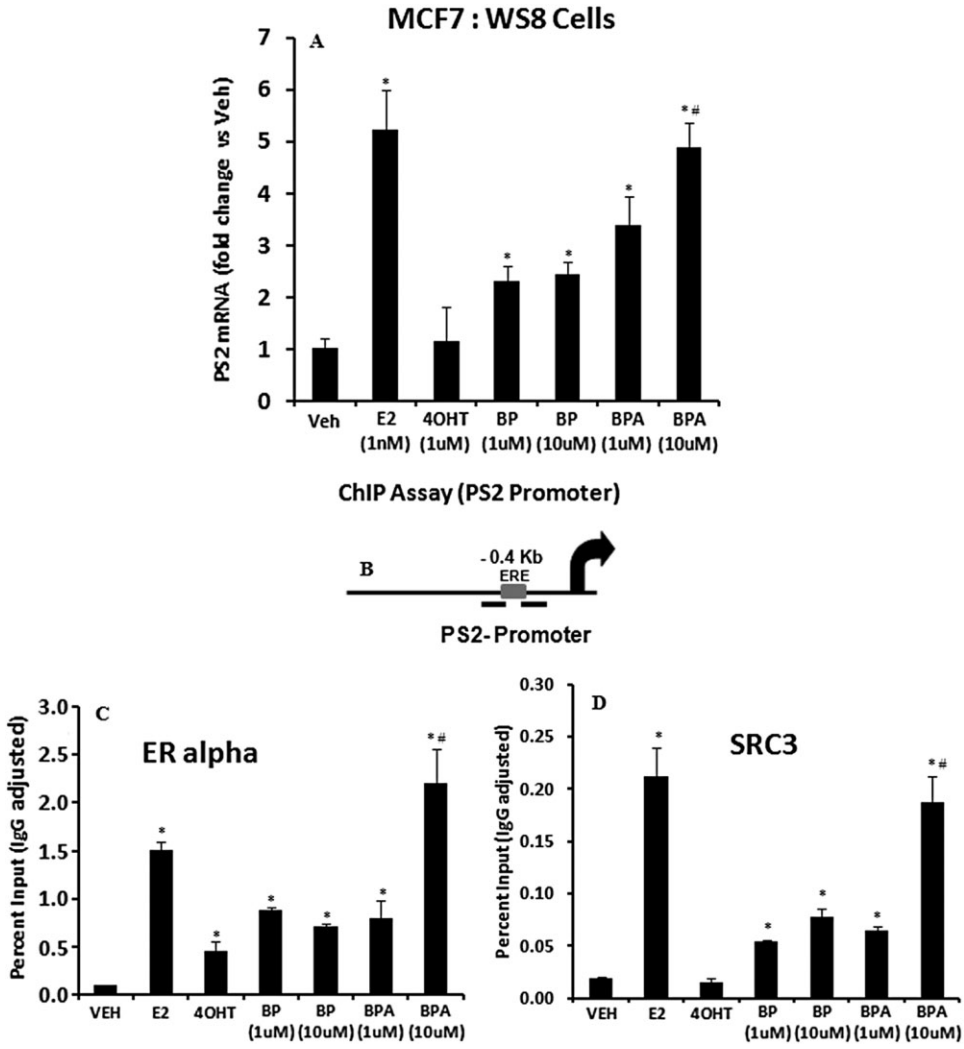


Figure 3

Regulation of *PS2* (*TFF1*) gene by bisphenol (BP), bisphenol A (BPA) compared with 17β-oestradiol (E2) and 4-hydroxy-tamoxifen (4OHT) and recruitment of oestrogen receptor alpha (ER alpha) and steroid receptor coactivator-3 (SRC3) at the oestrogen-responsive element (ERE) of proximal promoter of *PS2* gene followed by 45 min treatments of bisphenol (BP), bisphenol A (BPA) compared with 17β-oestradiol (E2) and 4-hydroxy-tamoxifen (4OHT) in MCF7 cells. (A) MCF7 cells were treated with indicated treatments for 4 h and harvested for total RNA. Total RNA was reverse transcribed and assessed for *PS2* gene expression levels using RT-PCR. *36B4* gene was used as an internal control. All values are represented in terms of fold difference versus vehicle treatment. (*$P < 0.05$ vs. vehicle treatment; $^{\#}P < 0.05$ vs. 1 μM BPA and 10 μM BP treatment) (B) Schematic representation of the *PS2* proximal promoter containing an ERE (grey box) and the black bars represent the primers used for RT-PCR. (C) Recruitment of ERα at the *PS2* proximal promoter, by ChIP assay after 45 min of indicated treatment. (*$P < 0.05$ vs. vehicle treatment; $^{\#}P < 0.05$ vs. 1 μM BPA and 10 μM BP treatment) (D) Recruitment of SRC3 at the *PS2* proximal promoter, by ChIP assay after 45 min of indicated treatment. All the values are representated as percent input of the starting chromatin material and after subtracting the IgG control for each sample. (*$P < 0.05$ vs. vehicle treatment; $^{\#}P < 0.05$ vs. 1 μM BPA and 10 μM BP treatment.)

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

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

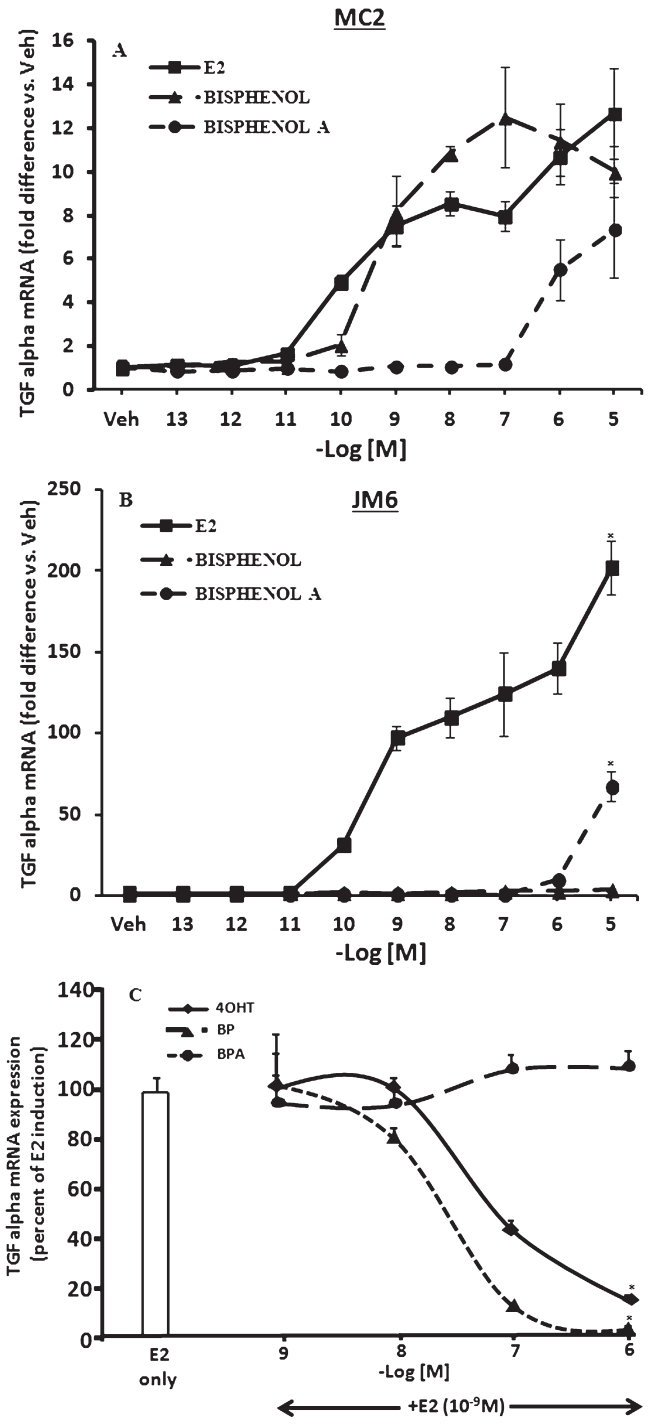


Figure 4

Induction of TGFα mRNA by 17β-oestradiol (E2), bisphenol (BP) and bisphenol A (BPA) in MDA : MB 231 cells stably transfected with wild type ERα (MC2 cells) or D351G mutant ERα (JM6 cells). (A) MC2 cells were treated with (E2), (BP) or BPA at indicated concentration for 48 h and cells were harvested for total RNA. Total RNA was reverse transcribed and real time PCR (RT-PCR) was performed to assess the expression of *TGFα* using *36B4* as an internal control. The values are presented as fold difference versus vehicle treated cells. (B) JM6 cells were treated with (E2), (BP) or (BPA) at indicated concentrations for 48 h and cells were harvested for total RNA. Total RNA was reverse transcribed and RT-PCR was performed to assess the expression of *TGFα* using *36B4* as an internal control. The values are presented as fold difference versus vehicle treated cells. (**P* < 0.05 vs.10^{-5} M BP treatment) (C) JM6 cells were treated with E2 alone or in combination with different concentration of BP, BPA or 4OHT as indicated for 48 h. The values are presented as percentage of expression of *TGFα* mRNA considering the E2-induced levels as 100%. (**P* < 0.05 vs. 1 nM E2 and 1 nM E2 +10^{-6} M BPA treatment.)

the hydroxyl groups of BPA and amino acids G521, E353 and R394 (Figure 5F). Apart from the H-bonds formation, the methyl groups are involved in hydrophobic contacts with amino acids L346, F404 and L428. Also, this binding mode has been encountered for 6 out of 10 poses resulted from the docking of BPA into the experimental structure 1GWR.

The predicted binding modes of BP to the open and closed conformation of ER are similar, forming the H-bond network between E353, R394 and the highly ordered water molecule and an additional H-bond with the hydroxyl group of T347 (Figure 5G–I). The composite score, Emodel, shows that BP is better accommodated in the binding site of the open or antagonist conformation of ERα and it is more likely for the ligand to bind at this conformation of ER. Similar results have been obtained using the induced fit docking method, which accounts for both the ligand and protein flexibility (Maximov *et al.*, 2010).

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

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

We thereafter determined the effect of BP and BPA treatment in regulating the apoptosis related genes in MCF7 : 5C cells and compared it with E2 and 4OHT as a positive and negative inducer of apoptosis respectively. We used the RT-PCR profiler assay kits for apoptosis from a commercial vendor which uses 384 well plates to profile the expression of 370 apoptosis related human genes (Qiagen; SABiosciences Corp.; Cat#330231 PAHS-3012E). To select a single time point of treatment with the ligands, we first treated the MCF7 : 5C cells with E2 (10^{-9} M) for 24, 48 and 72 h (in triplicate) and created an apoptotic gene signature throughout these time points after comparing them with vehicle treatment (Supporting Information Figure S1A, B, C and Supporting Information Table S1). This gene signature was generated by comparing the expression level of all the genes with vehicle

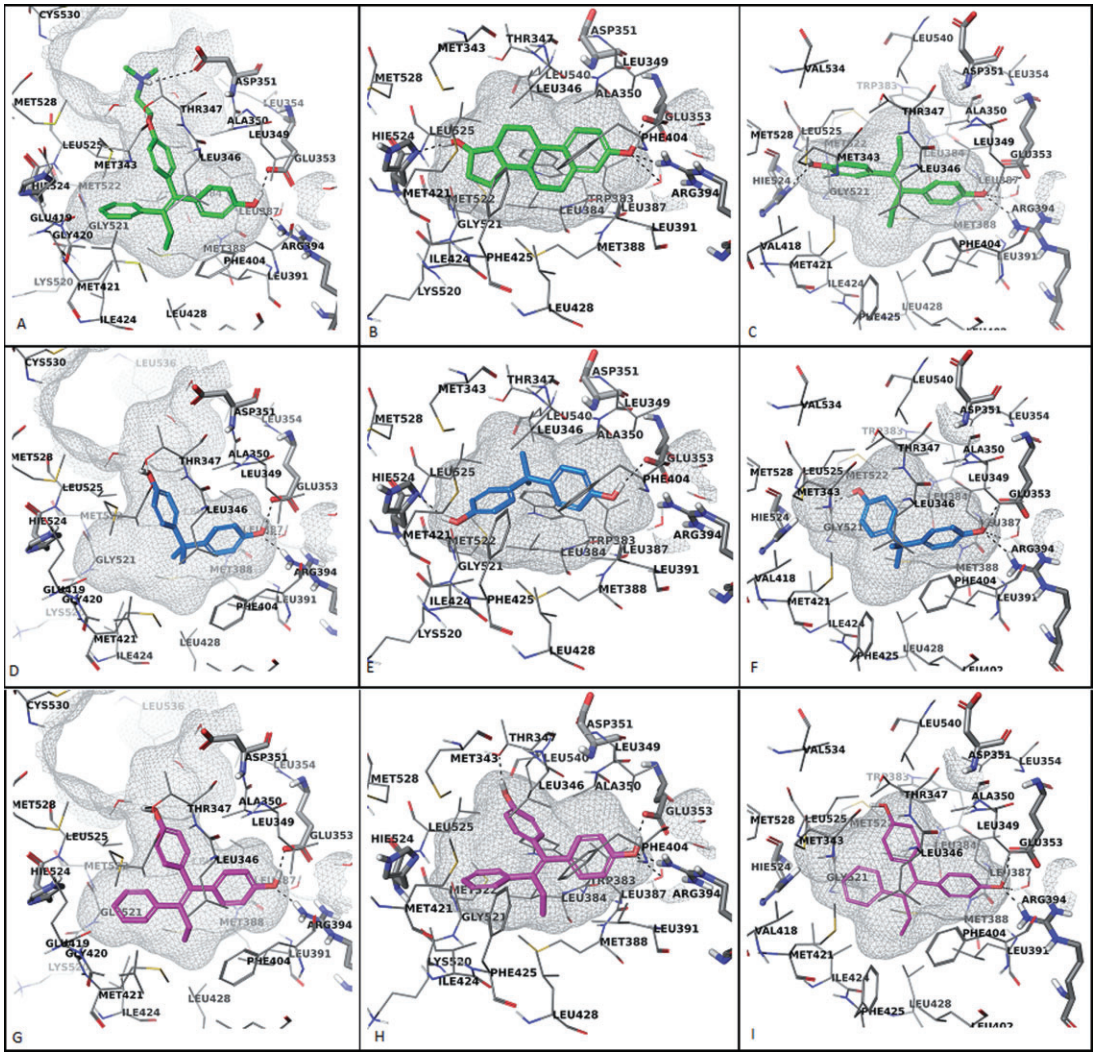


Figure 5

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

treatment and selecting the genes which were at least 2.5-fold overexpressed or underexpressed as compared to vehicle treated cells. The fold change was calculated by delta-delta Ct method using the web based tool, RT^2 profile PCR array data analysis version 3.5 (Qiagen; SABiosciences Corp.).

After carefully analysing the gene list generated by E2 treatments over the above said time period, we selected 48 h as the time point to treat MCF7 : 5C cells with BP, BPA and 4OHT and compare the expression of the apoptosis related genes with the gene signature of the E2 treatment at 48 h. This particular time point was selected because the MCF7 : 5C cells undergo apoptotic changes after E2 treatment during this time period (Lewis *et al.*, 2005) and also because after 48 h of E2 treatment, the cells are committed to apoptosis, as 4OHT treatment cannot rescue these cells after this time point (unpublished observations).

Next, we analysed the changes in the overall expression profiles of apoptotic genes by E2, 4OHT, BP and BPA versus vehicle (Veh) treatment at 48 h (Supporting Information Figure S2A, B, C and D respectively) using the same apoptosis RT profiler. For any gene to be considered as differentially expressed, we set the cut-off as 2.5-fold up- or down-regulation versus the vehicle treatment. Using this criterion, we created a gene list for up-regulated and down-regulated genes for each treatment group (Supporting Information Table S2). We thereafter generated a heat map (Figure 6) in which we selected all the genes which were at least 2.5-fold up- or down-regulated by E2 treatment and compared it with other ligand treatments. This heat map clearly demonstrates that the genes which are up-regulated at least 2.5-fold after 48 h of E2 treatment are not up-regulated in 4OHT or BP treatment. In contrast, the majority of the genes up-regulated

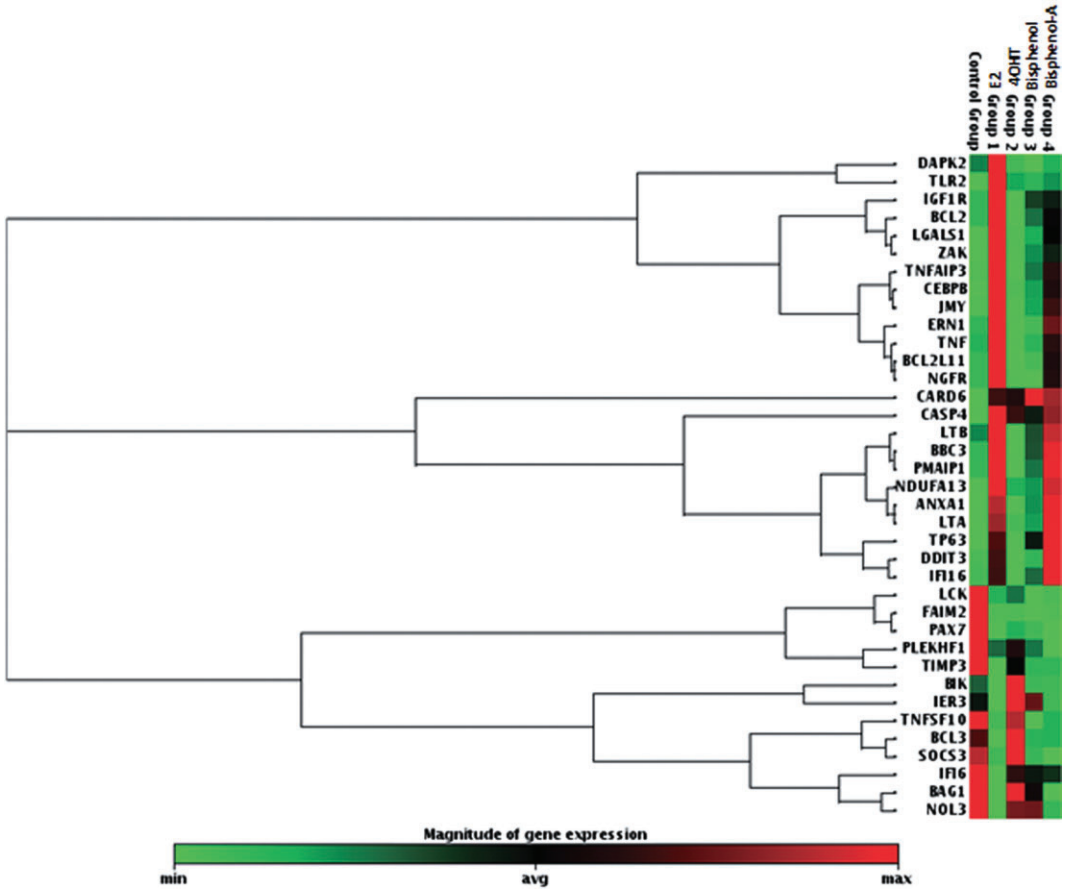


Figure 6

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

by BPA treatment were shown to be the same genes up-regulated by the E2 treatment. Many of these genes are up-regulated by BPA to the similar extent as E2 and others show a distinct trend of overexpression as compared to vehicle (Figure 6). Nevertheless, down-regulated genes follow a different pattern. The pattern of genes down-regulated by BP treatment resembles the pattern observed with E2 and BPA treatment and not with the pattern of 4OHT treatment (Figure 6 and Supporting Information Table S2). Approximately, 53 and 61% of down-regulated genes are in common with E2 treatment and with the treatment of BP and BPA respectively (Supporting Information Table S2).

Discussion

The chemical structures of the ligands which bind to ERα are critical in determining the biological effects in the oestrogen-responsive cells and tissues. Minor changes in the ligand structures can alter the way these ligands interact with the ERα protein and transform the conformation of the liganded –ERα complex in the cells. Structure-function relationships have been studied extensively using various biological end-points, such as modulation of prolactin gene expression in primary cell cultures of rat pituitary glands (Jordan and Lieberman, 1984; Jordan *et al.*, 1984; 1986), or *TGFα* activation in stably transfected wt and mutant ERα in MDA : MB 231 cells (Jordan *et al.*, 2001). The current study dissects, compares and contrasts the mechanism of action of BP and BPA, two structurally similar ligands of ERα, which have opposing effects on apoptosis but not on the growth of oestrogen-responsive breast cancer cells.

The results of this study established that unlike BPA and E2, BP was not functioning as an oestrogen-agonist in inducing apoptosis in MCF7 : 5C cells while both compounds (BPA and BP) were oestrogenic in inducing growth in MCF7 cells. This clearly indicated differential requirement of ERα mediated molecular action to achieve two distinct physiological responses in the breast cancer cells. Activation of oestrogen-responsive gene *PS2* by these compounds in MCF7 cells suggested that higher concentrations of BPA was as effective as E2 but BP treatment failed to achieve E2-like stimulation,

even with higher concentration. This phenomenon was observed because BP has a high ERα binding affinity and can maximally induce *PS2* gene at lower concentration and raising the concentration did not enhance the induction because it failed to recruit sufficient coactivator (SRC3) at the *PS2* gene promoter. This was most likely due to insufficient ERα recruitment at the promoter and inaccessibility of the coactivator interacting surface of BP-liganded ERα. A recent study (Bourgoin-Voillard *et al.*, 2010) however, suggested that BP-liganded ERα cannot bind to a peptide containing the coactivator interacting domain. This discrepancy can be attributed to the fact that our studies were performed in live cells chromatin as opposed to using an *in vitro* ELISA based system. This indicates that binding of liganded ERα and its interaction with other coregulators can be modulated by other factors involved in transcriptional complex.

On the other hand, BPA at higher concentration engaged SRC3 to a similar level as E2 treatment. The fact that higher concentration of BPA was required to recruit ERα and SRC3 to the similar levels as E2 treatment is because its binding affinity with ERα is very low (RBA, 0.073) (Routledge *et al.*, 2000) and therefore higher concentrations of the ligand is required to drive the kinetics towards the activated state. In the case of BP, it has a strong binding affinity to the ERα (RBA, 96.0) (Jordan *et al.*, 1984) and therefore maximal activation is achieved at lower concentration and increasing concentration do not enhance the activation. Overall, these results indicate that binding mode of BPA and E2 are similar whereas BP might bind differently to ERα. Indeed, our molecular docking studies determined that BPA binds to the ERα in two possible ways, both similar to agonistic mode of binding. Also docking scores calculated in this study predicted very low binding affinity of BPA to ERα, which is in excellent agreement with previous reports (Gould *et al.*, 1998; Kuiper *et al.*, 1998; Kitamura *et al.*, 2005). In contrast, modelling studies suggested antagonistic mode of binding (as in 4OHT) for BP to the ERα. To confirm the molecular modelling, we used a biological model system which can distinguish between planar and angular oestrogen ligands (Jordan *et al.*, 2001; Bentrem *et al.*, 2003) by measuring the transcriptional activation of *TGFα* in MDA : MB 231 cells stably transfected with wt ERα (MC2 cells) or mtERα (D351G) (JM6 cells). Results (Figure 4B) show that BP treatment failed to activate *TGFα* transcription similar to 4OHT (Jordan *et al.*, 2001) in JM6 cells whereas BPA treatment was similar to E2 action, albeit with lower potency. This consolidated our finding that the mode of action of BP is more like 4OHT rather than E2. Importantly, the structure of BP is identical to 4OHT except for the basic dimethylamine-ethoxy side chain. The absence of the side chain contributes towards the enhanced oestrogenic properties of BP with AF-1 fully engaged in ER responses to stimulate growth, as H12 of the ERα protein liganded with BP may not be properly restrained. This contrasts with 4OHT or RAL, where the restricted structure of the coactivator-interacting interface for binding of SRC3 or the other coactivators now has limited AF-1 and AF-2 activity for growth. Of note, 4OHT and BP-liganded ERα was less efficiently recruited to the *PS2* promoter ERE which may also contribute towards lesser recruitment of SCR3 for BP as recruitment of ERα precedes the coactivator binding (Metivier *et al.*, 2003).

The fact that SRC3 is essential for E2-induced apoptosis in the MCF7 : 5C cells (Hu *et al.*, 2011) as well as E2-mediated growth of MCF7 cells (List *et al.*, 2001) coupled with the findings of this study, leads to the hypothesis that the oestrogen-mediated growth of MCF7 cells is more sensitive and can be induced even if the conformation of the liganded-ERα complex allows only partial interaction of coactivators as in case of BP binding. In contrast, complete and robust interaction of coactivator with the liganded-ERα complex must be needed for rapid induction of apoptosis in MCF7 : 5C cells.

Indeed, using an 'apoptosis' pathway focused RT-PCR based profiler consisting of 370 genes, this study further illustrated that apoptosis related genes were similarly up-regulated by E2 and BPA treatments after 48 h of treatment whereas BP and 4OHT showed very few up-regulated genes and the TPE based compounds did not have a similar profile of up-regulated genes during this time frame. By comparing the gene list (Supporting Information Table S2), which includes all the genes up- or down-regulated at least 2.5-fold by the treatments, it is evident that 66% of up-regulated genes are common between E2 and BPA treatment, whereas only 8% genes are commonly up-regulated by BP or 4OHT treatment.

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

By employing structurally related ligands and using MCF7 : 5C and parental MCF7 cells, we have demonstrated that depending upon the biological response, the same molecule can function as an E2-antagonist or agonist respectively. Based on these data, it is reasonable to speculate that genistein and related phytoestrogens may also induce apoptosis in MCF7 : 5C cells as their binding to ERα LBD is similar as E2 and DES (Gao *et al.*, 2012) and function as type I oestrogens (Bentrem *et al.*, 2003). In conclusion, this study provides evidence that binding of ERα with different ligands that programme conformational changes of the liganded-ERα, determines the transcriptional profile of the responsive genes by virtue of interaction with coregulators.

Acknowledgements

This work (V.C.J.) was supported by the Department of Defense Breast Program under Award number W81XWH-06-1-0590 Center of Excellence; subcontract under the SU2C (AACR) Grant number SU2C-AACR-DT0409; the Susan G Komen For The Cure Foundation under Award number SAC100009 and the Lombardi Comprehensive Cancer Center Support Grant (CCSG) Core Grant NIH P30 CA051008. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

Conflicts of interest

None.

References

Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P *et al.* (2011). Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci U S A 108: 18879–18886.

Bentrem D, Fox JE, Pearce ST, Liu H, Pappas S, Kupfer D *et al.* (2003). Distinct molecular conformations of the estrogen receptor alpha complex exploited by environmental estrogens. Cancer Res 63: 7490–7496.

Bourgoin-Voillard S, Gallo D, Laios I, Cleeren A, Bali LE, Jacquot Y *et al.* (2010). Capacity of type I and II ligands to confer to estrogen receptor alpha an appropriate conformation for the recruitment of coactivators containing a LxxLL motif-Relationship with the regulation of receptor level and ERE-dependent transcription in MCF-7 cells. Biochem Pharmacol 79: 746–757.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O *et al.* (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389: 753–758.

Gao L, Tu Y, Agren H, Eriksson LA (2012). Characterization of agonist binding to His524 in the estrogen receptor alpha ligand binding domain. J Phys Chem B 116: 4823–4830.

Gould JC, Leonard LS, Maness SC, Wagner BL, Conner K, Zacharewski T *et al.* (1998). Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. Mol Cell Endocrinol 142: 203–214.

Hu ZZ, Kagan BL, Ariazi EA, Rosenthal DS, Zhang L, Li JV *et al.* (2011). Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. PLoS ONE 6: e20410.

Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC (1992). An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 90: 77–86.

Jordan VC (1993). Fourteenth Gaddum Memorial Lecture. A current view of tamoxifen for the treatment and prevention of breast cancer. Br J Pharmacol 110: 507–517.

Jordan VC, Lieberman ME (1984). Estrogen-stimulated prolactin synthesis in vitro. Classification of agonist, partial agonist, and antagonist actions based on structure. Mol Pharmacol 26: 279–285.

Jordan VC, O'Malley BW (2007). Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 25: 5815–5824.

Jordan VC, Lieberman ME, Cormier E, Koch R, Bagley JR, Ruenitz PC (1984). Structural requirements for the pharmacological activity of nonsteroidal antiestrogens in vitro. Mol Pharmacol 26: 272–278.

Jordan VC, Koch R, Mittal S, Schneider MR (1986). Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. Br J Pharmacol 87: 217–223.

Jordan VC, Schafer JM, Levenson AS, Liu H, Pease KM, Simons LA *et al.* (2001). Molecular classification of estrogens. Cancer Res 61: 6619–6623.

Kitamura S, Suzuki T, Sanoh S, Kohta R, Jinno N, Sugihara K *et al.* (2005). Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. Toxicol Sci 84: 249–259.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT *et al.* (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139: 4252–4263.

Labhart P, Karmakar S, Salicru EM, Egan BS, Alexiadis V, O'Malley BW *et al.* (2005). Identification of target genes in breast cancer cells directly regulated by the SRC-3/AIB1 coactivator. Proc Natl Acad Sci U S A 102: 1339–1344.

Levenson AS, Catherino WH, Jordan VC (1997). Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. J Steroid Biochem Mol Biol 60: 261–268.

Levenson AS, Tonetti DA, Jordan VC (1998). The oestrogen-like effect of 4-hydroxytamoxifen on induction of transforming growth factor alpha mRNA in MDA-MB-231 breast cancer cells stably expressing the oestrogen receptor. Br J Cancer 77: 1812–1819.

Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T *et al.* (2005). Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97: 1746–1759.

List HJ, Lauritsen KJ, Reiter R, Powers C, Wellstein A, Riegel AT (2001). Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells. J Biol Chem 276: 23763–23768.

Liu XF, Bagchi MK (2004). Recruitment of distinct chromatin-modifying complexes by tamoxifen-complexed estrogen receptor at natural target gene promoters in vivo. J Biol Chem 279: 15050–15058.

MacGregor Schafer J, Liu H, Bentrem DJ, Zapf JW, Jordan VC (2000). Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. Cancer Res 60: 5097–5105.

Maximov P, Sengupta S, Lewis-Wambi JS, Kim HR, Curpan RF, Jordan VC (2011). The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: a hypothesis. Horm Mol Biol Clin Investig 5: 27–34.

Maximov PY, Myers CB, Curpan RF, Lewis-Wambi JS, Jordan VC (2010). Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. J Med Chem 53: 3273–3283.

Metivier R, Stark A, Flouriot G, Hubner MR, Brand H, Penot G *et al.* (2002). A dynamic structural model for estrogen receptor-alpha activation by ligands, emphasizing the role of interactions between distant A and E domains. Mol Cell 10: 1019–1032.

Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M *et al.* (2003). Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 115: 751–763.

Routledge EJ, White R, Parker MG, Sumpter JP (2000). Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) alpha and ERbeta. J Biol Chem 275: 35986–35993.

Sengupta S, Jordan VC (2008). Selective estrogen modulators as an anticancer tool: mechanisms of efficiency and resistance. Adv Exp Med Biol 630: 206–219.

Sengupta S, Sharma CG, Jordan VC (2010). Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells. Horm Mol Biol Clin Investig 2: 235–243.

Shang Y, Brown M (2002). Molecular determinants for the tissue specificity of SERMs. Science 295: 2465–2468.

Shao W, Keeton EK, McDonnell DP, Brown M (2004). Coactivator AIB1 links estrogen receptor transcriptional activity and stability. Proc Natl Acad Sci U S A 101: 11599–11604.

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA *et al*. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95: 927–937.

Wolf DM, Jordan VC (1994). The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31: 129–138.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Representation of E2 (1 nM) regulated apoptotic genes in MCF7 : 5C cells at 24, 48 and 72 h of treatment versus vehicle treatment using volcano plots.

Figure S2 Representation of 17β-oestradiol 10^{-9} M (E2), 4-hydroxy tamoxifen, 10^{-6} M (4OHT), bisphenol, 10^{-6} M (BP) and bisphenol A, 10^{-6} M (BPA) regulated apoptotic genes in MCF7 : 5C cells after 48 h of treatment versus vehicle using volcano plots.

Figure S3 Dose dependent effect of BP (at various concentrations between 10^{-8} M and 10^{-7} M) on E2 (1 nM)-induced apoptosis in MCF7 : 5C cells, treated over a 6-day period. The growth is measured as percent of DNA present in each well; vehicle treated cells were considered as 100%.

Table S1 Gene list of E2 (1 nM) regulated apoptotic genes in MCF7 : 5C cells at 24, 48 and 72 h of treatment versus vehicle treatment.

Table S2 Gene list of 17β-oestradiol, 10^{-9} M (E2), 4-hydroxy tamoxifen, 10^{-6} M (4OHT), bisphenol, 10^{-6} M (BP) and bisphenol A, 10^{-6} M (BPA) regulated apoptotic genes in MCF7 : 5C cells after 48 h of treatment versus vehicle.

Menopause: The Journal of The North American Menopause Society
Vol. 20, No. 4, pp. 372-382
DOI: 10.1097/gme.0b013e31828865a5
© 2013 by The North American Menopause Society

2012 NAMS/Pfizer — Wulf H. Utian Endowed Lecture

Scientific rationale for postmenopause delay in the use of conjugated equine estrogens among postmenopausal women that causes reduction in breast cancer incidence and mortality

Ifeyinwa Obiorah, MBBS, MSc, and V. Craig Jordan, OBE, PhD, DSc, FMedSci

Abstract

High-dose synthetic estrogens were the first successful chemical therapy used in the treatment of metastatic breast cancer in postmenopausal women, and this approach became the standard of care in postmenopausal women with metastatic breast cancer between the 1950s and the end of the 1970s. The most recent analysis of the Women's Health Initiative estrogen-alone trial in hysterectomized women revealed a persistently significant decrease in the incidence of breast cancer and breast cancer mortality. Although estrogens are known to induce the proliferation of breast cancer cells, we have shown that physiologic concentrations induce apoptosis in breast cancer cells with long-term estrogen deprivation. We have developed laboratory models that illustrate the new biology of estrogen-induced apoptosis or growth to explain the effects of estrogen therapy. The key to the success of estrogen therapy lies in a sufficient period of withdrawal of physiologic estrogens (5-10 y) and the subsequent regrowth of nascent breast tumor cells that survive under estrogen-deprived conditions. These nascent tumors are now vulnerable to estrogen-induced apoptosis.

Key Words: Breast cancer – Estradiol – Women's Health Initiative – Apoptosis.

Despite the extensive progress made in the management of breast cancer, it still remains the most common cause of cancer and the second leading cause of cancer deaths in women in the United States. An estimated 230,480 new cases of invasive breast cancer and an estimated 57,650 cases of breast carcinoma in situ were projected to occur in 2011.[1] In addition, approximately 39,520 women were expected to die of breast cancer in 2011.[1] Multiple risk factors for breast cancer have been established, and estrogen is a key growth stimulus in the development and progression of the disease. Beatson[2] in 1896 provided the first medical evidence of the estrogen dependency of breast cancer. The conclusion that a woman's ovaries provided the fuel that maintained breast cancer was based on the observed remission of advanced breast tumors in a premenopausal woman who underwent bilateral oophorectomy. Boyd[3] surveyed all known cases in 1900 and concluded a 30% response rate of breast cancer to any anti–hormone therapy (HT)—a figure that has stood the test of time. Animal models provided further evidence on the role of estrogens in breast cancer growth. Lathrop and Loeb[4] in 1916 observed a decrease in the occurrence of mammary carcinomas in castrated immature female mice. Estrogen, an ovarian hormone, was subsequently extracted and purified, and it induced vaginal cornification in ovariectomized mice.[5] This advancement elucidated the biological properties of synthetic estrogens using ovariectomized mice, therefore establishing a connection between the mitogenic potential of estrogens and breast cancer. The strategy of targeting the estrogen receptor (ER) has led to the discovery of endocrine therapies that either block estrogen action by using

Received November 27, 2012; revised and accepted January 16, 2013.

From the Lombardi Comprehensive Cancer Center, Department of Oncology, Georgetown University Medical Center, Washington, DC.

The content of this article was presented by Dr. V. Craig Jordan as the NAMS/Pfizer — Wulf H. Utian Endowed Lecture on October 6, 2012, in Orlando, FL at the Annual Meeting of The North American Menopause Society (NAMS).

An endowment to NAMS from Pfizer established this annual lectureship, with faculty selected by The North American Menopause Society Scientific Program Committee.

Funding/support: This work was supported by the Department of 1090 Defense Breast Program Center of Excellence (award W81XWH-06-1-0590 to V.C.J.), the Susan G. Komen for the Cure Foundation (award SAC100009), and the Lombardi Comprehensive Cancer 1095 Center Support Grant (core grant NIH P30 CA051008).

The views and opinions of the authors do not reflect those of the US Army or the Department of Defense.

Financial disclosure/conflicts of interest: None reported.

Address correspondence to: V. Craig Jordan, OBE, PhD, DSc, FMedSci, Georgetown University Medical Center, Suite E501, Research Building, 3970 Reservoir Road NW, Washington, DC 20057. E-mail: vcj2@georgetown.edu

Copyright © 2013 The North American Menopause Society. Unauthorized reproduction of this article is prohibited.

selective ER modulators (SERMs) or deprive the ER of estrogens by using aromatase inhibitors (AIs).[6] Anti-HTs remain the gold standard of care in the treatment and prevention of ER-positive breast cancer.[7]

WOMEN'S HEALTH INITIATIVE: RISKS AND BENEFITS

The use of HT continues to be a source of controversial debate. The Women's Health Initiative (WHI)[8] is a set of clinical studies designed to investigate and develop strategies for the prevention and control of common causes of morbidity and mortality in postmenopausal women. The WHI was initiated in 1991 with a tentative end date in 2007 to provide research findings on the effects of postmenopausal HT, calcium and vitamin D supplements, and diet modification on cardiovascular disease, osteoporosis, breast cancer, and colorectal cancer. The HT arm of the study includes a random assignment of 27,500 women either to placebo, estrogen plus progestin (HT), or estrogen alone (estrogen therapy [ET]; for hysterectomized women). The principal outcomes of the study were the incidences of coronary heart disease (CHD) and osteoporosis, with breast cancer as a potential adverse outcome.[8] To date, this is the largest, randomized, placebo-controlled trial that conducted parallel studies to assess the outcomes of combined HT and estrogen-alone therapy.[9]

Estrogen-plus-progestin therapy

Treatment with HT was associated with elevated overall risks. CHD is a leading cause of death in postmenopausal women, and previous animal studies have shown that estrogen treatment has the potential to prevent the development of coronary atherosclerosis.[10] Therefore, the results of the effect of HT on CHD were highly awaited. Women received conjugated equine estrogens (CEE) 0.625 mg/day plus medroxyprogesterone or placebo 2.5 mg/day. After a mean follow-up of 5.2 years, the trial was terminated because not only was the combination therapy not cardioprotective but HT also elevated the risk of CHD (hazard ratio [HR], 1.24; 95% CI, 1-1.54), which was most apparent at 1 year of therapy.[11] Furthermore, HT was associated with a doubled risk of venous thrombosis[12] and an increased risk of stroke,[13] and it did not confer protection against peripheral arterial disease,[14] dementia, and cognitive decline.[15] Combined HT increased total breast cancer (HR, 1.24; $P < 0.001$) and invasive breast cancer (HR, 1.24; $P = 0.003$) compared with placebo after 5 years of therapy.[16] The breast cancers in the group receiving HT were diagnosed initially at a slightly lower rate during the first 2 years of the study but subsequently increased throughout the intervention period. The elevated risk of breast cancer markedly declined soon after the cessation of combined HT.[17] Short-term use of HT was associated with a decrease in colorectal cancer cases when compared with placebo ($P = 0.0003$), but no protective effect against colorectal cancer mortality was observed during the 8-year intervention period and the follow-up period.[18] Although HT did not increase lung cancer rates,[19] more women from the combined therapy group died of lung cancer, in particular from non–small cell lung cancer. In addition, there was no significant difference in the incidences of endometrial cancer and ovarian cancer in both treatment arms.[20] The benefits of HT include a significantly decreased incidence of bone fractures.[21] Seven hundred thirty-three women (8.6%) in the estrogen-plus-progestin group and 896 women (11.1%) in the placebo group developed a fracture (HR, 0.76; 95% CI, 0.69-0.83). Total hip bone mineral density increased by 3.7% after 3 years of therapy with HT compared with 0.14% in the control group ($P < 0.001$). Current recommendations[22] include the use of individualized HT. HT can be initiated around the time of menopause to treat menopause-related symptoms and to prevent osteoporosis in high-risk women. Treatment should be considered in conjunction with personal risk factors, such as risk of venous thrombosis, CHD, stroke, and breast cancer.

Estrogen-alone treatment

Between 1993 and 1998, 10,739 postmenopausal women aged 50 to 79 years who had had a hysterectomy were treated with 0.625 mg of either CEE or placebo.[23] Despite the early termination of the combined hormone trial, the WHI ET study continued under careful scrutiny. However, in February 2004, the National Institutes of Health decided to terminate the intervention phase of the trial before the scheduled closeout interval from October 2004 to March 2005. The primary outcomes of the trial were the rate of CHD, the incidence of invasive breast cancer, and the incidences of stroke, pulmonary embolism, colorectal cancer, hip fractures, and death from other causes. After a mean follow-up of 6.8 years, no significant effect of ET on CHD rates was observed compared with placebo. During the active intervention period, a reduction in coronary events occurred in women assigned to ET (HR, 0.95; 95% CI, 0.79-1.16).[24] The reduction was more significant in women aged 50 to 59 years (HR, 0.63; 95% CI, 0.36-1.08). However, a 39% increase in the incidence of stroke was observed in the ET group ($P = 0.07$), whereas the risk of venous thromboembolism (VTE), including deep venous thrombosis and pulmonary embolism, increased by 33% in the ET arm, but only the increased rate of deep venous thrombosis was statistically significant ($P = 0.03$).[23] The increased risk for VTE was most apparent in the first 2 years, and the increased risk was less than that observed for the estrogen-plus-progestin study.[25] Therefore, ET provided no overall protection against cardiovascular disease in healthy postmenopausal women. Interestingly, invasive breast cancer was diagnosed at a 23% lower rate in the ET group (26 vs 33 per 10,000 person-years); however, this did not reach statistical significance ($P = 0.06$).[23] No statistical differences in colorectal cancer rates or total cancer rates were observed. The major positive finding in the ET trial in 2004 was a 30% to 39% reduction in the rates of fractures (HR, 0.70; 95% CI, 0.63-0.79). In addition, ET did not significantly affect overall mortality rate or cause-specific mortality. Results from the final analysis of the WHI ET trial[26] showed that a persistent decrease in the risk of breast cancer was associated with

Copyright © 2013 The North American Menopause Society. Unauthorized reproduction of this article is prohibited.

ET and was 0.27% per year compared with 0.35% per year in the placebo arm, reaching statistical significance (HR, 0.77; 95% CI, 0.62-0.95) after a median follow-up of 11.8 years. There was no difference between intervention HR and post-intervention HR ($P = 0.76$). Breast cancer risk reduction in the ET arm was most apparent in women without benign breast disease ($P = 0.01$) or a family history of breast cancer ($P = 0.02$). Breast cancer mortality was reduced in the ET group (six deaths, 0.009% per year) compared with controls (16 deaths, 0.024% per year; HR, 0.37; 95% CI, 0.13-0.91; $P = 0.03$). Fewer women in the ET group died of any cause after a breast cancer diagnosis than did women in the placebo arm ($P = 0.04$). Although breast cancer rates and mortality were lower for women who received ET, beneficial effects are yet to be determined in high-risk groups, and adverse effects of stroke and VTE remain problematic. HT seemed to have more risks, and the only clinical benefit was the reduction of osteoporosis, whereas ET, in addition to fracture prevention, decreased the incidence of and mortality from breast cancer. The question that needs to be addressed is whether it is possible to decipher the paradox that HT and ET produce completely different biological results (ie, HT increases, whereas ET reduces, the incidence of breast cancer). If clarity is possible, perhaps this knowledge can be used appropriately to help women.

CHEMICAL THERAPY FOR THE TREATMENT OF BREAST CANCER

The first successful chemical therapy used to treat cancer was discovered by Sir Alexander Haddow, a British-born physician. Haddow[27] grew up in Broxburn, a small town 10 miles west of Edinburgh, Scotland. He became motivated to study medicine and biology after he was admitted to a hospital for a perforated appendix and had the marvelous opportunity to observe the daily visits of great Edinburgh surgeons who were inspired to make a difference in an era when public health and hygiene were far from being developed. Upon graduation from medical school, he assisted with routine investigation of infections from the entire southeast of Scotland. While studying bacterial colony formation, he realized its resemblance to the formation of chemical tumors in higher forms.[28] He went on to study the influence of carcinogenic substances on normal and malignant growth, as well as the drug resistance of cells to resultant tumors. Incidentally, he found that many carcinogenic hydrocarbons also retarded the growth of malignant tumors.[29] To elucidate the molecular mechanism of these compounds, we paid particular attention to the inhibitory action of synthetic estrogens. In that era, reviews of animal experiment showed that treatment of animals with estrogens induced carcinoma of certain organs such as the cervix, uterus, and breast. The paradoxical action of estrogens showing growth properties, induction of tumors, and growth-retarding effects in certain circumstances led to the first ever reported clinical trial[30] in 1944. Seventy-three women with advanced cancer were recruited to the study. Forty postmenopausal women with metastatic breast cancer and 30 cases of malignant disease in other organs received treatment with synthetic estrogens: triphenylchlorethylene, triphenylmethylethylene, or stilbestrol. Ten of 22 women with advanced breast cancer treated with triphenylchlorethylene showed significant regression of the tumors. Breast cancer patients treated with stilbestrol showed that 5 of 14 cases underwent a regression of tumors similarly noted with triphenylchlorethylene. Among four cases of breast cancer treated with triphenylmethylethylene, only one showed a favorable response. Thirty cases of advanced cancer—excluding breast cancer but including cancer of the skin, maxillary antrum, urinary bladder, ovary, and prostate, and leukemia—were treated with triphenylchlorethylene; only carcinomas of the prostate and bladder showed partial regression of the tumors. Data from the clinical study suggest that the success of ET in breast cancer was dependent on the menopausal state of the women. Haddow and David[31] stated, "When the various reports were assembled at the end of that time, it was fascinating to discover that rather general impression, not sufficiently strong from the relatively small numbers in any single group, became reinforced to the point of certainty; namely, the beneficial responses were three times more frequent in women over the age of 60 years than in those under that age; that estrogens may, on the contrary, accelerate the course of mammary cancer in younger women, and that their therapeutic use should be restricted to cases 5 years beyond the menopause. Here was an early and satisfying example of the advantages which may accrue from cooperative clinical trial." Therefore, the longer that a woman is postmenopausal, the greater is the probability of tumor regression in metastatic breast cancer. However, "…the extraordinary extent of tumor regression observed in perhaps 1% of postmenopausal cases (with estrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us…."[31] Therefore, at this point in 1970, the underlying mechanism of estrogen-induced tumor regression still remained unanswered.

TIME TO TREATMENT FAILURE AND TRANSITION TO TAMOXIFEN

In the 1960s, based on the data from clinical trials, high-dose stilbestrol became the mainstay of treatment in postmenopausal women with advanced breast cancer. However, the estrogen treatment was not without pitfalls. It was imperative that ET not be instituted until ovarian secretion has ceased in a woman. The overall objective remission rate for estrogen treatment in 407 women with advanced breast cancer was 31%.[32] The remission rate was associated with the increasing number of years after menopause (Table 1). The rate of regression was 9% in women who were less than 5 years postmenopausal, whereas the rate increased to 35% in women who have been postmenopausal for more than 5 years, corresponding with what was observed by Haddow and David.[31] A remarkable feature of ET observed in this setting was the "withdrawal response." Stoll[32] previously described that when tumor response to estrogen administration was lost, 30% of

 © 2013 The North American Menopause Society

Copyright © 2013 The North American Menopause Society. Unauthorized reproduction of this article is prohibited.

TABLE 1. *Objective response rates in postmenopausal women with metastatic breast cancer who are using high-dose estrogen therapy*

Age since menopause	Patients, n	Regression, %
0-5 y (postmenopausal)	63	9
>5 y (postmenopausal)	344	35

The 407 patients are divided in relation to menopause status.[32] The objective remission rate of breast cancer tumors was higher in women more than 5 years postmenopausal.

cases on treatment withdrawal underwent a second but shorter period of tumor remission, indicating that women can be palliated over many years by intermittent estrogen and subsequent withdrawal. The introduction of tamoxifen, a nonsteroidal antiestrogen, in the late 1970s revolutionized the clinical practice of endocrine treatment of ER-positive breast cancer.[33] The evidence supporting the antiestrogenic action of tamoxifen was based on its antitumor action using carcinogen-induced rat mammary tumor models[34,35] and subsequent athymic mice transplanted with human breast cancer cell lines.[36] The clinical efficacy of tamoxifen was first evaluated in women with late or recurrent carcinoma of the breast.[37] Results from this study were compared with unpublished data from breast cancer patients who were treated with diethylstilbestrol (DES) at the same hospital. Although response rates were similar, women from the DES arm experienced more severe adverse effects. Similarly, Ingle et al[38] directly compared the use of either tamoxifen or DES in the treatment of advanced breast cancer in postmenopausal women. Analysis of the study revealed that there was no statistically significant difference between the efficacies of both treatments; however, similar to the study by Cole et al,[37] toxicity was greater for the women receiving DES and was severe enough for some women who dropped out of the study. Based on these data, DES fell out of favor for the treatment of metastatic breast cancer, and tamoxifen became the preferred agent. Tamoxifen subsequently became the standard of care in the adjuvant treatment and prevention of breast cancer. Several clinical trials investigated the long-term benefits of adjuvant tamoxifen therapy. An overview[39] of 55 randomized trials that compared the use of adjuvant tamoxifen versus no tamoxifen in breast cancer patients worldwide revealed that the reduction in recurrence for 1-year, 2-year, and 5-year trials during about 10 years of follow-up were 21%, 29%, and 47%, respectively. A highly significant trend toward a greater effect, based on longer treatment, was observed. A corresponding reduction in mortality of 12%, 17%, and 26%, respectively, was observed, and this trend was also significant ($P = 0.003$). A subsequent report of the meta-analysis[40] showed that 5 years of adjuvant tamoxifen decreased the annual breast cancer mortality rate by 31% at 15-year follow-up, irrespective of the use of chemotherapy, age, progesterone receptor status, or other tumor characteristics in ER-positive breast cancer patients. Furthermore, the reduction observed at 5 years is significantly ($P < 0.00001$ for recurrence; $P = 0.01$ for breast cancer mortality) more effective when compared with 1 to 2 years of adjuvant tamoxifen. More recently, results from the Adjuvant Tamoxifen—Longer Against Shorter (ATLAS) trial[41] showed that 10 years of adjuvant treatment with tamoxifen produced a further reduction in breast cancer recurrence and mortality when compared with 5 years of tamoxifen therapy. It is perhaps instructive to point out that the main effect of the decrease in mortality with a decade of tamoxifen occurs in the decade after tamoxifen treatment is stopped. This further suggests the hypothesis originally proposed in the early 1990s—that a woman's own estrogen destroys the appropriately sensitive tamoxifen-resistant micrometastasis.[42] Thus, the study of the evolution of anti–hormone drug resistance to tamoxifen ironically provided an insight into the mechanism of estrogen-induced apoptosis studied today. Nevertheless, the current recommendation for the adjuvant endocrine treatment of ER-positive breast cancer is that tamoxifen be used as a first-line treatment in premenopausal or perimenopausal women but that postmenopausal women take AIs as a primary agent for 5 years or for 2 to 3 years after tamoxifen[43] for a total of 5 years of initial anti-HT. The latter is based on several studies where AIs have shown some superiority to tamoxifen as first-line agents in the treatment of postmenopausal women with breast cancer, as well as a significant reduction in endometrial cancer.[44-46] Furthermore, 5 years of AI therapy have been shown to be highly beneficial as an extended adjuvant treatment in postmenopausal women who had previously received 5 years of tamoxifen therapy, showing a 2.9% improvement in disease-free survival at 4 years (HR, 0.68; $P = 0.0001$) when compared with placebo.[47,48]

EVOLUTION OF ANTI–HORMONE DRUG RESISTANCE

Despite the ability of long-term adjuvant tamoxifen to improve survival, some women develop disease recurrence owing to acquired drug resistance. Early laboratory models were created to understand the development of drug resistance and subsequent deployment of second-line therapies. Treatment of ovariectomized athymic mice transplanted with ER-positive MCF-7 tumors with tamoxifen initially caused tumor regression, but subsequent regrowth of tumors occurred despite continuous tamoxifen treatment.[49] Retransplantation of the resistant tumors into athymic mice or rats led to tumor growth in response to tamoxifen and estradiol (E_2).[50] Evaluation of these tumors showed that the tamoxifen-stimulated tumors contained twice the ER content of E_2-induced tumors.[50,51] However, continuous treatment of transplanted MCF-7 tamoxifen-resistant tumors with either a pure antiestrogen or no treatment in nude mice results in no tumor growth.[52] Because AIs deprive the ER of estrogens and fulvestrant degrades the ER, the findings from these studies presaged the clinical use of these drugs as second-line agents after failure of tamoxifen treatment.[53] However, the early models of drug resistance to SERMs are based on short-term treatments and replicate the failure of tamoxifen after 1 or 2 years of treatment in advanced breast cancer, and this represents phase 1 SERM resistance. To mimic 5 years of adjuvant tamoxifen therapy for micrometastatic breast cancer,

Copyright © 2013 The North American Menopause Society. Unauthorized reproduction of this article is prohibited.

we created laboratory models to induce phase 2 resistance to SERMs by serially transplanting tamoxifen-stimulated MCF-7 tumors into tamoxifen-treated athymic mice for more than 5 years.[54] Interestingly, on stopping tamoxifen, the tamoxifen-stimulated MCF-7 tumors rapidly regressed in response to physiologic E_2, although about 50% of tumors regrew after E_2 treatment. The paradoxical E_2-induced apoptosis suggests that a woman's own estrogen may produce an antitumor effect on presensitized micrometastatic tumors after 5 years of adjuvant tamoxifen.[42] Failure of tumor regression after exhaustive anti-HT with a paradoxical E_2-inhibited growth (phase 3 resistance) indicates a potential treatment plan using E_2 as third-line endocrine therapy.[55] Tumors that regrow after E_2-induced apoptosis revert back to the original cancer phenotype and are again sensitive to the antitumor actions of tamoxifen or AIs.[54]

ET IN METASTATIC BREAST CANCER

In more recent years, the use of estrogens continues to show clinical benefit to postmenopausal women with advanced breast cancer in an estrogen-deprived setting. Lonning et al[56] treated with high-dose DES (5 mg TID) 32 women who had previously taken multiple endocrine therapies. Four women achieved complete response, whereas four women achieved partial response. In addition, five women had an objective response lasting more than 52 weeks, whereas two patients had stable disease for more than 6 months. Six patients dropped out of the study owing to severe adverse effects. However, one of the patients who had complete regression of cytologically confirmed chest wall relapse and 5 years of DES therapy remained disease-free for 10 years and 6 months after starting treatment. A long-term follow-up of the study of Ingle et al[38] that compared DES therapy to tamoxifen showed that the 5-year survival was 35% for DES and 16% for tamoxifen ($P = 0.039$).[57] However, DES treatment was associated with nausea, edema, and vaginal bleeding problems, whereas hot flushes were more commonly observed with tamoxifen. Another 2009 clinical study[58] reported findings on the treatment of postmenopausal women who had AI-resistant metastatic breast cancer with low-dose E_2 (6 mg) and high-dose E_2 (30 mg). Clinical benefit rates were 28% (95% CI, 18-41) and 29% (95% CI, 19-42) in the high-dose arm and low-dose arm, respectively, but adverse event rate was higher in the 30-mg group when compared with the 6-mg group. Six patients who were estrogen-responsive were retreated with AIs, among which two had partial response and one had stable disease. This indicates resensitization to estrogen deprivation and correlates with the hypothesis on SERM resistance.[54]

EXPERIMENTAL APPROACH TO DECIPHERING THE MECHANISM OF E_2-INDUCED APOPTOSIS

A novel cell model[59] was developed by our laboratory to address concerns on acquired resistance to long-term estrogen deprivation. An ER-positive/progesterone receptor–negative hormone-independent breast cancer cell line, MCF-7:5C (a variant clone of wild-type MCF-7 cells), was obtained by culturing MCF-7 cells continuously in estrogen-free media. Treatment with physiologic E_2 for 6 days caused a dramatic 90% reduction in the growth of MCF-7:5C cells.[60] The growth inhibition observed was confirmed by annexin V and 4′,6-diamidino-2-phenylindole staining to be apoptosis. Fulvestrant also reduced the growth of MCF-7:5C cells, but the growth inhibition was not caused by apoptosis.[61] Furthermore, these cells were resistant to 4-hydroxytamoxifen (4-OHT). The tumorigenic potential of MCF-7:5C cells was examined by injecting cells into ovariectomized athymic mice, and these cells were found to spontaneously grow into tumors in the absence of E_2.[61] In contrast, MCF-7:5C tumors in mice treated with E_2 regressed in a time-dependent manner and became undetectable after 8 weeks of treatment. Similarly, fulvestrant also decreased the growth of MCF-7:5C tumors, but the reduction was statistically significantly less when compared with that of E_2 ($P < 0.001$). MCF-7:2A cells[62,63]—another long-term estrogen-deprived cell line derived from MCF-7 cells—is more resistant to E_2-induced apoptosis. Based on clinical data showing that only about 30% of patients respond to estrogens after anti–hormone resistance, it seemed imperative to see whether E_2-induced apoptosis could be enhanced in anti–hormone-resistant cells. Overexpression of Bcl-2 elevates cellular glutathione (GSH) level, which is associated with increased resistance to chemotherapy apoptosis,[64,65] whereas restoration of apoptosis occurs in Bcl-2–expressing cells depleted of GSH.[66] MCF-7:2A cells express high levels of GSH synthetase and GSH peroxidase 2, which are involved in GSH synthesis.[67] Exposure of MCF-7:2A cells to a combination therapy of E_2 and buthionine sulfoximine (BSO; a GSH inhibitor) for 48 to 96 hours produced a sevenfold increase in apoptosis, whereas individual treatments had no significant effect on growth. The in vitro findings correlated with in vivo data from a mouse xenograft model in which daily administration of BSO either as a single agent or in combination with E_2 significantly decreased the tumor growth of MCF-7:2A cells. Thus, this provides a potential strategy for future clinical trials involving combination therapy with BSO and low-dose estrogen to improve response in patients with anti–hormone-resistant advanced breast cancer.[68]

CONJUGATED EQUINE ESTROGENS

Extensive progress in the production of estrogen preparations for commercial use was made by scientists at Wyeth Pharmaceuticals Canada (then Ayerst), who extracted conjugated estrogens from a pregnant horse's urine.[69] In 1942, US Food and Drug Administration approval[70] was obtained for the clinical use of CEE (premarin) for the treatment of menopausal symptoms and related conditions. There was an initial worldwide acceptance of CEE in the 1960s; however, increased risks of developing endometrial cancer led to a decline in prescriptions to postmenopausal women.[71,72] In the 1980s, a new surge of interest in the use of ET for the treatment of

© 2013 The North American Menopause Society

Copyright © 2013 The North American Menopause Society. Unauthorized reproduction of this article is prohibited.

osteoporosis led to clinical studies of women receiving either estrogen-alone therapy or estrogen-plus-progestin therapy. Women on estrogen and progestin treatment had a lower incidence of endometrial cancer,[73,74] indicating that progestin blocked the proliferative effect of estrogens on the endometrial lining. As a result, CEE were approved for the treatment and prevention of osteoporosis; women with an intact uterus were given progestin in addition to estrogens. CEE are made up of conjugated estrogens, and the tablet consists of at least 10 estrogens (Fig. 1), including estrone (59.2%), equilin (26.9%), 17α-dihydroequilin (16.3%), 17α-estradiol (4.32%), 17β-dihydroequilin (1.76%), 17α-dihydroequilenin (1.76%), 17β-dihydroequilenin (3.36%), equilenin (2.4%), 17β-estradiol (0.8%), and $\Delta^{8,9}$-dehydroestrone (4.16%). Generic synthetic versions of CEE are not currently approved by the Food and Drug Administration based on inadequacies noted in their active ingredients, bioequivalence, safety, and effectiveness.[75]

EFFECT OF CEE ON BREAST CANCER CELLS

Long-term concentrations of estrogen-deprived MCF-7 breast cancer cells undergo apoptosis upon treatment with physiologic E_2.[61] Based on the preliminary results of the WHI CEE study, we decided to elucidate the biological properties of the main estrogens in CEE in two different models of breast cancer cells. Estrogens have been shown to regulate the growth of ER-positive MCF-7 breast cancer cells. To study the biological activity of the actual estrogens, namely, equilin, estrone, and equilenin, we tested their ability to induce proliferation in MCF-7:WS8 cells, which contain ER and have retained estrogen responsiveness for a sustained period of continuous cell culture.[76] MCF-7 cells were grown in estrogen-free media for 3 days and treated with various concentrations of equilin, estrone, and equilenin, and their effects were compared with E_2 (Fig. 2A). All three estrogens were able to induce the cell growth of MCF-7 cells to the maximal level as E_2 in a dose-dependent manner. Equilin and estrone induced cell proliferation with maximal stimulation occurring at 0.1 nM, whereas equilenin reached maximal stimulation at 1 nM as compared with 0.01 nM for E_2. Next, we investigated the growth properties of equilin, estrone, and equilenin in long-term estrogen-deprived MCF-7:5C cells in comparison with E_2. Figure 2B shows that equilin, estrone, and equilenin drastically inhibited the growth of MCF-7:5C cells at comparable concentrations to E_2. Maximal growth inhibition was achieved with E_2 at 0.1 M, whereas equilin and estrone/equilenin

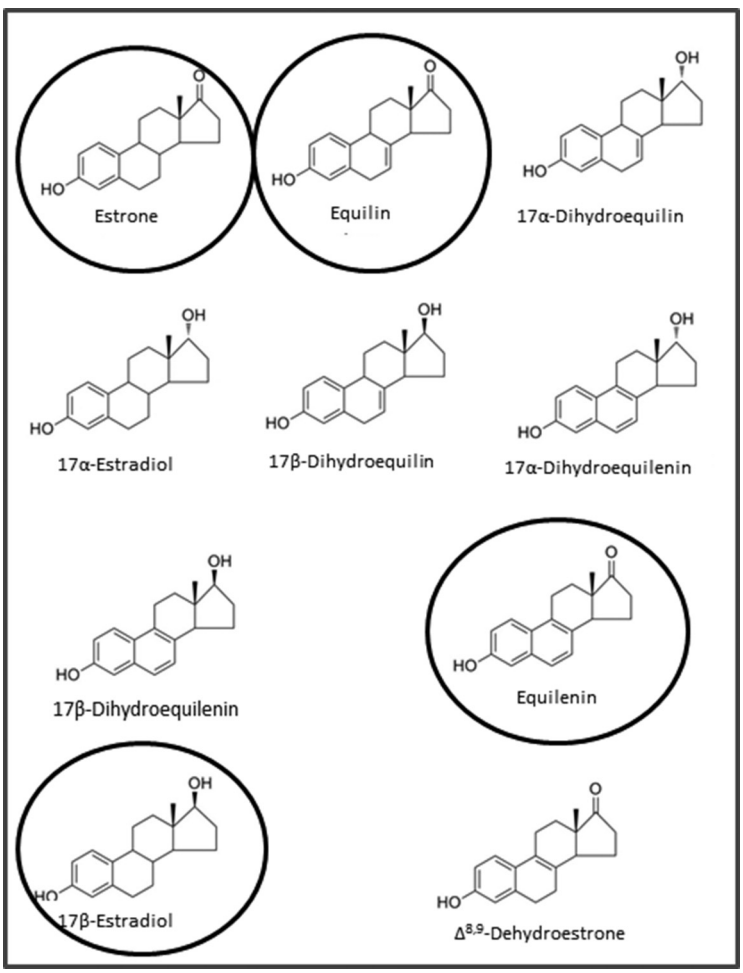


FIG. 1. Structures of the estrogenic constituents of premarin. Estradiol, equilin, estrone, and equilenin were used in our experimental studies.

Copyright © 2013 The North American Menopause Society. Unauthorized reproduction of this article is prohibited.

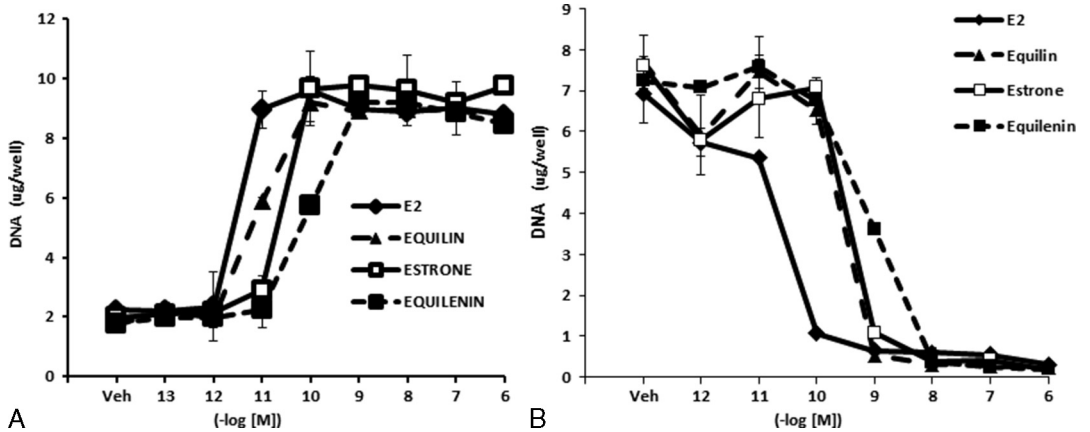


FIG. 2. Cell proliferation assay analysis of the biological properties of active steroids in conjugated equine estrogens in breast cancer cells. **A:** MCF-7 cells were grown in estradiol (E_2)–stripped media for 3 days; treated with various concentrations of E_2, equilin, estrone, and equilenin for 7 days; and compared with vehicle-treated cells (Veh; control). **B:** Equilin, estrone, and equilenin drastically inhibited the growth of MCF-7:5C cells similarly to E_2. The experiments were completed in triplicate and performed as previously described.[61]

reached maximal growth inhibition at 1 and 10 nM, respectively, after 7 days of treatment. To determine if the observed estrogen-induced growth inhibition of MCF-7:5C cells was caused by apoptosis, we used MCF-7:5C cells as controls, or E_2, equilin, estrone, or equilenin for 72 hours, and we measured apoptosis level using annexin V staining. E_2, equilin, estrone, and equilenin all showed increased apoptotic staining compared with control-treated cells (Fig. 3). The ability of conjugated estrogens to inhibit growth and to induce apoptosis in MCF-7:5C cells, and not parental MCF-7 cells, suggests that these biological properties are dependent on the duration of estrogen deprivation in breast cancer cells.

MOLECULAR MECHANISMS OF ESTROGEN-INDUCED APOPTOSIS

To decipher the precise series of events that precede estrogen-induced apoptosis, we interrogated differential gene expression in response to E_2 using Affymetrix-based microarray analysis.[63]

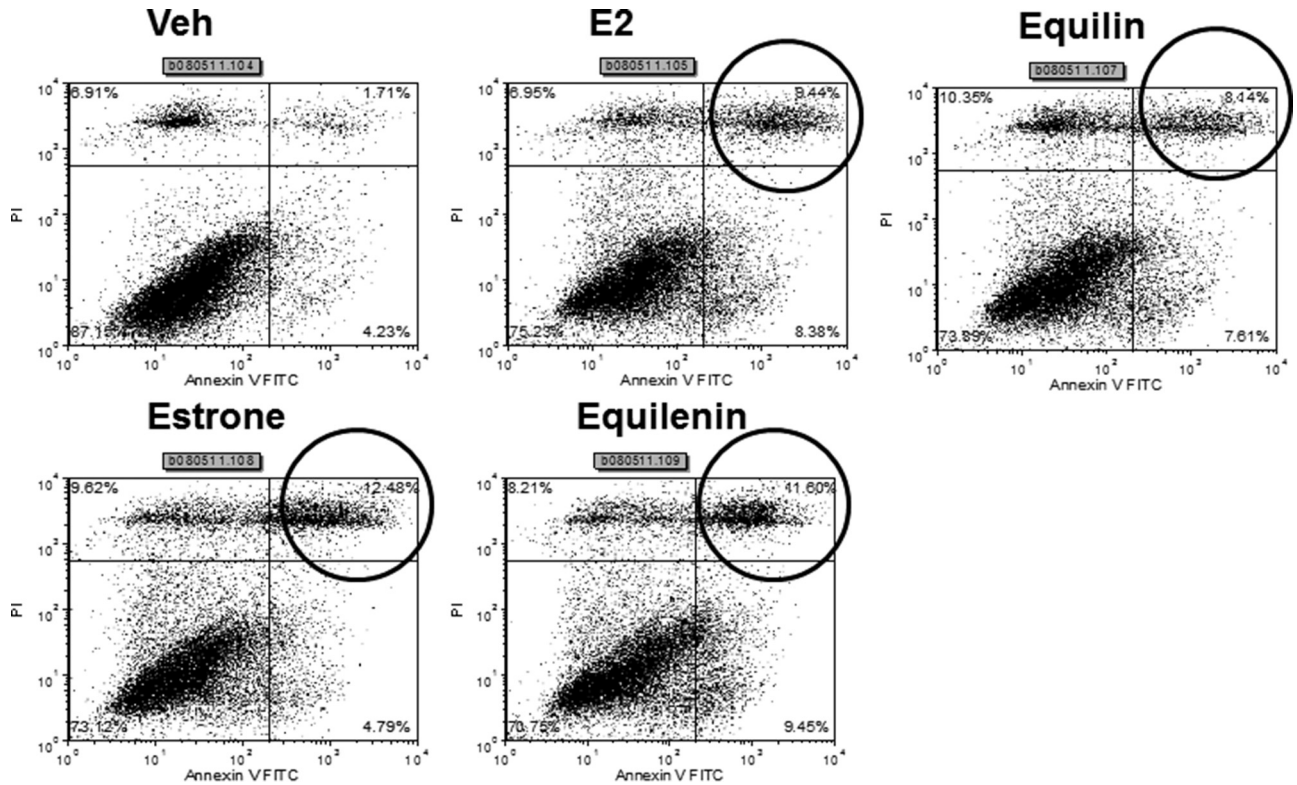


FIG. 3. Effects of estradiol (E_2) and active estrogens in conjugated equine estrogens on apoptosis in MCF-7:5C cells. MCF-7:5C cells were seeded in 100-mm plates and treated with vehicle-treated cells (Veh; control), 1 nM E_2, 1 nM equilin, 1 nM estrone, and 1 μM equilenin for 72 hours. Cells were stained with fluorescein isothiocyanate (FITC)–annexin V and propidium iodide (PI) and analyzed by flow cytometry as previously described.[61] The upper right box of Veh have less apoptotic cells (1.71%), whereas this fraction is increased for all estrogens (circled upper right-hand box).

© 2013 The North American Menopause Society

Copyright © 2013 The North American Menopause Society. Unauthorized reproduction of this article is prohibited.

Specific genes were identified for MCF-7:5C, indicating that E_2 induced endoplasmic reticulum stress (ERS) and inflammatory stress responses that led to apoptosis. Identified ERS genes indicated that E_2 inhibited protein folding, leading to accumulation of unfolded proteins and widespread inhibition of protein translation with subsequent induction of cell death. In response to severe ERS, Bim (*B*cl-2–*i*nteracting *m*ediator of cell death; BCL211) was induced. Further evidence of the involvement of the mitochondrial pathway in E_2-induced apoptosis was reported by Lewis et al,[61] who showed increased expression of several proapoptotic proteins, including, Bax, Bak, Bim, Noxa, Puma, and p53, in E_2-treated MCF-7:5C cells. Reversal of the apoptotic effect of E_2 on these cells was observed with the blockade of Bax and Bim expression using short interfering RNAs. The involvement of the Fas/Fasl death signaling (extrinsic) pathway in the apoptotic effect of E_2 has been investigated. Osipo et al[77] demonstrated that E_2-induced regression of tamoxifen stimulated breast cancer tumors by activating the death receptor Fas and by suppressing the antiapoptotic/prosurvival factors nuclear factor-κB and HER2/neu. Similarly, the growth of raloxifene-resistant MCF-7 cells in vitro and in vivo was attenuated by E_2 by increasing Fas expression and by reducing nuclear factor-κB activity.[78] Studies are currently ongoing to determine the sequence of events that occur before E_2 induces apoptosis in MCF-7:5C cells.

The resolution of the crystal structure provided insight into the activation of the ER by E_2 and silencing by antiestrogens[79,80] and provides insight into the "trigger" mechanism for the ER complex. The shape that the ligands make with the ER is imperative to their ability to induce apoptosis in MCF-7:5C cells. E_2 is sealed within the hydrophobic pocket of the ligand binding domain of the ER by helix 12 and is bound by coactivators, leading to activation of apoptotic genes. On the other hand, 4-OHT pushes back helix 12 and prevents coactivator binding, and this may be responsible for its ability to block estrogen-induced apoptosis in MCF-7:5C cells. Knockdown of coactivator AIB1/SRC3 in MCF-7:5C cells led to a loss of the apoptosis-inducing effect of E_2, suggesting that AIB1 is a significant control hub of E_2 in apoptosis induced in these breast cancer cells.[81] Structure function studies show that the shape of estrogen[82] can modulate the shape of the estrogen-ER complex to induce apoptosis.[83] Hydroxylated triphenylethylenes, which are structurally similar to 4-OHT and have estrogenic properties in MCF-7 cells, have been shown to block E_2-induced apoptosis.[84] The antiestrogenic shape they make with the ER may be responsible

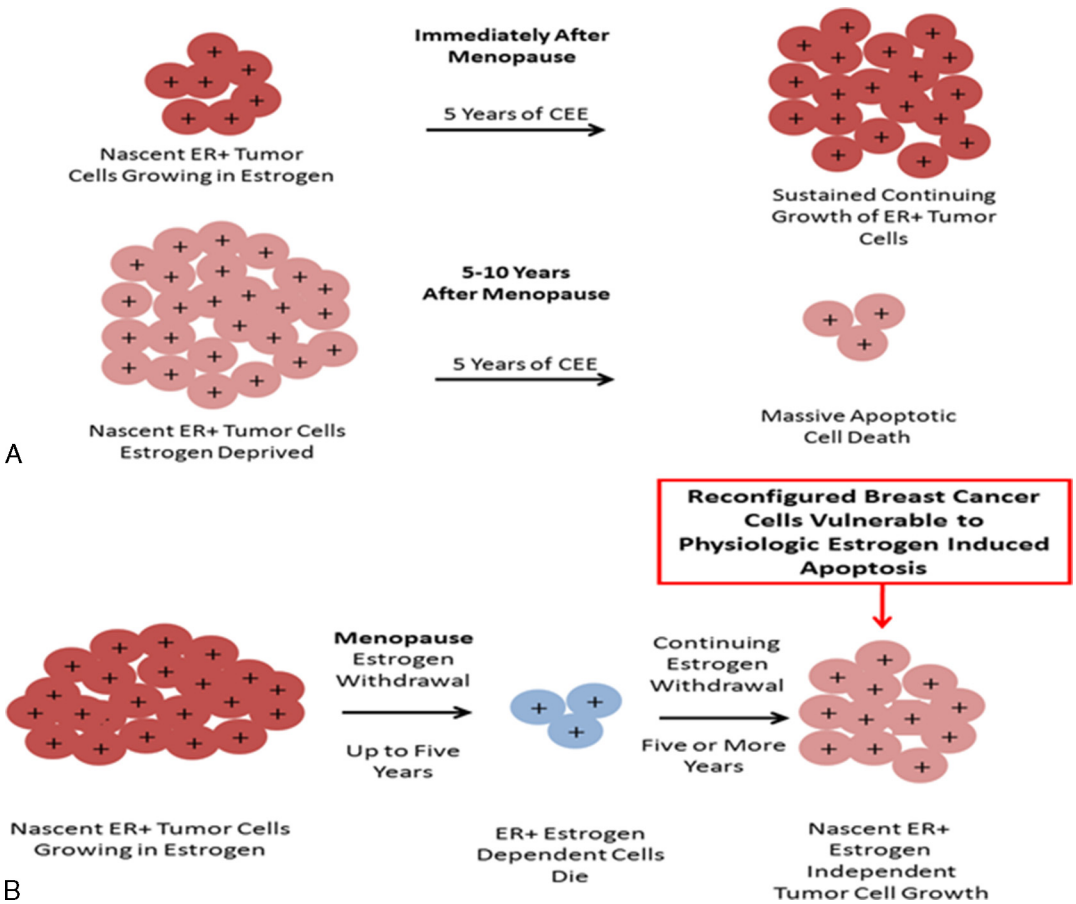


FIG. 4. The success of estrogen therapy is dependent on a woman's menopause status. **A:** Treatment of women with conjugated equine estrogens (CEE) immediately after menopause results in sustained growth of nascent estrogen receptor (ER)–positive tumors, whereas treatment 5 years after menopause causes apoptotic cell death. **B:** Estrogen withdrawal in postmenopausal women causes ER-positive cells to die, but some cells continue to grow independently of estrogen.

Copyright © 2013 The North American Menopause Society. Unauthorized reproduction of this article is prohibited.

for the delayed apoptotic effect of triphenylethylenes on MCF-7:5C cells. These pharmacologic studies are currently undergoing investigation and will be the focus of further reports.

DISCUSSION

Before the clinical use of anti-ET, high-dose estrogens were deemed to be effective in the induction of tumor regression in metastatic breast cancer.[30,32] In more recent times, ET shows significant clinical benefits on postmenopausal women who have undergone extensive antihormone treatment. Development of tamoxifen-stimulated tumors in athymic mice after a 5-year treatment with tamoxifen suggests that the development of anti–hormone resistance during years of treatment reconfigures the survival mechanism of breast cancer so that estrogen is no longer a potent mitogen that stimulates cell proliferation but rather becomes a death signal. Preclinical data clearly show that long-term estrogen deprivation of ER-positive MCF-7 breast cancers and subsequent treatment of cells with E_2 cause apoptosis of these cells. Creation of an estrogen-deprived environment either by withdrawal of estrogen treatment[32] or by exhaustive anti-HT increases the sensitivity of breast tumors to the ET, subsequently inducing tumor regression. Similarly, CEE alone reduce the incidence of breast cancer in hysterectomized postmenopausal women. This protective effect is not observed in women who receive addition progesterone therapy, suggesting that progestin may play a potential role in the increased breast cancer cases observed among postmenopausal women who received combined HT. To explain the aforementioned clinical data, laboratory studies show that estrogens in CEE were able to cause the proliferation of MCF-7 cells after these cells were grown in an estrogen-free medium for 3 days. This cell population is adapted to an environment rich in estrogen; thus, naturally, all cells grow with a "resupply" of natural steroidal estrogens. However, these same estrogens induce apoptosis to a similar extent as E_2 in MCF-7 cells that have been deprived of estrogen treatment for many years. The ability of ET to treat or prevent tumors is related to the menopause status of women and how long they have been physiologically deprived of estrogen. In the data by Stoll[32] (Table 1), the rate of remission of advanced breast cancer was significantly less in women who were less than 5 years postmenopausal (9%), and there was a 35% remission rate in women who were more than 5 years postmenopausal. It is important to stress that the majority of the women in the WHI CEE trial were older than 60 years, and the mean age at screening was 63.6 years. Here, the overall result was a reduction in breast cancer and mortality. There is a need for an "estrogen holiday" before starting ET. Induction of menopause in women gradually deprives the cells of estrogen. However, immediate treatment with estrogens may cause the growth of nascent ER-positive breast tumors that may increase breast cancer risk (Fig. 4A). The cells vulnerable to death with estrogens in CEE have been selected because estrogen deprivation at menopause causes estrogen-dependent nascent breast cancers to die, but not all die. Remaining cells that survive learn to grow without estrogen (Fig. 4B). These cells will continue to grow to produce breast cancer, unless exogenous estrogens induce apoptotic death. Therefore, 5 years of CEE treatment immediately after menopause will cause sustained continuing growth of ER-positive tumor cells. Because nascent ER-positive tumor cells have been estrogen-deprived in women who are 5 to 10 years postmenopausal, 5 years of CEE therapy induces massive apoptotic cell death, subsequent tumor cell death, and enhanced patient survival.

CONCLUSIONS

High-dose estrogen treatment is effective in causing tumor regression in metastatic breast cancer. The mechanism for this treatment was a paradox and was unknown for 60 years but is now being deciphered.[63] Objective tumor remission is observed in women who are more than 5 years postmenopausal.[30,32] ET administered to women in their late 60s causes a sustained decrease in breast cancer incidence and a decrease in mortality.[26] The question was "Why?" The key is the long-term estrogen deprivation of ER-positive breast cancer cells. We have created long-term estrogen deprivation breast cancer cell lines and, for the first time, have described the mechanism of estrogen-induced apoptosis. This new biology of estrogen-induced apoptosis can now be used to explain the effects of ET on reducing breast cancer incidence and mortality among women in their 60s.

REFERENCES

1. American Cancer Society. *Breast Cancer Facts & Figures 2011-2012*. Atlanta, GA: American Cancer Society Inc., 2011.
2. Beatson G. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. *Lancet* 1896;2:162-165.
3. Boyd S. On oophorectomy in cancer of the breast. *BMJ* 1900;2:1161-1167.
4. Lathrop AE, Loeb L. Further investigations on the origin of tumors in mice, 3: On the part played by internal secretion in the spontaneous development of tumors. *J Cancer Res* 1916;1:1-19.
5. Allen E, Doisy EA. An ovarian hormone: preliminary report on its localization, extraction and partial purification, and action in test animals. *JAMA* 1923;81:819-821.
6. Jordan VC, Brodie AMH. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* 2007;72:7-25.
7. Obiorah I, Jordan VC. Progress in endocrine approaches to the treatment and prevention of breast cancer. *Maturitas* 2011;70:315-321.
8. Design of the Women's Health Initiative clinical trial and observational study. The Women's Health Initiative Study Group. *Control Clin Trials* 1998;19:61-109.
9. Marjoribanks J, Farquhar C, Roberts H, Lethaby A. Long term hormone therapy for perimenopausal and postmenopausal women. *Cochrane Database Syst Rev* 2012;7:CD004143.
10. Karas RH. Animal models of the cardiovascular effects of exogenous hormones. *Am J Cardiol* 2002;90:22F-25F.
11. Manson JE, Hsia J, Johnson KC, et al. Estrogen plus progestin and the risk of coronary heart disease. *N Engl J Med* 2003;349:523-534.
12. Cushman M, Kuller, LH, Prentice, R, et al. Estrogen plus progestin and risk of venous thrombosis. *JAMA* 2004;292:1573-1580.
13. Wassertheil-Smoller S, Hendrix, S Limacher M, et al. Effect of estrogen plus progestin on stroke in postmenopausal women: the Women's Health Initiative: a randomized trial. *JAMA* 2003;289:2673-2684.
14. Hsia J, Criqui MH, Rodabough RJ, et al, for the Women's Health Initiative Investigators. Estrogen plus progestin and the risk of peripheral arterial disease. *Circulation* 2004;109:620-626.
15. Shumaker S, Legault C Rapp SR, et al. Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal

© 2013 The North American Menopause Society

Copyright © 2013 The North American Menopause Society. Unauthorized reproduction of this article is prohibited.

women: the Women's Health Initiative Memory Study: a randomized controlled trial. *JAMA* 2003;289:2651-2662.
16. Chlebowski R, Hendrix SL, Langer RD, et al. Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative randomized trial. *JAMA* 2003; 289:3243-3253.
17. Chlebowski RT, Kuller LH, Prentice RL, et al. Breast cancer after use of estrogen plus progestin in postmenopausal women. *N Engl J Med* 2009;360:573-587.
18. Chlebowski RT, Wactawski-Wende J, Ritenbaugh C, et al. Estrogen plus progestin and colorectal cancer in postmenopausal women. *N Engl J Med* 2004;350:991-1004.
19. Chlebowski RT, Schwartz AG, Wakelee H, et al. Oestrogen plus progestin and lung cancer in postmenopausal women (Women's Health Initiative trial): a post-hoc analysis of a randomised controlled trial. *Lancet* 2009;374:1243-1251.
20. Anderson G, Judd HL, Kaunitz AM, et al. Effects of estrogen plus progestin on gynecologic cancers and associated diagnostic procedures: the Women's Health Initiative randomized trial. *JAMA* 2003;290:1739-1748.
21. Cauley J, Robbins J, Chen Z, et al. Effects of estrogen plus progestin on risk of fracture and bone mineral density: the Women's Health Initiative randomized trial. *JAMA* 2003;290:1729-1738.
22. The North American Menopause Society. The 2012 hormone therapy position statement of The North American Menopause Society. *Menopause* 2012;19:257-271.
23. Anderson GL, Limacher M, Assaf AR, et al. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *JAMA* 2004;291:1701-1712.
24. Hsia J, Langer RD, Manson JE, et al. Conjugated equine estrogens and coronary heart disease: the Women's Health Initiative. *Arch Intern Med* 2006;166:357-365.
25. Curb J, Prentice RL, Bray PF, et al. Venous thrombosis and conjugated equine estrogen in women without a uterus. *Arch Intern Med* 2006;166: 772-780.
26. Anderson GL, Chlebowski RT, Aragaki AK, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *Lancet Oncol* 2012;13: 476-486.
27. Haddow A. An autobiographical essay. *Cancer Res* 1974;34:3159-3164.
28. Haddow A. The influence of carcinogenic substances on sarcomata induced by the same and other compounds. *J Pathol Bacteriol* 1938;47: 581-591.
29. Haddow A. Influence of certain polycyclic hydrocarbons on the growth of the Jensen rat sarcoma. *Nature* 1935;136:868-869.
30. Haddow A, Watkinson JM, Patterson E. Influence of synthetic oestrogens upon advanced malignant disease. *BMJ* 1944;2:393-398.
31. Haddow A, David A. Karnosky Memorial Lecture: thoughts on chemical therapy. *Cancer Res* 1970;26:737-754.
32. Stoll B. Palliation by castration or by hormone administration. In: Stoll B, ed. *Breast Cancer Management Early and Late*. London, England: William Herman Medical Books Ltd., 1977:133-146.
33. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2003;2:205-213.
34. Jordan VC. Antitumor activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)–induced rat mammary carcinoma model. *J Steroid Biochem* 1974;5:354.
35. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. *Eur J Cancer* 1976;12: 419-424.
36. Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. *Fed Proc* 1987;46:1870-1874.
37. Cole M, Jones C, Todd I. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. *Br J Cancer* 1971;25:270-275.
38. Ingle J, Ahmann D, Green S, et al. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *N Engl J Med* 1981;304:16-21.
39. Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;351: 1451-1467.
40. Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005; 365:1687-1717.
41. Davies C, Pan H, Godwin J, et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor–positive breast cancer: ATLAS, a randomised trial [published online ahead of print December 4, 2012]. *Lancet* doi: 10.1016/S0140-6736(12)61963-1.
42. Wolf D, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res* 1993;127:23-33.
43. Burstein HJ, Prestrud AA, Seidenfeld J, et al. American Society of Clinical Oncology clinical practice guideline: update on adjuvant endocrine therapy for women with hormone receptor–positive breast cancer. *J Clin Oncol* 2010;28:3784-3796.
44. Cuzick J, Sestak I, Baum M, et al. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 10-year analysis of the ATAC trial. *Lancet Oncol* 2010;11:1135-1141.
45. Mouridsen H, Giobbie-Hurder A, Goldhirsch A, et al. Letrozole therapy alone or in sequence with tamoxifen in women with breast cancer. *N Engl J Med* 2009;361:766-776.
46. Jones S, Seynaeve C, Hasenburg A, et al. Results of the first planned analysis of the TEAM (Tamoxifen Exemestane Adjuvant Multinational) prospective randomized phase III trial in hormone sensitive postmenopausal early breast cancer. *Cancer Res* 2009;69:15.
47. Goss PE, Ingle JN, Martino S, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. *N Engl J Med* 2003;349:1793-1802.
48. Ingle JN, Tu D, Pater JL, et al. Intent-to-treat analysis of the placebo-controlled trial of letrozole for extended adjuvant therapy in early breast cancer: NCIC CTG MA.17. *Ann Oncol* 2008;19:877-882.
49. Osborne C, Coronado EB, Robinson JP. Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol* 1987;23:1189-1196.
50. Gottardis M, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term anti-estrogen administration. *Cancer Res* 1988;48:5183-5187.
51. Gottardis MM, Wagner RJ, Borden EC, Jordan VC. Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro. *Cancer Res* 1989;49:4765-4769.
52. Gottardis MM, Jiang S-Y, Jeng M-H, Jordan VC. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. *Cancer Res* 1989;49:4090-4093.
53. Howell A, Robertson JFR, Abram P, et al. Comparison of fulvestrant versus tamoxifen for the treatment of advanced breast cancer in postmenopausal women previously untreated with endocrine therapy: a multinational, double-blind, randomized trial. *J Clin Oncol* 2004;22:1605-1613.
54. Yao K, Lee E-S, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;6:2028-2036.
55. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;5:207-213.
56. Lonning P, Taylor P, Anker G, et al. High-dose estrogen treatment in post-menopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 2001;67:111-116.
57. Peethambaram P, Ingle J, Suman V, et al. Randomized trial of diethylstilbestrol vs. tamoxifen in postmenopausal women with metastatic breast cancer. An updated analysis. *Breast Cancer Res Treat* 1999; 54:117-122.
58. Ellis M, Gao F, Dehdashti F, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor–positive, aromatase inhibitor–resistant advanced breast cancer: a phase 2 randomized study. *JAMA* 2009;302:774-780.
59. Jiang SY, Wolf DM, Yingling JM, et al. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* 1992;90:77-86.
60. Lewis J, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 2005;94:131-141.
61. Lewis J, Meeke K, Osipo C, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 2005;97:1746-1759.
62. Pink JJ, Jiang S-Y, Fritsch M, Craig Jordan VC. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor–related protein. *Cancer Res* 1995;55: 2583-2590.

Copyright © 2013 The North American Menopause Society. Unauthorized reproduction of this article is prohibited.

63. Ariazi EA, Cunliffe HE, Lewis-Wambi JS, et al. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *Proc Natl Acad Sci* 2011;108:18879-18886.
64. Voehringer D. BCL-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity. *Free Radic Biol Med* 1999;27:945-950.
65. Meredith MJ, Cusick CL, Soltaninassab S, et al. Expression of Bcl-2 increases intracellular glutathione by inhibiting methionine-dependent GSH efflux. *Biochem Biophys Res Commun* 1998;248:458-463.
66. Rudin CM, Yang Z, Schumaker LM, et al. Inhibition of glutathione synthesis reverses Bcl-2–mediated cisplatin resistance. *Cancer Res* 2003; 63:312-318.
67. Lewis-Wambi J, Kim H, Wambi C, et al. Buthionine sulfoximine sensitizes anti–hormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* 2008;10:R104.
68. Lewis-Wambi J, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? *Breast Cancer Res* 2009;11:206.
69. Watkins E. *The estrogen elixir: a history of hormone replacement therapy in America*. Baltimore, MD: The John Hopkins University Press, 2007:10-132.
70. FDA. *FDA Backgrounder on Conjugated Estrogens*. Silver Spring, MD: US Food and Drug Administration Center for Drug Evaluation and Research, 2005.
71. Smith DC, Prentice R, Thompson DJ, Herrmann WL. Association of exogenous estrogen and endometrial carcinoma. *N Engl J Med* 1975;293: 1164-1167.
72. Ziel HK, Finkle WD. Increased risk of endometrial carcinoma among users of conjugated estrogens. *N Engl J Med* 1975;293:1167-1170.
73. Greenblatt R, Gambrell RD, Stoddard LD. The protective role of progesterone in the prevention of endometrial cancer. *Pathol Res Pract* 1982;174:297-318.
74. Gambrell R. The prevention of endometrial cancer in postmenopausal women with progestogens. *Maturitas* 1978;1:107-112.
75. FDA. *Conjugated Estrogens—Letter from Dr Janet Woodcock. Memorandum*, Silver Spring, MD: US Food and Drug Administration Center for Drug Evaluation and Research, 1997.
76. Soule HD, Vazquez J, Long A, et al. A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst* 1973; 51:1409-1416.
77. Osipo C, Gajdos C, Liu H, et al. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* 2003;95:1597-1608.
78. Liu H, Lee E, Gajdos C, et al. Apoptotic action of 17β-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. *J Natl Cancer Inst* 2003;95:1586-1597.
79. Brzozowski AM, Pike ACW, Dauter Z, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 1997;389: 753-758.
80. Shiau AK, Barstad D, Loria P, et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 1998;95:927-937.
81. Hu ZZ, Kagan BL, Ariazi EA, et al. Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. *PLoS One* 2011;6:e20410.
82. Jordan VC, Schafer JM, Levenson AS, et al. Molecular classification of estrogens. *Cancer Res* 2001;61:6619-6623.
83. Maximov PY, Myers CB, Curpan RF, et al. Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. *J Med Chem* 2010;53:3273-3283.
84. Maximov P, Sengupta S, Lewis-Wambi JS. The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: a hypothesis. *Horm Mol Biol Clin Invest* 2011;5:27-34.

 © 2013 The North American Menopause Society

Copyright © 2013 The North American Menopause Society. Unauthorized reproduction of this article is prohibited.

Send Orders of Reprints at reprints@benthamscience.net

Current Clinical Pharmacology, **2013**, *8,* 135-155

The Discovery and Development of Selective Estrogen Receptor Modulators (SERMs) for Clinical Practice

Philipp Y. Maximov[1], Theresa M. Lee[2] and V. Craig Jordan[1,*]

[1]*Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, 3970 Reservoir Rd NW, Research Building, Suite E204A, Washington, DC 20057, USA;* [2]*Division of Hematology and Oncology, Georgetown University Medical Center, 3970 Reservoir Rd NW, Washington, DC 20057, USA*

Abstract: Selective estrogen receptor modulators (SERMs) are structurally different compounds that interact with intracellular estrogen receptors in target organs as estrogen receptor agonists or antagonists. These drugs have been intensively studied over the past decade and have proven to be a highly versatile group for the treatment of different conditions associated with postmenopausal women's health, including hormone responsive cancer and osteoporosis. Tamoxifen, a failed contraceptive is currently used to treat all stages of breast cancer, chemoprevention in women at high risk for breast cancer and also has beneficial effects on bone mineral density and serum lipids in postmenopausal women. Raloxifene, a failed breast cancer drug, is the only SERM approved internationally for the prevention and treatment of postmenopausal osteoporosis and vertebral fractures. However, although these SERMs have many benefits, they also have some potentially serious adverse effects, such as thromboembolic disorders and, in the case of tamoxifen, uterine cancer. These adverse effects represent a major concern given that long-term therapy is required to prevent osteoporosis or prevent and treat breast cancer.

The search for the 'ideal' SERM, which would have estrogenic effects on bone and serum lipids, neutral effects on the uterus, and antiestrogenic effects on breast tissue, but none of the adverse effects associated with current therapies, is currently under way. Ospemifene, lasofoxifene, bazedoxifene and arzoxifene, which are new SERM molecules with potentially greater efficacy and potency than previous SERMs, have been investigated for use in the treatment and prevention of osteoporosis. These drugs have been shown to be comparably effective to conventional hormone replacement therapy in animal models, with potential indications for an improved safety profile. Clinical efficacy data from ongoing phase III trials are available or are awaited for each SERM so that a true understanding of the therapeutic potential of these compounds can be obtained.

In this article, we describe the discovery and development of the group of medicines called SERMs. The newer SERMs in late development: ospemifene, lasofoxifene, bazedoxifene, are arzoxifene are described in detail.

Keywords: Arzoxifene, bazedoxifene, lasofoxifene, ospemifene, raloxifene, selective estrogen receptor modulator, tamoxifen.

THE QUEST TO PREVENT BREAST CANCER

The idea of using a chemical to prevent (chemo-prevention) breast cancer is a noble goal that has achieved significant successes in the past three decades. This is however not a new concept as Professor Antoine Lacassagne [1] had the vision which he stated at the Annual Meeting of the American Association for Cancer Research in 1936:

> *"If one accepts the consideration of adenocarcinoma of the breast as a consequence of a special hereditary sensibility to the proliferative action of oestrone, one is led to imagine a therapeutic preventive for subjects predisposed by their heredity to this cancer, to stop the congestion of oestrone in the breast."*

*Address correspondence to this author at the Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, 3970 Reservoir Rd NW, Research Building, Suite E204A, Washington, DC 20057, USA; Tel: 202.687.3207; Fax: 202.687.7505; E-mail: vcj2@georgetown.edu

However, his vision was based on his laboratory experiments with oophorectomy to prevent or estrogen replacement to enhance, tumorigenesis in strains of mice with a high incidence of mammary cancer. Most importantly, chemoprevention could not advance in humans because therapeutic knowledge was not available in the 1930's. The first antiestrogens would not be reported until the late 1950's more than 20 years later [2].

The non-steroidal antiestrogens initially had no major clinical impact during the first decade since the discovery of the first non-steroidal antiestrogen MER25 [3] in 1958. The early compounds were studied as antifertility agents in the laboratory, but clomiphene did the opposite in humans, so it was used successfully to induce ovulation in subfertile women. Clomiphene, a mixture of estrogenic (zuclomiphene) and antiestrogenic (enclomiphene) geometric isomer has been used for over 50 years for the induction of ovulation [4, 5]. This therapeutic advance set the scene for the subsequent breakthroughs in molecular pharmacology and medicines seen in the latter half of the 20th century (Fig. **1**). The

2212-3938/13 $58.00+.00 **© 2013 Bentham Science Publishers**

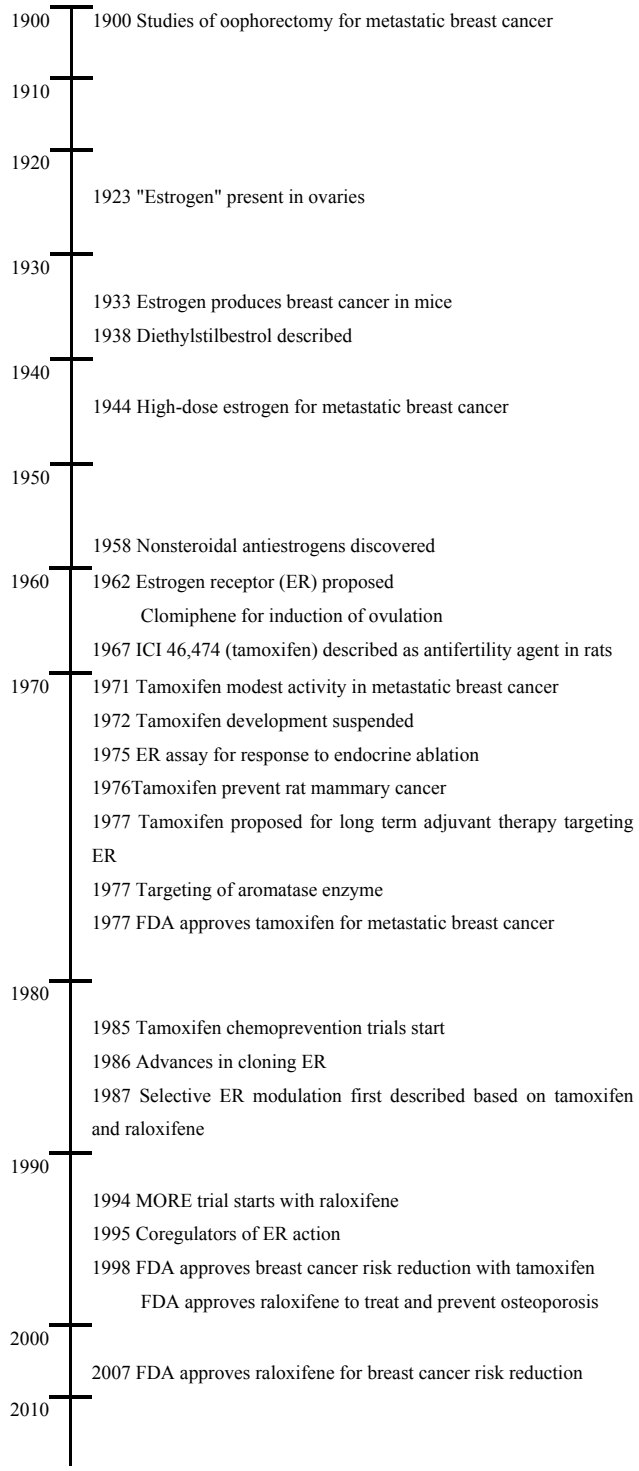


Fig. (1). Timeline of the major landmarks in estrogen action, anti-estrogens and SERMs for the treatment and prevention of breast cancer, and osteoporosis.

endocrinology of clomiphene was studied in some detail [6], for the obvious reason that the medicine was used to induce ovulation in healthy women, but toxicological issues prevented further drug development for other potential applications in women's health eg. breast cancer treatment and prevention. Then came tamoxifen, ICI 46,474, the failed contraceptive [7, 8] and orphan drug looking for a therapeutic application. Initial clinical studies demonstrated that it was safe and effective for the induction of ovulation in subfertile women [9, 10] and for the treatment of metastatic breast cancer in postmenopausal women [11, 12].

The story of the reinvention of tamoxifen to become the gold standard for the adjuvant treatment of breast cancer and the pioneering medicine for the reduction of breast cancer incidence in high risk women, has been told in detain elsewhere [13, 14]. Suffice to say the translational laboratory research work in the 1970's [15] that catalyzed tamoxifen's move from orphan drug resulted in tamoxifen becoming the standard of care for the long term adjuvant therapy of estrogen receptor (ER) positive breast cancer and, as a result, extended the lives of millions of women worldwide. The approvals for the use of tamoxifen are unique amongst anticancer agents and include the treatment of metastatic breast cancer, adjuvant therapy with chemotherapy, adjuvant therapy alone, the treatment of ductal carcinoma in situ, risk reduction in high risk pre- and postmenopausal women and breast cancer treatment in men. The advance was achieved based on the premise that tamoxifen, the pure *trans* isomer of a triphenylethylene was the lead member of the group of drugs known as nonsteroidal antiestrogens [16]. If estrogen was indicated in the growth of some breast cancer then an antiestrogenic drug would be effective as a treatment. But fashions in science and medicine change and this was about to happen in the 1980's with a new approach to the management of breast cancer: chemoprevention

Professor Trevor Powles was the first to initiate a pilot study for the chemoprevention of breast cancer in a small group of high risk women using tamoxifen. He selected women with a first degree relative that had already had breast cancer. His pilot toxicology study was initiated in 1985 and published in 1989 [17]. However, there were significant toxicological issues that had to be addressed in the laboratory and translated to clinical trial before an "antiestrogen" could be considered to be tested in large populations of healthy women for the chemoprevention of breast cancer. Tamoxifen was noted in the laboratory [18] and clinic [19] to increase the growth and incidence of endometrial cancer. Also at that time in the 1980's it was believed, that estrogen was useful to protect women from coronary heart disease and osteoporosis. Clearly there would be no advantage of using a drug classified as a "non-steroidal antiestrogen" to block estrogen mediated breast carcino-genesis in the few, but expose the whole experimental population to crushing osteoporosis or an elevation of the incidence of coronary heart disease. Studies conducted at the University of Wisconsin Comprehensive Cancer Center [2, 18, 20-26] were instrumental in providing clarity to these questions and created the new drug group – Selective ER Modulators or SERMs.

The mention of "modulation" at an ER target site first occurred with the examination of the structure function relationships of estrogenic triphenylethylene derivatives of tamoxifen at a prolactin gene target *in vitro* [27]. The

estrogenic compounds could activate or suppress prolactin synthesis by altering the shape of the ER complex between the extremes of an "antiestrogenic" or an "estrogenic" conformation [28]. This idea of the molecular modulation of the receptor at a single target site was then expanded to consider the physiologic responses that occurred with nonsteroidal antiestrogen at multiple target sites in the body – simultaneously.

A cluster of translational studies focused on the uterus, breast (mammary gland) and bone together created the data base for further confirmatory studies and the clinical trials by the pharmaceutical industry that resulted in the reinvention of the failed breast cancer drug keoxifene to become raloxifene the first clinically available SERM to prevent both osteoporosis and breast cancer [29-32]. Each of the laboratory studies provided an interlocking network of knowledge relevant to the practical application of a new drug group in medical practice. The fundamental concept of SERMs action described first in the late 1980s [2, 23] and later refined and defined as a balance of receptors and coregulators (Fig. **2**) is similar to the subsequent description of Protean agonists of the G-protein-coupled receptors [33].

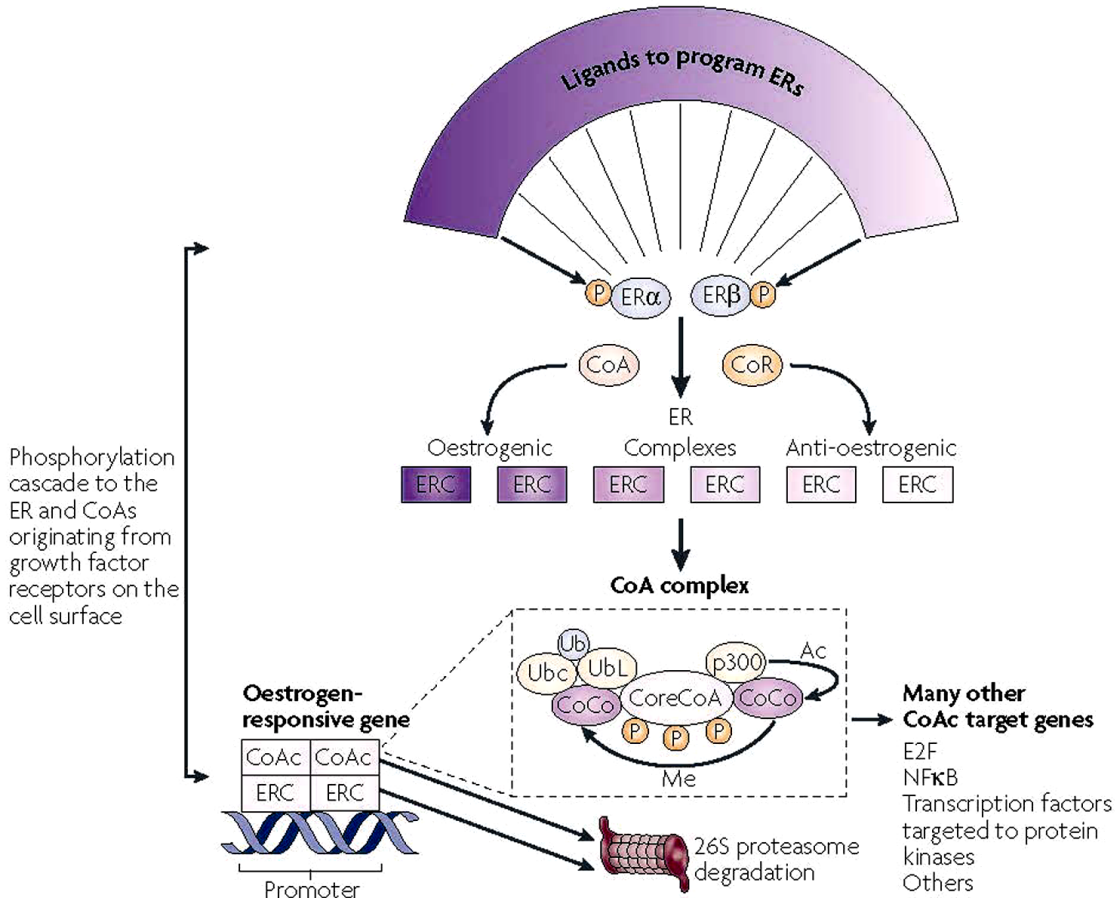


Fig. (2). Molecular networks potentially influence the expression of SERM action in a target tissue. The shape of the ligands that bind to the estrogen receptors (ERs)α and β programmes the complex to become an estrogenic or anti-estrogenic signal. The context of the ER complex (ERC) can influence the expression of the response through the numbers of co-repressors (CoR) or coactivators (CoA). In simple terms, a site with few CoAs or high levels of CoRs might be a dominant anti-estrogenic site. However, the expression of estrogenic action is not simply the binding of the receptor complex to the promoter of the estrogen-responsive gene, but a dynamic process of CoA complex assembly and destruction [101]. A core CoA, for example, steroid receptor coactivator protein 3 (SRC3), and the ERC are influenced by phosphorylation cascades that phosphorylate target sites on both complexes. The core CoA then assembles an activated multiprotein complex containing specific co-co-activators (CoCo) that might include p300, each of which has a specific enzymatic activity to be activated later. The CoA complex (CoAc) binds to the ERC at the estrogen-responsive gene promoter to switch on transcription. The CoCo proteins then perform methylation (Me) or acetylation (Ac) to activate dissociation of the complex. Simultaneously, ubiquitiylation by the bound ubiquitin-conjugating enzyme (Ubc) targets ubiquitin ligase (UbL) destruction of protein members of the complex through the 26S proteasome. The ERs are also ubiquitylated and destroyed in the 26S proteasome. Therefore, a regimented cycle of assembly, activation and destruction occurs on the basis of the preprogrammed ER complex [101]. However, the co-activator, specifically SRC3, has ubiquitous action and can further modulate or amplify the ligand-activated trigger through many modulating genes [215] that can consolidate and increase the stimulatory response of the ERC in a tissue. Therefore, the target tissue is programmed to express a spectrum of responses between full estrogen action and anti-estrogen action on the basis of the shape of the ligand and the sophistication of the tissue-modulating network. NFκB, nuclear factor κB. This figure is published with permission from Nature Publishing group. Jordan, V.C. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nature Reviews Cancer, 2007 Jan; 7(1): 46-53.

The first public description of the clinical concept of SERMs as useful medicines for women's health was at the First International Chemoprevention meeting in New York in 1987. The vision was stated as follows: "The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused on the identification of a population of high-risk women to test 'chemopreventive' agents. But, are resources being used less than optimally? An alternative would be to seize on the developing clues provided by an extensive clinical investigation of available antiestrogens. Could analogues be developed to treat osteoporosis or even retard the development of atherosclerosis? If this proved to be true, then a majority of women in general would be treated for these conditions as soon as menopause occurred. Should the agent also retain antibreast tumor actions, then it might be expected to act as a chemosuppressive on all developing breast cancers if these have an evolution from hormone-dependent disease to hormone independent disease. A bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century [23]".

Subsequently the "roadmap" for the pharmaceutical industry was refined and defined more precisely in the Cain Memorial Award lecture presented before the American Association for Cancer Research in 1989 for advances in laboratory research leading to the discovery and development of new therapeutic agents for the treatment of cancer. "We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids, so apparently, derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high-risk group to prevent breast cancer [2]".

Indeed, the discovery that tamoxifen and raloxifene had target site selective estrogenic and antiestrogenic actions around the body would stimulate all subsequent research on SERMs [34].

PHARMACOKINETICS OF TAMOXIFEN AND RALOXIFENE

Tamoxifen a long acting drug with a long biological half-life that is metabolically activated, whereas raloxifene is a very short acting drug that is rapidly conjugated and then excreted through the biliary tract. The metabolism, pharmacogenomics and pharmacokinetics of SERMs continue to present challenges. Just when everything appears to be straightforward, old drugs create unanticipated surprises and in contrast ideas to alter the pharmacokinetics of raloxifene from a short to a long acting drug do not result in success. Initially, there was little pharmacologic information or interest in the metabolism of tamoxifen in animals and man; this was not a major requirement to register a drug to treat advanced breast cancer in the 1970's [14]. The situation remained the same during the 1980's when tamoxifen was about to become the standard of care as the adjuvant antihormonal treatment of ER positive breast cancer and studies were planned to evaluate the worth of tamoxifen to prevent the breast cancer in high risk women [14]. At that time, it was accepted that tamoxifen was either metabolically activated to 4-hydroxytamoxifen [35, 36], a minor metabolite with high binding affinity to the ER but with a short biological half-life [37] or was demethylated to N-desmethyltamoxifen, a compound with low binding affinity for the ER but a long biological half-life. N-Desmethyltamoxifen was further demethylated to desdimethyltamoxifen and subsequently deaminated to the weakly antiestrogenic glycol derivative of tamoxifen referred to as metabolite Y [38]. These antiestrogenic metabolites deactivate the ER but based on concentrations of metabolites and their affinity, all were considered to play a role in blocking estrogen action.

The ubiquitous application of tamoxifen as a long-term, well tolerated treatment for breast cancer during the past two decades and its use as a preventive in high risk women, resulted in the close examination of symptom management, especially hot flashes, to enhance compliance. Selective serotonin reuptake inhibitors (SSRIs) are effective in controlling hot flashes experienced by up to 45% of treated patients. However, the identification and characterization [39-41] of the high affinity metabolite of tamoxifen 4-hydroxy-N-desmethyltamoxifen (endoxifen) and the finding that endoxifen levels are reduced by the co-administration of SSRIs [42-44] is an important observation that has potential therapeutic implications. It follows that since SSRIs block CYP2D6, thereby inhibiting the metabolism of tamoxifen to endoxifen, then the efficacy of tamoxifen as an anticancer agent (treatment or chemoprevention) could be impaired by either the ubiquitous use of SSRIs to prevent hot flashes or the administration of tamoxifen to women with a defect in the CYP2D6 enzyme that no longer converts tamoxifen to endoxifen. Preliminary evidence suggests that this might be the case [44, 45]. However, the proposition that patients should be genotyped to identify poor metabolizers who will be less likely to respond to tamoxifen remains controversial. Be as it may, it is probably unwise to use SSRI to reduce hot flashes in patients taking tamoxifen. Venlafaxine, a drug with low potential to interact with the CYP2D6 enzyme, is the agent of choice for symptom control.

The knowledge that tamoxifen was metabolically activated to hydroxylated metabolites with high affinity for the ER [35] created the opportunity for chemists in the pharmaceutical industry to design the high affinity SERMs, raloxifene, basedoxifene and lasofoxifene. However, the pharmacokinetics and pharmacodynamics of these polyphenolic compounds now creates a complex new set of problems to get an orally active drug constantly to the breast tissues to prevent estrogen-stimulated growth Raloxifene and other SERM members that are benzothiophene derivatives, are short acting [46-48]. However, raloxifene has a plasma elimination half-life of approximately 27 hours which apparently results from reversible Phase II metabolism which conjugates the polyphenolic drugs prior to excretion as sulphates and glucuronides. There appear to be two

aspects for consideration for a polyphenolic SERM to be an effective chemopreventive for breast cancer. Firstly, raloxifene is conjugated by the human intestinal enzymes UGTIA8 and UGTIA10 [49] but it is the dynamic relationship between absorption, Phase II metabolism and excretion in the intestine [50] that controls the 2% bioavailability of raloxifene [48]. The second aspect for consideration is the retention of raloxifene in the target tissue. This depends on local sulphation which inactivates the SERM prior to diffusion out of the tissue. Here again, there are disparities in the efficacy of multiple sulphation enzymes (sulphotransferases, SULTs) to terminate bioactivity of raloxifene in a target site. By way of example: 4-hydroxytamoxifen [35] is only sulphated by three of seven SULT isoforms whereas raloxifene is sulphated by all seven [51]. Additionally, SULTIEI, which sulphates raloxifene in endometrial tissue, is only expressed in the secretory phase [51] of the menstrual cycle following ovulation [52]. All these issues promted chemists in industry to improve the breast cancer treatment potential of SERMs by improving the pharmacokinetics by designing the long acting "raloxifene" named arzoxifene (see later section). Similarly lasofoxifene creates a very interesting innovation in enhanced pharmacokinetics. Lasofoxifene is extensively metabolized in rats and monkeys with tissues achieving maximal concentrations within one hour of oral administration of ^{14}C labeled lasofoxifene [53]. There was greater than 95% of lasofoxifene and metabolites excreted in feces through the biliary route with only a small amount of glucoronide. It is reasoned that increased oral bioavailability results from the fact that the non-planar lasofoxifene is a poor substrate for glucuronidation. Lasofoxifene exists in two enantiomer; the l-enantiomer has high ER binding and increased bioavailability, compared to the d-enantiomer [54]. This property of the molecule improves pharmacokinetics so that a clinical dose of 0.5mg daily is proven effective in clinical trial to prevent bone loss and prevent breast cancer [55]. This is $1/100^{th}$ the daily dose of raloxifene!

With this background of the challenges that the medicinal chemist faces and must solve to create a successful SERM, we now turn to the story that evolved during the 1980's that formed the basis for all future drug discoveries by the pharmaceutical industry. Simply stated; what were the circumstances that created the SERMs, what were the challenges for the clinical community and where did the new SERMs we study today have their origins?

THE BIOLOGICAL BASIS OF SERM ACTION: TARGET TISSUE SPECIFIC ACTIONS

In this section we will present the translational data, obtained primarily during the 1980's that proved to be the database that created the concept to move forward to clinical testing and advance novel SERMs for clinical applications. We will cluster each estrogen target tissue group studied in the 1980's that advanced the new SERM concept [2, 23] into clinical testing and validation during the 1990's.

Uterus, Breast and Endometrial Cancer

The development of the athymic (immune deficient) mouse models provided an invaluable opportunity to study human tumor cell lines *in vivo*. The ER positive breast cancer cell line MCF-7 [56] can be inoculated into ovariectomized athymic mice and will grow into tumors in response to the administration of sustained release physiologic estradiol. However, the pharmacology of tamoxifen is species specific; the compound is classified as an anti-estrogen in the rat but an estrogen in the mouse [7]. Administration of tamoxifen to athymic mice implanted with MCF-7 tumors demonstrated that only estradiol would cause the human breast tumor to grow, tamoxifen did not [22]. Nevertheless, the ovariectomized mouse uterus grew in response to either tamoxifen or estradiol. There was target site specificity and the conclusions in a pivotal paper [22] clearly stated the idea "The species differences observed with tamoxifen are the result of differences in the interpretation of the drug-ER complex by the cell. The drug-ER complex is perceived as either a stimulatory or an inhibitory signal in the different target tissues from different species". Nevertheless, the results could have been the result of species differences in pharmacology and not tissue specific pharmacology. To address this question two approaches were taken 1) the target site specificity of two human tumors were compared and contrasted implanted in the same athymic mouse and 2) inbred strains of mice with a high incidence of mammary tumors were used to determine whether there was target site specificity to prevent mammary cancer in the same species of rodent.

Bitransplantation of ovariectomized mice with a MCF-7 breast tumor in one axillary fat pad and an EnCa101 human endometrial tumor in the other provides an ideal translational model to evaluate the responsiveness of two human tumors in the same therapeutic environment. The analogy would be the responsiveness of the breast cancer patient to adjuvant tamoxifen but with an occult endometrial tumor. At the time of the experiments in 1987 there were no reports of an increase in endometrial cancer incidence in any adjuvant clinical trials. The laboratory study demonstrated that tamoxifen blocked breast tumor growth but tamoxifen enhanced estrogen-stimulated endometrial cancer growth [18].

Even before the start of the tamoxifen chemoprevention trials in the early 1990's it was clear that a new approach to the chemoprevention of breast cancer was necessary. Firstly the targeted population for preventing breast cancer was only a small percent of the potential population at risk ie: only about 8-10 women will develop breast cancer per 1000 high risk women per year. However, all women will be exposed to the side effects of tamoxifen. An increased risk of developing endometrial cancer was obviously significant to women so a solution needed to be addressed. Another medicine was necessary but clues were already in the refereed literature to formulate a strategy for the new drug class – the SERMs. An important clue was to be found using the 'nonsteroidal antiestrogen' keoxifene abandoned by Eli Lilly following its failure in testing as a breast cancer drug competitor to tamoxifen in 1987. Keoxifene was not as estrogen-like as tamoxifen in the rodent uterus [57] but was used as a comparator compound to illustrate that different antiestrogens would modulate the growth of human endometrial carcinoma implanted in to athymic mice [58]. Keoxifene did not have the same efficacy as tamoxifen to

enhance the growth of human endometrial carcinoma under laboratory conditions. Indeed keoxifene could block full tamoxifen stimulated endometrial carcinoma growth [58]. This was important pharmacological evidence published in the refereed literature years before raloxifene (a.k.a. keoxifene) advanced the path for progress in women's health after 1992.

The additional important target site specific evidence to support the clinical development of SERMs for women's health was the use of inbred strains of mice with a high incidence of spontaneous mammary cancer. The question to be addressed was whether tamoxifen could prevent mouse mammary carcinogenesis if the drug was classified as an estrogen in the mouse. Professor Antoine Lacassagne had used this model to support his hypothesis stating earlier that "Therapeutic compounds could be found to stop the congestion of oestrone in the breast" [1]. However, tamoxifen was classified as an estrogen in the mouse [7]. Studies comparing and contrasting tamoxifen and oophorectomy in the C3H/OUJ mouse strain demonstrated that long term tamoxifen treatment was effective in preventing mouse mammary tumorigenesis, was superior to oophorectomy, and that tamoxifen's action as an estrogen in the uterus was target site specific in the same species [59, 60]. Overall these mouse studies (athymic and high incidence mammary cancer strains) demonstrated "targeted estrogenic and antiestrogenic actions".

Summary and Conclusion

As a result of the finding in the laboratory [18], Fornander and colleagues [19] reported a significant increase in the risk of developing endometrial cancer during tamoxifen therapy. Practice changes occurred immediately and regular gynecologic examinations were recommended for women taking tamoxifen. It is important to note, however, that the risk of developing endometrial cancer is only elevated in postmenopausal women. The laboratory testing and reinvention of raloxifene as an antiestrogen with no uterine effects was to be critical to exploit the discovery of the estrogen-like effects of tamoxifen and raloxifene in bone.

Bone and Mammary Tumorigenesis

The fact that estrogens build bone and estrogen deprivation during the postmenopausal period enhances the risk of osteoporosis was a major concern for implementing a safe strategy of breast chemoprevention with the nonsteroidal antiestrogen tamoxifen. An antiestrogenic drug may prevent breast cancer in a few but enhance the risk of osteoporosis in the majority. Laboratory research and clinical translation would change that perspective and deliver the SERMs as a new drug group.

An early report using clomiphene (the mixture of estrogenic *cis* and antiestrogen *trans* isomers) in the ovariectomized rats [61] concluded that clomiphene builds bone. However, the study was flawed because clomiphene is a mixture of estrogenic and antiestrogenic isomers. It may have been that the estrogenic isomer built bone in the administered mixture of clomiphene isomers. In contrast, the first study in the ovariectomized rats with the nonsteroidal antiestrogens tamoxifen and keoxifene (ie: raloxifene) only used pure compounds based on a *trans* or "antiestrogenic" conformation. Both compounds blocked estradiol-induced increases in uterine weight but retarded decreases in bone loss and did not block estradiol induced increases in bone density [21]. The results with tamoxifen were immediately confirmed by others in the rat [62, 63] and these laboratory data were used to test the concept that tamoxifen is estrogen-like in bone in the Wisconsin Tamoxifen Study. Tamoxifen maintained and built bone in postmenopausal women with node negative (low risk recurrence) breast cancer [25] This result demonstrated, for the first time in a prospective randomized clinical trial, that the principle of "selective estrogenic (bone) and antiestrogenic (breast) action" occurred in humans. Also the laboratory data suggested that the target site specificity of the 'nonsteroidal antiestrogens' was not unique to tamoxifen but was a class effect. The initial discovery with the bone building effects of tamoxifen and raloxifene [21] coupled with the demonstration of the inhibition of rat mammary carcinogenesis with either tamoxifen and raloxifene [20] prompted the description of a vision for the future use of the new class of drugs [2, 23]. However, the rat mammary carcinogenesis studies with tamoxifen and raloxifene showed that the effect of raloxifene was not superior to tamoxifen and would not be long lasting [23]. This would be demonstrated subsequently in postmenopausal women in the STAR trial [32].

SUMMARY AND CONCLUSIONS

The laboratory and clinical data which demonstrated that tamoxifen is estrogen-like by increasing rat bone density and bone density in postmenopausal women was reassuring to move forward with the chemoprevention trials with tamoxifen in the 1990's. However, the fact that keoxifene maintained bone density in the ovariectomized rat [21] (but without an estrogen-like effect in the uterus seen with tamoxifen) triggered the hypothesis that drugs of this class could be used to treat osteoporosis and atherosclerosis, and prevent breast cancer at the same time [2, 23]. The development of raloxifene was the result to prevent both osteoporosis and to reduce the incidence of breast cancer.

There is a long and sustained decrease in breast cancer incidence for a decade (at least) after tamoxifen stops [64-66]. This is not true for raloxifene in the STAR trial after treatment stops. Raloxifene is recommended to be used continuously to prevent the developing breast cancers [32].

Concepts in the Control of Coronary Heart Disease (CHD)

In the days before atorvastatin (or 'statins'; HMG CoA reductase inhibitors) was proven to reduce low density lipoprotein (LDL) cholesterol [67] and as a result reduce the risk of coronary heart disease due to atherosclerosis [68-70], a variety of drugs that interfered with cholesterol metabolism were evaluated. One such compound triparanol blocked cholesterol biosynthesis [71] but became a *cause célèbre* as the buildup in desmosterol was linked to cataract formation in young women taking the medicine [72]. The Merrell company in Cincinnati who manufactured and marketed triparanol subsequently chose to avoid development of any

drug that increases circulating desmosterol. The subsequent discovery and investigation of clomiphene by Merrell also showed an increase in desmosterol, so long term treatment with clomiphene was subsequently avoided [14].

A related compound, ICI 46,464, is the pure trans isomer of triphenylethylene but does not increase desmosterol despite the fact that circulating cholesterol is lowered in the rat [7]. A safer toxicology profile predetermined the drug as a useful antiestrogen to use in long term therapy for a disease such as breast cancer. Indeed the fact that tamoxifen lowered circulating cholesterol in the rat was included in the patent. The application for tamoxifen stated, "The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolaemic activity".

Subsequent clinical studies [24, 26, 73, 74] demonstrated a decrease in LDL cholesterol thereby holding out the promise that drugs of this class might reduce atherosclerosis and reduce the risk of CHD. Although several individual reports have noted decreases in CHD in patients taking long-term adjuvant tamoxifen [75, 76] and a recent study found that taking tamoxifen for the recommended 5 years reduces the risk of cardiovascular disease and death as a result of a cardiovascular event [77], particularly among those age 50 to 59 years, the Overview Analyses of all data does not support cardioprotection [78].

Overall, with antiestrogenic effects in the breast, estrogen-like effects in the bone, and an action that lowered circulating cholesterol, the stage was set to create a new drug group the SERMs with an evidenced based roadmap for future drug development [2].

Although tamoxifen is the pioneering SERM, raloxifene is the medicine that first exploited the "roadmap" successfully starting in 1992 [79]. Scientists at Eli Lilly [80] confirmed the concept in animal models measuring bone density, uterine weights and circulating cholesterol (tamoxifen had been patented as a hypocholesterolemia drug in the early 1960's and related compounds also affected cholesterol metabolism and biosynthesis so the Lilly scientists confirmed the class effect of the drug group) and initiated the Multiple Outcomes of Raloxifene Evaluation or MORE trial. Raloxifene would be the first SERM to be approved for two of the three properties of the "ideal SERM": reduction in the incidence of fractures from osteoporosis and the reduction in the incidence of breast cancer [29-31]. Although raloxifene lowers circulating cholesterol in postmenopausal women, raloxifene does not reduce the risk of CHD in women at high risk [81].

SUMMARY AND CONCLUSION

The tantalizing clues that the nonsteroidal antiestrogens tamoxifen and raloxifene can lower total circulating cholesterol in ovariectimized rats and LDL cholesterol in postmenopausal women did not, for these compounds translate to decreasing CHD. This goal would, however, be achieved with a new agent lasofoxifene (see section on new SERMs under investigation).

MOLECULAR MECHANISMS OF SERM ACTION

There are two ERs referred to as α and β [82-84]. Each receptor protein is encoded on different chromosomes, and have homology as members of the steroid receptor superfamily. There are distinct patterns of distribution and distinct and subtle differences in structure and ligand binding affinity [85]. The ratio of ERα and ERβ at a target site may be an additional dimension for tissue modulation. A high ERα: ERβ ratio correlates well with high levels of cellular proliferation whereas the predominance of functional ERβ over ERα correlates with repression of proliferation [86-89]. Indeed, the ratio of ERs in normal and neoplasic breast tissue could be important for the long-term success of chemoprevention with SERMs.

The functional differences between ERα and ERβ can be traced to the differences in the Activating Function 1 (AF-1) domain located in the amino terminus of the ER. The amino acid homology of AF-1 is poorly conserved between ERα and ERβ (only 20%). In contrast, the AF-2 region located at the C terminus of the ligand binding domain, differs only by one amino acid: D545 in ERα and N496 in ERβ. Together the AF-1 and AF-2 are important for the interaction with other co-regulatory proteins that control gene transcription. Studies using chimeras of ER α and β by switching the AF-1 regions demonstrates the cell and promoter specific differences in transcriptional activity [90, 91]. In general, SERMs can partially activate engineered genes regulated by an estrogen response element through ERα but not ERβ [92]. In contrast, 4-hydroxytamoxifen and raloxifene can stimulate activating protein-1 (AP-1) regulated reporter genes with both ERα and ERβ in a cell dependent fashion [93].

The simple model for estrogen action, with either ERα or ERβ initiating estrogen action in the nucleus, has now evolved to a new dimension of protein partners that modulate gene transcription (Fig. **2**). Since the first steroid receptor coactivator (SRC-1) was described by O'Malley's group [94] there are now hundreds of coactivator and corepressor molecules (Fig. **2**) [95].

The finding that there are two ERs, has resulted in the synthesis of a range of receptor specific ligands to switch on or switch off a particular receptor [96]. It is, however, the external shape of the resulting complex that becomes the catalyst for changing the response to a SERM at a tissue target. Kraichely and co-workers[97] demonstrated the important observation that agonists for ERα and ERβ produce subtle quantitative differences with the interaction of members of the SRC family (SRC 1, 2 and 3) and that the coactivator can enhance ligand affinity for the ER.

It is reasonable to ask how the ligand programs the receptor complex to interact with other proteins? X-ray crystallography of estrogens or antiestrogens locked in the ligand binding domains of the ER demonstrates the mechanics where ligands promote coactivator binding or prevent coactivator binding based on the shape of the estrogen or antiestrogen receptor complex [98, 99]. Evidence has now accumulated to document that the broad spectrum of ligands that bind to the ER can create a broad range of ER complexes that are either fully estrogenic or antiestrogenic at a particular target site [100]. Thus a mechanistic model of

estrogen action and antiestrogen action (Fig. **2**) has emerged based on the shape of the ligand that programs the complex for future action. But how is the response initiated?

Not surprisingly, the coactivator model of steroid hormone action has now become enhanced into multiple layers of complexity thereby amplifying the molecular mechanisms of modulation. It appears that coactivators are not simply protein partners that connect one site to another in a complex [101]. The coactivators actively participate in modifying the activity of the complex. Post translational modification of coactivators *via* multiple kinase pathways initiated by cell surface growth factor receptors (e.g. epidermal growth factor receptor, insulin-like growth factor receptor 1 and ERBB2, also known as HER2) can result in a dynamic model of steroid hormone action. The core coactivator e.g. SRC3 (Fig. **2**) first recruits a specific set of co-coactivators e.g. p300 and ubiquitin-conjugating ligases under the direction of numerous protein remodelers (e.g. the peptidyl-prolyl isomerase Pin1, heat shock proteins and proteasome ATPases) to form a multi-protein coactivator complex that interacts with the phosphorylated ER at the specific gene promoter site [101]. Most importantly, the proteins assembled by the core coactivator as the core coactivated complex have individual enzymatic activities to acetylate or methylate adjacent proteins. Multiple cycles of the reaction can polyubiquitinate a substrate i.e. ER or a CoA, or, depending on the ubiquitin-ubiquitin linkage proteins can either to be activated further (K63 linkage) or degraded by the 26S proteasome (K48 linkage) [102].

Thus for effective gene transcription, programmed and targeted by the shape and phosphorylation status of the ER and coactivators, a dynamic and cyclic process of remodeling capacity is required for transcriptional assembly [103] that is immediately followed by the routine destruction of transcription complexes by the proteasome. Estrogen and SERM-ER complexes have distinct accumulation patterns in the target cell nucleus [104, 105] because they are destroyed at different rates [106].

These fundamental mechanisms [101, 107] in physiology also apply to the development of acquired drug resistance to SERMs in breast cancer. Model systems have demonstrated the conversion of the tamoxifen ER complex from an anti-estrogenic signal to an estrogenic signal in an environment enhanced for phosphorylation by overexpression of the ERBB2cell surface receptor and an increase in SRC3 (AIB1) [108, 109]. The enhanced level of coactivators and its enhanced phosphorylation state derived from an activated ERBB2 phosphorylation pattern will enhance the estrogen-like activity of tamoxifen at the ER. Clearly, issues of SERM action at target tissues and the eventual development of acquired drug resistance in breast cancer will be amplified for tumor cell survival as the duration of SERM use extends from a few years to perhaps decades [52].

THE CURRENT AND NEXT GENERATION OF SERMS

Tamoxifen and Raloxifene

There are currently 2 main chemical classes of SERMs approved for clinical use: the first-generation triphenylethylene derivatives, tamoxifen [110] and toremifene [111, 112], which are used in the treatment and in the case of tamoxifen in the prevention of breast cancer [65, 113]; and raloxifene, a second-generation benzothiopene derivative indicated for the treatment and prevention of osteoporosis [29] and the reduction of breast cancer incidence in high risk post-menopausal women [31]. All 3 compounds also have beneficial effects on serum lipids, but are still associated with adverse effects such as hot flushes and an increase in the risk of venous thromboembolism (VTE). Raloxifene is the only SERM compound approved worldwide for the prevention and treatment of postmenopausal osteoporosis and fragility fractures. The pivotal registration MORE (Multiple Outcomes of Raloxifene Evaluation) trial was a multicentered, randomized, blinded, placebo-controlled trial that included 7705 women aged 31-80 years from 25 countries. Results of the trial showed significantly reduced vertebral fractures in the raloxifene group (RR 0.60; 95% CI 0.50 to 0.70; $p < 0.01$) [29]. Raloxifene did not significantly reduce nonvertebral fractures with either 60 or 120 mg/day [29]. BMD increased by 0.4 to 1.20% at the lumbar spine; these effects have been documented further for at least 7 years in the CORE (Continuing Outcomes Relevant to Evista) trial [114]. All participants received 500 mg of calcium and 400-600 IU of vitamin D each day, in addition to study treatments. It is also important to stress that continuous treatment with raloxifene effectively controls the development of breast cancer [115].

Raloxifene lacks estrogenic activity in the uterus and has not demonstrated tamoxifen-like effects in the uterus either histopathologically or ultrasonographically [116], but it has been associated with adverse effects such as VTE and vasomotor symptoms, including hot flushes. In addition, both preclinical and clinical reports suggest that these ER agonists are considerably less potent than estrogen for the treatment of osteoporosis. The goal, therefore, became to create a “Designer Estrogen” [117] and enhance the value of the new multifunctional medicines. Newer generation SERMs being investigated for the prevention and treatment of osteoporosis in postmenopausal women include ospemifene (Ophena; QuatRx Pharmaceuticals), lasofoxifene (Fablyn; Pfizer), bazedoxifene (Viviant; Wyeth Pharmaceuticals), and Arzoxifene (LY353381, Lilly) which are in Phase III clinical trials or have undergone regulatory review (Fig. **3**, Table **1**). Other SERMS have had clinical trials suspended prematurely: levormeloxifene, for causing urinary incontinence and uterine prolapse, and idoxifene, for producing increased endometrial thickness on ultrasonography but without significant histologic abnormalities [116].

The four SERMs we will consider in detail have all achieved significant clinical evaluation. Some have moved forward to be approved in some countries, others have not been advanced. It is, however, important from a drug development perspective to state the idea for each structure was an improvement on the original discovery of the core structure, in some cases, 50 years ago. The links with the original pharmacologic discoveries is illustrated in Fig. (**4**), but the goal is to find the ideal SERM (Fig. **5**). Ospemifene is the direct result of the discovery of a weak anti-estrogenic metabolite of tamoxifen Metabolite Y, formed by

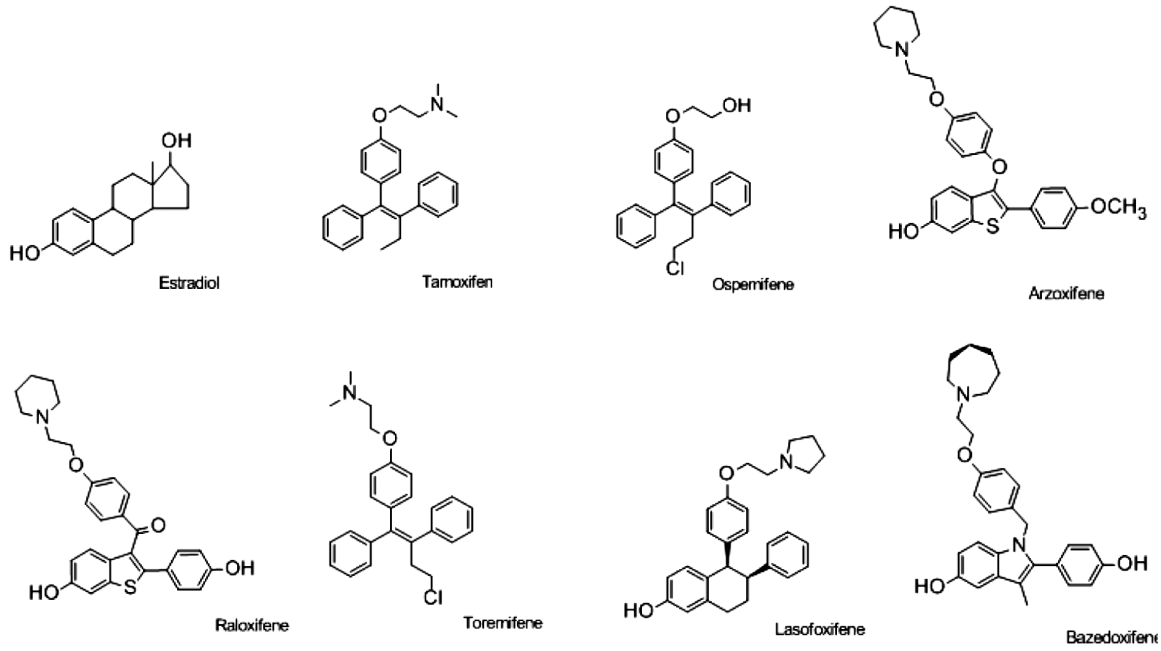


Fig. (3). Chemical structure of estradiol and selective estrogen receptor modulators (SERMs); raloxifene, tamoxifen, toremifene, ospemifene, lasofoxifene, arzoxifene and bazedoxifene.

Table 1. Current Status of New SERMs

Drug Name	Category (Structure)	Effects	Preclinical Results	Clinical Status
Ospemifene*	Tamoxifen-like	Vaginal atrophy treatment Osteoporosis treatment Breast cancer prevention	Estrogenic effects on vaginal epithelium that is not observed with tamoxifen or raloxifene [130, 131, 134] Inhibits tumor growth in animal models as effective as tamoxifen [137, 138]	Phase III trial (826 women) relieves vaginal dryness Phase II trial (118 women): Comparable to or slightly better than raloxifene [135] Phase III trial planned (detail not available) Not available
Arzoxifene* (LY353381)	Raloxifene-like	Breast cancer treatment Breast cancer prevention	Antiestrogenic in breast and endometrium, estrogenic in bone and lipids [172] Effective to prevent ER-positive and ER-negative mammary tumors especially in combination with LG100268 [138, 216]	Phase III trial (200 patients) inferior to tamoxifen [217] Phase I trials (50 and 76 women) low toxicity and favorable biomarker profile [218]
Lasofoxifene* (CP-336156, Fablyn)	Raloxifene-like	Osteoporosis treatment and prevention Vaginal atrophy treatment Breast cancer treatment and prevention Heart disease prevention	Higher potency than tamoxifen and raloxifene [139]; higher oral bioavailability than raloxifene [54] Effects similar to tamoxifen to prevent and treat NMU-induced mammary tumor in rats [219]	Phase III trial (1,907 women) significantly increases bone mineral density compared to placebo, no endometrial effects, no association with thromboembolic disorder [142] Phase III trial to compare with raloxifene (CORAL trial, details not available) Phase III trail (445 patients) improves vaginal atrophy compared to placebo Phase III trial (PEARL trial with 8,556 women), reduces ER-positive breast cancer incidence compared to placebo; slightly decreases major coronary disease risk; reduces vertebral and non-vertebral fractures; increases risks of venous thromboembolic events but not stroke; no endometrial effects [SABCS 2008, abstract 11]

Table 1. contd….

Drug Name	Category (Structure)	Effects	Preclinical Results	Clinical Status
Bazedoxifene* (TSE-424 WAY-140424)	Raloxifene-like	Osteoporosis treatment and prevention Breast cancer prevention	Increases bone density with little uterine or vasomotor effects Inhibits estrogen-stimulated breast cancer cells growth [154]	Phase III trial (7,492 women) reduces vertebral and non-vertebral fracture incidences, while raloxifene is not effective against non-vertebral fracture [160] Phase III trial (497 women) reduces endometrial thickness, unique property among known SERMs [220] Not available

*Ospemifene- not approved by the FDA, *Arzoxifene- not approved by the FDA, trials terminated by Eli Lilly, *Lasofoxifene- not approved by the FDA, approved in the EU, *Bazedoxifene- not approved by the FDA, approved in the EU.

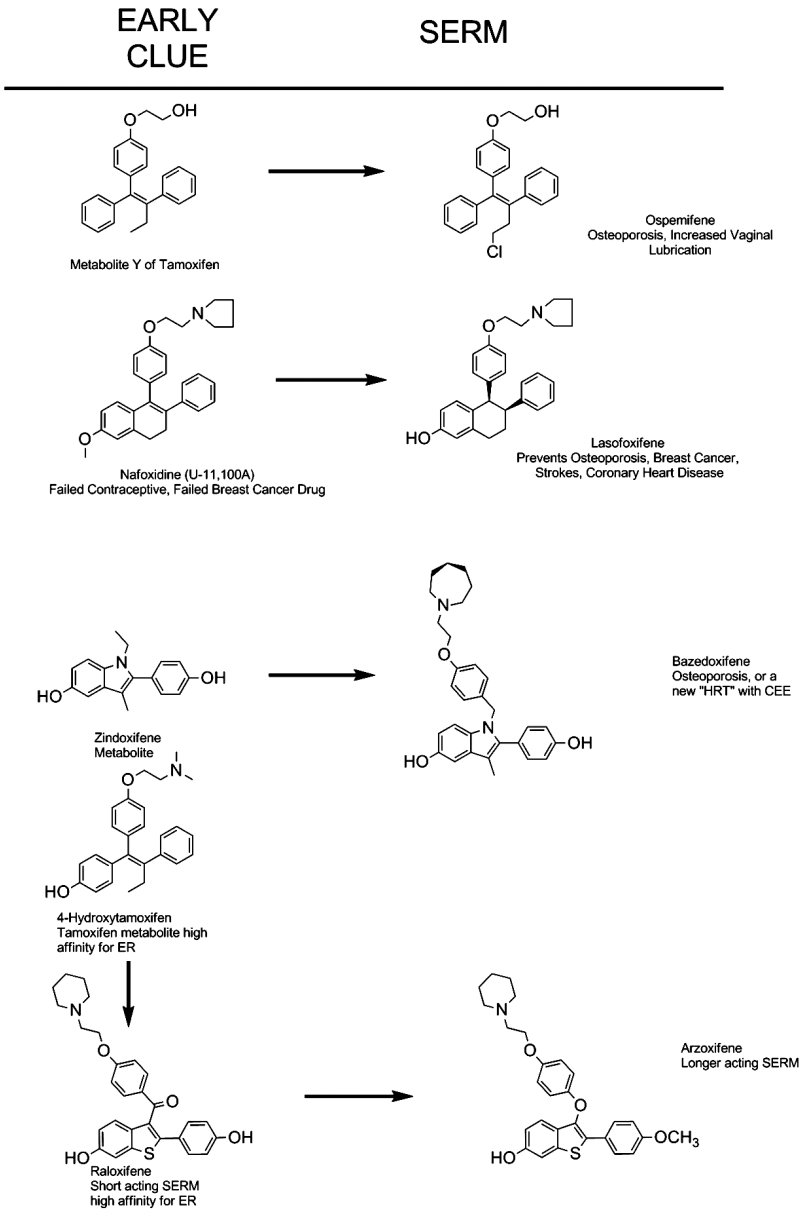


Fig. (4). Origins of current selective ER modulators for earlier nonsteroidal antiestrogens. Ospemifene is a known metabolite of the breast metabolite of the breast cancer drug toremifene. The metabolite of toremifene was found because an analogous metabolite Y was discovered for tamoxifen in the early 1980's [119]. Lasofoxifene has its origins with failed antifertility agent discovered in the early 1960's U-11, 100A [121]. The compound renamed nafoxidine was tested as a drug for the treatment of breast cancer but again failed because of serious side effects [123]. Bazedoxifene is an adaptation of an estrogenic metabolite from a failed breast cancer drug Zindoxifene [124]. Arzoxifene is the final compound in the lineage to find the optimal long acting SERM from the discovery that the hydroxylated metabolite of tamoxifen 4-hydroxytmaoxifen has a very high binding affinity for ER [35]. Raloxifene was a direct result of this discovery which became a successful SERM in clinical practice.

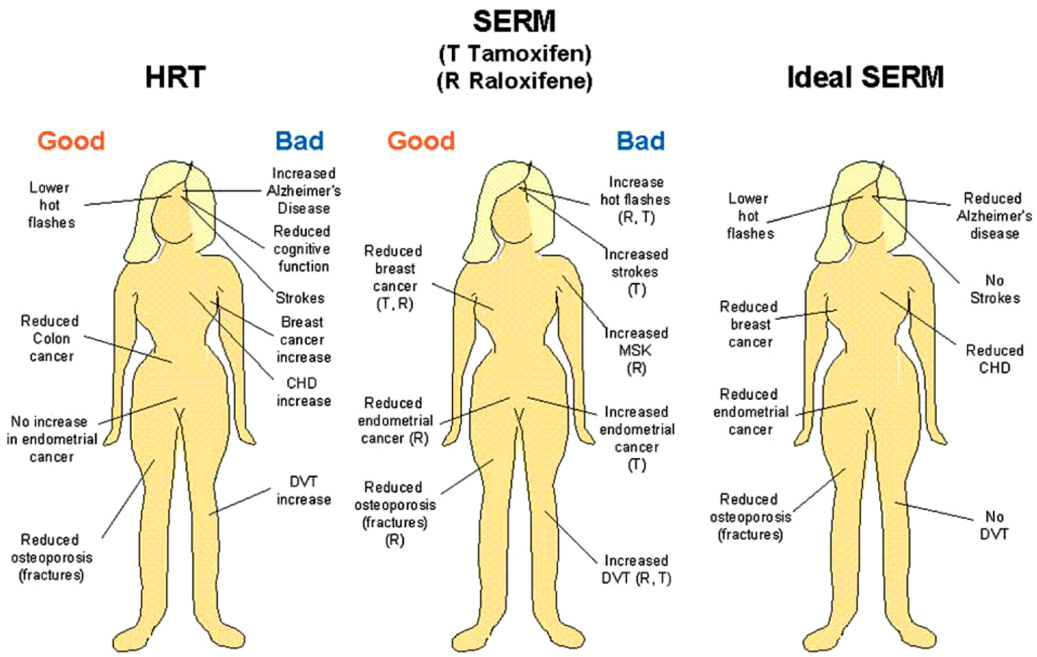


Fig. (5). Progress toward an ideal SERM. The overall good or bad aspects of administering hormone replacement therapy to postmenopausal women compared with the observed site-specific actions of the selective estrogen receptor modulators tamoxifen and raloxifene. The known beneficial or negative actions of SERMs have opened the door for drug discovery to create the ideal SERM or targeted SERMs to either improve quality of life or prevent diseases associated with aging in women. This figure is published with permission from Elsevier. Jordan, V.C. Selective estrogen receptor modulation: Concept and consequences in cancer. Cancer Cell, 2004 Mar; 5(3): 207-213.

demethylation, and deamination to a glycol side chain [118, 119]. The analogous metabolite was found for toremifene and became ospemifene. Unlike tamoxifen toremifine is not a rat hepatocarcinogen [120] so ospemifene would be a safer SERM. Lasofoxifene is derived from nafoxidine (U11, 100A) which was discovered as an antifertility compound in rodents [121, 122], that evolved to be an experimental breast cancer drug but was too toxic [123]. Basedoxifene is related to a metabolite of a failed breast cancer drug zindoxifene [124] and arzoxifene is the end product in the line of 4-hydroxytamoxifen [35], the antiestrogen is a metabolite of tamoxifen with high affinity for the ER but poor antitumor activity [37], to raloxifene (also with a poor antitumor activity [125]) and then to arzoxifene in an attempt to improve pharmacokinetics and develop a better breast cancer drug. We will consider the clinical evaluation of each.

Ospemifene

Ospemifene, is an antiestrogenic triphenylethylene derivative structurally similar to tamoxifen and toremifene. The story of the structure is of interest. In 1982/83 a new metabolite of tamoxifen was reported and shown to be a weak antiestrogen [38, 118]. Subsequently, the related metabolite of toremifene was found and reported. This metabolite is now known as ospemifene. Ospemifene was initially designed to treat vaginal atrophy in postmenopausal women; however, it may also be useful for the prevention and treatment of osteoporosis. Ospemifene binds to both ERs, though binds to the ERα more strongly. Similar to 17β-estradiol and tamoxifen, its estrogen-like effects are noted to occur in bone *via* enhanced osteoblastic proliferation and differentiation, but not osteoclast apoptosis. Raloxifene, in contrast, is noted to induce osteoclast apoptosis. Increased mineralization and bone nodule formation have been demonstrated in bone marrow cultures [126]. In an ovariectomised rat model, ospemifene's role in improved bone strength and density has been compared to estradiol and other SERMs, and at a dose of 10mg/kg, ospemifene has been found to prevent bone loss and increase bone strength on the femoral neck and lumbar vertebrae similar to the bone agonist effects observed in estradiol (at 50 μg/kg), raloxifene (3 mg/kg) and droloxifene (10 mg/kg) [127].

In the immature rat uterus, ospemifene has been shown to be of the order of 200- to 1000-fold less estrogenic than estradiol [127]. Notably, even at doses sufficient to prevent bone loss, ospemifene was found to induce weak antagonistic activity in the uterus and may even preserve normal endometrium. At doses 5-10 times higher than that required to prevent bone loss, however, ospemifene does appear to have estrogenic effects at the uterus similar to that seen with 1mg/kg of tamoxifen [127].

Tamoxifen appears to induce liver carcinogenesis *via* the creation of DNA adduct, but this does not occur with ospemifene in rats. This fact has led to the belief that ospemifene's carcinogenic potential is lower than that noted in tamoxifen [127, 128].

Data pooled from at least seven clinical trials have shown ospemifene has a favorable toxicity profile and is generally well tolerated [129-135]. Headache was the most commonly reported adverse event, with rates similar to that of placebo (15% and 12.8%, respectively) [129]. Likewise, endometrial

effects produced by ospemifene are comparable to that seen with raloxifene, and are less than that observed with tamoxifen [130, 131, 134]. In the vagina, however, ospemifene does have more estrogenic effects, thereby improving vaginal dryness more effectively than either raloxifene or tamoxifen [130, 134]. Similarly, ospemifene has been shown to have a positive, or at least neutral effect on hot flashes. Moreover, even at doses far exceeding that used in phase II and III clinical trials, phase 1 data has shown no significant toxicity.

Despite promising data in the ovariectomized mouse model, long-term data on the bone-protective effect in humans with ospemifene are lacking. A short-term, 3-month, phase II comparative study found ospemifene at doses 30, 60, or 90 mg/day compared with raloxifene, had similar to slightly better effects on bone as measured by markers of bone resorption, and comparable efficacy in lowering LDL-cholesterol [135]. The effects on bone varied across the groups, potentially due to the non-osteoporotic nature of the study population and to the short period of both treatment and follow-up [135]. A second phase II trial demonstrated that varying doses of ospemifene administration for three months did, in a dose-dependent manner, reduce markers for bone turnover compared with placebo [133]. Notably, however, the long-term prevention of bone loss and the prevention of osteoporotic fractures in women treated with ospemifene are not under study.

Data *in vitro* and *in vivo* suggest that ospemifene may have breast chemopreventive activity in breast tissue in much the same way as toremifene or raloxifene [127, 128, 136-138], but randomized clinical trials have not addressed this issue.

Lasofoxifene

Collaborative effort of Pfizer and Ligand Pharmaceuticals to synthesize novel SERMs with good oral bioavalability and higher potency for treatment of vaginal atrophy and osteoporosis resulted in the discovery of lasofoxifene. Lasofoxifene is a naphthalene derivative, a third generation SERM with high selective affinity for both the ERα and ERβ subtypes. IC50 of lasofoxifene is similar to that of estradiol, and 10 times higher than that of raloxifene and 4-hydroxytamoxifen. Lasofoxifene is able to inhibit osteoclastogenesis, reduced bone turnover, and prevented bone loss in preclinical studies [139, 140]. Lasofoxifene causes significant improvement in markers of bone turnover and bone mineral density in preclinical studies, as well as phase II and III trials [141-144]. One particular phase II study, which enrolled 394 healthy postmenopausal women, lasofoxifene 0.017, 0.05, 0.15, and 0.5 mg/day was compared with supplementation with calcium and vitamin D [145]. After six months of therapy, women receiving the two highest doses of lasofoxifene were noted to have statistically significant improvement in maintenance or gain of bone mineral density compared with the calcium plus vitamin D arm ($p<0.01$), and at one year of treatment all groups of lasofoxifene had significant improvement over the calcium plus vitamin D cohort. Across groups, 85-98% of women treated with lasofoxifene either had no loss of, or had improvement in BMD after one year.

Three separate phase III studies have also been completed. The first, OPAL (Older People And n-3 Long-chain polyunsaturated fatty acids), was actually a collection of multiple trials [146, 147]. In this study, 1907 nonosteoporotic postmenopausal women with lumbar spine T-scores from 0 to -2.5, all of whom received calcium and vitamin D supplementation, were randomized to receive lasofoxifene 0.025, 0.25, or 0.5 mg/day or placebo for 2 years. At six, twelve, and twenty-four months, lasofoxifene at all doses were shown to increase bone mineral density compared with a decrease observed in the placebo group, and at six and twenty-four months decrease bone turnover was observed compared with placebo. The groups treated with lasofoxifene also underwent bone biopsies which showed normal quality bones.

CORAL, a 2-year randomized, double-blind, placebo-controlled, and active treatment-controlled study, enrolled 410 women with lumbar spine BMD between +2 and -2.5 standard deviations of age-matched controls (Z-score) and compared indices of bone health in groups treated with lasofoxifene at either 0.25 or 1 mg/day, raloxifene 60 mg/day, or placebo [148]. All groups received calcium and vitamin D supplementation. Evaluated endpoints included percent change from baseline BMD in the lumbar-spine at 2 years (primary endpoint), as well as total hip BMD, LDL-cholesterol, safety, and biochemical markers of bone turnover including N-telopeptide, deoxypyridinoline crosslinks, bone-specific alkaline phosphatase, and osteocalcin. Lasofoxifene at both doses was superior to raloxifene and placebo at increasing lumbar spine BMD, though lasofoxifene at both doses and raloxifene were similar in increasing total hip BMD compared with placebo. Both agents decreased biochemical markers of bone turnover compared with placebo, though lasofoxifene did so to a greater extent. An editorial written by Goldstein considered lasofoxifene, therefore, superior to raloxifene to increase BMD and decrease markers of bone turnover [116].

PEARL, a large, 8556 women, 5-year, randomized, double blind, placebo-controlled, parallel-assignment study that evaluated safety and efficacy of 0.25mg/day and 0.5mg/day of lasofoxifene combined with 1000 mg calcium and 400-800 IU vitamin D daily [149]. Patients were women with osteoporosis with lumbar spine or femoral neck BMD <2.5 SD or less and the study evaluated efficacy in preventing new vertebral fractures. Though initially due to be completed in March 2006, the trial was extended to early 2008 in order to include 2 additional coprimary endpoints, nonvertebral fracture and ER-positive breast cancer. Results of the study were notable as the 0.2mg/day dose was found to reduce only vertebral fractures ($p < 0.001$) but the higher dose 0.5mg/day significantly decreased both vertebral ($p < 0.001$) and nonvertebral fractures ($p = 0.002$). Importantly, the lasofoxifene 0.5 mg dose also showed decreased risk of ER positive breast cancer [150], coronary heart disease, and stroke, though an increased risk for VTE, and long term data confirms the safety and efficacy of the agent [55].

Lasofoxifene has shown decrease in bone turnover markers, coronary heart disease, serum lipids, and stroke incidence [55]. Lasofoxifene, unlike many other SERMs, has been shown to reduce vaginal pH and decrease vaginal

dryness [151], but over 5 years it has been shown to be associated with endometrial hypertrophy, a finding which warrants close monitoring [55]. Long-term efficacy data comparing lasofoxifene with raloxifene and hormone-replacement therapy to elucidate whether lasofoxifene is superior for the prevention and treatment of postmenopausal osteoporosis and osteoporosis-related fractures is still lacking. Further studies should also be completed to elucidate whether it ought to play a role in menopause symptom control.

Bazedoxifene

Bazedoxifene (BZA, TSE-424), an indole-based ER ligand which has been carefully selected for its better side effect profile compared with its predecessors, is being developed for use both alone for the prevention and treatment of osteoporosis in postmenopausal women, and in combination with conjugated equine estrogens for menopausal symptoms [152-154]. Already approved by the European Union in April, 2009, it is in the late phases of review by the US FDA. It binds to both ERα and ERβ, though with slightly higher affinity for ERα, is less selective for ERα than raloxifene, and in fact has a nearly 10-fold lower affinity for ERα than 17β-estradiol [152, 154]. It is tissue-specific, and in both *in vitro* and *in vivo* preclinical models, has been shown to positively affect lipid profiles and skeletal-related markers *via* antiresorptive affects, and displays estrogen receptor interaction without stimulating the endometrium, causing breast cancer cell proliferation, or negatively affecting the central nervous system.

Even at low doses, bazedoxifene maintains bone mass, and reaches maximal significant efficacy at a dose of 0.3mg/kg/day, and this dose has been shown to maintain vertebral compressive strength better than or equivalent to sham-operated animals [152, 154]. Efficacy on maintaining skeletal parameters have been shown to be similar among bazedoxifene, raloxifene, and lasofoxifene [80, 139], and recently, bazedoxifene has been shown in ovariectomized monkeys to partially preserve bone densimetry- measured bone mass, as well as preserve bone strength and reduce bone turnover at a dose up to 25mg/kg/day for 18 months [155]. Further, in preclinical *in vivo* studies, an improved uterine profile for bazedoxifene compared with raloxifene was noted, as well as lack of adverse effect on plasma lipids or reproductive tract histology [152]. Bazedoxifene is well tolerated, and both increases endothelial nitric oxide synthase activity and does not antagonize the effect of 17β-estradiol on vasomotor symptoms, both of which are improvements over raloxifene [152-154].

When bazedoxifene was coadministered with CEEs such as Premarin® or human parathyroid hormone (hPTH), preclinical studies utilizing ovariectomized mice noted that at doses 7- to 10-fold higher than the bone efficacious dose, bazedoxifene antagonized the uterine stimulation by Premarin® but did not change the uterine weight compared with ovariectomized controls [156]. Further, BMD and cancellous bone compartments were similar between animals treated with bazedoxifene 3 mg/kg/day and Premarin® 2.5 mg/kg/day versus sham-operated animals. When combined with bone efficacious doses of CEEs, bazedoxifene, compared with raloxifene and lasofoxifene, showed no difference in skeletal parameters [157]. Further, lasofoxifene 0.1 mg/kg/day has been shown in another study to enhance reversal of osteopenia when coadministered with hPTH 10 μg/kg/day similarly to bazedoxifene, raloxifene, or risedronic acid and greater than hPTH monotherapy [158].

Taken together, bazedoxifene may then emerge as a promising new treatment for osteoporosis, either as monotherapy or combined with conjugate estrogens, with an improved side effect profile given the reduced uterine and vasomotor effects over SERMs currently available. In fact, bazedoxifene has been studied in the prevention and treatment of postmenopausal osteoporosis. Two phase III trials showed bazedoxifene at varying doses to improve skeletal parameters [159-161]. The first found that in postmenopausal women at risk for osteoporosis, the drug (at 10, 20, and 40mg) prevented bone loss and reduced bone turnover, with a favorable endometrial, breast, and ovarian safety profile [159, 160]. The second study recruited postmenopausal women who already had osteoporosis, showed bazedoxifene at 20 and 40 mg significantly reduced the risk of new vertebral fractures compared with placebo without any evidence of endometrial or breast stimulation, and in a higher risk group, bazedoxifene 20 mg significantly decreased the risk of nonvertebral fracture compared with both placebo and raloxifene 60mg [160]. In studies that followed women for five years, no breast or endometrial stimulation was seen at either 3 or 5 years and generally the medication was well tolerated, with rates of adverse events and discontinuations due to adverse events similar to placebo [162]. However, hot flushes and leg cramps, most of which were mild and did not lead to cessation of the medication, were noted more frequently at 5 years in patients treated with bazedoxifene compared with placebo [160].

The major adverse effect of bazedoxifene is venous thromboembolism, the majority of which occur in the first two years [163]. The increased risk of VTE with bazedoxifene over five years is similar to that seen with longterm evaluation with raloxifene [164]. Raloxifene [81, 164] has a much higher risk of VTE in the first two years than bazedoxifene. Additionally, there is a slightly increased risk for fatal stroke when raloxifene is compared with placebo over 5.6 years of followup, though the overall stroke risk is not statistically different from placebo [81]. Similarly, the risk of PE or RVT, as well as cardiac events is similar among the bazedoxifene and placebo groups.

Multiple studies have demonstrated favorable breast and endometrial safety profiles over 5 years [163]. In fact, not only is the incidence of breast and endometrial-related adverse effects similar between placebo and bazedoxifene, but there were fewer cases of endometrial carcinoma in the bazedoxifene group compared with placebo. Incidence of breast cancer and fibrocystic breast disease was not different between bazedoxifene [31] and placebo groups [162, 163], though the risk of breast cancer is decreased with tamoxifen and raloxifene [31].

Therefore, bazedoxifene has shown favorable effects on bone parameters in postmenopausal women, and has been shown to be relatively safe and well tolerated. It exhibits no

breast or endometrial stimulation and the small increase in VTE is better in the first two years, and similar in the longer-term to other SERMs.

Arzoxifene

Arzoxifene is a benzothiophene analogue in which the carbonyl hinge of raloxifene has been replaced by an ether (Fig. **3**). Additionally, there is a protective methyl ether on one of the phenolic hydroxyls. These features lead to increased antiestrogen properties, greater bioavailability, and increased binding affinity for the ERα compared with raloxifene [165-177]. Preclinical data has shown favorable estrogenic effects on bone and lipid metabolism, while exerting antiestrogen effects on breast and uterine tissue [174]. In fact, preclinical studies which compared equivalent doses of arzoxifene, tamoxifen, and raloxifene showed arzoxifene inhibits tumor growth to a greater extent than the other two agents [170, 172, 177, 178].

Phase I data has shown that in patients with metastatic breast cancer, arzoxifene at varying dosages (10, 20, 50 or 100 mg/day) was tolerated well, had no dose limiting toxicities, and was even found to decrease osteocalcin, which suggested a bone health benefit [179]. The drug was even tolerated well in women with liver disease, and the most common side effect was hot flashes, reported in 56% of women regardless of the dose taken. In a study of patients with advanced hormone receptor positive endometrial cancer, 34% of women treated with arzoxifene 20mg daily showed favorable response with minimal toxicity [180]. Further, data from healthy volunteers showed doses as low as 10 mg/day is biologically active, and doses from 25 to 100 mg daily showed similar effects on bone markers, lipoprotein levels, and gonadotropin levels [172].

In ovariectomized rats, long-term treatment with arzoxifene showed a protective effect on cancellous bone mass, architecture, and strength and did not stimulate endometrium proliferation [181]; in young rats, it entirely inhibited uterine growth [168]. At bone protective doses of 0.1 and 0.5 mg/kg/day, arzoxifene also exerts a positive effect on serum lipids [181]. Further, in ovariectomized mice, arzoxifene plus PTH increased bone mass at trabecular bone sites both more quickly and to a greter extent than PTH alone, PTH plus equine estrogens, or PTH plus raloxifene [182].

Recent data has shown that in postmenopausal women with osteoporosis and invasive breast cancer, treatment with arzoxifene for 4 years significantly reduced the risk of vertebral fractures. Neither raloxifene, bazedoxifene, nor arzoxifene reduced the risk of nonvertebral fractures in the same study [160]. Lasofoxifene 0.5 mg/day did reduce the risk of nonvertebral fractures, but it reduced markers of bone turnover to a similar amount as arzoxifene in the same study [55].

A different phase II study found that during 6 months of arzoxifene, lumbar spine bone mineral density showed dose response relationships [183], though this was not seen with raloxifene. Further, a phase III study of postmenopausal women with osteoporosis found improved bone turnover markers and increased spine and hip bone density in patients treated with arzoxifene 20 mg/day [184]. Two larger studies, FOUNDATION [185] and GENERATIONS [184] found that in women with at-risk or low bone density, arzoxifene 20mg/daily significantly increased BMD and reduced bone turnover markers compared with placebo. Data taken from the GENERATIONS study note that arzoxifene, however, has no improved clinical efficacy in preventing fractures over raloxifene as arzoxifene has some vertebral, but not nonvertebral fracture risk-reduction. All antiresorptive agents seem to exert non-vertebal fracture risk reduction, but only alendronate, risedronate, zoledronic acid, lasofoxifene, and denosumab have demonstrated some nonvertebral risk-reduction in postmenopausal women with osteoporosis [55, 186-189]. It is hypothesized that arzoxifene, despite improved BMD and markers of bone turnover over raloxifene, may not have enough antiresorptive potency to significantly improve non-vertebral fractures in patients enrolled in the GENERATIONS trial.

Along a different vein, with the exception of bazedoxifene, SERMs as a class have been shown to reduce the risk of invasive breast cancer, as arzoxifene, tamoxifen, raloxifene, and 0.5 mg/day of lasofoxifene have all been shown to reduce invasive breast cancer risk [30, 55, 81, 113, 150, 190].

Arzoxifene, like raloxifene, does not seem to have adverse effects on cardiovascular health in postmenopausal women [183, 184]. Additionally, lasofoxifene has even been shown to decrease the incidence of coronary events and stroke compared with placebo [55]. However, tibolone and tamoxifen increase the risk of stroke, and CEE with medroxyprogesterone increases the risk of Coronary Artery Disease (CAD) and stroke [113, 191, 192]. Perhaps the reason for this difference in effect is related to differences on the agents' effect on inflammation as the agents influence C-reactive protein (CRP) differently. Estrogen and tibolone increase levels of CRP [192], raloxifene and arzoxifene have no effect on CRP levels, and lasofoxifene decreases CRP levels [55]. All decrease LDL levels. Major side effects of arzoxifene include VTE (a side effect common among all agents with any estrogen receptor agonist effects), hot flushes, muscle cramps, vaginal discharge, vulvovaginitis, and increased reports of endometrial cancer and hyperplasia, though the last two failed to reach statistical significance [185]. Also, several SERMs, including arzoxifene, increase the risk of cholecystitis as estrogen has known lithogenic effects on bile [193]. Further, increased pulmonary complications including coughing, pneumonia, increased reports of upper respiratory infections, and serious COPD related events have been reported with treatment with arzoxifene [190]. Although previous trials of SERMs, estrogen, and tibolone have not reported increased pulmonary complications, bronchial epithelium and alveolar macrophages do express ER [194, 195]. Therefore, inhibition of ER increases expression of inflammatory lung markers, including tumor necrosis factor α (TNF-α) [194, 195]. In fact, there was a small increased risk of lung metastases, but not primary lung tumors, with treatment with arzoxifene, though given the lack of biologic basis for pulmonary susceptibility to metastases, this finding may be due to chance alone [190].

Arzoxifene in similar to other SERMs in that it reduces the risk of invasive breast cancer, reduces bone resorption, increase BMD modestly, and decrease the risk of vertebral, but not nonvertebral fractures [190]. Yet it increases the risk of venous thromboembolic events and adverse gyenocologic events. Results from a five year clinical study were released by Lilly in 2009 that arzoxifene met its primary endpoints of reduction in vertebral fractures and breast cancer in postmenopausal women [185]. However, due to lack of successfully meeting the study's planned secondary endpoints including reduction in non-vertebral fractures and cardiovascular events and improvements in cognitive function, Lilly announced they were discontinuing development of the drug and would not seek regulatory approval.

Tissue Selective Estrogen Complex (TSEC)

Currently, research is advancing to establish the optimal balance between ER agonist and antagonist activity for an ideal menopausal therapy. An approach, termed the tissue-selective estrogen complex, blends tissue-selective activities of a SERM with an estrogen. For example, bazedoxifene in combination with conjugated equine estrogens (CEE) has been studied for the treatment of both hot flushes and vulvar vaginal atrophy, with positive results on both menopausal symptoms [196, 197].

One study involving 3397 women either 1-5 years post menopause or >5 years post menopause enrolled in the Osteoporosis Prevention I and II Substudies aimed to evaluate the efficacy of the tissue-selective estrogen complex bazedoxifene/CEE to prevent osteoporosis [198]. The study used bazedoxifene (10, 20, or 40 mg) with CEEs (0.625 or 0.45 mg), raloxifene (60 mg), or placebo, and was administered daily for 2 years. The primary outcome was change in bone mineral density at the lumbar spine, though hip bone mineral density was also measured.

For women 1-5 years postmenopause, all bazedoxifene/CEE treatment groups showed greater percent increase in lumbar spine BMD from baseline to 2 years compared with raloxifene ($p < 0.05$). BMD significantly improved relative to raloxifene ($p < 0.05$) with both lower doses of bazedoxifene/CEE doses for women >5 years. In substudy I, mean percent increases in total hip BMD were significantly higher from baseline to month 24 with bazedoxifene (10 mg)/CEEs (0.625 or 0.45 mg) and bazedoxifene (20 mg)/CEEs (0.625 mg) compared with raloxifene. Further, total hip BMD was significantly higher with all doses of bazedoxifene/CEE doses from baseline at months 12 and 24 compared with decreases observed with placebo [198].

In substudy II, total hip BMD was higher in all bazedoxifene/CEE doses compared with placebo at both months 12 and 24, and for femoral neck BMD, the same superiority of bazedoxifene/CEE doses over placebo was true except for bazedoxifene (40 mg)/CEEs (0.45 mg) at month 12 [198]. Additionally, at both time points, median percent changes from baseline in serum osteocalcin and C-telopeptide were significantly greater with all bazedoxifene/CEE doses than with placebo ($p <0.001$). Total hip BMD was significantly better ($p < 0.05$) for bazedoxifene (10 mg)/CEEs (0.625 or 0.45 mg) over raloxifene, and bazedoxifene (20 mg)/CEEs (0.45 mg) at month 24 over raloxifene. In terms of side effects, rates of serious side effects including myocardial infarction, venous thromboembolism, superficial thrombosis or phlebitis, coronary artery disease, and breast pain were all similar between azedoxifene/CEEs groups and placebo [198]. This study highlighted the potential for a SERM/CEE combination that may provide the benefits of hormone therapy in a symptomatic postmenopausal woman with her uterus without the need for a progestin.

CONCLUSIONS

The original SERM idea [2] has now been proven in clinical trial to have benefit for women in routine clinical practice. The past 50 years has seen the rise and fall of hormone replacement therapy (HRT) [191, 199, 200] as the answer to postmenopausal women's health (Fig. **5**). In its place, the development of first tamoxifen and then the first true SERM raloxifene advanced the concept towards the ideal SERM (Fig. **5**). The agents currently in development or the process of approval and launch each edge towards an optimal multifunctional medicine for postmenopausal women's health.

Tamoxifen, the pioneering medicine that led the transition from "nonsteroidal antiestrogen" to become the first SERM in clinical practice, was the gold standard for the antihormonal therapy for two decades [14, 110] and pioneered chemoprevention [65, 113]. Nevertheless, the discovery and development of the aromatase inhibitors [201], resulted in improvements in adjuvant therapy outcomes and a reduction in side effects for postmenopausal breast cancer patients [202]. Now tamoxifen remains the standard of care for the premenopausal patients and for risk reduction in both premenopausal and postmenopausal women. Raloxifene is available for risk reduction in postmenopausal women with or without a uterus [203, 204], but unlike tamoxifen that is used for 5 years, raloxifene must be given indefinitely [32]. It should be mentioned that an aromatase inhibitor exemestane has been successfully tested to reduce breast cancer risk in postmenopausal women [205]. However, unlike the promise of a reduction of breast cancer incidence with SERMs, exemestane decreases bone density [206].

The development of novel SERMs targeted to the ER in recent years has led to significant progress in the identification of therapeutic agents for the management of postmenopausal conditions related to estrogen deficiency, particularly osteoporosis. The possibility of designing a single molecule that has all of the desired characteristics of an ideal SERM (Fig. **5**) seems to be unlikely, but progress has clearly been achieved with lasofoxifene [55] and the TSEC proposal is also innovative.

The benefits of tamoxifen use outweigh the associated risks in women who have already been diagnosed with breast cancer [110]. However, endometrial safety concerns outweigh the bone protection offered by SERMs in the development of postmenopausal osteoporosis. Because raloxifene has a good record of endometrial safety it is currently the only SERM approved for the prevention and treatment of postmenopausal osteoporosis, having demonstrated efficacy in preventing bone loss and fractures, with the added benefit of preventing breast cancer.

Clinical data on newer SERMs in development (Fig. **3**) indicate that these compounds may, or may not, have attributes that represent an improvement relative to currently available SERMs. Other SERMs have shown promise in treating the symptoms of menopause, such as vaginal atrophy, and are also undergoing investigation as possible agents for the prevention of breast cancer. A common adverse event associated with SERMs to date seems to be an increased incidence of hot flushes and warrants further study to determine a solution. There are several novel agents being evaluated to address hot flashes [207-210]. Bazedoxifene has been shown to maintain or increase BMD, reduce bone turnover, and decrease the risk of new vertebral fracture in postmenopausal women without evidence of endometrial or breast stimulation in large, prospective phase III studies [196-198]. In the global placebo- and active-controlled osteoporosis treatment study, bazedoxifene showed a significant reduction in nonvertebral fracture risk in a subgroup of more than 1,700 women at higher risk for fracture relative to both placebo and raloxifene. The TSEC containing bazedoxifene/CEEs had an acceptable endometrial profile, suggesting an alternative to the addition of a progestin to estrogens for endometrial protection [197]. The beneficial effects of bazedoxifene/CEEs on menopausal symptoms and bone loss as well as the bleeding profile and overall safety data may indicate a suitable option for symptomatic postmenopausal women. Clarification of other safety concerns (i.e., venous thromboembolic events) is needed to appropriately determine the benefit/risk balance of SERMs in development.

For the future, basic research is essential for further progress in exploiting this drug group. Basic knowledge of mechanisms must advance the original SERM concept [2, 23]. The subsets of ERα and ERβ specific agonists can be used to further define targets in other pathologic states [211-214]. Finally, we must embrace the molecular biology of coactivator/corepressor action in the molecular pharmacology drug discovery process [101, 211, 213, 214]. Forty years ago it would have been impossible to achieve the current clinical advances without laboratory findings to transform an orphan drug group the "nonsteroidal antiestrogens" [16] into the SERMs [2, 23]. This "road map" proved to be particularly prophetic and significantly advanced women's health in numerous disease states throughout the world.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This paper recognizes Russell E. McDaniel for his exemplary service to the Tamoxifen Team at Georgetown University. This paper is the result. This work (VCJ) was supported by the Department of Defense Breast Program under Award number W81XWH-06-1-0590 Center of Excellence; subcontract under the SU2C (AACR) Grant number SU2C-AACR-DT0409; the Susan G Komen for the Cure Foundation under Award number SAC100009 and the Lombardi Comprehensive Cancer 1095 Center Support Grant (CCSG) Core Grant NIH P30 CA051008. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

REFERENCES

[1] Lacassagne A. Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer 1936; 27: 217-25.
[2] Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 1990; 50: 4177-89.
[3] Lerner LJ, Holthaus FJ Jr., Thompson CR. A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol. Endocrinology 1958; 63: 295-318.
[4] Greenblatt RB, Barfield WE, Jungck EC, Ray AW. Induction of ovulation with MRL/41. Preliminary report. JAMA 1961; 178: 101-4.
[5] Greenblatt RB, Roy S, Mahesh VB. Induction of ovulation. Am J Obstet Gynecol 1962; 84: 900-12.
[6] Clark JH, Markaverich BM. The agonistic-antagonistic properties of clomiphene: a review. Pharmacol Ther 1981; 15: 467-519.
[7] Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 1967; 13: 101-19.
[8] Harper MJ, Walpole AL. Mode of action of I.C.I. 46,474 in preventing implantation in rats. J Endocrinol 1967; 37: 83-92.
[9] Klopper A, Hall M. New synthetic agent for the induction of ovulation: preliminary trials in women. Br Med J 1971; 1: 152-4.
[10] Williamson JG, Ellis JD. The induction of ovulation by tamoxifen. J Obstet Gynaecol Br Commonw1973; 80: 844-7.
[11] Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer 1971; 25: 270-5.
[12] Ward HW. Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J 1973; 1: 13-4.
[13] Jordan VC. Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 2006; 147(Suppl 1): S269-76.
[14] Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2003; 2: 205-13.
[15] Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 2008; 44: 30-8.
[16] Jordan VC. Biochemical pharmacology of antiestrogen action. Pharmacol Rev 1984; 36: 245-76.
[17] Powles TJ, Hardy JR, Ashley SE, *et al.* A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. Bri J cancer 1989; 60: 126-31.
[18] Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 1988; 48: 812-5.
[19] Fornander T, Rutqvist LE, Cedermark B, *et al.* Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1989; 1: 117-20.
[20] Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 1987; 47: 4020-4.
[21] Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 1987; 10: 31-5.
[22] Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc 1987; 46: 1870-4.
[23] Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 1988; 6: 589-95.
[24] Love RR, Wiebe DA, Newcomb PA, *et al.* Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann Intern Med 1991; 115: 860-4.
[25] Love RR, Mazess RB, Barden HS, *et al.* Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 1992; 326: 852-6.
[26] Love RR, Newcomb PA, Wiebe DA, *et al.* Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 1990; 82: 1327-32.

[27] Jordan VC, Koch R, Mittal S, Schneider MR. Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis *in vitro*. Br J Pharmacol 1986; 87: 217-23.

[28] Lieberman ME, Gorski J, Jordan VC. An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens *in vitro*. J Biol Chem 1983; 258: 4741-5.

[29] Ettinger B, Black DM, Mitlak BH, *et al*. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 1999; 282: 637-45.

[30] Cummings SR, Eckert S, Krueger KA, *et al.* The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA 1999; 281: 2189-97.

[31] Vogel VG, Costantino JP, Wickerham DL, *et al.* Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 2006; 295: 2727-41.

[32] Vogel VG, Costantino JP, Wickerham DL, *et al.* Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. Cancer Prev Res (Phila Pa) 2010; 3: 696-706.

[33] Kenakin T. Inverse, protean, and ligand-selective agonism: matters of receptor conformation. FASEB J 2001; 15: 598-611.

[34] Miller CP. SERMs: evolutionary chemistry, revolutionary biology. Curr Pharm Des 2002; 8: 2089-111.

[35] Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 1977; 75: 305-16.

[36] Allen KE, Clark ER, Jordan VC. Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 1980; 71: 83-91.

[37] Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 1980; 16: 239-51.

[38] Jordan VC, Bain RR, Brown RR, Brown RR, Gosden B, Santos MA. Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient during therapy for advanced breast cancer. Cancer Res 1983; 43: 1446-50.

[39] Johnson MD, Zuo H, Lee KH, *et al.* Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. Breast Cancer Res Treat 2004; 85: 151-9.

[40] Lim YC, Desta Z, Flockhart DA, Skaar TC. Endoxifen (4-hydroxy-N-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen. Cancer Chemother Pharmacol 2005; 55: 471-8.

[41] Lim YC, Li L, Desta Z, *et al.* Endoxifen, a secondary metabolite of tamoxifen, and 4-OH-tamoxifen induce similar changes in global gene expression patterns in MCF-7 breast cancer cells. J Pharmacol Exp Ther 2006; 318: 503-12.

[42] Stearns V, Johnson MD, Rae JM, *et al.* Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. J Natl Cancer Inst 2003; 95: 1758-64.

[43] Jin Y, Desta Z, Stearns V, *et al.* CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. J Natl Cancer Inst 2005; 97: 30-9.

[44] Borges S, Desta Z, Li L, *et al.* Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. Clin Pharmacol Ther 2006; 80: 61-74.

[45] Goetz MP, Rae JM, Suman VJ, *et al.* Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. J Clin Oncol 2005; 23: 9312-8.

[46] Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea- induced rat mammary carcinoma model. Cancer Res 1987; 47: 4020-4.

[47] Jordan VC, Gosden B. Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 1983; 113: 463-8.

[48] Snyder KR, Sparano N, Malinowski JM. Raloxifene hydrochloride. Am J Health Syst Pharm 2000; 57: 1669-75; quiz 76-78.

[49] Kemp DC, Fan PW, Stevens JC. Characterization of raloxifene glucuronidation *in vitro*: contribution of intestinal metabolism to presystemic clearance. Drug Metab Dispos 2002; 30: 694-700.

[50] Jeong EJ, Lin H, Hu M. Disposition mechanisms of raloxifene in the human intestinal Caco-2 model. J Pharmacol Exp Ther 2004; 310: 376-85.

[51] Falany JL, Pilloff DE, Leyh TS, Falany CN. Sulfation of raloxifene and 4-hydroxytamoxifen by human cytosolic sulfotransferases. Drug Metab Dispos 2006; 34: 361-8.

[52] Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nat Rev Cancer 2007; 7: 46-53.

[53] Prakash C, Johnson KA, Schroeder CM, Potchoiba MJ. Metabolism, distribution, and excretion of a next generation selective estrogen receptor modulator, lasofoxifene, in rats and monkeys. Drug Metab Dispos 2008; 36: 1753-69.

[54] Rosati RL, Da Silva Jardine P, *et al.* Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryltetrahydronaphthalene. J Med Chem 1998; 41: 2928-31.

[55] Cummings SR, Ensrud K, Delmas PD, *et al.* Lasofoxifene in postmenopausal women with osteoporosis. N Engl J Med 2010; 362: 686-96.

[56] Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res 1997; 57: 3071-8.

[57] Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 1983; 32: 1031-6.

[58] Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 1990; 50: 3189-92.

[59] Jordan VC, Lababidi MK, Mirecki DM. Anti-oestrogenic and anti-tumour properties of prolonged tamoxifen therapy in C3H/OUJ mice. Eur J Cancer 1990; 26: 718-21.

[60] Jordan VC, Lababidi MK, Langan-Fahey S. Suppression of mouse mammary tumorigenesis by long-term tamoxifen therapy. J Natl Cancer Inst 1991; 83: 492-6.

[61] Beall PT, Misra LK, Young RL, Spjut HJ, Evans HJ, LeBlanc A. Clomiphene protects against osteoporosis in the mature ovariectomized rat. Calcif Tissue Int 1984; 36: 123-5.

[62] Turner RT, Wakley GK, Hannon KS, Bell NH. Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. J Bone Miner Res 1987; 2: 449-56.

[63] Turner RT, Wakley GK, Hannon KS, Bell NH. Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. Endocrinology 1988; 122: 1146-50.

[64] Cuzick J, Forbes JF, Sestak I, *et al.* Long-term results of tamoxifen prophylaxis for breast cancer--96-month follow-up of the randomized IBIS-I trial. J Natl Cancer Inst 2007; 99: 272-82.

[65] Fisher B, Costantino JP, Wickerham DL, *et al.* Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 2005; 97: 1652-62.

[66] Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. J Natl Cancer Inst 2007; 99: 283-90.

[67] Nawrocki JW, Weiss SR, Davidson MH, *et al.* Reduction of LDL cholesterol by 25% to 60% in patients with primary hypercholesterolemia by atorvastatin, a new HMG-CoA reductase inhibitor. Arterioscler Thromb Vasc Biol 1995; 15: 678-82.

[68] Gould AL, Rossouw JE, Santanello NC, Heyse JF, Furberg CD. Cholesterol reduction yields clinical benefit: impact of statin trials. Circulation 1998; 97: 946-52.

[69] LaRosa JC, He J, Vupputuri S. Effect of statins on risk of coronary disease: a meta-analysis of randomized controlled trials. JAMA 1999; 282: 2340-6.

[70] Pignone M, Phillips C, Mulrow C. Use of lipid lowering drugs for primary prevention of coronary heart disease: meta-analysis of randomised trials. BMJ 2000; 321: 983-6.

[71] Steinberg D, Avigan J, Feigelson EB. Effects of Triparanol (Mer-29) on Cholesterol Biosynthesis and on Blood Sterol Levels in Man. J Clin Invest 1961; 40: 884-93.

[72] Kirby TJ. Cataracts produced by triparanol. (MER-29). Trans Am Ophthalmol Soc 1967; 65: 494-543.

[73] Love RR, Mamby CC, Feyzi JM. Tamoxifen-induced decreases in total cholesterol with 2 weeks of treatment. J Natl Cancer Inst 1993; 85: 1344-5.

[74] Love RR, Wiebe DA, Feyzi JM, Newcomb PA, Chappell RJ. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women after 5 years of treatment. J Natl Cancer Inst 1994; 86: 1534-9.

[75] Rutqvist LE, Mattsson A. Cardiac and thromboembolic morbidity among postmenopausal women with early-stage breast cancer in a randomized trial of adjuvant tamoxifen. The Stockholm Breast Cancer Study Group. J Natl Cancer Inst 1993; 85: 1398-406.

[76] McDonald CC, Alexander FE, Whyte BW, Forrest AP, Stewart HJ. Cardiac and vascular morbidity in women receiving adjuvant tamoxifen for breast cancer in a randomised trial. The Scottish Cancer Trials Breast Group. BMJ 1995; 311: 977-80.

[77] Hackshaw A, Roughton M, Forsyth S, *et al.* Long-term benefits of 5 years of tamoxifen: 10-year follow-up of a large randomized trial in women at least 50 years of age with early breast cancer. J Clin Oncol 2011; 29: 1657-63.

[78] Esteva FJ, Hortobagyi GN. Comparative assessment of lipid effects of endocrine therapy for breast cancer: implications for cardiovascular disease prevention in postmenopausal women. Breast 2006; 15: 301-12.

[79] Lewis L, Jordan V, 103-21. Comprehensive Medicinal Chemistry. In: Taylor J, Triggle D, Eds. Case Histories: Raloxifene. Oxford, UK: Elsevier Limited 2006; pp. 103-21.

[80] Black LJ, Sato M, Rowley ER, *et al.* Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 1994; 93: 63-9.

[81] Barrett-Connor E, Mosca L, Collins P, *et al.* Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med 2006; 355: 125-37.

[82] Jensen EV, Jacobson HI. Basic guides to the mechanism of estrogen action. Recent Progr Hormone Res 1962; 18: 387-414.

[83] Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J. Sequence and expression of human estrogen receptor complementary DNA. Science 1986; 231: 1150-4.

[84] Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 1996; 93: 5925-30.

[85] Kuiper GG, Carlsson B, Grandien K, *et al.* Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 1997; 138: 863-70.

[86] Roger P, Sahla ME, Makela S, Gustafsson JA, Baldet P, Rochefort H. Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. Cancer Res 2001; 61: 2537-41.

[87] Shaaban AM, O'Neill PA, Davies MP, *et al.* Declining estrogen receptor-beta expression defines malignant progression of human breast neoplasia. Am J Surg Pathol 2003; 27: 1502-12.

[88] Paruthiyil S, Parmar H, Kerekatte V, Cunha GR, Firestone GL, Leitman DC. Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. Cancer Res 2004; 64: 423-8.

[89] Acconcia F, Totta P, Ogawa S, *et al.* Survival versus apoptotic 17beta-estradiol effect: role of ER alpha and ER beta activated non-genomic signaling. J Cell Physiol 2005; 203: 193-201.

[90] Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J, Nilsson S. Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. Mol Pharmacol 1998; 54: 105-12.

[91] McInerney EM, Weis KE, Sun J, Mosselman S, Katzenellenbogen BS. Transcription activation by the human estrogen receptor subtype beta (ER beta) studied with ER beta and ER alpha receptor chimeras. Endocrinology 1998; 139: 4513-22.

[92] Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 1999; 140: 5566-78.

[93] Paech K, Webb P, Kuiper GG, *et al.* Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites [see comments]. Science 1997; 277: 1508-10.

[94] Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 1995; 270: 1354-7.

[95] Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocrine Rev 2004; 25: 45-71.

[96] Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. J Med Chem 2003; 46: 1081-111.

[97] Kraichely DM, Sun J, Katzenellenbogen JA, Katzenellenbogen BS. Conformational changes and coactivator recruitment by novel ligands for estrogen receptor-alpha and estrogen receptor-beta: correlations with biological character and distinct differences among SRC coactivator family members. Endocrinology 2000; 141: 3534-45.

[98] Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. The structural basis of estrogen receptor/co-activator recognition and the antagonism of this interaction by tamoxifen. Cell 1998; 95: 927-37.

[99] Brzozowski AM, Pike AC, Dauter Z, *et al.* Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997; 389: 753-8.

[100] Wijayaratne AL, Nagel SC, Paige LA, *et al.* Comparative analyses of mechanistic differences among antiestrogens. Endocrinology 1999; 140: 5828-40.

[101] Lonard DM, O'Malley BW. The expanding cosmos of nuclear receptor coactivators. Cell 2006; 125: 411-4.

[102] Sun Y. E3 Ubiquitin Ligases as Cancer Targets and Biomarkers. Neoplasia 2006; 8: 645-54.

[103] Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 2000; 103: 843-52.

[104] Jordan VC, Dix CJ, Rowsby L, Prestwich G. Studies on the mechanism of action of the nonsteroidal antioestrogen tamoxifen (I.C.I. 46,474) in the rat. Mol Cell Endocrinol 1977; 7: 177-92.

[105] Horwitz KB, McGuire WL. Nuclear mechanisms of estrogen action. Effects of estradiol and anti- estrogens on estrogen receptors and nuclear receptor processing. J Biol Chem 1978; 253: 8185-91.

[106] Wijayaratne AL, McDonnell DP. The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. J Biol Chem 2001; 276: 35684-92.

[107] Wu RC, Smith CL, O'Malley BW. Transcriptional Regulation by Steroid Receptor Coactivator Phosphorylation. Endocrine Rev 2006; 26: 393-9.

[108] Osborne CK, Bardou V, Hopp TA, *et al.* Role of the estrogen receptor coactivator AIB1 (SRC3) and HER2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst 2003; 95: 353-61.

[109] Shou J, Massarweh S, Osborne CK, *et al.* Mechanisms of Tamoxifen Resistance: Increased Estrogen Receptor-HER2/neu Cross-Talk in ER/HER2–Positive Breast Cancer. J Natl Cancer Inst 2004; 96: 926-35.

[110] Davies C, Godwin J, Gray R, *et al.* Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 2011; 378: 771-84.

[111] Sawaki M, Wada M, Sato Y, *et al.* High-dose toremifene as first-line treatment of metastatic breast cancer resistant to adjuvant aromatase inhibitor: A multicenter phase II study. Oncol Lett 2012; 3: 61-5.

[112] Holli K, Valavaara R, Blanco G, *et al.* Safety and efficacy results of a randomized trial comparing adjuvant toremifene and tamoxifen in postmenopausal patients with node-positive breast cancer. Finnish Breast Cancer Group. J Clin Oncol 2000; 18: 3487-94.

[113] Fisher B, Costantino JP, Wickerham DL, *et al.* Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998; 90: 1371-88.

[114] Siris ES, Harris ST, Eastell R, *et al.* Skeletal effects of raloxifene after 8 years: results from the continuing outcomes relevant to Evista (CORE) study. J Bone Miner Res 2005; 20: 1514-24.

[115] Martino S, Cauley JA, Barrett-Connor E, *et al.* Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 2004; 96: 1751-61.
[116] Goldstein SR. Not all SERMs are created equal. Menopause 2006; 13: 325-7.
[117] Jordan VC. Designer estrogens. Sci Am 1998; 279: 60-7.
[118] Bain RR, Jordan VC. Identification of a new metabolite of tamoxifen in patient serum during breast cancer therapy. Biochem Pharmacol 1983; 32: 373-5.
[119] Jordan VC, Bain RR, Brown RR, Gosden B, Santos MA. Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. Cancer Res 1983; 43: 1446-50.
[120] Hard GC, Iatropoulos MJ, Jordan K, *et al.* Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. Cancer Res 1993; 53: 4534-41.
[121] Lednicer D, Lyster SC, Aspergren BD, Duncan GW. Mammalian antifertility agents. 3. 1-Aryl-2-phenyl-1,2,3,4-tetrahydro-1-naphthols, 1-aryl-2-phenyl-3,4-dihydronaphthalenes, and their derivatives. J Med Chem 1966; 9: 172-6.
[122] Lednicer D, Lyster SC, Duncan GW. Mammalian antifertility agents. IV. Basic 3,4-dihydronaphthalenes and 1,2,3,4-tetrahydro-1-naphthols. J Med Chem 1967; 10: 78-84.
[123] Legha SS, Slavik M, Carter SK. Nafoxidine--an antiestrogen for the treatment of breast cancer. Cancer 1976; 38: 1535-41.
[124] Robinson SP, Koch R, Jordan VC. *In vitro* estrogenic actions in rat and human cells of hydroxylated derivatives of D16726 (zindoxifene), an agent with known antimammary cancer activity *in vivo*. Cancer Res 1988; 48: 784-7.
[125] Gradishar W, Glusman J, Lu Y, Vogel C, Cohen FJ, Sledge GW Jr. Effects of high dose raloxifene in selected patients with advanced breast carcinoma. Cancer 2000; 88: 2047-53.
[126] Qu Q, Harkonen PL, Vaananen HK. Comparative effects of estrogen and antiestrogens on differentiation of osteoblasts in mouse bone marrow culture. J Cell Biochem 1999; 73: 500-7.
[127] Qu Q, Zheng H, Dahllund J, *et al.* Selective estrogenic effects of a novel triphenylethylene compound, FC1271a, on bone, cholesterol level, and reproductive tissues in intact and ovariectomized rats. Endocrinology 2000; 141: 809-20.
[128] Hellmann-Blumberg U, Taras TL, Wurz GT, DeGregorio MW. Genotoxic effects of the novel mixed antiestrogen FC-1271a in comparison to tamoxifen and toremifene. Breast Cancer Res Treat 2000; 60: 63-70.
[129] DeGregorio MW, Wurz GT, Taras TL, Erkkola RU, Halonen KH, Huupponen RK. Pharmacokinetics of (deaminohydroxy)toremifene in humans: a new, selective estrogen-receptor modulator. Eur J Clin Pharmacol 2000; 56: 469-75.
[130] Voipio SK, Komi J, Kangas L, Halonen K, DeGregorio MW, Erkkola RU. Effects of ospemifene (FC-1271a) on uterine endometrium, vaginal maturation index, and hormonal status in healthy postmenopausal women. Maturitas 2002; 43: 207-14.
[131] Rutanen EM, Heikkinen J, Halonen K, Komi J, Lammintausta R, Ylikorkala O. Effects of ospemifene, a novel SERM, on hormones, genital tract, climacteric symptoms, and quality of life in postmenopausal women: a double-blind, randomized trial. Menopause 2003; 10: 433-9.
[132] Ylikorkala O, Cacciatore B, Halonen K, *et al.* Effects of ospemifene, a novel SERM, on vascular markers and function in healthy, postmenopausal women. Menopause 2003; 10: 440-7.
[133] Komi J, Heikkinen J, Rutanen EM, Halonen K, Lammintausta R, Ylikorkala O. Effects of ospemifene, a novel SERM, on biochemical markers of bone turnover in healthy postmenopausal women. Gynecol Endocrinol 2004; 18: 152-8.
[134] Komi J, Lankinen KS, Harkonen P, *et al.* Effects of ospemifene and raloxifene on hormonal status, lipids, genital tract, and tolerability in postmenopausal women. Menopause 2005; 12: 202-9.
[135] Komi J, Lankinen KS, DeGregorio M, *et al.* Effects of ospemifene and raloxifene on biochemical markers of bone turnover in postmenopausal women. J Bone Miner Metab 2006; 24: 314-8.
[136] Taras TL, Wurz GT, DeGregorio MW. *In vitro* and *in vivo* biologic effects of Ospemifene (FC-1271a) in breast cancer. J Steroid Biochem Mol Biol 2001; 77: 271-9.
[137] Namba R, Young LJ, Maglione JE, *et al.* Selective estrogen receptor modulators inhibit growth and progression of premalignant lesions in a mouse model of ductal carcinoma in situ. Breast Cancer Res 2005; 7: R881-9.
[138] Wurz GT, Read KC, Marchisano-Karpman C, *et al.* Ospemifene inhibits the growth of dimethylbenzanthracene-induced mammary tumors in Sencar mice. J Steroid Biochem Mol Biol 2005; 97: 230-40.
[139] Ke HZ, Paralkar VM, Grasser WA, *et al.* Effects of CP-336,156, a new, nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. Endocrinology 1998; 139: 2068-76.
[140] Maeda T, Ke HZ, Simmons H, Thompson D. Lasofoxifene, a next generation estrogen receptor modulator: preclinical studies. Clin Calcium 2004; 14: 85-93.
[141] Gennari L. Lasofoxifene (Pfizer). Curr Opin Investig Drugs 2005; 6: 1067-78.
[142] Gennari L. Lasofoxifene: a new type of selective estrogen receptor modulator for the treatment of osteoporosis. Drugs Today (Barc) 2006; 42: 355-67.
[143] Gennari L, Merlotti D, Martini G, Nuti R. Lasofoxifene: a third-generation selective estrogen receptor modulator for the prevention and treatment of osteoporosis. Expert Opin Investig Drugs 2006; 15: 1091-103.
[144] Goldstein SR. Not all selective estrogen response modulators are created equal: update on lasofoxifene. Int J Gynecol Cancer 2006; 16 Suppl 2: 504.
[145] Moffett A, Ettinger M, Bolognese M, *et al.* Lasofoxifene, a next generation SERM, is effective in preventing loss of BMD and reducing LDL-C in postmenopausal women. J Bone Min Res 2004; 19: S96.
[146] Davidson M, Moffett A, Welty F, *et al* Extraskeletal effects of lasofoxifene on postmenopausal women. J Bone Min Res 2005; 20: S173.
[147] McClung M, Siris E, Cummings S. Lasofoxifene increased BMD of the spine and hip and decreased bone turnover markers in postmenopausal women with low or normal BMD. J Bone Min Res 2005; 20: F429.
[148] McClung MR, Siris E, Cummings S, *et al.* Prevention of bone loss in postmenopausal women treated with lasofoxifene compared with raloxifene. Menopause 2006; 13: 377-86.
[149] Cummings S, Eastell R, Ensrud K. The effects of lasofoxifene on fractures and breast cancer: 3 year results from the PEARL trial. J Bone Miner Res 2008; 23: S81.
[150] LaCroix AZ, Powles T, Osborne CK, *et al.* Breast cancer incidence in the randomized PEARL trial of lasofoxifene in postmenopausal osteoporotic women. J Natl Cancer Inst 2010; 102: 1706-15.
[151] Gennari L. Lasofoxifene, a new selective estrogen receptor modulator for the treatment of osteoporosis and vaginal atrophy. Expert Opin Pharmacother 2009; 10: 2209-20.
[152] Miller CP, Collini MD, Tran BD, *et al.* Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. J Med Chem 2001; 44: 1654-7.
[153] Gruber C, Gruber D. Bazedoxifene (Wyeth). Curr Opin Investig Drugs 2004; 5: 1086-93.
[154] Komm BS, Kharode YP, Bodine PV, Harris HA, Miller CP, Lyttle CR. Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity. Endocrinology 2005; 146: 3999-4008.
[155] Smith S, Minck D, Jolette J, *et al.* Bazedoxifene prevents ovariectomy-induced bone loss in the Cynomolgus monkey. J Bone Miner Res 2005; 20: S174.
[156] Komm B, Kharode Y, Bodine P, *et al.* Bazedoxifene + conjugated estrogens: a balanced combination to provide optimal estrogenic "safety" and efficacy. J Bone Miner Res 2003; 18: S272.
[157] Komm B, Kharode Y, Bodine P, *et al.* Combining a SERM with conjugated estrogens (CE) to improve the SERM profile: not all SERMs may succeed. J Bone Miner Res 2003; 25: S273.
[158] Kharode Y, Green P, Marzolf J, *et al.* Comparison of the effects of bazedoxifene, raloxifene, lasofoxifene and risedronate, co-treatment on h-PTH-induced reversal of established osteopenia in ovariectomized rats. J Bone Miner Res 2003; 18: S273.
[159] Miller PD, Chines AA, Christiansen C, *et al.* Effects of bazedoxifene on BMD and bone turnover in postmenopausal women: 2-yr results of a randomized, double-blind, placebo-, and active-controlled study. J Bone Miner Res 2008; 23: 525-35.

[160] Silverman SL, Christiansen C, Genant HK, *et al.* Efficacy of bazedoxifene in reducing new vertebral fracture risk in postmenopausal women with osteoporosis: results from a 3-year, randomized, placebo-, and active-controlled clinical trial. J Bone Miner Res 2008; 23: 1923-34.

[161] Pinkerton JV, Archer DF, Utian WH, *et al.* Bazedoxifene effects on the reproductive tract in postmenopausal women at risk for osteoporosis. Menopause 2009; 16: 1102-8.

[162] Archer DF, Pinkerton JV, Utian WH, *et al.* Bazedoxifene, a selective estrogen receptor modulator: effects on the endometrium, ovaries, and breast from a randomized controlled trial in osteoporotic postmenopausal women. Menopause 2009; 16: 1109-15.

[163] de Villiers TJ, Chines AA, Palacios S, *et al.* Safety and tolerability of bazedoxifene in postmenopausal women with osteoporosis: results of a 5-year, randomized, placebo-controlled phase 3 trial. Osteoporos Int 2011; 22: 567-76.

[164] Grady D, Ettinger B, Moscarelli E, *et al.* Safety and adverse effects associated with raloxifene: multiple outcomes of raloxifene evaluation. Obstet Gynecol 2004; 104: 837-44.

[165] Dodge JA, Lugar CW, Cho S, *et al.* Evaluation of the major metabolites of raloxifene as modulators of tissue selectivity. J Steroid Biochem Mol Biol 1997; 61: 97-106.

[166] Grese TA, Cho S, Finley DR, *et al.* Structure-activity relationships of selective estrogen receptor modulators: modifications to the 2-arylbenzothiophene core of raloxifene. J Med Chem 1997; 40: 146-67.

[167] Grese TA, Sluka JP, Bryant HU, *et al.* Molecular determinants of tissue selectivity in estrogen receptor modulators. Proc Natl Acad Sci USA 1997; 94: 14105-10.

[168] Palkowitz AD, Glasebrook AL, Thrasher KJ, *et al.* Discovery and synthesis of [6-hydroxy-3-[4-[2-(1-piperidinyl)ethoxy]phenoxy]-2-(4-hydroxyphenyl)]b enzo[b]thiophene: a novel, highly potent, selective estrogen receptor modulator. J Med Chem 1997; 40: 1407-16.

[169] Grese TA, Pennington LD, Sluka JP, *et al.* Synthesis and pharmacology of conformationally restricted raloxifene analogues: highly potent selective estrogen receptor modulators. J Med Chem 1998; 41: 1272-83.

[170] Sato M, Turner CH, Wang T, Adrian MD, Rowley E, Bryant HU. LY353381.HCl: a novel raloxifene analog with improved SERM potency and efficacy *in vivo*. J Pharmacol Exp Ther 1998; 287: 1-7.

[171] Bryant HU, Glasebrook AL, Yang NN, Sato M. An estrogen receptor basis for raloxifene action in bone. J Steroid Biochem Mol Biol 1999; 69: 37-44.

[172] Munster PN. Arzoxifene: the development and clinical outcome of an ideal SERM. Expert Opin Investig Drugs 2006; 15: 317-26.

[173] Dardes RC, Bentrem D, O'Regan RM, Schafer JM, Jordan VC. Effects of the new selective estrogen receptor modulator LY353381.HCl (Arzoxifene) on human endometrial cancer growth in athymic mice. Clin Cancer Res 2001; 7: 4149-55.

[174] Suh N, Glasebrook AL, Palkowitz AD, *et al.* Arzoxifene, a new selective estrogen receptor modulator for chemoprevention of experimental breast cancer. Cancer Res 2001; 61: 8412-5.

[175] Detre S, Riddler S, Salter J, A'Hern R, Dowsett M, Johnston SR. Comparison of the selective estrogen receptor modulator arzoxifene (LY353381) with tamoxifen on tumor growth and biomarker expression in an MCF-7 human breast cancer xenograft model. Cancer Res 2003; 63: 6516-22.

[176] Licun W, Tannock IF. Selective estrogen receptor modulators as inhibitors of repopulation of human breast cancer cell lines after chemotherapy. Clin Cancer Res 2003; 9: 4614-8.

[177] Freddie CT, Larsen SS, Bartholomaeussen M, Lykkesfeldt AE. The effect of the new SERM arzoxifene on growth and gene expression in MCF-7 breast cancer cells. Mol Cell Endocrinol 2004; 219: 27-36.

[178] Morello KC, Wurz GT, DeGregorio MW. SERMs: current status and future trends. Crit Rev Oncol Hematol 2002; 43: 63-76.

[179] Munster PN, Buzdar A, Dhingra K, *et al.* Phase I study of a third-generation selective estrogen receptor modulator, LY353381.HCL, in metastatic breast cancer. J Clin Oncol 2001; 19: 2002-9.

[180] McMeekin DS, Gordon A, Fowler J, *et al.* A phase II trial of arzoxifene, a selective estrogen response modulator, in patients with recurrent or advanced endometrial cancer. Gynecol Oncol 2003; 90: 64-9.

[181] Ma YL, Bryant HU, Zeng Q, *et al.* Long-term dosing of arzoxifene lowers cholesterol, reduces bone turnover, and preserves bone quality in ovariectomized rats. J Bone Miner Res 2002; 17: 2256-64.

[182] Sato M, Zeng GQ, Rowley E, Turner CH. LY353381 x HCl: an improved benzothiophene analog with bone efficacy complementary to parathyroid hormone-(1-34). Endocrinology 1998; 139: 4642-51.

[183] Downs RW Jr, Moffett AM, Ghosh A, Cox DA, Dowsett SA, Harper K. Effects of arzoxifene on bone, lipid markers, and safety parameters in postmenopausal women with low bone mass. Osteoporos Int 2010; 21: 1215-26.

[184] Kendler DL, Palacios S, Cox DA, *et al.* Arzoxifene versus raloxifene: effect on bone and safety parameters in postmenopausal women with osteoporosis. Osteoporos Int 2012; 23(3): 1091-109.

[185] Bolognese M, Krege JH, Utian WH, *et al.* Effects of arzoxifene on bone mineral density and endometrium in postmenopausal women with normal or low bone mass. J Clin Endocrinol Metab 2009; 94: 2284-9.

[186] Black DM, Cummings SR, Karpf DB, *et al.* Randomised trial of effect of alendronate on risk of fracture in women with existing vertebral fractures. Fracture Intervention Trial Research Group. Lancet 1996; 348: 1535-41.

[187] Harris ST, Watts NB, Genant HK, *et al.* Effects of risedronate treatment on vertebral and nonvertebral fractures in women with postmenopausal osteoporosis: a randomized controlled trial. Vertebral Efficacy With Risedronate Therapy (VERT) Study Group. JAMA 1999; 282: 1344-52.

[188] Pols HA, Felsenberg D, Hanley DA, *et al.* Multinational, placebo-controlled, randomized trial of the effects of alendronate on bone density and fracture risk in postmenopausal women with low bone mass: results of the FOSIT study. Fosamax International Trial Study Group. Osteoporos Int 1999; 9: 461-8.

[189] Black DM, Delmas PD, Eastell R, *et al.* Once-yearly zoledronic acid for treatment of postmenopausal osteoporosis. N Engl J Med 2007; 356: 1809-22.

[190] Cummings SR, McClung M, Reginster JY, *et al.* Arzoxifene for prevention of fractures and invasive breast cancer in postmenopausal women. J Bone Miner Res 2011; 26: 397-404.

[191] Rossouw JE, Anderson GL, Prentice RL, *et al.* Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA 2002; 288: 321-33.

[192] Cummings SR, Ettinger B, Delmas PD, *et al.* The effects of tibolone in older postmenopausal women. N Engl J Med 2008; 359: 697-708.

[193] Cirillo DJ, Wallace RB, Rodabough RJ, *et al.* Effect of estrogen therapy on gallbladder disease. JAMA 2005; 293: 330-9.

[194] Ivanova MM, Mazhawidza W, Dougherty SM, Minna JD, Klinge CM. Activity and intracellular location of estrogen receptors alpha and beta in human bronchial epithelial cells. Mol Cell Endocrinol 2009; 305: 12-21.

[195] Vegeto E, Cuzzocrea S, Crisafulli C, *et al.* Estrogen receptor-alpha as a drug target candidate for preventing lung inflammation. Endocrinology 2010; 151: 174-84.

[196] Archer DF, Lewis V, Carr BR, Olivier S, Pickar JH. Bazedoxifene/conjugated estrogens (BZA/CE): incidence of uterine bleeding in postmenopausal women. Fertil Steril 2009; 92: 1039-44.

[197] Pinkerton JV, Stovall DW. Bazedoxifene when paired with conjugated estrogens is a new paradigm for treatment of postmenopausal women. Expert Opin Investig Drugs 2010; 19: 1613-21.

[198] Lindsay R, Gallagher JC, Kagan R, Pickar JH, Constantine G. Efficacy of tissue-selective estrogen complex of bazedoxifene/conjugated estrogens for osteoporosis prevention in at-risk postmenopausal women. Fertil Steril 2009; 92: 1045-52.

[199] Crandall CJ, Aragaki AK, Cauley JA, *et al.* Breast tenderness and breast cancer risk in the estrogen plus progestin and estrogen-alone women's health initiative clinical trials. Breast Cancer Res Treat 2012; 132: 275-85.

[200] Narod SA. Hormone replacement therapy and the risk of breast cancer. Nat Rev Clin Oncol 2011; 8: 669-76.

[201] Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007; 72: 7-25.

[202] Dowsett M, Cuzick J, Ingle J, *et al.* Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 2010; 28: 509-18.

[203] Vogel VG. Raloxifene: a selective estrogen receptor modulator for reducing the risk of invasive breast cancer in postmenopausal women. Womens Health (Lond Engl) 2007; 3: 139-53.

[204] Vogel VG, Costantino JP, Wickerham DL, *et al.* Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. Cancer Prev Res (Phila) 2010; 3: 696-706.

[205] Goss PE, Ingle JN, Ales-Martinez JE, Cheung AM, Chlebowski RT, Wactawski-Wende J, *et al.* Exemestane for breast-cancer prevention in postmenopausal women. N Engl J Med 2011; 364: 2381-91.

[206] Mieog JS, Morden JP, Bliss JM, Coombes RC, van de Velde CJ. Carpal tunnel syndrome and musculoskeletal symptoms in postmenopausal women with early breast cancer treated with exemestane or tamoxifen after 2-3 years of tamoxifen: a retrospective analysis of the Intergroup Exemestane Study. Lancet Oncol 2012.

[207] Watanabe N, Ikeno A, Minato H, Nakagawa H, Kohayakawa C, Tsuji J. Discovery and preclinical characterization of (+)-3-[4-(1-piperidinoethoxy)phenyl]spiro[indene- 1,1'-indane]-5,5'-diol hydrochloride: a promising nonsteroidal estrogen receptor agonist for hot flush. J Med Chem 2003; 46: 3961-4.

[208] Wallace OB, Lauwers KS, Dodge JA, *et al.* A selective estrogen receptor modulator for the treatment of hot flushes. J Med Chem 2006; 49: 843-6.

[209] Jain N, Xu J, Kanojia RM, *et al.* Identification and structure-activity relationships of chromene-derived selective estrogen receptor modulators for treatment of postmenopausal symptoms. J Med Chem 2009; 52: 7544-69.

[210] Jain N, Kanojia RM, Xu J, *et al.* Novel chromene-derived selective estrogen receptor modulators useful for alleviating hot flushes and vaginal dryness. J Med Chem 2006; 49: 3056-9.

[211] Meegan MJ, Lloyd DG. Advances in the science of estrogen receptor modulation. Curr Med Chem 2003; 10: 181-210.

[212] Arevalo MA, Santos-Galindo M, Lagunas N, Azcoitia I, Garcia-Segura LM. Selective estrogen receptor modulators as brain therapeutic agents. J Mol Endocrinol 2011; 46: R1-9.

[213] Nilsson S, Gustafsson JA. Estrogen receptors: therapies targeted to receptor subtypes. Clin Pharmacol Ther 2011; 89: 44-55.

[214] Rosano C, Stec-Martyna E, Lappano R, Maggiolini M. Structure-based approach for the discovery of novel selective estrogen receptor modulators. Curr Med Chem 2011; 18: 1188-94.

[215] O'Malley BW. Molecular biology. Little molecules with big goals. Science 2006; 313: 1749-50.

[216] Liby K, Rendi M, Suh N, *et al.* The combination of the rexinoid, LG100268, and a selective estrogen receptor modulator, either arzoxifene or acolbifene, synergizes in the prevention and treatment of mammary tumors in an estrogen receptor-negative model of breast cancer. Clin Cancer Res 2006; 12: 5902-9.

[217] Deshmane V, Krishnamurthy S, Melemed AS, Peterson P, Buzdar AU. Phase III double-blind trial of arzoxifene compared with tamoxifen for locally advanced or metastatic breast cancer. J Clin Oncol 2007; 25: 4967-73.

[218] Fabian CJ, Kimler BF, Anderson J, *et al.* Breast cancer chemoprevention phase I evaluation of biomarker modulation by arzoxifene, a third generation selective estrogen receptor modulator. Clin Cancer Res 2004; 10: 5403-17.

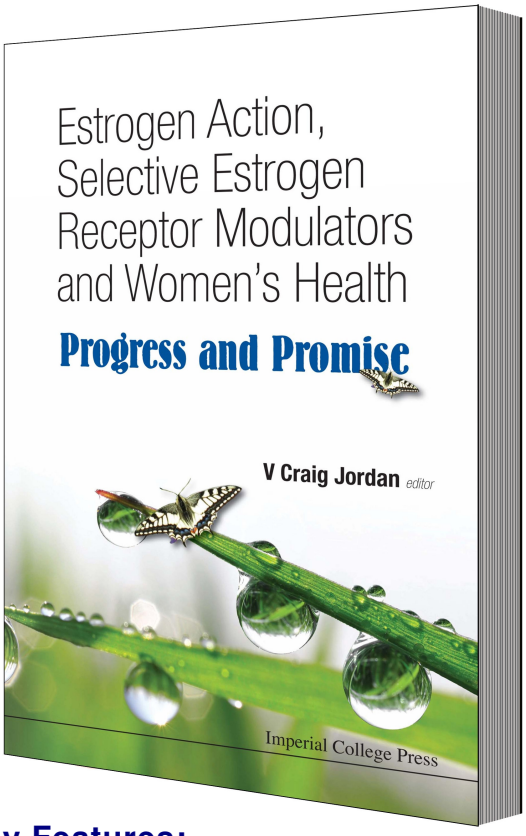
[219] Cohen LA, Pittman B, Wang CX, Aliaga C, Yu L, Moyer JD. LAS, a novel selective estrogen receptor modulator with chemopreventive and therapeutic activity in the N-nitroso-N-methylurea-induced rat mammary tumor model. Cancer Res 2001; 61: 8683-8.

[220] Ronkin S, Northington R, Baracat E, *et al.* Endometrial effects of bazedoxifene acetate, a novel selective estrogen receptor modulator, in postmenopausal women. Obstet Gynecol 2005; 105: 1397-404.

Received: March 01, 2012 Revised: July 11, 2012 Accepted: October 03, 2012

Connecting Great Minds

25% Discount



Estrogen Action, Selective Estrogen Receptor Modulators and Women's Health

Progress and Promise

edited by **V Craig Jordan** *(Georgetown University, USA)*

Key Features:

- A unique easy-to-read book for clinicians, biological faculty, informed patients, scientists and medical professionals
- Focuses on the evolution of estrogen action in women's health and changes in healthcare through scientific research
- A concise summary of progress
- No other competing titles of the same caliber
- Written by the pioneers in the field

This volume presents the evolution of the authors' ideas about estrogen action and its modulation by a new group of drugs called SERMs (Selective Estrogen Receptor Modulators). The pioneering SERMs — tamoxifen and raloxifene — are known to have saved the lives of millions of women around the world and improved the health of millions more. Estrogen is the central hormone of women's health and reproduction. The book is a journey through 40 years of discovery and success in advancing women's health, with the prospect of improved innovation through medicinal chemistry for the future.

Readership: Clinicians, biological faculty, informed patients, scientists and students in biology and medicine, healthcare and medical professionals, breast cancer survivors.

600pp
978-1-84816-957-9

Jul 2013
~~US$179~~ ~~£118~~
US$134.25 **£88.5**

Scan to Order

Contents:

- Advocate Foreword (Nancy Brinker)
- Scientific Foreword (Bert O'Malley)
- Centennial Article Cancer Research 2009. A Century of Deciphering the Control Mechanisms of Sex Steroid Action in Breast and Prostate Cancer: The Origins of Targeted Therapy and Chemoprevention (V C Jordan)
- Diethylstilbestrol: The Pioneering Cancer Treatment and Tragedy in Reproductive Endocrinology (J Lewis-Wambi & V C Jordan)
- Contraception and Women's Health (V D Castracane & V C Jordan)
- Hormone Replacement Therapy and Women's Health (J Eng-Wong et al.)
- Induction of Ovulation by Non-Steroidal Antiestrogens (I Obiorah & V C Jordan)
- Clues About Estrogen and Antiestrogen Action from X-ray Crystallography (G Greene & V C Jordan)
- Co-Regulator Activity and Their Role in Selective Estrogen Receptor Modulator Action (C D Chien et al.)
- Role of Estrogen in Familial Breast Cancer (E Rosen & C Isaacs)
- An Advance with Aromatase Inhibitors (S Chumsri et al.)
- Adjuvant Antihormone Therapy (A Howell & V C Jordan)
- Pharmacogenomics of Tamoxifen (H Brauch & V C Jordan)
- Antihormone Drug Resistance (R Clarke & V C Jordan)
- Novel Selective Estrogen Receptor Modulators (S Sengupta & V C Jordan)
- Chemoprevention and Breast Cancer (T Powles et al.)
- Selective Androgen Receptor Modulators (C C Coss & J T Dalton)
- Emerging Principle: Selective Nuclear Receptor Modulators (P Fan & V Craig Jordan)
- Appendix 1: Scientific Survival Suggestions
- Appendix 2: Decades of Discovery
- Appendix 3: The "Tamoxifen Team" over a 30-year Period: An Account of Students Obtaining a PhD Degree (or MD as a Medical Science Researcher in the British System for Medical Doctors)

25% Discount on Books till **31st Aug 2013** by indicating discount code **"WSP86825"**.
Order your copy at **http://www.worldscientific.com/worldscibooks/10.1142/8525**

World Scientific
Connecting Great Minds

ICP Imperial College Press
www.icpress.co.uk

Preferred Publisher for Leading Thinkers

ORDER FORM

Please complete the form and send it to any of our offices below or order online @ www.worldscientific.com.
Alternatively, you can send your order directly to your regular book supplier.

- **NORTH & SOUTH AMERICA** — **World Scientific Publishing Co. Inc.**
  27 Warren Street, Suite 401-402, Hackensack, NJ 07601, USA Toll-free fax: 1 888 977 2665 Toll-free: 1 800 227 7562 Email: sales@wspc.com
- **EUROPE & THE MIDDLE EAST** — **World Scientific Publishing (UK) Ltd.**
  c/o Marston Book Services, P O Box 269, Abingdon, Oxon OX14 4YN, UK Fax: 44 (0) 123 546 5555 Tel: 44 (0) 123 546 5500 Email: direct.orders@marston.co.uk
- **ASIA & THE REST OF THE WORLD** — **World Scientific Publishing Co. Pte. Ltd.**
  Farrer Road, P O Box 128, SINGAPORE 912805 Fax: 65 6467 7667 Tel: 65 6466 5775 Email: sales@wspc.com.sg

TITLE SELECTION

TITLE(S)	ISBN	QTY	PRICE (US$/£)
ESTROGEN ACTION, SELECTIVE ESTROGEN RECEPTOR MODULATORS AND WOMEN'S HEALTH	978-1-84816-957-9		

MODE OF DELIVERY

Please select your preferred mode of delivery. For delivery charges, please contact any of our worldwide offices.

	USA	Canada	UK	Europe	Asia	Mexico & South America	Middle East & Others
☐ Air Mail	N/A	1-2 weeks	1-2 weeks	1-3 weeks	1-2 weeks	N/A	3-4 weeks
☐ Surface Mail	N/A	2-3 weeks	1-2 weeks	3-6 weeks	4-8 weeks	4-6 weeks	8-10 weeks
☐ UPS	1-2 weeks	N/A	N/A	N/A	N/A	N/A	N/A

METHOD OF PAYMENT

☐ Cheque/Bank draft enclosed for **US$/£** _____

- For cheque payment in North and South America, please make cheque payable to "**World Scientific Publishing Co. Inc.**"
- For cheque payment in Europe and the Middle East, please make cheque payable to "**Marston Book Services**"
- For cheque payment from the rest of the world, please make cheque payable to "**World Scientific Publishing Co. Pte. Ltd.**"

☐ Charge my ☐ VISA ☐ MC ☐ Amex

Card No: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐ CVV: ☐☐☐ Exp. Date: ☐☐☐☐

☐ Bill my company/institution: _____
(please attach purchase order)

Signature: _____ Tel: _____

- **Special Prices** are available to developing countries and some Eastern European countries. Please write in for further details.
- Prices subject to change without prior notice.

CONTACT INFORMATION

Title & Name: _____

Organization: _____

Address: _____

_____

City/State/Zip: _____ Country: _____

Email: _____

☐ Email me the new publications by World Scientific and Imperial College Press.

Fields of interest: _____

Join over *400,000 subscribers* who receive our email newsletters.

Participate in:
- The Times Higher Education Supplement World University Rankings Survey

Get alerted about:
- Subscriber Discount
- New and forthcoming publications
- Free online chapters
- New book reviews
- Podcasts and videos of author interviews
- Useful tips in research

Sign up now @
http://www.worldscientific.com/mailinglist.html

Biochemical Pharmacology xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells☆

Gregory Segala [a,f], Philippe de Medina [a,b], Luigi Iuliano [d], Chiara Zerbinati [d], Michael R. Paillasse [a,b], Emmanuel Noguer [a,b], Florence Dalenc [a,f], Bruno Payré [e], V. Craig Jordan [c], Michel Record [a,f], Sandrine Silvente-Poirot [a,f,1,*], Marc Poirot [a,f,1,*]

[a] UMR 1037 INSERM-University Toulouse III, Cancer Research Center of Toulouse, France
[b] Affichem, Toulouse, France
[c] Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, USA
[d] Department of Medico-Surgical Sciences & Biotechnologies, Sapienza University of Rome, Latina, Italy
[e] Centre de Microscopie Electronique Appliquée à la Biologie, Toulouse, France
[f] Institut Claudius Regaud, Toulouse, France

ARTICLE INFO

Article history:
Received 27 December 2012
Accepted 27 February 2013
Available online xxx

Keywords:
Cholesterol epoxide
Sulfation
Breast cancer
AEBS
ChEH

ABSTRACT

Tamoxifen (Tam) is a selective estrogen receptor modulator (SERM) that remains one of the major drugs used in the hormonotherapy of breast cancer (BC). In addition to its SERM activity, we recently showed that the oxidative metabolism of cholesterol plays a role in its anticancer pharmacology. We established that these effects were not regulated by the ER but by the microsomal antiestrogen binding site/cholesterol-5,6-epoxide hydrolase complex (AEBS/ChEH). The present study aimed to identify the oxysterols that are produced under Tam treatment and to define their mechanisms of action. Tam and PBPE (a selective AEBS/ChEH ligand) stimulated the production and the accumulation of 5,6α-epoxy-cholesterol (5,6α-EC), 5,6α-epoxy-cholesterol-3β-sulfate (5,6-ECS), 5,6β-epoxy-cholesterol (5,6β-EC) in MCF-7 cells through a ROS-dependent mechanism, by inhibiting ChEH and inducing sulfation of 5,6α-EC by SULT2B1b. We showed that only 5,6α-EC was responsible for the induction of triacylglycerol (TAG) biosynthesis by Tam and PBPE, through the modulation of the oxysterol receptor LXRβ. The cytotoxicity mediated by Tam and PBPE was triggered by 5,6β-EC through an LXRβ-independent route and by 5,6-ECS through an LXRβ-dependent mechanism. The importance of SULT2B1b was confirmed by its ectopic expression in the SULT2B1b(-) MDA-MB-231 cells, which became sensitive to 5,6α-EC, Tam or PBPE at a comparable level to MCF-7 cells. This study established that 5,6-EC metabolites contribute to the anticancer pharmacology of Tam and highlights a novel signaling pathway that points to a rationale for re-sensitizing BC cells to Tam and AEBS/ChEH ligands.

© 2013 Elsevier Inc. All rights reserved.

☆ This work is part of the PhD thesis of GS.

Abbreviations: AEBS, antiestrogen binding site; ChEH, cholesterol epoxide hydrolase; Tam, tamoxifen, *trans*-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-*N,N*-dimethylethylamine; 4OH-tam, 4-[(Z)-1-[4-(2-dimethylaminoethyloxy)phenyl]-2-phenylbut-1-enyl]phenol; Ralox, raloxifene, [6-hydroxy-2-(4-hydroxyphenyl)-1-benzothiophen-3-yl]-[4-(2-piperidin-1-ylethoxy)phenyl]methanone; Clom, clomiphene, 2-[4-[(Z)-2-chloro-1,2-di(phenyl)ethenyl]phenoxy]-N,N-diethylethanamine; BZA, bazedoxifene1-[[4-[2-(azepan-1-yl)ethoxy]phenyl]methyl]-2-(4-hydroxyphenyl)-3-methylindol-5-ol; Tesm, tesmilifene (DPPE), N,N′-diethylamino-4-(phenylmethylphenoxy)ethanamine-HCl; PBPE, 1-(2-(4-benzylphenoxy)ethyl)pyrrolidin-HCl; PCPE, 1-(2-(4-(2-phenylpropan-2-yl)phenoxy)ethyl)pyrrolidine; MBPE, 4-(2-(4-benzylphenoxy)ethyl)morpholine; MCPE, 4-(2-(4-(2-phenylpropan-2-yl)phenoxy)ethyl)morpholine; BD-1008, N-(3,4-dichlorophenethyl)-N-methyl-2-(pyrrolidin-1-yl)ethanemine; SR-31747A, (E)-N-(4-(3-chloro-4-cyclohexylphenyl) but-3-enyl)-N-ethylcyclohexanamine; FPT, flupenthixol, 2-[4-[(3Z)-3-[2-(trifluoromethyl)thio xanthen-9-ylidene]propyl]piperazin-1-yl]ethanol; CLP, chlorpromazine, 3-(2-chlorophenothiazin-10-yl)-N,N-dimethylpropan-1-amine; TFP, trifluoroperazine, 10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)phenothiazine; U-18666A, 3-beta-(2-(diethylamino)ethoxy)androst-5-en-17-one; AY-9944, *trans*-1,4-Bis(2-chlorobenzaminomethyl)cyclohexane; Triparanol, 2-(4-chlorophenyl)-1-(4-(2-(diethylamino)ethoxy)phenyl)-1-p-tolylethanol; D8D7I, 3β-hydroxysterol-Δ^8-Δ^7-isomerase; DHCR7, 3β-hydroxysterol-Δ^7-reductase; 7-KC, 7-ketocholesterol; 7α-HC, 7α-hydroxycholesterol; 7β-HC, 7β-hydroxycholesterol; 4β-HC, 4β-hydroxycholesterol; 5,6-EC, 5,6-epoxy-cholesterol; CT, cholestane-3β,5α,6β-triol; 5,6-αEC, 5,6α-epoxy-5α-cholestestan-3β-ol; 5,6β-EC, 5,6β-epoxy-5β-cholestestan-3β-ol; 25-HC, 25-hydroxycholesterol; 27-HC, 27-hydroxycholesterol; 5,6-ECS, 5,6α-epoxy-5α-cholestestan-3β-sulfate; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; LXRα, Liver-X-Receptor alpha; LXRβ, Liver-X-Receptor beta; SULT2B1, Steroid sulfotransferase 2B1; SCD1, Stearoyl-CoA desaturase 1; ACC, Acetyl-CoA carboxylase; siRNA, small interfering RNA; siSC, siRNA scrambled.

* Corresponding authors at: Team "Sterol metabolism and therapeutic innovations in oncology", UMR 1037 INSERM-University Toulouse III, Cancer Research Center of Toulouse, Institut Claudius Regaud, 20 rue du Pont Saint Pierre, 31052, Toulouse Cedex, France. Tel.: +33 561424648; fax: +33 561424631.
E-mail addresses: poirot.sandrine@hotmail.fr (S. Silvente-Poirot), marc.poirot@inserm.fr (M. Poirot).

[1] Both authors contributed equally to this work.

0006-2952/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bcp.2013.02.031

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

1. Introduction

Breast cancer (BC) is the most common cancer in women affecting more than 1 million women world-wide and with about 400,000 deaths due to this disease every year [1]. Tamoxifen (Tam) is one of the major drugs used as an adjuvant treatment to prevent BC recurrence and as a therapy to extend the lives of patients with metastatic disease [2]. Tam is a selective estrogen receptor modulator (SERM) that can compete with 17β-estradiol (E2) at the estrogen receptor (ER) on ER positive breast cancers and block its mitogenic action. This mechanism constitutes the rational for its clinical use [3]. It is now emerging that Tam displays a complex pharmacology and may exert additional ER-independent mechanisms [4]. Cholesterol metabolism is reportedly involved in breast cancer development in mice [5–7] and in resistance to Tam in patients [8,9]. At the molecular level, Tam has been shown to modulate cholesterol metabolism through its interaction with the microsomal antiestrogen binding site (AEBS) [4]. The AEBS binds selective estrogen modulators (SERMs) such as Tam, 4-hydroxytamoxifen, raloxifene and clomiphene [10]. Diphenylmethane (DPM) compounds such as 1-(2-(4-benzylphenoxy)ethyl)pyrrolidin-HCl (PBPE) and N,N'-diethylamino-4-(phenylmethylphenoxy)ethanamine-HCl (tesmilifene) have been developed to selectively bind to the AEBS/ChEH complex [11–14]. DPM were used for the molecular characterization the AEBS/ChEH complex [15–21] and the definition of its functional role in the pharmacology of its cognate ligands [12–14,16,22–27]. Despite a lack of a clear understanding of its mechanism of action at that time, tesmilifene was evaluated positively for the treatment of breast and prostate cancer in phase II and II clinical trials [28–31]. However, a pivotal phase III clinical trial was aborted because of the lack of a therapeutic outcome [32]. It is clear that a better understanding of its mechanism of action would have warranted a better selection of patients and an improved clinical response to tesmilifene.

We established that the AEBS is a hetero-oligomeric complex consisting of 3β-hydroxysteroid-Δ^8-Δ^7-isomerase (D8D7I, EBP) and 3β-hydroxysteroid-Δ^7-reductase (DHCR7) [16] with both enzymes being involved in the post-lanosterol cholesterol biosynthesis pathway. In addition, we showed that the AEBS carried out cholesterol-5,6-epoxide hydrolase (ChEH) activity [15]. ChEH catalyzes the *trans*-hydration of 5,6α-epoxy-cholesterol (5,6α-EC) and 5,6β-epoxy-cholesterol (5,6β-EC) into cholestane-3β,5α,6β-triol (CT) [33]. We showed that the interaction of the AEBS/ChEH complex with its cognate ligands induced: (1) the intracellular accumulation of free cholesterol precursors due to a non-competitive inhibition of cholesterogenic enzymes that are involved in the AEBS [8,16]; (2) the competitive inhibition of ChEH that could lead to the accumulation of 5,6-EC [15,33]. We established that free sterols accumulated in cells in multilamellar bodies (MLB) [23,24] and were responsible for the induction of a survival autophagy [8,10,23,34,35].

We previously showed that SERMs and other AEBS/ChEH ligands induced BC cell differentiation and cytotoxicity in a concentration- and time-dependent manner [12,23–25]. We found that these effects occurred independently of the ER through the modulation of the oxidative metabolism of cholesterol, and these effects were inhibited by the antioxidant vitamin E (Vit E, alpha-tocopherol) [10,23,24,34]. We found that the exposure of MCF-7 cells to 1–5 μM Tam or 10–20 μM PBPE for 3 days led to the appearance of BC cell characteristics of differentiation with no cytotoxicity [24], while 10 μM Tam and 40 μM PBPE triggered cell death [23,24]. Cytotoxicity required the expression of new genes and new proteins in BC cells establishing that transcription factors were involved in this process [23].

ROS include different reactive oxygen species, and some of them are known to produce different oxysterols [36]. NAD(P)H oxidase (NOX) is a ROS producing enzyme that induces the production of superoxide anion ($O_2^{\bullet -}$) which can be transformed by superoxide dismutase into H_2O_2 [37]. H_2O_2 produces 5,6-ECs as major cholesterol oxidation products [38]. We reported earlier that Tam and PBPE stimulated ROS production in MCF-7 cells [23] and other groups have reported that Tam induced ROS production in different cell lines including BC cells such as MCF-7 and MDA-MB-231 cells [39–42]. Consistent with these data, it was recently reported that the induction of TAG by Tam in MCF7 was inhibited by catalase [40], the enzyme that destroys H_2O_2, suggesting the formation of unknown endogenous mediator. We postulated that the stimulation of ROS by Tam can induce the production of 5,6-ECs. One of the major characteristics of BC cell differentiation induced by AEBS ligands is the stimulation of triacylglycerol (TAG) biosynthesis [23,24,40,43]. TAG biosynthesis is known to be under control of the oxysterol receptors Liver-X-Receptors (LXR) [44], and LXR were shown to be modulated by 5,6α-EC [45], suggesting that LXR could contribute to the oxysterol-dependent activity of Tam. The impact of Tam and AEBS/ChEH ligands on 5,6-EC metabolism in BC cells remains to be studied. 5,6-EC are known to be present in low amounts in mammals [36] and their presence in BC cells has never been studied. We designed the present study to identify the cholesterol autoxidation species that are produced under Tam- and AEBS ligand-treatment and to determine the molecular pathways involved in the induction of TAG biosynthesis by these oxysterols and Tam and PBPE and cytotoxicity in BC cells.

2. Materials and methods

2.1. Chemicals and reagents

Raloxifene, RU-39411 and SR-31747A were from Sanofi-Aventis. Triparanol was from Dr C Wolf (University Paris 06, France). BD-1008 was kindly given by Pr W D Bowen (Brown University, RI, USA). Bazedoxifene was synthesized as previously described [46] as were other compounds [11,15]. 5,6α-EC, 5,6β-EC, d7-5,6α-EC, d7-5,6β-EC, [^{14}C]-5,6α-EC and [^{14}C]-5,6β-EC were synthesized as described previously [15,47]. Other deuterated oxysterols were from Avanti Polar Lipids (Alabaster, AL). All other chemicals were from Sigma–Aldrich (St. Louis, MO).

2.2. Detection and quantification of 5,6-EC

Cells were grown to 70% confluence, and then pre-treated for 30 min with the appropriate amount of drug or solvent vehicle in the presence or absence of 500 μM Vit E. After a 48 h incubation, the cells were washed and scraped in cold PBS and the neutral lipids were extracted with chloroform/methanol/8.8% aqueous KCl (2:1:1 v/v) as reported previously [48]. 10^8 cells were used for analysis. The organic phase was evaporated to dryness under an argon stream and the residues dissolved in 50 μl of ethanol. 100 pmol of deuterated oxysterols as internal standards (IS) were added under argon flux, the samples saponified with KOH 1 N for 1 h at 55 °C, and oxysterols extracted with chloroform/methanol/8.8% aqueous KCl (2:1:1, v/v/v). Oxysterol purification was accomplished using a 100 mg Sep-Pak Silica Vac RC Cartridge equilibrated with hexane. Samples were applied to the silica cartridge, washed with hexane (5 ml), 12% methylterbutyl ether (MTBE) in hexane (5 ml), 23% MTBE in hexane (7 ml), 40% MTBE in acetone (5 ml) and the oxysterol fraction was eluted with 5 × 2 ml of MeOH. Under these conditions of preparation, no 5,6-EC were formed as artifacts. We measured a 98% yield in 5,6-EC recovery using [^{14}C]-5,6-EC without cholesterol and vit E contaminations.

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

Oxysterols were derivatized using pyridine–hexamethyldisilazane–trimethylchlorosilane (3:2:1) and analyzed by GC/MS. Quantification of oxysterols was carried out using stable isotope dilution mass spectrometry. For GC–MS analysis, samples were redissolved in 100 μl methylene chloride and 1 μl was used for analysis in a trace gas chromatographer (Thermo Fisher Scientific, Austin, TX) coupled to a mass spectrometer (Polaris Q, Thermo Fisher Scientific) (GC–MS). Samples were separated on an RTX-5MS fused silica column (15 m × 250 μm × 0.25 μm). The oven temperature program was as follows: 180 °C for 1 min, 20 °C/min to 250 °C and then 5 °C/min to 300 °C where the temperature was kept for 6 min. Helium was used as the carrier gas, with a flow rate of 1 ml/min. The molecules were ionized by electron impact at 70 eV. 5,6-EC were monitored with ions at mass/charge ratio (*m*/*z*) 472 (7-KC), 479 (*d*7-KC); 474 (5,6α-EC and 5,6β-EC), 481 (*d*7-5,6α-EC and *d*7-5,6β-EC); 546 (CT), 553 (*d*7-CT); 456 (27-HC, 25-HC), 462 (*d*6-27-HC, *d*6-25-HC); 456 (4β-HC, 7α-HC, 7β-HC), 463 (*d*7-4β-HC, *d*7-7α-HC, *d*7-7β-HC). Quantitative GC/MS determinations were calculated from triplicate injections and from the linear response range of standard curves established for oxysterol/IS pairs.

2.3. Cholesterol oxidation analysis

MCF-7 cells were seeded in 100 mm plates at 0.5×10^6, then incubated with [^{14}C]-5,6-EC (final concentration 0.6 μM; 20 μCi/μmol) in the presence of solvent vehicle (EtOH 0.1%), and 5 μM Tam for 72 h. The cells were scraped and pelleted by centrifugation for 10 min at 1500 rpm, and then extracted as described above. Samples were spotted onto Fluka 20 × 20 silica gel plates previously heated for 1 h at 100 °C and developed using chloroform/acetone/MeOH:2/acetic acid/H_2O (8/4/2/2/1). The radioactive metabolites were identified on TLC plates by co-migration with authentic standards by autoradiography using Kodak Biomax MS film (Sigma–Aldrich) and quantified by liquid scintillation counting of the 5,6-EC and CT regions. The *Rf* for 5,6-EC and CT were 0.94 and 0.82 respectively.

2.4. Measurement of ChEH activity

Cells were seeded in 6-well plates (100,000 cells/well) in RPMI 1640 medium with 5% FCS. Cells were incubated for 24 h with 0.6 μM [^{14}C]-5,6α-EC in the presence of increasing concentrations of drugs. Cells were scraped, resuspended in PBS and pelleted by centrifugation 10 min at 800 × *g* and then extracted with 200 μl of chloroform/methanol (2:1). The organic layer was reduced to dryness under a flux of argon, and the residue was resuspended in 30 μl of ethanol. More than 95% of the radioactivity was recovered in the organic layers. Samples were applied to TLC plates that had been heated previously for 1 h at 100 °C and were developed using ethyl acetate. The radioactive metabolites were visualized by autoradiography using Kodak Biomax MS film (Sigma–Aldrich) and quantified by liquid scintillation counting of the 5,6-EC region. The concentration of a compound required to inhibit ChEH by 50% (IC_{50}) was calculated using Graphpad Prism software, version 4.0 (GraphPad Software Inc., San Diego, CA). The IC_{50} values were calculated with data from triplicate assays at each drug concentration.

2.5. Cell culture

MCF-7 and MDA-MB-231 cells were from the American Type Culture Collection (ATCC) and cultured until passage 30. MCF-7 and MDA-MB-231 cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), penicillin and streptomycin (50 U/ml) in a humidified atmosphere with 5% CO_2 at 37 °C.

2.6. Luciferase assay

One day after seeding in 100-mm dishes, MCF-7 or MDA-MB-231 cells were transfected with the LXRE-luciferase construct using polyethyleneimine (PEI). For each dish, a transfection solution with 4.3 μg of PEI and 5 μg of the construct in 2 ml of OptiMEM was prepared and incubated with the cells. After 5 h, the medium was replaced by RPMI 1640 with 5% FCS. Cells were incubated at 37 °C in a humidified 5% CO_2 incubator. One day after transfection, cells were seeded in 12-well plates (50,000 cells/well) in RPMI 1640 with 5% FCS. After 4 h, the cells were treated with the test compounds dissolved in ethanol. Before treatment with the test compounds the medium was replaced by phenol red-free medium without FCS. At the end of the treatment, the cells were lysed in 100 μl of passive lysis buffer (Promega, Charbonnières, France). Luciferase (Luc) activity was measured using the Luc assay reagent (Promega), according to the manufacturer's instructions. Protein concentrations were measured using the Bradford technique to normalize the Luc activity data as reported earlier [49]. For each condition, the mean Luc activity was calculated from the data of three independent wells.

2.7. Western blot analysis

Immunoblotting was carried out as previously described [23]. Proteins were separated on 10% SDS-PAGE gels, electro-transferred onto PVDF membranes and incubated overnight at 4 °C with goat anti-LXRβ (Y-16, sc-34341, Santa Cruz) or anti-SULT2B1b (AB82865, Abcam) or anti-Actin (C4, MAB1501, Millipore). Visualization was carried out using an ECL plus kit (Pierce), and chemiluminescence was detected by autoradiography (Amersham Biosciences).

2.8. Oil Red O staining procedures and TAG quantification

A stock solution was prepared as follows: 0.5 g of Oil Red O (ORO) (Sigma) was dissolved in isopropanol. Before use, the solution was diluted 3:2 with distilled water and then filtered through Whatman paper. Cells were grown on glass coverslips and treated with drugs for 48 h and then fixed with 3.7% paraformaldehyde for 1 h at room temperature followed by washing twice with PBS (Euromedex). Cells were incubated with 2 ml of 60% (v/v) isopropyl alcohol in water for 5 min. 2 ml of ORO working solution was added to each well for 5 min. Cells were rinsed with tap water until the rinsing water was clear. Finally, the cells were counter-stained with 2 ml hematoxilin stain for 1 min. Quantifications of lipid accumulation and TAG quantification were done as exactly as previously reported [24].

2.9. Transmission electron microscopy

Cells were fixed with 2% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.4) for 1 h and washed with the Sorensen's phosphate buffer (0.1 M) for 12 h. The cells were then post-fixed with 1% OsO_4 in Sorensen's phosphate buffer (Sorensen's phosphate 0.05 M, glucose 0.25 M, OsO_4 1%) for 1 h, washed twice with distilled water, and pre-stained with an aqueous solution of 2% uranyl acetate for 12 h. Samples were then treated exactly as previously described [24].

2.10. RNA isolation and qPCR analysis

The detailed procedures have been published previously [50]. Total RNA was extracted from cultured cells using an RNA Extraction kit (Qiagen) according to manufacturer's instructions. LXRα: forward primer 5′-ACACCTACATAGCGTCGCAAG-3′, reverse primer 5′-GACGAGCTTCTCGATCATAGCC-3′; LXRβ: forward primer

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

5′-CTACAGCAAGGACGACTT-3′, reverse primer 5′-AGATAGTTAGATAGGCGATAGAG-3′; Sterol Regulatory Element Binding Protein 1c (SREBP-1c): forward primer 5′-CAGCCCCACTTCATCAAGG-3′, reverse primer 5′-ACTAGTTAGCCAAGATAGGTTCCG-3′; Stearoyl-Coenzyme A Desaturase 1 (SCD1): forward primer 5′-ACCGCTCTTACAAAGCTCGG-3′, reverse primer 5′-CCACGTCGGGAATTATAGAGGAT-3′; Acetyl-Coenzyme A Carboxylase (ACC): forward primer 5′-GCCCACGGTTATCATAGGACC-3′, reverse primer 5′-GTCAGGCGAATAGTTAGATTTTCAG-3′; Sulfotransferase 2B1 (SULT2B1): forward primer 5′-CGGGACGACGACATCTTTAT-3′, reverse primer 5′-CACCCACAATAGGTCTCACAC-3′; ATP-Binding Cassette Transporter A1 (ABCA1): forward primer 5′-ATAGAGGACAACAACTACAAAGCC-3′, reverse primer 5′-GGGAAAGAGGACTAGACTCCAAA-3′; ATP-Binding Cassette Transporter G1 (ABCG1): forward primer 5′-TTTAGAGGGATTTAGGGTCTAGAAC-3′, reverse primer 5′-CCCCTTTAATCGTTTTAGTCTAGCT-3′; low density lipoprotein receptor (LDLR): forward primer 5′-GGACCAACGAATAGCTTAGGACA-3′, reverse primer 5′-CTAGGCACTTAGTAGCCACCC-3′; Steroid Sulfatase (STS): forward primer 5′-TAGGGATCTCTTTAGACCAATCTAGA-3′, reverse primer 5′-CAGCAAGGGTAAGGAGGGTAG-3′; Cylophilin A (CycA): forward primer 5′-GCATACGGGTCCTAGGCATCTTAGTC-3′, reverse primer 5′-ATAGGTAGATCTTCTTAGCTAGGTCTTAGC-3′. First-strand cDNA was synthesized with iScript Reverse Transcriptase (Bio-Rad). 25 ng of cDNA were amplified using SyBR Supermix (Bio-Rad). Quantitative PCR analyses were performed on an iCycler (Bio-Rad). The threshold cycle (Ct) values of genes of interest were normalized with the Ct values of CycA.

2.11. Knock-down of LXRβ and SULT2B1b by siRNA

Gene expression of endogenous LXRβ or SULT2B1b was suppressed with a pool of 4 siRNAs for LXRβ (siLXRβ, M-003412, Dharmacon) or 4 siRNAs for SULT2B1b (siSULT2B1b, M-009488, Dharmacon) along with a control scrambled sequence siRNA (siSC, D-001210, Dharmacon). MCF-7 cells were seeded in 100-mm dishes in RPMI medium containing 5% FBS. After 24 h of seeding, cells were transfected in Opti-MEM with 50 nM siSC, siLXRβ or siSULT2B1b using DharmaFECT1 (T-2001, Dharmacon) following the procedure recommended by the manufacturer. After transfection, the cells were grown in RPMI 1640 5% FCS at 37 °C in a humidified 5% CO_2 incubator.

2.12. Transfection of MDA-MB-231 cells with SULT2B1b

One day after seeding in 100-mm dishes, MDA-MB-231 cells were transfected with the construct pCMV6-XL5-SULT2B1b (SULT2B1b) (SC123353, Origene) or the empty pCMV6-XL5 (Mock) using polyethyleneimine (PEI) as described above. One day after transfection, cells were seeded in 12-well plates (50,000 cells/well) in RPMI 1640 with 5% FCS and grown at 37 °C in a humidified 5% CO_2 incubator.

2.13. Cell death assay

Cells were seeded in RPMI 1640 with 5% FBS into 6-well plates at 100,000 cells/well. The cells were then treated with solvent vehicle (0.1% ethanol), 10 μM Tam or 40 μM PBPE for 72 h. Cell death was determined by the trypan blue exclusion assay. The cells were scraped and resuspended in the trypan blue solution (0.25%, w/v in PBS) and counted in a Malassez cell under a light microscope.

2.14. Chemical synthesis of oxysterol-sulfates

5,6α-Epoxy-5α-cholestestan-3β-sulfate (5,6-ECS) and cholestane-5α,6β-diol-3β-sulfate (CTS) were synthesized using a published method [51]. The purity of the synthesized steroids was determined by thin-layer chromatography, nuclear magnetic resonance spectroscopy, and mass spectrometry and was greater than 95%.

2.15. Biosynthesis of oxysterol-sulfates in cells

0.5×10^6 cells were incubated with [^{14}C]-5,6α-EC (final concentration 0.6 μM; 20 μCi/μmol) in the presence of solvent vehicle (EtOH 0.1%), 5 μM Tam or 10 μM PBPE for 72 h. The cells were then scraped and pelleted by centrifugation for 10 min at 1500 rpm, and then extracted under the conditions previously described for 5,6-EC. Samples were spotted onto Fluka 20 × 20 silica gel plates previously heated for 1 h at 100 °C and developed using chloroform/acetone/MeOH/acetic acid/H_2O (8/4/2/2/1). The radioactive metabolites were identified and quantified as described above. The *Rf* for 5,6-EC, CT, 5,6-ECS and CTS were 0.94, 0.82, 0.56 and 0.46 respectively.

2.16. Statistical analysis

Values are the mean ± S.E. of three independent experiments each carried out in duplicate. Statistical analysis was carried out using a Student's *t*-test for unpaired variables. * and ** in the figures refer to statistical probabilities (*P*) of <0.001 and <0.0001, respectively, compared with control cells that received solvent vehicle alone.

3. Results

3.1. Tam and PBPE stimulate the production and the accumulation of 5,6α-EC, and 5,6β-EC in MCF-7 cells

We investigated the nature of sterol oxidation products induced by Tam and PBPE in MCF-7 cells using isotope dilution gas chromatography–mass spectrometry (GC–MS) to monitor the different oxysterol species. The treatment of cells for 3 days with 5 μM Tam and 10 μM PBPE (a concentration required for the induction of BC cell differentiation) drastically increased the content of 5,6α-EC and 5,6β-EC. Other ring B oxysterols such as 7-KC, 7α-HC and 7β-HC (Fig. 1A) were detectable but did not increase under Tam or PBPE treatment. 4β-HC, 25-HC and 27-HC were not detectable in MCF-7 cells at the basal level and were not increased under Tam or PBPE treatment. This increase in 5,6-EC biosynthesis by Tam or PBPE was inhibited by the antioxidant Vit E establishing that 5,6-EC were produced through a ROS-mediated mechanism induced by Tam and PBPE (Fig. 1A). 5,6α-EC and 5,6β-EC are products of epoxidation on the delta 5 double bond of cholesterol (Fig. 1A). Kinetic analysis indicated (Fig. 1B) that a 6 h treatment with Tam was sufficient to induce cholesterol epoxidation. The production of 5,6α-EC increased up to 12 h and then decreased after 24 h suggesting possible metabolism of 5,6α-EC in MCF-7 cells. The production of 5,6β-EC increased up to 24 h and then plateaued at 72 h. The absence of an increase in CT, the product of hydration of 5,6-EC by ChEH, is consistent with the inhibition of ChEH by Tam and PBPE in BC cells (Fig. 1A). Indeed, we previously reported that Tam and AEBS ligands were potent inhibitors of the rat liver microsomal ChEH and that MCF-7 cells expressed ChEH [15]. We next showed that Tam and PBPE inhibited ChEH in intact MCF-7 cells in a concentration-dependent manner giving IC_{50} of 48.5 ± 7 nM and 1.23 ± 0.9 μM respectively (Fig. 1C) explaining why no increase in CT was found in treated cells despite the increase in 5,6-EC. These data established that Tam and PBPE induced the accumulation of 5,6α-EC and 5,6β-EC in MCF-7 cells through a dual mechanism involving a ROS-mediated cholesterol epoxidation and the inhibition of ChEH (Fig. 1D).

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

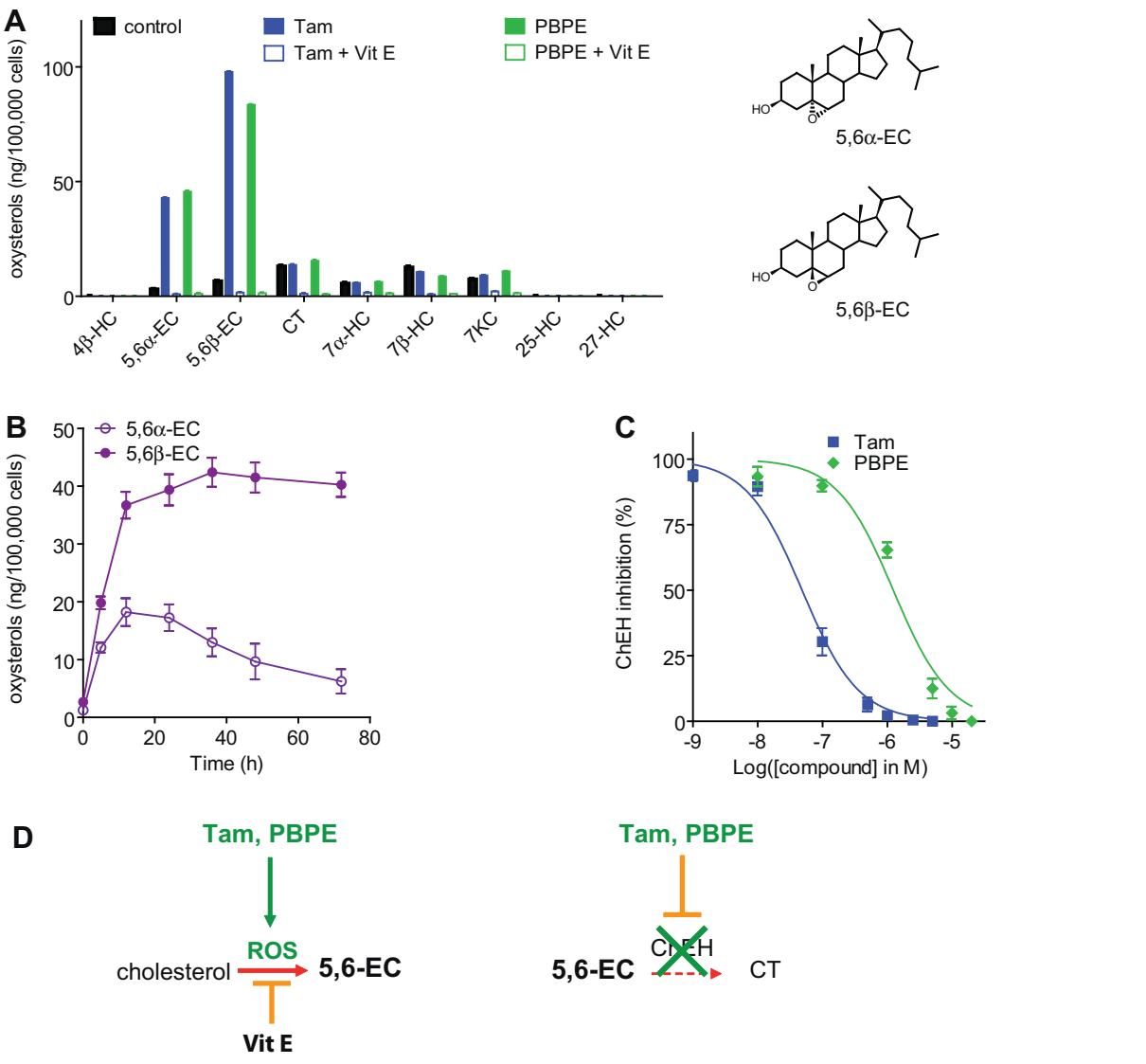


Fig. 1. Analyses of the ROS-dependent stimulation of oxysterol biosynthesis by Tam and PBPE in MCF-7 cells. (A) Quantification of oxysterol in MCF-7 cells incubated for 48 h with 10 μM Tam or 40 μM PBPE in the absence or presence of 500 μM Vit E. 9 different oxysterols were quantified by gas chromatography using a dilution isotope methodology as described in Section 2. 4β-HC: 4β-hydroxycholesterol; 5,6α-EC: 5,6α-epoxy-cholesterol; 5,6β-EC: 5,6β-epoxy-cholesterol; CT: cholestane-3β,5α,6β-triol; 7α-HC: 7α-hydroxycholesterol; 7β-OH: 7β-hydroxycholesterol; 7KC: 7-ketocholesterol; 25-HC: 25-hydroxycholesterol; 27-HC: 27-hydroxycholesterol. The results are reported in ng of oxysterol per 10^5 cells. 5,6α-EC and 5,6β-EC are drawn the right part of the figure. (B) Kinetic study on the accumulation 5,6α-EC and 5,6β-EC in MCF-7 cells after treatment with Tam for 0, 6, 12, 24, 48 and 72 h. 8×10^7 to 10^8 cells were used per condition. (C) Inhibition of cholesterol-5,6-epoxide hydrolase activity (ChEH) by Tam and PBPE in MCF-7 cells. Cells were incubated with [^{14}C]-5,6-EC (0.6 μM; 20 μCi/μmol) and were treated with increasing concentrations of Tam or PBPE ranging from 10 nM to 10 μM over 24 h. The positions of the 5,6-EC and CT were determined using [^{14}C]-5,6-EC and [^{14}C]-CT as standards. The data presented are the means ± S.E. of three independent experiments. (D) Scheme summarizing 5,6-EC formation and accumulation under Tam and PBPE treatment in MCF-7 cells.

3.2. 5,6α-EC, Tam and PBPE stimulated an LXRβ-dependent TAG biosynthesis in MCF-7 cells

AEBS/ChEH ligands were reported to stimulate TAG biosynthesis in MCF-7 cells, which can be revealed by the accumulation of ORO positive vesicles [23,24]. TAG biosynthesis can be controlled at the transcriptional level by nuclear receptors such as LXRα and LXRβ [52] that are known to be modulated by 5,6α-EC [45]. Since Tam and PBPE stimulate the accumulation of 5,6α-EC, we evaluated whether 5,6-EC, Tam and PBPE stimulate TAG biosynthesis in an LXR-dependent manner. We initially examined whether LXR isoforms were expressed in MCF-7 cells and found that LXRβ was predominant in MCF-7 cells ($Ct_{LXR\alpha}$ = 32.1, $Ct_{LXR\beta}$ = 25.1) and that LXRα was not detectable at the protein level (data not shown). MCF-7 cells were transfected with a plasmid encoding a luciferase (Luc) coding gene under the control of a promoter containing an LXR-response element (LXRE). Analysis at 12 h of treatment showed that the LXR agonist T0901317 activated the expression of Luc with an EC_{50} of 0.21 ± 0.06 μM and established that 5,6α-EC stimulated Luc activity in a concentration-dependent manner with an EC_{50} of 8.9 ± 0.1 μM (Fig. 2B). In contrast, 5,6β-EC was inefficient at stimulating Luc activity. Tam and PBPE stimulated Luc activity, but as opposed to direct LXR modulators such as T0901317 and 5,6α-EC, the stimulation required 48 h of treatment (Fig. 2C). Both Tam and PBPE stimulated Luc activity dose-dependently with EC_{50} values of 1.5 ± 0.3 μM and 9.9 ± 0.4 μM respectively at 48 h of treatment (Fig. 2D). The stimulation of Luc activity by Tam and PBPE was completely blocked by vit E, whereas vit E did not inhibit

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

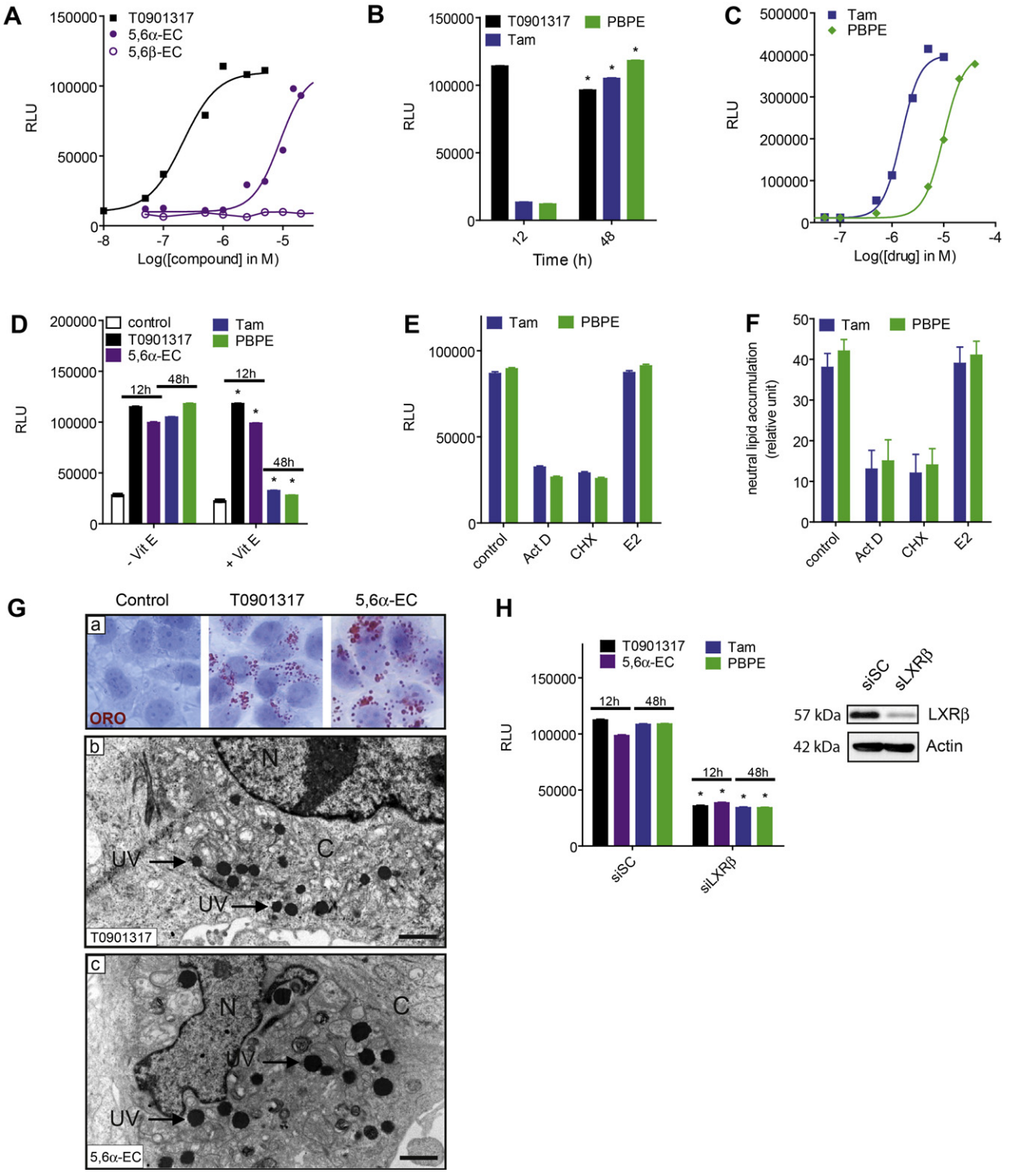


Fig. 2. Impact of 5,6α-EC, 5,6β-EC, Tam and PBPE treatment on the LXR-dependent transcription in MCF-7 cells. (A) Effect of T0901317, 5,6α-EC, and 5,6β-EC on LXRE-Luc. MCF-7 cells transfected with an LXRE-Luc plasmid were treated with increasing concentrations of T0901317, 5,6α-EC, 5,6β-EC for 12 h and assayed for luciferase activity (Luc) and expressed as relative luciferase unit (RLU) as described in Section 2. (B) Effect of T0901317, Tam, and PBPE on LXRE-Luc at 12 h and 48 h. Cells were incubated with 1 μM T0901317, 5 μM Tam and 20 μM PBPE and assayed for Luc activity at 12 h and 48 h after treatment. (C) Dose–response study of Tam and PBPE on MCF-7 cells transfected with an LXRE-Luc plasmid. Cells were incubated with solvent vehicle or increasing concentrations (50 nM–50 μM) of each drug for 40 h and assayed for Luc activity. (D) Effect of Vit E on LXRE-luc activity stimulated by solvent vehicle (control), T0901317, 5,6α-EC, Tam and PBPE in MCF-7 cells. MCF-7 cells were treated for 12 h with 1 μM T0901317 or 20 μM 5,6α-EC, 48 h with 5 μM Tam or 10 μM PBPE, with or without 500 μM Vit E, and assayed for Luc activity. Effect of actinomycin D (Act D), cycloheximide (CHX), and 17β-estradiol (E2) on the stimulation of LXR-dependent Luc activity by Tam and PBPE. MCF-7 cells were incubated for 40 h with 5 μM Tam and 20 μM PBPE in the absence or in the presence of 1 μg/ml Act D, 2.5 μg/ml CHX or 100 nM E2 and (E) assayed for Luc activity and F) for ORO staining and quantification. (G) The effect of LXR ligands T0901317 and 5,6α-EC on neutral lipid accumulation was assessed by ORO staining and ultrastructure analyses were done by electron microscopy. (a) Staining of neutral lipids with ORO in MCF-7 cells treated with solvent vehicle, 1 μM T0901317, or 20 μM 5,6α-EC for 24 h. Cells were stained with ORO and counterstained with Meyer's hematoxylin as described in Section 2. Electron micrographs of MCF-7 cells treated with: (b) 1 μM T0901317, (c) 20 μM 5,6α-EC for 24 h. Unilamellar vesicles (UV) were found in the cytoplasm of treated cells. *N*, nucleus; *C*, cytoplasm. Bars, 6.6 μm. (F) Effect of T0901317, 5,6α-EC, Tam and PBPE on the LXRβ-dependent stimulation of Luc activity in MCF-7 cells. MCF-7 cells were transfected with siRNA scrambled (siSC) or siRNA targeting LXRβ (siLXRβ). LXRβ expression was verified by Western blot

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

the stimulation of Luc by the direct LXR modulators T0901317 and 5,6α-EC (Fig. 2E). We found that actinomycin D (Act D) and cycloheximide (CHX) inhibited the stimulation of LXR-dependent Luc activity (Fig. 2E) and the stimulation of ORO positive vesicles by Tam and PBPE in MCF-7 cells (Fig. 2F), while co-treatment with E2 did not interfere with drug effects (Fig. 2E–F). This indicates that transcription and translation were required for induction of TAG biosynthesis by Tam and PBPE (Fig. 2A) while the ER was not involved. These data support the implication of 5,6α-EC as the endogenous mediator of the stimulation of Luc by Tam and PBPE. T0901317 and 5,6α-EC induced the accumulation of neutral lipid in MCF-7 cells as judged by the appearance of cytoplasmic ORO-positive vesicles (Fig. 2G(a)) as previously observed with MCF-7 cells treated with Tam or PBPE [24]. Ultrastructure analysis of MCF-7 cells by electron microscopy showed the accumulation of unilamellar vesicles (UV) in cells treated with T0901317 (Fig. 2G(b)) or 5,6α-EC (Fig. 2G(c)) which reflect the accumulation of neutral lipids such as TAG [24]. We next performed the knock-down of LXRβ in MCF-7 cells using an siRNA approach to define the implication of this receptor in the LXR-dependent stimulation of Luc activity by 5,6-EC and drugs. Knock-down of LXRβ was confirmed by Western blotting (Fig. 2H, insert) and drastically decreased the stimulation of Luc activity by T0901317, 5,6α-EC, Tam and PBPE (Fig. 2H). ORO staining of MCF-7 cells transfected with the scrambled control siRNA (siSC) showed that treatment with Tam or PBPE induced the accumulation of ORO-positive vesicles, while in cells transfected with siRNA against LXRβ, Tam and PBPE did not stimulate the accumulation of ORO-positive vesicles (Fig. 3A). Lipid analysis showed that knock-down of LXRβ completely inhibited the production of TAG induced by T0901317, 5,6α-EC, Tam and PBPE (Fig. 3B). We next used qPCR to investigate if Tam and PBPE modulated LXR-responsive genes and found that Tam and PBPE slightly repressed the expression of ABCA1, SREBP-1c and stimulated the expression of SCD1, ACC, ABCG1 and LDLR (Fig. 3C) showing a modulatory activity rather than an agonist activity as reported by Berrodin et al. in other cell lines [45]. This modulation of gene expression was abrogated in the presence of vit E consistent with what was seen for ORO positive accumulation and TAG biosynthesis [23,24] and for LXRβ-dependent Luc activity (Fig. 2E). We next studied the expression by qPCR of LXR-responsive genes in MCF-7 cells treated with the LXR modulator T0901317 and 5,6α-EC or the inducors of 5,6-EC accumulation, Tam and PBPE. We found that T0901317 stimulated the expression of ABCA1, SREBP-1c, SCD1, ACC, ABCG1, and repressed the expression of LDLR (Fig. 3D). 6 h of treatment of MCF-7 cells with 5,6α-EC induced a repression of the expression of ABCA1, SREBP-1c, had little impact on ACC expression and stimulated the expression of SCD1 and LDLR (Fig. 3D), consistent with the peculiar modulatory activity of LXR-dependent transcription reported with 5,6α-EC [45]. Knock down of LXRβ abrogated these effects showing that these transcriptional modulations were LXRβ-dependent (Fig. 3D). Tam and PBPE slightly repressed the expression of ABCA1, SREBP-1c and stimulated the expression of SCD1, ACC, ABCG1 and LDLR (Fig. 3D) similarly to what we found with 5,6α-EC in an LXRβ-dependent manner. Altogether, these data demonstrate the role of LXRβ in the induction of TAG biosynthesis by T0901317, 5,6α-EC, Tam and PBPE in MCF-7 cells. The induction of TAG biosynthesis by Tam and PBPE required a longer treatment (48 h) than 5,6α-EC (24 h), and the production of ROS which are involved in 5,6-EC production, strongly suggesting that 5,6α-EC was the endogenous mediator in Tam and PBPE stimulation of TAG biosynthesis.

3.3. 5,6α-EC is sulfated by SUL2B1b in MCF-7 cells treated with Tam and PBPE

MCF-7 cells have been reported to over-express the sterol sulfotransferase (SULT2B1b), and 5,6α-EC was shown to be the preferred substrate of this enzyme [53] to form 5,6α-epoxy-5α-cholestestan-3β-sulfate (5,6-ECS) that can be produced in MCF-7 cells treated with Tam or PBPE. Since our data suggested a possible metabolism of 5,6α-EC (Fig. 1B), we studied 5,6α-EC metabolism in MCF-7 cells and observed that 5,6α-EC was metabolized into 5,6-ECS when ChEH was inhibited by Tam or PBPE (Fig. 4A). The knock-down of SULT2B1b expression and activity (Fig. 4B) confirmed the inhibition of 5,6-ECS biosynthesis thus establishing the implication of SULT2B1b in 5,6-ECS formation in MCF-7 cells. We next evaluated the impact of 5,6-ECS on LXR-responsive genes in MCF-7 cells (Fig. 4C) and found that 5,6-ECS gave a similar profile as 5,6α-EC (Fig. 3D). However, the knock-down of SULT2B1b did not inhibit the stimulation of TAG biosynthesis by 5,6α-EC, Tam and PBPE (Fig. 4C) showing that the production of 5,6-ECS was not necessary for the induction of TAG biosynthesis by 5,6α-EC, Tam or PBPE. These data established that the inhibition of ChEH by Tam and PBPE induced the accumulation of 5,6α-EC that is metabolized into 5,6-ECS by SULT2B1b (Fig. 4E).

3.4. Importance of LXRβ and SULT2B1b in the cytotoxicity induced by Tam, PBPE, 5,6α-EC, 5,6β-EC and 5,6-ECS in MCF-7 cells

We next compared the cytotoxicity of Tam, PBPE and 5,6-EC metabolites in MCF-7 cells in which LXRβ (MCF7/siLXRβ) or SULT2B1b (MCF7/siSULT) were knocked down by transfection with small interfering RNA (siRNA) and compared to MCF-7 transfected with control scrambled siRNA (MCF7/siSC). Tam, PBPE, 5,6α-EC, 5,6-ECS and 5,6β-EC induced cytotoxicity in MCF-7 with EC_{50} of 2.5, 10.9, 22.4, 10.4 and 12.6 μM respectively (Table 1). MCF7/siLXRβ showed a 2.2- and 1.7-fold decrease in sensitivity to Tam and PBPE compared to the control MCF7/siSC demonstrating that LXRβ was involved in their cytotoxicity (Table 1). The loss of sensitivity to 5,6α-EC and 5,6-ECS in MCF7/siLXRβ indicated that LXRβ mediated their cytotoxicity. We observed a 2.3- and 1.8-fold diminution of sensitivity to Tam and PBPE respectively in MCF7/siSULT compared to MCF7/siSC showing that the presence of SULT2B1b contributed to the cytotoxicity of the drugs (Table 1). Interestingly, the contribution of SULTB1b was equivalent to that of LXRβ in the cytotoxicity induced by Tam and PBPE suggesting that the sulfation of 5,6α-EC into 5,6-ECS was required for cytotoxicity. This was confirmed by the observation that MCF7/siSULT cells that did not produce 5,6-ECS (Fig. 4B) lost their sensitivity to 5,6α-EC ($EC_{50} > 40$ μM) (Table 1). No change in the sensitivity to 5,6β-EC was measured in MCF7/siLXRβ and MCF7/siSULT compared to MCF7/siSC demonstrating that the cytotoxicity of 5,6β-EC was independent of LXRβ and SULT2B1b (Table 1). We had previously shown that Tam and PBPE activity was diminished by the over-expression of the anti-apoptotic protein Bcl2 in MCF-7 cells (MCF7/Bcl2) [23]. Measurement of their EC_{50} on MCF7/Bcl2 showed a 4.9- and 3.2-fold decreased sensitivity to Tam and PBPE respectively compared to control cells (MCF-7/Neo)

using anti-LXRβ antibodies. 24 h after cell transfection with siRNA, cells were transfected with the LXRE-luc plasmid. Cells were treated with solvent vehicle, 12 h with 1 μM T0901317, 12 h with 20 μM 5,6α-EC, 48 h with 5 μM Tam or 20 μM PBPE and assayed for Luc activity. H) Effect of T0901317, 5,6α-EC, Tam and PBPE on the LXRβ-dependent stimulation of TAG biosynthesis in MCF-7 cells. MCF-7 cells transfected with siSC or siLXRβ were treated with solvent vehicle, 12 h with 1 μM T0901317, 12 h with 20 μM 5,6α-EC, 48 h with 5 μM Tam or 20 μM PBPE. TAG quantification was done as described in Section 2. In all experiments the values are the mean ± S.E. of three independent experiments performed in triplicate. $^{*}P < 0.001$.

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

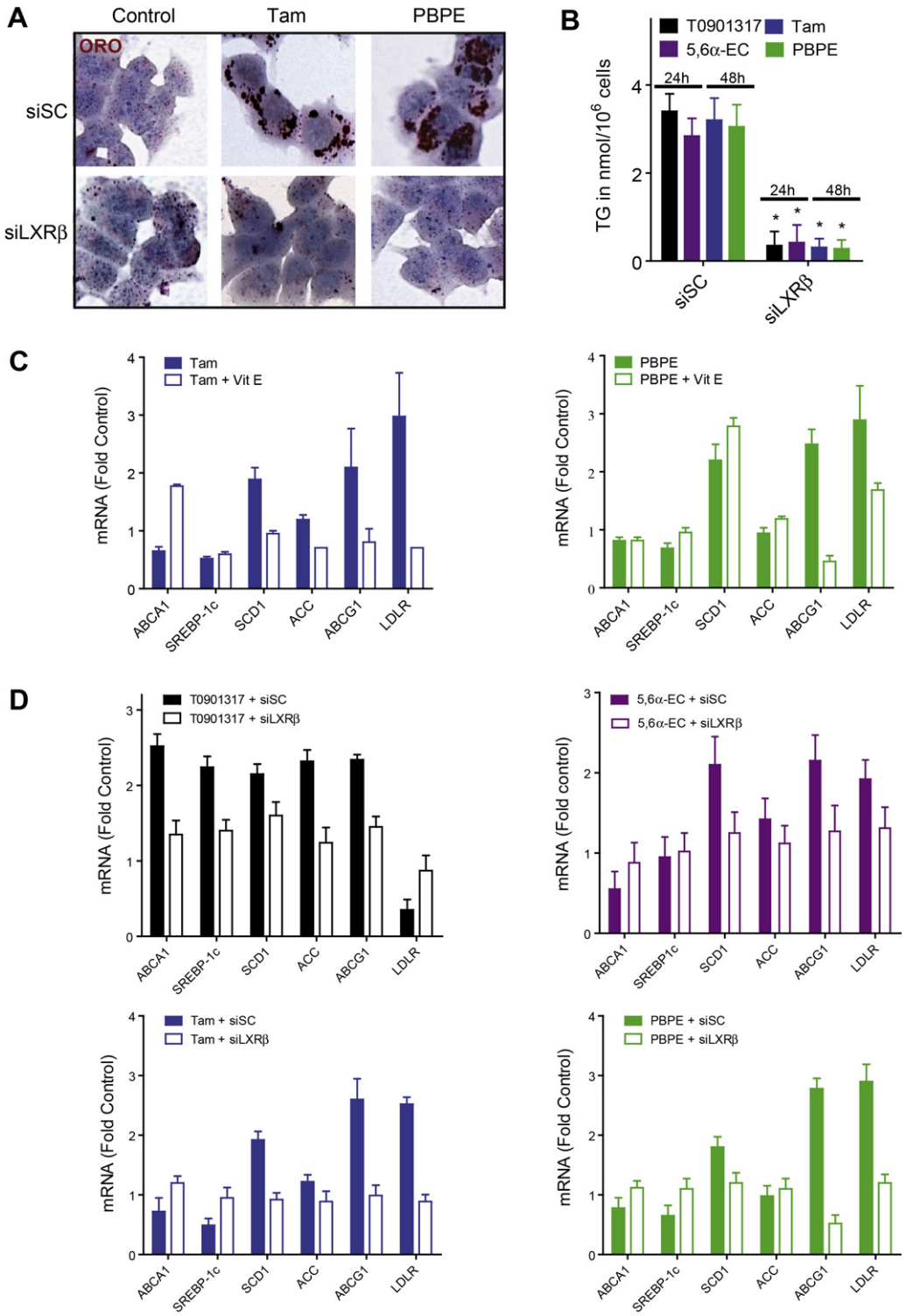


Fig. 3. Importance of LXRβ in the stimulation by Tam and PBPE of TAG biosynthesis in MCF-7 cells. (A) MCF-7 cells were transfected with siRNA scrambled (siSC) or siRNA targeting LXRβ (siLXRβ) and incubated 48 h with 5 μM Tam or 10 μM PBPE. Cells were stained for neutral lipids with ORO and countersained with Meyer's hematoxylin as described in Section 2. (B) MCF-7 cells transfected with siSC or siLXRβ were incubated 24 h with 1 μM T0901317, 20 μM 5,6α-EC or incubated 48 h with 5 μM Tam or 10 μM PBPE. TAG quantification was done as described in Section 2. (C) Importance of oxidation in the regulation of the expression of LXR-responsive genes by Tam and PBPE. MCF-7 cells were treated 40 h with 5 μM Tam or 10 μM PBPE in the presence or absence of 500 μM vit E. Expression of the LXR target was measured by quantitative RT-PCR as described in Section 2. (D) Importance of LXRβ in the regulation of the expression of LXR-responsive genes by T01901317, 5,6α-EC, Tam and PBPE. MCF-7 cells transfected with siSC or siLXRβ were incubated 6 h with 1 μM T0901317, 20 μM 5,6α-EC or incubated 40 h with 5 μM Tam or 10 μM PBPE. The expression of LXR-responsive genes was measured by quantitative RT-PCR as described in Section 2. Values are means of three independent experiments.

(Table 1). There were no changes in sensitivity to 5,6α-EC and 5,6-ECS in MCF7/Bcl2 cells, establishing that the Bcl2-controlled cytotoxicity of Tam and PBPE did not involve the production of 5,6α-EC and 5,6-ECS (Table 1). MCF7/Bcl2 cells showed decreased sensitivity to 5,6β-EC ($EC_{50} > 40$ μM) establishing that 5,6β-EC was responsible for the Bcl2-controlled cytotoxicity of Tam and PBPE (Table 1).

Altogether, these data established that, in MCF-7 cells, the cytotoxicity of Tam and PBPE can be decomposed into two

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

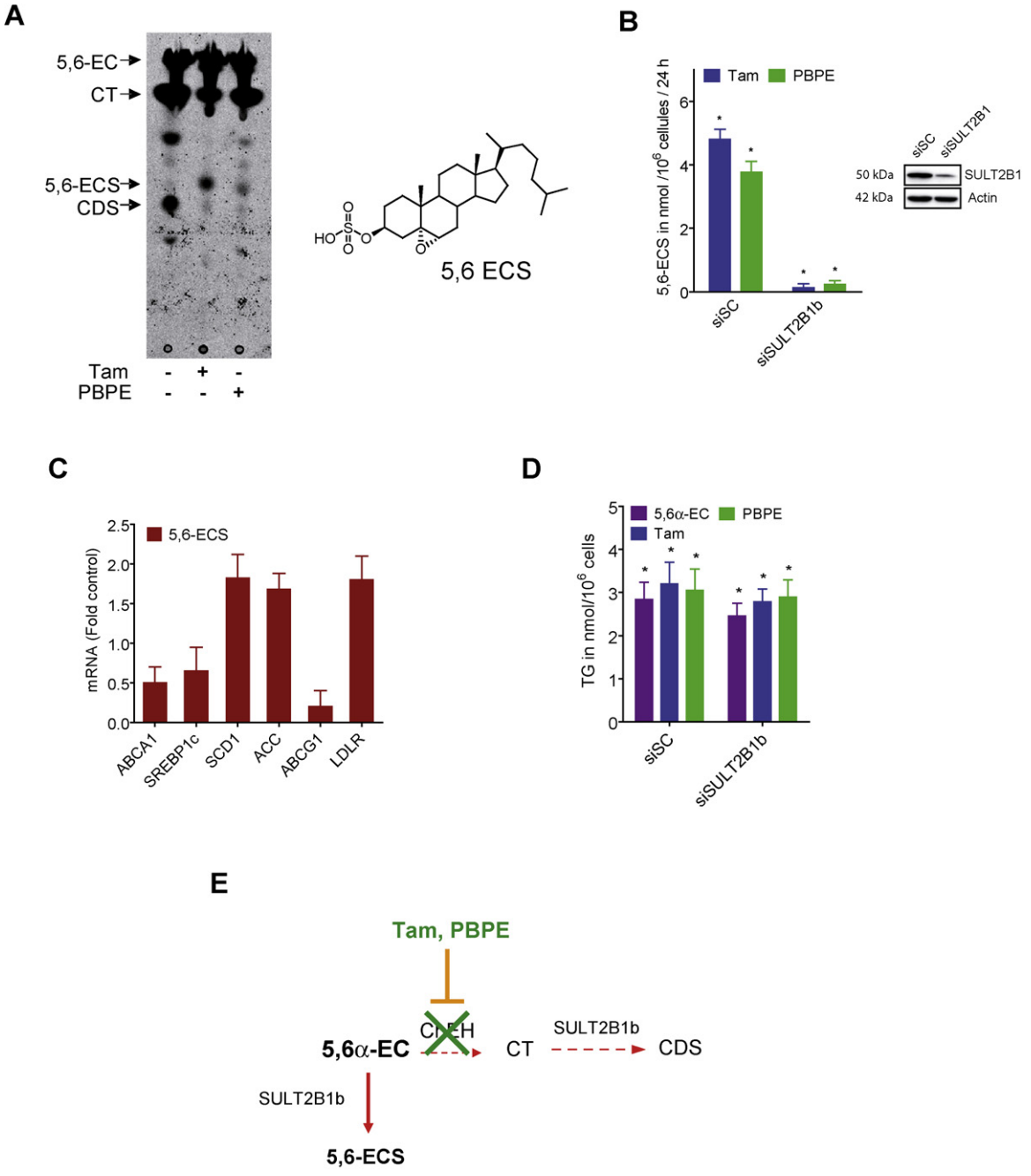


Fig. 4. Effect of Tam and PBPE on 5,6-EC sulfation in MCF-7 cells, the impact of 5,6-ECS on LXRβ-dependent TAG biosynthesis, and the importance of SULT2B1b. (A) MCF-7 cells were incubated with [^{14}C]-5,6-EC (0.6 μM; 20 μCi/μmol) in the presence of solvent vehicle (EtOH 0.01%), 5 μM Tam or 20 μM of PBPE for 48 h. The lipids were extracted and separated by silica TLC plates as described in Section 2. The TLC plates were developed by autoradiography and the positions of 5,6α-EC metabolites were determined using authentic standards. A representative autoradiogram of the TLC from three independent experiments is shown. The chemical structure of 5,6-ECS is given. (B) Impact of the knock-down of SULT2B1b on SULT2B1b protein expression and 5,6-ECS biosynthesis in MCF-7 cells. MCF-7 cells were transfected with siSC or siSULT2B1b. SULT2B1b expression was verified by Western blot using an anti-SULT2B1b antibody. 24 h after transfection, the cells were incubated with [^{14}C]-5,6-EC (0.6 μM; 20 μCi/μmol) for 48 h. 5,6-ECS biosynthesis was quantified as described in Section 2. Values are the mean ± S.E. of three independent experiments performed in triplicate. *$P < 0.001$. (C) Effect of 5,6-ECS on the modulation of endogenous responsive genes in MCF-7 cells. Cells were treated 6 h in the presence of 20 μM 5,6-ECS. The expression of LXR-responsive genes was measured by quantitative RT-PCR as described in Section 2. Values are means of three independent experiments. (D) Impact of the knock-down of SULT2B1b on TAG biosynthesis induced by 5,6α-EC, Tam and PBPE in MCF-7 cells. 24 h after MCF-7 cells were transfected with siSC or siSULT2B1b they were treated for 24 h with 20 μM 5,6α-EC or 48 h with 5 μM Tam or 20 μM PBPE for TAG quantification. Quantifications were performed as described in the caption of Fig. 2. Values are the mean ± S.E. of three independent experiments performed in triplicate. *$P < 0.001$. (E) Scheme summarizing 5,6-ECS formation under Tam and PBPE treatment in MCF-7 cells.

mechanisms: (1) a SULT2B1b- and LXRβ-dependent cytotoxicity mediated by 5,6-ECS, the sulfated metabolite of 5,6α-EC and (2) a Bcl2-controlled cytotoxicity mediated by 5,6β-EC. These data show that 5,6-EC epimers are cytotoxic through different mechanisms and account for both the mechanisms responsible for the cytotoxicity induced by Tam and PBPE against MCF-7 cells.

3.5. AEBS/ChEH ligands of different structural and pharmacological classes had similar effects on MCF-7 cells as Tam and PBPE

MCF-7 cells were treated with other AEBS/ChEH ligands showing they were potent inducers of 5,6-EC and 5,6-ECS accumulation (Table 1). We established that drugs that are known

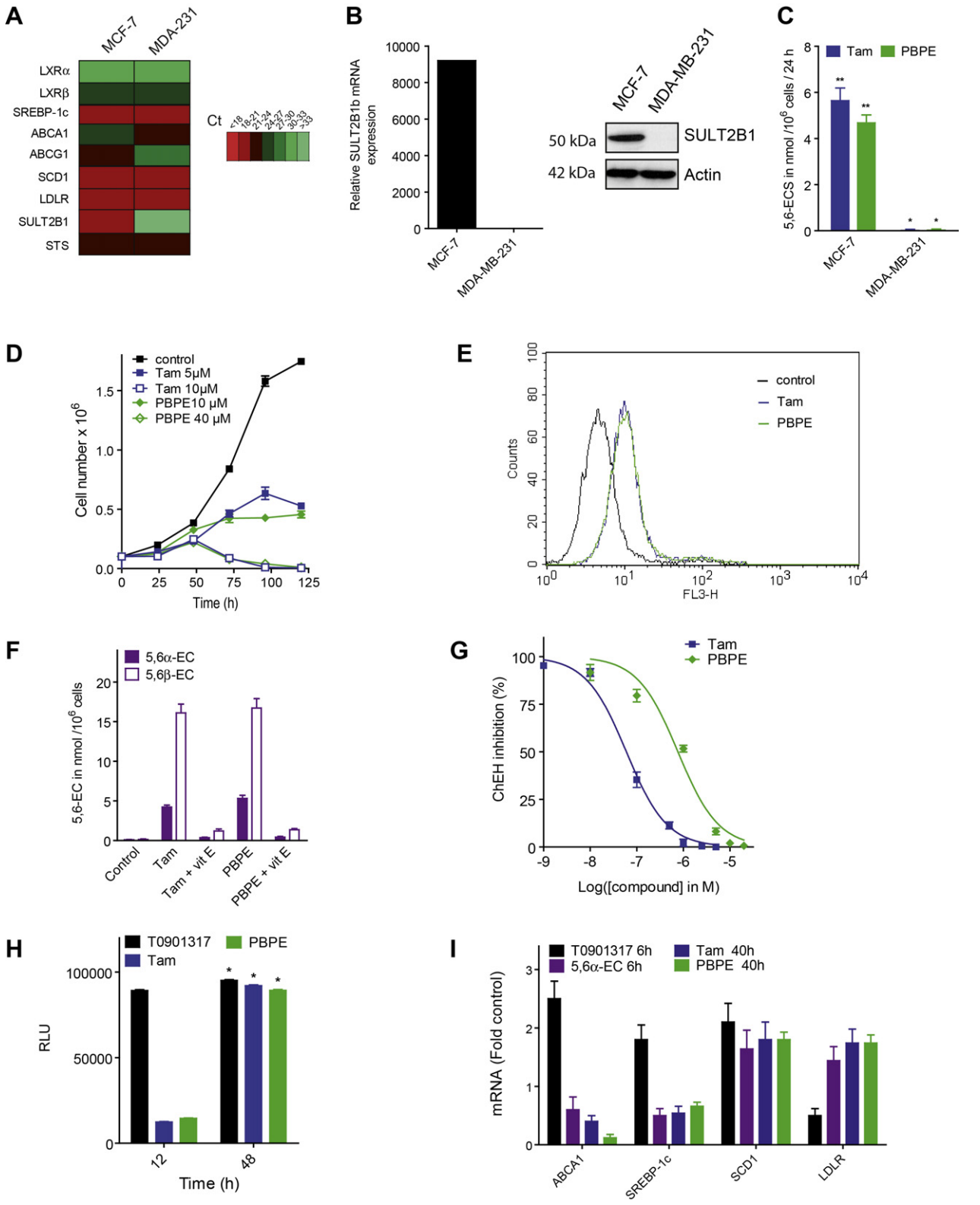


Fig. 5. Impact of Tam and PBPE on the modulation of cholesterol oxidative metabolism in MDA-MB-231 cells and of the modulation of 5,6-ECS. (A) Expression of mRNA encoding LXR isoforms, LXR-responsive genes, sterol sulfotransferase SULT2B1b and steroid sulfatase (STS) in MCF-7 cells and MDA-MB-231 cells. The expression of mRNA was measured by qPCR. (B) Expression of SULT2B1b in MCF-7 and MDA-MB-231 cells was measured at the mRNA level by qPCR and at the protein level by Western blotting. (C) The sulfation of 5,6α-EC into 5,6-ECS was measured in MCF-7 and MDA-MB-231 cells by incubating cells with 5,6α-EC for 48 h. Quantification was done as described in Section 2. (D) Measurement of the antiproliferative index. MDA-MB-231 cells were plated into 6-well plates and treated 48 h after plating with 5 and 10 μM Tam or 10 and 40 μM PBPE or the solvent vehicule (0.1% EtOH) for 5 days. The drugs and media were changed every 48 h. Cells were counted daily. Cells were harvested by trypsinization and counted on a Coulter counter. Experiments were repeated in triplicate. (E) Tam and PBPE induced the production of reactive oxygen species in MDA-MB-231 cells. MDA-MB-231cells were treated for 48 h with the solvent vehicle (0.1% EtOH), 5 μM Tam or 20 μM PBPE. ROS production was determined by flow cytometry analysis on cells stained with dihydroethidine probe (2.5 μM) as described in Section 2. Experiments were repeated at least three times in duplicate with comparable results. (F) Quantification of oxysterol in MDA-MB-231 cells incubated for 48 h with 10 μM Tam or 40 μM PBPE in the absence or presence of 500 μM Vit E. The results are reported in ng

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

Table 1
Effect of AEBS/ChEH ligands on 5,6-EC and 5,6-ECS biosynthesis, neutral lipid accumulation, and inhibition of ChEH on MCF-7 cells after 48 h treatment with drugs. 5,6-EC (5,6α-EC and 5,6β-EC) biosynthesis were performed by studying the metabolism of [^{14}C]-cholesterol in MCF-7 cells treated for 48 h with 10 μM of drugs as described in Section 2. 5,6-ECS biosynthesis was measured as described in the legend of Fig. 2. Stimulation of neutral lipid accumulation was monitored after treatment of MCF-7 cells and revealed by staining with Oil Red O (ORO) and visualized by light microscopy. Cytotoxicity (Cytx) was measure by the trypan blue exclusion methodology for a 72 h exposure of MCF-7 cells with 10 μM (SERMs), 20 μM (selective AEBS/ChEH ligands (AEBS/ChEH), sigma receptor ligands (σ-R) and cholesterol biosynthesis inhibitors (CBI)). The inhibition by Vit E (−) included the production 5,6-EC, 5,6-ECS, neutral lipid accumulation and cytotoxicity. + means a stimulation, − means inhibition.

Compound	Class	5,6-EC	5,6-ECS	ORO	Cytx	Vit E
Tam	SERM	+	+	+	+	−
4OHTam		+	+	++	+	−
Ralox		+	+	+	+	−
BZA		+	++	+	+	−
Clom		+	+	+	+	−
RU-39411		+	+	++	+	−
PBPE	AEBS/ChEH	+	+	+	+	−
PCPE		+	+	+	+	−
Tesm		+	+	+	+	−
MBPE		+	+	+	+	−
MCPE		+	+	+	+	−
BD-1008	Misc	+	+	+	+	−
SR-31747A		+	+	+	+	−
FPT		+	+	+	+	−
CLP		+	+	+	+	−
TFP		+	+	+	+	−
U-18666A	CBI	+	+	+	+	−
AY-9944		+	+	+	+	−
Triparanol		+	+	++	+	−

to inhibit ChEH [15,33], stimulated the biosynthesis of TAG and were cytotoxic to MCF-7 cells (Table 1). SERMs such as raloxifene and clomiphene compounds of the DPN family such as tesmilifene, cholesterol biosynthesis inhibitors such as U-18666A, triparanol and AY-9944, sigma receptor ligands BD-1008 and SR-31747A and tricyclic antidepressants such as trifluoroperazine, flupenthixol and chlorpromazine showed similar characteristics as Tam and PBPE on MCF-7 cells (Table 1). Altogether these data established that AEBS/ChEH ligands belonging to different structural and pharmacological classes induced the accumulation of 5,6-EC and 5,6-ECS, stimulated the accumulation of neutral lipid and were cytotoxic to MCF-7 cells. All these effects were inhibited by vit E.

3.6. Effect of Tam and PBPE on MDA-MB-231 cells

To confirm the importance of SULT2B1b in Tam and PBPE activity, we tested them on MDA-MB-231 cells. MDA-MB-231 cells were found to be similar to MCF-7 cells in their expression level of LXRβ, LXRα, SREBP-1c, SCD1, and LDLR. They expressed a higher amount of ABCA1 and a weaker amount of ABCG1 compared to MCF-7 cells (Fig. 5A). Interestingly, in contrast to MCF-7 cells, MDA-MB-231 did not express SULT2B1b while both cell lines expressed the steroid sulfatase (STS) in equal amount. STS being responsible for the de-sulfation of steroid- and sterol-sulfates [54]. The absence of SULT2B1b at the protein level was confirmed by Western blotting while SULT2B1b was detected in MCF-7 cells (Fig. 5B). Analyses of 5,6-EC sulfation in cells showed that, while in the presence of Tam and PBPE, MCF-7 cells produced 5,6-ECS, no production of 5,6-ECS was found under the same conditions of treatment in MDA-MB-231 cells (Fig. 5C). To study further the sensitivity of MDA-MB-231 to Tam and PBPE, kinetic and dose response studies were carried out. In Fig. 5D it can be seen that drugs induced a concentration- and time-dependent growth control and cytotoxicity. Treatment of cells with Tam and PBPE induced the production of ROS (Fig. 5D) as observed in MCF-7 cells [23]. We found that Tam and PBPE stimulated the biosynthesis of 5,6-EC in MDA-MB-231 cells (Fig. 5F). The antioxidant Vit E inhibited the stimulation of 5,6-EC production by Tam and PBPE (Fig. 5F) strongly suggesting that this epoxidation was ROS-dependent. Tam and PBPE were found to inhibit ChEH with IC_{50} of 59.9 ± 8 nM and 765 ± 12 nM respectively (Fig. 5G). To determine if LXRβ was modulated by Tam and PBPE in MDA-MB-231 cells, cells were transfected with the LXRE-Luc plasmid. T0901317, but neither Tam nor PBPE activated the expression of Luc at 12 h (Fig. 5H). At 48 h of treatment, Tam and PBPE stimulated Luc activity, as observed in MCF-7 cells (Fig. 2B). We next used qPCR to study the expression of endogenous LXR-responsive genes in MDA-MB-231 cells treated with the LXR modulator T0901317, 5,6α-EC, Tam and PBPE. We found that T0901317 stimulated the expression of ABCA1, SREBP-1c, SCD1, and repressed the expression of LDLR (Fig. 5I). 6 h of treatment of cells with 5,6α-EC and 40 h treatment with Tam or PBPE induced a similar effect of repression ABCA1 and SREBP-1c, and stimulation of the expression of SCD1 and LDLR (Fig. 5I).

Looking at neutral lipid metabolism, we found that Tam and PBPE induced the accumulation of ORO positive vesicles in MDA-MB-231 cells and knock-down of LXRβ inhibited this stimulation (Fig. 6A) establishing that this stimulation was LXRβ-dependent. We next showed that Tam and PBPE stimulated the accumulation of TAG in MDA-MB-231 cells (Fig. 6B) and it was ROS- and LXRβ-dependent (Fig. 6C). The LXR modulators T0901317 and 5,6α-EC stimulated the biosynthesis of TAG in an LXR-dependent manner in MDA-MB-231 cells (Fig. 6C). The expression of SULT2B1b did not modify the induction of TAG biosynthesis by 5,6α-EC, Tam or PBPE, indicating that TAG biosynthesis does not necessarily require SULT2B1b and the production of 5,6-ECS as observed in MCF-7 cells. 5,6-ECS was found to be cytotoxic to these cells and improved the cytotoxicity of Tam and PBPE (Fig. 6E). Altogether, these data established that MDA-MB-231 are SULT2B1b negative cells that cannot produce 5,6-ECS. We established that Tam and PBPE induced similar events in these cells as in MCF-7 cells in terms of ROS, 5,6-EC, and the induction of LXR-dependent TAG biosynthesis. The addition of 5,6-ECS to cells strongly sensitized MDA-MB-231 cells to Tam and PBPE.

3.7. Expression of SULT2B1b in MDA-MB-231 cells sensitized them to cytotoxicity induced by Tam and PBPE

MDA-MB-231 cells were found 1.9- and 2.1-fold less sensitive than in MCF-7 cells to Tam and PBPE (Table 2). These cells were insensitive to 5,6α-EC while their sensitivity to 5,6-ECS and 5,6β-EC was equivalent to that measured in MCF-7 cells (Table 2). Interestingly, the sensitivity of MDA-MB-231 to Tam and PBPE was similar to that of MCF-7/siLXRβ and MCF-7/siSULT suggesting an

of oxysterol per 10^6 cells. (G) Inhibition of ChEH activity by Tam and PBPE in MDA-MB-231 cells. Cells were incubated with [^{14}C]-5,6-EC (0.6 μM; 20 μCi/μmol) and were treated with increasing concentrations of Tam or PBPE ranging from 10 nM to 10 μM over 24 h. The positions of the 5,6-EC and CT were determined using [^{14}C]-5,6-EC and [^{14}C]-CT as standards. (H) Effect of T0901317, Tam, and PBPE on LXRE-Luc at 12 h and 48 h. MDA-MB-231 cells transfected with an LXRE-Luc plasmid were incubated with 1 μM T0901317, 5 μM Tam and 20 μM PBPE and assayed for Luc activity at 12 h and 48 h after treatment (Luc), expressed as relative luciferase unit (RLU) as described in Section 2. (I) Effect of T0901317, 5,6α-EC, Tam and PBPE on the expression of LXR-responsive genes in MDA-MB-231. MDA-MB-231 cells were incubated 6 h with 1 μM T0901317, 20 μM 5,6α-EC or incubated 40 h with 5 μM Tam or 10 μM PBPE. The expression of LXR-responsive genes was measured by quantitative RT-PCR as described in Section 2. Values are means of three independent experiments.

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

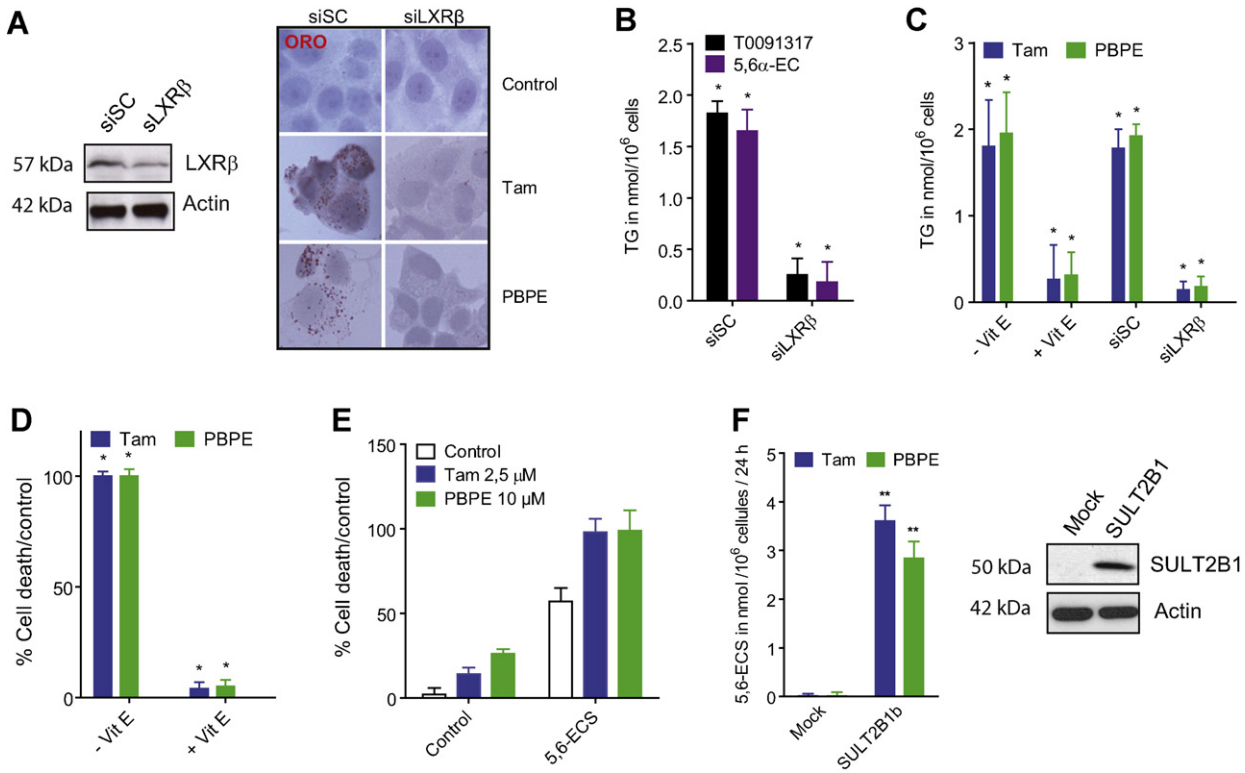


Fig. 6. Importance of LXRβ and ROS in the induction of TAG by Tam and PBPE in MDA-MB-231cells and effect of 5,6-ECS complementation on the cytotoxicity induced by Tam and PBPE. (A) Importance of LXRβ in the induction by Tam and PBPE of neutral lipid accumulation in MDA-MB-231 cells. Cells were transfected with siSC or siLXRβ and incubated 48 h with 5 μM Tam or 10 μM PBPE. LXRβ expression was verified by Western blot using anti-LXRβ antibodies. Cells were stained for neutral lipids with ORO and counterstained with Meyer's hematoxylin as described in Section 2. (B) Effect of Tam and PBPE on ROS- and LXRβ-dependent stimulation of TAG biosynthesis in MDA-MB-231 cells. MDA-MB-231 cells were transfected or not with siSC or siLXRβ and 24 h later treated for 48 h with solvent vehicle, 5 μM Tam or 20 μM PBPE in the presence or absence of 500 μM Vit E. TAG quantification was done as described in Section 2. (C) Effect of T0901317 and 5,6α-EC on the LXRβ-dependent stimulation of TAG biosynthesis in MDA-MB-231 cells. MDA-MB-231 cells were transfected with siSC or siLXRβ. TAG quantification was done as described in Section 2. (D) Effect of Vit E on the induction of cytotoxicity by Tam and PBPE in MDA-MB-231 cells. MDA-MB-231 cells were incubated 72 h in the presence of 10 μM Tam or 40 μM PBPE in the presence or absence of 500 μM Vit E. Cell death was quantified using the trypan blue exclusion methodology as described in Section 2. (E) Effect of 5,6-ECS on the induction of cytotoxicity by Tam and PBPE in MDA-MB-231 cells. Cells were treated 72 with solvent vehicle, 2.5 μM Tam or 10 μM PBPE in the absence or presence of 10 μM 5,6-ECS. Cell death was quantified using the trypan blue exclusion methodology as described in Section 2. (F) Ectopic expression of SULT2B1b activity in MDA-MB-231 cells. Cells were transfected with a plasmid encoding human SULT2B1b. Expression of SULT2B1b was verified by Western blotting and the functionality of the enzymes was confirmed by transformation of 5,6α-EC into 5,6-ECS as described above. The data presented here are the means ± S.E. of four independent experiments in triplicate. $^{*}P < 0.001$, $^{**}P < 0.0001$.

absence of LXRβ-dependent cytotoxicity due to the absence SULT2B1b expression and the absence of 5,6-ECS formation. Knock-down of LXRβ (MDA-MB-231/siLXRβ) confirmed the absence of LXRβ-depentent cytotoxicity of Tam and PBPE showing no significant impact on the EC_{50} of Tam and PBPE compared to MDA-MB-231 control cells (MDA-MB-231/siSC). Interestingly a loss of 5,6-ECS sensitivity was found in MDA-MB-231/siLXRβ ($EC_{50} > 40$ μM) confirming the LXRβ-dependent cytotoxicity of 5,6-ECS. As expected, there was no change in the cytotoxicity induced by 5,6β-EC in MDA-MB-231/siLXRβ compared to MDA-MB-231/siSC showing that 5,6β-EC induced a similar LXRβ-independent cytotoxicity in MDA-MB-231 as in MCF-7 cells (Table 2). To determine the importance of SULT2B1b in Tam, PBPE and 5,6α-EC cytotoxicity, MDA-MB-231 cells were transfected with a plasmid encoding SULT2B1b (MDA-MB-231/SULT) which led to the expression of the enzyme at the protein level (Fig. 6F) and enabled the production of 5,6-ECS when the cells were treated with Tam or PBPE (Fig. 6F). SULT2B1b expression sensitized cells by 1.9- and 2.2-fold to Tam and PBPE respectively compared to control cells (MDA-MB-231/MOCK) and MDA-MB-231/SULT became sensitive to 5,6α-EC (Table 2). Interestingly, MDA-MB-231/SULT cells were as sensitive to Tam, PBPE and 5,6α-EC as MCF-7 cells (Table 2) establishing a sensitization of cells to Tam, PBPE and 5,6α-EC. Altogether, these data showed that the induction of cytotoxicity by Tam and PBPE in MDA-MB-231 is LXR-independent and is mediated by 5,6β-EC. Importantly, the ectopic expression of SULT2B1b in MDA-MB-231 cells restored the sensitivity of cells to Tam and PBPE to the same level as MCF-7 cells.

4. Discussion

The aim of the present study was first to identify the cholesterol autoxidation species that are produced under Tam- and AEBS/ChEH ligands-treatment of BC cells and to determine the molecular pathways involved in the induction of TAG biosynthesis and cytotoxicity.

Here we report for the first time that Tam and other AEBS ligands induced the production 5,6-EC diastereoisomers, 5,6α-EC and 5,6β-EC in a 1/3 ratio in MCF-7 cells. No other oxysterols from the series studied were found to be stimulated. The production of 5,6-EC diastereoisomers was totally blocked by Vit E establishing that they were produced through a ROS mediated mechanism.

Tam has been reported to stimulate NOX in MCF-7 and MDA-MB-231 cells [41] which can induce H_2O_2 production required for cholesterol epoxidation. The mechanism involved in NOX activation has not been studied in BC cells but a hypothesis can be formulated. AEBS/ChEH ligands have been shown to inhibit cholesterol biosynthesis at the AEBS level which led to the accumulation of free sterol in cells [16], producing the appearance of multilamellar bodies [23,24]. The presence of MLB recapitulates

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

Table 2
Evaluation of the cytotoxicity of Tam, PBPE, 5,6α-EC, 5,6-ECS and 5,6β-EC on wild type and genetically modified MCF-7 cells and MDA-MB-231 cells. Cells were exposed 72 h to drugs (Tam, PBPE) or oxysterols (5,6α-EC, 5,6-ECS, 5,6β-EC) with increasing concentrations ranging from 1 to 100 μM. Cytotoxicity was measure by the trypan blue exclusion methodology. EC_{50} corresponds to the concentration required to kill 50% of cells. MCF-7/*siSC*, MCF-7/*siLXRβ* and MCF-7/*siSULT* are MCF-7 cells transfected with a scrambled small interfering RNA (siRNA), an siRNA against LXRβ and with an siRNA against SULT2B1b respectively. MCF7/*neo* and MCF-7/*bcl2* are MCF-7 cells transfected permanently with the pZip-*neo* and pZip-*bcl2* vectors [23]. MDA-MB-231/*siSC* and MDA-MB-231/*siLXRβ* are MDA-MB-231 cells transfected with siSC or siLXRβ. MDA-MB-231/MOCK and MDA-MB-231/SULT are MDA-MB-231 cells transfected with an empty pCMV6-XL5 plasmid or a pCMV6-XL5-SULT2B1b encoding the human SULT2B1b. Results are the mean of 3 independent experiments in triplicate.

	Tam	PBPE	5,6α-EC	5,6-ECS	5,6β-EC
			EC_{50} in μM		
MCF-7	2.5 ± 0.6	10.9 ± 2.1	22.4 ± 1.5	10.4 ± 0.6	12.6 ± 0.6
MCF-7/*siSC*	2.2 ± 0.8	10.2 ± 1.8	21.6 ± 2.2	11.4 ± 1.4	12.4 ± 1.7
MCF-7/*siLXRβ*	4.8 ± 0.8	17.8 ± 1.8	>40	>40	12.5 ± 2.5
MCF-7/*siSULT*	5.1 ± 0.7	18.1 ± 1.7	>40	11.1 ± 1.3	13.5 ± 2.6
MCF-7/*neo*	2.1 ± 0.8	10.1 ± 1.7	22.4 ± 1.5	9.1 ± 1.3	11.5 ± 3.6
MCF-7/*bcl2*	10.2 ± 1.1	32. ± 2.1	25.6 ± 3.2	12. 1 ± 1.4	>40
MDA-MB-231	4.8 ± 1.3	23.2 ± 1.1	>40	9.6 ± 1.8	14.2 ± 1.1
MDA-MB-231/*siSC*	4.7 ± 1.2	21.0 ± 1.3	>40	10.1 ± 1.5	13.6 ± 1.6
MDA-MB-231/*siLXRβ*	4.6 ± 1.4	22.1 ± 1.4	>40	>40	13.4 ± 1.8
MDA-MB-231/*MOCK*	4.9 ± 1.3	23.5 ± 1.4	>40	9.4 ± 2.1	13.9 ± 1.4
MDA-MB-231/*SULT*	2.6 ± 1.4	10.6 ± 1.3	24.4 ± 2.5	9.2 ± 2.8	14.1 ± 2.5

Niemann-Pick C (NPC) diseases characterized by the accumulation of free sterols in cells [55] and which is known to be induced by U18666A [56], an AEBS/ChEH ligand [15]. NPC1 diseases are associated with a stimulation of oxidative stress and with the production in the blood of patients [57] of increased amounts of 5,6-EC and CT, produced by hydration by ChEH NPC diseases are associated with the accumulation of lipid rafts in cells [58], and these lipid rafts were reported to control the activation of NOX in MCF-7 cells [59].

Altogether, these data strongly suggested the implication of free sterol accumulation in NOX activation and ROS production as a result of the treatment of BC cells by Tam and other AEBS/ChEH ligands. Therefore, the precise determination of the mechanism involved in ROS formation in BC cells treated with Tam and PBPE deserves further investigations.

We reported that 5,6-EC are extensively hydrated by ChEH in MCF-7 cells to give CT [15]. We have previously shown that Tam and PBPE were potent inhibitors of ChEH from rat liver microsomes and show in the present study that these drugs were also potent inhibitors of the human ChEH present in MCF-7 and MDA-MB-231 cells. As observed for ChEH from rat liver microsomes, AEBS ligands inhibited the human ChEH from BC cells at pharmacologic and therapeutic concentrations. The consequence of ChEH inhibition is the blockage of CT formation and the accumulation of 5,6-EC diastereoisomers. CT has been reported to be metabolized into a tumor promoter suggesting that blocking its production might *per se* constitute a protection against tumorigenic processes [33,38]. Since the sterol and oxysterol sulfotransferase SULT2B1b was reported to be over expressed in MCF-7 cells [60] and used 5,6α-EC as preferred substrate among cholesterol and several ring B oxysterol [53], we studied 5,6α-EC metabolism in BC cells. As expected we found that under Tam and PBPE treatment, 5,6α-EC was sulfated into 5,6-ECS. We then evaluated whether the accumulation of 5,6-EC and 5,6-ECS was involved in the ROS dependent induction of TAG biosynthesis and the cytotoxicity triggered by Tam and AEBS/CHEH ligands in BC cells. Activation of TAG biosynthesis constitutes one the major markers of BC re-differentiation which is indicative of the reactivation of lactation [24,40,43]. TAG biosynthesis is tightly regulated and several enzymes involved in its biosynthesis have been reported to be under the transcriptional control of nuclear receptors including members of the oxysterol liver-X-receptors (LXRα and LXRβ) [61]. We established that Tam, AEBS/ChEH ligands, and 5,6α-EC induced TAG biosynthesis through an LXRβ-dependent mechanism leading to the up-regulation of lipogenic enzymes involved in TAG biosynthesis (ACC and SCD1). The fact that the inhibition of 5,6α-EC biosynthesis by Vit E and the knock-down of LXRβ blocked the biosynthesis of TAG induced by AEBS/ChEH ligands established that 5,6α-EC is the oxysterol that mediates TAG biosynthesis in BC cells stimulated by Tam and PBPE.

5,6α-EC was reported to be an LXRα and LXRβ modulator that displays agonist, antagonist and inverse agonist properties in a cell type-dependent manner [45]. We found here that 5,6α-EC showed a similar effect on the regulation of LXR-responsive genes in MCF-7 cells confirming the observations that Berrodin et al. made in other cell lines [45]. 5,6-ECS had a similar profile of gene regulation as 5,6-EC on BC cells. This established that if 5,6-ECS is a direct regulator of LXR, than this compound is not a full antagonist on LXR as reported earlier [51,62], but rather a modulator depending on the target gene.

In this current study, we demonstrate that the cytotoxicity induced by Tam and AEBS ligands implicated both 5,6α-EC and 5,6β-EC as endogenous mediators but through different mechanisms. 5,6α-EC was cytotoxic in an LXRβ-dependent manner after being sulfated by SULT2B1b to produce 5,6-ECS in MCF-7 cells. Consistent with these data, 5,6α-EC was not cytotoxic in SULT2B1b negative cells such as MDA-MB-231 cells. The cytotoxicity of 5,6-ECS was found to be LXRβ-dependent establishing that 5,6-ECS was the oxysterol that mediates the LXRβ-dependent cytotoxicity induced by Tam and AEBS/ChEH ligands in MCF-7 cells. 5,6β-EC was the most prominent oxysterol produced in both MCF-7 and MDA-MB-231 cells. Its cytotoxicity was not LXRβ- and SULT2B1b-dependent. 5,6β-EC has been reported to induce apoptosis in

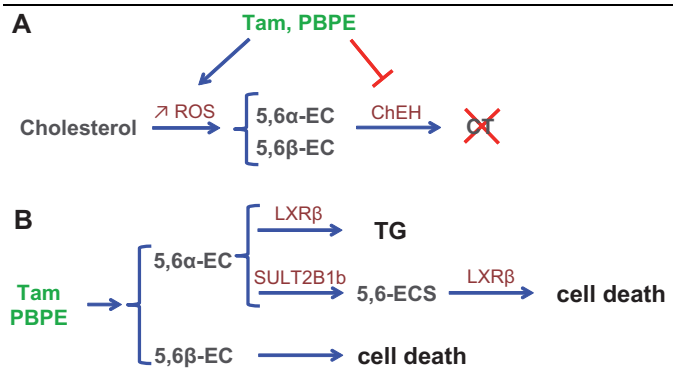


Fig. 7. Proposed molecular mechanism explaining the induction of TAG biosynthesis and cytotoxicity by Tam and PBPE in MCF-7 cells.

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

tumor cells through a mechanism involving mitochondria [63] but the determination of the precise mechanism of action of 5,6β-EC deserves further studies.

Several groups have shown that Vit E blocked the cytoxicity of Tam on breast cancer cell lines such as MCF-7 cells and MDA-MB-231 [23,24,64,65] and the mechanism described here gives a rationale to explain these effects. Fig. 7 provides a scheme describing the molecular mechanisms of the action of Tam and PBPE in the stimulation of TAG biosynthesis and cytotoxicity. These data indicate that the LXR-dependent cytotoxicity of Tam and AEBS/ChEH ligands depends on the presence of 5,6-EC and its product of sulfation (5.6-ECS) by SULT2B1b. On the other hand, we showed for the first time that the expression of SULT2B1b in the triple negative and SULT2B1b negative MDA-MB-231 cells, sensitizes the cells to the cytotoxicity of Tam and AEBS/ChEH ligands to the level of MCF-7 cells.

We found that sulfation of 5,6α-EC into 5,6-ECS by SULT2B1b was possible when 5,6α-EC accumulated in cells when ChEH was inhibited. Together these data established that 5,6-ECS formation contributes to the sensitivity of BC cells to Tam cytotoxicity. The fact that the ectopic expression of SUT2B1b in MDA-MB-231 cells or addition of 5,6-ECS sensitizes cells to Tam and PBPE strongly suggests that the expression of SULT2B1b in cells could be a predictor of the sensitivity of BC cells to Tam and AEBS/ChEH ligands. Furthermore the association of SERMs or DPM compounds with 5,6-ECS could represent an alternative to treat SULT2b1b negative triple negative untreatable BC. Further investigations will be carried to determine if these *in vitro* observations are reproduced *in vivo* on BC cell xenographs in mice.

The transcriptional modulation of 5,6-ECS was found comparable to that of 5,6α-EC suggesting that other LXR-responsive genes could be differentially modulated and this could explain the higher cytotoxicity of 5,6-ECS compared to 5,6-EC. This is supported by the observation that 5,6-ECS was found to be an antagonist in LXRE-Luc (data not shown and [51,62]) despite a similar modulation of several LXR-responsive genes, but further investigations are warranted.

The effect of other ChEH/AEBS selective inhibitors was observed with tesmilifene on the induction of BC cell differentiation and death. Tesmilifene was shown to significantly improve the overall survival in a phase III randomized trial for metastatic breast cancer when given with doxorubicin [32]. These effects were proposed to be due to the killing of tumor initiating cells (TIC) observed at therapeutic doses in four different models of breast cancer [22]. Since tesmilifene is a selective AEBS ligand and inhibitor of ChEH [8,15], the mechanisms detailed in the present study are likely to be involved in these effects giving a rationale for its use.

Tam has been reported in some clinical studies to induce a reversible stimulation of TAG production during the time of treatment and to lower circulating LDL cholesterol [66,67]. The liver is one of the tissues richest in AEBS/ChEH [15] and a tissue that produces 5,6α-EC [36], thus it is reasonable to propose that Tam could cause a decrease in LDL cholesterol through a previously observed inhibition of cholesterol esterification [68] and a stimulation of the expression of LDLR [69] through an LXR-dependent mechanism. On the other hand the hyper-triacylglycerolemia can be explained by the LXR-dependent mechanism we report on this paper, because LXRβ is known to control the biosynthesis of TAG [70]. This is supported by the observation that Vit E blocks hyper-triacylglycerolemia in patients treated with Tam [71], which is similar to what we found in MCF-7 cells in the present study. Thus, variations in the control of the LXR signaling pathway could explain the variations in the severity of the hyper-triacylglycerolemia observed in patients and again this deserves further study.

This current study established for the first time that 5,6-EC metabolites and LXRβ play a role in the induction of cell differentiation and death by Tam, other SERMs and AEBS/ChEH ligands in BC cells.These mechanisms now have to be taken into account in the development of new SERMs or other AEBS/ChEH ligands for anticancer applications.

Competing interest

PDM and MRP are employed by the company Affichem.

Acknowledgments

We thank David J Mangelsdorf (University of Texas, Dallas, TX, USA) for kindly providing plasmids encoding human LXR and LXRE-luc. MP and SSP are supported by internal grants from the "Institut National de la Santé et de la Recherche Medicale", the University of Toulouse III and an external grant from the "Fondation de France". VCJ is supported by P30CA051008, SSP is in charge of research at the Centre National de la Recherche Scientifique. GS is funded by the "Ministere de la Recherche" and the "Association pour la Recherche sur le Cancer".

References

[1] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225–49.
[2] Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ. Strategies for subtypes—dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 2011;22:1736–47.
[3] Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nat Rev Cancer 2007;7:46–53.
[4] de Medina P, Favre G, Poirot M. Multiple targeting by the antitumor drug tamoxifen: a structure-activity study. Curr Med Chem Anticancer Agents 2004;4:491–508.
[5] Llaverias G, Danilo C, Mercier I, Daumer K, Capozza F, Williams TM, et al. Role of cholesterol in the development and progression of breast cancer. Am J Pathol 2011;178:402–12.
[6] Danilo C, Frank PG. Cholesterol and breast cancer development. Curr Opin Pharmacol 2012;12:677–82.
[7] Alikhani N, Ferguson RD, Novosyadlyy R, Gallagher EJ, Scheinman EJ, Yakar S, et al. Mammary tumor growth and pulmonary metastasis are enhanced in a hyperlipidemic mouse model. Oncogene 2013;32:961–7.
[8] Poirot M, Silvente-Poirot S, Weichselbaum RR. Cholesterol metabolism and resistance to tamoxifen. Curr Opin Pharmacol 2012;12:683–9.
[9] Pitroda SP, Khodarev NN, Beckett MA, Kufe DW, Weichselbaum RR. MUC1-induced alterations in a lipid metabolic gene network predict response of human breast cancers to tamoxifen treatment. Proc Natl Acad Sci USA 2009;106:5837–41.
[10] de Medina P, Paillasse MR, Segala G, Khallouki F, Brillouet S, Dalenc F, et al. Importance of cholesterol and oxysterols metabolism in the pharmacology of tamoxifen and other AEBS ligands. Chem Phys Lipids 2011;164:432–7.
[11] Poirot M, De Medina P, Delarue F, Perie JJ, Klaebe A, Faye JC. Synthesis, binding and structure-affinity studies of new ligands for the microsomal anti-estrogen binding site (AEBS). Bioorg Med Chem 2000;8:2007–16.
[12] Delarue F, Kedjouar B, Mesange F, Bayard F, Faye JC, Poirot M. Modifications of benzylphenoxy ethanamine antiestrogen molecules: influence affinity for antiestrogen binding site (AEBS) and cell cytotoxicity. Biochem Pharmacol 1999;57:657–61.
[13] Fargin A, Bayard F, Faye JC, Traore M, Poirot M, Klaebe A, et al. Further evidence for a biological role of anti-estrogen-binding sites in mediating the growth inhibitory action of diphenylmethane derivatives. Chem Biol Interact 1988;66:101–9.
[14] Brandes LJ. A diphenylmethane derivative selective for the anti-estrogen binding site may help define its biological role. Biochem Biophys Res Commun 1984;124:244–9.
[15] de Medina P, Paillasse MR, Segala G, Poirot M, Silvente-Poirot S. Identification and pharmacological characterization of cholesterol-5,6-epoxide hydrolase as a target for tamoxifen and AEBS ligands. Proc Natl Acad Sci USA 2010;107:13520–25.
[16] Kedjouar B, de Medina P, Oulad-Abdelghani M, Payre B, Silvente-Poirot S, Favre G, et al. Molecular characterization of the microsomal tamoxifen binding site. J Biol Chem 2004;279:34048–61.
[17] Mesange F, Sebbar M, Capdevielle J, Guillemot JC, Ferrara P, Bayard F, et al. Identification of two tamoxifen target proteins by photolabeling with 4-(2-morpholinoethoxy)benzophenone. Bioconjug Chem 2002;13:766–72.
[18] Mesange F, Sebbar M, Kedjouar B, Capdevielle J, Guillemot JC, Ferrara P, et al. Microsomal epoxide hydrolase of rat liver is a subunit of theanti-oestrogen-binding site. Biochem J 1998;334(Pt 1):107–12.

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

[19] Poirot M, Chailleux C, Mesange F, Bayard F, Faye JC. Characterization of the membranous antiestrogen binding protein: II. Purification to homogeneity. J Recept Res 1994;14:37–46.
[20] Chailleux C, Poirot M, Mesange F, Bayard F, Faye JC. Characterization of the membranous antiestrogen binding protein: I. Partial purification of the protein in its active state. J Recept Res 1994;14:23–35.
[21] Poirot M, Chailleux C, Fargin A, Bayard F, Faye JC. A potent and selective photoaffinity probe for the anti-estrogen binding site of rat liver. J Biol Chem 1990;265:17039–43.
[22] Deng T, Liu JC, Pritchard KI, Eisen A, Zacksenhaus E. Preferential killing of breast tumor initiating cells by N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine/tesmilifene. Clin Cancer Res 2009;15:119–30.
[23] de Medina P, Payre B, Boubekeur N, Bertrand-Michel J, Terce F, Silvente-Poirot S, et al. Ligands of the antiestrogen-binding site induce active cell death and autophagy in human breast cancer cells through the modulation of cholesterol metabolism. Cell Death Differ 2009;16:1372–84.
[24] Payre B, de Medina P, Boubekeur N, Mhamdi L, Bertrand-Michel J, Terce F, et al. Microsomal antiestrogen-binding site ligands induce growth control and differentiation of human breast cancer cells through the modulation of cholesterol metabolism. Mol Cancer Ther 2008;7:3707–18.
[25] Kedjouar B, Daunes S, Vilner BJ, Bowen WD, Klaebe A, Faye JC, et al. Structural similitudes between cytotoxic antiestrogen-binding site (AEBS) ligands and cytotoxic sigma receptor ligands. Evidence for a relationship between cytotoxicity and affinity for AEBS or sigma-2 receptor but not for sigma-1 receptor. Biochem Pharmacol 1999;58:1927–39.
[26] Poirot M, Garnier M, Bayard F, Riviere I, Traore M, Wilson M, et al. The antiproliferative properties of 4-benzylphenoxy ethanamine derivatives are mediated by the anti-estrogen binding site (ABS), whereas the anti-estrogenic effects of trifluopromazine are not. Biochem Pharmacol 1990;40:425–9.
[27] Brandes LJ, Gerrard JM, Bogdanovic RP, Lint DW, Reid RE, LaBella FS. Correlation of the antiproliferative action of diphenylmethane-derivative antiestrogen binding site ligands with antagonism of histamine binding but not of protein kinase C-mediated phosphorylation. Cancer Res 1988;48:3954–8.
[28] Raghavan D, Brandes LJ, Klapp K, Snyder T, Styles E, Tsao-Wei D, et al. Phase II trial of tesmilifene plus mitoxantrone and prednisone for hormone refractory prostate cancer: high subjective and objective response in patients with symptomatic metastases. J Urol 2005;174:1808–13 [discussion 13].
[29] Khoo K, Brandes L, Reyno L, Arnold A, Dent S, Vandenberg T, et al. Phase II trial of N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine.HCl and doxorubicin chemotherapy in metastatic breast cancer: a National Cancer Institute of Canada clinical trials group study. J Clin Oncol 1999;17:3431–7.
[30] Brandes LJ, Bracken SP, Ramsey EW. N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine in combination with cyclophosphamide: an active, low-toxicity regimen for metastatic hormonally unresponsive prostate cancer. J Clin Oncol 1995;13:1398–403.
[31] Brandes LJ, Simons KJ, Bracken SP, Warrington RC. Results of a clinical trial in humans with refractory cancer of the intracellular histamine antagonist, N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine-HCl, in combination with various single antineoplastic agents. J Clin Oncol 1994;12:1281–90.
[32] Reyno L, Seymour L, Tu D, Dent S, Gelmon K, Walley B, et al. Phase III study of N,N-diethyl-2-[4-(phenylmethyl) phenoxy]ethanamine (BMS-217380-01) combined with doxorubicin versus doxorubicin alone in metastatic/recurrent breast cancer: National Cancer Institute of Canada Clinical Trials Group Study MA.19. J Clin Oncol 2004;22:269–76.
[33] Silvente-Poirot S, Poirot M. Cholesterol epoxide hydrolase and cancer. Curr Opin Pharmacol 2012;12:696–703.
[34] de Medina P, Silvente-Poirot S, Tamoxifen Poirot M. AEBS ligands induced apoptosis and autophagy in breast cancer cells through the stimulation of sterol accumulation. Autophagy 2009;5:1066–7.
[35] Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 2012;8:445–544.
[36] Schroepfer Jr. GJ. Oxysterols: modulators of cholesterol metabolism and other processes. Physiol Rev 2000;80:361–554.
[37] Cai H. NAD(P)H oxidase-dependent self-propagation of hydrogen peroxide and vascular disease. Circ Res 2005;96:818–22.
[38] Poirot M, Silvente-Poirot S. Cholesterol-5,6-epoxides. Chemistry, biochemistry, metabolic fate and cancer. Biochimie 2013;95:622–31.
[39] Shah VP, Chegini HA, Vishneski SR, Weatherman RV, Blackmore PF, Dobrydneva Y. Tamoxifen promotes superoxide production in platelets by activation of PI3-kinase and NADPH oxidase pathways. Thromb Res 2012;129:36–42.
[40] Chakraborty A, Bodipati N, Demonacos MK, Peddinti R, Ghosh K, Roy P. Long term induction by pterostilbene results in autophagy and cellular differentiation in MCF-7 cells via ROS dependent pathway. Mol Cell Endocrinol 2012;355:25–40.
[41] Kallio A, Zheng A, Dahllund J, Heiskanen KM, Harkonen P. Role of mitochondria in tamoxifen-induced rapid death of MCF-7 breast cancer cells. Apoptosis 2005;10:1395–410.
[42] Lee YS, Kang YS, Lee SH, Kim JA. Role of NAD(P)H oxidase in the tamoxifen-induced generation of reactive oxygen species and apoptosis in HepG2 human hepatoblastoma cells. Cell Death Differ 2000;7:925–32.
[43] Munster PN, Troso-Sandoval T, Rosen N, Rifkind R, Marks PA, Richon VM. The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces differentiation of human breast cancer cells. Cancer Res 2001;61:8492–7.
[44] Jakobsson T, Treuter E, Gustafsson JA, Liver Steffensen KR. X receptor biology and pharmacology: new pathways, challenges and opportunities. Trends Pharmacol Sci 2012;33:394–404.
[45] Berrodin TJ, Shen Q, Quinet EM, Yudt MR, Freedman LP, Nagpal S. Identification of 5{alpha},6{alpha}-epoxycholesterol as a novel modulator of liver X receptor activity. Mol Pharmacol 2010;78:1046–58.
[46] Lewis-Wambi JS, Kim H, Curpan R, Grigg R, Sarker MA, Jordan VC. The selective estrogen receptor modulator bazedoxifene inhibits hormone-independent breast cancer cell growth and down-regulates estrogen receptor alpha and cyclin D1. Mol Pharmacol 2011;80:610–20.
[47] de Medina P, Paillasse MR, Payre B, Silvente-Poirot S, Poirot M. Synthesis of new alkylaminooxysterols with potent cell differentiating activities: identification of leads for the treatment of cancer and neurodegenerative diseases. J Med Chem 2009;52:7765–77.
[48] Paillasse MR, de Medina P, Amouroux G, Mhamdi L, Poirot M, Silvente-Poirot S. Signaling through cholesterol esterification: a new pathway for the cholecystokinin 2 receptor involved in cell growth and invasion. J Lipid Res 2009;50:2203–11.
[49] de Medina P, Casper R, Savouret JF, Poirot M. Synthesis and biological properties of new stilbene derivatives of resveratrol as new selective aryl hydrocarbon modulators. J Med Chem 2005;48:287–91.
[50] de Medina P, Genovese S, Paillasse M, Mazaheri M, Subra-Caze S, Bystricky K, et al. Auraptene is an inhibitor of cholesterol esterification and a modulator of estrogen receptors. Mol Pharmacol 2010;78:827–36.
[51] Song C, Hiipakka RA, Liao S. Auto-oxidized cholesterol sulfates are antagonistic ligands of liver X receptors: implications for the development and treatment of atherosclerosis. Steroids 2001;66:473–9.
[52] Viennois E, Mouzat K, Dufour J, Morel L, Lobaccaro JM, Baron S. Selective liver X receptor modulators (SLiMs): what use in human health. Mol Cell Endocrinol 2012;351:129–41.
[53] Fuda H, Javitt NB, Mitamura K, Ikegawa S, Strott CA. Oxysterols are substrates for cholesterol sulfotransferase. J Lipid Res 2007;48:1343–52.
[54] Reed MJ, Purohit A, Woo LW, Newman SP, Potter BV. Steroid sulfatase: molecular biology, regulation, and inhibition. Endocr Rev 2005;26:171–202.
[55] Liao G, Yao Y, Liu J, Yu Z, Cheung S, Xie A, et al. Cholesterol accumulation is associated with lysosomal dysfunction and autophagic stress in Npc1 −/− mouse brain. Am J Pathol 2007;171:962–75.
[56] Pacheco CD, Kunkel R, Lieberman AP. Autophagy in Niemann-Pick C disease is dependent upon Beclin-1 and responsive to lipid trafficking defects. Hum Mol Genet 2007;16:1495–503.
[57] Porter FD, Scherrer DE, Lanier MH, Langmade SJ, Molugu V, Gale SE, et al. Cholesterol oxidation products are sensitive and specific blood-based biomarkers for Niemann-Pick C1 disease. Sci Transl Med 2010;2:56ra81.
[58] Simons K, Gruenberg J. Jamming the endosomal system: lipid rafts and lysosomal storage diseases. Trends Cell Biol 2000;10:459–62.
[59] Rao Malla R, Raghu H, Rao JS. Regulation of NADPH oxidase (Nox2) by lipid rafts in breast carcinoma cells. Int J Oncol 2010;37:1483–93.
[60] He D, Meloche CA, Dumas NA, Frost AR, Falany CN. Different subcellular localization of sulphotransferase 2B1b in human placenta and prostate. Biochem J 2004;379:533–40.
[61] Wagner M, Zollner G, Trauner M. Nuclear receptors in liver disease. Hepatology 2011;53:1023–34.
[62] Argmann CA, Edwards JY, Sawyez CG, O'Neil CH, Hegele RA, Pickering JG, et al. Regulation of macrophage cholesterol efflux through hydroxymethylglutaryl-CoA reductase inhibition: a role for RhoA in ABCA1-mediated cholesterol efflux. J Biol Chem 2005;280:22212–21.
[63] Ryan L, O'Callaghan YC, O'Brien NM. Comparison of the apoptotic processes induced by the oxysterols 7beta-hydroxycholesterol and cholesterol-5beta,6-beta-epoxide. Cell Biol Toxicol 2004;20:313–23.
[64] Mandlekar S, Yu R, Tan TH, Kong AN. Activation of caspase-3 and c-Jun NH2-terminal kinase-1 signaling pathways in tamoxifen-induced apoptosis of human breast cancer cells. Cancer Res 2000;60:5995–6000.
[65] Gundimeda U, Chen ZH, Gopalakrishna R. Tamoxifen modulates protein kinase C via oxidative stress in estrogen receptor-negative breast cancer cells. J Biol Chem 1996;271:13504–14.
[66] Love RR, Newcomb PA, Wiebe DA, Surawicz TS, Jordan VC, Carbone PP, et al. Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 1990;82:1327–32.
[67] Love RR, Wiebe DA, Feyzi JM, Newcomb PA, Chappell RJ. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women after 5 years of treatment. J Natl Cancer Inst 1994;86:1534–9.
[68] de Medina P, Payre BL, Bernad J, Bosser I, Pipy B, Silvente-Poirot S, et al. Tamoxifen is a potent inhibitor of cholesterol esterification and prevents the formation of foam cells. J Pharmacol Exp Ther 2004;308:1165–73.
[69] Suarez Y, Fernandez C, Gomez-Coronado D, Ferruelo AJ, Davalos A, Martinez-Botas J, et al. Synergistic upregulation of low-density lipoprotein receptor activity by tamoxifen and lovastatin. Cardiovasc Res 2004;64:346–55.
[70] Patel R, Patel M, Tsai R, Lin V, Bookout AL, Zhang Y, et al. LXRbeta is required for glucocorticoid-induced hyperglycemia and hepatosteatosis in mice. J Clin Invest 2011;121:431–41.
[71] Babu JR, Sundravel S, Arumugam G, Renuka R, Deepa N, Sachdanandam P. Salubrious effect of vitamin C and vitamin E on tamoxifen-treated women in breast cancer with reference to plasma lipid and lipoprotein levels. Cancer Lett 2000;151:1–5.

Please cite this article in press as: Segala G, et al. 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells. Biochem Pharmacol (2013), http://dx.doi.org/10.1016/j.bcp.2013.02.031

Molecular Endocrinology. First published ahead of print May 9, 2013 as doi:10.1210/me.2012-1353

Transcriptional repression of AIB1 by FoxG1 leads to apoptosis in breast cancer cells

Jordan V. Li[1], Christopher D. Chien[1], Jason P. Garee[1], Jianming Xu[2], Anton Wellstein[1], and Anna T. Riegel[1]

[1]Departments of Pharmacology and Oncology, Lombardi Cancer Center, Georgetown University Medical Center, WA, DC 20007; [2]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030

The oncogene nuclear receptor coactivator amplified in breast cancer 1 (AIB1) is a transcriptional coactivator that is overexpressed in various types of human cancers. However, the molecular mechanisms controlling AIB1 expression in the majority of cancers remain unclear. In this study, we identified a novel interacting protein of AIB1, forkhead-box protein G1 (FoxG1), which is an evolutionarily conserved forkhead-box transcriptional corepressor. We show that FoxG1 expression is low in breast cancer cell lines, and that low levels of FoxG1 are correlated with a worse prognosis in breast cancer. We also demonstrate that transient overexpression of FoxG1 can suppress endogenous levels of AIB1 mRNA and protein in MCF-7 breast cancer cells. Exogenously expressed FoxG1 in MCF-7 cells also leads to apoptosis that can be rescued in part by AIB1 overexpression. Using chromatin immunoprecipitation (ChIP), we determined that FoxG1 is recruited to a region of the AIB1 gene promoter previously characterized to be responsible for AIB1-induced, positive auto-regulation of transcription through the recruitment of an activating, multiprotein complex, involving AIB1, E2F1 and Sp1. Increased FoxG1 expression significantly reduces the recruitment of AIB1, E2F1 and p300 to this region of the endogenous AIB1 gene promoter. Our data imply that FoxG1 can function as a pro-apoptotic factor in part through suppression of AIB1 coactivator transcription complex formation, thereby reducing the expression of the AIB1 oncogene.

Amplified in breast cancer 1 (AIB1, ACTR, RAC3, SRC3, NCOA3 and p/CIP[b]) belongs to the p160 family of steroid receptor coactivators and is found to be frequently amplified in multiple human cancers (1). Similar to the other p160 coactivators, AIB1 can associate with hormone-bound nuclear receptors, and potentiate transcriptional activation by enhancing transcriptional complex assembly and through local chromatin remodeling (2–4). AIB1 is an oncogene and has been strongly implicated in the development of hormone-responsive and nonresponsive cancers (5, 6) by coactivating not only nuclear receptors but also nonreceptor transcription factors such as E2F1, nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and PEA3 (7–10). In mouse models, AIB1 overexpression results in the development of mammary hyperplasia and tumorigenesis (11). The overexpression of AIB1 has been observed in 30%–60% of human breast tumors and a strong correlation exists between high levels of AIB1 and high HER2 levels, larger tumor size, higher tumor grade, increased cancer reoccurrence and worse prognosis (12).

AIB1 expression can be controlled at multiple levels. AIB1 protein levels are regulated by a number of proteasomal degradation pathways (13–15). In terms of AIB1 mRNA, we have previously reported that all-*trans* retinoic acid, antiestrogens and tamoxifen, and TGF-β can upregulate AIB1 transcripts, whereas estrogen can suppress AIB1 gene expression (16). In addition, a recent study demonstrates that transcription of the AIB1 gene is controlled by regulatory sequences within the bp –250 to

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.
Copyright © 2013 by The Endocrine Society
Received October 30, 2012. Accepted April 29, 2013.

Abbreviations: HER2, Epidermal growth factor receptor 2; p300, E1A-binding protein p300; CBP, CREB-binding protein; TGF-β, transforming growth factor-beta; TLE, transcriptional corepressor of the groucho/transducin-like enhancer of split; PR, progesterone receptor; ANOVA, analysis of variance.

Copyright (C) 2013 by The Endocrine Society

EARLY RELEASE: Molecular Endocrinology
The Endocrine Society

+350 region of its promoter which enable AIB1 to autoregulate and enhance the expression of its own gene (7, 17). In these studies, an Sp1-binding site down-stream of exon 1 was described within the –250/+350 region that also recruited E2F1. This enables AIB1 to complex with E2F1, and this Sp1-associated transcription complex significantly increases the coactivation of the AIB1 gene (17).

AIB1 is also known to directly bind to other coactivators such as histone acetyltransferase p300/CBP, p300/CBP-associated cofactor p/CAF, and arginine methyltransferase CARM 1, and enhances transcriptional activation by bringing these potent cofactors capable of modifying chromatin organization to the target gene promoter (3, 18, 19). The ability to interact with a wide range of transcriptional cofactors allows AIB1 to act as a potent coactivator (12). In contrast, only a few transcriptional corepressors that interact with the SRC family proteins are known (20, 21). Therefore, we conducted broad screens of AIB1-interacting proteins using mass spectrometry (MS) to detect low abundance AIB1 binding partners that may potentially suppress AIB1 function and negatively regulate the AIB1 gene expression (reviewed in 22). We focused on AIB1-interacting proteins that segregated under the category of "transcriptional repressors," and here we demonstrate that the winged-helix, DNA-binding transcriptional corepressor FoxG1 (also known as brain factor 1, BF1) which we identified as an AIB1 interacting protein, can downregulate AIB1 promoter activity and suppress both AIB1 transcript and protein expression in MCF-7 cells. FoxG1 belongs to the forkhead-box family of transcriptional regulators, and is a protein mainly expressed in the brain and testis in human (23, 24). FoxG1 controls the development of the telencephalon and cerebral corticogenesis (23) and is shown to interact with global transcriptional corepressors and histone deacetylases to potentiate transcriptional repression (25). FoxG1 can also directly interact with androgen receptor (AR) and suppress AR-mediated transactivation (24). While FoxG1 knockout mice develop cerebral hypoplasia and die at birth, humans with FoxG1 haploinsufficiency show severe mental retardation and microcephaly (23, 26, 27). The prominent developmental phenotype associated with FoxG1 pathology has focused most investigations of FoxG1 function on the brain and neurogenesis, therefore not much is known about the role of FoxG1 in cancer and the molecular mechanism underlying FoxG1 function.

Our data indicate that FoxG1 is directly recruited to the AIB1 promoter. We report a mechanism by which FoxG1 overexpression compromises the integrity of an Sp-1-associated activating transcriptional complex. This complex is required for the upregulation of AIB1 gene expression, and FoxG1 reduces its recruitment by disassembly and detachment of the activating complex from the AIB1 promoter. We also show that FoxG1 downregulates AIB1 expression which leads to apoptosis in human breast cancer cells.

Results

AIB1 interacts with the transcriptional corepressor FoxG1

To identify proteins that interact with AIB1, we performed AIB1-specific immunoprecipitations (IP) of lysates from HEK293T cells transfected with a FLAG-tagged AIB1 construct. After IP with a FLAG antibody, we isolated FLAG-associated immunocomplexes by denaturing gel electrophoresis, followed by Coomassie Blue staining. Twelve visible bands were excised, and extracted proteins were subjected to MS analysis (described in (22)). FoxG1 was identified as a candidate AIB1-interacting protein through our MS evaluation. It was of interest for further investigation since it is known to function in certain contexts as a transcriptional repressor (25) and could potentially regulate AIB1 function and gene expression. To verify AIB1 interaction with FoxG1, we performed coimmunoprecipitation (co-IP) experiments. FLAG-AIB1 was expressed by transient transfection together with HA-FoxG1 in HEK293T cells and HA-FoxG1 was detected in the immunoprecipitates of FLAG-AIB1 from whole cell lysates (Figure 1A). We also confirmed the interaction of endogenous AIB1 and FoxG1 in MCF-7 breast cancer cells which harbor the 20q AIB1 gene amplicon and express high levels of AIB1 protein and a detectable amount of FoxG1 (Figure 1B). These IP results confirm the MS data and demonstrate that FoxG1 is present in complexes that coimmunoprecipitate with AIB1.

FoxG1 is predominantly expressed in the brain and its non-neuronal expression in normal tissues is low (23, 24). The expression of FoxG1 in human cancer has not been widely reported. In a comparison of normal breast cells and breast cancer cell lines, we found that the mRNA expression level of FoxG1 was significantly lower in breast cancer cell lines, irrespective of their estrogen receptor (ER) status, as compared to FoxG1 expression in the normal human mammary epithelial cells (HMEC) (Figure 1C). These data suggest a loss of FoxG1 expression from normal to cancerous transition (Figure 1C) and indicate that reduced FoxG1 expression might have prognostic significance in human breast cancer. Our reanalysis of published microarray data (www.oncomine.org) from

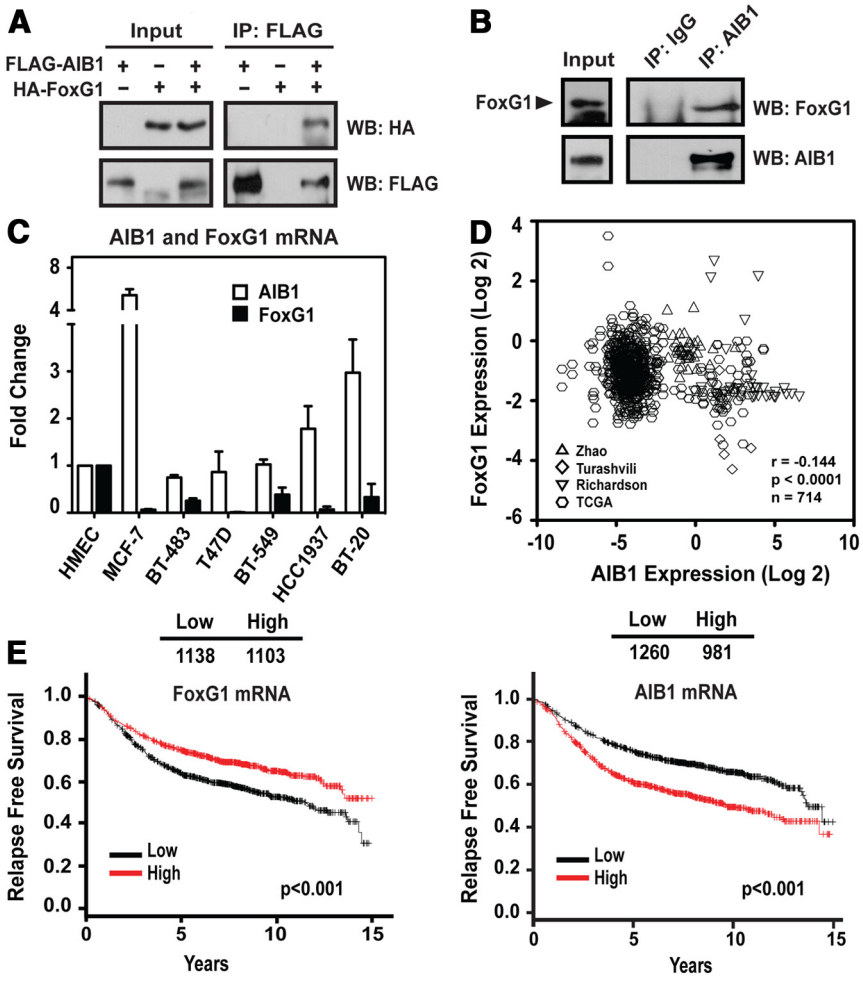


FIGURE 1. AIB1 and FoxG1 interact in mammalian cells. A, AIB1 interacts with FoxG1 in HEK293T cells. FLAG-AIB1 was cotransfected with HA-FoxG1 constructs. 48 h post transient transfection, whole cell lysates were collected and used for immunoprecipitation (IP) and western blot (WB) analysis with anti-FLAG and anti-HA antibodies as indicated. B, Interaction of endogenous AIB1 with FoxG1 in MCF-7 breast cancer cells. Nuclear lysates were prepared from MCF-7 cells and immunoprecipitated with an AIB1 antibody or control IgG. FoxG1 protein associated with AIB1 in the IP was detected by WB as indicated. C, AIB1 and FoxG1 mRNA expression levels in breast cancer cell lines. Total RNA was harvested from breast cancer cell lines to determine the relative gene expression for AIB1 and FoxG1. D, FoxG1 and AIB1 mRNA expression are inversely correlated. Data from Zhao et al. (28), Turashvili et al. (29), Richardson et al. (30), and TCGA (The Cancer Genome Atlas - Invasive Breast Carcinoma Gene Expression Data) were analyzed using Oncomine (www.oncomine.org). Higher expression of FoxG1 coincides with lower expression of AIB1 and vice versa. E, Analysis of the levels of AIB1 and FoxG1 mRNA on a gene expression microarray of breast cancer samples from patients with known relapse-free survival (RFS) times provided by Kaplan-Meier Plotter (http://www.kmplot.com) (KM analysis parameters are described in *Material and Methods*) (31).

human breast cancer clinical samples of four independent studies (Zhao et al. (28), Turashvili et al. (29), Richardson et al. (30), and The Cancer Genome Atlas - Invasive Breast Carcinoma Gene Expression Data/TCGA) further supported this hypothesis and showed a significant ($P < .0001$) negative correlation between AIB1 and FoxG1 mRNA expression over 714 samples, where higher expression of FoxG1 coincided with lower expression of AIB1 and vice versa (Figure 1D).

In addition, we used unbiased gene expression data compiled by Kaplan-Meier (KM) Plotter (http://kmplot.com) (31) from a series of suitable studies that allow for an analysis of clinical outcomes correlated with a single gene expression. We saw that higher FoxG1 mRNA levels, in 2241 breast cancer samples, correlated with increased relapse-free survival rate (Figure 1E) (KM analysis parameters are described in *Materials and Methods*). In contrast, in the same data set, elevated AIB1 transcript levels correlated with reduced relapse-free survival (RFS) (Figure 1E), which is consistent with previous reports on AIB1 prognostic significance in human breast cancer (reviewed in (12)).

FoxG1 induces apoptosis in MCF-7 cells

The expression pattern for AIB1 and FoxG1 in MCF-7 cells was of interest because these cells significantly overexpress AIB1 and show low levels of FoxG1 mRNA expression compared to HMEC (Figure 1C). We therefore chose to study the phenotypic effect of FoxG1 overexpression on MCF-7 cells. Twenty-four hours after expression vector transfection, overexpressing FoxG1 led to cell detachment from culture dishes, and the induction of apoptosis as determined by annexin V staining (Figure 2A, upper panel). The early and total apoptosis average indices for duplicate samples of 14.5 and 28.64% in FoxG1-expressing cells were significantly elevated compared to MCF-7 cells transfected with the control empty vector (EV) with average apoptosis indices of 1.68 and 9.4%, respectively (Figure 2A, lower panel). Increased expression of FoxG1 in MCF-7 cells was correlated with the apoptotic response.

Previous studies reported increased incidence of apoptosis in MCF-7 cells when AIB1 expression is downregulated by small-interfering RNA (siRNA)-directed gene silencing (32). Thus, since AIB1 and FoxG1 form a complex (Figure 1A and B), we conjectured that a portion

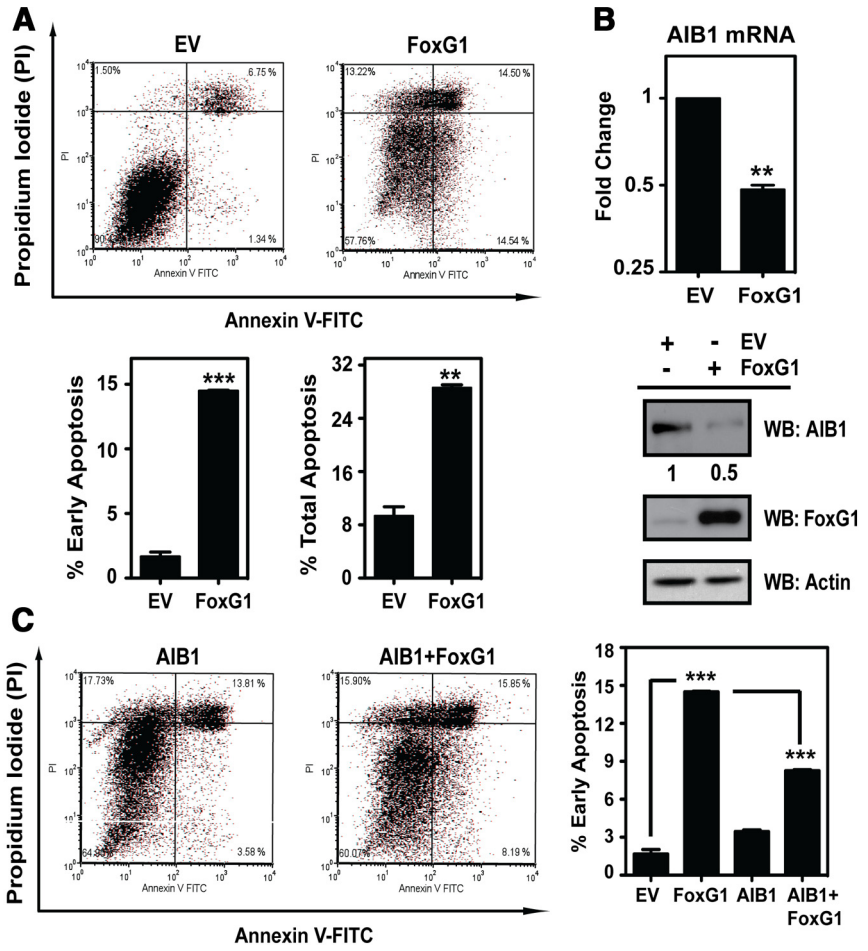


FIGURE 2. FoxG1 induces apoptosis and down-regulates AIB1 expression in MCF-7 cells. A, MCF-7 cells were transfected with either an empty vector (EV) control or FoxG1 constructs. 24 h after transfection, cells were subjected to Annexin V apoptosis analysis. The percentages of cells in early- and late apoptosis are represented by bottom right- and top right quadrants of the FACS analysis, respectively. Percent total apoptosis was the total percentage of cells in both early- and late apoptosis. The mean ± SEM values were obtained from duplicate samples from each transfection condition. ***, $P < .001$; **, $P < .01$ relative to EV. Statistical analysis was done by Student's *t* test. B, Analysis of endogenous AIB1 expression in MCF-7 cells overexpressing FoxG1. Cells were transfected with EV or FoxG1 as in (A). Total RNA and whole cell lysates were collected to determine the relative levels of mRNA and protein for AIB1. Cells transfected with EV were arbitrarily set at 1 and cells expressing FoxG1 were analyzed in reference to it. Student's *t* test. **, $P < .01$ relative to EV. Relative protein levels were determined by WB with antibodies as indicated. C, AIB1 rescues MCF-7 cells from FoxG1-induced apoptosis. MCF-7 cells were transfected separately with expression vectors for either EV control, FoxG1, AIB1, or AIB1 and FoxG1 together. Cells were assessed for apoptosis as in (A). ***, $P < .001$, EV vs. FoxG1; or FoxG1 vs. AIB1+FoxG1. One-way ANOVA with Bonferroni post test.

of the FoxG1-induced apoptotic effect might be mediated through changes in AIB1 expression. Consistent with this notion, FoxG1 overexpression caused a twofold decrease in both AIB1 mRNA and protein expression levels (Figure 2B). We also observed a similar effect of FoxG1 on AIB1 expression in the metastatic MDA-MB-231 cells. In these cells, FoxG1 overexpression caused a 3% to 6% increase in early apoptosis and a near 60% reduction in the levels of AIB1 protein (Supplemental Figure 1A and B). This indicates that FoxG1 can have a negative regulatory effect on AIB1 expression also in other different subtypes of breast cancer cells that are estrogen receptor (ER) negative.

We next asked whether the FoxG1 induction of apoptosis was mediated directly by the loss of AIB1 by determining whether exogenously expressed AIB1 was sufficient to rescue these cells from FoxG1-induced apoptosis. Our analysis revealed that AIB1 coexpression with FoxG1 allowed an approximately 50% rescue from apoptosis compared to cells transfected with only FoxG1 (Figure 2C, right panel; compare second bar with the fourth bar). Thus, our phenotypic studies argue that a significant portion of the FoxG1 induction of apoptosis in MCF-7 cells is mediated through down-regulation of AIB1 expression.

FoxG1 represses AIB1 promoter activity

Our data demonstrate that FoxG1 overexpression represses the levels of AIB1 transcript (Figure 2B), thus, we hypothesized that FoxG1 could directly regulate the AIB1 promoter. Previous studies have identified a region in the AIB1 gene promoter responsible for the positive auto-regulation of transcription through the recruitment of an activating transcriptional protein complex involving AIB1, E2F1, and Sp1 (17). This critical positive regulatory sequence covers a –250 to +350 span up and downstream of the transcription start site of the AIB1 gene. An intronic Sp1 binding site, marked by a GC box at bp +150/+160 is required for the AIB1 promoter activation by E2F1 and AIB1. Moreover, the Sp1 binding sequence serves as a docking site for the recruitment of the AIB1-E2F1 complex, through which AIB1 can act as a coactivator on its own promoter, establishing a positive auto-regulatory loop of AIB1 gene expression (Figure 3A) (17).

To determine whether FoxG1 impacted AIB1 promoter activity we cotransfected MCF-7 cells with an

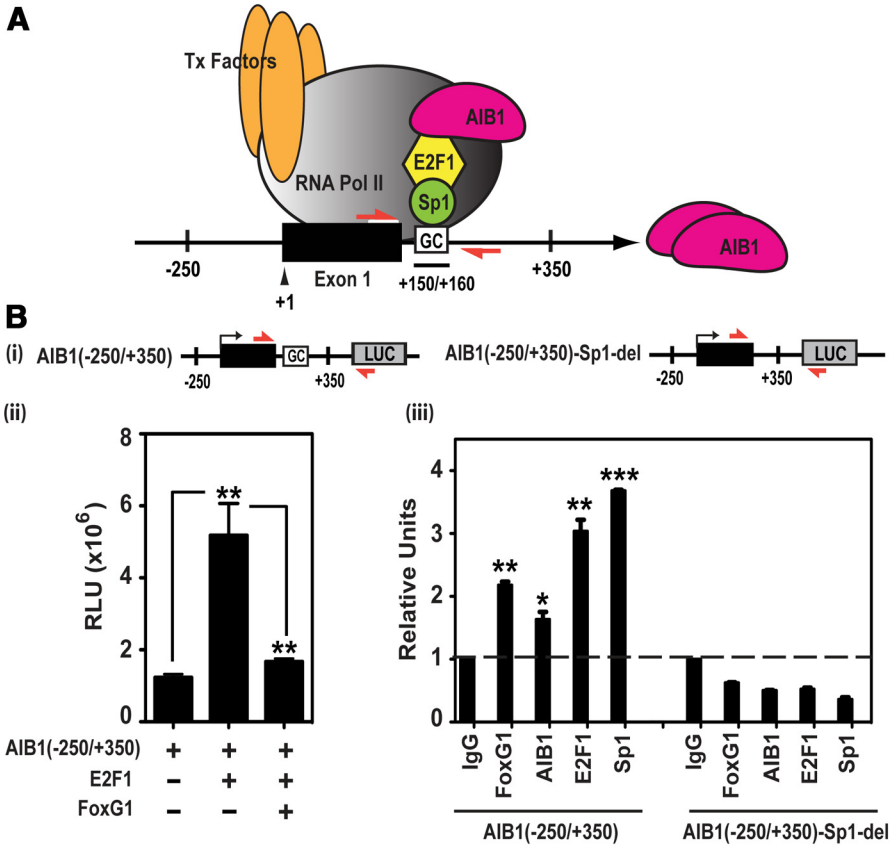


FIGURE 3. FoxG1 represses AIB1 gene promoter activity. A, Model of the AIB1 gene promoter. Model showing an activating transcriptional complex consisting of AIB1, E2F1 and Sp1, anchored to DNA through a Sp1-binding site, the *GC box,* which is down stream of exon 1 (black box) in the –250 to +350 base pair (bp) region of the AIB1 promoter. The red arrows represent the locations and orientations of the AIB1 promoter-specific primers. B, *panel i,* AIB1 wild type (WT) and mutant Sp1 site-deleted promoter luciferase reporters. The red arrows are primers that specifically detect these reporters. *Panel ii*, FoxG1 represses the activity of the AIB1 promoter reporter. MCF-7 cells were transfected with WT AIB1(-250/+350) reporter alone, or together with E2F1 in the presence or absence of FoxG1. A representative graph is shown from two independent experiments and data were analyzed by one-way ANOVA with Bonferroni post test. **, $P < .01$ when E2F1 is compared to promoter alone; or FoxG1 and E2F1 together relative to E2F1. *Panel iii*, Protein association to the Sp1 binding site in the transfected AIB1 gene promoter. HEK293T cells were transfected with either the WT AIB1 reporter or the mutant reporter where the Sp1 binding sequence is deleted. Cells were processed for ChIP 6 h post transfection. Recruitment of FoxG1, AIB1, E2F1 and Sp1 to both the WT- and mutant reporters was assessed with a pair of primers that specifically detect the transfected reporter DNA (*panel i*, red arrows). The IgG-ChIP was arbitrarily set as 1 and all the samples were analyzed and plotted in reference to IgG. Data represent two independent experiments and were analyzed by Student's *t* test. ***, $P < .001$; **, $P < .01$; *, $P < .05$ compared with IgG control.

E2F1 expression vector in the presence or absence of FoxG1 and a wild-type (WT) AIB1 promoter-luciferase reporter containing the intact positive regulatory sequence of the AIB1 gene promoter (Figure 3B i). E2F1 significantly enhanced the AIB1 promoter reporter activity, as shown previously (Figure 3B ii) (17), whereas the addition of FoxG1 suppressed E2F1-induced AIB1 promoter activation back to the basal level, indicating an inhibitory role for FoxG1 in AIB1 gene expression (Figure 3B ii).

Because the intronic GC-rich, Sp1 binding sequence is essential for the recruitment of Sp1, AIB1 and E2F1 (17), we next tested whether FoxG1 is also recruited to the AIB1 gene promoter through this element. We performed chromatin immunoprecipitation (ChIP) assays using HEK293T cells transfected with either the WT luciferase reporter AIB1(-250/+350) or the same reporter with a deleted Sp1 site - AIB1(-250/+350)-Sp1-del (Figure 3B i). PCR primers with a forward primer positioned on exon 1 of the AIB1 gene and a reverse primer in the luciferase sequence were used to specifically detect and distinguish the transfected luciferase vectors from the endogenous AIB1 promoter (Figure 3B i, red arrows). Our ChIP analysis showed that FoxG1 is associated with the WT AIB1(-250/+350) reporter but not the mutant reporter AIB1(-250/+350)-Sp1-del (Figure 3B iii). Consistent with published literature (17), our data also demonstrated that AIB1, E2F1 and Sp1 bind to the WT AIB1(-250/+350) reporter, whereas the Sp1 site deletion abolished recruitment of these proteins (Figure 3B iii). These results indicate that the Sp1 binding site is not only required for Sp1,

E2F1 and AIB1 binding to the AIB1 promoter, but it is also critical for the recruitment of FoxG1.

FoxG1 forms a complex with AIB1 and E2F1 on the endogenous AIB1 gene promoter

We next performed ChIP assays to investigate whether FoxG1 is directly recruited to the –250/+350 region of the endogenous AIB1 gene promoter in MCF-7 cells. Using a pair of AIB1 promoter-specific primers where the forward primer is positioned on exon 1 of the AIB1 gene and the reverse primer is situated down-stream of the Sp1 binding sequence (Figure 3A, red arrows), we found that an antibody specific to FoxG1, but not the IgG control, successfully immunoprecipitated endogenous FoxG1 on the AIB1 promoter (Figure 4A). As a negative control we demonstrated no specific FoxG1 binding relative to the IgG control to a region in the coding sequence of exon 4 of the AIB1 gene (Figure 4A). We also show that FoxG1 is not recruited specifically to the nontarget albumin promoter (Figure 4A). Together, these ChIP data support FoxG1 specific recruitment to the AIB1 promoter.

AIB1 has been shown to directly interact with E2F1 and the AIB1-E2F1 complex is essential for E2F1-regulated gene transcription (7). Individual binding of AIB1 and E2F1 to the –250/+350 region of the endogenous AIB1 gene promoter is well established (17), and our goal was to determine whether AIB1 and E2F1 are recruited to this region as a complex. We performed reciprocal ChIP-reChIP (reChIP) assays to address this question. Cross-linked and sonicated chromatin was prepared from MCF-7 cells, incubated and immunoprecipitated first with either AIB1 or E2F1 antibodies. The AIB1- or E2F1 ChIP, followed by "release" of the protein-enriched chromatin, was subjected to a subsequent ChIP with antibodies specific to either E2F1 or AIB1, respectively. As a control, isotype IgG was used for the first round of ChIP followed by E2F1- or AIB1 reChIP. The two-step reciprocal reChIP successfully precipitated the endogenous AIB1 promoter, indicating that AIB1 and E2F1 form a protein complex at the –250/+350 region of the AIB1 promoter (Figure 4B).

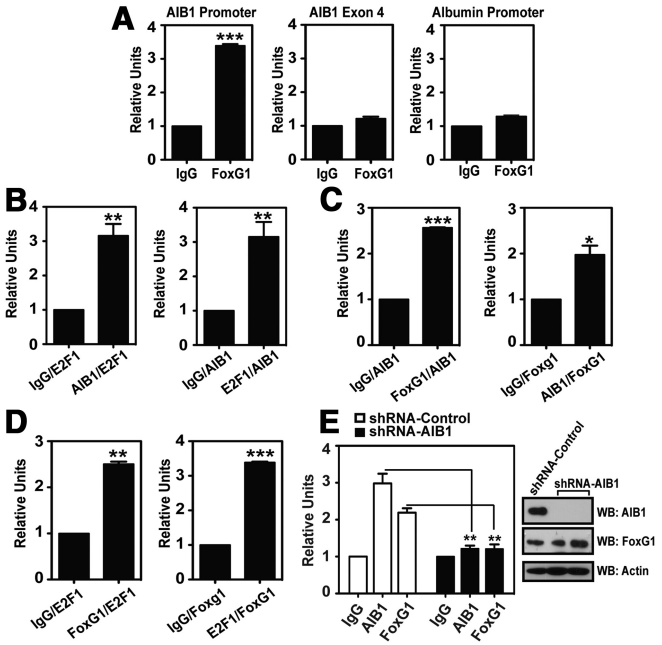


FIGURE 4. FoxG1 forms a complex with AIB1 and E2F1 on the AIB1 gene promoter. A, FoxG1 is recruited to the endogenous AIB1 promoter. ChIP assays were performed in MCF-7 cells, where endogenous FoxG1-DNA complex was immunoprecipitated with anti-FoxG1 antibody or isotype IgG. Protein-enriched DNA was analyzed by RT-PCR using AIB1 promoter-specific primers (Figure 3A, red arrows) or primers that will either amplify a region in exon 4 of the AIB1 gene or the albumin promoter. ChIP results were analyzed by Student's *t* test, where ***, $P < .001$ relative to IgG. B to D Two-step ChIP-reChIP assays were performed in MCF-7 cells, and all DNA samples were subjected to two rounds of ChIPs. Sonicated chromatin was immunoprecipitated first with (B) AIB1 or E2F1-, (C) FoxG1 or AIB1-, or (D) FoxG1 or E2F1 antibodies, followed by reChIP with antibodies specific to (B) E2F1 or AIB1, (C) AIB1 or FoxG1, or (D) E2F1 or FoxG1. As a negative control, isotype IgG was used for the first-round ChIPs followed by reChIP of the respective second-round antibodies. The endogenous AIB1 promoter bound to each immunocomplex as indicated in the figure was analyzed by RT-PCR using the AIB1 promoter-specific primers. Student's *t* test. ***, $P < .001$; **, $P < .01$; *, $P < .05$ when compared with each respective IgG-reChIP control. E, FoxG1 recruitment to the endogenous AIB1 promoter is dependent on AIB1. Endogenous AIB1 was depleted by infection of MCF-7 cells with lentiviral vectors expressing shRNAs targeting a distinct sequence in AIB1 (shRNA-AIB1) or control scrambled shRNA (shRNA-Control). 96 h post lentiviral infection, cells were subjected to ChIP analyses where protein-DNA complexes were immunoprecipitated with antibodies against either AIB1 or FoxG1, or an isotype IgG. Student's *t* test. **, $P < .01$; shRNA-Control vs. shRNA-AIB1. The amount of AIB1 protein knocked down 96 h post infection was assessed by Western blot with antibodies as indicated.

We next assayed for endogenous FoxG1 participation in the AIB1-E2F1 complex by reciprocal reChIP assays. We found that the endogenous AIB1 promoter was precipitated from FoxG1/AIB1 and FoxG1/E2F1 reChIP immunoprecipitates, as well as AIB1/FoxG1 and E2F1/FoxG1 reChIP immunoprecipitates (Figure 4C and D). These data suggest simultaneous chromatin co-occupancy of the three proteins, and indicate that there is basal level recruitment of endogenous AIB1, E2F1 and FoxG1 to the –250/+350 regulatory sequence of the AIB1 promoter.

We have confirmed an interaction between AIB1 and FoxG1 (Figure 1A and B), and shown that the two proteins are recruited to the endogenous AIB1 gene promoter as a complex (Figure 4C). To examine whether FoxG1 occupancy at the endogenous AIB1 promoter is dependent on the corecruitment of AIB1, we infected MCF-7 cells with lentiviral vectors expressing shRNAs targeting AIB1 (shRNA-AIB1) or control scrambled shRNAs (shRNA-Control). Depletion of AIB1 protein by shRNA silencing led to a threefold decrease in AIB1 occupancy at the endogenous AIB1 gene promoter as well as a twofold reduction in the recruitment of FoxG1 to the promoter (Figure 4E). This decrease in FoxG1 occupancy at the AIB1 promoter was not due to a reduction in FoxG1 expression since its protein levels remained unchanged while the levels of AIB1 protein were significantly reduced (Figure 4E, Western Blot). Together our data indicate that the presence of AIB1 is required for the corecruitment of FoxG1 to the AIB1 gene promoter.

FoxG1 compromises the integrity of the activating complex on the AIB1 gene promoter

We next performed ChIP assays to investigate the effect of overexpressing FoxG1 (at levels that suppress AIB1 mRNA expression and cause apoptosis as shown in Figure 2A and B) on the transcription complex present on the endogenous AIB1 promoter at bp +150/+160 (Figure 3A). ChIP assays were performed from MCF-7 cells that had been transfected with either an EV control or a FoxG1-expression vector. We demonstrate that increased expression of FoxG1 resulted in $> 50\%$ decline in the promoter-binding activities of AIB1, E2F1, p300 and RNA polymerase II (Pol II), although there was no significant change in Sp1 recruitment at the promoter level (Figure 5A). The loss of promoter binding was not due to FoxG1-induced changes in gene expression since protein levels of these factors were either unchanged or increased (e.g., E2F1) when FoxG1 was overexpressed (Figure 5B). Interestingly, we did not observe increased chromatin occupancy of FoxG1 when it was overexpressed (Figure 5A) suggesting that once cellular expression of FoxG1 is above a threshold level, it can promote rapid disassembly of the Sp1-associated complex without affecting the direct binding of Sp1 to the +150/+160 AIB1 promoter binding element. To test this, we performed reciprocal reChIP experiments to assess the integrity of the transcriptional protein complex with increased expression of FoxG1. We show that FoxG1 overexpression in MCF-7 cells caused significant reduction in the recruitment of protein complexes comprising Sp1 and E2F1, AIB1 and FoxG1 respectively, or E2F1 and Sp1, AIB1 and FoxG1 respectively (Figure 5C and D). Most interestingly, we observed that overexpressing FoxG1 led to a near three- to twelvefold decrease (depending on the orientation of the reChIP) in the corecruitment of E2F1 complexed with Sp1 to the AIB1 promoter (Figure 5C and D; compare "Sp1/E2F1" and "E2F1/Sp1" between EV, white bar and FoxG1, black bar).

The recruitment of p300 to the +150/+160 Sp1-associated complex has not been described previously and overexpression of FoxG1 also reduces its association with the complex at the AIB1 promoter (Figure 5A), without a reduction in p300 protein expression (Figure 5B). The binding of p300 to AIB1 is known to promote and stabilize transcriptional complex formation, and exert a positive effect on gene transcription (3, 33, 34). Therefore, we wanted to determine whether overexpressing FoxG1 had any effect on the recruitment of the AIB1-p300 complex. We assessed co-occupancy of AIB1 and p300 at the AIB1 promoter by reciprocal reChIP, and discovered a five- to eightfold reduction in the recruitment of AIB1-p300 complex to the AIB1 promoter in MCF-7 cells transfected with FoxG1 as compared to control (Figure 5E).

FoxG1 disrupts AIB1's coactivator function

We next tested if the effect of FoxG1 on AIB1-containing transcription complexes was limited to the Sp1 binding site in the AIB1 gene promoter or if other promoter elements known to involve AIB1 were also affected. AIB1 has previously been shown to coactivate NF-κB and AP-1 (8, 10). Therefore we examined the impact of FoxG1 on promoters containing these transcription factor binding sites. As reported previously (35), AIB1 overexpression induced transcription from all these reporters (Figure 6A to C) but concomitant FoxG1 overexpression caused a significant reduction in the AIB1-induced transcription of the AP-1 and NF-κB promoters (Figure 6A and B). We also observed a near-complete reversal of AIB1 coactivation on the estrogen-responsive promoter (ERE) reporter in the presence of FoxG1 and estrogen (Figure 6C). Interestingly, FoxG1 expression caused a slight decrease in AIB1 coactivation on the promoter-reporters in the empty vector transfected cells (Figure 6A to C). HEK293T cells

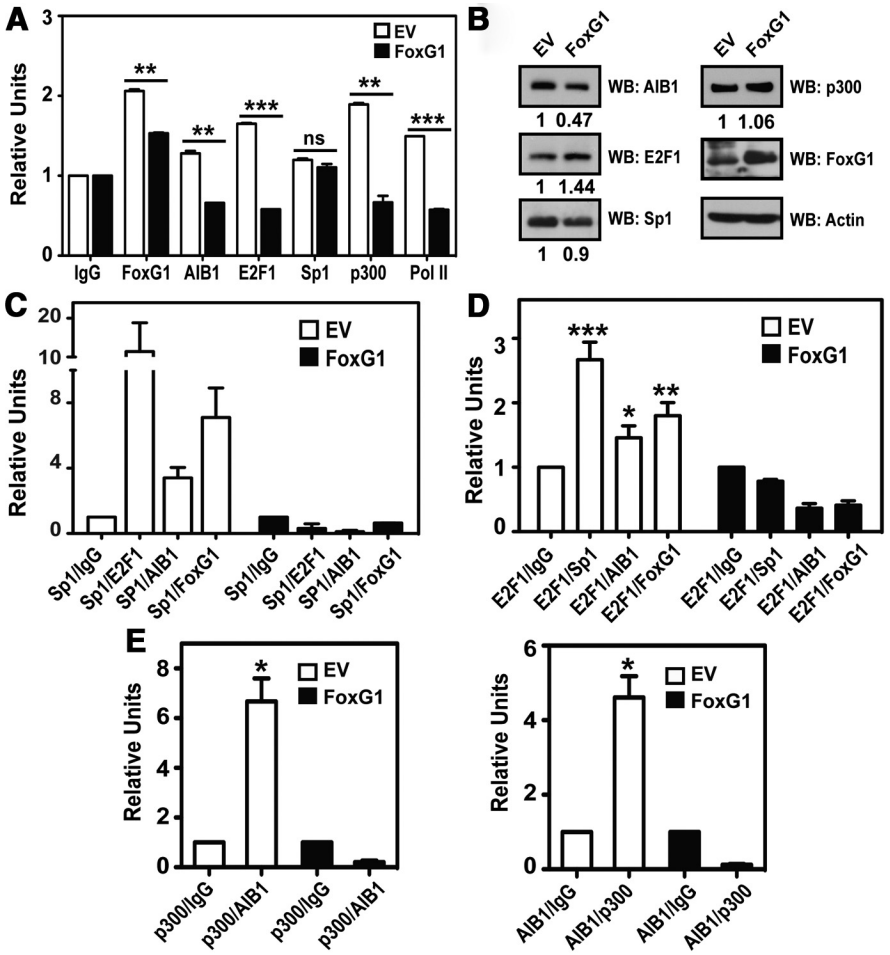


FIGURE 5. FoxG1 destabilizes the Sp1-assoicated transcription complex on the AIB1 gene promoter. A, FoxG1 overexpression leads to decreased recruitment of the members of the transcriptional complex to the endogenous AIB1 promoter. ChIP assays were performed in MCF-7 cells transfected with EV or FoxG1 vectors, by enriching protein-bound endogenous AIB1 promoter with antibodies as indicated. Student's *t* test, where ***, $P < .001$; **, $P < .01$ were FoxG1-expressing cells (black bars) relative to EV (white bars). B, Relative protein levels after FoxG1 transfection in MCF-7 cells are shown by WB and probed with antibodies as indicated. C and D, Overexpressing FoxG1 compromises the integrity of the transcription complex. The immunocomplexes associated with the AIB1 promoter were assessed by ChIP-reChIP experiments, where chromatin was immunoprecipitated sequentially first with an anti-Sp1 antibody, followed by reChIP with antibodies specific to either E2F1, AIB1, or FoxG1; or first with an anti-E2F1 antibody, followed by reChIP with antibodies specific to either Sp1, AIB1, or FoxG1. The Sp1-ChIP and E2F1-ChIP were also followed by a reChIP of IgG as a negative control. ***, $P < .001$; **, $P < .01$; *, $P < .05$ relative to E2F1/IgG. Student's *t* test. E, Overexpressing FoxG1 causes reduction in p300-AIB1 co-occupancy at the AIB1 promoter. MCF-7 cells were transfected with EV or FoxG1 as in (A), and harvested for reChIP experiments by performing reciprocal and sequential ChIPs using antibodies specific to p300, followed by AIB1, or to AIB1, followed by p300. The AIB1 promoter-specific primers were used to assess the relative occupancy of the AIB1–p300 complex at the endogenous AIB1 promoter. *, $P < .05$ relative to p300/IgG or AIB1/IgG. Student's *t* test.

express endogenous AIB1 protein, which may coactivate the reporters and affect transcription from the empty vector control. Thus, FoxG1 overexpression should dampen basal AIB1 coactivating activity. To assess the impact of FoxG1 expression on endogenous genes in MCF-7 cells we used quantitative RT-PCR to generate a mRNA expression profile of 168 genes that are known to participate in or respond to ER or NF-κB signaling. In addition, five housekeeping genes were included as a loading control (ACTB, B2M, GAPDH, HPRT1, RPLP0). Using a cutoff of gene expression changes of > 1.5 fold and $P < .01$, we found that in the NF-κB gene set, 30 genes were upregulated and 14 were downregulated (40 were unchanged); in the ER responsive gene set, 8 genes were upregulated and 42 genes were downregulated (34 were unchanged) after overexpression of FoxG1. In supplemental Table 1 we detailed some of the notable genes involved in breast cancer pathogenesis that were downregulated by FoxG1 from the NF-κB and ER gene array sets. However, the repressive effect of FoxG1 on AIB coactivated gene promoters was not universal since we found in parallel experiments that FoxG1 overexpression had no impact on endogenous E2F1-regulated genes such as CDK2, CDC25a, MCM7, E2F1 and CDC6 in MCF-7 cells (Figure 6D). These data indicate that FoxG1 is important not only for the control of AIB1 promoter, but

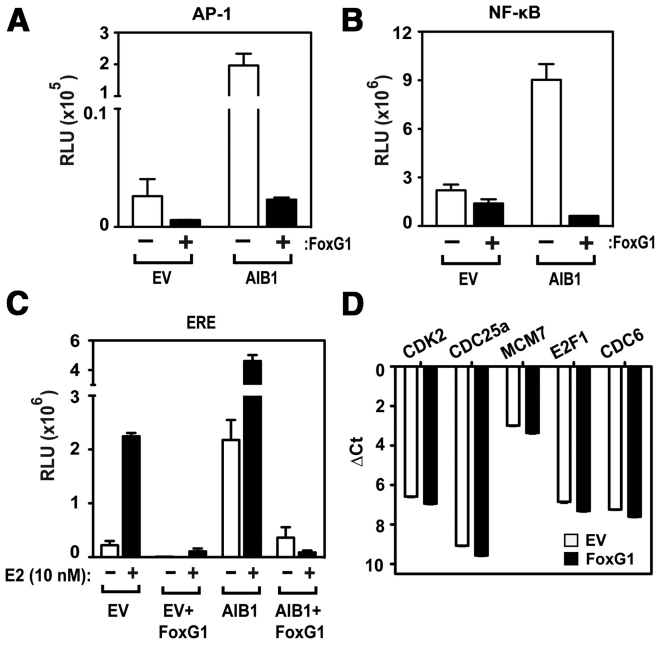


FIGURE 6. FoxG1 disrupts AIB1's coactivator function. A and B, FoxG1's effect on steroid-independent promoters. HEK293T cells were transfected with AIB1 expression constructs as indicated with either (A) a multimerized AP-1 reporter or (B) a multimerized NF-κB reporter, in the presence or absence of FoxG1. c-fos and c-jun expression vectors were also cotransfected with the AP-1 reporter. 24 h after transfection, cells were lysed to measure luciferase activity. C, FoxG1's effect on estrogen-stimulated transcription. AIB1 was cotransfected with ERα and estrogen-responsive promoter reporter (ERE) constructs into hormone-stripped HEK293T cells, with or without cotransfection of FoxG1. Cells were treated with ethanol (-) or 10 nM estradiol (E2) (+) for 24 h and analyzed for reporter activity. Results are expressed as changes in the level of activation compared with EV-transfected cells. D, FoxG1 has no effect on E2F1-regulated gene expression. MCF-7 cells were transfected with EV or FoxG1 and total RNA was harvested from cells to determine the relative gene expression for CDK2, CDC25A, MCM7, E2F1, and CDC6. The Ct values were normalized to actin expression as control.

also for some AIB1 regulated steroid-dependent and -independent transcription, although the impact of FoxG1 is dependent on the promoter context.

Discussion

The control of the overall levels and activity of the nuclear receptor coactivator AIB1 in a cell occurs at multiple levels including control of AIB1 levels of gene transcription (17, 36), control of AIB1 mRNA stability and degradation (with e.g., *miRNA-17–5p* (37)), control of protein modification including phosphorylation (35, 38–40), acetylation (3) and sumoylation (41) and control of proteasomal degradation of AIB1 protein (13, 14, 42). In the current study we have determined that FoxG1 can control levels of AIB1 mRNA by directly influencing the transcription of the AIB1 gene. Based on our data we propose a model (Figure 7) whereby the Sp1 site at bp +150/+160 of the AIB1 gene promoter is directly repressed by increasing levels of FoxG1. AIB1 can complex with E2F1 and together regulate the activity of its own promoter (17, 36). E2F1 can regulate AIB1 promoter activity by interacting with Sp1 bound at bp +150/+160 which, via direct binding to DNA, appears to anchor the E2F1-AIB1 coactivating complex to the AIB1 gene promoter (Figure 7) (17). This allows AIB1 to coactivate and enhance the transcriptional activity of its own promoter. Our data show a reduction in the recruitment of the "anchorage complex", E2F1-Sp1, as well as the essential "coactivating complex", E2F1-AIB1 to the AIB1 promoter when FoxG1 is overexpressed (Figure 5C and D). Our data also indicate that p300 is recruited as part of the activating complex necessary for high levels of AIB1 gene transcription (Figure 5A and 7). CBP/p300 can bind AIB1 directly, promote stable formation of the transcription complex and has strong histone acetylase activity necessary for altering local chromatin structure and activating transcription (3, 34). We show that overexpressing FoxG1 led to a dramatic reduction in the recruitment of p300-AIB1 complex to the AIB1 promoter (Figure 5E). Our data indicate that as FoxG1 levels rise in the cell, the Sp1-associated transcription complex is disrupted, causing E2F1, AIB1 and p300 to dissociate from Sp1, thus reducing AIB1 gene transcription. Interestingly, Hsia et al. have shown that E2F family proteins together with AAA+ nuclear coregulator cancer associated (ANCCAs) proteins are recruited to the AIB1 gene promoter by binding to multiple noncanonical E2F binding sequences within the

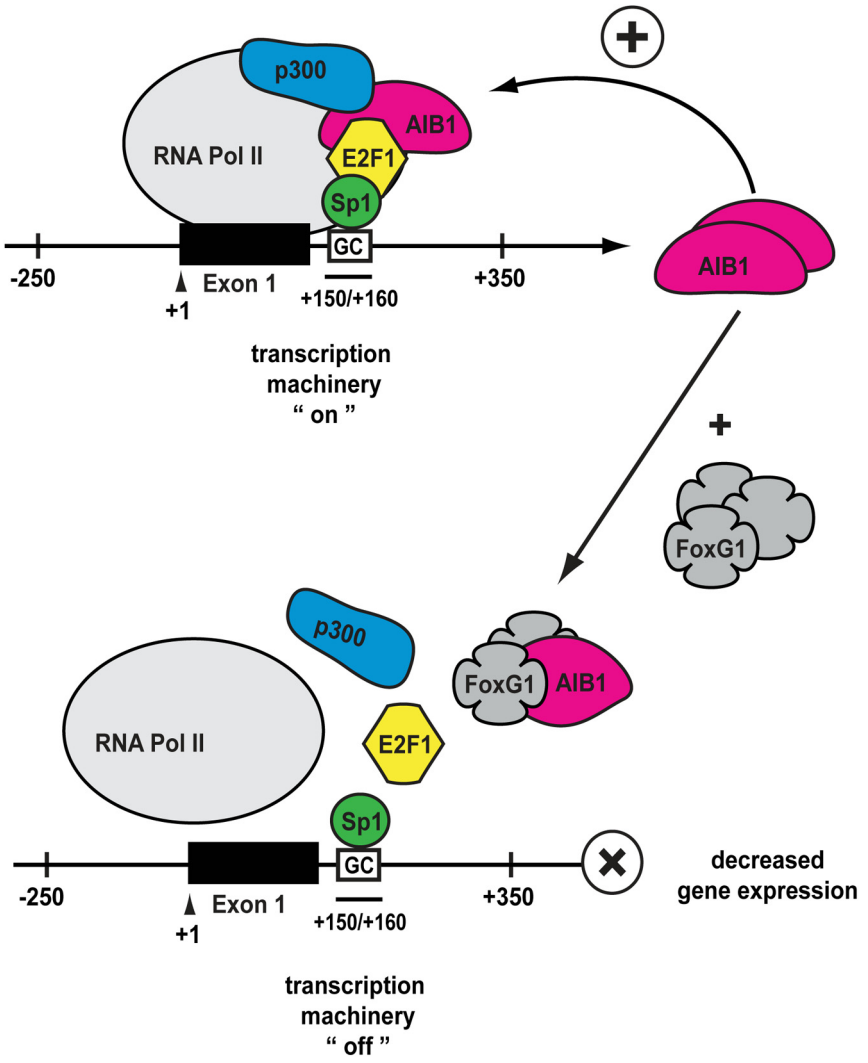


FIGURE 7. A proposed model for the role of FoxG1 in regulating AIB1 gene expression. FoxG1 binds to, and reduces AIB1 binding to the components of the activating transcription complex that is required for the upregulation of AIB1 gene expression. In the presence of increased FoxG1 levels, the activating complex disassembles and disassociates from the AIB1 promoter, leading to reduced AIB1 gene transcription.

AIB1 first exon and intron regions, and are able to directly control AIB1 expression in breast cancer cells (43). However, we believe that the inhibition of AIB1 gene transcription by FoxG1 requires no other elements of the AIB1 gene promoter since deletion of the Sp1 binding sequence alone effectively prevents recruitment of FoxG1, AIB1, E2F1, and Sp1 to the AIB1 promoter reporter (Figure 3B iii).

Previous studies have shown that FoxG1 can cause transcriptional repression by binding DNA directly (44) and nucleating a repressosome by recruiting histone deacetylase 1 (HDAC1) and TLE family proteins (25). However, a search (http://www.ncbi.nlm.nih.gov/nuccore) of the region flanking the Sp1 site in the AIB1 gene promoter using AIB1 genomic DNA (GenBank accession no. AL353777) shows no match for the consensus FoxG1 binding sequence, AATGTAAACA, which is evolutionarily conserved and commonly shared by avian, rat and human FoxG1 gene (45). Furthermore, the interaction of AIB1 with FoxG1 in cell lysates occurs in the absence of tethering DNA. This suggests that in the context of the Sp1 binding site in the AIB1 promoter, FoxG1 inhibits transcription by disrupting an activating transcription complex bearing *histone acetylase* activity, rather than by forming a de novo repressosome after direct DNA-binding to the AIB1 gene promoter. Also consistent with this paradigm of FoxG1-induced repression mechanism is that, in our ChIP assays in cells overexpressing FoxG1, we did not observe increased FoxG1 binding to the AIB1 promoter near the Sp1 binding site. In fact, as exogenous FoxG1 levels increase in the cell, the amount of FoxG1 present in the Sp1-associated transcription complex decreases along with the loss of AIB1 and E2F1. This suggests that at higher concentrations of FoxG1, there is an increase in its access or affinity for AIB1 binding, possibly though dimerization, and this in turn would accelerate degeneration and disassembly of the activating transcription complex. Similar models of repression have been seen with Foxp1, a transcriptional repressor of the forkhead protein family which has been shown to be tumor suppressive in several types of cancers (46). Foxp1 can homo- and heterodimerize with Foxp2 and Foxp4, and dimerization is required for interacting with other transcription cofactors and for executing transcriptional repression (47).

Our data also suggest that FoxG1 may fall under the category of "short-range repressors," which generally act within 100 bp of, or bind adjacently to a transcriptional activator, causing inhibition through "quenching" (48–50). Short-range repressors may also directly interact with an activating cofactor and interfere with its activity or block its access to the basal transcriptional machinery (51, 52). It is been demonstrated that several members of the short-range repressors mediate transcriptional repression in a repressor concentration-dependent manner, in

which higher repressor protein levels (as compared to low levels) are sufficient to switch a gene from an active to an inactive state (52–54). Therefore, it is possible that activation of the AIB1 gene promoter occurs when FoxG1 protein levels are lowered or lost in a cell. Since high AIB1 expression can lead to uncontrolled cell proliferation and tumorigenesis (11), it is possible that cells employ FoxG1 to control and dampen AIB1 transcription. FoxG1 thus may serve as a short-range repressor for the AIB1 promoter, as we have shown that FoxG1 binding to AIB1 leads to the detachment of the critical activating protein complexes from the AIB1 gene promoter which subsequently causes the disintegration of the Sp1-associated positive regulatory transcription complex (Figure 5C and D). In this sense, FoxG1 acts like a tumor suppresser and this would be consistent with the loss of FoxG1 expression as cells evolve from normal to cancerous (Figure 1C).

A question that arises from the proposed model in Figure 7 is: How does FoxG1 cause destabilization of the Sp1-associated transcription complex? One possibility is that through binding, FoxG1 induces a conformational change in AIB1 which leads to reduced affinity between AIB1 and the other members of the Sp1 transcription complex. Future studies will be directed to examine the domains of AIB1 and FoxG1 responsible for complex formation and repression of gene transcription. Another intriguing observation from our study is that, despite the disruptive effect FoxG1 exerts on AIB1-mediated coactivation, we found that this is not limited to, or exclusive to the Sp1 regulatory sequence. In fact, we observed dramatic reductions in AIB1 coactivation at NF-κB and AP-1 regulatory elements (Figure 6A and B). Although not all AIB1-associated promoter elements are influenced by FoxG1, since the transcription of a number of E2F1-driven genes was unaffected by increasing amounts of FoxG1 in the cell. This implies that FoxG1 repression of a gene promoter activity is context-specific for AIB1 in a transcription complex. A recent genome-wide location analysis of AIB1 chromatin affinity sites in 17β-estradiol (E2) -treated MCF-7 cells demonstrated a significant overlap of AIB1 with FoxA1 binding sites in the breast cancer cell DNA (55). FoxA1 is another member of the forkhead family and a determining factor for estrogen receptor function and endocrine response (56). It would be interesting to investigate the portion of AIB1 genomic binding sites that are also engaged by FoxG1, and to determine whether such a population represent a subset of FoxG1- regulated genes.

Reintroducing AIB1 into FoxG1-induced apoptotic MCF-7 cells was only able to partially restore viability in these cells (Figure 2C). This could indicate that FoxG1 induction of apoptosis also involves genes that are not directly regulated by AIB1. However, complete replenishment of endogenous AIB1 levels is difficult to achieve after knockdown and also the temporal response of different AIB1 regulated genes can be variable. Global ChIP assays have revealed that AIB1 is widely distributed in the genome (55) and our data have shown that FoxG1 downregulates AIB1 coactivation of AP-1 and NF-κB transcription (Figure 6A and B). AP-1 is known to promote the expression of genes involved in cell cycle progression (57), and NF-κB-dependent gene transcription is crucial for proproliferation and antiapoptosis signals (58). Thus reduction in AIB1 levels by FoxG1 repression likely has effects on multiple AIB1-regulated pathways which result in apoptosis. Of note is that FoxG1 is also known to antagonize TGF-β signaling by binding to, and blocking, the action of SMAD-3/4 proteins, both of which are major signal-transducers of TGF-β (59). Interestingly, AIB1 is one of a number of TGF-β responsive genes in A549 human lung carcinoma cells (60) and TGF-β can significantly upregulate AIB1 gene transcription in MCF-7 cells (16). Thus FoxG1 inhibition of TGF-β signaling might also be involved in the FoxG1-mediated apoptosis in MCF-7 cells.

The overexpression of the oncogene AIB1 is associated with worse disease outcome in multiple types of tumors (61). However, loss of AIB1 can also be pro-oncogenic in certain contexts, such as in B cell lymphoma (62). Similarly, although FoxG1 can interact with AR directly in vitro and acts as a corepressor to both AR- and PR-mediated transactivation (24), FoxG1 is also shown to be upregulated in ovarian cancer (63), and its gene amplification is associated with the development of bladder cancer and medulloblastoma (64, 65). This suggests that FoxG1 can be both pro- or antioncogenic, depending on the cellular environment. Our analysis of the microarray data generated from breast cancer populations indicates that lower levels of FoxG1 segregate with worse clinical outcome (Figure 1E). Since estrogen is reported to suppress cellular AIB1 expression in MCF-7 cells (16), it is possible that ER status may contribute to the better prognosis associated with higher FoxG1 levels in patients. However, the risk of relapse with low FoxG1 was not significantly different in the ER-positive and -negative patient populations analyzed in Figure 1E (data not shown). Overall our observations in the present study suggest that FoxG1 can act like a tumor suppressor in breast cancer, and downregulation of FoxG1 function could represent an important mechanism to drive AIB1-dependent survival and growth. Mimicking FoxG1 binding to AIB1 with small molecule inhibitors is therefore a possible therapeutic approach in AIB1 overexpressing cancers.

Materials and Methods

Plasmids

HA-p300, E2F1 and IRES-FoxG1 constructs were kindly provided by Dr. Maria L. Avantaggiati (Georgetown University), Dr. Hongwu Chen (University of California, Davis) and from Dr. Joan Massague (Sloan-Kettering Institute), respectively. FLAG-AIB1 plasmid was previously described (35, 66). HA-FoxG1 was generated first by restriction enzyme digestion of the IRES-FoxG1 construct with *Eco*RI and *Bam*HI, followed by insertion of the excised FoxG1 coding region into phCMV2.

Cell Lines and Transient Transfection

HEK293T, MDA-MB-231 and MCF-7 were obtained from the Tissue Culture Shared Resource at Georgetown University. HEK293T and MDA-MB-231 were grown in Dulbecco modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) and MCF-7 was cultured in phenol red-free Iscove's modified Eagle's medium (IMEM, Invitrogen). All the mediums were supplemented with 10% FBS. The human mammary epithelial cells (HMEC) were purchased and cultured in commercially supplied medium (BulletKit, Lonza, Walkersville, MD). Transient transfection was performed in HEK293T and MCF-7 with FuGENE 6 (Roche, Indianapolis, IN) and FuGENE HD (Promega, Madison, WI), respectively.

Western Blot, Nuclear Extraction and Immunoprecipitation (IP)

(i) For interaction of AIB1 with FoxG1 in HEK293T, cells were transfected with 8 μg of either FLAG-AIB1, HA-FoxG1, or FLAG-AIB1 and HA-FoxG1 together. 48 h post transfection, cells were washed with cold 1X PBS and lysed in 1% Nonidet P-40 lysis buffer containing 1 mM NaO_3VO_4 and 1X Complete protease inhibitor tablet (Roche). Whole cell lysates were subjected to IP with anti-FLAG M2 affinity gel (Sigma, St Louis, MO) as described previously (67) and samples were subjected to SDS-PAGE. WB was probed with antibodies against FLAG (M2, Sigma) or HA (Y-11, Santa Cruz Biotechnology, Santa Cruz, CA).

(ii) For the endogenous interaction of AIB1 with FoxG1, MCF-7 cells were plated in 15-cm dishes and nuclear lysates were prepared from cells as per the protocol recommended by the CelLytic NuCLEAR Extraction kit (NXTRACT, Sigma). 2 mg of nuclear lysates were used to immunoprecipitate AIB1 with anti-AIB1 antibody (611105, BD Biosciences, San Jose, CA). The amount of FoxG1 associated with AIB1 was detected with a FoxG1 antibody (ab3394, Abcam, Cambridge, MA), and the precipitated AIB1 was probed using FLAG M2 antibody (Sigma).

(iii) For protein expression levels in MCF-7 cells overexpressing FoxG1, cells were transfected with an empty vector (EV) control or FoxG1 constructs for 24 h. Whole cell lysates were prepared as indicated in (i), and relative protein levels were assessed with the following antibodies: AIB1 (5E11, Cell Signaling Technology Inc, Danvers, MA); E2F1 (KH95, Santa Cruz Biotechnology); Sp1 (PEP2, Santa Cruz Biotechnology); p300 (C-20, Santa Cruz Biotechnology); FoxG1 (ab3394, Abcam) and human actin (C4, Millipore, Billerica, MA).

Kaplan-Meier (KM) Analysis

KM survival curves are generated from Kaplan-Meier Plotter (http://kmplot.com) (31). Analysis parameters used to generate FoxG1 and AIB1 mRNA KM plots are the following: Affymetrix IDs of "206018 at" and "209062 x at" were used for FoxG1 and RAC3/AIB1 respectively; data were plotted for relapse free survival (RFS) at 15 y follow-up threshold; patient data were split and analyzed by median using all probe sets per gene; database "n = 2361" was used to generate all the KM plots in this study.

Annexin V Apoptosis Assay and Flow Cytometry

MCF-7 cells were grown in 10-cm dishes with IMEM containing 10% FBS and transfected with an EV control or FoxG1-expressing constructs. After 24 h, cells were trypsinized and stained with FITC-conjugated Annexin V and propidium iodide (PI) as per the protocol recommended by the TACS Annexin V kit (Trevigen Inc, Gaithersburg, MD). The percentage of cells in early and late apoptosis was determined using fluorescence-activated cell sorting (FACS) on a *Facstar-Plus Dual Laser* flow cytometer (Becton Dickinson, Franklin Lakes, NJ), provided by the Flow Cytometry Shared Resource at Georgetown University. Duplicate samples from each transfection condition were subjected to FACS analysis for apoptotic index.

ChIP and ChIP-reChIP

For ChIP assays using transfected HEK293T, cells in 15-cm dishes were transfected with 12 μg of either wild-type AIB1(-250/+350) or mutant AIB1(-250/+350)-Sp1-del promoter reporter constructs. 6 h after transfection, cells were fixed with 1% formaldehyde (3.7% formaldehyde, 100 mM NaCl, 50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA) for 10 min at 37°C and the reaction was stopped by 0.125 M of glycine solution for 5 min at room temperature. ChIP procedures were carried out essentially as described previously (68). 1 mg of total protein was immunoprecipitated overnight with 5 μg of antibodies against either FoxG1 (ab18259, Abcam), AIB1/NCoA3 (C-20, Santa Cruz Biotechnology), E2F1 (1:1 mixture of C-20 & KH95, Santa Cruz Biotechnology), Sp1 (PEP2x, Santa Cruz Biotechnology) or IgG (as negative control). After reversal of cross-links and protein digestion, DNA was purified using GENECLEAN Turbo kit (Obiogene Inc, Carlsbad, CA). Real time PCR (RT-PCR) (iCycler, Bio-Rad, Hercules, CA) was performed in triplicates using IQ SYBR Green Supermix (Bio-Rad) and 2μl purified ChIP DNA to examine protein recruitment to the WT and mutant reporter constructs with the following primers: 5′-GCGAGTTTCCGATTTAAAGC (complementary to the 5′AIB1 promoter sequence) and 5′-CTTTATGTTTTTGGCGTCTTCCA (complimentary to the 5′ reporter sequence) (17).

For the association of FoxG1 with the endogenous AIB1 promoter, MCF-7 cells were plated in 15-cm dishes and grown until 70%–80% confluence. Cells were cross-linked and fragmented for ChIP assays as described above. The Protein-DNA complexes were immunoprecipitated with 5 μg of negative control rabbit IgG (Santa Cruz Biotechnology) or anti-FoxG1 antibody. Purified DNA was analyzed by RT-PCR with a pair of AIB1 promoter-specific primers: 5′- GCGAGTTTCCGATTTAAAGC and 5′-GCCTTGGCAGATCTGAAG (17). To further verify the specificity of FoxG1 binding to the AIB1 pro-

moter, ChIP DNA samples were also analyzed by RT-PCR with primers amplifying an region in exon 4 of the AIB1 gene (5′-AGACGGGAGCAGGAAAGTAA and 5′-CGCACATTTATCTGGTTTGACATTG) or primers that detect the albumin promoter (5′-TGGGGTTGACAGAAGAGAAAAGC and 5′-TACATTGACAAGGTCTTGTGGAG) (17). To investigate protein recruitment to the AIB1 promoter in MCF-7 cells overexpressing FoxG1, cells were transfected with either an EV control or FoxG1 constructs. 24 h after transfection, cells were collected, sonicated and crude chromatin solution was diluted and incubated overnight at 4°C with specific antibodies against FoxG1, AIB1, E2F1, Sp1, p300 (C-20x, Santa Cruz Biotechnology), Pol II (C-21x, Santa Cruz Biotechnology) and IgG as negative control. Purified DNA was analyzed by RT-PCR using the AIB1 promoter-specific primers.

The two-step ChIP-reChIP (reChIP) experiments were performed in MCF-7 cells. Chromatin was precleared with 20 μl Magna ChIP Protein A+G Magnetic Beads (16663, Millipore) and 2 mg chromatin DNA was immunoprecipitated with 40 μl beads and 5 μg of either AIB1, E2F1 or FoxG1 antibodies in the first round of ChIPs. The ChIP precipitates were gently washed as in the usual ChIP assay and the chromatin-protein complexes were eluted from the beads in 75 μl TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) containing 1X Complete protease inhibitor (Roche) and 10 mM DTT for 30 min at 37°C. After centrifugation, the supernatant from each sample was diluted with 1.5 ml ChIP dilution buffer (20 mM Tris, pH 8.0, 2 mM EDTA, pH 8.0, 150 mM NaCl, 1% Triton X-100) containing 1X Complete protease inhibitor and subjected to the second round of ChIP with 40 μl beads and 5 μg antibodies specifically against either E2F1, AIB1, or FoxG1. All first-round ChIPs were also followed by an IgG ChIP as a negative control. Cross-links were reversed in the precipitated complexes with 200 mM NaCl for 16 h at 65°C and proteins digested with 1 μg of proteinase K for 1 h at 45°C. For reChIP assays performed in MCF-7 cells with exogenously expressed FoxG1, sonicated chromatin was prepared from cells transfected with either an EV control or FoxG1 vectors, and subjected to reChIP procedures as described above with the exception of first-round immunoprecipitations using anti-E2F1 antibody, followed by second-round ChIPs with either IgG (negative control), Sp1, AIB1 or FoxG1 antibodies. Recovered ChIP DNA was purified and analyzed by RT-PCR with the AIB1 promoter-specific primers.

Data (Ct values obtained from RT-PCR) collected from all ChIP and reChIP experiments in this study were first calculated as percentage of their respective inputs. The IgG-ChIPs and -reChIPs were then arbitrarily set as 1 and all the samples were analyzed and plotted in reference to IgG.

Short Hairpin RNA Constructs and Lentivirus Infection

shRNA-AIB1 (5-TGGTGAATCGAGACGGAAACA-3) was subcloned into the EcoRI and AgeI restriction sites in PLKO.1 puro (Addgene, Cambridge, MA) (35). Control scrambled short hairpin RNA (shRNA-Control) was purchased from Addgene. Lentivirus production was performed as described previously using the recommended protocols for production of lentiviral particles with packaging plasmid (pCMV-dR8.2 dvpr) and envelope plasmid (pCMV-VSVG) (Addgene) (69).

Luciferase Reporter Assay

Cells were plated in triplicates for all the luciferase reporter assays. 50,000 MCF-7 cells per well in a 24-well dish were transfected in serum-free IMEM with 0.5 μg WT AIB1(-250/+350) promoter reporter construct alone, or together with 0.25 μg E2F1, with or without cotransfection of 0.25 μg FoxG1. 24 h post transfection, cells were lysed in 100 μl 1X passive lysis buffer (Promega) and incubated at room temperature for 30 min on a rocker. Luciferase values were measured using the luciferase reporter assay kit (Promega). Protein concentration for each sample was determined using the BCA protein assay (Pierce, Rockford, IL) and luciferase values were normalized with their protein concentrations. Reporter assays using HEK293T cells were performed as descried above. Cells were transfected in DMEM without serum with AIB1 expression plasmids and either 0.2 μg multimerized AP-1 or NF-κB reporter constructs (Stratagene, Santa Clara, CA), in the presence or absence of 0.5 μg FoxG1. 25 ng of c-fos and c-jun expression vectors were also cotransfected with the AP-1 reporter. Hormone-stripped HEK293T cells used for estrogen-responsive promoter (ERE) reporter assays were transfected with AIB1-(0.5 μg), ERα (20 ng) constructs, and ERE luciferase vector (0.2 μg), with or without 0.5 μg FoxG1 for 24 h. Cells were then treated with hormone for 24 h before assessing for luciferase activity.

RNA Extraction and Real Time PCR

Total RNA was harvested using RNeasy mini kit (Qiagen, Valencia, CA) and reverse-transcribed with iScript can synthesis kit (Bio-Rad) using 1 μg of total RNA. cDNA fragments were amplified in triplicates by RT-qPCR (iCycler, Bio-Rad) of 45 cycles with primers listed in Table 1 (7, 36). For AIB1 and FoxG1 gene expression in the 7 tested cell lines, frozen cell pellets of BT-483, T47D, BT-549, HCC-1937 and BT-20 cell lines were obtained from the Tissue Culture Shared Resource at Georgetown University. HMEC and MCF-7 cells were plated in

Table 1. RT-PCR primers used in this study

Primers	Forward sequence	Reverse Sequence
AIB1	AGACGGGAGCAGGAAAGTAA	CGCACATTTATCTGGTTTGACATTG
FoxG1	AGAAGAACGGCAAGTACGAGA	TGTTGAGGGACAGATTGTGGC
CDK2	TTTGCTGAGATGGTGACTCGC	CACTGGAGGAGGGGTGAGATTAG
CDC25a	TGAAGAATGAGGAGGAGACCCC	CTGATGTTTCCCAGCAACTGTATG
MCM7	AAGCCAGGAGTGCCAAACCAAC	GCAGCAGTGCCTTCTTCACATC
E2F1	CGCATCTATGACATCACCAACG	GAAAGTTCTCCGAAGAGTCCACG
CDC6	AAAGAGAATGGTCCCCCTCACTC	AGTTTTTCCAGTTCCAGGAGCAC
Actin	CCTGGCACCCAGCACAAT	GCCGATCCACACGGAGTACT

10-cm dishes and grown in culture until 70%–80% confluence. The fold change in AIB1 and FoxG1 gene expression was normalized first to the human actin gene, then calculated by the comparative Ct method, with relative transcript levels determined as $y = 2^{\wedge -\Delta CT}$. AIB1 and FoxG1 mRNA expression of the 6 cancer cell lines was further normalized to the normal HMEC (set as 1), which expresses the lowest levels of AIB1 in the group. For E2F1-regulated gene expression in MCF-7 cells overexpressing FoxG1, cells were transfected with an EV control or FoxG1 constructs. After 24 h of transfection, the ΔCt values of E2F1-regulated genes were obtained by normalizing to actin. Gene expression data for NF-κB and ER signaling and target genes were collected using NF-κB Signaling Targets RT (2) Profiler PCR Array and ER Signaling Pathway Activity RT (2) Profiler PCR Array (Qiagen). Expression of each gene was first normalized to the housekeeping genes and then to EV.

Statistical Analysis

All experiments were performed independently for at least two times and results are presented as means ± SEM. Data were analyzed by unpaired Student's *t* test for comparison of two groups or one-way ANOVA with Bonferroni post test for comparison of more than two groups. Statistical significance is indicated in each figure by asterisks: ***, $P < .001$; **, $P < .01$; *, $P < .05$.

Acknowledgments

This work was supported, in whole or in part, by National Institutes of Health Grant R01 CA113477 from NCI (to A. T. R.). This work was also supported by Center of Excellence Grant W81XWH-06–10590 (to V. C. Jordan, A. W., and A. T. R.). Flow cytometry was performed by Lombardi Comprehensive Cancer Center core facilities, supported in part by cancer center support grant CA051008.

Received October 30, 2012. Accepted April 29, 2013.

Address all correspondence and requests for reprints to: Mailing address: Department of Oncology, Lombardi Cancer Center, Georgetown University, Research Building E307, 3970 Reservoir Rd. NW, WA, DC 20007-2197. Phone: (202) 687-1479. Fax: (202) 687-4821. E-mail: ariege01@georgetown.edu.

Disclosure Summary: The authors have nothing to disclose.

This work was supported by in whole or in part, by National Institutes of Health Grant R01 CA113477 from NCI (to A. T. R.). This work was also supported by Center of Excellence Grant W81XWH-06–10590 (to V. C. Jordan, A. W., and A. T. R.). Flow cytometry was performed by Lombardi Comprehensive Cancer Center core facilities, supported in part by cancer center support grant CA051008.

References

1. **Xu J, Wu R-C, O'Malley BW.** Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. *Nat Rev Cancer*. 2009;9:615–630.
2. **Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM, Meltzer PS.** AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science*. 1997;277:965–968.
3. **Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, Privalsky ML, Nakatani Y, Evans RM.** Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell*. 1997;90:569–580.
4. **Suen CS, Berrodin TJ, Mastroeni R, Cheskis BJ, Lyttle CR, Frail DE.** A transcriptional coactivator, steroid receptor coactivator-3, selectively augments steroid receptor transcriptional activity. *J. Biol. Chem*. 1998;273:27645–27653.
5. **Xu J, Li Q.** Review of the in vivo functions of the p160 steroid receptor coactivator family. *Molecular Endocrinology*. 2003;17:1681–1692.
6. **Hsia EYC, Zou JX, Chen H-W.** The roles and action mechanisms of p160/SRC coactivators and the ANCCA coregulator in cancer. *Progress in Molecular Biology and Translational Science* 2009;87:261–298.
7. **Louie MC, Zou JX, Rabinovich A, Chen H-W.** ACTR/AIB1 functions as an E2F1 coactivator to promote breast cancer cell proliferation and antiestrogen resistance. *Molecular and Cellular Biology*. 2004;24:5157–5171.
8. **Yan J, Yu CT, Ozen M, Ittmann M, Tsai SY, Tsai MJ.** Steroid receptor coactivator-3 and activator protein-1 coordinately regulate the transcription of components of the insulin-like growth factor/AKT signaling pathway. *Cancer Research*. 2006;66:11039–11046.
9. **Qin L, Liao L, Redmond A, Young L, Yuan Y, Chen H, O'Malley BW, Xu J.** The AIB1 oncogene promotes breast cancer metastasis by activation of PEA3-mediated matrix metalloproteinase 2 (MMP2) and MMP9 expression. *Molecular and Cellular Biology*. 2008;28:5937–5950.
10. **Werbajh S, Nojek I, Lanz R, Costas MA.** RAC-3 is a NF-kappa B coactivator. *FEBS Lett*. 2000;485:195–199.
11. **Torres-Arzayus MI, de Mora JF, Yuan J, Vazquez F, Bronson R, Rue M, Sellers WR, Brown M.** High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. *Cancer Cell*. 2004;6:263–274.
12. **Lahusen T, Henke RT, Kagan BL, Wellstein A, Riegel AT.** The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer. *Breast Cancer Res Treat*. 2009;116:225–237.
13. **Mani A, Oh AS, Bowden ET, Lahusen T, Lorick KL, Weissman AM, Schlegel R, Wellstein A, Riegel AT.** E6AP mediates regulated proteasomal degradation of the nuclear receptor coactivator amplified in breast cancer 1 in immortalized cells. *Cancer Research*. 2006;66:8680–8686.
14. **Wu R-C, Feng Q, Lonard DM, O'Malley BW.** SRC-3 coactivator functional lifetime is regulated by a phospho-dependent ubiquitin time clock. *Cell*. 2007;129:1125–1140.
15. **Li C, Ao J, Fu J, Lee D-F, Xu J, Lonard D, Malley BWOA.** Tumor-suppressor role for the SPOP ubiquitin ligase in signal-dependent proteolysis of the oncogenic co-activator SRC-3/AIB1. *Oncogene*. 2011;30:1–15.
16. **Lauritsen KJ, List H-J, Reiter R, Wellstein A, Riegel AT.** A role for TGF-beta in estrogen and retinoid mediated regulation of the nuclear receptor coactivator AIB1 in MCF-7 breast cancer cells. *Oncogene*. 2002;21:7147–7155.
17. **Mussi P, Yu C, O'Malley BW, Xu J.** Stimulation of steroid receptor coactivator-3 (SRC-3) gene overexpression by a positive regulatory loop of E2F1 and SRC-3. *Molecular Endocrinology*. 2006;20:3105–3119.
18. **Teyssier C, Chen D, Stallcup MR.** Requirement for multiple domains of the protein arginine methyltransferase CARM1 in its transcriptional coactivator function. *J. Biol. Chem*. 2002;277:46066–46072.
19. **Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M.** Cofactor dynam-

ics and sufficiency in estrogen receptor-regulated transcription. *Cell*. 2000;103:843–852.
20. **Li X, Kimbrel EA, Kenan DJ, McDonnell DP.** Direct interactions between corepressors and coactivators permit the integration of nuclear receptor-mediated repression and activation. *Molecular Endocrinology*. 2002;16:1482–1491.
21. **Karmakar S, Gao T, Pace MC, Oesterreich S, Smith CL.** Cooperative activation of cyclin D1 and progesterone receptor gene expression by the SRC-3 coactivator and SMRT corepressor. *Molecular Endocrinology*. 2010;24:1187–1202.
22. **Hu Z-Z, Kagan BL, Ariazi EA, Rosenthal DS, Zhang L, Li JV, Huang H, Wu C, Jordan VC, Riegel AT, Wellstein.** A Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. *PLoS ONE*. 2011;6:e20410.
23. **Xuan S, Baptista CA, Balas G, Tao W, Soares VC, Lai E.** Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. *Neuron*. 1995;14:1141–1152.
24. **Obendorf M, Meyer R, Henning K, Mitev YA, Schröder J, Patchev VK, Wolf SS.** FoxG1, a member of the forkhead family, is a corepressor of the androgen receptor. *The Journal of Steroid Biochemistry and Molecular Biology*. 2007;104:195–207.
25. **Yao J, Lai E, Stifani S.** The winged-helix protein brain factor 1 interacts with groucho and hes proteins to repress transcription. *Molecular and Cellular Biology*. 2001;21:1962–1972.
26. **Hanashima C, Li SC, Shen L, Lai E, Fishell G.** Foxg1 suppresses early cortical cell fate. *Science*. 2004;303:56–59.
27. **Shoichet SA, Kunde S-A, Viertel P, Schell-Apacik C, Voss von H, Tommerup N, Ropers H-H, Kalscheuer VM.** Haploinsufficiency of novel FOXG1B variants in a patient with severe mental retardation, brain malformations and microcephaly. *Hum. Genet*. 2005;117:536–544.
28. **Zhao H, Langerød A, Ji Y, Nowels KW, Nesland JM, Tibshirani R, Bukholm IK, Kåresen R, Botstein D, Børresen-Dale A-L, Jeffrey SS.** Different gene expression patterns in invasive lobular and ductal carcinomas of the breast. *Mol. Biol. Cell*. 2004;15:2523–2536.
29. **Turashvili G, Bouchal J, Baumforth K, Wei W, Dziechciarkova M, Ehrmann J, Klein J, Fridman E, Skarda J, Srovnal J, Hajduch M, Murray P, Kolar Z.** Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis. *BMC Cancer*. 2007;7:55.
30. **Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, Liao X, Iglehart JD, Livingston DM, Ganesan S.** X chromosomal abnormalities in basal-like human breast cancer. *Cancer Cell*. 2006;9:121–132.
31. **Győrffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, Szallasi Z.** An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast Cancer Res Treat*. 2009;123:725–731.
32. **Oh A, List H-J, Reiter R, Mani A, Zhang Y, Gehan E, Wellstein A, Riegel AT.** The nuclear receptor coactivator AIB1 mediates insulin-like growth factor I-induced phenotypic changes in human breast cancer cells. *Cancer Research*. 2004;64:8299–8308.
33. **Korzus E, Torchia J, Rose DW, Xu L, Kurokawa R, McInerney EM, Mullen TM, Glass CK, Rosenfeld MG.** Transcription factor-specific requirements for coactivators and their acetyltransferase functions. *Science*. 1998;279:703–707.
34. **Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, Rosenfeld MG.** The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature*. 1997;387:677–684.
35. **Oh AS, Lahusen JT, Chien CD, Fereshteh MP, Zhang X, Dakshanamurthy S, Xu J, Kagan BL, Wellstein A, Riegel AT.** Tyrosine phosphorylation of the nuclear receptor coactivator AIB1/SRC-3 is enhanced by Abl kinase and is required for its activity in cancer cells. *Molecular and Cellular Biology*. 2008;28:6580–6593.
36. **Louie MC, Revenko AS, Zou JX, Yao J, Chen H-W.** Direct control of cell cycle gene expression by proto-oncogene product ACTR, and its autoregulation underlies its transforming activity. *Molecular and Cellular Biology*. 2006;26:3810–3823.
37. **Hossain A, Kuo MT, Saunders GF.** Mir-17–5p regulates breast cancer cell proliferation by inhibiting translation of AIB1 mRNA. *Molecular and Cellular Biology*. 2006;26:8191–8201.
38. **Wu RC, Qin J, Hashimoto Y, Wong J, Xu J, Tsai SY, Tsai MJ, O'Malley BW.** Regulation of SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) coactivator activity by IkB kinase. *Molecular and Cellular Biology*. 2002;22:3549–3561.
39. **Wu R-C, Qin J, Yi P, Wong J, TSAI SY, TSAI M-J, O'Malley BW.** Selective phosphorylations of the SRC-3/AIB1 coactivator integrate genomic responses to multiple cellular signaling pathways. *Molecular Cell*. 2004;15:937–949.
40. **Font de Mora J, Brown M.** AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Molecular and Cellular Biology*. 2000;20:5041–5047.
41. **Wu H, Sun L, Zhang Y, Chen Y, Shi B, Li R, Wang Y, Liang J, Fan D, Wu G, Wang D, Li S, Shang Y.** Coordinated regulation of AIB1 transcriptional activity by sumoylation and phosphorylation. *J. Biol. Chem*. 2006;281:21848–21856.
42. **Naeem H, Cheng D, Zhao Q, Underhill C, Tini M, Bedford MT, Torchia J.** The activity and stability of the transcriptional coactivator p/CIP/SRC-3 are regulated by CARM1-dependent methylation. *Molecular and Cellular Biology*. 2006;27:120–134.
43. **Hsia EYC, Kalashnikova EV, Revenko AS, Zou JX, Borowsky AD, Chen HW.** Deregulated E2F and the AAA+ coregulator ANCCA drive proto-oncogene ACTR/AIB1 overexpression in breast cancer. *Molecular Cancer Research*. 2010;8:183–193.
44. **Li J, Thurm H, Chang HW, Iacovoni JS, Vogt PK.** Oncogenic transformation induced by the Qin protein is correlated with transcriptional repression. *Proc. Natl. Acad. Sci*. 1997;94:10885–10888.
45. **Li J, Chang HW, Lai E, Parker EJ, Vogt PK.** The oncogene qin codes for a transcriptional repressor. *Cancer Research*. 1995;55:5540–5544.
46. **Banham AH, Beasley N, Campo E, Fernandez PL, Fidler C, Gatter K, Jones M, Mason DY, Prime JE, Trougouboff P, Wood K, Cordell JL.** The FOXP1 winged helix transcription factor is a novel candidate tumor suppressor gene on chromosome 3p. *Cancer Research*. 2001;61:8820–8829.
47. **Li S, Weidenfeld J, Morrisey EE.** Transcriptional and DNA binding activity of the Foxp1/2/4 family is modulated by heterotypic and homotypic protein interactions. *Molecular and Cellular Biology*. 2004;24:809–822.
48. **Gray S, Levine M.** Short-range transcriptional repressors mediate both quenching and direct repression within complex loci in Drosophila. *Genes, Development*. 1996;10:700–710.
49. **Arnosti DN, Gray S, Barolo S, Zhou J, Levine M.** The gap protein knirps mediates both quenching and direct repression in the Drosophila embryo. *EMBO J*. 1996;15:3659–3666.
50. **Barolo S, Levine M.** hairy mediates dominant repression in the Drosophila embryo. *EMBO J*. 1997;16:2883–2891.
51. **Li C, Manley JL.** Even-skipped represses transcription by binding TATA binding protein and blocking the TFIID-TATA box interaction. *Molecular and Cellular Biology*. 1998;18:3771–3781.
52. **Sauer F, Jäckle H.** Concentration-dependent transcriptional activation or repression by Krüppel from a single binding site. *Nature*. 1991;353:563–566.
53. **Kosman D, Small S.** Concentration-dependent patterning by an ectopic expression domain of the Drosophila gap gene knirps. *Development*. 1997;124:1343–1354.
54. **Hewitt GF, Strunk BS, Margulies C, Priputin T, Wang XD, Amey R, Pabst BA, Kosman D, Reinitz J, Arnosti DN.** Transcriptional repression by the Drosophila giant protein: cis element positioning provides an alternative means of interpreting an effector gradient. *Development*. 1999;126:1201–1210.
55. **Lanz RB, Bulynko Y, Malovannaya A, Labhart P, Wang L, Li W,**

Qin J, Harper M, O'Malley BW. Global characterization of transcriptional impact of the SRC-3 coregulator. *Molecular Endocrinology*. 2010;24:859–872.
56. **Hurtado A, Holmes KA, Ross-Innes CS, Schmidt D, Carroll JS.** FOXA1 is a key determinant of estrogen receptor function and endocrine response. *Nature Genetics*. 2010;43:27–33.
57. **Liu Y, Lu C, Shen Q, Munoz-Medellin D, Kim H, Brown PH.** AP-1 blockade in breast cancer cells causes cell cycle arrest by suppressing G1 cyclin expression and reducing cyclin-dependent kinase activity. *Oncogene*. 2004;23:8238–8246.
58. **Biswas DK, Shi Q, Baily S, Strickland I, Ghosh S, Pardee AB, Iglehart JD.** NF-kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. *Proc. Natl. Acad. Sci*. 2004;101:10137–10142.
59. **Seoane J, Le H-V, Shen L, Anderson SA, Massagué J.** Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell*. 2004;117:211–223.
60. **Akiyama N, Matsuo Y, Sai H, Noda M, Kizaka-Kondoh S.** Identification of a Series of Transforming Growth Factor beta -Responsive Genes by Retrovirus-Mediated Gene Trap Screening. *Molecular and Cellular Biology*. 2000;20:3266–3273.
61. **Ma G, Ren Y, Wang K, He J.** SRC-3 has a role in cancer other than as a nuclear receptor coactivator. *Int. J. Biol. Sci*. 2011;7:664–672.
62. **Coste A, Antal MC, Chan S, Kastner P, Mark M, O'Malley BW, Auwerx J.** Absence of the steroid receptor coactivator-3 induces B-cell lymphoma. *EMBO J*. 2006;25:2453–2464.
63. **Chan DW, Liu VWS, To RMY, Chiu PM, Lee WYW, Yao KM, Cheung ANY, Ngan HYS.** Overexpression of FOXG1 contributes to TGF-beta resistance through inhibition of p21WAF1/CIP1 expression in ovarian cancer. *British Journal of Cancer*. 2009;101:1433–1443.
64. **Kim T-H, Jo S-W, Lee YS, Kim Y-J, Lee S-C, Kim W-J, Yun SJ.** Forkhead box O-class 1 and Forkhead box G1 as Prognostic Markers for Bladder Cancer. *J Korean Med Sci*. 2009;24:468.
65. **Adesina AM, Nguyen Y, Mehta V, Takei H, Stangeby P, Crabtree S, Chintagumpala M, Gumerlock MK.** FOXG1 dysregulation is a frequent event in medulloblastoma. *J Neurooncol*. 2007;85:111–122.
66. **Reiter R, Wellstein A, Riegel AT.** An isoform of the coactivator AIB1 that increases hormone and growth factor sensitivity is overexpressed in breast cancer. *J. Biol. Chem*. 2001;276:39736–39741.
67. **Lahusen T, Fereshteh M, Oh A, Wellstein A, Riegel AT.** Epidermal growth factor receptor tyrosine phosphorylation and signaling controlled by a nuclear receptor coactivator, amplified in breast cancer 1. *Cancer Research*. 2007;67:7256–7265.
68. **Chien CD, Kirilyuk A, Li JV, Zhang W, Lahusen T, Schmidt MO, Oh AS, Wellstein A, Riegel AT.** Role of the nuclear receptor coactivator AIB1-Delta 4 splice variant in the control of gene transcription. *J. Biol. Chem*. 2011;286:26813–26827.
69. **Al-Otaiby M, Tassi E, Schmidt MO, Chien CD, Baker T, Salas AG, Xu J, Furlong M, Schlegel R, Riegel AT, Wellstein A.** Role of the nuclear receptor coactivator AIB1/SRC-3 in angiogenesis and wound healing. *AJPA*. 2012;180:1474–1484.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Cancer Research

c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells

Ping Fan, Obi L. Griffith, Fadeke Agboke, et al.

Cancer Res Published OnlineFirst May 23, 2013.

Updated version	Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-4152
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2013/05/23/0008-5472.CAN-12-4152.DC1.html
Author Manuscript	Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells

Ping Fan[1], Obi L Griffith[2,3], Fadeke Agboke[1], Pavana Anur[4], Xiaojun Zou[1], Russell E McDaniel[1], Karen Creswell[1], Sung Hoon Kim[4], John A Katzenellenbogen[4], Joe W Gray[2,5], V Craig Jordan[1*]

*Address correspondence to this author: Scientific Director of Lombardi Comprehensive Cancer Center, Georgetown University, E507A Research Bldg, 3970 Reservoir RD NW, Washington D.C. 20057. Tel:(202) 687 2897; Email:vcj2@georgetown.edu

Authors' Affiliation:

[1] Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington D.C. 20057

[2] Lawrence Berkeley National Laboratory, Life Sciences Division, Cancer & DNA Damage Responses, Berkeley CA 94720

[3] Current address: Department of Medicine, Division of Oncology, The Genome Institute, Washington University, St. Louis MO 63108

[4] Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana IL 61801

[5] Biomedical Engineering Department, Oregon Health and Science University, Portland OR 97239

Running title: c-Src mediates estrogen-induced stress and apoptosis

Key words: c-Src, endoplasmic reticulum stress, oxidative stress, apoptosis, estrogen receptor

There are no conflicts to disclose.

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Abstract The emergence of antiestrogen resistance in breast cancer is an important clinical phenomenon affecting long-term survival in this disease. Identifying factors that convey cell survival in this setting may guide improvements in treatment. Estrogen (E_2) can induce apoptosis in breast cancer cells that have been selected for survival after E_2 deprivation for long periods (MCF-7:5C cells), but the mechanisms underlying E_2-induced stress in this setting have not been elucidated. Here, we report that the c-Src kinase functions as a key adapter protein for the estrogen receptor (ER, ESR1) in its activation of stress responses induced by E_2 in MCF-7:5C cells. E_2 elevated phosphorylation of c-Src which was blocked by 4-hydroxytamoxifen (4-OHT), suggesting that E_2 activated c-Src through the ER. We found that E_2 activated the sensors of the unfolded protein response (UPR), IRE1α (ERN1) and PERK kinase (EIF2AK3), the latter of which phosphorylates eukaryotic translation initiation factor-2α (eIF2α). E_2 also dramatically increased reactive oxygen species (ROS) production and up-regulated expression of heme oxygenase HO-1 (HMOX1), an indicator of oxidative stress, along with the central energy sensor kinase AMPK (PRKAA2). Pharmacological or RNAi-mediated inhibition of c-Src abolished the phosphorylation of eIF2α and AMPK, blocked E_2-induced ROS production, and inhibited E_2-induced apoptosis. Together, our results establish that c-Src kinase mediates stresses generated by E_2 in long-term E_2-deprived cells that trigger apoptosis. This work offers a mechanistic rationale for a new approach in the treatment of endocrine-resistant breast cancer.

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Introduction

Developing drugs that target the estrogen receptor (ER) either directly (tamoxifen) or indirectly (aromatase inhibitors) has improved the prognosis of breast cancer (1,2). Although aromatase inhibitors show considerable advantages over tamoxifen with respect to patient disease free survival and tolerability, acquisition of resistance to all forms of endocrine treatments is inevitable (3,4). Multiple mechanistic changes are involved in antihormone resistance which provides the scientific rationale for the clinical development of additional targeted therapies (5,6). It is well known that the biological actions of E_2 are mediated through the ER, which functions in the nucleus as ligand-dependent transcription factors to promote gene transcription and stimulation of cell growth (7). Paradoxically, laboratory evidence demonstrates that E_2 can induce apoptosis in sensitive antihormone-resistant cells *in vivo* (8-10). This new targeted strategy provides novel therapeutic approaches to endocrine resistant breast cancer. A recent phase II clinical trial reports that E_2 provides a clinical benefit for patients with aromatase inhibitor-resistant advanced breast cancer (11). Additionally, the laboratory results on E_2-induced apoptosis using antihormone treated MCF-7 cells have been used to explain the reduction of breast cancer and the reduction in mortality observed in postmenopausal hysterectomized women in their 60's treated with conjugated equine estrogen (CEE) when compared to a placebo treated control (12). The antitumor action of CEE is observed, not only during CEE treatment but for 6 years after treatment. These data suggest a cidal effect for CEE and has been noted recently (13). These encouraging clinical results prompted us to investigate the mechanisms underlying E_2-induced apoptosis, to increase the therapeutic benefits of E_2 in aromatase inhibitor resistant breast cancer.

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Experimental evidence has established the oncogene, c-Src, as a critical component of multiple signaling pathways that regulate proliferation, survival, angiogenesis and metastasis (14,15). Increased c-Src activity is believed to play an important role in the development and progression of breast cancer (16), and c-Src has been considered as a survival signal for endocrine resistant breast cancer cells (17). Therefore, a c-Src inhibitor administered as a single agent or in combination with other anti-hormone therapy has the potential to enhance the inhibitory effects of antihormones and delay antihormone resistance (18). These observations highlight c-Src as an important therapeutic target for the treatment of human breast cancer.

Mitochondria are important intracellular organelles involved in apoptosis via an intrinsic pathway (19). Although the molecular mechanisms of E_2-induced apoptosis are not fully understood, evidence indicates that mitochondrial related caspase pathways are involved (20, 21). Similarly, a variety of events in apoptosis focus on mitochondria, including the loss of mitochondrial transmembrane potential, release of cytochrome *c*, and participation of pro- and antiapoptotic Bcl-2 family proteins (22,23). However, accumulating evidence suggests that the endoplasmic reticulum where members of the Bcl-2 family of proteins localize, is also a major point of integration of pro-apoptotic signaling or damage sensing (24,25). The endoplasmic reticulum senses local stress such as unfolded protein through a set of pathways known as the unfolded protein response (UPR) (26), which activates three transmembrane sensors PRK-like endoplasmic reticulum kinase (PERK), inositol-requiring 1 alpha (IRE-1α), and activating transcription factor 6 (ATF-6) in endoplasmic reticulum (26). Depending on the duration and degree of stress, the UPR can provide either survival signals by activating adaptive and antiapoptotic signals, or death signals by inducing cell death programs (27, 28).

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

We have found that E_2 changes the cell number according to the treatment period in long-term E_2 deprived breast cancer cell lines MCF-7:5C and MCF-7:2A (25). E_2 has the capacity to decrease around 80 percentage of cell number in MCF-7:5C cells after 7 days treatment whereas in MCF-7:2A cells after two weeks treatment (29). Unexpectedly, the c-Src inhibitor effectively rescues the decreasing of cell number by E_2 in two long-term E_2 deprived cell lines (29). The goal of this study is to identify the mechanisms underlying the early stage of E_2-induced apoptosis and the function of c-Src in the process of E_2-initiated apoptosis. To that end, we demonstrate that E_2 triggers endoplasmic reticulum stress and oxidative stress which activate two main apoptotic pathways, the mitochondrial ('intrinsic') and death receptor ('extrinsic') pathways, whereas c-Src plays an essential role in mediating stress responses induced by E_2 in MCF-7:5C cells. These findings have important clinical implications for the appropriate application of combination therapies in advanced aromatase inhibitor resistant breast cancer.

Materials and Methods

Materials

Estradiol was purchased from Sigma-Aldrich (St. Louis, MO). c-Src inhibitor PP2 was purchased from CalBiochem (San Diego, CA). ERα antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Total MAPK, phosphorylated MAPK, phosphorylated c-Src, phosphorylated eIF2α, total eIF2α, IRE1 α antibodies were from Cell Signaling Technology (Beverly, MA). Total c-Src mouse antibody was from Millipore (Temecula, CA). Estrogen dendrimer conjugate (EDC) was a kind gift by Dr. Katzenellenbogen (University of Illinois at Urbana-Champaign).

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Cell culture conditions and cell proliferation assays

Estrogen-deprived MCF-7:5C cells were maintained in estrogen-free RPMI 1640 medium supplemented with 10% dextran-coated charcoal-stripped fetal bovine serum as previously described (20). The DNA fingerprinting pattern of cell line is consistent with the report by the ATCC (29). The DNA content of the cells, a measure of proliferation, was determined by using a DNA fluorescence Quantitation kit (29).

Cell cycles analysis

Briefly, MCF-7:5C cells were treated with vehicle (0.1% EtOH) and E_2 (10^{-9} mol/L) respectively. Cells were harvested and gradually fixed with 75% EtOH on ice. After staining with propidium iodide (PI), cells were analyzed using a FACSort flow cytometer (Becton Dickinson, San Jose, CA), and the data were analyzed with ModFit software.

Annexin V analysis of apoptosis

The FITC Annexin V Detection Kit I (BD Pharmingen, San Diego, CA) was used to quantify apoptosis by flow cytometry according to the manufacturer's instructions. In brief, MCF-7:5C cells were treated with different compounds respectively. Cells were suspended in 1x binding buffer and 1 x 10^5 cells were stained simultaneously with FITC-labeled annexin V (FL1-H) and propidium iodide (PI) (FL2-H). Cells were analyzed using FACSort flow cytometer (Becton Dickinson, San Jose, CA).

Mitochondrial/Transmembrane potential *($\Delta\psi_m$)* detection

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Mitochondrial membrane potential was measured by flow cytometry using the cationic lipophilic green fluorochrome rhodamine-123 (Rh123) (Molecular Probes) as previously described (20). Disruption of $\Delta\Psi_m$ is associated with a lack of Rh123 retention and a decrease in fluorescence.

Detection of oxidative stress

Intracellular ROS were detected by fluorescent dye 2',7'-dichlorofluorescein diacetate (H_2DCFDA, Invitrogen) (30). Briefly, MCF-7:5C cells were treated with E_2 for different time points using vehicle (0.1% EtOH) cells as control. Cells were loaded with 1 μM CM-H_2DCFDA for 10 min and washed with PBS twice. Then, cells were monitored at fluorescence 530 nm and an excitation wavelength of 488 nm through flow cytometry.

Immunoblotting

Proteins were extracted in cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with Protease Inhibitor Cocktail (Roche, Indianapolis, IN) and Phosphatase Inhibitor Cocktail Set I and Set II (Calbiochem, San Diego, CA). The immunoblotting was performed as previously described (29).

Transient transfection reporter gene assays

Transient transfection assay was performed using a dual-luciferase system (Promega, Madison, WI). To determine ER transcriptional activity, cells were transfected with an estrogen response element (ERE)-regulated (pERE (5x) TA-ffLuc plus pTA-srLuc) dual-luciferase reporter gene sets. The cells were treated with E_2 for 24 hours following the transfection. Then,

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

the cells were harvested and processed for dual-luciferase reporter activity, in which the firefly luciferase activity was normalized by renilla luciferase activity.

Quantitative real-time RT-PCR

Total RNA, isolated with an RNeasy Micro kit (Qiagen, Valencia, CA), was converted to first-strand cDNA using a kit from Applied Biosystem (Foster City, CA). Quantitative real-time PCR assays were done with the SYBR Green PCR Master Mixes (Applied Biosystems, Foster City, CA) and a 7900HT Fast Real-time PCR System (Applied Biosystems, Foster City, CA). All primers were synthesized in Integrated DNA Technologies (San Diego, CA). The sequence of primers was shown in the Supplementary table S1. All the data were normalized by 36B4.

RNA sequencing (RNA-seq) analysis

MCF-7:5C cells were treated with different compounds for 72 hours. Cells were harvested in TRIzol. Total RNA was isolated with an RNeasy Micro kit. These long RNA samples were first converted into a library of cDNA fragments. Sequencing adaptors were subsequently added to each cDNA fragment and a 2x100 bp paired-end sequence was obtained from each cDNA using high-throughput sequencing technology (Illumina GAII). An average of 73.8 million such reads was produced for each sample. The resulting sequence reads were aligned to reference genome build hg19 using TopHat 1.3.0 (31), a splice junction aligner. Transcript abundance were estimated as Fragments Per Kilobase of exon per Million fragments mapped (FPKM), using Cufflinks 1.0.3 (32). Additional analysis was performed with the alternative expression analysis by sequencing (Alexa-seq) software package as previously described (33). Gene expression measures were compared between Cufflinks and Alexa-seq for the set of 17993 overlapping

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

genes. Correlations were excellent with Spearman correlations of 0.955 to 0.971 for the six samples. Pathway analysis was performed with DAVID (34) on lists of differentially expressed gene lists.

Statistical analysis

All reported values are the means ± SE. Statistical comparisons were determined with two-tailed Student's *t* tests. Results were considered statistically significant if the *P* value was <0.05.

Results

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

It is well documented that E_2 stimulates growth and prevents apoptosis in wild-type breast cancer cells and estrogen-responsive osteoblast cells (35,36). In contrast, physiological concentrations of E_2 induce apoptosis in long-term E_2 deprived breast cancer cells (20,21). c-Src plays a critical role in relaying ER signaling pathways in breast cancer cells (37). To investigate the function of E_2 and c-Src in long-term E_2-deprived breast cancer cells MCF-7:5C, a specific c-Src tyrosine kinase inhibitor, PP2, was utilized to block phosphorylation of c-Src (Fig. 1A). It also effectively abolished the growth pathways including the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT pathways in MCF-7:5C cells (Fig. 1A). E_2 activated c-Src through ER since 4-hydroxytamoxifen (4-OHT) completely suppressed phosphorylation of c-Src (Fig. 1B). Although our previous finding showed that E_2 initiates apoptosis in MCF-7:5C cells (20), E_2 was able to activate non-genomic (Supplementary Fig. S1A) and genomic pathways in MCF-7:5C cells (Fig. 1C). These actions were blocked by the c-Src inhibitor, PP2 (Fig. 1C and Supplementary Fig. S1A). Even though the characteristic E_2-induced apoptosis occurs after 72 hours treatment (20), cell numbers were initially increased by E_2 with a high percentage in S

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

phase (Fig.1D). All of these results suggested that E_2 caused an imbalance between growth and apoptosis in MCF-7:5C cells.

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

We have shown that long-term E_2 deprivation increases c-Src activity (29). Therefore, we addressed the question of whether the c-Src inhibitor, PP2, in combination with E_2 would enhance apoptosis in MCF-7:5C cells. Unexpectedly, the c-Src inhibitor blocked apoptosis initiated by E_2 (Fig. 2A and Supplementary Fig. S1D). To confirm that inhibition of c-Src could block E_2-induced apoptosis, a specific siRNA was utilized to knock down c-Src in MCF-7:5C cells (Fig. 2B), which reduced the percentage of Annexin V binding induced by E_2 (Fig. 2C). Further experiments showed that E_2 disrupted mitochondrial membrane potential ($\Delta\Psi_m$) after 48 hours treatment which was measured by flow cytometry using rhodamine 123 (Rh123) (Fig. 2D). The c-Src inhibitor PP2 and 4-OHT both prevented reduction of Rh123 retention induced by E_2 (Fig. 2D). These data demonstrated that E_2-triggered apoptosis utilize the c-Src tyrosine kinase pathway. To evaluate the role of the non-genomic pathway in E_2-induced apoptosis, studies were completed with a synthetic ligand, estrogen dendrimer conjugate (EDC), that only activates the non-genomic pathway at certain concentration (38). The results demonstrated that EDC (10^{-8} mol/L) activated the non-genomic pathway incorporating c-Src (Supplementary Fig. S2). Importantly, EDC had no capacity to activate endogenous E_2 target gene pS2 and did not induce apoptosis in MCF-7:5C cells (Supplementary Fig. S2). All of these findings suggested that the non-genomic pathway does not play a critical role in triggering E_2-induced apoptosis.

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

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

The ER is the initial site for E_2 to induce apoptosis since anti-estrogens ICI 182,780 and 4-OHT completely block apoptosis triggered by E_2 (reference 20 and Supplementary Fig. S3A). In addition to the mediation of ER growth pathways, c-Src is involved in the process of ligand-activated ER ubiquitylation (39). Therefore, blockade of c-Src tyrosine kinase with PP2 further increased ERα protein and mRNA expression levels in MCF-7:5C cells (Fig. 3A). E_2 activated estrogen response element (ERE) activity which could be blocked by 4-OHT but not by PP2 (Fig. 3B). It was interesting to find that the c-Src inhibitor alone could up-regulate E_2 inducible gene pS2 and was additive with E_2 to elevate pS2 mRNA level (Fig. 3C). Another important ER target gene progesterone receptor (PR) has been regarded as an indicator of a functional ER pathway, since expression of PR is regulated by E_2. Although the c-Src inhibitor alone did not elevate PR expression, it dramatically synergized with E_2 to up-regulate PR mRNA (Fig. 3D). All of these results demonstrated that blockade of c-Src increased expression of classical ER target genes. It also implied that classical ER pathway might not directly involve in the E_2-induced apoptosis.

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

To further investigate the mechanisms of the suppression of E_2-induced apoptosis by PP2, RNA-seq analysis was performed to examine the genes regulated by E_2 to trigger apoptosis in MCF-7:5C cells. A wide range of apoptosis-related genes were activated by E_2 (Fig.4A), which were functionally classified into three groups: TP53-related genes (such as TP63, PMAIP1, and CYFIP2), stress-related genes (such as HMOX1, PPP1R15A, ZAK, NUAK2 *etc.*), and inflammatory response-related genes (such as LTB, FAS, TNFRSF21, and CXCR4 *etc.*). Most were stress-related genes (Supplementary Fig. S3B). Consistent with the biological experiments,

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

4-OHT and PP2 both blocked apoptosis-related genes induced by E_2 but to a different extent in MCF-7:5C cells (Fig.4A). The majority of these apoptosis-related genes were confirmed by real-time PCR with similar changes noted as in RNA-seq analysis (Fig. 4B, 4C, 4D and Supplementary Fig. S4). E_2 dramatically increased p63 mRNA levels (Fig. 4B) but did not arrest cells in the G1 phase. In fact, S phase was markedly elevated in MCF-7:5C cells (Fig. 1D). Heme oxygenase 1 (HMOX1) which is active at high concentrations of heme, catalyzes the degradation of heme and is thought to function as an oxidative stress indicator (40). In breast cancer cells, cytochrome *c* is a major source of heme protein found in the inner membrane of the mitochondrion. E_2 markedly increased HMOX1 in MCF-7:5C cells (Fig. 4C) thereby confirming that E_2 may damage the mitochondria and caused cytochrome *c* release. In contrast to MCF-7:5C cells, E_2 decreased HMOX1 levels in wild-type MCF-7 cells (Supplementary Fig. S5A) and clearly did not change HMOX1 expression in another long-term E_2 deprived cell line MCF-7:2A (Supplementary Fig. S5B), both of MCF-7 and MCF-7:2A do not undergo apoptosis after exposure to E_2 in the first three days. Additionally, E_2 upregulated tumor necrosis factor (TNF) family members (such as TNFα, LTA, and LTB), which were abolished by 4-OHT and PP2 (Fig. 4D and Supplementary Fig. S6A and S6B). Low dose of TNFα activated pro-apoptotic pathways in MCF-7:5C cells and inhibited cell growth (Supplementary Fig. S6C and S6D). All of these data suggested that E_2 widely activated intrinsic and extrinsic apoptosis pathways and c-Src was directly involved in mediating apoptosis.

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

Reactive oxygen species (ROS) are the product of oxidative stress by mitochondria, whereas an increase in ROS contributes to degenerative changes in mitochondrial function (41). Under physiological conditions, cellular ROS levels are tightly controlled by low-molecular-weight

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

radical scavengers and by a complex intracellular network of enzymes such as catalases (CAT) and superoxide dismutases (SOD). Under conditions of lethal stress, ROS are considered as key effectors of cell death (42). Intracellular ROS were detected by CM-H_2DCFDA through flow cytometry (Fig.5A). Detectable ROS appeared after 48 hours of treatment with E_2. The production of ROS reached a peak after 72 hours treatment (Fig.5A and 5B). Blocking ER (by 4-OHT) and c-Src (by PP2) abolished ROS generation induced by E_2 (Fig.5C), indicating that both ER and c-Src were upstream signals of ROS. Free radical scavengers Mn-TBAP, catalase, and sodium formate (SF) which respectively act on superoxide radical (O_2^-), H_2O_2, and hydroxyl radical (OH^-), were utilized to suppress the production of ROS. Our results suggested that H_2O_2 and OH^- were the major sources of ROS induced by E_2. This conclusion was based on the observation that catalase and sodium formate inhibited E_2-induced apoptosis, whereas Mn-TBAP was less effective (Fig. 5D). The RNA-seq analysis demonstrated that E_2 did not significantly regulate antioxidant enzymes such as catalases (CAT) and superoxide dismutases (SOD) in MCF-7:5C cells (data not shown). Our results suggest that E_2 has the potential to damage mitochondria to cause oxidative stress.

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

Our previous global gene array data show that E_2 activates genes related to endoplasmic reticulum stress in MCF-7:5C cells (25). To relieve stress, sensors of unfolded protein responses (UPR) are activated as initial responses (43). In this study, a significant induction of UPR sensors, inositol-requiring protein 1 alpha (IRE1α) and PERK/eukaryotic translation initiation factor-2α (eIF2α), by E_2 occurred after 24 hours of treatment and was further increased by prolonging treatment times in MCF-7:5C cells (Fig. 6A). The antiestrogen 4-OHT completely abolished the response (Fig. 6A). The PERK inhibitor blocked phosphorylation of eIF2α and

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

prevented E_2-induced apoptosis (Fig. 6B and 6C), confirming that endoplasmic reticulum stress was important in the apoptosis initiated by E_2. Phosphorylated eIF2α closely associates with an important cellular energy sensor, adenosine monophosphate (AMP)-activated protein kinase (AMPK), to regulate protein translation and apoptosis (44). AMPK, which phosphorylates many metabolic enzymes to stimulate catabolic pathways and increases the capacity of cells to produce ATP (45), was significantly activated after 48 hours treatment with E_2 (Fig. 6D). The c-Src inhibitor, PP2, blocked the phosphorylation of eIF2α but not IRE1α induced by E_2 (Fig. 6E). PP2 also prevented the activation of AMPK after E_2 treatment (Fig. 6F). All of these data indicate that c-Src acts as an important transducer in the protein kinase pathways (eIF2α and AMPK) of stress response (Fig. 6E and 6F) that result in apoptosis.

Discussion

We have previously investigated the inhibitory effects of E_2 on long-term endocrine resistant breast cancer tumor growth *in vivo* (8-10). And we have confirmed this therapeutic effect is related with the apoptosis induced by E_2 (20). This scientific discovery has been used in the clinical trials to treat aromatase inhibitor resistant breast cancer patients and 30% of patients receive benefit (11). The potential limitation on translational research in the treatment of hormone responsive breast cancer is that only four ER positive breast cancer cell lines are available to use routinely (46). Only MCF-7 of the four produces the phenotype of E_2-induced apoptosis observed clinically (20,21). The purpose of establishing long-term E_2 deprivation *in vitro* models is to mimic administration of an aromatase inhibitor that reduces levels of circulating estrogen in clinical studies (47). After a period of proliferative quiescence lasting a few months, the return of proliferation is similar to the relapses observed 12-18 months after primary hormonal therapy in patients. Multiple pathways are involved in the adaptive response to

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

the pressure of E_2 deprivation (48). Although MCF-7 cells grown long term have been shown to differ substantially in various properties depending upon the number of passages and geographic source of the cell lines, induction of apoptosis by physiological concentrations of E_2 is the common characteristic of these *in vitro* model systems (20,21). Nevertheless, how E_2 induces apoptosis is at present unclear. Our new observation (29) that a c-Src inhibitor paradoxically can block E_2-induced apoptosis naturally demands further study. We examined this aspect of c-Src pharmacology to describe fully this phenomenon and gain an insight into the convergence of ER and c-Src pathways for the modulations of an apoptotic trigger in breast cancer. Here, for the first time we document that c-Src participates in the mediation of stress responses induced by E_2 to widely activate apoptosis-related genes involved in the intrinsic and the extrinsic apoptosis pathways.

The ER is the initial point for E_2 to induce apoptosis since anti-estrogens ICI 182,780 and 4-OHT completely block apoptosis triggered by E_2 (reference 20 and Supplementary Fig. S3A). Contradictory to the traditional apoptosis mechanism caused by cytotoxic chemotherapy with cell cycles arrest, E_2-induced apoptotic cells simultaneously undergo proliferation with an increased S phase of cell cycle resulting in increased cell number despite p53 family members being up-regulated (Fig.1D and 4B). E_2 exerts a dual function on MCF-7:5C cells, with both initial proliferation and the apoptosis. In other words, the initial response of E_2 to stimulate growth is the up-regulating of classical transcriptional activity by ER (Fig. 3B) without any detected apoptotic changes in the first 24 hours. Activation of apoptotic genes appeared after 48 hours treatment with E_2 (data not shown), and reached a peak by 72 hours (Fig. 4B, 4C, and 4D). Consistently, characteristic apoptosis occurred at 72 hours (Fig. 2A). These data suggest that the higher rate of proliferation by E_2 might activate other pathways to trigger apoptosis. Our data

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

demonstrate that E_2 caused endoplasmic reticulum stress which activated the unfolded protein response (UPR) within 24 hours (Fig. 6A). The initial aim of UPR is to restore normal function of the cell, however, if the damage is too severe to repair, the UPR ultimately initiates cell death through activation of the apoptotic pathway (49).

c-Src functioned as an important downstream signal of ER in MCF-7:5C cells, which was activated by E_2 (Fig.1B, Supplementary Fig. S1A, S1B, and S1C) and demonstrated multiple levels of association with ER (Fig. 1B, 1C, 2A, 3A, 3C, and 3D). An important finding in this study is that c-Src tyrosine kinase is critical for E_2-induced apoptosis (Fig. 2A, 2C, and 2D). This, therefore, raised the question of the actual role played by c-Src in the process of apoptosis induced by E_2. c-Src mediated PI3K/AKT and MAPK growth pathways by E_2 (Fig. 1C). However, specific inhibitors of PI3K/Akt (LY294002) and MAPK (U0126) could inhibit cell growth but not prevented E_2-induced apoptosis in MCF-7:5C cells (Supplementary Fig. S7), which imply that MAPK/Akt growth pathways are not directly involved in the apoptosis-induced by E_2. In MCF-7:5C cells, E_2 activated the non-genomic pathway after 10 minutes treatment and the c-Src inhibitor blocked the non-genomic pathway (Supplementary Fig. S1A and S1B). Detectable elevation of c-Src phosphorylation appeared after 30 mins treatment with E_2 (Supplementary Fig. S1B). Consistent stimulation of c-Src appeared after 24 hours treatment and gradually increased when extending to 48 hours (Fig. 1B and Supplementary Fig. S1C). All of these data suggest that c-Src activation is a direct effect resulting from E_2. To further explore the function of the non-genomic pathway in the process of E_2-induced apoptosis, EDC was used to treat MCF-7:5C cell which is very ineffective in stimulating transcription of endogenous E_2 target genes (38). The EDC (10^{-8} mol/L) activated the non-genomic pathway but without capacity to activate genomic pathway and did not induce apoptosis in MCF-7:5C cells

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

(Supplementary Fig. S2). All of these results suggest that the non-genomic pathway does not play a critical role in the E_2-induced apoptosis. Interestingly, the EDC could continuously activate c-Src and Akt but without any effect on MAPK after 24 hours treatment (Supplementary Fig. S2E), which may be resulted from enhanced association between ERα and membrane growth factor receptor (48).

Additionally, E_2 activated classical ERE activity but the c-Src inhibitor could not block the response (Fig. 3B). Furthermore, the c-Src inhibitor collaborated with E_2 to up-regulate endogenous ER target genes pS2 and PR (Fig. 3C and 3D). All of these results imply that classical ER transcriptional pathways are not directly involved in E_2-induced apoptosis. Similarly, Zhang *et al* reported that the inhibitory effects of E_2 on cell growth are independent of the classical ERE regulated transcriptional genes (50)**.** Our global gene array data suggest that E_2 signaling can occur through a non-classical transcriptional pathway involving the interaction of ER with other transcription factors such as activator protein-1 (AP-1) and Sp1, which may regulate stress responses (25). In the present study, E_2 initiated UPR (Fig. 6A), increased ROS production (Fig. 5A), and widely activated apoptosis related genes (Fig. 4A). The c-Src was involved in the stress responses and inhibition of c-Src decreased the expression of apoptosis related genes induced by E_2, which are critical mechanisms for the blockade of c-Src to prevent E_2-induced apoptosis.

Overall, E_2 induces endoplasmic reticulum and mitochondrial stresses in MCF-7:5C cells, which subsequently up-regulates apoptosis-related genes to activate intrinsic and extrinsic apoptotic pathways. Unexpectedly, c-Src tyrosine kinase plays a critical role in the stress response induced by E_2. These data clearly raise a concern regarding the ubiquitous use of c-Src

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

inhibitors to treat patients with advanced aromatase inhibitor-resistant breast cancer, thereby undermining the beneficial effects of E_2-induced apoptosis.

Grants support

VCJ is supported by the Department of Defense Breast Program under Award number W81XWH-06-1-0590 Center of Excellence; subcontract under the SU2C (AACR) Grant number SU2C-AACR-DT0409; the Susan G Komen For The Cure Foundation under Award number SAC100009; GHUCCTS CTSA (Grant # UL1RR031975) and the Lombardi Comprehensive Cancer Center Support Grant (CCSG) Core Grant NIH P30 CA051008. JWG is supported by the SU2C (AACR) Grant (SU2C-AACR-DT0409). JAK is supported by NIH grant (PHS 5R37DK015556). OLG was supported by a fellowship from the Canadian Institutes of Health Research (CIHR). The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

References

1. Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 2009; 69:1243-54.

2. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007; 72:7-25.

3. Sabnis G, Schayowitz A, Goloubeva O, Macedo L, Brodie A. Trastuzumab reverses letrozole resistance and amplifies the sensitivity of breast cancer cells to estrogen. Cancer Res 2009; 69:1416-28.

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

4. Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 2007; 25:5815-24.

5. Massarweh S, Osborne CK, Creighton CJ, Qin L, Tsimelzon A, Huang S, et al. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. Cancer Res 2008; 68:826-33.

6. Sabnis G, Goloubeva O, Jelovac D, Schayowitz A, Brodie A. Inhibition of the phosphatidylinositol 3-kinase/Akt pathway improves response of long-term estrogen-deprived breast cancer xenografts to antiestrogens. Clin Cancer Res 2007; 13:2751-7.

7. McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 2002; 108:465-74.

8. Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 2000; 6:2028-36.

9. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 2003 ;95:1597-608.

10. Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, et al. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 2003; 95:1586-97.

11. Ellis MJ, Gao F, Dehdashti F, Jeff DB, Marcom PK, Carey LA, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 2009; 302:774-80.

12. Anderson GL, Chlebowski RT, Aragaki AK, Kuller LH, Manson JE, Gass M, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. Lancet Oncol 2012; 13:476-86.

13. Obiorah I, Jordan VC. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. Menopause 2013 (*in press*).

14. Thomas SM, Brugge JS. Cellular functions regulated by Src family kinases. Ann Rev Cell Dev Biol 1997; 13:513-609.

15. Pascoe D, Oursler MJ. The Src signaling pathway regulates osteoclast lysosomal enzyme secretion and is rapidly modulated by estrogen. J Bone Miner Res 2001; 16:1028-36.

16. Jallal H, Valentino ML, Chen G, Boschelli F, Ali S, Rabbani SA. A Src/AbI Kinase inhibitor, SKI-606, blocks breast cancer invasion, growth, and metastasis in vitro and in vivo. Cancer Res 2007; 67:1580-8.

17. Fan P, Wang J, Santen RJ, Yue W. Long-term treatment with tamoxifen facilitates translocation of estrogen receptor alpha out of the nucleus and enhances its interaction with EGFR in MCF-7 breast cancer cells. Cancer Res 2007; 67:1352-60.

18. Hiscox S, Jordan NJ, Smith C, James M, Morgan L, Taylor KM, et al. Dual targeting of SRC and ER prevents acquired antihormone resistance in breast cancer cells. Breast Cancer Res Treat 2009; 115:57-67.

19. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998; 281:1309-12.

20. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 2005; 97:1746-59.

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

21. Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 beta-estradiol. J Natl Cancer Inst 2001; 93:1714-23.

22. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science 2004; 305: 626-9.

23. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. Cell 2005; 120:483-95.

24. Ferri KF, Kroemer G. Organelle-specific initiation of cell death pathways. Nat Cell Biol 2001; 3:E255-63.

25. Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, et al. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci USA 2011;108:18879-86.

26. Chakrabarti A, Chen AW, Varner JD. A review of the mammalian unfolded protein response. Biotechnol Bioeng 2011; 108:2777-93.

27. Tsai YC, Weissman AM. The Unfolded Protein Response, Degradation from Endoplasmic Reticulum and Cancer. Genes Cancer 2010; 1:764-78.

28. Roberts CG, Gurisik E, Biden TJ, Sutherland RL, Butt AJ. Synergistic cytotoxicity between tamoxifen and the plant toxin persin in human breast cancer cells is dependent on Bim expression and mediated by modulation of ceramide metabolism. Mol Cancer Ther 2007; 6:2777-85.

29. Fan P, McDaniel RE, Kim HR, Clagett D, Haddad B, Jordan VC. Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines. Eur J Cancer 2012; 48:3488-98.

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

30. Tobaben S, Grohm J, Seiler A, Conrad M, Plesnila N, Culmsee C. Bid-mediated mitochondrial damage is a key mechanism in glutamate-induced oxidative stress and AIF-dependent cell death in immortalized HT-22 hippocampal neurons. Cell Death Differ 2011; 18:282-92.

31. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-seq. Bioinformatics 2009; 25:1105-11.

32. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, Baren MJ, et al. Transcript assembly and quantification by RNA-seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature Biotechnology 2010; 28:511-5.

33. Griffith M, Griffith OL, Mwenifumbo J, Goya R, Morrissy AS, Morin RD, et al: Alternative expression analysis by RNA sequencing. Nat Methods 2010; 7:843-7.

34. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nature Protoc 2009; 4:44-57.

35. Fan P, Yue W, Wang JP, Aiyar S, Li Y, Kim TH, et al. Mechanisms of resistance to structurally diverse antiestrogens differ under premenopausal and postmenopausal conditions: evidence from in vitro breast cancer cell models. Endocrinology 2009; 150:2036-45.

36. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, et al. Nongenotropic, sex-onspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 2001; 104:719-30.

37. Shupnik MA. Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. Oncogene 2004; 23:7979-89.

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

38. Harrington WR, Kim SH, Funk CC, Madak-Erdogan Z, Schiff R, Katzenellenbogen JA, et al. Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. Mol Endocrinol 2006; 20:491-502.

39. Chu I, Arnaout A, Loiseau S, Sun J, Seth A, McMahon C, et al. Src promotes estrogen-dependent estrogen receptor alpha proteolysis in human breast cancer. J Clin Invest 2007; 117:2205-15.

40. Araujo JA, Zhang M, Yin F. Heme oxygenase-1, oxidation, inflammation, and atherosclerosis. Front Pharmacol 2012; 3:119.

41. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, et al. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 2000; 404:787-90.

42. Matsuda S, Umeda M, Uchida H, Kato H, Araki T. Alterations of oxidative stress markers and apoptosis markers in the striatum after transient focal cerebral ischemia in rats. J Neural Transm 2009; 116:395-404.

43. Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell 2003; 11:619-33.

44. Dagon Y, Avraham Y, Berry EM. AMPK activation regulates apoptosis, adipogenesis, and lipolysis by eIF2*a* in adipocytes Biochem Biophys Res Commun 2006; 340:43-7.

45. Birnbaum MJ**.** Activating AMP-activated protein kinase without AMP. Mol Cell 2005; 19:289-90.

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

46. Sweeney EE, McDaniel RE, Maximov PY, Fan P, Jordan VC. Models and Mechanisms of Acquired Antihormone Resistance in Breast Cancer: Significant Clinical Progress Despite Limitations. Horm Mol Biol Clin Investig 2012; 9:143-63.

47. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 1992; 90:77-86.

48. Santen RJ, Song RX, Zhang Z, Kumar R, Jeng MH, Masamura S, et al. Adaptive hypersensitivity to estrogen: Mechanisms and clinical relevance to aromatase inhibitor therapy in breast cancer treatment. J Steroid Biochem Mol Biol 2005; 95:155-65.

49. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 2000; 403:98-103.

50. Zhang Y, Zhao H, Asztalos S, Chisamore M, Sitabkhan Y, Tonetti DA. Estradiol-induced regression in T47D:A18/PKCalpha tumors requires the estrogen receptor and interaction with the extracellular matrix. Mol Cancer Res 2009; 7:498-510.

Figure Legends

Figure 1. **c-Src mediated estrogen-activated growth pathways in MCF-7:5C cells.** A, MCF-7:5C cells were treated with vehicle (0.1% DMSO) and PP2 (5×10^{-6}mol/L) for different durations. Phosphorylated c-Src, MAPK, and Akt were detected by immunoblotting. Total c-Src, MAPK, and Akt were used for loading controls. B, MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9}mol/L), 4-OHT (10^{-6}mol/L), E_2 (10^{-9}mol/L) plus 4-OHT (10^{-6}mol/L), PP2 (5×10^{-6}mol/L), E_2 (10^{-9}mol/L) plus PP2 (5×10^{-6}mol/L) respectively for 48 hours. Phosphorylated c-Src was detected by immunoblotting. Total c-Src was used for loading control. C, MCF-7:5C cells were treated with E_2 or combined with PP2 respectively for 24 hours. Phosphorylated

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

MAPK and Akt were examined by immunoblotting. Total MAPK and Akt were used for loading controls. D, MCF-7:5C cells were treated with vehicle and E_2 for different durations. Total DNA was determined using a DNA fluorescence quantitation kit. As a parallel experiment, MCF-7:5C cells were treated with vehicle and E_2 for 72 hours. Cells were fixed for cell cycles analysis. $P<0.05$, * compared with respective control.

Figure 2. **Inhibition of c-Src suppressed estrogen-induced apoptosis in MCF-7:5C cells.** A, MCF-7:5C cells were treated with different compounds respectively as above for 72 hours and Annexin V binding assay was used to detect apoptosis. B, MCF-7:5C cells were transfected with siRNA of c-Src for 72 hours using non-target siRNA as control. c-Src was detected by immunoblotting. The β-actin was used for loading control. C, MCF-7:5C cells were transfected with c-Src siRNA and non-target siRNA as above. Then, they were treated with vehicle (0.1% EtOH) and E_2 (10^{-9}mol/L) respectively for 72 hours. Apoptosis was detected through Annexin V binding assay. $P<0.05$, * compared with control. D, MCF-7:5C cells were treated with different compounds respectively as above for 48 hours and cells were harvested to detect mitochondrial potential through Rh123. $P<0.001$, ** compared with control.

Figure 3. **Suppression of E_2-induced apoptosis by the c-Src inhibitor was independent of the classical ERE regulated transcriptional genes in MCF-7:5C cells.** A, MCF-7:5C cells were treated with vehicle (0.1% DMSO) and PP2 (5×10^{-6}mol/L) respectively for 24 hours. ERα protein was detected by immunoblotting. ERα mRNA was quantified with qPCR. $P<0.05$, * compared with control. B, MCF-7:5C cells were transfected with ERE firefly luciferase plasmid plus renilla luciferase plasmid. Then, cells were treated with different compounds respectively for 24 hours to detect ERE activity. $P<0.001$, ** compared with control. C, MCF-7:5C cells were treated with different compounds respectively for 24 hours. The pS2 mRNA was quantified

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

with qPCR. *P*<0.001, ** compared with control. D, MCF-7:5C cells were treated with different compounds respectively for 72 hours. The PR mRNA was quantified with qPCR. *P*<0.001, ** compared with control.

Figure 4. **c-Src was involved in the process of triggering apoptosis-related genes by E_2 in MCF-7:5C cells.** A, MCF-7:5C cells were treated with vehicle and different compounds respectively as above for 72 hours. Cells were harvested in TRIzol for RNA-seq analysis. B, MCF-7:5C cells were treated with different compounds as above. TP63 mRNA was quantified with qPCR. *P*<0.001, ** compared with control. C, HMOX1 mRNA was quantified with qPCR. *P*<0.001, ** compared with control. D, TNFα mRNA was quantified with qPCR. *P*<0.001, ** compared with control.

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

Figure 6. **c-Src was involved in estrogen-induced endoplasmic reticulum stress in MCF-7:5C cells.** A, MCF-7:5C were treated with E_2 (10^{-9}mol/L) or combined with 4-OHT (10^{-6}mol/L) for different durations. IRE1α and phosphorylated eIF2α were used as indicators of UPR

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

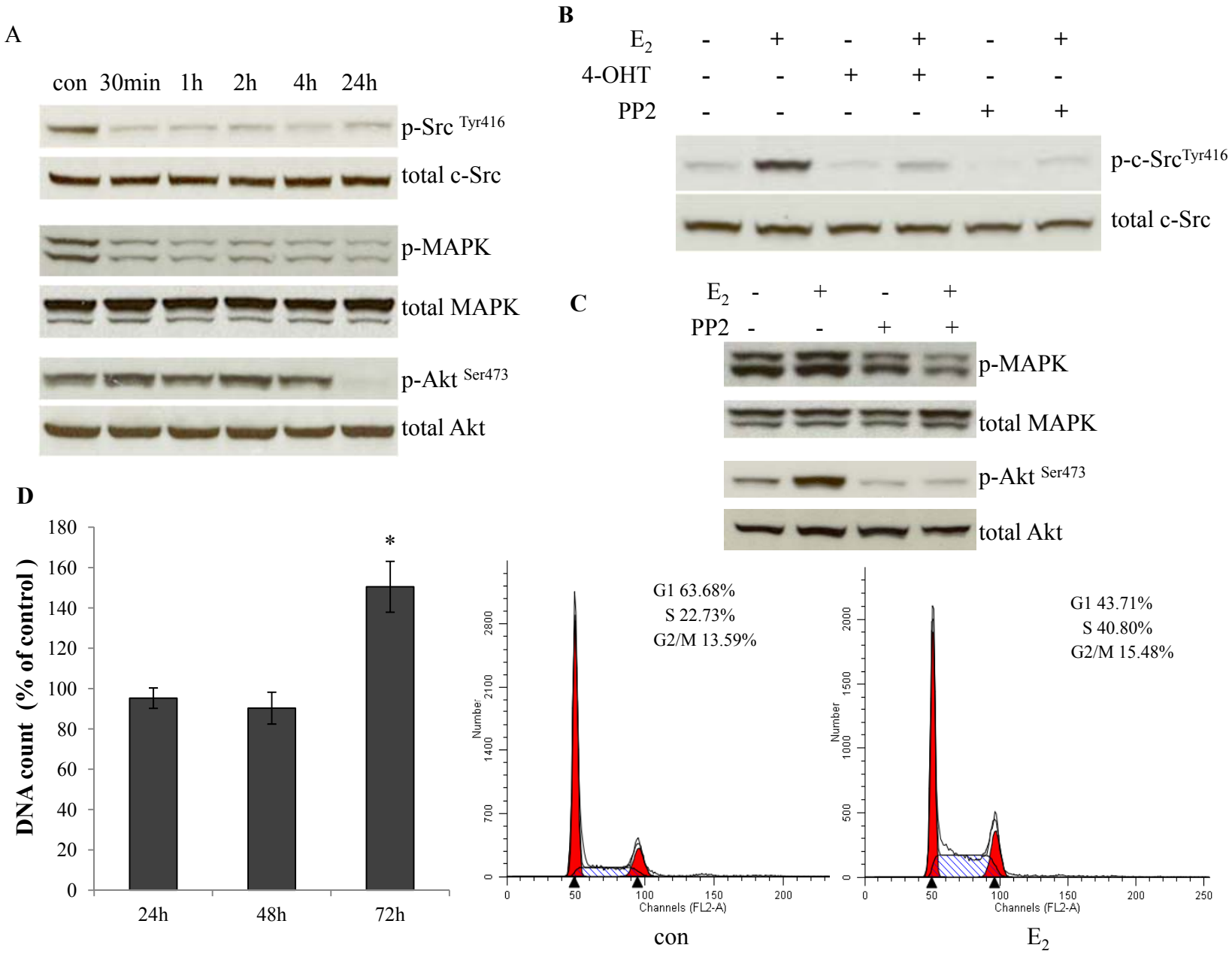
activation. B, MCF-7:5C cells were treated with vehicle (0.1% DMSO), E_2 (10^{-9}mol/L), PERK inhibitor (1×10^{-5}mol/L), E_2 (10^{-9}mol/L) plus PERK inhibitor (1×10^{-5}mol/L) respectively for 24 hours. Phosphorylated eIF2α was examined as the downstream of PERK. Total eIF2α was determined for loading control. C, MCF-7:5C cells were treated with E_2 or combined with PERK inhibitor respectively for 72 hours. Apoptosis was detected through Annexin V binding assay. D, MCF-7:5C cells were treated with E_2 or combined with 4-OHT as above. Phosphorylated AMPK was examined by immunoblotting. Total AMPK was determined for loading control. E, MCF-7:5C cells were treated with E_2 or combined with PP2 for 24 hours. IRE1α and phosphorylated eIF2α were examined by immunoblotting. Total eIF2α and β-actin were determined for loading controls. F, MCF-7:5C cells were treated with E_2 or combined with PP2 for 48 hours. Phosphorylated AMPK and total AMPK were examined by immunoblotting.

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

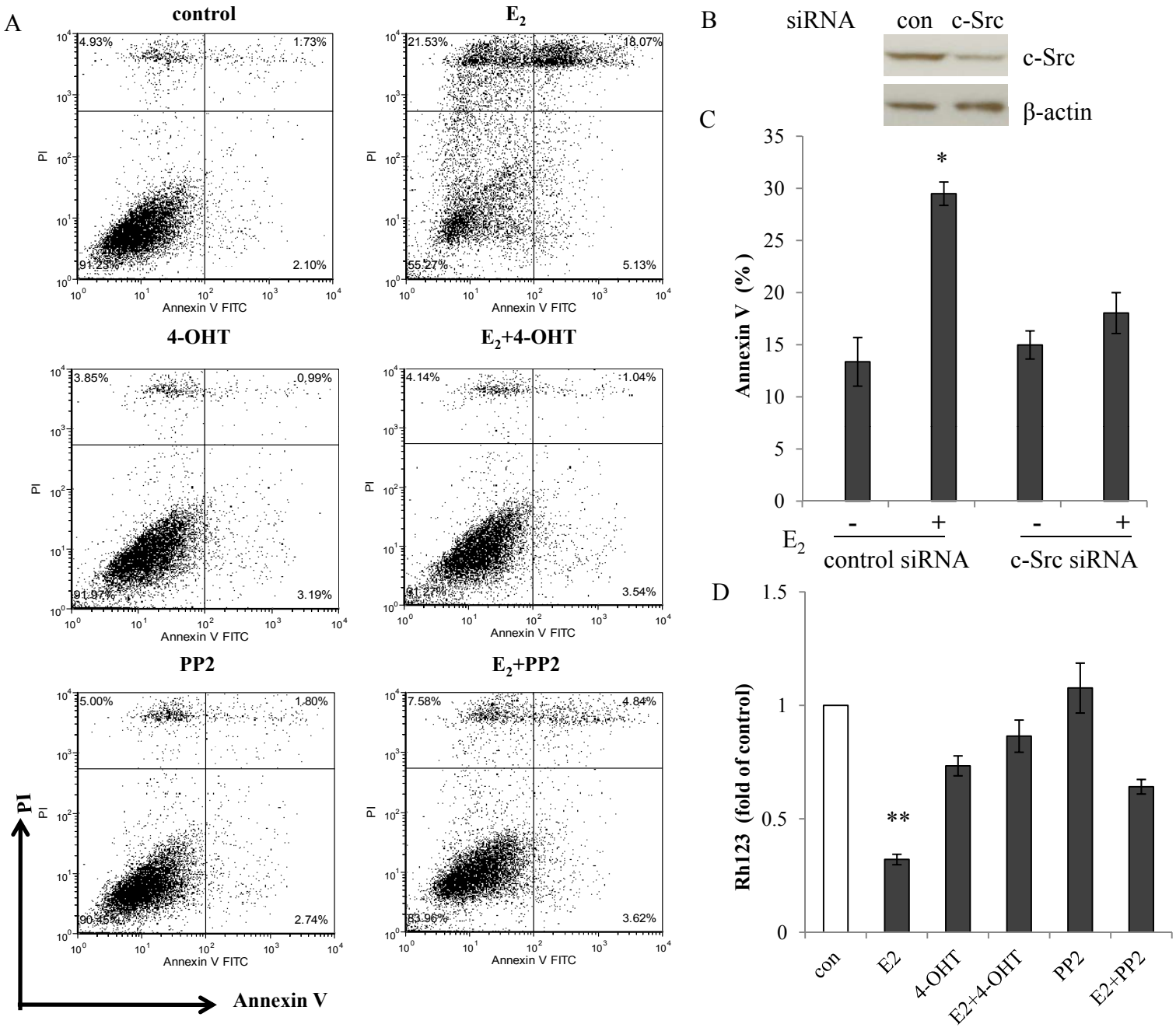
Figure 1



Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

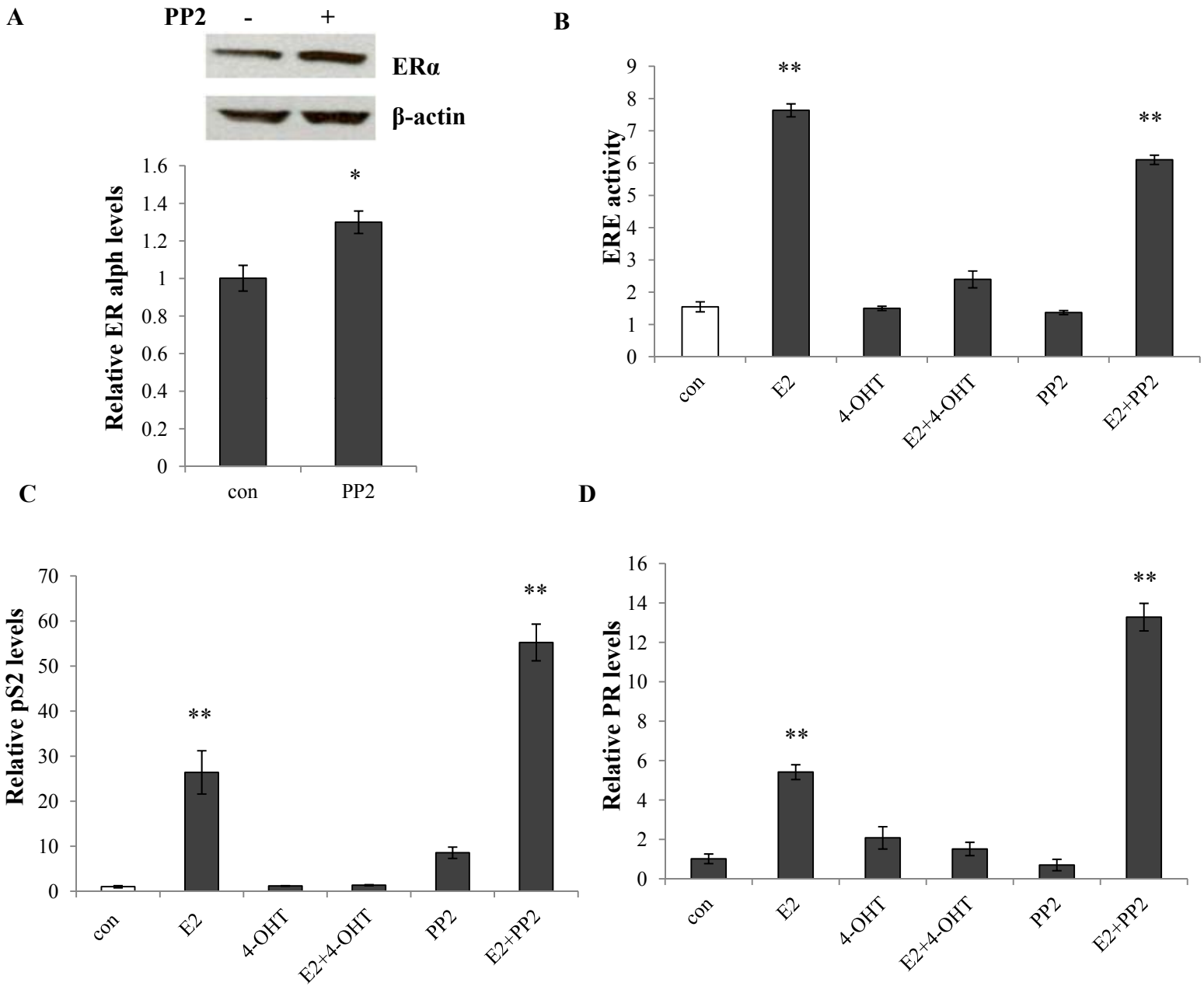
Figure 2



Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

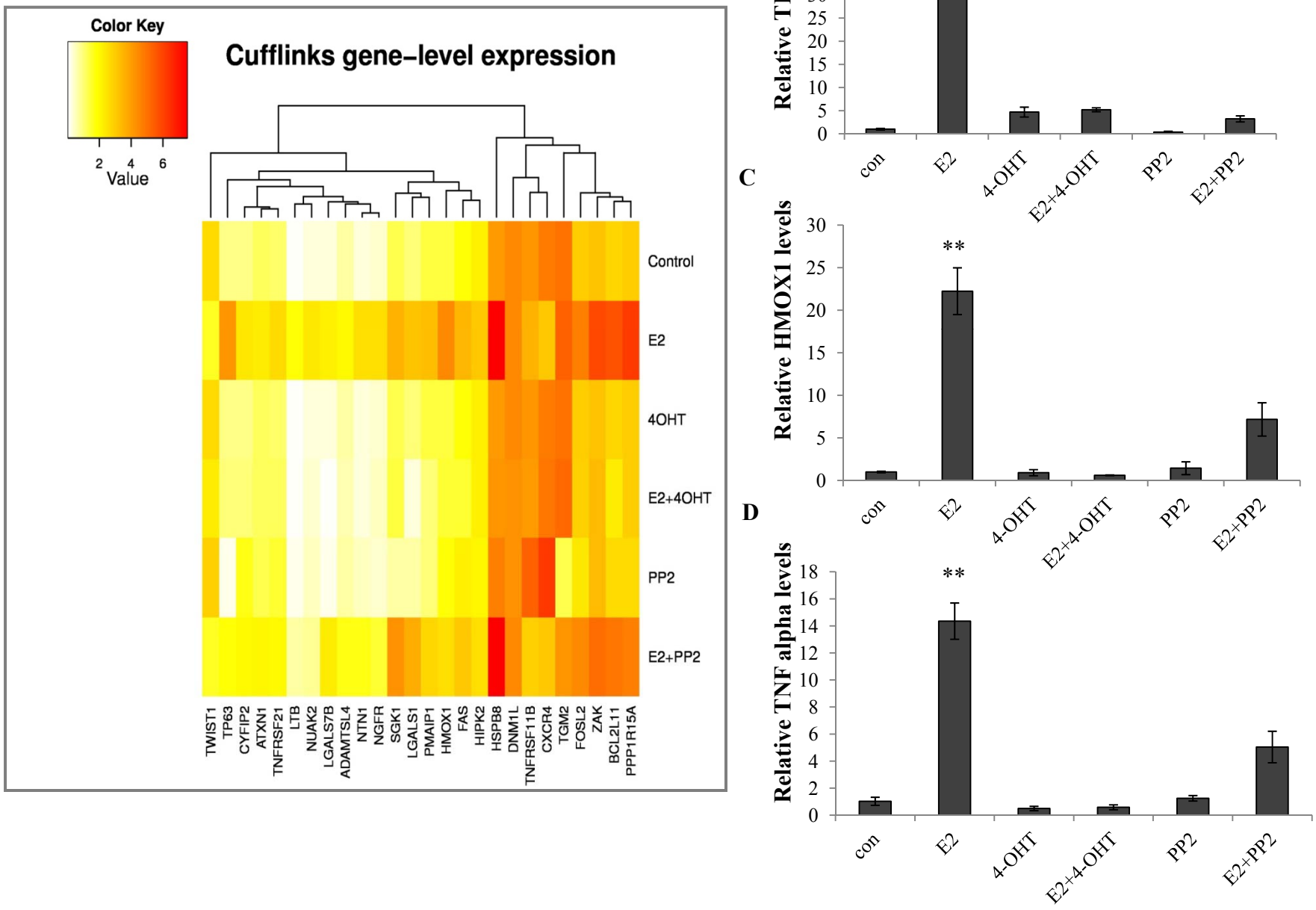
Figure 3



Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

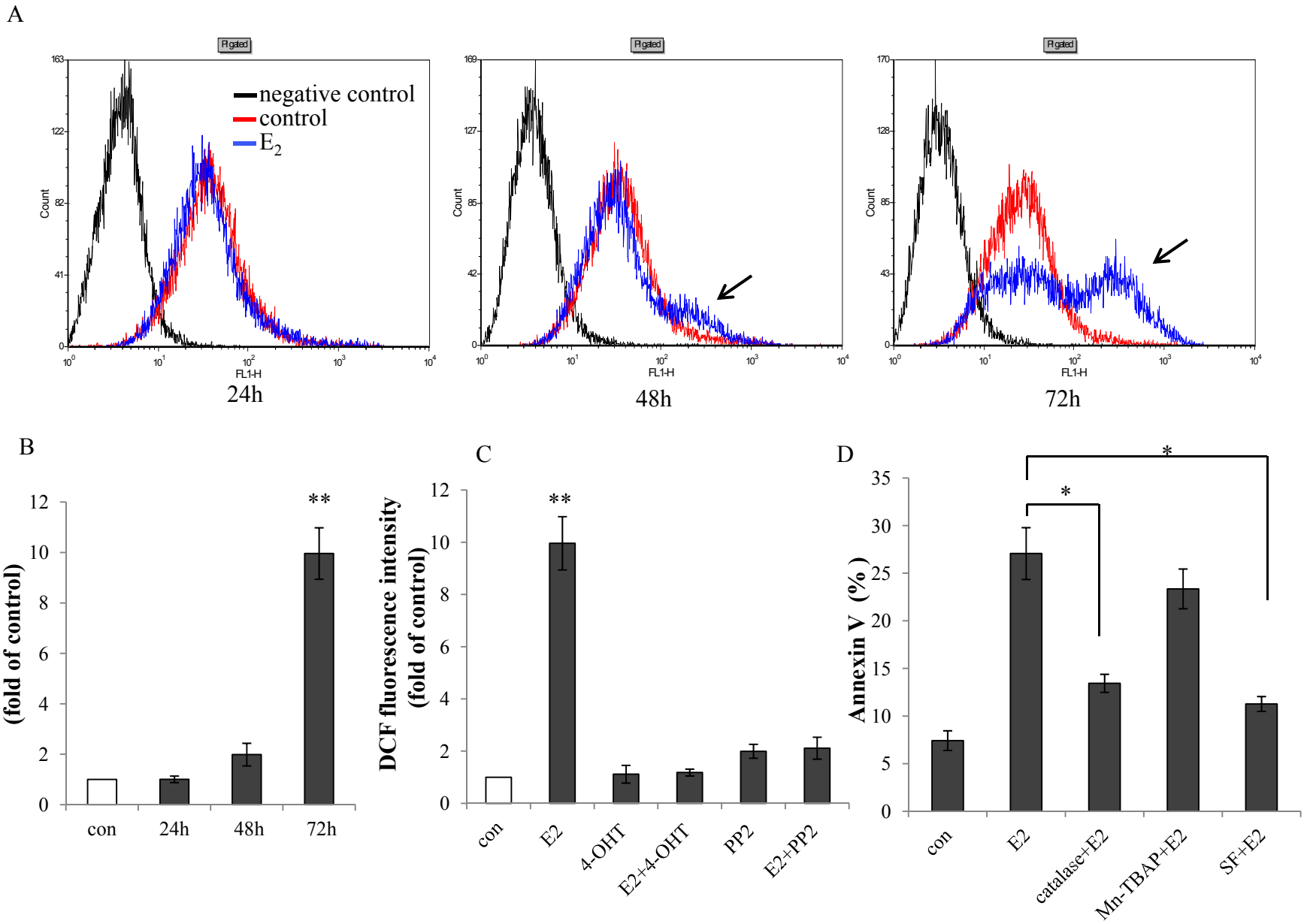
Figure 4



Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

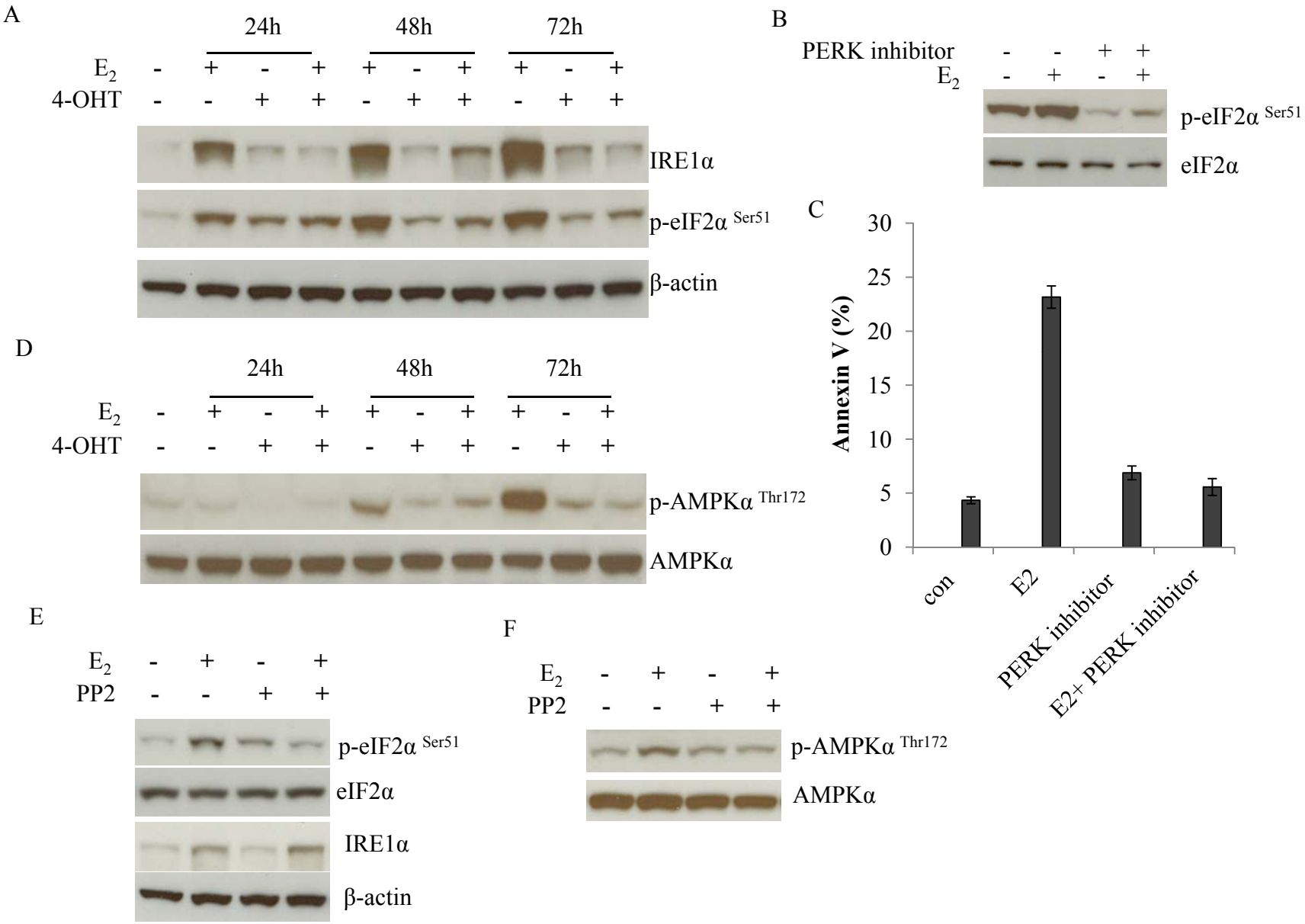
Figure 5



Downloaded from cancerres.aacrjournals.org on May 30, 2013. © 2013 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on May 23, 2013; DOI: 10.1158/0008-5472.CAN-12-4152
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 6



To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

AUTHOR QUERY FORM

	**Book Series: Vitamins and Hormones, 93** **Chapter: 7**	**Please e-mail your responses and any corrections to:** **E-mail: P.Simonraj@elsevier.com**

Dear Author,

Any queries or remarks that have arisen during the processing of your manuscript are listed below and are highlighted by flags in the proof. (AU indicates author queries; ED indicates editor queries; and TS/TY indicates typesetter queries.) Please check your proof carefully and answer all AU queries. Mark all corrections and query answers at the appropriate place in the proof (e.g., by using on-screen annotation in the PDF file http://www.elsevier.com/authors/book-authors) or compile them in a separate list, and tick off below to indicate that you have answered the query.

Please return your input as instructed by the project manager.

Uncited references: References that occur in the reference list but are not cited in the text. Please position each reference in the text or delete it from the reference list.

Robson and Schonberg, 1938

Missing references: References listed below were noted in the text but are missing from the reference list. Please make the reference list complete or remove the references from the text.

Location in Chapter	**Query / remark**	
AU:1, page 142	Yang L (2005) has been changed to Yang, Parkin, Ferlay, Li, and Chen (2005) to match the reference list. Please check.	☐
AU:2, page 143	Please check the edits made in the sentence "Stage IIIC tumors. . ."	☐
AU:3, page 146	Robson (1938) has not been found in the reference list. Please supply full details for this reference.	☐

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

AU:4, page 155	Please check the sentence "In the absence. . ." for clarity/completeness.	☐
AU:5, page 157	Please check the edits made in the sentence "However, the strategy. . ."	☐
AU:6, page 162	Please check the sentence "No treatment. . ." for completeness.	☐
AU:7, page 163	Please check the edits made in the sentence "They also suggested. . ."	☐
AU:8, page 173	Please check the edits made in the sentence "The population of women. . ."	☐
AU:9, page 175	Please check the inserted volume number and the modified last page number.	☐
AU:10, page 175	Please check the inserted year of publication, volume number, and page range.	☐
AU:11, page 176	Please check the inserted author names "W. Lawson" and "R. Robinsox."	☐
AU:12, pages 176, 177, 179, 183, 184, 185	Please provide publisher location.	☐
AU:13, page 177	Please check the inserted volume number and page range.	☐
AU:14, page 179	Please provide volume number.	☐
AU:15, page 179	Please provide expanded form of the journal title.	☐
AU:16, page 182	Please check the expanded journal title.	☐
AU:17, page 187	Please check the inserted author name "D. Parkin."	☐

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

CHAPTER SEVEN

C0035 # Estrogen-Mediated Mechanisms to Control the Growth and Apoptosis of Breast Cancer Cells: A Translational Research Success Story

Russell E. McDaniel, Philipp Y. Maximov, V. Craig Jordan[1]

Department of Oncology, Georgetown University, Lombardi Comprehensive Cancer Center, Washington, District of Columbia, USA

[1]Corresponding author: e-mail address: vcj2@georgetown.edu

UNCORRECTED PROOF

Contents

1. Introduction 140
2. Clinical Presentation of Breast Cancer 141
3. Targeted Therapy 144
   3.1 Foundations of chemical therapy 144
   3.2 The first chemical therapy to treat cancer 145
   3.3 Nonsteroidal antiestrogens 150
4. Transition to Tamoxifen 153
5. Selective Estrogen Receptor Modulation 158
6. Acquired Drug Resistance and the Surprise of SERMs 160
7. Estrogen-Induced Apoptosis: Back to the Beginning 162
8. The Legacy of Tamoxifen 167
Acknowledgments 173
References 173

Abstract

The treatment and prevention of solid tumors have proved to be a major challenge for medical science. The paradigms for success in the treatment of childhood leukemia, Hodgkin's disease, Burkett's lymphoma, and testicular carcinoma with cytotoxic chemotherapy did not translate to success in solid tumors—the majority of cancers that kill. In contrast, significant success has accrued for patients with breast cancer with antihormone treatments (tamoxifen or aromatase inhibitors) that are proved to enhance survivorship, and remarkably, there are now two approved prevention strategies using either tamoxifen or raloxifene. This was considered impossible 40 years ago. We describe the major clinical advances with nonsteroidal antiestrogens that evolved into selective estrogen receptor modulators (SERMs) which successfully exploited the ER

Vitamins and Hormones, Volume 93
ISSN 0083-6729
http://dx.doi.org/10.1016/B978-0-12-416673-8.00007-1

© 2013 Elsevier Inc.
All rights reserved.

VH, 978-0-12-416673-8

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:12 Page Number: 139

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

target selectively inside a woman's body. The standard paradigm that estrogen stimulates breast cancer growth has been successfully exploited for over 4 decades with therapeutic strategies that block (tamoxifen, raloxifene) or reduce (aromatase inhibitors) circulating estrogens in patients to stop breast tumor growth. But this did not explain why high-dose estrogen treatment that was the standard of care to treat postmenopausal breast cancer for 3 decades before tamoxifen caused tumor regression. This paradox was resolved with the discovery that breast cancer resistance to long-term estrogen deprivation causes tumor regression with physiologic estrogen through apoptosis. The new biology of estrogen action has been utilized to explain the findings in the Women's Health Initiative that conjugated equine estrogen alone given to postmenopausal women, average age 68, will produce a reduction of breast cancer incidence and mortality compared to no treatment. Estrogen is killing nascent breast cancer cells in the ducts of healthy postmenopausal women. The modulation of the ER using multifunctional medicines called SERMs has provided not only significant improvements in women's health and survivorship not anticipated 40 years ago but also has been the catalyst to enhance our knowledge of estrogen's apoptotic action that can be further exploited in the future.

1. INTRODUCTION

p0005 Translation research is a conversation between the laboratory and clinical practice. Pharmacology has always been by definition translational research. The goal in the laboratory is to discover a weakness in the disease that can be exploited selectively to kill the infection (or at least stop disease progression and the death of the host), but without injuring the normal tissue. The key word here is "selectively," as the proposed strategy for disease treatment leaves the safety of the laboratory to enter the uncertain world of treating patients.

p0010 At the outset, we will consider the disease to be controlled and the relentless threat to the patient the disease presents. Breast cancer is unique with its most important drug target, the estrogen receptor (ER). What is unique is the fact that the ER is not tumor specific. The ER is ubiquitous in one form or another (ERα or ERβ) within a woman's body. Nevertheless, the most progress during the past 40 years in patient survivorship has been made by targeting the ER in breast cancer. We will examine two ideas that have been essential to reduce the death rate from breast cancer: first, how do we develop drugs to treat disease? Second, how do we ensure selectivity, that is, kill the disease and not the patient. The story will advance rapidly through the twentieth century, but as with all journeys of discovery, surprises were in store along the way and dogma destroyed. These surprises

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:12 Page Number: 140

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

are at the heart of our conversation with nature that is necessary for progress in medical science to save lives.

p0015 We will first describe the stages of breast cancer and its incidence in various countries. This is important not only to appreciate the extent of the disease worldwide but also to provide a basis to understand how fashions in treatment have evolved. The first fashion was to treat what could be seen, that is, metastatic breast cancer (stage IV) by endocrine ablative surgery or the empirical use of high-dose hormone therapy (Kennedy, 1965a). Endocrine therapy was palliative and no significant gains were anticipated. After the palliative use of endocrine approaches to treat stage IV breast cancer for 70 years, by the 1970s, nobody cared about palliative endocrine therapy. By the 1960s, combination cytotoxic chemotherapy was showing dramatic promise for the treatment of stage IV breast cancer so combination cytotoxic chemotherapy was used as an adjuvant to destroy micrometastases (stages I and II) that could not be seen but were predicted to grow and cause a recurrence of the disease. Regrettably, success was modest and cures elusive. However, the change in fashion to embrace long-term adjuvant therapy with antihormones saved millions of lives worldwide. The subsequent discovery and development of selective estrogen receptor modulators (SERMs) (Jordan, 2001) was the key step in developing a practical approach to reduce the incidence of breast cancer but, at the same time, maintained a hope to be able to reduce the morbidity produced by other diseases such as osteoporosis, coronary heart disease, strokes, and endometrial cancer. It has therefore been possible over the past 40 years to address effectively the targeted treatment of all stages of breast cancer and prevent the disease. As a result prognosis, survivorship has been enhanced and breast cancer incidence can now be reduced not only in the high-risk population but also in the general population.

s0010 2. CLINICAL PRESENTATION OF BREAST CANCER

p0020 Of the 275,370 American women that are estimated to die in 2012 from cancer, 39,510 of them (or approximately 14%) are projected to die due to cancer of the breast (Howlader et al., 2009). Of the baby girls born today, 12.38% will be diagnosed with breast cancer at some point in their lifetime; 2.76% will die from breast cancer (Howlader et al., 2009). With the exception of skin cancers, breast cancer is the most common of all cancers in women, accounting for about one-third of all diagnoses in the United States (Breast Cancer Facts & Figures, 2011–2012). In recent years, 124.3

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:13 Page Number: 141

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

out of 100,000 women per year have been diagnosed with invasive breast cancer in the United States (31.4 out of 100,000 women per year have been diagnosed with *in situ* breast cancer; 23 out of 100,000 died; Howlader et al., 2009). The District of Columbia has had the highest number of deaths due to invasive breast cancer in women with 27.96 out of 100,000 (Howlader et al., 2009). Louisiana, New Jersey, Ohio Mississippi, Missouri, Maryland, and Virginia all have relatively high death rates (above 24.18 per 100,000) (Howlader et al., 2009). While White American women have the highest rate of breast cancer diagnosis, African American women have an increased mortality rate from breast cancer, with 31.6 out of 100,000 dying (Howlader et al., 2009).

p0025 According to the American Cancer Society, 89% of women with breast cancer will still be living 5 years after their diagnosis (Breast Cancer Facts & Figures, 2011–2012). In fact, as of 2008, there were about 2.6 million alive in America who had at one time been diagnosed with breast cancer (Breast Cancer Facts & Figures, 2011–2012).

p0030 Breast cancer also accounts for about 14% of cancer deaths among Canadian women, second only to lung cancer (Canadian Cancer Statistics, 2012). In Canada, in 2012, there will be an estimated 96 cases of breast cancer per 100,000 women or about 22,700 new diagnoses, with Ontario and Nova Scotia having the highest incidences. Five thousand one-hundred Canadian women will die in 2012 from breast cancer—out of 36,000 total female cancer deaths—with Prince Edward Island having the highest breast cancer mortality rate (Canadian Cancer Statistics, 2012).

p0035 In Brazil, in 2008, there were 49,400 new cases of breast cancer with 50.7 cases per 100,000 women (EISRCM, 2006) representing 28% of cancers in women (INCA, 2006). In 2006, there were 10,834 deaths due to breast cancer (INCA, 2006). Malignant breast cancer is the seventh leading cause of death in Brazilian women (INCA, 2006). In the European Union, breast cancer represented about 30% of cancer incidences in women (Ferlay, Parkin, & Steliarova-Foucher, 2010), and about 16.6% of all female cancer deaths (Ferlay et al., 2010). In China, 168,013 new cases of breast cancer in women were estimated in 2005 (Yang, Parkin, Ferlay, Li, & Chen, 2005). Au1

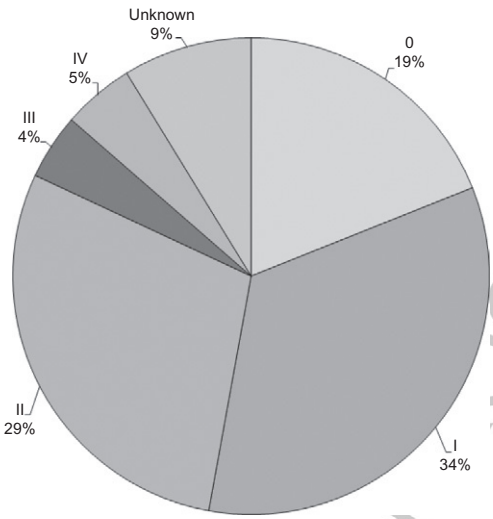
p0040 Breast cancer cases are divided into several stages, depending on the development of the disease. The population distribution of this relentlessly moving target, as it first occurs in the breast and subsequently breaks out, is illustrated in Fig. 7.1. Invasive breast cancer—or cancer cells from the breast that have overrun tissue beyond their origin, be it breast or other parts of the body—is divided into four stages. Potentially cancerous, abnormally

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:13 Page Number: 142

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007



f0005 **Figure 7.1** Percentage of each stage of breast cancer as recorded by SEER between 2002 and 2008 (Howlader et al., 2009; Ries, Eisner, & Kosary, 2001). (See Color Insert.)

growing cells in the wall of the breast duct called ductal carcinoma *in situ*, or DCIS, is often referred to as stage 0 (Breast Cancer Survival Rates by Stage, 2011).

p0045 Stage I breast cancer is the first stage where the cancerous cells have spread into breast tissue away from the duct. This type of tumor is confined to the breast, and its diameter is no more than 2 cm. Stage II breast tumors have either spread to the lymph nodes under the arm or grown to be more than 2 cm in diameter (Breast Cancer Survival Rates by Stage, 2011).

p0050 Stage III breast cancer is known as "locally advanced cancer" and is divided into three subsections. Stage IIIA is when the tumor spreads to underarm lymph nodes that are attached to other bodily features (including other lymph nodes). Stage III also comprises tumors of greater than 5 cm diameter that have spread to isolated underarm lymph nodes. Stage IIIB is any breast tumor that has grown into the skin of the breast or into the chest wall. The size of the tumor is unimportant in stage IIIB classification. Stage IIIC tumors have either spread to the lymph nodes above or below the Au2 collarbone, or spread to the lymph nodes under the arm and behind the breastbone (Breast Cancer Survival Rates by Stage, 2011).

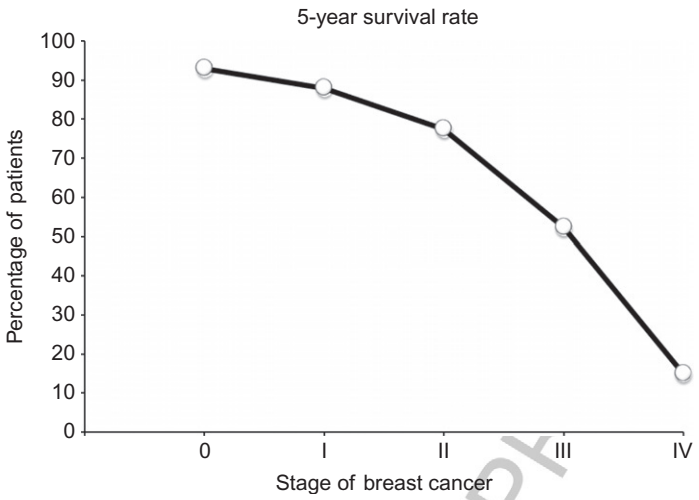
p0055 Metastatic breast cancer is known as stage IV. This cancer has spread from the breast to other organs. The brain, bones, and liver are frequent locations

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:13 Page Number: 143

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007



f0010 **Figure 7.2** Five-year survival rates among the various stages of breast cancer (Breast Cancer Survival Rates by Stage, 2011).

for secondary breast cancers. Stage IV breast cancer has a poor prognosis with a 15% 5-year survival rate (Breast Cancer Survival Rates by Stage, 2011).

p0060 It is, therefore, important to stress that all "breast cancer" is not the same. We now know this from the molecular fingerprints from individual tumors that can be classified into subgroups (Hu et al., 2006; Perou et al., 2000; Sorlie et al., 2003). But personalized medicine has not yet arrived. Early detection and staging remain essential for survival (Fig. 7.2). Treatments with endocrine therapies have proved to be more successful the sooner they are deployed. But how did this happen?

s0015 ## 3. TARGETED THERAPY

s0020 ### 3.1. Foundations of chemical therapy

p0065 In 1908, Professor Paul Ehrlich was awarded the Nobel Prize for Medicine. In his Nobel Prize Lecture (Baumler, 1984), he described his work on antitoxins for diphtheria toxin and alluded to his side chain theory of receptors. However, he also alluded to his new studies on arsenicals (Baumler, 1984). He stated, "I want to show you that we are approaching the problem of obtaining an insight into the nature of the effects produced by drugs by following these points systematically, it will be easier than before to develop planned synthesis for pharmaceuticals targeted to requirements" (Baumler, 1984). He died of a heart attack and kidney failure on the afternoon of

UNCORRECTED PR

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:14 Page Number: 144

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

August 20th, 1915, so he was not to receive his second Nobel Prize for his discovery that changed pharmacology and the treatment of disease forever. Based upon his early experience discovering dyes that stain bacteria but not human cells, he conceived of the idea that chemicals could be synthesized to kill the disease-causing bacteria specifically. Through his research, he created the process of synthesizing analogues of known toxic chemicals, testing the efficacy and safety of chemicals in appropriate animal models of human disease, and a suitable candidate could then be tested in clinical trial.

p0070 Sahachiro Hata, in Erlich's team, created the appropriate animal models of disease and ultimately discovered that chemical 606 was completely effective against laboratory models of syphilis. Ehrlich approached Hoescht to enter into mass production for clinical trials. These trials worked spectacularly to cure a fatal disease and Salvarsan became the first specific chemical therapy (or chemotherapy). Professor Ehrlich had created the roadmap for drug discovery by the pharmaceutical industry, but he also turned from the treatment of infections to cancer research. In 1909, the press announced, "The beginning of the end of the cancer problem is in sight," and an editorial in *Scientific American* in 1912 stated, "Unquestionably, their [Ehrlich and Wasseman's] investigations justify the hope of a cure for human cancer" (Schrek, 1960). However, in 1915, Ehrlich admitted defeat and stated, "I have wasted 15 years of my life in experimental cancer" (Schrek, 1960). So it would remain for the next 30 years, but this stagnation would change with the first successful use of a chemical therapy to treat metastatic breast cancer (stage IV) (Haddow, Watkinson, Paterson, & Koller, 1944).

s0025 3.2. The first chemical therapy to treat cancer

p0075 The link between estrogen and the growth of breast cancer is a fascinating tale. The interconnected research ventures in endocrinology and chemistry during the first 40 years of the twentieth century would create a new dimension in therapeutics, result in the use of high doses of synthetic estrogens to treat some metastatic breast cancers successfully, but also create a paradox. If ovarian estrogens fuel the growth of breast cancer, why does a high dose of estrogen kill breast cancer cells in postmenopausal women? This paradox has only recently been solved and we will use this chapter to illustrate how the twists and turns of endocrine therapy have both revolutionized patient care and exposed a new biology of estrogen action: estrogen-induced apoptosis.

p0080 In 1896, George Beatson reported the first case of oophorectomy as a treatment for breast cancer (Beatson, 1896). Although it is often said that he performed the operation empirically, he actually relied on his knowledge

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:14 Page Number: 145

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

that farmers had discovered there was a link between the ovary and the lactating mammary gland. In 1900, Boyd (1900) collected all-known cases of oophorectomy from hospitals around Britain and discovered there was a 30% response rate. This is perhaps the first "clinical trial" and gave the medical community new knowledge that has stood the test of time. The response rate to any endocrine therapy is 30%. The work during the early decades of the twentieth century on laboratory mouse models of breast cancer by Lathrop and Loeb (1916) and Lacassagne (1933) would be valuable to advance knowledge about hormones and breast cancer growth. However, an understanding of why oophorectomy was beneficial to treat breast cancer and which tumor would be responsive would remain a mystery until the 1960s. The first clues that the ovaries contained a substance that causes responses in a target organ were reported by Allen and Doisey (1923). They named their substance in pig ovary estrogen. They determined the biological effect by ovariectomizing mice to stop the estrous cycles and discovered that the vaginal epithelium would undergo replication and cornification when pig ovarian extract was injected. The animal model in the mouse (referred to henceforth as the "Allen–Doisy test") would be the essential test system to discover synthetic estrogens a decade later during the 1930s.

p0085 The story of the discovery of potent nonsteroidal estrogens is remarkable (Jordan, Mittal, Gosden, Koch, & Lieberman, 1985). With only a few early clues that simple synthetic molecules could initiate mouse vaginal cornification, two major groups of potent estrogenic compounds were described in the 1930s: the stilbenes (Dodds, 1938; Dodds, Goldberg, Lawson, & Robinsox, 1938) of which diethylstilbestrol (Fig. 7.3) would become a key compound and used clinically, and the longer acting triphenylethylenes (Robson, 1937, 1938; Robson & Schonberg, 1942; Thompson & Werner, Au3
1953). These two classes of compounds would be the essential tools with which to change breast cancer therapy but most of the therapeutic advances over the decades between 1930 and 1980 would be almost by chance. Remarkably, the successful translational research would enhance survival from breast cancer and significantly improve women's health. Two practical facts emerged during this period: estrogens support mammary and breast tumorigenesis and growth but estrogen was used routinely to treat and cause regression of some metastatic breast cancers. This paradox would lie dormant until rediscovery during the past decade.

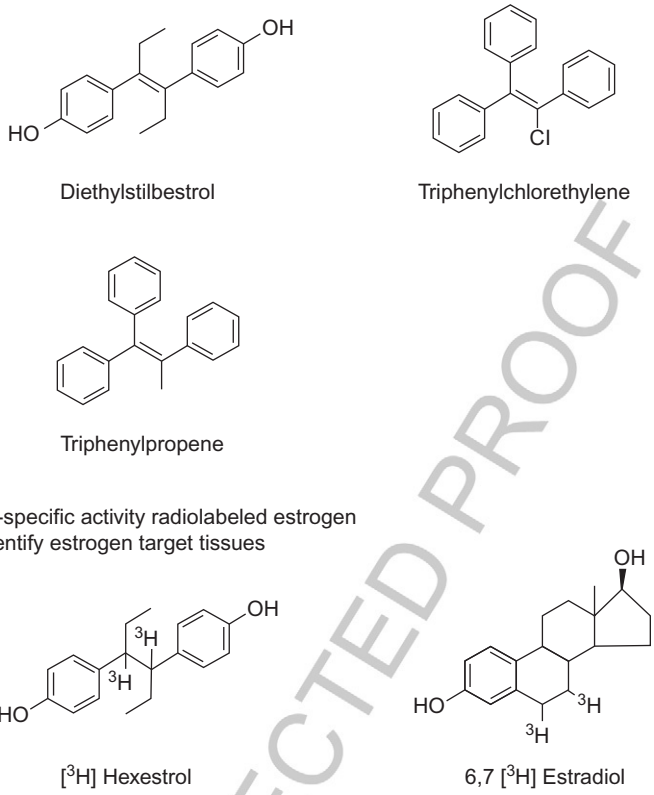
p0090 Lacassagne (1936a, 1936b), Shimkin and Wyman (1945, 1946), and Shimkin, Wyman, and Andervont (1946) contributed evidence that estrogens could increase mouse mammary tumorigenesis. Lacassagne (1936b)

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:14 Page Number: 146

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007



f0015 **Figure 7.3** Compounds used by Haddow as the first "chemical therapy for cancer" (Haddow et al., 1944) and tritiated DES (hexestrol) and estradiol used in the first studies of retention of the estrogen in target tissues (Glascock & Hoekstra, 1959; Jensen & Jacobson, 1962).

went one step further at the Annual Meeting of the American Association of Cancer Research in Boston in 1936 by stating that

> *If one accepts the consideration of adenocarcinoma of the breast as the consequence of a special hereditary sensibility to the proliferative actions of oestrone, one is led to imagine a therapeutic preventative for subjects predisposed by their heredity to this cancer. It would consist—perhaps in the very near future when the knowledge and use of hormones will be better understood—in the suitable use of a hormone antagonistic or excretory, to prevent the stagnation of oestrone in the ducts of the breast.*

p0095 Unfortunately, there would be no "therapeutic antagonist" to use clinically until tamoxifen started its journey as an antiestrogen for the treatment of breast cancer (Jordan, 2003c, 2008b) some 40 years later!

p0100 In the first half of the twentieth century, breast cancer treatment was severe and unsuccessful. Radical mastectomy was the standard of care, radiation

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:15 Page Number: 147

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

therapy was advancing from an art to a science, and nonspecific cytotoxic chemotherapy started to be introduced to treat cancer in general after the end of the Second World War. Prospects for the patient in general were abysmal and the examination of the state-of-the-art breast cancer treatment in 1977 (Stoll, 1977b) was not too much more hopeful. Nevertheless, with the wisdom of insight, one counterintuitive observation in the 1940s was to act as a catalyst for the eventual discovery of targeted cancer therapies. Alexander Haddow, conducting laboratory studies, discovered that carcinogenic polycyclic hydrocarbons actually caused tumor regression in animals but clearly one could not apply this "translational therapy" to patient care. However, he reasoned that the polycyclic synthetic estrogens had a similar sort of structure as the carcinogens (scary but true!), so following testing in the laboratory he compared and contrasted high-dose DES and triphenylethylenes (Fig. 7.3) as treatments for prostate cancer, breast cancer, and "other cancers." Prostate cancer responded as did metastatic breast cancer (stage IV) (30%) but none of the "other cancers" responded (Haddow et al., 1944). The application of high-dose estrogen therapy to provide palliative treatment for some postmenopausal women with metastatic breast cancer was the first chemical therapy to treat any cancer successfully. This approach became the standard of medical care in both the United Kingdom and the United States of America (Kennedy, 1965b; Kennedy & Nathanson, 1953) for the next 30 years until the resurrection of the triphenylethylene-based antiestrogen tamoxifen (Jordan, 2003c). In 1970, Sir Alexander Haddow FRS, during the inaugural Karnofsky (Haddow, 1970) lecture (the highest honor bestowed by the American Society for Clinical Oncology), stated his concerns for the future of specific and effective cancer therapy.

> *In the first place, the fact that the cancer cell is but a modification of the normal somatic cell holds out little prospect of a chemotherapiaspecifica in Ehrlich's sense, whereby chemical substances which, on the one hand, are taken up by certain parasites and are able to kill them, are, on the other hand, tolerated well by the organism itself, or at any rate without too great damage.*
>
> *(Haddow, 1970)*

p0105 In his Karnofsky lecture, Haddow also mentioned the importance of the few breast tumors that just melted away during high-dose estrogen therapy. However, he stated,

> *. . . the extraordinary extent of tumour regression observed in perhaps 1% of postmenopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us . . .*
>
> *(Haddow, 1970)*

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:16 Page Number: 148

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

p0110 It is also important to stress that, at the time of Haddow's Karnofsky lecture in 1970, bacteria were routinely grown in the laboratory for testing antibiotic sensitivity; the right antibiotic could then be used appropriately to treat the right disease. No such tests existed for cancer. Practice was to give the drug and hope it might work. Therefore, the definition of the anticancer mechanism of DES in some breast tumors was the essential first step to determine which tumors will respond and which will not. What is the target for drug sensitivity or in Ehrlich's terms—the receptor? One study in 1949 by Walpole and Paterson (1949) declared defeat but the answer to the question "why" was to come ultimately from DES itself. The stilbene can be hydrogenated with tritium across the double bond to produce high-specific activity [^{3}H] hexestrol (Fig. 7.3). Hexestrol is a potent estrogen. Glascock and Hoekstra (1959) in fact showed the binding of [^{3}H] hexestrol in the estrogen target tissues of sheep and goats. The idea was subsequently translated to a clinical study in patients with metastatic breast cancer. Those patients whose breast tumor retained [^{3}H] hexestrol were more likely to respond to endocrine ablation (Folca, Glascock, & Irvine, 1961). These very preliminary findings were refined first by Jensen and Jacobson (1962) using [^{3}H] estradiol (Fig. 7.3) to describe the binding and retention of estradiol in the estrogen target tissues (uterus, vagina, pituitary gland) of the immature rat. Tritiated estradiol was bound initially, but not retained in tissues (muscle, lung) that were not targets of estrogen action. Gorski's group subsequently extracted and identified the soluble ER from the immature rat uterus (Toft & Gorski, 1966; Toft, Shyamala, & Gorski, 1967). These data were rapidly translated to identify the ER in breast tumors (Jordan, Wolf, Mirecki, Whitford, & Welshons, 1988) and there was a spectrum of none to a lot. Gorski's group discovered (Toft et al., 1967) that the extracted ER from target tissues could subsequently be liganded with [^{3}H] estradiol *in vitro*, so there was no need to inject radioactive estrogens into patients. The Jensen group went on to establish sucrose density gradient analysis as the method of choice to identify the breast tumor ER in the United States. In 1974 (McGuire, Carbone, & Vollmer, 1975), an NCI conference to consider the value of the ER assay to predict responsiveness of metastatic breast cancer to endocrine ablation or DES concluded that the absence of ER in a breast tumor predicted that the tumor would not respond to endocrine ablation or DES. If ER was present, there was about a 60% probability of an objective response. Thus, patients with ER-negative tumors should not be treated with endocrine ablation surgery; it would be worthless. At that time, in the mid-1970s, medical practice changed in America with a requirement that all patients with a diagnosis of breast cancer should have an ER assay on their tumor

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:17 Page Number: 149

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

tissue. By the end of the 1970s, ER assay laboratories were springing up at most academic institutions (V.C.J. was involved in establishing one at the Worchester Foundation for Experimental Biology, Massachusetts in the early 1970s and was director of the steroid receptor laboratory at the Ludwig Unit in Bern, Switzerland (1979), organizing international quality control for the Ludwig clinical trials group, and the steroid receptor laboratory at the University of Wisconsin Clinical Cancer Center in the 1980s).

p0115 It should again be stressed that during the 1960s and 1970s the therapeutics of breast cancer was primitive. Only metastatic disease (stage IV) was addressed with therapy and this stage is fatal within a few years (Fig. 7.2). But the therapeutic options slowly evolved and this story again has its origins in the interest in synthetic estrogens. The synthetic estrogens stilbenes or triphenylethylenes used by Haddow in the 1940s (Haddow et al., 1944) (Fig. 7.3) were synthesized by Imperial Chemical Industries (ICI) Ltd. (now Astra Zeneca) but they were not alone in their interest in estrogens. Numerous pharmaceutical companies during the 1950s were interested in synthetic estrogens primarily because of the revolution in therapeutics that occurred with the development of the oral contraceptive that emanated from the vision of Gregory Pincus at the Worcester Foundation (Speroff, 2009). His chemical method stopped ovulation in the woman. No egg—no baby. It was reasoned by chemists in the pharmaceutical industry that if only another novel chemical method of contraception could be discovered, then the use of chemicals to prevent pregnancy, which was not a disease, could be expanded.

p0120 Leonard Lerner, a young scientist in the pharmaceutical industry in the 1950s, would take the next conceptual advance in reproduction research; that step would fail, but open the door for others to create the first targeted therapy for any cancer, the first endocrine therapy to save hundreds of thousands of women's lives, and the first chemical therapy approved to reduce the incidence of breast cancer in women of high risk for the disease. This did not occur because there was a specific plan by the pharmaceutical industry. The advance with tamoxifen would come from ICI Pharmaceuticals Division where their fertility control program would discover and then abandon ICI 46,474 to be resurrected and advanced by individuals with close friendships and who were in the right place at the right time and ready to exploit a unique opportunity.

s0030 3.3. Nonsteroidal antiestrogens

p0125 Leonard Lerner was tasked within the William S. Merrell Company to study nonsteroidal estrogens. At the time, the company marketed

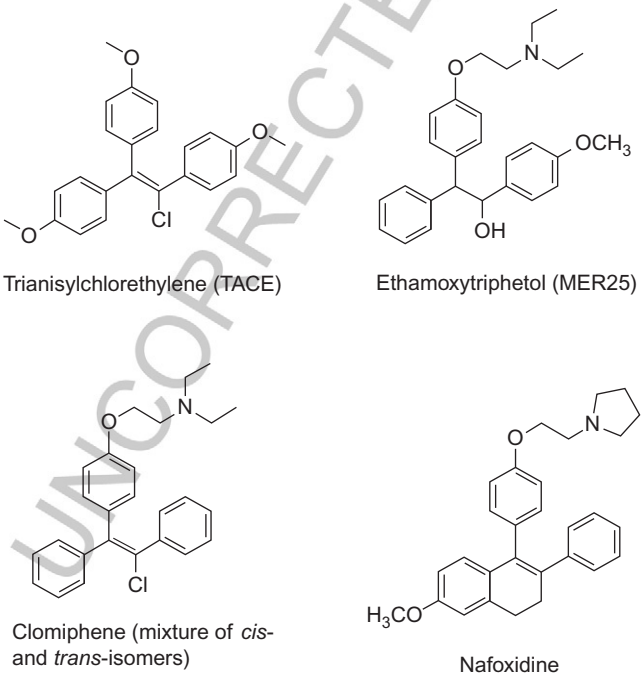
UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:18 Page Number: 150

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

trianisylchlorethylene (TACE) (Fig. 7.4), but Lerner noticed a compound in the cardiovascular program was similar in structure—MER25 (Fig. 7.4) (Lerner, 1981). He tested the triphenylethanol and could detect no estrogenic activity in any species tested but it was a weak blocker of estrogen action (Lerner, Holthaus, & Thompson, 1958). However, what electrified the pharmaceutical industry was that MER25 and its successor clomiphene (Fig. 7.4) were postcoital antifertility agents in rats. Unfortunately, in clinical trial, the nonsteroidal antiestrogens were effective in inducing ovulation in subfertile women, so hopes of making a blockbuster drug disappeared. Clomiphene was tested as a breast cancer drug in metastatic disease (Hecker et al., 1974), as was nafoxidine (Legha, Slavik, & Carter, 1976), but development was abandoned because of concerns about toxic side effects (Fig. 7.4). No one was recommending careers in failed antifertility drugs or cancer therapy. Arthur Walpole was the head of the Fertility Control Program at ICI Pharmaceuticals Division in Alderley Park, Cheshire. He was interested in cancer research but was tasked to improve the toxicology profile of clomiphene that increased circulating desmosterol. Desmosterol was



f0020 **Figure 7.4** Structures of nonsteroidal estrogens and antiestrogens mentioned in the text.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:18 Page Number: 151

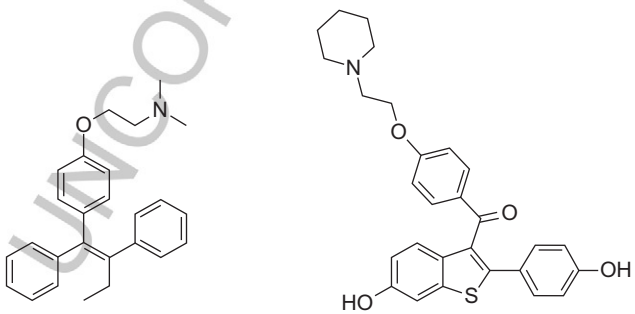
To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

associated with cataract formation in women (Laughlin & Carey, 1962). The result of the antifertility program at Alderley Park in the 1960s was ICI 46,474, the *trans*-isomer of a substituted triphenylethylene (Fig. 7.5) that was antiestrogenic with postcoital antifertility properties in the rat (Harper & Walpole, 1967a, 1967b). The patent application read,

> *The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolaemic activity (Jordan, 2003c).*

p0130 Preliminary clinical studies demonstrated modest anticancer activity in metastatic breast cancer in postmenopausal women (Cole, Jones, & Todd, 1971) and the induction of ovulation in subfertile women (Klopper & Hall, 1971). However, after a review of all the data at Alderley Park in 1972, the Research Director decided to terminate clinical development—there was no financial future in ICI 46,474 (Jordan, 2006). However, Walpole reasoned that the company should put ICI 46,474 on the market as an orphan drug for the treatment of metastatic breast cancer and the induction of ovulation for subfertile women and "outsource" work to discover a strategy for the clinical use of tamoxifen. Walpole had recently met and examined the Ph.D. thesis of a young graduate student, Craig Jordan, in the Department of Pharmacology at the University of Leeds. Jordan was now spending 2 years as a visiting scientist as the Worcester foundation. Why not sponsor his research with an unrestricted grant? Let Jordan develop a clinical strategy for a nonsteroidal antiestrogen for the treatment of breast cancer.



Tamoxifen
(ICI 46,474 a failed "morning after pill")

Raloxifene
(formerly keoxifene a failed breast cancer drug)

f0025 **Figure 7.5** Structures of tamoxifen and raloxifene.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:19 Page Number: 152

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

Scholarships were made available for Jordan students, technician's salaries were provided, and hundreds of laboratory rats were chauffeured from Alderley Park to the University of Leeds. This personal story has recently been told elsewhere (Poirot, 2011), but it is time now to focus on the pioneering medicine tamoxifen and how it not only changed breast cancer therapy but also acted as the catalyst to create new knowledge about the pharmacology of nonsteroidal antiestrogens that sequentially led to selective ER modulators, chemoprevention, the science of acquired drug resistance to antihormone therapy, and the new biology of estrogen-induced apoptosis.

s0035

4. TRANSITION TO TAMOXIFEN

p0135

Tamoxifen is unique in the annals of cancer therapeutics. While it is the first targeted therapy to treat cancer (a nonsteroidal antiestrogen targeted to the ER to stop estrogen-stimulated growth), the selective toxicity of tamoxifen was lucky. There are ERs all around the postmenopausal women's body, but as it turned out, these ERs do not appear to play a significant role in physiological homeostasis. Indeed, it was lucky that tamoxifen was also an antitumor agent in the premenopausal women without significant actions on normal physiology. Tamoxifen is approved by the FDA for the treatment of all stages of breast cancer, DCIS, male breast cancer, and for the reduction of breast cancer risk in both high-risk pre- and postmenopausal women. No other cancer therapy has such a spectrum of approved applications. At the outset of the translational research studies in the early years of the 1970s, it could not have been anticipated that a palliative medicine, FDA approved in December 1977 for the short-term (1–2 years) disease control of one in three postmenopausal patients with metastatic breast cancer, could so dramatically change the prognosis and survivorship for millions of women with ER-positive early breast cancer. During the 1970s, a laboratory strategy was put in place that would ultimately revolutionize thinking about the approach to treating breast cancer by targeting the tumor, killing the cancer cells not the patient, and treating earlier stages of the disease or even women only at risk for developing breast cancer (Jordan, 2008b). In the 1970s, the new fashion in therapeutics was combination cytotoxic chemotherapy that declared victory in childhood leukemia and was in the process of mopping up Hodgkin's disease (Stoll, 1977a). Justifiably, cytotoxic chemotherapy was king and only the appropriate acronym of drugs had now to be discovered to cure breast cancer. By contrast, no one in the

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:19 Page Number: 153

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

pharmaceutical industry or clinical oncology was advocating a massive effort in endocrine therapy—or in fact any effort. Few cared.

p0140 The three publications that presaged the future clinical promise of tamoxifen as a pioneering medicine were all published in the *European Journal of Cancer* (Jordan, 2008b). The idea that tamoxifen blocked estrogen-stimulated breast cancer growth through blocking estradiol binding to the ER was controversial but was demonstrated both biochemically (Jordan & Koerner, 1975) and in cell culture (Lippman & Bolan, 1975). However, although these data were embraced in the United States, the same was not true for the United Kingdom where no clear clinical correlations between ER and tumor response could be demonstrated in clinical trial for the next 15 years (NATO, 1983; SCTO, 1987). Conceptually, this was important because the Europeans tended toward palliative applications with endocrine therapy, whereas in the United States, the goal was cure with combination cytotoxic chemotherapy. Simply stated, nobody cared about the mechanism of tamoxifen action but the good news was that in the United Kingdom everyone with breast cancer was to receive tamoxifen. This inadvertent policy was perhaps the correct decision for the wrong reason that ensured survivorship for tens of thousands of women in the United Kingdom.

p0145 The second conceptual advance was the finding that two sustained release subcutaneous injections of tamoxifen at the same time—as oral administration of dimethylbenzanthracene (DMBA) to 50-day-old female Sprague–Dawley rats, would prevent the initiation and growth of mammary carcinogenesis (Jordan, 1976). This observation was expanded (Jordan, Allen, & Dix, 1980; Jordan, Naylor, Dix, & Prestwich, 1980) and subsequently used as important laboratory evidence by Dr. Trevor Powles to explore the potential of tamoxifen to be use in the chemoprevention of breast cancer in high-risk women (Powles et al., 1989). The new dimension of the chemoprevention of breast cancer arrived in 1998 with the FDA approval of the pioneer tamoxifen for reducing the incidence of breast cancer in pre- and postmenopausal women at high risk (Fisher et al., 1998; Powles et al., 1998; Veronesi et al., 1998).

p0150 The third paper and advance that translated to clinical trial ultimately extended the survivorship of perhaps millions of women receiving long-term adjuvant tamoxifen therapy to prevent the recurrence of ER-positive breast cancer in patients with node-positive or node-negative breast cancer (stages I and II). In the early 1970s, the dilemma was when to use combination cytotoxic chemotherapy in the treatment plan for

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:20 Page Number: 154

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

breast cancer. There was great enthusiasm that the use of combination cytotoxic chemotherapy would eventually lead to the cure of breast cancer. Very good results had been noted during the late 1960s (Cooper, 1969) and now a new strategy was considered: adjuvant therapy to destroy micrometastatic disease that had spread systemically after the woman had a mastectomy and local radiation. The strategy seemed sound that combination cytotoxic would cure patients with a low tumor burden. Regrettably, early results were modest (Bonadonna et al., 1976; Fisher et al., 1975) with the best effect noted in premenopausal patients. However, subsequent work demonstrated that cytotoxic chemotherapy destroys the ovary so the treatment could reasonably be interpreted as an aggressive ovarian ablation (Jordan, 1991). With the slow development of the antiestrogen tamoxifen during the 1970s, attentions started to focus not on the palliative use of tamoxifen for metastatic breast cancer but on the idea that tamoxifen might have potential as an adjuvant therapy. In the laboratory, the DMBA rat mammary carcinoma model was considered to be "state of the art" for the study of the endocrine treatment of breast cancer. Huggins, Grand, and Brillantes (1961) first showed that a single oral administration of 20 mg DMBA to 50-day-old female Sprague–Dawley rats would produce multiple mammary carcinomas in all rats within 150 days after DMBA treatment. The development of tumors was endocrine dependent; the tumors contained ER and regressed in response to ovariectomy (Welsch, 1985). In the absence of any other experimental options to Au4 explore adjuvant therapy with tamoxifen, different durations of tamoxifen (or its potent metabolite 4-hydroxytamoxifen discovered around this time(Jordan, Collins, Rowsby, & Prestwich, 1977; Jordan, Dix, Naylor, Prestwich, & Rowsby, 1978) as tamoxifen actions was the sum of its antiestrogenic metabolites) to determine if a short course of the antiestrogen for a month (equivalent to a year in women as adjuvant therapy) would be cidal (Lippman & Bolan, 1975) or whether longer durations would be necessary to prevent tumor development. The idea was to destroy the early transformed cells, not unlike adjuvant therapy. The profound conclusion was that longer adjuvant therapy was going to be a better clinical strategy (Jordan, 1978; Jordan & Allen, 1980; Jordan, Allen, et al., 1980; Jordan, Dix, & Allen, 1979). The laboratory studies also derived another conclusion that was to have ramifications for the later use of polar nonsteroidal antiestrogen for the treatment of breast cancer. Tamoxifen was metabolically activated to 4-hydroxytamoxifen (Jordan et al., 1977, 1978). This was not a requirement for antiestrogenic activity but an

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:20 Page Number: 155

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

advantage (Jordan & Allen, 1980). Polar nonsteroidal antiestrogens may be better at blocking estrogen actions at the ER but they had poor bioavailability and were rapidly excreted (Jordan & Allen, 1980). The subsequent idea that tamoxifen needed to be metabolically activated by hydroxylation of the primary metabolite *N*-desmethyltamoxifen to endoxifen was to preoccupy pharmacogenomics research on tamoxifen during the past decade with arguments both for and against the critical role of different CYP2D6 genotypes (Brauch et al., 2013; Dieudonne et al., 2009; Kiyotani et al., 2010; Lammers et al., 2010; Lash et al., 2011; Madlensky et al., 2011; Rae et al., 2012; Regan et al., 2012; Schroth et al., 2009). Simply stated, if CYP2D6 was aberrant then there is low metabolism to endoxifen and a lower probability of a response of the patients tumor to tamoxifen. Be that as it may, the fundamental issue in the 1970s was to select an appropriate duration of adjuvant tamoxifen therapy to test in breast cancer clinical trials.

p0155 The clinical community selected a 1-year course of adjuvant tamoxifen therapy in all early clinical trials (LBCSG, 1984; Rose et al., 1985). This was an obvious choice based on the limited effectiveness of tamoxifen to treat metastatic breast cancer. Tamoxifen is only effective for about 1 year (Ingle et al., 1981) so there was an understandable concern that longer adjuvant tamoxifen therapy would precipitate early drug resistance and recurrent disease that would now be fatal. But the studies with the DMBA rat mammary carcinoma model did not comply with clinical "predictions" based on the treatment of metastatic breast cancer. Short-term therapy (1 month equivalent to a year in a patient) was unable to control tumorigenesis in the rat but continuous therapy for six months (6 years in a patient) was 90% effective in controlling tumorigenesis (Jordan, Allen, et al., 1980). The DMBA rat model was to be proved to predict accurately subsequent clinical trials data. Five years of adjuvant tamoxifen therapy became the standard of care for the treatment of breast cancer for 20 years and remains so for the premenopausal patient.

p0160 There are several notable features of adjuvant tamoxifen therapy that were exposed during clinical trials and these data were enhanced and amplified by the regular review of ongoing adjuvant clinical trials through the Oxford Overview Analysis process. The survival advantage for these women taking long-term tamoxifen therapy is profound, whereas short term (1 year of treatment) is not of significance in premenopausal patients (Davies et al., 2011; EBCTCG, 1998, 2005). Most importantly, and we will examine this clinical observation in more detail during the discussions of acquired tamoxifen

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:21 Page Number: 156

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

resistance, is the sustained and decrease mortality noted *after* 5 years of adjuvant tamoxifen. This was a surprising observation that now has a plausible scientific explanation. The science will be considered in Section 7.

p0165 The next step in the tamoxifen tale was the evaluation of its worth to prevent breast cancer in high-risk women. The evidence to support this decision to test the hypothesis in clinical trial was solid. The expanding database on tamoxifen as the endocrine adjuvant therapy of choice during 1980s and 1990s was reassuring for clinicians. Most important in this regard was the use of adjuvant tamoxifen therapy for the treatment of node-negative breast cancer because 80% of patients are cured by surgery and local radiotherapy, which meant that an increasing proportion of "cured" patients were already being treated with 5 years of adjuvant therapy (Fisher et al., 1989; SCTO, 1987). The fact that adjuvant tamoxifen reduced contralateral breast cancer (primary breast cancer) by 50% (Cuzick & Baum, 1985) was proof of principle primary presentation would be successful and the earlier knowledge that tamoxifen prevented mammary tumorigenesis in rodents (Jordan, 1976) enhanced the opportunities for the clinical trials community.

p0170 Overall, the placebo-controlled clinical trials of chemoprevention demonstrated a significant decrease in the incidence of breast cancer following tamoxifen therapy that was sustained even when the drug treatment was terminated (Cuzick, Forbes, & Howell, 2006; Fisher et al., 2005, 1998; Powles, Ashley, Tidy, Smith, & Dowsett, 2007). However, the strategy was flawed as Au5 only a few women (2–5 per thousand per year) has their breast cancer prevented but hundreds of women per thousand would experience significant side effects such as menopausal symptoms and there would be an increased risk of deep vein thrombosis in postmenopausal women. Perhaps more serious was the finding in the laboratory that tamoxifen increased the growth of human endometrial cancer implanted in athymic mice but did block estrogen-stimulated growth of breast cancer completely in the same athymic mouse (Gottardis, Robinson, Satyaswaroop, & Jordan, 1988). These observations moved rapidly from the laboratory to clinical care within 3 years once the laboratory findings were confirmed in a placebo-controlled clinical trial (Fornander et al., 1989). Clinical findings demonstrated a three- to fivefold increase in the risk of developing endometrial cancer in postmenopausal women who now, as a treatment population, would have regular gynecological examinations when using tamoxifen. Although endometrial cancer was not significant for the treatment of breast cancer as the decreases in mortality were profound (EBCTCG, 2005), for the well women, this was a troubling side effect. It was said "one cancer was being substituted for

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:22 Page Number: 157

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

another." In the prevention setting, this and the emerging new laboratory knowledge during the early 1990s that tamoxifen was a hepatocarcinogen in rats (Greaves, Goonetilleke, Nunn, Topham, & Orton, 1993; Hard et al., 1993) (this laboratory observation has never translated to patient populations—fortunately) mandated that a profoundly different strategy was essential, if chemoprevention was ever to be accepted as a reality in clinical practice.

5. SELECTIVE ESTROGEN RECEPTOR MODULATION

p0175 Up until the mid-1970s, the nonsteroidal antiestrogens were initially potential and then failed postcoital contraceptives. The antiestrogens became agents of interest to be exploited in gynecology. Both clomiphene and tamoxifen were successful for the induction of ovulation in subfertile women. A review by Lunan and Klopper (1975) focuses almost entirely on the potential applications in gynecology and there is only passing references to breast cancer treatment. By the mid-1980s, with tamoxifen FDA approved in December 1977 and adjuvant clinical trials well underway it was now time to consolidate all the information about the nonsteroidal antiestrogens as pharmacological agents (Jordan, 1984), so that further effective translational research could help patients. It was time also to review all that was known about tamoxifen (Furr & Jordan, 1984). After all, tamoxifen was, and is, the only nonsteroidal antiestrogen to be approved for the therapeutics of all stages of breast cancer and chemoprevention. It is, however, of interest to mention that tamoxifen had not been granted patent protection in the United States because of the perceived primacy of the earlier Merrel patents in the 1960s (Jordan, 2003c). That all changed in 1986 almost exactly at the time that long-term adjuvant tamoxifen therapy was the treatment strategy of choice for patients with ER-positive breast cancer (Consensus conference, Adjuvant chemotherapy for breast cancer, 1985). But it was the move toward using tamoxifen to prevent breast cancer in high-risk populations of women that now became the driving force behind understanding the "good, bad, and the ugly" of tamoxifen pharmacology. A surprise was in store.

p0180 It was reasoned at the time that, if estrogen was important to maintain bone density, then a nonsteroidal antiestrogen may prevent breast cancer in the few but create osteoporosis in the majority. The same argument was articulated about coronary heart disease and atherosclerosis, but it was already known in tamoxifen's patent (earlier described in Section 3.3) that circulating cholesterol was lowered by the drug (Harper & Walpole, 1967b).

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:23 Page Number: 158

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

p0185 The question of bone loss with nonsteroidal antiestrogen was addressed in the ovariectomized and intact rat using tamoxifen and the failed breast cancer drug keoxifene (Fig. 7.5), also a nonsteroidal antiestrogen (Black, Jones, & Falcone, 1983). Both nonsteroidal antiestrogens actually prevented bone loss from ovariectomy and a combination with estrogen further improved bone density (Jordan, Phelps, & Lindgren, 1987). These breakthrough data were confirmed (Turner, Evans, & Wakley, 1993; Turner, Wakley, Hannon, & Bell, 1987, 1988; Turner et al., 1998), but initially, these data in the refereed literature were ignored by the pharmaceutical industry. They did, however, act as preliminary data to initiate a prospective placebo-controlled clinical trial with tamoxifen in postmenopausal breast cancer patients with node-negative breast cancer. This trial was initiated at a time when node-negative breast cancer patients did not receive adjuvant therapy as a standard of care. The Wisconsin Tamoxifen Study demonstrated that tamoxifen lowered low-density lipoprotein (bad) cholesterol, did not substantially reduce high-density lipoprotein (good) cholesterol (Love et al., 1990, 1991), and improved bone density measured by dual photon absorptiometry (Love et al., 1992).Thus, not only did the animal studies unexpectedly translate to potential clinical benefit but also a new concept and vision was about to change medicine.

p0190 The laboratory studies with keoxifene and tamoxifen on bone density showed estrogen-like actions, but parallel studies at the same time demonstrated that tamoxifen and keoxifene could prevent rat mammary carcinogenesis (Gottardis & Jordan, 1987), an antiestrogenic effect. Thus, this class of compounds including clomiphene (Fig. 7.4), which was mixed isomers that are estrogenic or antiestrogenic (Beall et al., 1985), had all shown a similar effect on bone in the rat. So the potential new drug group had the potential to turn on and turn off sites around the body. At this time, it was already known that tamoxifen was more estrogenic in the rodent uterus (Harper & Walpole, 1967b) and human endometrial cancer would grow with tamoxifen (Gottardis et al., 1988) so this again illustrated the target site specific actions. The complex of the "antiestrogen" with the ER was being interpreted differently at different sites around the body (Jordan & Robinson, 1987). The endometrial cancer issue clearly was a "bad" for tamoxifen but others in the class, like keoxifene, were less estrogen like in the uterus (Black et al., 1983), less likely to stimulate endometrial cancer in patients (Gottardis, Ricchio, Satyaswaroop, & Jordan, 1990), and were already known to maintain or build bone (Jordan et al., 1987). A road map for industry was proposed and simply stated (Lerner & Jordan, 1990):

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:23 Page Number: 159

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

Is this the end of the possible applications for antiestrogens? Certainly not. We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer.

p0195 In 1993, keoxifene was renamed raloxifene (Fig. 7.5) with patent protection to treat and prevent osteoporosis in postmenopausal women. The pivotal registration trial call Multiple Outcomes Relative to Evista was to demonstrate that raloxifene simultaneously could prevent fractures of the lumbar spine by about 50% (Ettinger et al., 1999) and reduce the incidence of ER-positive breast cancer by about 80% (Cummings et al., 1999) with no increase in endometrial cancer. Raloxifene became the first multifunctional medicine in women health because of the positive results of the Study of Tamoxifen and Raloxifene (Vogel et al., 2006) where both drugs, now called selective ER modulators or SERM, reduced breast cancer incidence in high-risk postmenopausal women by 50%. Two diseases, osteoporosis and breast cancer, were controlled by one drug. However, it was later shown (Vogel et al., 2010) that a 5-year course of raloxifene is not sufficient to maintain long-term benefit for the prevention of breast cancer-like tamoxifen. Raloxifene is approved by the FDA for indefinite administration for the prevention and treatment of osteoporosis, and breast cancer reduction is sustained during extended treatment (Martino et al., 2004). Again, the unanticipated merits of tamoxifen to sustain antitumor actions in chemoprevention (Powles et al., 2007) would raise the question why? A plausible answer would occur through serendipity and the examination of acquired drug resistance to SERMs in the laboratory.

s0045 6. ACQUIRED DRUG RESISTANCE AND THE SURPRISE OF SERMS

p0200 During the 1970s, the concept of acquired resistance to antihormone therapy was simple. Breast tumors were considered to be a mixture of cells: some were ER negative and some ER positive. The concentration of ER in a tumor was therefore an average of total tumor ER per unit protein, for

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:24 Page Number: 160

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

example, 150 fm ER per mg tumor protein. This was measured by extracting the unoccupied tumor ER, and following some competitive binding assay with tritiated estradiol plus/minus a massive excess of non-radioactive ligand, which was either an estrogen or an antiestrogen (Jordan et al., 1988), the total ER tumor concentrations was established. Based on the 1974 Conference in Bethesda (McGuire et al., 1975), it was decided that tumors would be classified as either ER positive (above 10 fm/mg cytosol protein) or ER negative (below 10 fm/mg cytosol protein). Tumors that were ER positive would most likely respond to endocrine ablation or high-dose estrogen therapy but ER-negative tumors were unlikely to respond (McGuire et al., 1975).

p0205 Failure of endocrine therapy (Ingle et al., 1981) usually occurs after about a year or two of treatment in metastatic breast cancer (stage IV). The received wisdom was that the ER-positive cells were dying and the tumor was being repopulated with ER-negative cells. However, this did not explain the fact that clinicians could identify an endocrine therapy responsive tumor that would respond and fail but then respond again to a different endocrine therapy. This could continue for several cycles and is referred to as the "endocrine cascade." Clearly some other mechanism of acquired resistance was occurring. The adaptations of the tumor to the environment during treatment were illustrated by the responses of some tumors to high-dose DES therapy. We noted earlier that Haddow observed that some tumors melted away, but during the 1960s and 1970s, Basil Stoll showed that some tumors would regress but they would regrow during DES therapy only to regress again once DES treatment was stopped (Stoll, 1977b). This was called a "withdrawal response." There was no explanation for all these events.

p0210 During the 1980s, with the general acceptance by the clinical community that clinical trials had to be started to test long-term adjuvant tamoxifen therapy, it became clear that there was a need for realistic laboratory models of acquired drug resistance to tamoxifen. These would be necessary to assess mechanisms of resistance and subvert the process, but more importantly, in the short term, to discover effective second-line therapies for patients that prematurely recur during adjuvant tamoxifen treatment.

p0215 Tamoxifen blocks estradiol-stimulated MCF-7 tumor growth when cells are inoculated into athymic mice (Osborne, Hobbs, & Clark, 1985). However, tamoxifen cannot control tumor growth indefinitely; eventually, MCF-7 tumors grow despite continuing tamoxifen treatment (Osborne, Coronado, & Robinson, 1987). This situation was examined from another

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:24 Page Number: 161

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

perspective using serial transplantations of MCF-7 tumors with acquired tamoxifen resistance. Remarkably, the growth of tumors with acquired tamoxifen resistance is dependent upon tamoxifen (Gottardis & Jordan, 1988; Gottardis, Wagner, Borden, & Jordan, 1989) or indeed any SERM such as raloxifene or toremifene (O'Regan et al., 2002). Physiologic estrogen treatment also caused tumors to grow so the ER mechanism was reconfigured in the breast cancer cells to grow with either estrogen or tamoxifen as the binding ligand. No treatment or treatment with a pure steroidal antiestrogen ICI 164,384 (Gottardis, Jiang, Jeng, & Jordan, 1989) (the lead compound for the series that became the clinically approved drug fulvestrant). These data in the laboratory presaged the subsequent clinical findings that either an aromatase inhibitor (no estrogen) or fulvestrant would be appropriate second-line therapies following treatment failure with tamoxifen (Howell et al., 2004; Osborne et al., 2002).

p0220 The issue of the development of acquired resistance to tamoxifen within a year or two when used for the treatment of metastatic breast cancer (stage IV) appeared to be replicated in the laboratory (Gottardis & Jordan, 1988), but the fact that adjuvant tamoxifen treatment could be continued for 5 years without rapid early treatment recurrences was not explained by the laboratory models developed in the 1980s. Again serendipity intervened with a chance observation that opened up the study of a new biology of estrogen-induced apoptosis.

s0050 7. ESTROGEN-INDUCED APOPTOSIS: BACK TO THE BEGINNING

p0225 The MCF-7 tumor model of acquired resistance to tamoxifen was a significant advance for the study of SERM resistance, but the tumor biology could only be retained *in vivo*, through repeated transplantation into generations of athymic mice every 4 or 5 months. Cell culture models of antihormone therapy were becoming available (Sweeney, McDaniel, Maximov, Fan, & Jordan, 2012) once it was realized that the MCF-7 cell line, that had actually been derived from a patient treated with high-dose DES, was subsequently grown and propagated *in vitro* in a media rich in an estrogen as a contaminant of the phenol red redox indicator (Berthois, Katzenellenbogen, & Katzenellenbogen, 1986; Bindal, Carlson, Katzenellenbogen, & Katzenellenbogen, 1988; Bindal & Katzenellenbogen, 1988). Studies removing all estrogens from media initially cause MCF-7 cells to die but remaining cells adapt and grow

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:25 Page Number: 162

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

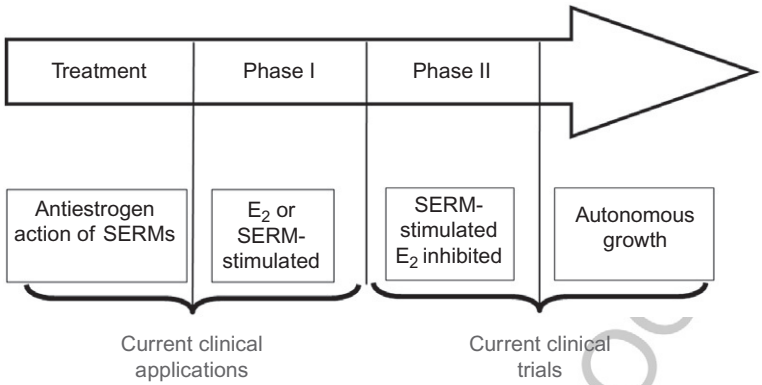
independent of estrogen but retain the ER (i.e., do not become ER negative) (Katzenellenbogen, Kendra, Norman, & Berthois, 1987; Welshons & Jordan, 1987). These early studies would replicate the action of aromatase inhibitor on the ER-positive tumor. During the next decade, numerous cell lines would be created (Herman & Katzenellenbogen, 1994; Jiang, Wolf, Yingling, Chang, & Jordan, 1992; Masamura, Santner, Heitjan, & Santen, 1995; Pink, Jiang, Fritsch, & Jordan, 1995; Shim et al., 2000) that would yield further insights into estrogen action in the twenty-first century. However, the breakthrough in the understanding of the evolution of acquired resistance to tamoxifen was to come from the years of retransplantation of MCF-7 tumors into tamoxifen-treated athymic mice. Continuous retransplantation into tamoxifen-treated mice over a 5-year period changes the tumor cell response to physiological estrogen treatment from a survival signal to a trigger of apoptosis (Wolf & Jordan, 1993; Yao et al., 2000). Small tumors do not grow with physiologic estradiol treatment but melt away completely. Large tumors undergo dramatic regression but eventually start to regrow vigorously with continuing estradiol treatment. Retransplantation of the growing tumors into new athymic mice demonstrates growth is dependent upon estrogen treatment, no treatment results in no tumor growth, and tamoxifen again inhibits estradiol-stimulated growth (Fig. 7.6). The estrogen destroys cells with acquired resistance to tamoxifen with the remaining tumor tissue again responsive to tamoxifen treatment. These laboratory findings were reproducible and exhibited a cyclical pattern of sensitivity and resistance indicating a plasticity in the tumor cell population (Balaburski et al., 2010; Yao et al., 2000). They also Au7
suggested a mechanism to explain the sustained and enhanced antitumor effect of tamoxifen *after* a long duration of the SERM had been administered (at least 5 years). It was proposed that acquired drug resistance evolves through Phase I resistance where both estrogen and tamoxifen stimulate tumor growth and then the survival mechanisms are reconfigured so that, in Phase II that occurs before 5 years, only tamoxifen supports the survival of micrometastases; physiologic estrogen causes tumor cell death (Fig. 7.6) (Jordan, 2004). The use of adjuvant tamoxifen for 5 years prepares the micrometastatic disease to be destroyed by the woman's own estrogen once the tamoxifen is stopped (Wolf & Jordan, 1993). Mortality continues to decrease as micrometastatic disease is eradicated (EBCTCG, 2005). The same events would explain the sustained effects of long-term tamoxifen treatment in the chemoprevention setting (Cuzick et al., 2006; Fisher et al., 2005; Powles et al., 2007).

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:25 Page Number: 163

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007



f0030 **Figure 7.6** The evolution of drug resistance (Jordan, 2004). (See Color Insert.)

t0005 **Table 7.1** Response rates to high-dose DES treatment of breast cancer patients in Lonning et al. (2001)

Response		
Complete	**Partial**	**Stable disease**
4[a]/32	6/32	2/32

np0005 [a]One patient remains disease-free 10 years and 6 months after commencing DES treatment.

p0230 These laboratory data (Wolf & Jordan, 1993; Yao et al., 2000) also proposed the potential use of high- or low-dose estrogen treatment as a salvage therapy for patients who had received exhaustive (i.e., the endocrine cascade) antihormone therapy. This strategy has been evaluated clinically. Lonning et al. (2001) noted an overall 30% response rate to high-dose DES (15 mg daily) (Table 7.1) and one patient had a remarkable response continuing to last now for over 10 years.

> *One of the patients (AO) who achieved a complete response of a 16 × 16 mm cytological confirmed chest wall relapse, received DES treatment for five years, where after she been subject to regular follow-up without active treatment. To this day, she remains disease-free 10 years and six months after commencing DES treatment.*
>
> *(Lonning, 2009)*

p0235 Recently, Ellis et al. (2009) have tested the "low-dose" estrogen therapy hypothesis (Yao et al., 2000) and noted a similar clinical benefit (~29%) for women receiving either 6 mg estradiol daily or 30 mg estradiol daily, after failing an aromatase inhibitor. Responses were not as profound in the Ellis study (Ellis et al., 2009) compared with the Lonning study

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:26 Page Number: 164

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

(Lonning et al., 2001) probably because the patients in the Ellis study were not treated "exhaustively" with antihormones and had therefore not evolved to Phase II endocrine resistance.

p0240 The "Phase II" tamoxifen resistance model also taught another interesting lesson. The pure antiestrogen fulvestrant produced tumoristasis, whereas physiologic estrogen causes profound tumor regression starting after about 1 week (Osipo, Gajdos, Liu, Chen, & Jordan, 2003). However, a combination of fulvestrant plus physiologic estrogen causes dramatic tumor growth (Osipo et al., 2003). These data imply that a combination of fulvestrant and aromatase inhibitor as endocrine therapy following failure of long-term tamoxifen treatment may produce better tumor control than fulvestrant alone (presupposing one does not use physiologic estrogen to treat patients alone first!) Although results are not exactly optimal, two large treatment trials have recently been published using similar dosage regimens. One shows significant PFS and survival advantages for the combination (Mehta et al., 2012), whereas the other does not (Bergh et al., 2012). However, neither trial uses optimal fulvestrant therapy, that is, 500 mg, or twice the recommended monthly dose of 250 mg (Di Leo et al., 2010).

p0245 With the relentless rise of interest in the development of an aromatase inhibitor to replace tamoxifen as the long-term adjuvant therapy of choice for postmenopausal women, studies of resistance moved naturally to study the effect of estrogen withdrawal on ER-positive cells *in vitro*. The experimental results (Song et al., 2001) were to dovetail nicely into results from prior studies with tamoxifen *in vivo* (Yao et al., 2000). Long-term estrogen-deprived (LTED) MCF-7 cells could initially gain a "supersensitivity" to estrogen in the environment once the main source of estrogen had been removed. In other words, the original studies (Katzenellenbogen et al., 1987; Welshons & Jordan, 1987) that demonstrated initial cell death when MCF-7 cells were exposed to an estrogen-free environment, but then a population of cells grew spontaneously. This "estrogen-free growth" was interpreted as the cells being selected that were "hypersensitive" to extremely low estrogen concentrations (Masamura et al., 1995; Shim et al., 2000). But further examinations of concentration response relationship showed that estrogen-induced apoptosis occurred in these cells (Song et al., 2001), but not just at high concentrations but at low concentrations as predicted by the MCF-7 tamoxifen-resistant model *in vivo* (Jordan, Liu, & Dardes, 2002). Specific clones of MCF-7 cells generated from populations of LTED MCF-7 cells (Jiang et al., 1992; Pink et al., 1995) can undergo immediate estrogen-induced apoptosis (MCF-7:5C)

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:26 Page Number: 165

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

(Lewis et al., 2005) or apoptosis induced by estrogen a week later (MCF-7:2A) (Ariazi et al., 2011).

p0250 Several facts are emerging to understand the new biology of estrogen-induced apoptosis. The molecular events to trigger apoptosis with physiologic estrogen initiate the mitochondrial or intrinsic pathway first and then for the final execution there is recruitment of the extrinsic pathway (Lewis et al., 2005). This process is fundamentally different to cytotoxic chemotherapy that immediately causes a G1 blockade with a commitment to program cell death within 12 h. Massive DNA disruption requires immediate action by the cell.

p0255 What then is the physiologic trigger for estrogen-induced apoptosis? Apoptosis caused by estrogen can be modulated and is dependent upon the shape of the ligand ER complex. Estrogens are classified (Jordan et al., 2001) into Class I or planar estrogens such as estradiol or DES and Class II or angular estrogens such as hydroxylated triphenylethylenes. Both classes of estrogen *cause* cell replication but only Class I estrogens, which permit the ligand to be sealed within the ligand-binding domain (Brzozowski et al., 1997; Shiau et al., 1998), can initiate immediate estrogen-induced apoptosis in the correctly configured estrogen-deprived breast cancer cell. Coactivators must bind to the ER complex for growth or apoptosis (Hu et al., 2011). By contrast, an estrogenic triphenylethylene in Class II alters the shape of the ER complex "so that it temporarily adapts to the shape of an antiestrogenic ER complex" (Maximov et al., 2011) which cannot adequately bind coactivators to delay estrogen-induced apoptosis. These data dramatically illustrate the promiscuous nature of cell replication and survival with almost any signal input that is minimally adequate to bind to the ER. The signal for death must be precise because it is final for the cell.

p0260 One obvious application for the discovery of the cellular mechanisms that *prevent* estrogen-induced apoptosis is to deploy a companion therapy to neutralize resistance to apoptosis and enhance responsiveness to estrogen. Looked at simply, it would be an advantage to enhance apoptosis and convert clinical responses of 30% for estrogen-treated patients following exhaustive antihormone therapy to over 50%. Two approaches have addressed the goal of enhancing response rates to physiologic estrogen-induced apoptosis. First, the MCF-7:2A cells have a delayed response to estrogen-induced apoptosis and also have an enhanced glutathione synthetic pathway (Ariazi et al., 2011). Glutathione protects against oxidative stress. The administration of buthioninesulphoximine that blocks the synthesis of glutathione causes rapid estrogen-induced apoptosis (Lewis-Wambi, Swaby, Kim, & Jordan, 2009; Lewis-Wambi et al., 2008). Second, it was believed that blocking the cSrc

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:26 Page Number: 166

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

oncogene, which is present in 70% of human breast cancer, would further enhance estrogen-induced apoptosis of the breast cancers treated exhaustively with antihormone therapy. In fact, blocking cSrc actually also blocked estrogen-induced apoptosis (Fan et al., 2012). This was not the anticipated result, but this is new knowledge that must, in the future, be considered when dissecting the trigger mechanism of estrogen-induced apoptosis. It could not have been predicted that cSrc was essential for estrogen-induced apoptosis. The discovery of the precise triggering mechanism for estrogen-induced apoptosis will, because it is biologically unique, provide additional approaches to discover new targeted therapies.

8. THE LEGACY OF TAMOXIFEN

p0265 In 1970, there was no tamoxifen, only ICI 46,474, a failed "morning after pill," that was abandoned by the pharmaceutical industry in 1972 (Jordan, 2003c, 2006). By a series of fortunate friendship and the key individuals being in the right place at the right time, the first target drug in breast cancer therapy, tamoxifen, was reinvented (Jordan, 2008b) to become a life-saving medicine, the first SERM, the first chemopreventive drug to reduce the risk of any cancer and the drug that would throw light on the "mechanism of estrogen-induced apoptosis" solving Haddow's paradox when he deployed the first chemical therapy, high-dose estrogen, to treat breast cancer successfully (Jordan, 2008a).

p0270 There are two additional therapeutic advances that tamoxifen catalyzed: the aromatase inhibitors and the development of the SERM principle as a multifunctional drug group.

p0275 Angela Brodie's dedicated and pioneering work (Brodie & Longcope, 1980; Brodie, Marsh, & Brodie, 1979; Brodie, Schwarzel, Shaikh, & Brodie, 1977; Coombes, Goss, Dowsett, Gazet, & Brodie, 1984) was essential as proof of principle that a selective aromatase inhibitor could be discovered with clinical efficacy. The problem with her discovery, 4-hydroxyandrostenedione, was that it was an injectable rather than a more convenient oral preparation. However, the fact that the failed "morning after pill" ICI 46,474 was transformed successfully into the "gold standard" tamoxifen for the adjuvant treatment of breast cancer provided a new target (the aromatase enzyme) to improve antihormonal therapy in breast cancer. With profits expanding from sales of tamoxifen in the United States after 1990, the key issue for the successful drug development of an aromatase inhibitor would be satisfied: profits. The patent from tamoxifen would

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:27 Page Number: 167

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

run out in America, and aromatase inhibitors be substituted. Three orally active third-generation aromatase inhibitors were subsequently successfully developed for adjuvant therapy: anastrazole, letrozole, and exemestane. Each was demonstrated to have a small but consistent improvement over 5 years of tamoxifen alone whether given instead of tamoxifen in postmenopausal patients, after 5 years of tamoxifen or switching after a couple of years of tamoxifen (Baum et al., 2002; Boccardo et al., 2005; Coates et al., 2007; Coombes et al., 2004; Goss et al., 2003, 2005; Howell et al., 2005; Thurlimann et al., 2005). There has even been a successful trial of exemestane as a preventive in postmenopausal high-risk women (Goss et al., 2011). However, it is hard to see how this approach would be superior to a sophisticated third-generation SERM functioning as a multifunctional medicine in women's health.

p0280 The advantages of aromatase inhibitors for postmenopausal patients are clear in large population trials and for health-care systems. Patents for aromatase inhibitors are running out or have run out and cheap generics are becoming available. (The aromatase inhibitors were initially priced extremely high compared to tamoxifen to compensate for each only securing about one-third of the original tamoxifen market.) A disease-free survival advantage is noted for adding an aromatase inhibitor to the treatment plan compared to tamoxifen alone (Dowsett et al., 2010) and concerns about endometrial cancer and blood clots are diminished. Current clinical studies to improve endocrine response rates seek to exploit emerging knowledge about the molecular mechanisms of antihormone resistance to aromatase inhibitors (Roop & Ma, 2012). Combinations of letrozole and lapatinib, an inhibitor of the HER2 pathway, show some advantages over letrozole alone in ER-positive and HER-positive metastatic breast cancer (Riemsma et al., 2012). A similar improvement in responsiveness to aromatase inhibitors is noted with a combination with the mTor inhibitor everolimus (Bachelot et al., 2012; Baselga et al., 2012, 2009).

p0285 The second major advance in therapeutics catalyzed by tamoxifen is the SERM group of medicines. The cluster of laboratory findings in the 1980s that described the fact that the "nonsteroidal antiestrogens" were actually targeted estrogens and antiestrogens in select estrogen target tissues (Jordan, 2001) prompted a significant effort by the pharmaceutical industry to exploit the concept with new SERMs (Jordan, 2003a, 2003b). This, in large measure, was because both tamoxifen and raloxifene were so successful economically. The osteoporosis market is much bigger than the endocrine treatment of breast cancer.

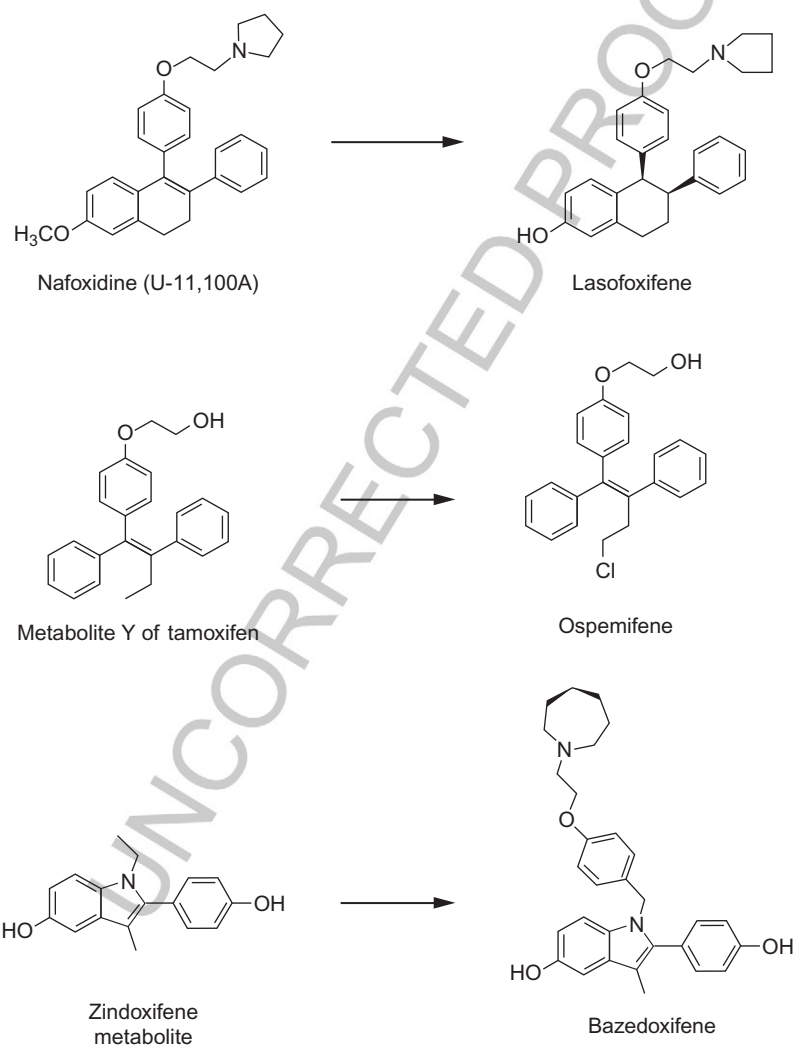
UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:27 Page Number: 168

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

p0290 Today considerable scientific success has been achieved with new SERMs, but it remains a challenge to create a drug with absolute safety guarantees for all women. The bar is now very high by necessity as prevention of multiple diseases implies that subjects who are the target population are in fact currently well. We will comment briefly on three compounds: lasofoxifene, basedoxifene, and ospemifene, but first it is worth mentioning that the molecules each have a "history" (Fig. 7.7). Lasofoxifene started its



f0035 **Figure 7.7** The historical origins of the new SERMs.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:28 Page Number: 169

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

molecular odyssey from its origins in the fertility control program at Upjohn in Kalamazoo in the early 1960s (Lednicer, Emmert, Duncan, & Lyster, 1967; Lednicer, Lyster, & Duncan, 1967). The antiestrogen/postcoital contraceptive, U11,100A, was discovered and initially discovered by the fertility control program. But U11,100A was reinvented to become nafoxidine for the treatment of breast cancer (Legha et al., 1976). Unfortunately, this program was abandoned because of the severe side effect of photophobia. Lasofoxifene is a very potent SERM with an effective dose of 0.5 mg daily being recommended for the treatment and prevention of osteoporosis (Cummings et al., 2010). This is 1/100th the recommended daily dose of raloxifene (60 mg daily). Medicinal chemists discovered that the levorotatory enantiomer is resistant to glucuronidation so that the molecule is not readily excreted (Rosati et al., 1998). The registration trial of postmenopausal evaluation and risk-reduction with lasofoxifene documented significant decreases in ER-positive breast cancer, coronary heart disease, strokes, and endometrial cancer (Cummings et al., 2010). Basically, all that the original roadmap (Lerner & Jordan, 1990) predicted for a SERM to prevent breast cancer (and endometrial cancer) as beneficial side effect for the prevention of osteoporosis and coronary heart disease. Lasofoxifene is approved in the European Union, but not in the United States.

p0295 Bazedoxifene (Fig. 7.7) (Gruber & Gruber, 2004; Komm et al., 2005; Miller et al., 2001), evolved from the metabolite of an earlier compound zindoxifene that failed to have antitumor activity in clinical trial (Stein et al., 1990), actually showed estrogen-like activity in laboratory studies (Robinson, Koch, & Jordan, 1988). Introduction of the appropriate phenylalkylaminoethoxy side chain created an important new SERM. Bazedoxifene is of interest as it has not only been tested as a SERM for the prevention of osteoporosis (Kawate & Takayanagi, 2011; Silverman et al., 2012) but also has been evaluated as a new kind of hormone replacement therapy, that is, bazedoxifene plus conjugated equine estrogens (CEE) (Kagan, Williams, Pan, Mirkin, & Pickar, 2010; Lindsay, Gallagher, Kagan, Pickar, & Constantine, 2009; Pinkerton, Pickar, Racketa, & Mirkin, 2012). There is an additive effect on bone density, but the SERM blocks breast and endometrial actions of estrogen. Clearly, this is an innovation application of SERMs that clearly avoids the tumorigenic effect of both CEE and synthetic progestin (Crandall et al., 2012).

p0300 Last, there is ospemifene (Fig. 7.7). The history of ospemifene is interesting as it has also evolved from a previously researched predecessor. A new metabolite of tamoxifen (metabolite Y) was reported in 1982–1983 and was

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:28 Page Number: 170

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

shown to have weak antiestrogenic properties (Bain & Jordan, 1983; Jordan, Bain, Brown, Gosden, & Santos, 1983). Later, a similar metabolite was found for another antiestrogen toremifene, and this metabolite is now known as ospemifene. Originally, ospemifene was developed to treat vaginal atrophy in postmenopausal women, but it also can be useful for the prevention and treatment of osteoporosis. In clinical trials, ospemifene was shown to be well tolerated and have a safe toxicity profile (DeGregorio et al., 2000; Rutanen et al., 2003; Voipio et al., 2002). However, there is still not enough data from the trials to assess the effectiveness of ospemifine in regard with osteoporosis or breast cancer prevention.

p0305 However, with all the SERMs, the principal issue can be the quality of life for the patient. Hormone replacement therapy solves the menopausal symptom of hot flashes and night sweats. This is an important issue for those with severe symptoms. The SERMs, at present, are known to exacerbate rather than resolve this issue. However, prompted by potential markets, pharmaceutical chemists are attempting to decipher the complexities of this important SERM side effect to allow therapeutic compliance with SERMs to become optimal (Jain et al., 2006, 2009; Wallace et al., 2006; Watanabe et al., 2003).

p0310 So how far can the SERM concept go? Already medicinal chemists have created selective agonist/antagonist for all members of the nuclear receptor superfamily (Fan & Jordan, 2013), and there is the promise of the further understanding of selective ERα/ERβ modulators (Sengupta & Jordan, 2013). The products, should they find applications in the clinic, hold the promise of treating diseases selectively that could never have been imagined 40 years ago.

p0315 But all is not resolved with SERMs and one discovery in the early 1980s remains a work in progress. The availability of [^{3}H] tamoxifen allowed the identification of an "antiestrogen-binding protein" by Sutherland et al. (1980). It was hypothesized that it could be linked with antiestrogen action, but in recent years, compelling evidence has been presented that it plays a role in cholesterol metabolism (Payre et al., 2008) and is identified in mice as membranous epoxide hydrolase. The biology is complex but there are suggestions that this may be a mechanism for tumoricidal action (Delarue et al., 1999; Payre et al., 2008).

p0320 In coming to the end of our story, we return to the beginning of chemical therapy for cancer. Sir Alexander Haddow FRS, it is fair to state, actually became the catalyst for change in the chemical treatment of cancer. In 1970, there were no tests to establish the sensitivity of a tumor to chemical therapy.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:29 Page Number: 171

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

It was empirical medicine of trial and error in patient based on often suspect clinical experience rather than rigorously controlled clinical trials. Haddow advanced clinical certainty during his career from individual experience by organizing a small clinical trial to obtain preliminary data (Haddow et al., 1944), with a subsequent large multicentered trial to ensure a valid result was being promoted to improve clinical practice. To prove the validity and reproducibility of these preliminary data, a collaborative clinical trial was organized with a dozen centers throughout the United Kingdom organized by the Royal Society of Medicine (Haddow was the President of the Section of Oncology at the Royal Society of Medicine). He stated his discovery during his 1970 Karnofsky lecture:

> *When the various reports were assembled at the end of that time, it was fascinating to discover that rather general impression, not sufficiently strong from the relatively small numbers in any single group, became reinforced to the point of certainty; namely, the beneficial responses were three times more frequent in women over the age of 60 years than in those under that age; that oestrogens may, on the contrary, accelerate the course of mammary cancer in younger women, and that their therapeutic use should be restricted to cases 5 years beyond the menopause. Here was an early and satisfying example of the advantages which may accrue from cooperative clinical trial.*
>
> *(Haddow, 1970)*

p0325 A similar conclusion was noted by Stoll (1977b) through a review of his lifetime experience with 407 postmenopausal patients with stage IV breast cancer treated with high-dose estrogen (Table 7.2). It is clear a prolonged period of estrogen deprivation after the menopause is needed for the optimal apoptotic activity of estrogen to develop.

p0330 These early data have relevance to solve a current paradox in women's health that has major significance. The Women's Health Initiative (WHI) Study of combination CEE and the synthetic progestin medroxyprogesterone acetate (HRT) (to prevent endometrial cancer) was initiated to assess the effects of HRT on improving women's health, that is, preventing fractures, coronary heart disease, and Alzheimer's, and balancing

t0010 **Table 7.2** Objective response rates in postmenopausal women with metastatic breast cancer using high-dose estrogen therapy

Age since menopause	Patient #	Regression (%)
Postmenopausal 0–5 years	63	9
Postmenopausal >5 years	344	35

The 407 patients are divided in relation to menopause (Stoll, 1977b).

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:29 Page Number: 172

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

this with the known side effects of increasing the incidence of breast cancer and thromboembolic disorders. The study did show a decrease in osteoporotic fractures but no benefit for coronary heart disease or for Alzheimer's disease (Rossouw et al., 2002). Breast cancer incidence was increased (Chlebowski et al., 2003; Shumaker et al., 2003). However, the examination of the second WHI trial of CEE alone versus placebo in hysterectomized postmenopausal women showed an initial decrease in breast cancer incidence (Anderson et al., 2004; Prentice et al., 2008), and then a further decrease that was sustained for 5 years after CEE treatment was terminated (LaCroix et al., 2011). A recent analysis demonstrates rather remarkably not only a sustained decrease in breast cancer incidence but also all cancers and a significant decrease in mortality (Anderson et al., 2012). The population of Au8 women were aged an average of 68 years, that is, following a long period of estrogen deprivation CEE causes a tumoricidal action which fits nicely with the Haddow/Stoll explanation of needing an "estrogen holiday" to create the correct antitumor sensitivity to estrogen. In other words, estrogen should not be given alone straight after menopause as an ERT. These data obtained in the modern era close the circle on our current understanding of estrogen action in the life and death of breast cancer cells (Jordan, 2008a). The saga of SERMs not only advanced women's health, dramatically improving survivorship and preventing both breast cancer and osteoporosis but also created the opportunity to discover the new biology of estrogen-induced apoptosis. This natural mechanism is programmed in a completely different way than the cellular response to cytotoxic therapy. Our ability to decipher the actual trigger of estrogen-induced apoptosis may open up new opportunities in targeted cancer therapeutics.

ACKNOWLEDGMENTS

This work (V. C. J.) was supported by the Department of Defense Breast Program under Award number W81XWH-06-1-0590 Center of Excellence, subcontract under the SU2C (AACR) Grant number SU2C-AACR-DT0409, the Susan G. Komen for the Cure Foundation under Award number SAC100009, and the Lombardi Comprehensive Cancer 1095 Center Support Grant (CCSG) Core Grant NIH P30 CA051008. The views and opinions of the author(s) do not reflect those of the U.S. Army or the Department of Defense.

REFERENCES

Allen, E., & Doisey, E. A. (1923). An ovarian hormone: Preliminary reports on its localization, extraction and partial purification and action in test animals. *Journal of the American Medical Association*, *81*(10), 819–821.

Anderson, G. L., Chlebowski, R. T., Aragaki, A. K., Kuller, L. H., Manson, J. E., Gass, M., et al. (2012). Conjugated equine oestrogen and breast cancer incidence and mortality in

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:29 Page Number: 173

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

postmenopausal women with hysterectomy: Extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *The Lancet Oncology*, *13*(5), 476–486.

Anderson, G. L., Limacher, M., Assaf, A. R., Bassford, T., Beresford, S. A., Black, H., et al. (2004). Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: The Women's Health Initiative randomized controlled trial. *Journal of the American Medical Association*, *291*(14), 1701–1712.

Ariazi, E. A., Cunliffe, H. E., Lewis-Wambi, J. S., Slifker, M. J., Willis, A. L., Ramos, P., et al. (2011). Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(47), 18879–18886.

Bachelot, T., Bourgier, C., Cropet, C., Ray-Coquard, I., Ferrero, J. M., Freyer, G., et al. (2012). Randomized phase II trial of everolimus in combination with tamoxifen in patients with hormone receptor-positive, human epidermal growth factor receptor 2-negative metastatic breast cancer with prior exposure to aromatase inhibitors: A GINECO study. *Journal of Clinical Oncology*, *30*(22), 2718–2724.

Bain, R. R., & Jordan, V. C. (1983). Identification of a new metabolite of tamoxifen in patient serum during breast cancer therapy. *Biochemical Pharmacology*, *32*(2), 373–375.

Balaburski, G. M., Dardes, R. C., Johnson, M., Haddad, B., Zhu, F., Ross, E. A., et al. (2010). Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: A unifying concept in anti-hormone resistance. *International Journal of Oncology*, *37*(2), 387–398.

Baselga, J., Campone, M., Piccart, M., Burris, H. A., 3rd., Rugo, H. S., Sahmoud, T., et al. (2012). Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. *The New England Journal of Medicine*, *366*(6), 520–529.

Baselga, J., Semiglazov, V., van Dam, P., Manikhas, A., Bellet, M., Mayordomo, J., et al. (2009). Phase II randomized study of neoadjuvant everolimus plus letrozole compared with placebo plus letrozole in patients with estrogen receptor-positive breast cancer. *Journal of Clinical Oncology*, *27*(16), 2630–2637.

Baum, M., Budzar, A. U., Cuzick, J., Forbes, J., Houghton, J. H., Klijn, J. G., et al. (2002). Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: First results of the ATAC randomised trial. *The Lancet*, *359*(9324), 2131–2139.

Baumler, E. (1984). *Paul Ehrlich: Scientist for life*. (1st ed.). New York: Holmes & Meier Publishers.

Beall, P. T., Misra, K. L., Young, R. L., Spjut, H. J., Evan, H. J., & LeBlanc, A. (1985). Clomiphene protects against osteoporosis in the mature ovariectomized rat. *Calcified Tissue International*, *36*, 123–125.

Beatson, G. T. (1896). On the treatment of inoperable cases of carcinoma of the mamma: Suggestions for a new method of treatment, with illustrative cases. *The Lancet*, *148*, 104–107.

Bergh, J., Jonsson, P. E., Lidbrink, E. K., Trudeau, M., Eiermann, W., Brattstrom, D., et al. (2012). FACT: An open-label randomized phase III study of fulvestrant and anastrozole in combination compared with anastrozole alone as first-line therapy for patients with receptor-positive postmenopausal breast cancer. *Journal of Clinical Oncology*, *30*(16), 1919–1925.

Berthois, Y., Katzenellenbogen, J. A., & Katzenellenbogen, B. S. (1986). Phenol red in tissue culture media is a weak estrogen: Implications concerning the study of estrogen-responsive cells in culture. *Proceedings of the National Academy of Sciences of the United States of America*, *83*(8), 2496–2500.

Bindal, R. D., Carlson, K. E., Katzenellenbogen, B. S., & Katzenellenbogen, J. A. (1988). Lipophilic impurities, not phenolsulfonaphthalein, account for the estrogenic activity in commercial preparations of phenol red. *Journal of Steroid Biochemistry*, *31*(3), 287–293.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:30 Page Number: 174

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

Bindal, R. D., & Katzenellenbogen, J. A. (1988). Bis(4-hydroxyphenyl)[2-(phenoxysulfonyl) phenyl]methane: Isolation and structure elucidation of a novel estrogen from commercial preparations of phenol red (phenolsulfonphthalein). *Journal of Medicinal Chemistry*, *31*(10), 1978–1983.

Black, L. J., Jones, C. D., & Falcone, J. F. (1983). Antagonism of estrogen action with a new benzothiophene derived antiestrogen. *Life Sciences*, *32*(9), 1031–1036.

Boccardo, F., Rubagotti, A., Puntoni, M., Guglielmini, P., Amoroso, D., Fini, A., et al. (2005). Switching to anastrozole versus continued tamoxifen treatment of early breast cancer: Preliminary results of the Italian Tamoxifen Anastrozole Trial. *Journal of Clinical Oncology*, *23*(22), 5138–5147.

Bonadonna, G., Brusamolino, E., Valagussa, P., Rossi, A., Brugnatelli, L., Brambilla, C., et al. (1976). Combination chemotherapy as an adjuvant treatment in operable breast cancer. *The New England Journal of Medicine*, *294*(8), 405–410.

Boyd, S. (1900). On oophorectomy in cancer of the breast. *British Medical Journal*, *2*, 1161–1167. Au9

Brauch, H., Schroth, W., Goetz, M. P., Mürdter, T. E., Winter, S., Ingle, J. N., et al. (2013). Tamoxifen use in postmenopausal breast cancer: CYP2D6 matters. *Journal of Clinical Oncology*, *31*, 176–180. Au10

Breast Cancer Facts and Figures (2011–2012). Atlanta, American Cancer Society: American Cancer Society, Inc.

Breast Cancer Survival Rates by Stage (2011). American Cancer Society.

Brodie, A. M., & Longcope, C. (1980). Inhibition of peripheral aromatization by aromatase inhibitors, 4-hydroxy- and 4-acetoxy-androstene-3,17-dione. *Endocrinology*, *106*(1), 19–21.

Brodie, A. M., Marsh, D., & Brodie, H. J. (1979). Aromatase inhibitors. IV. Regression of hormone-dependent, mammary tumors in the rat with 4-acetoxy-4-androstene-3,17-dione. *Journal of Steroid Biochemistry*, *10*(4), 423–429.

Brodie, A. M., Schwarzel, W. C., Shaikh, A. A., & Brodie, H. J. (1977). The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. *Endocrinology*, *100*(6), 1684–1695.

Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., et al. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*, *389*(6652), 753–758.

Canadian Cancer Statistics (2012). Canadian Cancer Society.

Chlebowski, R. T., Hendrix, S. L., Langer, R. D., Stefanick, M. L., Gass, M., Lane, D., et al. (2003). Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: The Women's Health Initiative Randomized Trial. *Journal of the American Medical Association*, *289*(24), 3243–3253.

Coates, A. S., Keshaviah, A., Thurlimann, B., Mouridsen, H., Mauriac, L., Forbes, J. F., et al. (2007). Five years of letrozole compared with tamoxifen as initial adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer: Update of study BIG 1-98. *Journal of Clinical Oncology*, *25*(5), 486–492.

Cole, M. P., Jones, C. T., & Todd, I. D. (1971). A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. *British Journal of Cancer*, *25*(2), 270–275.

Consensus conference (1985). Adjuvant chemotherapy for breast cancer. *Journal of the American Medical Association*, *254*(24), 3461–3463.

Coombes, R. C., Goss, P., Dowsett, M., Gazet, J. C., & Brodie, A. (1984). 4-Hydroxyandrostenedione in treatment of postmenopausal patients with advanced breast cancer. *The Lancet*, *2*(8414), 1237–1239.

Coombes, R. C., Hall, E., Gibson, L. J., Paridaens, R., Jassem, J., Delozier, T., et al. (2004). A randomized trial of exemestane after two to three years of tamoxifen therapy in

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:30 Page Number: 175

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

postmenopausal women with primary breast cancer. *The New England Journal of Medicine*, *350*(11), 1081–1092.

Cooper, R. G. (1969). Combination chemotherapy in hormone resistant breast cancer. *Proceeding of the American Association for Cancer Research*, *10*, 15.

Crandall, C. J., Aragaki, A. K., Cauley, J. A., McTiernan, A., Manson, J. E., Anderson, G., et al. (2012). Breast tenderness and breast cancer risk in the estrogen plus progestin and estrogen-alone women's health initiative clinical trials. *Breast Cancer Research and Treatment*, *132*(1), 275–285.

Cummings, S. R., Eckert, S., Krueger, K. A., Grady, D., Powles, T. J., Cauley, J. A., et al. (1999). The effect of raloxifene on risk of breast cancer in postmenopausal women: Results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *Journal of the American Medical Association*, *281*(23), 2189–2197.

Cummings, S. R., Ensrud, K., Delmas, P. D., LaCroix, A. Z., Vukicevic, S., Reid, D. M., et al. (2010). Lasofoxifene in postmenopausal women with osteoporosis. *The New England Journal of Medicine*, *362*(8), 686–696.

Cuzick, J., & Baum, M. (1985). Tamoxifen and contralateral breast cancer. *The Lancet*, *2*(8449), 282.

Cuzick, J., Forbes, J. F., & Howell, A. (2006). Re: Tamoxifen for the prevention of breast cancer: Current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *Journal of the National Cancer Institute*, *98*(9), 643, author reply 643–644.

Davies, C., Godwin, J., Gray, R., Clarke, M., Cutter, D., Darby, S., et al. (2011). Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: Patient-level meta-analysis of randomised trials. *The Lancet*, *378*(9793), 771–784.

DeGregorio, M. W., Wurz, G. T., Taras, T. L., Erkkola, R. U., Halonen, K. H., & Huupponen, R. K. (2000). Pharmacokinetics of (deaminohydroxy)toremifene in humans: A new, selective estrogen-receptor modulator. *European Journal of Clinical Pharmacology*, *56*(6–7), 469–475.

Delarue, F., Kedjouar, B., Mesange, F., Bayard, F., Faye, J. C., & Poirot, M. (1999). Modifications of benzylphenoxy ethanamine antiestrogen molecules: Influence affinity for antiestrogen binding site (AEBS) and cell cytotoxicity. *Biochemical Pharmacology*, *57*(6), 657–661.

Di Leo, A., Jerusalem, G., Petruzelka, L., Torres, R., Bondarenko, I. N., Khasanov, R., et al. (2010). Results of the CONFIRM phase III trial comparing fulvestrant 250 mg with fulvestrant 500 mg in postmenopausal women with estrogen receptor-positive advanced breast cancer. *Journal of Clinical Oncology*, *28*(30), 4594–4600.

Dieudonne, A. S., Lambrechts, D., Claes, B., Vandorpe, T., Wildiers, H., Timmerman, D., et al. (2009). Prevalent breast cancer patients with a homozygous mutant status for CYP2D6*4: Response and biomarkers in tamoxifen users. *Breast Cancer Research and Treatment*, *118*(3), 531–538.

Dodds, E. (1938). Biological effects of the synthetic oestrogenic substance 4:4′-dihydroxy-alpha:beta-diethylstilbene. *The Lancet*, *232*(6015), 1389–1391.

Dodds, E. C., Goldberg, L., Lawson, W., & Robinsox, R. (1938). Oestrogenic activity of esters of diethyl stilboestrol. *Nature*, *142*(3587), 211–212. Au11

Dowsett, M., Cuzick, J., Ingle, J., Coates, A., Forbes, J., Bliss, J., et al. (2010). Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. *Journal of Clinical Oncology*, *28*(3), 509–518.

EBCTCG, (1998). Tamoxifen for early breast cancer: An overview of the randomised trials. Early Breast Cancer Trialisits' Collaborative Group. *The Lancet*, *351*(9114), 1451–1467.

EBCTCG, (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. *The Lancet*, *365*(9472), 1687–1717.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:31 Page Number: 176

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

EISRCM, (2006). *O Câncer de Mama no Brasil Situacão epidemiológica e rastreamento*. Encontro Internacional Sobre Rasteamento de Cancer de Mama. Au12

Ellis, M. J., Gao, F., Dehdashti, F., Jeffe, D. B., Marcom, P. K., Carey, L. A., et al. (2009). Lower-dose vs. high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: A phase 2 randomized study. *Journal of the American Medical Association*, *302*(7), 774–780.

Ettinger, B., Black, D. M., Mitlak, B. H., Knickerbocker, R. K., Nickelsen, T., Genant, H. K., et al. (1999). Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: Results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. *Journal of the American Medical Association*, *282*(7), 637–645.

Fan, P., & Jordan, V. C. (2013). An emerging principle: Selective nuclear receptor modulators. In V. C. Jordan (Ed.), *Estrogen actions, SERMs, and women's health*. World Scientific. Au12

Fan, P., McDaniel, R. E., Kim, H. R., Clagett, D., Haddad, B., & Craig Jordan, V. (2012). Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines. *European Journal of Cancer*, *48*, 3488–3498. Au13

Ferlay, J., Parkin, D. M., & Steliarova-Foucher, E. (2010). Estimates of cancer incidence and mortality in Europe in 2008. *European Journal of Cancer*, *46*, 765–781.

Fisher, B., Carbone, P., Economou, S. G., Frelick, R., Glass, A., Lerner, H., et al. (1975). 1-Phenylalanine mustard (L-PAM) in the management of primary breast cancer. A report of early findings. *The New England Journal of Medicine*, *292*(3), 117–122.

Fisher, B., Costantino, J., Redmond, C., Poisson, R., Bowman, D., Couture, J., et al. (1989). A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. *The New England Journal of Medicine*, *320*(8), 479–484.

Fisher, B., Costantino, J. P., Wickerham, D. L., Cecchini, R. S., Cronin, W. M., Robidoux, A., et al. (2005). Tamoxifen for the prevention of breast cancer: Current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *Journal of the National Cancer Institute*, *97*(22), 1652–1662.

Fisher, B., Costantino, J. P., Wickerham, D. L., Redmond, C. K., Kavanah, M., Cronin, W. M., et al. (1998). Tamoxifen for prevention of breast cancer: Report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *Journal of the National Cancer Institute*, *90*(18), 1371–1388.

Folca, P. J., Glascock, R. F., & Irvine, W. T. (1961). Studies with tritium-labelled hexoestrol in advanced breast cancer. Comparison of tissue accumulation of hexoestrol with response to bilateral adrenalectomy and oophorectomy. *The Lancet*, *2*(7206), 796–798.

Fornander, T., Rutqvist, L. E., Cedermark, B., Glas, U., Mattsson, A., Silfversward, C., et al. (1989). Adjuvant tamoxifen in early breast cancer: Occurrence of new primary cancers. *The Lancet*, *1*(8630), 117–120.

Furr, B. J., & Jordan, V. C. (1984). The pharmacology and clinical uses of tamoxifen. *Pharmacology and Therapeutics*, *25*(2), 127–205.

Glascock, R. F., & Hoekstra, W. G. (1959). Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep. *The Biochemical Journal*, *72*, 673–682.

Goss, P. E., Ingle, J. N., Ales-Martinez, J. E., Cheung, A. M., Chlebowski, R. T., Wactawski-Wende, J., et al. (2011). Exemestane for breast-cancer prevention in postmenopausal women. *The New England Journal of Medicine*, *364*(25), 2381–2391.

Goss, P. E., Ingle, J. N., Martino, S., Robert, N. J., Muss, H. B., Piccart, M. J., et al. (2003). A randomized trial of letrozole in postmenopausal women after five years of tamoxifen

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:31 Page Number: 177

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

therapy for early-stage breast cancer. *The New England Journal of Medicine*, *349*(19), 1793–1802.

Goss, P. E., Ingle, J. N., Martino, S., Robert, N. J., Muss, H. B., Piccart, M. J., et al. (2005). Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: Updated findings from NCIC CTG MA.17. *Journal of the National Cancer Institute*, *97*(17), 1262–1271.

Gottardis, M. M., Jiang, S. Y., Jeng, M. H., & Jordan, V. C. (1989). Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal anti-estrogens. *Cancer Research*, *49*(15), 4090–4093.

Gottardis, M. M., & Jordan, V. C. (1987). Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Research*, *47*(15), 4020–4024.

Gottardis, M. M., & Jordan, V. C. (1988). Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Research*, *48*(18), 5183–5187.

Gottardis, M. M., Ricchio, M. E., Satyaswaroop, P. G., & Jordan, V. C. (1990). Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. *Cancer Research*, *50*(11), 3189–3192.

Gottardis, M. M., Robinson, S. P., Satyaswaroop, P. G., & Jordan, V. C. (1988). Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Research*, *48*(4), 812–815.

Gottardis, M. M., Wagner, R. J., Borden, E. C., & Jordan, V. C. (1989). Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro. *Cancer Research*, *49*(17), 4765–4769.

Greaves, P., Goonetilleke, R., Nunn, G., Topham, J., & Orton, T. (1993). Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. *Cancer Research*, *53*(17), 3919–3924.

Gruber, C., & Gruber, D. (2004). Bazedoxifene (Wyeth). *Current Opinion in Investigational Drugs*, *5*(10), 1086–1093.

Haddow, A. (1970). David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer*, *26*(4), 737–754.

Haddow, A., Watkinson, J. M., Paterson, E., & Koller, P. C. (1944). Influence of synthetic oestrogens on advanced malignant disease. *British Medical Journal*, *2*(4368), 393–398.

Hard, G. C., Latropoulos, M. J., Jordan, K., Radi, L., Kaltenberg, O. P., Imondi, A. R., et al. (1993). Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. *Cancer Research*, *53*(19), 4534–4541.

Harper, M. J., & Walpole, A. L. (1967a). Mode of action of I.C.I. 46,474 in preventing implantation in rats. *The Journal of Endocrinology*, *37*(1), 83–92.

Harper, M. J., & Walpole, A. L. (1967b). A new derivative of triphenylethylene: Effect on implantation and mode of action in rats. *Journal of Reproduction and Fertility*, *13*(1), 101–119.

Hecker, E., Vegh, I., Levy, C. M., Magin, C. A., Martinez, J. C., Loureiro, J., et al. (1974). Clinical trial of clomiphene in advanced breast cancer. *European Journal of Cancer*, *10*(11), 747–749.

Herman, M. E., & Katzenellenbogen, B. S. (1994). Alterations in transforming growth factor-alpha and -beta production and cell responsiveness during the progression of MCF-7 human breast cancer cells to estrogen-autonomous growth. *Cancer Research*, *54*(22), 5867–5874.

Howell, A., Cuzick, J., Baum, M., Buzdar, A., Dowsett, M., Forbes, J. F., et al. (2005). Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *The Lancet*, *365*(9453), 60–62.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:32 Page Number: 178

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

Howell, A., Robertson, J. F., Abram, P., Lichinitser, M. R., Elledge, R., Bajetta, E., et al. (2004). Comparison of fulvestrant versus tamoxifen for the treatment of advanced breast cancer in postmenopausal women previously untreated with endocrine therapy: A multinational, double-blind, randomized trial. *Journal of Clinical Oncology*, *22*(9), 1605–1613.

Howlader, N., Noone, A. M., Krapcho, M., Neyman, N., Aminou, R., Altekruse, S. F., et al. (2009). *SEER cancer statistics review, 1975–2009 (Vintage 2009 populations)*. Bethesda, MD: National Cancer Institute. *http://seer.cancer.gov/csr/1975_2009_pops09/*.

Hu, Z., Fan, C., Oh, D. S., Marron, J. S., He, X., Qaqish, B. F., et al. (2006). The molecular portraits of breast tumors are conserved across microarray platforms. *BMC Genomics*, *7*, 96.

Hu, Z. Z., Kagan, B. L., Ariazi, E. A., Rosenthal, D. S., Zhang, L., Li, J. V., et al. (2011). Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. *PLoS One*, *6*(6), e20410.

Huggins, C., Grand, L. C., & Brillantes, F. P. (1961). Mammary cancer induced by a single feeding of polymucular hydrocarbons, and its suppression. *Nature*, *189*, 204–207.

INCA (2006). *A Situacao do Cancer no Brasil*. Instituto Nacional de Cancer. Au12

Ingle, J. N., Ahmann, D. L., Green, S. J., Edmonson, J. H., Bisel, H. F., Kvols, L. K., et al. (1981). Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *The New England Journal of Medicine*, *304*(1), 16–21.

Jain, N., Kanojia, R. M., Xu, J., Jian-Zhong, G., Pacia, E., Lai, M. T., et al. (2006). Novel chromene-derived selective estrogen receptor modulators useful for alleviating hot flushes and vaginal dryness. *Journal of Medicinal Chemistry*, *49*(11), 3056–3059.

Jain, N., Xu, J., Kanojia, R. M., Du, F., Jian-Zhong, G., Pacia, E., et al. (2009). Identification and structure-activity relationships of chromene-derived selective estrogen receptor modulators for treatment of postmenopausal symptoms. *Journal of Medicinal Chemistry*, *52*(23), 7544–7569.

Jensen, E. V., & Jacobson, H. I. (1962). Basic guides to the mechanism of estrogen action. *Recent Progress in Hormone Resistence*, *18*, 387–414.

Jiang, S. Y., Wolf, D. M., Yingling, J. M., Chang, C., & Jordan, V. C. (1992). An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Molecular and Cellular Endocrinology*, *90*(1), 77–86.

Jordan, V. C. (1976). Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. *European Journal of Cancer*, *12*(6), 419–424.

Jordan, V. C. (1978). Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen treatment as a potential adjuvant therapy. *Rev Endocr Rel Cancer*, (October Suppl.), 49–55. Au14 Au15

Jordan, V. C. (1984). Biochemical pharmacology of antiestrogen action. *Pharmacological Reviews*, *36*(4), 245–276.

Jordan, V. C. (1991). Are we giving tamoxifen for too long? *European Journal of Cancer*, *27*(9), 1070–1072.

Jordan, V. C. (2001). Selective estrogen receptor modulation: A personal perspective. *Cancer Research*, *61*(15), 5683–5687.

Jordan, V. C. (2003a). Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 1. Receptor interactions. *Journal of Medicinal Chemistry*, *46*(6), 883–908.

Jordan, V. C. (2003b). Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *Journal of Medicinal Chemistry*, *46*(7), 1081–1111.

Jordan, V. C. (2003c). Tamoxifen: A most unlikely pioneering medicine. *Nature Reviews. Drug Discovery*, *2*(3), 205–213.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:32 Page Number: 179

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

Jordan, V. C. (2004). Selective estrogen receptor modulation: Concept and consequences in cancer. *Cancer Cell*, *5*(3), 207–213.

Jordan, V. C. (2006). Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. *British Journal of Pharmacology*, *147*(Suppl. 1), S269–S276.

Jordan, V. C. (2008a). The 38th David A. Karnofsky lecture: The paradoxical actions of estrogen in breast cancer—Survival or death? *Journal of Clinical Oncology*, *26*(18), 3073–3082.

Jordan, V. C. (2008b). Tamoxifen: Catalyst for the change to targeted therapy. *European Journal of Cancer*, *44*(1), 30–38.

Jordan, V. C., & Allen, K. E. (1980). Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *European Journal of Cancer*, *16*(2), 239–251.

Jordan, V. C., Allen, K. E., & Dix, C. J. (1980). Pharmacology of tamoxifen in laboratory animals. *Cancer Treatment Reports*, *64*(6–7), 745–759.

Jordan, V. C., Bain, R. R., Brown, R. R., Gosden, B., & Santos, M. A. (1983). Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. *Cancer Research*, *43*(3), 1446–1450.

Jordan, V. C., Collins, M. M., Rowsby, L., & Prestwich, G. (1977). A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *The Journal of Endocrinology*, *75*(2), 305–316.

Jordan, V. C., Dix, C. J., & Allen, K. E. (1979). The effectiveness of longterm treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In S. E. Salmone & S. E. Jones (Eds.), *Adjuvant therapy of cancer II* (pp. 19–26). New York: Grune and Stratton.

Jordan, V. C., Dix, C. J., Naylor, K. E., Prestwich, G., & Rowsby, L. (1978). Nonsteroidal antiestrogens: Their biological effects and potential mechanisms of action. *Journal of Toxicology and Environmental Health*, *4*(2–3), 363–390.

Jordan, V. C., & Koerner, S. (1975). Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *European Journal of Cancer*, *11*(3), 205–206.

Jordan, V. C., Liu, H., & Dardes, R. (2002). Re: Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 beta-estradiol and the two faces of Janus: Sex steroids as mediators of both cell proliferation and cell death. *Journal of the National Cancer Institute*, *94*(15), 1173, author reply 1173–1175.

Jordan, V. C., Mittal, S., Gosden, B., Koch, R., & Lieberman, M. E. (1985). Structure-activity relationships of estrogens. *Environmental Health Perspectives*, *61*, 97–110.

Jordan, V. C., Naylor, K. E., Dix, C. J., & Prestwich, G. (1980). Anti-oestrogen action in experimental breast cancer. *Recent Results in Cancer Research*, *71*, 30–44.

Jordan, V. C., Phelps, E., & Lindgren, J. U. (1987). Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Research and Treatment*, *10*(1), 31–35.

Jordan, V. C., & Robinson, S. P. (1987). Species-specific pharmacology of antiestrogens: Role of metabolism. *Federation Proceedings*, *46*(5), 1870–1874.

Jordan, V. C., Schafer, J. M., Levenson, A. S., Liu, H., Pease, K. M., Simons, L. A., et al. (2001). Molecular classification of estrogens. *Cancer Research*, *61*(18), 6619–6623.

Jordan, V. C., Wolf, M., Mirecki, D. M., Whitford, D., & Welshons, W. V. (1988). Hormone receptor assays: Clinical usefulness in the management of carcinoma of the breast. *CRC Critical Reviews in Clinical Laboratory Sciences*, *26*, 97–152.

Kagan, R., Williams, R. S., Pan, K., Mirkin, S., & Pickar, J. H. (2010). A randomized, placebo- and active-controlled trial of bazedoxifene/conjugated estrogens for treatment of moderate to severe vulvar/vaginal atrophy in postmenopausal women. *Menopause*, *17*(2), 281–289.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:33 Page Number: 180

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

Katzenellenbogen, B. S., Kendra, K. L., Norman, M. J., & Berthois, Y. (1987). Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Research*, *47*(16), 4355–4360.

Kawate, H., & Takayanagi, R. (2011). Efficacy and safety of bazedoxifene for postmenopausal osteoporosis. *Clinical Interventions in Aging*, *6*, 151–160.

Kennedy, B. J. (1965a). Hormone therapy for advanced breast cancer. *Cancer*, *18*(12), 1551–1557.

Kennedy, B. J. (1965b). Systemic effects of androgenic and estrogenic hormones in advanced breast cancer. *Journal of the American Geriatrics Society*, *13*, 230–235.

Kennedy, B. J., & Nathanson, I. T. (1953). Effect of intensive sex steroid hormone therapy in advanced breast cancer. *Journal of the American Medical Association*, *152*, 1135–1141.

Kiyotani, K., Mushiroda, T., Imamura, C. K., Hosono, N., Tsunoda, T., Kubo, M., et al. (2010). Significant effect of polymorphisms in CYP2D6 and ABCC2 on clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients. *Journal of Clinical Oncology*, *28*(8), 1287–1293.

Klopper, A., & Hall, M. (1971). New synthetic agent for the induction of ovulation: Preliminary trials in women. *British Medical Journal*, *1*(5741), 152–154.

Komm, B. S., Kharode, Y. P., Bodine, P. V., Harris, H. A., Miller, C. P., & Lyttle, C. R. (2005). Bazedoxifene acetate: A selective estrogen receptor modulator with improved selectivity. *Endocrinology*, *146*(9), 3999–4008.

Lacassagne, A. (1933). Influence d'un facteur familial dans la production par la folliculine, de cancers mammaires chez la souris male. *Comptes Rendus des Seances* ***de*** *la Societe de Biologie et des ses Filiales (Paris)*, *114*, 427–429.

Lacassagne, A. (1936a). A comparative study of the carcinogenic action of certain oestrogenic hormones. *American Journal of Cancer*, *28*, 735–740.

Lacassagne, A. (1936b). Hormonal pathogenesis of adenocarcinoma of the breast. *American Journal of Cancer*, *27*(2), 217–228.

LaCroix, A. Z., Chlebowski, R. T., Manson, J. E., Aragaki, A. K., Johnson, K. C., Martin, L., et al. (2011). Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: A randomized controlled trial. *Journal of the American Medical Association*, *305*(13), 1305–1314.

Lammers, L. A., Mathijssen, R. H., van Gelder, T., Bijl, M. J., de Graan, A. J., Seynaeve, C., et al. (2010). The impact of CYP2D6-predicted phenotype on tamoxifen treatment outcome in patients with metastatic breast cancer. *British Journal of Cancer*, *103*(6), 765–771.

Lash, T. L., Cronin-Fenton, D., Ahern, T. P., Rosenberg, C. L., Lunetta, K. L., Silliman, R. A., et al. (2011). CYP2D6 inhibition and breast cancer recurrence in a population-based study in Denmark. *Journal of the National Cancer Institute*, *103*(6), 489–500.

Lathrop, A. E., & Loeb, L. (1916). Further investigations on the origin of tumors in mice. III. On the part played by internal secretion in the spontaneous development of tumors. *Journal of Cancer Research*, *1*(1), 1–19.

Laughlin, R. C., & Carey, T. F. (1962). Cataracts in patients treated with triparanol. *Journal of the American Medical Association*, *181*, 369–370.

LBCSG (1984). Randomised trial of chemo-endocrine therapy, endocrine therapy, and mastectomy alone in postmenopausal patients with operable breast cancer and axillary node metastasis. *The Lancet*, *1*(8384), 1256–1260.

Lednicer, D., Emmert, D. E., Duncan, G. W., & Lyster, S. C. (1967). Mammalian antifertility agents. V. 5,6-Diarylhydronaphthalenones. *Journal of Medicinal Chemistry*, *10*(6), 1051–1054.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:33 Page Number: 181

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

Lednicer, D., Lyster, S. C., & Duncan, G. W. (1967). Mammalian antifertility agents. IV. Basic 3,4-dihydronaphthalenes and 1,2,3,4-tetrahydro-1-naphthols. *Journal of Medicinal Chemistry*, *10*(1), 78–84. Au16

Legha, S. S., Slavik, M., & Carter, S. K. (1976). Nafoxidine—An antiestrogen for the treatment of breast cancer. *Cancer*, *38*(4), 1535–1541.

Lerner, L. J. (1981). The first nonsteroidal antiestrogen—MER25. In R. L. Sutherland & V. C. Jordan (Eds.), *Nonsteroidal antiestrogens, molecular pharmacology and antitumor activity* (pp. 1–16). Sydney: Academic Press.

Lerner, L. J., Holthaus, F. J., Jr., & Thompson, C. R. (1958). A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol. *Endocrinology*, *63*(3), 295–318.

Lerner, L. J., & Jordan, V. C. (1990). Development of antiestrogens and their use in breast cancer: Eighth Cain memorial award lecture. *Cancer Research*, *50*(14), 4177–4189.

Lewis, J. S., Meeke, K., Osipo, C., Ross, E. A., Kidawi, N., Li, T., et al. (2005). Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *Journal of the National Cancer Institute*, *97*(23), 1746–1759.

Lewis-Wambi, J. S., Kim, H. R., Wambi, C., Patel, R., Pyle, J. R., Klein-Szanto, A. J., et al. (2008). Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Research*, *10*(6), R104.

Lewis-Wambi, J. S., Swaby, R., Kim, H., & Jordan, V. C. (2009). Potential of l-buthionine sulfoximine to enhance the apoptotic action of estradiol to reverse acquired antihormonal resistance in metastatic breast cancer. *The Journal of Steroid Biochemistry and Molecular Biology*, *114*(1–2), 33–39.

Lindsay, R., Gallagher, J. C., Kagan, R., Pickar, J. H., & Constantine, G. (2009). Efficacy of tissue-selective estrogen complex of bazedoxifene/conjugated estrogens for osteoporosis prevention in at-risk postmenopausal women. *Fertility and Sterility*, *92*(3), 1045–1052.

Lippman, M. E., & Bolan, G. (1975). Oestrogen-responsive human breast cancer in long term tissue culture. *Nature*, *256*(5518), 592–593.

Lonning, P. E. (2009). Additive endocrine therapy for advanced breast cancer—Back to the future. *Acta Oncologica*, *48*(8), 1092–1101.

Lonning, P. E., Taylor, P. D., Anker, G., Iddon, J., Wie, L., Jorgensen, L. M., et al. (2001). High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Research and Treatment*, *67*(2), 111–116.

Love, R. R., Mazess, R. B., Barden, H. S., Epstein, S., Newcomb, P. A., Jordan, V. C., et al. (1992). Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *The New England Journal of Medicine*, *326*(13), 852–856.

Love, R. R., Newcomb, P. A., Wiebe, D. A., Surawicz, T. S., Jordan, V. C., Carbone, P. P., et al. (1990). Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. *Journal of the National Cancer Institute*, *82*(16), 1327–1332.

Love, R. R., Wiebe, D. A., Newcomb, P. A., Cameron, L., Leventhal, H., Jordan, V. C., et al. (1991). Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Annals of Internal Medicine*, *115*(11), 860–864.

Lunan, C. B., & Klopper, A. (1975). Antioestrogens. A review. *Clinical Endocrinology*, *4*(5), 551–572.

Madlensky, L., Natarajan, L., Tchu, S., Pu, M., Mortimer, J., Flatt, S. W., et al. (2011). Tamoxifen metabolite concentrations, CYP2D6 genotype, and breast cancer outcomes. *Clinical Pharmacology and Therapeutics*, *89*(5), 718–725.

Martino, S., Cauley, J. A., Barrett-Connor, E., Powles, T. J., Mershon, J., Disch, D., et al. (2004). Continuing outcomes relevant to Evista: Breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *Journal of the National Cancer Institute*, *96*(23), 1751–1761.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:34 Page Number: 182

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

Masamura, S., Santner, S. J., Heitjan, D. F., & Santen, R. J. (1995). Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. *The Journal of Clinical Endocrinology and Metabolism*, *80*(10), 2918–2925.

Maximov, P., Sengupta, S., Lewis-Wambi, J. S., Kim, H. R., Curpan, R. F., & Jordan, V. C. (2011). The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: A hypothesis. *Hormonal Molecular Biology and Clinical Investigation*, *5*(1), 27–34.

McGuire, W. L., Carbone, P. P., & Vollmer, E. P. (Eds.), (1975). *Estrogen receptors in human breast cancer*. Raven Press. Au12

Mehta, R. S., Barlow, W. E., Albain, K. S., Vandenberg, T. A., Dakhil, S. R., Tirumali, N. R., et al. (2012). Combination anastrozole and fulvestrant in metastatic breast cancer. *The New England Journal of Medicine*, *367*(5), 435–444.

Miller, C. P., Collini, M. D., Tran, B. D., Harris, H. A., Kharode, Y. P., Marzolf, J. T., et al. (2001). Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. *Journal of Medicinal Chemistry*, *44*(11), 1654–1657.

NATO (1983). Controlled trial of tamoxifen as adjuvant agent in management of early breast cancer. Interim analysis at four years by Nolvadex Adjuvant Trial Organisation. *The Lancet*, *1*(8319), 257–261.

O'Regan, R. M., Gajdos, C., Dardes, R. C., De Los Reyes, A., Park, W., Rademaker, A. W., et al. (2002). Effects of raloxifene after tamoxifen on breast and endometrial tumor growth in athymic mice. *Journal of the National Cancer Institute*, *94*(4), 274–283.

Osborne, C. K., Coronado, E. B., & Robinson, J. P. (1987). Human breast cancer in the athymic nude mouse: Cytostatic effects of long-term antiestrogen therapy. *European Journal of Cancer & Clinical Oncology*, *23*(8), 1189–1196.

Osborne, C. K., Hobbs, K., & Clark, G. M. (1985). Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. *Cancer Research*, *45*(2), 584–590.

Osborne, C. K., Pippen, J., Jones, S. E., Parker, L. M., Ellis, M., Come, S., et al. (2002). Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: Results of a North American trial. *Journal of Clinical Oncology*, *20*(16), 3386–3395.

Osipo, C., Gajdos, C., Liu, H., Chen, B., & Jordan, V. C. (2003). Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. *Journal of the National Cancer Institute*, *95*(21), 1597–1608.

Payre, B., de Medina, P., Boubekeur, N., Mhamdi, L., Bertrand-Michel, J., Terce, F., et al. (2008). Microsomal antiestrogen-binding site ligands induce growth control and differentiation of human breast cancer cells through the modulation of cholesterol metabolism. *Molecular Cancer Therapeutics*, *7*(12), 3707–3718.

Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., et al. (2000). Molecular portraits of human breast tumours. *Nature*, *406*(6797), 747–752.

Pink, J. J., Jiang, S. Y., Fritsch, M., & Jordan, V. C. (1995). An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Research*, *55*(12), 2583–2590.

Pinkerton, J. V., Pickar, J. H., Racketa, J., & Mirkin, S. (2012). Bazedoxifene/conjugated estrogens for menopausal symptom treatment and osteoporosis prevention. *Climacteric*, *15*(5), 411–418.

Poirot, M. (2011). Four decades of discovery in breast cancer research and treatment—An interview with V. Craig Jordan. *The International Journal of Developmental Biology*, *55*, 703–712.

Powles, T. J., Ashley, S., Tidy, A., Smith, I. E., & Dowsett, M. (2007). Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. *Journal of the National Cancer Institute*, *99*(4), 283–290.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:34 Page Number: 183

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

Powles, T., Eeles, R., Ashley, S., Easton, D., Chang, J., Dowsett, M., et al. (1998). Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomised chemoprevention trial. *The Lancet*, *352*(9122), 98–101.

Powles, T. J., Hardy, J. R., Ashley, S. E., Farrington, G. M., Cosgrove, D., Davey, J. B., et al. (1989). A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *British Journal of Cancer*, *60*(1), 126–131.

Prentice, R. L., Chlebowski, R. T., Stefanick, M. L., Manson, J. E., Langer, R. D., Pettinger, M., et al. (2008). Conjugated equine estrogens and breast cancer risk in the Women's Health Initiative clinical trial and observational study. *American Journal of Epidemiology*, *167*(12), 1407–1415.

Rae, J. M., Drury, S., Hayes, D. F., Stearns, V., Thibert, J. N., Haynes, B. P., et al. (2012). CYP2D6 and UGT2B7 genotype and risk of recurrence in tamoxifen-treated breast cancer patients. *Journal of the National Cancer Institute*, *104*(6), 452–460.

Regan, M. M., Leyland-Jones, B., Bouzyk, M., Pagani, O., Tang, W., Kammler, R., et al. (2012). CYP2D6 genotype and tamoxifen response in postmenopausal women with endocrine-responsive breast cancer: The breast international group 1-98 trial. *Journal of the National Cancer Institute*, *104*(6), 441–451.

Riemsma, R., Forbes, C. A., Amonkar, M. M., Lykopoulos, K., Diaz, J. R., Kleijnen, J., et al. (2012). Systematic review of lapatinib in combination with letrozole compared with other first-line treatments for hormone receptor positive (HR+) and HER2+ advanced or metastatic breast cancer (MBC). *Current Medical Research and Opinion*, *28*(8), 1263–1279.

Ries, L. A. G., Eisner, M. P., & Kosary, C. L. (2001). *SEER cancer statistics review, 1973–1998*. Au12 National Cancer Institute.

Robinson, S. P., Koch, R., & Jordan, V. C. (1988). In vitro estrogenic actions in rat and human cells of hydroxylated derivatives of D16726 (zindoxifene), an agent with known antimammary cancer activity in vivo. *Cancer Research*, *48*(4), 784–787.

Robson, J. M. (1937). Oestrous reactions, including mating, produced by triphenyl ethylene. *Nature*, *140*(3535), 196.

Robson, J. M., & Schonberg, A. (1938). Duration of action of natural and synthetic oestrogens. *Nature*, *142*(3589), 292–293.

Robson, J. M., & Schonberg, A. (1942). A new synthetic oestrogen with prolonged action when given orally. *Nature*, *150*(3792), 22–23.

Roop, R. P., & Ma, C. X. (2012). Endocrine resistance in breast cancer: Molecular pathways and rational development of targeted therapies. *Future Oncology*, *8*(3), 273–292.

Rosati, R. L., Da Silva Jardine, P., Cameron, K. O., Thompson, D. D., Ke, H. Z., Toler, S. M., et al. (1998). Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryltetrahydronaphthalene. *Journal of Medicinal Chemistry*, *41*(16), 2928–2931.

Rose, C., Thorpe, S. M., Andersen, K. W., Pedersen, B. V., Mouridsen, H. T., Blichert-Toft, M., et al. (1985). Beneficial effect of adjuvant tamoxifen therapy in primary breast cancer patients with high oestrogen receptor values. *The Lancet*, *1*(8419), 16–19.

Rossouw, J. E., Anderson, G. L., Prentice, R. L., LaCroix, A. Z., Kooperberg, C., Stefanick, M. L., et al. (2002). Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results from the Women's Health Initiative randomized controlled trial. *Journal of the American Medical Association*, *288*(3), 321–333.

Rutanen, E. M., Heikkinen, J., Halonen, K., Komi, J., Lammintausta, R., & Ylikorkala, O. (2003). Effects of ospemifene, a novel SERM, on hormones, genital tract, climacteric symptoms, and quality of life in postmenopausal women: A double-blind, randomized trial. *Menopause*, *10*(5), 433–439.

Schrek, R. (1960). Fashions in cancer research. In W. B. Wartman (Ed.), *1959–1960 Year book* (pp. 26–39). Chicago: The Year Book Publishers.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:34 Page Number: 184

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

Schroth, W., Goetz, M. P., Hamann, U., Fasching, P. A., Schmidt, M., Winter, S., et al. (2009). Association between CYP2D6 polymorphisms and outcomes among women with early stage breast cancer treated with tamoxifen. *Journal of the American Medical Association*, *302*(13), 1429–1436.

SCTO (1987). Adjuvant tamoxifen in the management of operable cancer: The Scottish Trial. Report from the Breast Cancer Trials Committee, Scottish Cancer Trials Office (MRC), Edinburgh. *The Lancet*, *2*(8852), 171–175.

Sengupta, S., & Jordan, V. C. (2013). Novel selective estrogen receptor modulators. In V. C. Jordan (Ed.), *Estrogen actions, SERMs, and women's health*. World Scientific. Au12

Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., et al. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell*, *95*(7), 927–937.

Shim, W. S., Conaway, M., Masamura, S., Yue, W., Wang, J. P., Kmar, R., et al. (2000). Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. *Endocrinology*, *141*(1), 396–405.

Shimkin, M. B., & Wyman, R. S. (1945). Effect of adrenalectomy and ovariectomy on mammary carcinogenesis in strain C3H mice. *Journal of the National Cancer Institute*, *6*, 187–189.

Shimkin, M. B., & Wyman, R. S. (1946). Mammary tumors in male mice implanted with estrogen-cholesterol pellets. *Journal of the National Cancer Institute*, *7*(2), 71–75.

Shimkin, M. B., Wyman, R. S., & Andervont, H. B. (1946). Mammary tumors in mice following transplantation of mammary tissue. *Journal of the National Cancer Institute*, *7*(2), 77.

Shumaker, S. A., Legault, C., Rapp, S. R., Thal, L., Wallace, R. B., Ockene, J. K., et al. (2003). Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: The Women's Health Initiative Memory Study: A randomized controlled trial. *Journal of the American Medical Association*, *289*(20), 2651–2662.

Silverman, S. L., Chines, A. A., Kendler, D. L., Kung, A. W., Teglbjaerg, C. S., Felsenberg, D., et al. (2012). Sustained efficacy and safety of bazedoxifene in preventing fractures in postmenopausal women with osteoporosis: Results of a 5-year, randomized, placebo-controlled study. *Osteoporosis International*, *23*(1), 351–363.

Song, R. X., Mor, G., Naftolin, F., McPherson, R. A., Song, J., Zhang, Z., et al. (2001). Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *Journal of the National Cancer Institute*, *93*(22), 1714–1723.

Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., et al. (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(14), 8418–8423.

Speroff, L. (2009). *A good man, Gregory Goodwin Pincus: The man, his story, the birth control pill*. Arnica Publishing. Au12

Stein, R. C., Dowsett, M., Cunningham, D. C., Davenport, J., Ford, H. T., Gazet, J. C., et al. (1990). Phase I/II study of the anti-oestrogen zindoxifene (D16726) in the treatment of advanced breast cancer. A Cancer Research Campaign Phase I/II Clinical Trials Committee study. *British Journal of Cancer*, *61*(3), 451–453.

Stoll, B. (1977a). *Breast cancer management early and late*. London: William Herman Medical Books Ltd.

Stoll, B. A. (1977b). Palliation by castration or by hormone administration. In B. A. Stoll (Ed.), *Breast cancer management early and late* (pp. 133–146). London: W. Heinemann Medical Books Ltd.

Sutherland, R. L., Murphy, L. C., San Foo, M., Green, M. D., Whybourne, A. M., & Krozowski, Z. S. (1980). High-affinity anti-oestrogen binding site distinct from the oestrogen receptor. *Nature*, *288*(5788), 273–275.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:35 Page Number: 185

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

Sweeney, E. E., McDaniel, R. E., Maximov, P. Y., Fan, P., & Jordan, V. C. (2012). Models and mechanisms of acquired antihormone resistance in breast cancer: Significant clinical progress despite limitations. *Hormonal Molecular Biology and Clinical Investigation*, *9*(2), 143–163.

Thompson, C. R., & Werner, H. W. (1953). Fat storage of an estrogen in women following orally administered tri-p-anisyl chloroethylene. *Proceedings of the Society for Experimental Biology and Medicine*, *84*, 491–492.

Thurlimann, B., Keshaviah, A., Coates, A. S., Mouridsen, H., Mauriac, L., Forbes, J. F., et al. (2005). A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *The New England Journal of Medicine*, *353*(26), 2747–2757.

Toft, D., & Gorski, J. (1966). A receptor molecule for estrogens: Isolation from the rat uterus and preliminary characterization. *PNAS*, *55*(6), 1574–1581.

Toft, D., Shyamala, G., & Gorski, J. (1967). A receptor molecule for estrogens: Studies using a cell-free system. *Proceedings of the National Academy of Sciences of the United States of America*, *57*(6), 1740–1743.

Turner, R. T., Evans, G. L., Sluka, J. P., Adrian, M. D., Bryant, H. U., Turner, C. H., et al. (1998). Differential responses of estrogen target tissues in rats including bone to clomiphene, enclomiphene, and zuclomiphene. *Endocrinology*, *139*(9), 3712–3720.

Turner, R. T., Evans, G. L., & Wakley, G. K. (1993). Mechanism of action of estrogen on cancellous bone balance in tibiae of ovariectomized growing rats: Inhibition of indices of formation and resorption. *Journal of Bone and Mineral Research*, *8*(3), 359–366.

Turner, R. T., Wakley, G. K., Hannon, K. S., & Bell, N. H. (1987). Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. *Journal of Bone and Mineral Research*, *2*(5), 449–456.

Turner, R. T., Wakley, G. K., Hannon, K. S., & Bell, N. H. (1988). Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. *Endocrinology*, *122*(3), 1146–1150.

Veronesi, U., Maisonneuve, P., Costa, A., Sacchini, V., Maltoni, C., Robertson, C., et al. (1998). Prevention of breast cancer with tamoxifen: Preliminary findings from the Italian randomised trial among hysterectomised women. Italian Tamoxifen Prevention Study. *The Lancet*, *352*(9122), 93–97.

Vogel, V. G., Costantino, J. P., Wickerham, D. L., Cronin, W. M., Cecchini, R. S., Atkins, J. N., et al. (2006). Effects of tamoxifen vs. raloxifene on the risk of developing invasive breast cancer and other disease outcomes: The NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *Journal of the American Medical Association*, *295*(23), 2727–2741.

Vogel, V. G., Costantino, J. P., Wickerham, D. L., Cronin, W. M., Cecchini, R. S., Atkins, J. N., et al. (2010). Update of the national surgical adjuvant breast and bowel project study of tamoxifen and raloxifene (STAR) P-2 trial: Preventing breast cancer. *Cancer Prevention Research (Philadelphia, Pa.)*, *3*(6), 696–706.

Voipio, S. K., Komi, J., Kangas, L., Halonen, K., DeGregorio, M. W., & Erkkola, R. U. (2002). Effects of ospemifene (FC-1271a) on uterine endometrium, vaginal maturation index, and hormonal status in healthy postmenopausal women. *Maturitas*, *43*(3), 207–214.

Wallace, O. B., Lauwers, K. S., Dodge, J. A., May, S. A., Calvin, J. R., Hinklin, R., et al. (2006). A selective estrogen receptor modulator for the treatment of hot flushes. *Journal of Medicinal Chemistry*, *49*(3), 843–846.

Walpole, A. L., & Paterson, E. (1949). Synthetic oestrogens in mammary cancer. *The Lancet*, *2*(6583), 783–786.

Watanabe, N., Ikeno, A., Minato, H., Nakagawa, H., Kohayakawa, C., & Tsuji, J. (2003). Discovery and preclinical characterization of (+)-3-[4-(1-piperidinoethoxy)phenyl]

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:35 Page Number: 186

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

spiro[indene-1,1′-indane]-5,5′-diol hydrochloride: A promising nonsteroidal estrogen receptor agonist for hot flush. *Journal of Medicinal Chemistry*, *46*(19), 3961–3964.

Welsch, C. W. (1985). Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: A review and tribute to Charles Brenton Huggins. *Cancer Research*, *45*(8), 3415–3443.

Welshons, W. V., & Jordan, V. C. (1987). Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *European Journal of Cancer & Clinical Oncology*, *23*(12), 1935–1939.

Wolf, D. M., & Jordan, V. C. (1993). A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results in Cancer Research*, *127*, 23–33.

Yang, L., Parkin, D., Ferlay, J., Li, L., & Chen, Y. (2005). Estimates of cancer incidence in China for 2000 and projections for 2005. *Cancer Epidemiology, Biomarkers & Prevention*, *14*(1), 243–249. Au17

Yao, K., Lee, E. S., Bentrem, D. J., England, G., Schafer, J. I., O'Regan, R. M., et al. (2000). Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clinical Cancer Research*, *6*(5), 2028–2036.

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:35 Page Number: 187

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

To protect the rights of the author(s) and publisher we inform you that this PDF is an uncorrected proof for internal business use only by the author(s), editor(s), reviewer(s), Elsevier and typesetter SPi. It is not allowed to publish this proof online or in print. This proof copy is the copyright property of the publisher and is confidential until formal publication.

B978-0-12-416673-8.00007-1, 00007

Non-Print Items

Keywords: Selective estrogen receptor modulators, Tamoxifen, Raloxifene, Aromatase inhibitors, Chemoprevention, Estrogen-induced apoptosis

UNCORRECTED PROOF

Comp. by: CKarunaJothi Stage: Proof Chapter No.: 7 Title Name: VH
Date:18/5/13 Time:10:35:36 Page Number: 189

Metadata of the Book that will be visualized online

Book Title	Tamoxifen	
Book SubTitle	Pioneering Medicine in Breast Cancer	
Copyright Year	2013	
Copyright Holder	Springer Basel	

Author	Family Name	**Maximov**
	Particle	
	Given Name	**Philipp Y.**
	Suffix	
	Division	
	Organization	Georgetown University Medical Center
	Address	Reservoir Rd NW 3970, 20057, Washington, District of Columbia, USA
	Email	pm386@georgetown.edu
Author	Family Name	**McDaniel**
	Particle	
	Given Name	**Russell E.**
	Suffix	
	Division	
	Organization	Georgetown University Medical Center
	Address	Reservoir Rd NW 3970, 20057, Washington, District of Columbia, USA
	Email	rem74@georgetown.edu
Author	Family Name	**Jordan**
	Particle	
	Given Name	**V. Craig**
	Suffix	
	Division	, Lombardi Comprehensive Cancer Center
	Organization	Georgetown University Medical Center
	Address	Reservoir Rd NW 3970, 20057, Washington, DC, USA
	Email	vcj2@georgetown.edu

Author's Proof

Milestones in Drug Therapy

Series Editors

Michael J. Parnham, Fraunhofer IME & Goethe University Frankfurt, Germany,
Jacques Bruinvels, Bilthoven, The Netherlands

Series Editors

J.C. Buckingham, Imperial College School of Medicine, London, UK
R.J. Flower, The William Harvey Research Institute, London, UK
A.G. Herman, Universiteit Antwerpen, Antwerp, Belgium
P. Skolnick, National Institute on Drug Abuse, Bethesda, MD, USA

For further volumes:
http://www.springer.com/series/4991

Author's Proof

Author's Proof

Philipp Y. Maximov • Russell E. McDaniel •
V. Craig Jordan

Tamoxifen

Pioneering Medicine in Breast Cancer

Springer

Author's Proof

Philipp Y. Maximov
Russell E. McDaniel
Georgetown University Medical Center
Washington
District of Columbia
USA

V. Craig Jordan
Lombardi Comprehensive Cancer Center
Georgetown University Medical Center
Washington, DC
USA

ISBN 978-3-0348-0663-3 ISBN 978-3-0348-0664-0 (eBook)
DOI 10.1007/978-3-0348-0664-0
Springer Basel Heidelberg New York Dordrecht London

Library of Congress Control Number: XXXX

© Springer Basel 2013
This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed. Exempted from this legal reservation are brief excerpts in connection with reviews or scholarly analysis or material supplied specifically for the purpose of being entered and executed on a computer system, for exclusive use by the purchaser of the work. Duplication of this publication or parts thereof is permitted only under the provisions of the Copyright Law of the Publisher's location, in its current version, and permission for use must always be obtained from Springer. Permissions for use may be obtained through RightsLink at the Copyright Clearance Center. Violations are liable to prosecution under the respective Copyright Law.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
While the advice and information in this book are believed to be true and accurate at the date of publication, neither the authors nor the editors nor the publisher can accept any legal responsibility for any errors or omissions that may be made. The publisher makes no warranty, express or implied, with respect to the material contained herein.

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Author's Proof

Thanks to all the "Tamoxifen Teams" who translated ideas into life saved over the past 35 years

With the indispensable assistance of Fadeke Agboke, Puspanjali Bhatta, and Amy Botello

Author's Proof

Author's Proof

Foreword

I joined the Clinical Research Department of ICI Americas (ICI) in Wilmington, Delaware, in 1973, after competing in the World Championships for Rowing in Moscow, Russia, as a member of the first US women's rowing team. I mention this competition because as I was part of a team who was pioneering the international competition of women's crew, I was among the team at ICI who was pioneering the support and development of "targeted therapies," the first being tamoxifen. The operative word here is *team*. Having previously worked at the National Cancer Institute supporting the Breast Cancer Task Force, I was considered the most qualified individual at the time in the newly formed ICI to plan and organize the clinical investigation of the antiestrogen ICI46,474 in the United States!

I remember asking my director how long it takes to have a drug approved. He told me about 8 years; as a competitor, and not understanding all the aspects of pharmaceutical drug development, I said to myself, "We will do it *four years*." As it is known, the Food and Drug Administration (FDA) approved the labeling for tamoxifen on December 31, 1977, just 4 years and 5 months from the day I was hired. Thinking back over those early years, I recall a number of my colleagues as dedicated individuals who understood the importance of developing tamoxifen—Beverly Bach, Fran Ehrlich, David Sofi, and Bruce Decker—working in clinical research, regulatory affairs, market research, and marketing. Eventually, dozens of staff were all on the mission as a *team* to make tamoxifen available as quickly as possible to those patients who were most likely to benefit.

As you will read throughout this book, the early clinical development of tamoxifen was driven by clinical investigators and scientists in the United States, Canada, and Europe, who devoted their lives to the treatment of patients with breast cancer, such as Pierre Band, Harvey Lerner, and Lucien Israel. In fact, it was Harvey Lerner who demonstrated to Stuart Pharmaceuticals the urgency of continuing to develop this agent when the financial forecast was not compelling.

As you will read, the story of ICI46,474 began with its discovery in the fertility control program at ICI Pharmaceuticals, Alderley Park, Cheshire. It was an excellent morning-after pill in rats, but in fact stimulated ovulation in subfertile women. Although marketed in the United Kingdom for the induction of ovulation, the

Author's Proof

agent's main focus in America was to treat breast cancer. A few small clinical studies of ICI46,474 conducted in Europe had reported modest activity in metastatic breast cancer (Cole et al. *British Journal of Cancer*, 1971;25:270–275 and Ward *British Medical Journal*, 1973;5844:13–14).

In the early 1970s, US clinical trial cooperative groups were focusing on the use of combination cytotoxic chemotherapy with the goal of curing breast cancer. Endocrine therapy was largely viewed as palliative; so there was little possibility that this antiestrogen would make much of an impact in the treatment of metastatic breast cancer or provide reasonable financial returns for investment in clinical studies. Then, in 1973, I met Craig Jordan, one of the few people in the world with a background in, and understanding of, the pharmacology of nonsteroidal antiestrogens. I arranged with my management to provide Craig with an unrestricted research grant at the Worcester Foundation and visited him to discuss the progress as he reinvented the strategic therapeutic use of ICI46,474 to become the drug tamoxifen that we know today. Craig's laboratory studies supported the exclusive use of tamoxifen to treat estrogen receptor (ER)-positive tumors. We used his results, prior to their publication, in our "investigators brochure."

I suggested that Craig become our scientific advisor for tamoxifen and arranged for him to meet the senior leadership of the Eastern Cooperative Oncology Group (ECOG): Doug Tormey, head of the ECOG Breast Committee, and Paul Carbone, chairman of ECOG. ICI Americas continued supporting his research, and in the laboratory, Craig discovered the strategy used today, that of long-term adjuvant tamoxifen therapy specifically targeting ER-positive breast tumors.

Looking at "the good, the bad, and the ugly" of tamoxifen, Craig's laboratory raised the question of whether the agent would increase the incidence of endometrial cancer. It did. This led to the recruitment of gynecologists to the breast cancer patient's care team, an extremely valuable advance at the end of the 1980s, as tamoxifen was about to be tested as a chemopreventive agent in high-risk women.

On a personal note, Craig and I had numerous adventures over the years, coincident with various clinical trial meetings. Here, I relate a story that demonstrates his philosophy of honoring commitment. In 1979, Craig was to be the opening speaker at the tamoxifen meeting in Sorrento, Italy. He was working in Bern, Switzerland, and was scheduled to fly down on an Alitalia flight from Zurich to Naples on the evening before his talk. Craig had to leave Zurich on the last flight that evening, as he had a site visit at the Institute in Bern earlier in the day. Then disaster struck. I learned that Alitalia was to go on strike that evening and urged him to leave Bern at lunch time, if there was to be any hope of his presenting at the meeting. Craig declared, "But I have a room full of site visitors from America—not possible," followed by, "Don't worry, I will be there." After my call, Craig immediately contacted his technician Brigitte Haldemann to drive him through the night over the 730 miles to Sorrento. With an hour to spare and after a shower, he presented his talk.

To this day, tamoxifen remains in the news. The Adjuvant Tamoxifen Longer Against Shorter (ATLAS) trial shows that 10 years of adjuvant tamoxifen is superior to 5 years of tamoxifen (Davies C et al., *Lancet*, 2012; epub 12/12/

Author's Proof

2012). The therapeutic strategy is again being tested successfully, but the benefit in decreasing mortality occurs in the second decade after stopping longer-duration tamoxifen. This phenomenon (Wolf D, and Jordan VC, *Recent Results in Cancer Research*, 1993;127:23–33) led to the new biology of estrogen-induced apoptosis.

What happened to chemoprevention? Tamoxifen became the first agent to be approved by the Food and Drug Administration for reduction of breast cancer incidence in high-risk premenopausal and postmenopausal women. In January 2013, the National Institute for Health and Clinical Excellence (NICE) recommended tamoxifen be made available through the National Health Service in the United Kingdom for the chemoprevention of breast cancer.

This book tells the humanistic story of the development of tamoxifen. It is a tribute of gratitude to the tens of thousands of women and men who participated in clinical trials throughout the development of tamoxifen, which is now a therapeutic agent for the prevention as well as the treatment of minimal through advanced stages of breast cancer, depending on the patient's hormonal receptor status. It is also an acknowledgment of hundreds of clinical oncology health teams working to advance our understanding of the biology of breast cancer as well as thousands of clinicians caring for those with breast cancer.

I am amazed and so grateful that so many millions of lives have been extended and many more have benefited from the research and therapeutic strategies retold in this book. I am personally grateful to have played a role, minimal as it was and is, in the development of tamoxifen.

West Conshohocken, PA, USA

Lois Trench-Hines
Founder and Chief Executive Officer
Meniscus Limited



Pictured from *left* to *right*, George Hines, Lois Trench-Hines, Alexandra Jordan-Noel, and V. Craig Jordan. Photographed at a celebration at the Swiss Ambassador's Residence in Washington, DC, to celebrate the award of the St. Gallen Prize for Outstanding Accomplishments in the Adjuvant Treatment of Breast Cancer in 2011

This figure will be printed in b/w

Author's Proof

Author's Proof

Preface

The story of tamoxifen is unique. This pioneering medicine was not conceived as part of a major development plan in the pharmaceutical industry to create a blockbuster, but rather tamoxifen (ICI46,474) was an orphan product that had failed its first indication as a "morning-after pill." Breast cancer was a consideration, but the company terminated clinical development in 1972. The resurrection of the medicine then occurred and, after a period of dismissal by the clinical community in the mid-1970s, successes went from strength to strength.

The success of the product depended upon individuals being in the right place at the right time and a "gentleman's agreement" between industry (ICI Pharmaceuticals Division now AstraZeneca) and academia (Worcester Foundation and the Leeds University) to create a new strategy for the treatment and prevention of breast cancer. The gestation period for that strategy was the whole of the 1970s [1–4]. The principles conceived of targeting the tumor estrogen receptor (ER) and using long-term adjuvant endocrine therapy translated effectively to clinical trials that demonstrated dramatic and lasting reduction in mortality [5]. It is estimated that the hundreds of thousands, perhaps millions, of women are alive today because of the successful translation of research conducted in the 1970s.

Additionally, laboratory research on the prevention of mammary carcinogenesis [2, 3] in animals would translate to successful clinical trials [6–8] with tamoxifen being the first medicine to be approved by the Food and Drug Administration (FDA) for the reduction of the incidence of breast cancer in pre- and postmenopausal women at high risk. Tamoxifen was the first medicine to be approved to reduce the risk for any cancer.

Without the economic success of tamoxifen, there would have been no incentive to develop the aromatase inhibitors for the adjuvant treatment of ER-positive breast cancer in postmenopausal patients. Without the study of the "good, the bad, and the ugly" of the tamoxifen, there would be no selective ER modulators (SERMs). The chance finding that tamoxifen and also a failed breast cancer drug keoxifene (to be renamed 5 or 6 years later as raloxifene) would maintain bone density in ovariectomized rats [9] opened the door to the suggestion that

Author's Proof

> Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. [10]

Today, raloxifene is approved by the FDA for the prevention and treatment of osteoporosis in postmenopausal women and for the prevention of breast cancer in high-risk postmenopausal women [11]. However, tamoxifen became the pioneering SERM that switched on or switched off estrogen target sites around a woman's body. This new drug group also led to the idea of now being able to treat diseases via any member of the nuclear hormone receptor superfamily. Specificity would be enhanced and side effects reduced.

This monograph documents the milestones achieved during the curious twists and turns in the development of tamoxifen over the past 40 years. The story starts with the systemic synthesis of nonsteroidal estrogens that through serendipity suddenly gave us the nonsteroidal antiestrogens. The discovery by Leonard Lerner AU5 in the 1950s of MER25 (or ethamoxytriphetol) and subsequently clomiphene [10] and the finding that they were antifertility agents in rats [10] aroused the interest of the pharmaceutical industry to develop "morning-after pills." Nonsteroidal antiestrogens, however, were excellent contraceptives in rats but actually induced ovulation in subfertile women. Interest in nonsteroidal antiestrogens waned.

Cancer treatment was a consideration because of the known link between estrogen and the growth of some metastatic breast cancers. However, again there was no real enthusiasm from the pharmaceutical industry. Tamoxifen, after an unlikely start in the 1960s, advanced alone during the 1970s to become the "gold standard" for the antihormone treatment and prevention of breast cancer fro the next 20 years. Despite all the "ups and downs" of the story, tamoxifen remains a cheap and effective lifesaving drug around the world. Indeed, the concept first described by our studies in the 1970s that "longer was better" as the treatment strategy for adjuvant therapy with tamoxifen for patients with ER-positive breast cancer continues to go from strength to strength in clinical trial. Ten years AU6 of adjuvant therapy is now known to be superior to 5 years of adjuvant therapy, but the profound decrease in mortality occurs during the decade after stopping tamoxifen at 10 years [12]. Again, there is a prediction we made in the 1990s that tamoxifen causes the evolution of drug resistance in the undetected micrometastases that exposes a vulnerability to estrogen-induced apoptosis in the tumor cells [13].

Lois Trench-Hanes generously accepted my invitation to contribute our Foreword. She was there at the beginning of tamoxifen in America and was the one who AU7 recruited me, on Arthur Walpole's recommendation, to advance the science and to support clinical development. We had many adventures over the years but her attitude of "get the job done" was essential to the start of this milestone. She was a force to be reckoned with, that through her willingness to see the project succeed for AU8 her company by establishing the correct clinical contacts not only propelled tamoxifen forward but helped my career development. She and her husband George are

Author's Proof

lifelong friends and Lois is a godmother to my youngest daughter Alexandra (see pictures in Lois's Foreword).

This monograph has been assembled by my Tamoxifen Team (VCJ) at the Lombardi Comprehensive Cancer Center at Georgetown University, Washington, DC. It is intended to illustrate and document the real journey traveled by this milestone in medicine.

V. Craig Jordan
Russell E. McDaniel
Philipp Y. Maximov

References

1. Jordan VC, Koerner S (1975) Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 11:205–206
2. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
3. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
4. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44:30–38
5. Davies C, Godwin J, Gray R et al (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378:771–784
6. Fisher B, Costantino JP, Wickerham DL et al (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90:1371–1388
7. Fisher B, Costantino JP, Wickerham DL et al (2005) Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97:1652–1662
8. Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M (2007) Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. J Natl Cancer Inst 99:283–290
9. Jordan VC, Phelps E, Lindgren JU (1987) Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10:31–35
10. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189
11. Vogel VG, Costantino JP, Wickerham DL et al (2006) Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 295:2727–2741

Author's Proof

12. Davies C, Pan H, Godwin J et al (2013) Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. Lancet 381(9869):805–816
13. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127:23–33

Author's Proof

Acknowledgments



"We are in it for life"™
Tamoxifen Team
Georgetown University

Dr. Jordan wishes to thank all of his "Tamoxifen Teams," who for the past four decades have converted ideas into lives saved. He also wishes to thank the Department of Defense Breast Program under award number W81XWH-06-1-0590 Center of Excellence; subcontract under the SU2C (AACR) grant number SU2C-AACR-DT0409; the Susan G Komen for the Cure Foundation under award number SAC100009; and the Lombardi Comprehensive Cancer Center Support Grant (CCSG) Core Grant NIH P30 CA051008 for his current research funding. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

Author's Proof

Author's Proof

Contents

287

1 Discovery and Pharmacology of Nonsteroidal Estrogens and Antiestrogens 1 289
Introduction 1 290
Testing Methods for Estrogen 2 291
Structure-Activity Relationships of Estrogens 3 292
Estrogen Action 6 293
Nonsteroidal Antiestrogens 7 294
Structure-Activity Relationships in the Rat 7 295
Substituted Triphenylethylenes 7 296
Bicyclic Antiestrogens 9 297
The Molecular Modulation of the Estrogen Receptor by Nonsteroidal Antiestrogens 12 299
Effect of Antiestrogens in Different Species 16 300
Mouse 17 301
Rat 18 302
Chick 20 303
Conclusion 20 304
References 22 305

2 Tamoxifen Goes Forward Alone 31 306
Introduction 31 307
ICI 46,474: The Early Years 33 308
ICI 46,474 to Tamoxifen 37 309
Patenting Problems 39 310
Conclusion 40 311
References 42 312

3 Metabolites of Tamoxifen as the Basis of Drug Development 47 313
Introduction 47 314
Basic Mechanisms of Tamoxifen Metabolism 48 315
Metabolic Mimicry 52 316
Tamoxifen Metabolism Today 55 317

Author's Proof

Clinical Correlations . . . 57
References . . . 60

4 Adjuvant Therapy: The Breakthrough . . . 69
Introduction . . . 69
Adjuvant Therapy with Tamoxifen . . . 70
Studies in Premenopausal Women . . . 73
Overview of Clinical Trials . . . 74
Arrival of Aromatase Inhibitors as Adjuvant Therapy . . . 76
Increasing Survivorship Following 5 Years of Adjuvant Tamoxifen . . . 77
References . . . 80

5 The Wisconsin Story in the 1980s: Discovery of Target Site-Specific Estrogen Action . . . 85
Introduction . . . 85
Laboratory Studies on the Target Site-Specific Pharmacology of "Nonsteroidal Antiestrogens" . . . 86
The Wisconsin Tamoxifen Study . . . 89
Translational Research . . . 90
References . . . 96

6 Carcinogenesis and Tamoxifen . . . 101
Introduction . . . 101
Tamoxifen and the Endometrial Carcinoma . . . 102
Deaths from Endometrial Carcinoma . . . 103
Tamoxifen and the Stage of Endometrial Carcinoma . . . 104
Incidence of Endometrial Cancer with Tamoxifen . . . 105
Tamoxifen and Rat Liver Carcinogenesis . . . 107
Tamoxifen and DNA Adduct Formation . . . 108
Doses of Tamoxifen in Animals and Man . . . 109
Testing at Comparable Therapeutic Levels . . . 110
Conclusion . . . 111
References . . . 113

7 Chemoprevention: Cinderella Waiting for the Ball . . . 115
Introduction . . . 115
The Link Between Estrogen and Breast Cancer . . . 116
Prevention of Mammary Cancer in Rodents . . . 117
Tamoxifen: The First SERM for the Prevention of Breast Cancer in High-Risk Populations . . . 119
Royal Marsden Study . . . 120
NSABP/NCI P-1 Study . . . 122
Italian Study . . . 125
The International Breast Cancer Intervention Study (IBIS-I) . . . 126
Follow-Up of Chemoprevention Studies with Tamoxifen . . . 127

Author's Proof

Two Approaches to the Chemoprevention of Breast Cancer 127 360
Raloxifene: Abandoned and Resurrected 128 361
Conclusion 131 362
References 131 363

8 Tamoxifen and Raloxifene Head to Head: The STAR Trial 135 364
References 141 365

9 Acquired Resistance to Tamoxifen: Back to the Beginning 143 366
Introduction 143 367
The MCF-7 Breast Cancer Cell Line 144 368
Tamoxifen Metabolism Hypothesis 145 369
Growth Factor-Driven Acquired and Intrinsic Resistance 146 370
An Evolving Model of Acquired Resistance to SERMs and Aromatase 371
Inhibitors 147 372
Back to the Beginning 149 373
Mechanisms of Estrogen-Induced Apoptosis 150 374
A New Classification of Estrogens 152 375
Final Thoughts on Four Decades of Discovery to Advance the Value of 376
the ER Target in Breast Cancer 153 377
References 157 378

10 The Legacy of Tamoxifen 163 379
Introduction 163 380
Pure Antiestrogens 164 381
SERM Successes 166 382
Lasofoxifene (CP-336156, Fablyn) 167 383
Bazedoxifene (TSE-424, WAY-140424) 168 384
Ospemifene (FC-1271a) 168 385
Refining the SERM Concept Further 169 386
References 172 387

Appendix A: Four Decades of Discovery in Breast Cancer Research 388
and Treatment: An Interview with V. Craig Jordan 177 389

Appendix B: Selected Awards That Recognize the Contribution 390
of Tamoxifen and Raloxifene to Medicine 195 391

Author's Proof

Author's Proof

About the Authors



This figure will be printed in b/w

V. Craig Jordan, OBE, Ph.D., D.Sc., FMedSci, member of the National Academy of Sciences, is known as the "father of tamoxifen." He was educated in England, obtaining his Ph.D. in Pharmacology (1973) studying a group of failed antifertility agents called nonsteroidal antiestrogens. There was no interest in drug development, but his work in academia blossomed into tamoxifen. Over a 40-year career, he researched all aspects of antiestrogens and then SERMs using structure-function relationships to investigate molecular mechanisms, developed new models, studied metabolism, developed the first realistic models of SERM resistance in vivo, and translated all of his concepts into clinical trials. He was there for the birth of tamoxifen as he is credited for reinventing a "failed morning-after contraceptive" to become the "gold standard" for the treatment of breast cancer. During his work, Jordan has held professorships at Wisconsin (1985–1993), Northwestern (1993–2004) (also the Diana Princess of Wales Professor), the Fox Chase Cancer Center (2004–2009) (also the Alfred Knudson Professor), and currently Georgetown Lombardi Cancer Center where he is the scientific director. He has contributed more than 600 scientific articles with more than 23,000 citations. His work on SERMs has been recognized with the ACS Medal of Honor, the BMS Award, the Kettering Prize, the Karnofsky Award (ASCO), the Landon Award (AACR), and the St. Gallen Prize. He is a member of the National Academy of Sciences and the Academy of Medical Sciences (UK) and is one of the 90 honorary fellows of the Royal Society of Medicine worldwide, and he received the Order of the British Empire (OBE) for services to International Breast Cancer Research in 2002. The chapters described in this book are all written by Dr. Jordan as he contributed personally to every aspect of tamoxifen application in therapeutics and all aspects of tamoxifen's pharmacology. He discovered SERMs and the new biology of estrogen-induced apoptosis.

Author's Proof

Each chapter is a personal journey with a few decades of discovery that forever changed women's health.



This figure will be printed in b/w

Philipp Y. Maximov, M.D., Ph.D., graduated from the Russian National Research Medical University named after N. I. Pirogov (RNRMU) in Moscow, Russia, specializing in medical biochemistry in 2006, and completed his postgraduate program in 2010, receiving the Candidate of Medical Sciences degree (equivalent to Ph.D. in the USA) in medical biochemistry and molecular biology with a thesis titled "Structure-functional relationship of triphenylethylene estrogens and the estrogen receptor alpha in human breast cancer cells" under the mentorship of Dr. V. Craig Jordan, OBE, Ph.D., D.Sc. Dr. Maximov during his last year of medical school was chosen to be one of the top students in his class to represent his university in an exchange program between Fox Chase Cancer Center (FCCC) in Philadelphia, PA. From 2005 to 2010, Dr. Maximov was a graduate student at FCCC and, from 2006, was a Ph.D. student in Dr. Jordan's laboratory. After his Ph.D. thesis defense in Moscow, Dr. Maximov rejoined Dr. Jordan's lab as a Susan G. Komen for the Cure International Postdoctoral Fellow at Georgetown University in 2011. Dr. Maximov is a member of the American Association for Cancer Research (AACR) and the Royal Medical Society in London, UK.



This figure will be printed in b/w

Russell E. McDaniel received his BS in Biochemistry in 2007 from Temple University in Philadelphia, PA. He served in the Peace Corps for 2 years, teaching high school chemistry in Mozambique, before joining Dr. Jordan's laboratory. At present, he is pursuing a master's degree in biotechnology at Georgetown.

Author's Proof

Author Queries

Chapter No.:

Query Refs.	Details Required	Author's response
AU1	Please check if "in America" should be changed to "in the United States".	
AU2	Please mention the name of the institute in the phrase "the Institute in Bern".	
AU3	Please check if "terminated clinical development in 1972" can be edited as "terminated clinical development of the medicine in 1972".	
AU4	Should "postmenopausal patients" be changed to "postmenopausal women"?	
AU5	Please check if edit to sentence starting "The discovery. . ." is okay.	
AU6	Please check the phrase "Ten years of adjuvant therapy is now known to be superior to 5 years of adjuvant therapy" for correctness, if appropriate.	
AU7	Please check if "in America" should be changed to "in the United States".	
AU8	"though" has been changed to "through" in sentence starting "She was a force. . ." Please check if okay.	
AU9	Please check if "assembled" can be changed to "put together".	
AU10	Please check if edit to sentence starting "He also wishes. . ." is okay.	
AU11	Please check if the phrase "There was no interest in drug development" can be edited as "There was no interest in drug development until then".	
AU12	Please check if "Wisconsin" and "Northwestern" should be changed to "Wisconsin University" and "Northwestern University" in sentence starting "During his work. . ."	
AU13	Please check if edit to sentence starting "He is a member. . ." is okay.	

Author's Proof

AU14	Please check if edit to sentence starting “Philipp Y. Maximov. . .” is okay.	
AU15	Please check if edit to sentence starting “From 2005. . .” is okay.	

Author's Proof

Metadata of the chapter that will be visualized online

Chapter Title	Discovery and Pharmacology of Nonsteroidal Estrogens and Antiestrogens	
Copyright Year	2013	
Copyright Holder	Springer Basel	
Corresponding Author	Family Name	**Maximov**
	Particle	
	Given Name	**Philipp Y.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**McDaniel**
	Particle	
	Given Name	**Russell E.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**Jordan**
	Particle	
	Given Name	**V. Craig**
	Suffix	
	Division	Lombardi Comprehensive Cancer Center
	Organization	Georgetown University Medical Center
	Address	Washington, DC, USA
Abstract	The application of synthetic organic chemistry to establish the simplest basic structure of estrogenic compounds was a major triumph for medicinal chemistry in the 1930s. Two groups of compounds were discovered: the hydroxylated stilbenes with high potency and rapid excretion and the triphenylethylenes with high lipophilicity, metabolic activation, and a very long duration of action. A study of structure-function relationships in laboratory animals would result in the use of high-dose estrogen treatment for metastatic breast cancer in postmenopausal patients in the 1940s. The triphenylethylene-based antiestrogens would evolve into the nonsteroidal antiestrogens that in the 1960s were predicted to be potential postcoital contraceptives in women based on compelling rodent studies. This application did not succeed and enthusiasm for clinical development waned.	

Author's Proof

Chapter 1
Discovery and Pharmacology of Nonsteroidal Estrogens and Antiestrogens

Abstract The application of synthetic organic chemistry to establish the simplest basic structure of estrogenic compounds was a major triumph for medicinal chemistry in the 1930s. Two groups of compounds were discovered: the hydroxylated stilbenes with high potency and rapid excretion and the triphenylethylenes with high lipophilicity, metabolic activation, and a very long duration of action. A study of structure-function relationships in laboratory animals would result in the use of high-dose estrogen treatment for metastatic breast cancer in postmenopausal patients in the 1940s. The triphenylethylene-based antiestrogens would evolve into the nonsteroidal antiestrogens that in the 1960s were predicted to be potential postcoital contraceptives in women based on compelling rodent studies. This application did not succeed and enthusiasm for clinical development waned.

Introduction

It is now more than 75 years since the first compound, with known chemical structure, was shown to produce estrogenic effects in animals [1] (Fig. 1.1, compound 1). Since that time, thousands of compounds have been screened for estrogenic activity. During the past 50 years, the early events involved in the molecular mechanism of action of estrogens in their target tissues (e.g., vagina, uterus, pituitary gland, or breast), via the estrogen receptor (ER), have been described [2–4]. In this opening chapter, we will describe how the structure-function relationships of nonsteroidal estrogens set the stage for the serendipitous discoveries of nonsteroidal antiestrogens and the selective estrogen receptor modulators (SERMs). The story, with its twists and turns, is more about people and the exploitation of opportunities by individuals than a plan implicated in the drug development department of any pharmaceutical company.

Uncorrected Proof

P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
DOI 10.1007/978-3-0348-0664-0_1, © Springer Basel 2013

Author's Proof

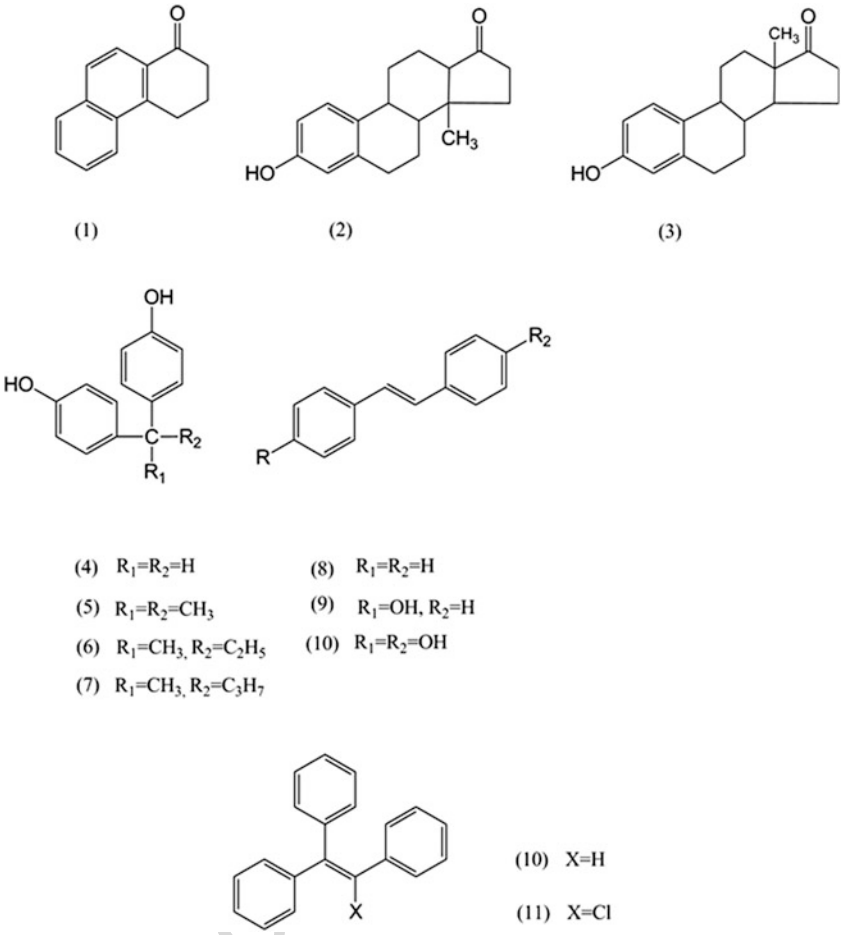


Fig. 1.1 Formulae of compounds found, in the 1930s, to have estrogenic activity in vivo. Compound 2 was believed to be the molecular structure of ketohydroxyestrin (estrone). This is now known to be incorrect and compound 3 is estrone

Testing Methods for Estrogen

To discover new knowledge about the control of fertility by hormones, animal models are required to detect target tissue-specific effects of test compounds. The Allen-Doisy test [5] depends upon the induction of vaginal cornification in castrate animals 60–80 h after the subcutaneous administration of estrogens. A colony of animals is ovariectomized and used for assays 2 weeks later. To maintain the sensitivity of the colony and retard atrophy of the uterus and vagina [6], the animals are primed with 1 μg estradiol (SC) every 6 weeks. The animals are not used for 2 weeks following either priming or experimental use. However, it is often wise to

Author's Proof

screen the animals by the vaginal smear technique to check for incomplete ovariectomy or test compounds with prolonged biological activity. This technique accurately identified a "principle" that Allen and Doisy called estrogen in ovarian follicular fluid [5].

Direct administration of estrogens into the vagina increases the sensitivity of the Allen-Doisy test and cornification occurs earlier since the response is not dependent upon distribution and metabolism [7, 8]. Emmens [7–10] assayed and evaluated the structural derivatives of stilbene and triphenylethylene by both intravaginal and systemic Allen-Doisy tests. This early work accurately established the relative potency of the test compounds.

Martin and Claringbold [11] developed the intravaginal assay to study the early events of estrogen stimulation by using the increase in vaginal mitoses and vaginal epithelium thickening as measures of the estrogenic response. Martin [12] further showed that the reduction of 2,3,5-triphenyltetrazolium chloride to formazan in epithelial cells of the vagina following the local application of estrogens could form the basis of a sensitive assay procedure for early estrogenic events.

The increase in uterine weight of young castrate rats was used to determine systemic estrogen and activity by Bülbring and Burn [13]. The preparation of castrate animals has been found to be an unnecessary step, and immature rats or mice are usually used [14, 15]. Estrogens induce a rapid early imbibition of water by the uterus, and this effect was used in the 6-h assay of estrogens by Astwood [16]. However, this technique cannot distinguish between full estrogens and partial agonists and also suffers from differences in the release of test compounds from the injection site which will ultimately affect the time course of the uterine response. Most assays utilize a 3-day injection technique to stimulate full uterine growth [17].

Potential estrogenic activity can be inferred for a compound by its ability to inhibit the binding of [^{3}H]estradiol to its target tissues in vivo [18, 19]. However, many nonsteroidal antiestrogens produce the same effect [20, 21] so this effect cannot be assumed to predict biological activity. Similarly, the ability of a compound to inhibit the binding of [^{3}H]estradiol to ERs in vitro suggests a potential mechanism of action via the ER but again this alone cannot predict biological activity, i.e., agonist or antagonist actions [22]. Armed with the bioassay technique in vivo, a host of compounds were screened during the 1930–1960s to find potential novel agents for clinical applications.

Uncorrected Proof

Structure-Activity Relationships of Estrogens

The pioneering studies by Sir Charles Dodds laid the foundation for all the subsequent research on the structure-activity relationships of nonsteroidal estrogens. The 1930s saw a remarkable expansion of knowledge that culminated in the description of the optimal structural requirements in a simple molecule to produce estrogen action. The first compound of known structure (1-keto-1,2,3,4-tertrahydrophenanthrene) (Fig. 1.1, compound 1) to be found to have estrogenic

Author's Proof

activity [1] was tested because of its structural similarity to the presumed structure of ketohydroxyestrin (Fig. 1.1, compound 1). As it turned out, the structure of the natural steroid (estrone) was incorrect (Fig. 1.1, compound 2), but this did not matter; the fact that nonsteroidal compounds can exhibit estrogenic properties was established. A phenanthrene nucleus was later found to be unnecessary for estrogenic activity [23]. Simple bisphenolic compounds are active (Fig. 1.1, compounds 4–7) and, as will be seen later in this chapter, this is a recurrent feature of many nonsteroidal estrogens. The finding that hydroxystilbenes (Fig. 1.1, compounds 8–10) possess potent estrogenic activity provided a valuable clue that stimulated a systematic investigation of analogs to optimize the potency. At this time, an interesting side issue occurred that deserves comment, as it illustrates how parallel research endeavors can eventually reach the same conclusions. Anol, a simple phenol derived from anethole (Fig. 1.2), was reported to possess extremely potent estrogenic activity with 1 μg capable of inducing estrus in all rats [24]. These results were not confirmed with different preparations of anol [25, 26], but it was found that dimerization of anol to dianol (Fig. 1.2) can occur and this impurity, which was known to have potent estrogenic [27] properties, was the compound responsible for the controversy [27]. At this time, Dodds reported [28–30] that diethyl substitution at the ethylenic bond of stilbestrol (Fig. 1.2) produces an extremely potent estrogen [31]; other substitutions produce less active compounds [28, 32]. The structural similarity between diethylstilbestrol and estradiol (the formula was established by 1938) was noted, but an attempt to mimic the rigid steroid structure by the synthesis of dihydroxyhexahydrochrysene (Fig. 1.2) resulted in a drop in estrogenic potency. Dihydroxyhexahydroxchrysene is approximately 1/2,000 as potent as diethylstilbestrol [23]. AU1

There was considerable interest in the development of a long-acting synthetic estrogen because of the potential for clinical application. The duration of action of diethylstilbestrol can be increased dramatically by esterification of the phenolic groups [28]. A 10-μg dose of diethylstilbestrol dipropionate can produce estrus for more than 50 days, while the phenol at the same dose is active for only 5 days. The simple hydrocarbon triphenylethylene (Fig. 1.1, compound 11) is a weakly active estrogen [33], but 10 mg can produce vaginal cornification in mice for up to 9 weeks. Replacement of the free ethylenic hydrogen with chlorine (Fig. 1.1, compound 12) increases the potency and duration of action by subcutaneous administration [34], but when administered orally, triphenylchloroethylene has a similar duration or action as diethylstilbestrol or estradiol benzoate. In the search for orally active agents, Robson and Schonberg [35] showed that DBE (Fig. 1.2) was very effective by the oral route. The long duration of action is related to depot formation in body fat [36], but DBE did not reach clinical trial. The related compound trianisylchloroethylene (TACE) became available clinically as a long-acting estrogen (Fig. 1.2). TACE is stored in body fat for prolonged periods [37–39]. It was around the mid-1940s and early 1950s that the discovery that high-dose synthetic estrogens could cause the regression of about 30 % of metastatic breast cancers in postmenopausal women became the standard of care for the treatment of breast cancer [40, 41]. This is interesting not only because this was the

Uncorrected Proof

Author's Proof

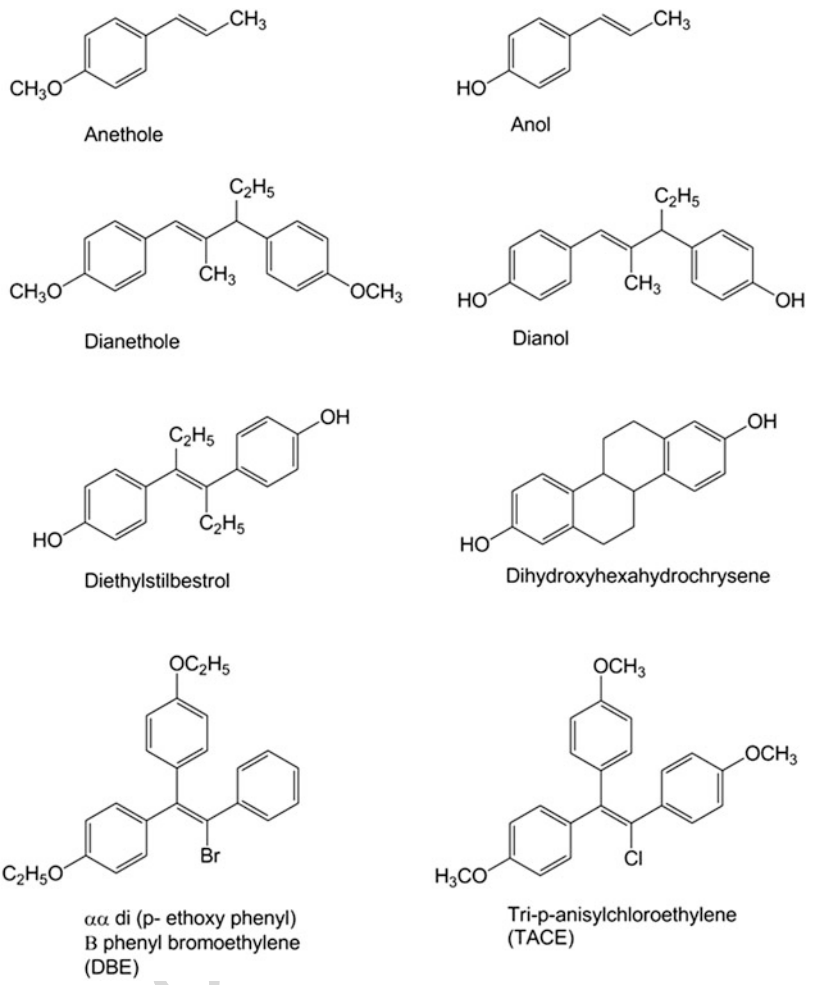


Fig. 1.2 Formulae of nonsteroidal compounds with estrogenic (or suspected) activity in vivo

first time a chemical therapy was shown to cause regression of cancer but also the compounds that Haddow used were made and provided by chemists at Imperial Chemical Industries (ICI). High-dose synthetic estrogen therapy was to remain the standard of care for the palliative treatment of breast cancer until the late 1970s early 1980s when another synthetic estrogen derivative, also produced by chemists at ICI pharmaceutical division. ICI 46,474 (later to be known as tamoxifen) would revolutionize breast cancer treatment and prevention. This is the story of this book.

The first 25 years established many of the important structural features that govern the potency and duration of action of estrogens. This is a remarkable feat of structure-functional relationships without knowledge of the ER target. We will now briefly consider the evolving subcellular mechanism of estrogen action in its target

Author's Proof

tissues before describing the structure-activity relationships and pharmacological properties of the nonsteroidal antiestrogens.

Estrogen Action

The reason for the target site specificity of the estrogens remained obscure until the synthesis of tritium-labeled compounds with high specific activity. The synthesis of [^{3}H]hexestrol (reduction of diethylstilbestrol with tritium and a palladium catalyst) by Glascock working with Sir Charles Dodds [42] and the subsequent observation that there was binding of hexestrol in the uterus, vagina, mammary glands, and pituitary gland of immature female goats and sheep [43] provided the first evidence for the target tissue localization of estrogens. The subsequent applications of [^{3}H] hexestrol to determine hormone responsiveness in metastatic breast cancer was a big step in our antiestrogen story [44]. The subsequent fundamental study by Jensen and Jacobson [2] of the distribution and binding of [^{3}H]estradiol in the immature rat demonstrated that estradiol selectively binds to, and is retained by, the uterus, vagina, and pituitary gland. These systematic studies suggested there is a specific receptor for estradiol in its target tissues. The biochemical identification of an estrogen-binding protein in the immature rat uterus and the observation that [^{3}H] estradiol becomes located in the receptor nucleus of the cell provided a model to describe the initiation of estrogen-stimulated events. The early evidence for an ER system has been described [3, 4]. Simply stated, the estrogen dissociates from plasma proteins and readily diffuses into the cell. Initially it was thought that the cytoplasmic ER binds the ligand and the resulting receptor complex is activated before translocation to the nucleus. Interaction with nuclear acceptors (now referred to as promoter regions of estrogen-responsive gene) results in the activation of RNA and DNA polymerases to initiate subsequent protein synthesis and cell proliferation, respectively. There were, however, an increasing number of observations that were inconsistent with the classical two-step hypothesis. These reports have been reviewed [45]. Two innovative approaches to the question of the actual subcellular localization of unoccupied ER deserve comment. These methods, which did not require cellular disruption, settle the issue of where the unoccupied receptors resided in the cell. Therefore, if it was, in fact, cell disruption that causes the unoccupied receptor to “fall out of the nucleus” but ER complexes are “stuck” in the nucleus, this would explain the early translocation model. Indeed, a series of studies with weakly binding antiestrogens injected into the immature rat arrived at the same conclusion [46]. Monoclonal antibodies raised to the ER were used as tags for immunohistochemical studies. The antibody is linked to a peroxidase enzyme system to visualize the receptor, which appears to be located exclusively in the nuclear compartment, even in the absence of estrogen [47]. The other approach was to enucleate ER-containing GH3 rat pituitary tumor cells with cytochalasin B. Unoccupied receptors are observed in nucleoplasts rather than cytoplasts [48]. Similar studies were subsequently published using estrogen-free culture of

Uncorrected Proof

Author's Proof

ER-positive MCF-7 breast cancer cells [49]. Although it was possible that these studies were generating artifactual results, the simplified model of estrogen action, i.e., unoccupied ER is a nuclear protein, is now considered to represent subcellular events in vivo.

Nonsteroidal Antiestrogens

The finding by Lerner and coworkers [50] that the compound ethamoxytriphetol (MER 25, Fig. 1.3) is an inhibitor of estrogen action provided a new tool for laboratory research and clinical investigation. It is of considerable interest that MER 25 had been synthesized as part of a cardiovascular pharmacology program and only found its way to endocrine testing at Dr. Lerner's request. Lerner had spotted that MER 25 looked like the nonsteroidal triphenylethylenes so he wanted to test it for estrogenic properties. There were none but he discovered the first nonsteroidal antiestrogen. MER 25 was subsequently found to have antifertility properties in the rat [51–53], so clinical use as an oral contraceptive seemed logical. Preliminary clinical trials with MER 25 were scientifically successful [54–56]; however, the clinical studies were discontinued because of low potency and toxic side effects. In the search for new compounds, a structural derivative of triphenylethylene, clomiphene (also called chloramiphene or MRL 41; in Fig. 1.3, the generic isomers enclomiphene and zuclomiphene are shown. Clomiphene is a mixture of isomers) was found to be a potent antifertility agent in rats [57, 58] and it became the forerunner of many structurally similar compounds that were synthesized and tested as potential postcoital antifertility agents [59–64]. The spectrum of compounds was reviewed by Emmens [65].

Structure-Activity Relationships in the Rat

There are no published reports specifically documenting the structure-activity relationships of MER 25. Apart from one triphenylethane MRL 37, with a hydrogen substituted for MER 25's alcoholic hydroxyl [53, 66], most interest has focused on compounds related to triphenylethylene. The original antiestrogens can be classified into two major groups: substituted triphenylethylenes and bicyclic antiestrogens.

Substituted Triphenylethylenes

Early studies with clomiphene used a mixture of geometric isomers [53, 57, 58]. The *cis* and *trans* isomers were separated [67] and each has been reported to

Uncorrected Proof

Author's Proof

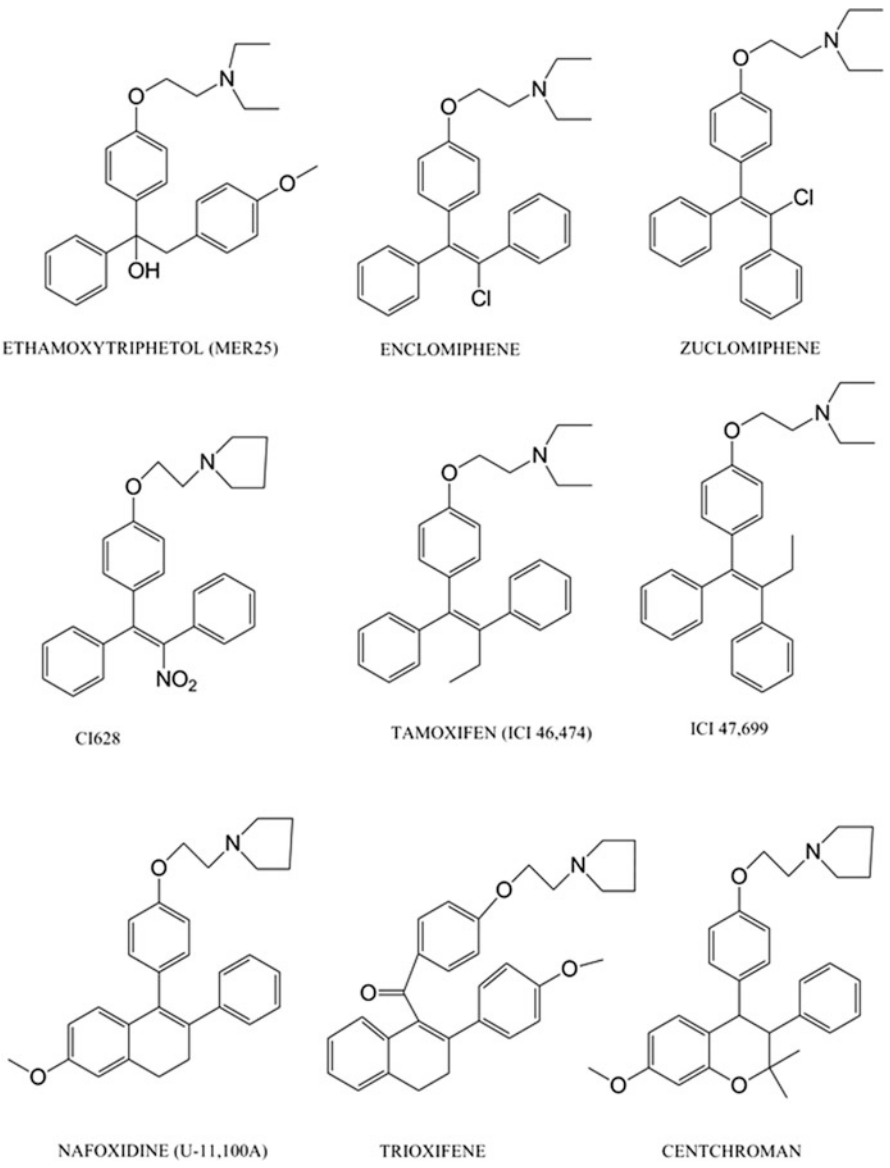


Fig. 1.3 The formulae of nonsteroidal antiestrogens mentioned in the text. Zuclomiphene and ICI 47,699 are the estrogenic geometric isomers of the antiestrogenic enclomiphene and ICI 46,474 (tamoxifen)

possess different biologic activities [68–70]; however, some controversy surrounded the designation of the isomers in relation to their observed biologic properties. They were originally labeled as geometric isomers! It is now clear that the *trans* isomer enclomiphene (originally named isomer B or *cis* clomiphene) has

Author's Proof

antiestrogenic properties in the rat, whereas the *cis* isomer zuclomiphene (originally named isomer A or *trans* clomiphene) is estrogenic (Fig. 1.3). Comparison of the isomers of tamoxifen and enclomiphene in the uterine weight test demonstrated only minor differences in their dose-response curves [71]. Parenthetically, in 1972, during the examination of my Ph.D. entitled "A study of the oestrogenic and anti-oestrogenic activities of some substituted triphenylethylenes and triphenylethanes," I was asked by my external examiner Dr. Arthur Walpole, head of the fertility program of ICI Pharmaceutical Division, why the biological properties of the geometric isomers of clomiphene and tamoxifen were opposite? I replied it was obviously the influence of the chlorine in clomiphene, never considering that the geometric isomers of clomiphene were misidentified. Walpole knew that!

The fundamental importance of the geometric shape of a molecule for antiestrogenic activity was realized after the report by Harper and Walpole [72] of the contrasting biological properties of the *cis* and *trans* isomers of substituted triphenylethylenes. Tamoxifen (ICI 46,474) and its *cis* isomer ICI 47,699 (Fig. 1.3) have been identified by nuclear magnetic resonance [73] and the structure of ICI 47,699 confirmed as the *cis* isomer by X-ray crystallography [74]. The simultaneous administration of tamoxifen with estradiol to immature rats prevents the increases in uterine wet weight or vaginal cornification observed with estradiol alone. In contrast, ICI 47,699 is only estrogenic in conventional tests [75]; however, very high doses have been shown to inhibit estradiol action in the uterus [71].

p-Methoxy-substituted derivatives of tamoxifen have been synthesized and tested [76] but this type of structural modification does not increase antiestrogenic activity.

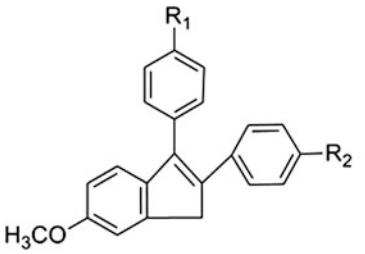
CI628 (CN-55, 945–27) (Fig. 1.3) is an estrogen antagonist in the rat [77]. The isomeric mixture was used only briefly for the experimental treatment of advanced breast cancer; however, there is a considerable literature on the use of CI628 in studies with the human breast cancer ER in vitro [78]. It is an antitumor agent in the rat mammary carcinoma model [79]. There is no information on the biological properties of the separated geometric isomers; both appear to be antiestrogenic [71].

Uncorrected Proof

Bicyclic Antiestrogens

Scientists at the Upjohn Company, Kalamazoo, MI, focused much attention on the structure-activity relationships and properties of bicyclic (fixed ring)-based nonsteroidal antiestrogens [59–61]. Simple hydroxylated indenes [80, 81] that are superficially related to the structure of DES are potent estrogens. The structure-activity relationships of the indene nucleus have been investigated in the search for potent antifertility relationships [59] (Fig. 1.4). The 6-methoxy group is an advantage for activity but potent antifertility activity is determined by the substituted amine ethoxy side chain. Optimal activity is observed with the pyrrolidino side chain (IND 1, Fig. 1.4) and other substituted side chains (IND 2, 3, 4) have reduced activity. A morpholino side chain (IND 5) produces a compound with

Author's Proof



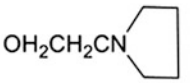
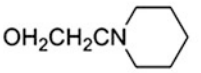
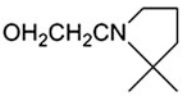
Compounds	R_1	R_2	
IND 1	OH_2CH_2CN 	H	Potent Antifertility Action
IND 2	OH_2CH_2CN 	H	
IND 3	OH_2CH_2CN 	H	Reduced Antifertility Activity
IND 4	HOH_2CH_2CN 	H	
IND 5	OH_2CH_2CN 	H	Low Antifertility Activity

Fig. 1.4 The relative antifertility activity of substituted indenes in the rat (Data adapted from Lednicer et al. [59])

253 approximately 1 % of the activity of IND 1 with the pyrrolidino side chain. In the
254 same study, Lednicer and coworkers [59] showed that the 6 phenols of IND 4 had
255 approximately 5 % of the potency of the methoxy compound. Hydroxylated

Author's Proof

derivative might be expected to have a shorter duration of action so that larger doses will be required to maintain adequate drug levels.

The 3,4-dihydronaphthalenes further exemplify the importance of the substituted side chain for optimal activity (Fig. 1.5). Nafoxidine (see Fig. 1.3 for comparison with other nonsteroidal antiestrogens) is the most potent compound of the series although the ether oxygen of the side chain can be replaced by carbon with very little loss of potency. However, decrease in the length of the side chain (NAF 1–3) (Fig. 1.5) reduces the antiestrogenic potency and in fact, removal of the side chain (NAF 6) results in the complete loss of the antagonist activity. The resulting compounds are estrogens [60, 61]. These observations led Lednicer et al. [60] to suggest that a basic group, at a given position in space, is required to obtain a molecule with estrogen antagonist activity. This point of view is further supported by the observation that dimethylation *ortho* to the aminoethoxy side chain in MER25 [82] and tamoxifen [83] reduces antiestrogen activity and receptor binding, respectively. The methyl substitutions reduce the number of positions in space that the side chain can adopt. A series of derivatives of tamoxifen with different polar side chains had been investigated [84]. The resulting biological activity related to structure is shown in Fig. 1.6. Trioxifene (available as the mesylate salt LY133314, Fig. 1.3) has been described [85] and phase I trials as a potential agent for breast cancer therapy were completed, but the drug was not developed. The unusual structural feature of trioxifene (Fig. 1.3) is the introduction of a ketone group linking the *p*-alkylaminoethoxyphenyl ring to the ethylenic bond. The structure therefore diverges from the usual triphenylethylene type. This, in the future, would turn out to be an important structural feature to create the antiestrogens with no estrogen-like actions in the uterus as raloxifene.

Centchroman (Fig. 1.3) has been studied in considerable detail in laboratory animals and women as it was investigated as a postcoital contraceptive agent [86, 87]. The structure-activity relationships of the chromans and the unsaturated chromenes have been given considerable attention. The structure with the greatest similarity to nafoxidine (a 3, 4-diphenylchromene) has very potent antifertility activity in rats. Substitution of hydrogen for two methyl groups at the 2 position gives a less active compound but reduction of the 3, 4 double bond restores potent antifertility activity (centchroman). It is important to note that two diastereoisomers are possible for the substituted chroman. Centchroman is the active *trans* isomer, whereas the *cis* isomer is virtually inactive [88, 89]. Like the 3, 4-dihydronaphthalenes, centchroman is antiestrogenic in the rat [90].

All the nonsteroidal antiestrogens have an alkylaminoethoxy side chain. As previously noted, moving the group further away from the double bond with the substitution of a ketone group (trioxifene) does not reduce antiestrogenic activity. Nevertheless, there seems to be a requirement for the nitrogen on the aminoethoxy side chain to be at a given position in space. A chain length of three atoms seems to be required to place the nitrogen group in the optimal position [60]. All of the studies in vivo with the structure-function relationships of antiestrogens as antifertility agents built up a strong conceptual model that the antiestrogens side chain was interacting actively with a select portion of the ER. Studies now evolved to

Uncorrected Proof

Author's Proof

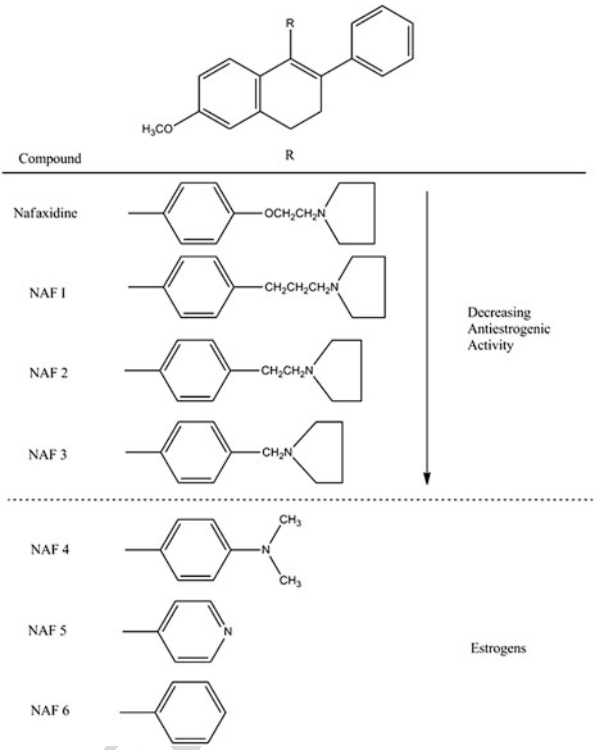


Fig. 1.5 The relative antiestrogenic activity of substituted 3,4-dihydronaphthalene in immature rats (Data adapted from Lednicer et al. [60, 61])

molecular mechanisms in the decades between 1970 and 2000 to predict efficacy of the ER ligand complex based on interrogation of ligand ER interactions.

The Molecular Modulation of the Estrogen Receptor by Nonsteroidal Antiestrogens

The description of the selective binding of [^{3}H]estradiol in the estrogen target tissues of the immature rat (uterus, vaginal) [2] and the subsequent isolation of the ER as an extractable protein from the rat uterus [91, 92] was not only an advance in molecular endocrinology but also an advance that would improve the therapeutics of breast cancer. The idea that by detecting the presence of the ER in a breast tumor would soon evolve from being a prediction test to decide the appropriateness of endocrine ablative surgery to become the target for antiestrogenic drugs was an important conceptual step [93]. Once it was found that the ER was extractable in the 1960s, it was possible to study and understand the binding of ligands to the ER and perhaps gain an insight into the mechanism of action of estrogens and antiestrogens. Early studies of the competitive binding of estrogens and antiestrogens with [^{3}H] estradiol for the ER in vitro [94, 95] were unable to distinguish between estrogens

Uncorrected

Author's Proof

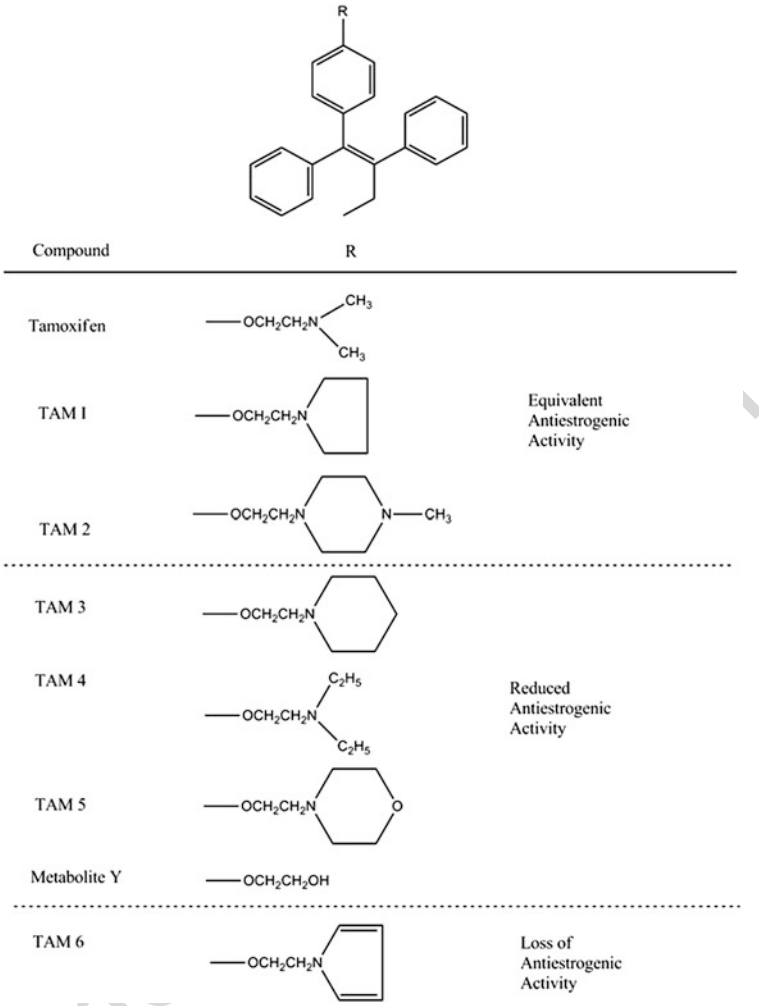


Fig 1.6 The effect of different side chains on the antiestrogenic activity of tamoxifen (Data adapted from Robertson et al. [84])

and antiestrogens biologically. All that could be concluded was that antiestrogens had low binding affinity for the ER and this, it was argued, was why such large doses were necessary to block estrogen action [94]. Also, it was concluded that the low affinity of antiestrogens for the ER was part of their mechanism of action: the ligand would not remain long enough bound to the receptor to activate estrogen action [94]. This proposal was all to change with the discovery of the pharmacological properties of 4-hydroxytamoxifen, a metabolite of tamoxifen then thought to be the principal metabolite of tamoxifen [96]. 4-Hydroxytamoxifen has a binding affinity for the ER equivalent to estradiols, so if it was possible to have high affinity

Author's Proof

antiestrogens, then low affinity was not the mechanism of antiestrogen action. The shape of the resulting complex was the key to efficacy and the subsequent modulation of signal transduction. 4-Hydroxytamoxifen was subsequently adopted as the standard laboratory antiestrogen in cell culture and 20 years later was used as an antiestrogenic ligand to be crystallized with the ligand-binding domain of the human ER [97].

In the 1970s, what was needed was a model cell system to study the structure-function relationships of ligands that bind to the ER. In this way, the intrinsic efficacy of the ligand ER complex could be deciphered, without concerns about pharmacokinetics and metabolism. The ER-positive breast cancer cell line MCF-7 had been described [98] but the fact that the cells apparently grew spontaneously in culture and would not respond to estradiol with growth but would when inoculated into athymic mice [99] led to considerable controversy in the field. Maybe estrogen was acting indirectly to promote breast cancer growth? Nevertheless, tamoxifen did block the spontaneous growth of MCF-7 cells and this blockade could be reversed with estradiol [100]. Interestingly enough, MCF-7 cells, or rather their ER, would be essential to create the first monoclonal antibodies to human ER [101, 102] and subsequently be the critical tool necessary to clone and sequence the human ER [103, 104].

The first cell system used to study the modulation of the ligand ER complex in vitro was primary cultures of the immature rat pituitary gland [105]. The target for the ER was the prolactin gene [22, 106]. The first publication validated the mechanism of actions of nonsteroidal antiestrogens at the ER to regulate estrogen-induced gene transcription as competitive inhibition of estradiol binding to the ER and that it was an advantage but not a requirement for an antiestrogen to be metabolically activated [106]. As with other drug receptor interactions, affinity and the intrinsic efficacy of the drug receptor complex are not interconnected for drug action. Numerous studies of structure-function relationships of triphenylethylenes described the structure-function relationships to modulate the ER complex between the extremes for estrogenic intrinsic efficacy and complete antiestrogen action [22, 107–111]. The structure-activity relationship [112] studies permitted the creation of a map of the hypothetical folding of the ER complex. However, it was the serendipitous advance in deciphering breast cancer cell replication in vitro that was to enhance the interpretation of all future laboratory studies.

In the mid-1980s, the Katzenellenbogen laboratory [113] made the critical discovery that ER-positive breast cancer cells had all been cultured in media containing high concentrations of a pH indicator, phenol red that contained a contaminant that was an estrogen (Fig. 1.7) [114, 115] (note: this is reminiscent of the anol-dianol controversy). Removal of the phenol red from media now permitted the structure-activity relationship studies of nonsteroidal antiestrogens to be extrapolated from prolactin gene modulation to the replication of breast cancer cell lines [116, 117].

However, the critical question to be addressed in molecular pharmacology was "what is the essential interaction of the antiestrogenic side chain with the ER that modulates estrogen-like and antiestrogen action?" A simple estrogen/antiestrogen

Uncorrected Proof

Author's Proof

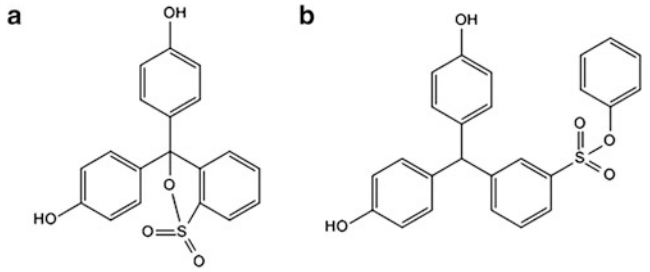


Fig. 1.7 pH indicator phenol red and contaminant bis(4-hydroxyphenyl)-[2-9phenoxy-sulfonul-phenyl]methane found in the growth medium that produced estrogenic effect of the MCF-7 cell line [114, 115]

model of the ER had been proposed as the "crocodile model" [118] with the jaws closed for estrogen action and the antiestrogen, a stick in the jaws to keep them open for antiestrogen action (Fig. 1.8). An antiestrogenic region (AER) that interacts with the appropriately positioned alkylaminoethoxy side chain on the ligand backbone had been proposed previously [22, 112, 118], but how to find it? Several advances were necessary before progress could occur. A model of acquired drug resistance to tamoxifen in athymic mice needed to be developed, the ER needed to be screened for mutations in drug resistant MCF-7 breast tumors, and ER needed to be stably transfected into ER-negative breast cancer cell and suitable gene modulated. All this was done to propose a hypothetical modulation of the antiestrogen ER complex prior to the crystallization of the ligand-binding domain with estradiol and raloxifene [119]. A biological clue was found in the human ER that would complement the structural knowledge of the ligand ER binding domain complex with functional information at a transforming growth factor-alpha (TGFα) gene target.

A mutation, asp 351 tyr, was noted in one MCF-7 tumor cell line with acquired resistance to tamoxifen [120]. The first transfection of the wild-type ER [121] into the ER-negative breast cancer cell line MDA-MB-231 eventually allowed any mutant ER to be transfected. The introduction of ERs with ligands could now be monitored at the estrogen-responsive binding domain with 4-hydroxytamoxifen and raloxifene [97, 119] indicating that while raloxifene's side chain shielded and possibly neutralized Asp351, the side chain of tamoxifen was shorter and barely interacted with Asp 351. To address the hypothesis that the side chain was preventing the interaction of Asp 351 with activating function 1 (AF-1) motif of the ER, ER complex was interrogated using mutations of Asp 351 and structural derivation of raloxifene [122–125] (Fig. 1.6). It was concluded that this amino acid was important to alter surface interactions with other co-regulators of hormone action.

The modulation of the ER complex through coactivator proteins went some way to explain SERM action, i.e., nonsteroidal antiestrogens switching on and switching off sites around a woman's body. But long before this concept was discovered and described in the mid-1980s [126], the literature was full of examples of the

Uncorrected Proof

Author's Proof

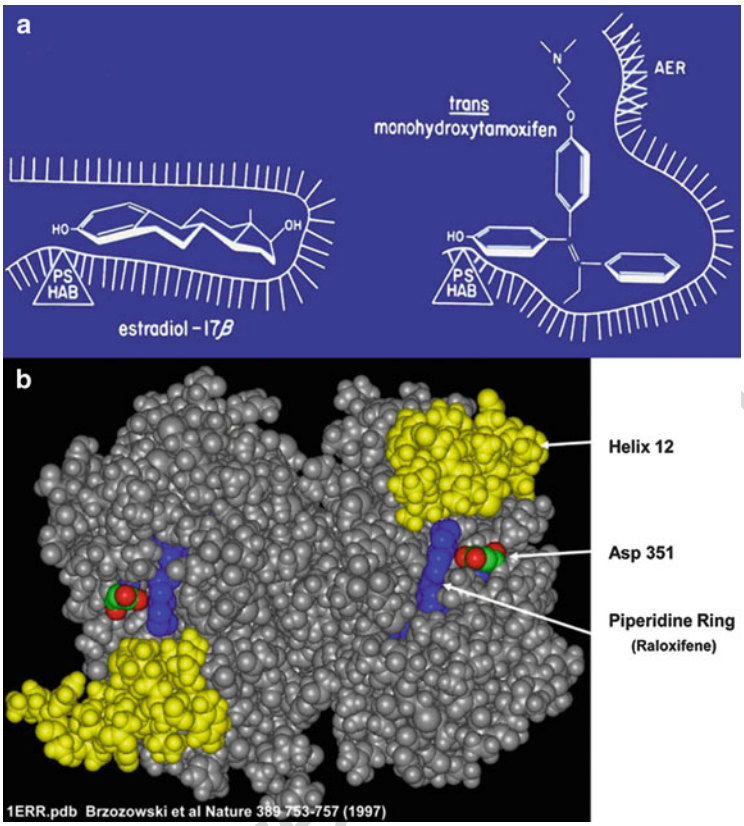


Fig. 1.8 The "crocodile" model of antiestrogenic action of 4-hydroxytamoxifen and its interaction with the antiestrogenic region of the ER [118], as well as the X-ray crystallography of the ligand-binding domain (LBD) of the ER interacting with the raloxifene piperidine ring via its Asp351 and thus producing an antagonistic conformation of the receptor and antiestrogenic biological effect (Front cover of [185])

species-specific pharmacology of nonsteroidal antiestrogens. We will illustrate this now but no adequate explanation has yet been offered or proven to explain the diverse pharmacology in different species.

Effect of Antiestrogens in Different Species

Lerner and coworkers [127] reported that the compound MER 25 antagonizes the actions of estradiol in rats and mice with no other demonstrable hormonal or antihormonal activity. In contrast, Emmens [128] found MER 25 to be only weakly active as an inhibitor of estradiol-stimulated vaginal cornification in the ovariectomized mouse. Nevertheless, the original claim of antiestrogenic activity has been

Author's Proof

adequately confirmed in a variety of interesting models. MER 25 inhibits diethylstilbestrol or estradiol-stimulated increases in the reticuloendothelial system [129] of the ovariectomized mouse. In the mature female rat, a large dose of MER 25 (20 mg) inhibits the estrogen-stimulated uterine ballooning observed at proestrus and doubling the dose also inhibits ovulation [130]. If MER 25 is administered after ovulation, there is inhibition of the estrogen-stimulated DNA, RNA, and protein synthesis that occurs during uterine decidualization [131]. The antiestrogenic action of MER 25 has also been reported at the level of the pituitary. Hypertrophy of the rat pituitary by continued estrogen administration is inhibited by the coadministration of MER 25 [132]. Similarly, estrogen-simulated prolactin release in the ovariectomized rat can be inhibited by large daily dose of MER 25 [133].

Although MER 25 is notable for its very low estrogenic activity in all species tested, some estrogenic responses in the uterus have been quantified. A single dose of MER 25 (5 mg) increases ovariectomized rat uterine glycogen, glucose, and percent water inhibition[134]. A striking short-lived increase in immature rat uterine glucose-6-phosphate dehydrogenase activity and a marked rise in uterine total lipid is observed after a single administration of MER 25 (10 mg) [135]. In ovariectomized mice, MER 25 has some estrogenic activity as evidenced by increases in uterine weight and a stimulation of the enzymes alkaline phosphatase and isocitrate dehydrogenase [76].

Overall, though, the pharmacology of MER 25 is established as an estrogen antagonist. Since the pharmacology of the related triphenylethylenes is so complex, this is presented in species-related groups.

Mouse

The antiestrogens based on triphenylethylene are generally considered to be estrogenic in the mouse. However, this statement is only true under precisely defined conditions. Tamoxifen (oral or SC) is typically estrogenic in the Allen-Doisy (vaginal smear) test using mature ovariectomized mice [75]. In comparative studies, tamoxifen [136, 137] and trioxifene [136] are estrogenic in the 3-day ovariectomized mouse uterine weight test. Similarly tamoxifen, ICI 47,699, enclomiphene, and zuclomiphene are fully uterotrophic in immature mice [138] and tamoxifen does not possess antiuterotrophic activity [139]. In contrast, nafoxidine [138] and trioxifene [136] are partially estrogenic with antiestrogenic properties in immature mice. It is of interest that trioxifene appears to be fully estrogenic in mature ovariectomized mice and antiestrogenic in immature mice, while tamoxifen is more estrogenic than trioxifene in both test systems [136]. Lee [140] pointed out that tamoxifen and nafoxidine are mitogenic in the ovariectomized mouse uterus and neither compound inhibits the mitogenic response to estrone. However, daily treatment of ovariectomized mice with tamoxifen for up to 14 days reduces estrone-stimulated uterine weight gain [141]. It is possible that the accumulation of tamoxifen may alter the pharmacology to produce an inhibitory effect.

Uncorrected Proof

Author's Proof

In this context, SC administration of a large dose of tamoxifen (or related *p*-methoxylated compounds) to ovariectomized mice produces a short period of estrogenic activity followed by a prolonged antiestrogenic and antifertility response [20, 142, 143]. The validity of the vaginal smear technique to assay prolonged antiestrogenic activity was initially questioned [144] although there is agreement about the reduced effectiveness of tamoxifen to produce a fully cornified vaginal epithelium [145].

Rat

The pharmacology of antiestrogens in the rat is dependent upon the target tissue or biochemical end point being studied. For this reason the effects of antiestrogens in different organs will be considered.

All of the nonsteroidal antiestrogens are able to stimulate a partial estrogenic response in the immature and ovariectomized rat uterus. Histological comparisons of estrogen and antiestrogen-stimulated uteri have demonstrated selective differences in both cell stimulation and mitotic activity. CI628 [146], tamoxifen, 4-hydroxytamoxifen [147], and nafoxidine [148] stimulate an enormous increase in the size of luminal epithelial cells. Estradiol increases the incorporation of [^{3}H] thymidine [146] and the mitotic activity [149] in luminal epithelial cells, whereas antiestrogens are much less active [146, 147]. In general antiestrogens produce hypertrophy rather than hyperplasia of luminal epithelial cells.

Much research with antiestrogens has focused on the estrogen control mechanisms of pituitary function. This, in part, is because of the early clinical applications of both clomiphene and tamoxifen as agents for the induction of ovulation in subfertile women [150, 151]. Estrogen-stimulated prolactin release in ovariectomized rats [152] is partially inhibited by nafoxidine [153] and tamoxifen [154]. Studies [155] have demonstrated that in the intact rat the cyclical release of prolactin at proestrus is inhibited by continuous tamoxifen therapy. This is consistent with the finding that tamoxifen, 4-hydroxytamoxifen, and trioxifene inhibit estrogen-stimulated prolactin synthesis by rat pituitary cells in culture [22]. Similarly, tamoxifen inhibits the growth and secretion of prolactin by the estrogen-induced pituitary tumor 7315a [156]. Furthermore, tamoxifen sensitizes the pituitary tumor cells to the inhibitory effects of bromocriptine on prolactin secretion in vitro [157].

Tamoxifen [158–160] and enclomiphene [158] (zuclomiphene is inactive) inhibit ovulation by blocking estrogen action at the level of the hypothalamus and pituitary. Gonadotropin release is inhibited in male and female rats by large doses of clomiphene (mixed isomers) [57] but it is possible that the estrogenic *cis* isomer is predominantly responsible for these effects. The ability of centchroman and clomiphene (mixed isomers) to alter serum FSH, LH, and prolactin in male and female rats has been compared [161]. Clomiphene lowers LH in male rats, slightly increases LH in female rates, but causes a large increase in prolactin in both species.

Uncorrected Proof

Author's Proof

Centchroman, with its rigid bicyclic structure, produces a similar effect to clomiphene on gonadotropin and prolactin levels in both sexes, although the compound is less estrogenic than clomiphene. Studies with the weakly estrogenic compound tamoxifen demonstrate that short-term (5 days) therapy of ovariectomized rats does not lower LH [162], whereas longer therapy (up to 4 weeks) results in a consistent decrease in LH levels [155]. Similarly, a large dose of tamoxifen is sufficiently estrogenic to decrease LH release in male rats [163].

Studies with rat pituitary cells in vitro demonstrates [164] that both estradiol and clomiphene (mixed isomers) sensitize the cells to the effects of LHRH (luteinizing hormone-releasing hormone). The antiestrogenic isomer enclomiphene is apparently only acting as an estrogen in this system. In contrast, Miller and Huang [165] observed that tamoxifen inhibits the estrogen sensitization of ovine pituitary cells to an LHRH analog. Furthermore, tamoxifen, CI628, and nafoxidine inhibit estrogen-stimulated LH release and reverse the inhibition of FSH release by estradiol in this system. To explain these contrasting results, it must be conceded that nothing is known about the pharmacology of antiestrogens in the sheep! Therefore, species differences may be responsible for differences in the action of the compounds. However, the fact that in vivo antiestrogens can cause increases or decreases in LH depending upon the physiological model used must point to the complex factors involved in the regulation of gonadotropin release.

Before considering other organ site effects of antiestrogens, one early observation with nonsteroidal estrogens in the pituitary is worthy of note. Continuous estradiol administration for several weeks can cause pituitary hypertrophy in the rat (F344), while administrating the estrogenic triphenylethylene TACE does not [37]. Of perhaps greater significance, TACE inhibits the hypertrophy of the pituitary produced by estradiol [166]. Current knowledge of the aberrant binding of triphenylethylene-based estrogen to the ER [167, 168] may actually be the reason for the different carcinogenic actions of differently shaped estrogens.

Several estrogen-modulated synthetic events in the liver have been considered as potential sites of antiestrogen action. For convenience, and because the effects of antiestrogens are similar, the rat and primate liver will be considered together. Rat and monkey liver have a well-defined ER system [169, 170], suggesting a mechanism for the effects of estrogen and antiestrogen. The continuous treatment of ovariectomized immature rats with tamoxifen or estradiol increases the synthesis of renin substrate [171]. Similarly, a comparison of ethinyl estradiol and nafoxidine has demonstrated that both are full agonists in stimulating plasma renin substrate in mature female rats [172]. During tamoxifen therapy, breast cancer patients have elevated circulating levels of sex hormone-binding globulin (SHBG) [173]. This observation is of interest because SHBG synthesis in the liver in under estrogen control. Overall, it seems that only estrogenic effects have been described for antiestrogens in the mammalian liver.

Uncorrected Proof

Author's Proof

Chick

Most studies with antiestrogens have focused on the effects in the oviduct and liver. The pharmacology of both tamoxifen [174] and 4-hydroxytamoxifen [175] in the oviduct is as a full antagonist. No estrogenic effects have been reported. In general, antiestrogens are full antagonists of estrogen action in the liver [176].

Conclusion

The purpose of our introductory chapter is to document the important evolutions of knowledge about nonsteroidal synthetic estrogens that really laid the foundation for all future work on nonsteroidal antiestrogens and then selective ER modulators (SERMs).

The intense interest in a study of nonsteroidal antiestrogens in the laboratory during the 1960s and 1970s as antifertility agents in different species or as laboratory tools to dissect estrogen action in its target tissues now slowly started to evolve from reproduction research to targeted cancer therapeutics. Tamoxifen would soon no longer be a laboratory tool and pharmacological curiosity, but an orphan breast cancer drug in search of an optimal strategy to best be deployed in the clinic. This now becomes the theme of our book.

Postscript. A series of chance meetings occurred at ICI Pharmaceuticals Division Alderley Park near my home in Cheshire. In 1967 I wanted to work in cancer research over my summer holiday (I had previously worked at the Yorkshire Cancer Research Campaign laboratory at Leeds University in the summer of 1966) so I went to ICI and I phoned up Dr. Steven Carter from the phonebook outside the laboratories in Alderley Park. He had just reported the unusual actions of cytochalasins in the journal Nature [177]. I asked him for a summer job and he asked me to set up an appointment. I said: "I'm outside ICI now," so he invited me in and the job was mine. Cytochalasins are a series of natural products but cytochalasin B caused polynuclear cells or at different concentration nuclear extrusion with a small cell membrane. This same natural product would later be used by Wayne Welshons to aid in discovering the actual location of the unoccupied ER [48, 178]. I had a great opportunity working in Steven Carter's laboratory on the electron microscopy of nuclear extrusions in mouse L cells. This was my introduction to ICI pharmaceuticals division. More importantly, I came to know Dora Richardson, the synthetic chemist who later would provide me with tamoxifen metabolites; Arthur (Walop) Walpole whose antifertility laboratory was opposite Steven Carter's; and Mike Barett, who was in charge of ICI's beta-blocker discovery program that was building on Jim Black's landmark discovery at ICI. Jim Black subsequently won the Nobel Prize in 1988 in Physiology and Medicine for "discoveries of important principles for drug treatment." Mike Barett's laboratory

Uncorrected Proof

Author's Proof

was next door to Steven Carter's. This again is where chance and opportunity take control, but you have to be ready to see the opportunity and be prepared to rise to the challenge. Mike Barett became the professor of Pharmacology at Leeds University. He apparently was impressed with my skill as a lecturer (I was a Ph.D. student at that time in the early 1970s) so he offered me a job as a lecturer in Pharmacology. I had no Ph.D. yet and no publications, but I was talent spotted!

I started my lifelong "love affair" with nonsteroidal estrogens and antiestrogens with the start of my Ph.D. thesis work entitled "A study of the oestrogenic and anti-oestrogenic activities of some substituted triphenylethylenes and triphenylethanes." I was supported by a Medical Research Council scholarship, which I only received by chance because I was originally on the waiting list. Someone declined their scholarship so there I was, a Ph.D. student in the Department of Pharmacology at the University of Leeds (1969–1972). I decided to study the ER with Dr. Edward Clark in the Department of Pharmacology at the University of Leeds. Dr. Jack Gorski had published an exciting series of reports showing that the ER could easily be extracted from the rat uterus and isolated by sucrose density gradient analysis. My project was going to be simple: I was to establish the new technique of sucrose density gradient analysis, isolate the receptor, and crystallize the protein with an estrogen and an antiestrogen. Through X-ray crystallography in the Astbury Department of Biophysics at the University of Leeds, we would establish the three-dimensional shape of the complexes to explain antiestrogenic action. The goal was to solve a fundamental question in pharmacology: What is the molecular mechanism of action for a drug? Progress was slow in establishing the receptor purification technique of sucrose gradient analysis, and I switched my thesis topic to study the structure-activity relationship of antiestrogens. As it turned out, this was a good, strategic decision, as it has taken the best efforts of the research community nearly 30 years to achieve success. The structure of the ER complex was solved by scientists at York University, England, in 1997. No one has yet succeeded in crystallizing the whole ER with an antiestrogen.

My study of failed contraceptives was less than inspiring as no one was recommending careers in a dead end. It was clear in the late 1960s that nonsteroidal antiestrogens would not be "morning-after pills." They were excellent in rats but did exactly the opposite in women. Also, as it turned out, the pharmaceutical industry chose to discontinue all their interest in these compounds because of too much toxicity or there was no money to be made. But chance meetings and my desire to be a part of developing a clinically useful drug for cancer would change that perspective.

The road to my Ph.D. was complicated in early 1972 as the university could not find an examiner. No one cared about failed contraceptives! Mike Barett solved the problem after Sir Charles Dodds declined with the words: "Sorry, I have not kept up with the literature during the past 20 years." He invited his former colleague Arthur Walpole to be my examiner, and after some grumbling by the university that it was inappropriate because "he was from industry," this set off a chain of events that would create tamoxifen as the gold standard for the treatment and prevention of

Uncorrected Proof

Author's Proof

breast cancer. This is the next chapter in our story as "Tamoxifen Goes Forward Alone."

During my Ph.D. studies, I learned all the names of the important players in estrogen action: Elwood Jensen, Jack Gorski, and Bill McGuire. Jensen and Gorski were world authorities on the ERs and my Ph.D. supervisor Ted Clark would often remark "look how many authors are on their papers; we cannot compete with these big groups." Bill McGuire was medically qualified and really drove the ER and progesterone receptor concept to predict the susceptibility of endocrine ablation into a clinical reality [179, 180]. Jensen and Gorski would eventually become my colleagues, coauthors, and then fellow members of the National Academy of Sciences. I remember well everyone congratulating Elwood Jensen at the pivotal meeting linking ER tumor level with response to ablative endocrine therapy in Bethesda, MD, in 1974 [181], when his election was announced. Elwood and I would receive the inaugural Dorothy P. Landon Award for translational research from the American Association for Cancer Research (AACR) in 2002, he for the ER target and me for the development of the "science of antiestrogens applied to cancer research" [182]. Bill McGuire and I would be close friends until his untimely death in 1992 [183, 184]. Each of these prominent scientists gave me help and support in the early years of my career with invitations to their laboratories to talk about my "orphan drug tamoxifen" or with letters of recommendation. Each was important for my career development as a young scientist in the 1970s.

In closing Chap. 1, I wish to state that my focused interest in the pharmacology of nonsteroidal antiestrogens started with Leonard Lerner's discovery of MER 25. I was thrilled to meet him at meetings in Mont-Tremblant, Canada, of "Recent Progress in Hormone Research" started by Gregory Pincus of the Worcester Foundation. Len and I talked endlessly about a "group of forgotten drugs," nonsteroidal antiestrogens. Two enthusiasts. I was more than thrilled to receive the Bruce Cain Award with Len from the AACR in 1989 [126]. At that time something that was nothing was being turned into something of medical significance.

References

1. Cook JW, Dodds EC, Hewett CL (1933) A synthetic oestrus-exciting compound. Nature 131:56
2. Jensen EV, Jacobson HI (1962) Basic guides to the mechanism of estrogen action. Recent Program Horm Res 18:387–414
3. Gorski J, Toft D, Shyamala G et al (1968) Hormone receptors: studies on the interaction of estrogen with the uterus. Recent Prog Horm Res 24:45–80
4. Jensen EV, DeSombre ER (1973) Estrogen-receptor interactions. Science 182:126–134
5. Allen E, Doisy EA (1923) An ovarian hormone. J Am Med Assoc 81:819–821
6. Kahnt LC, Doisy EA (1928) The vaginal smear method of assay of the ovarian hormone. Endocrinology 12:760–768
7. Emmens CW (1940/1941) Precursors of oestrogens. J Endocrinol 2:444–458
8. Emmens CW (1950) The intravaginal assay of naturally occurring oestrogens. J Endocrinol 6:302–307

Uncorrected Proof

Author's Proof

9. Emmens CW (1942) Oestrogens and pro-oestrogens related to stilbene and triphenylethylene. J Endocrinol 3:168–173
10. Emmens CW (1947) Halogen-substituted oestrogens related to triphenylethylene. J Endocrinol 5:170–173
11. Martin L, Claringbold PJ (1958) A highly sensitive assay for oestrogens. Nature 181:620–621
12. Martin L (1960) The use of 2-3-5-triphenyltetrazolium chloride in the biological assay of oestrogens. J Endocrinol 20:187–197
13. Bulbring E, Burn JH (1935) The estimation of oestrin and of male hormone in oily solution. J Physiol 85:320–333
14. Lauson HD, Heller OG, Golden JB, Sevringhaus EL (1939) The immature rat uterus in the assay of estrogenic substances and a comparison of estradiol, estrone, and estriol. Endocrinology 24:35–44
15. Evans JS, Varney RF, Koch FC (1941) The mouse uterine weight method for the assay of estrogens. Endocrinology 28:747–752
16. Astwood EB (1938) A six-hour assay for the quantitative determination of estrogen. Endocrinology 23:25–31
17. Rubin BL, Dorfman AS, Black L, Dorfman RI (1951) Bioassay of estrogens using the mouse uterine response. Endocrinology 49:429–439
18. Terenius L (1965) Uptake of radioactive oestradiol in some organs of immature mice. The capacity and structural specificity of uptake studied with several steroid hormones. Acta Endocrinol (Copenh) 50:584–596
19. Terenius L (1966) Effect of synthetic oestrogens and analogues on the uptake of oestradiol by the immature mouse uterus and vagina. Acta Pharmacol Toxicol (Copenh) 24:89–100
20. Jordan VC (1975) Prolonged antioestrogenic activity of ICI 46, 474 in the ovariectomized mouse. J Reprod Fertil 42:251–258
21. Jordan VC, Naylor KE (1979) The binding of [3H]-oestradiol-17 beta in the immature rat uterus during the sequential administration of non-steroidal anti-oestrogens. Br J Pharmacol 65:167–173
22. Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741–4745
23. Dodds EC, Lawson W (1936) Synthetic oestrogenic agents without the phenanthrene nucleus. Nature 137:996
24. Dodds EC, Lawson W (1937) A simple aromatic oestrogenic agent with an activity of the same order as that of oestrone. Nature 139:627
25. Zondek B, Bergmann E (1938) Phenol methyl ethers as oestrogenic agents. Biochem J 32:641–645
26. Dodds EC, Lawson W (1937) Oestrogenic activity of *p*-hydroxy propenyl benzene (Anol). Nature 139:1068
27. Campbell NR, Dodds EC, Lawson W (1938) Oestrogenic activity of anol: a highly active phenol isolated from the byproducts. Nature 142:1121
28. Campbell NR, Dodds EC, Lawson W (1938) Oestrogenic activity of dianol, a dimeride of *p*-propenyl-phenol. Nature 141:78
29. Dodds E (1938) Biological effects of the synthetic oestrogenic substance 4:4′-dihydroxy-alpha:beta-diethylstilbene. Lancet 232:1389–1391
30. Dodds EC, Goldberg L, Lawson W, Robinson R (1938) Oestrogenic activity of certain synthetic compounds. Nature 141:247–248
31. Dodds EC, Golberg L, Lawson W, Robinson R (1938) Oestrogenic activity of esters of diethylstilbestrol. Nature 142:211–212
32. Dodds EC, Golberg L, Lawson W, Robinson R (1938) Oestrogenic activity of alkylated stilbestrols. Nature 142:34
33. Robson JM, Schonberg A (1937) Oestrous reactions, including mating, produced by triphenylethylene. Nature 140:196

Uncorrected Proof

Author's Proof

34. Robson JM, Schonberg A, Fahim H (1938) Duration of action of natural and synthetic oestrogens. Nature 142:292–293
35. Robson JM, Schonberg A (1942) A new synthetic oestrogen with prolonged action when given orally. Nature 150:22–23
36. Robson JM, Ansari MY (1943) The fate of D.B.E. (alpha di-*p*-ethoxphenyl) (Beta-phenylbromoethylene) in the body. J Pharm Exptl Therap 79:340–345
37. Thompson CR, Werner HW (1951) Studies on estrogen tri-p-anisylchloroethylene. Proc Soc Exp Biol Med 77:494–497
38. Greenblatt RB, Brown NH (1952) The storage of estrogen in human fat after estrogen administration. Am J Obstet Gynecol 63:1361–1363
39. Thompson CR, Werner HW (1953) Fat storage of an estrogen in women following orally administered tri-p-anisylchloroethylene. Proc Soc Exp Biol Med 84:491–492
40. Haddow A, Watkinson JM, Paterson E, Koller PC (1944) Influence of synthetic oestrogens on advanced malignant disease. Br Med J 2:393–398
41. Kennedy BJ, Nathanson IT (1953) Effects of intensive sex steroid hormone therapy in advanced breast cancer. J Am Med Assoc 152:1135–1141
42. Dodds EC, Folley SJ, Glascock RF, Lawson W (1958) The excretion of microgram doses of hexoestrol by rabbits and rats. Biochem J 68:161–167
43. Glascock RF, Hoekstra WG (1959) Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep. Biochem J 72:673–682
44. Folca PJ, Glascock RF, Irvine WT (1961) Studies with tritium-labelled hexoestrol in advanced breast cancer. Comparison of tissue accumulation of hexoestrol with response to bilateral adrenalectomy and oophorectomy. Lancet 2:796–798
45. Tate AC, Jordan VC (1983) The estrogen receptor. In: Agarwal MK (ed) Textbook of Receptorology. Walter DeGruyter, Berlin, pp 381–463
46. Jordan VC, Tate AC, Lyman SD et al (1985) Rat uterine growth and induction of progesterone receptor without estrogen receptor translocation. Endocrinology 116:1845–1857
47. King WJ, Greene GL (1984) Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature 307:745–747
48. Welshons WV, Lieberman ME, Gorski J (1984) Nuclear localization of unoccupied oestrogen receptors. Nature 307:747–749
49. Welshons WV, Cormier EM, Wolf MF et al (1988) Estrogen receptor distribution in enucleated breast cancer cell lines. Endocrinology 122:2379–2386
50. Lerner LJ (1981) The first non-steroidal antioestrogen- MER 25. In: Sutherland RL, Jordan VC (eds) Non steroidal antioestrogens. Academic, Sydney, pp 1–16 AU5
51. Segal SJ, Nelson WO (1958) An orally active compound with anti-fertility effects in rats. Proc Soc Exp Biol Med 98:431–436 AU6
52. Chang MC (1959) Degeneration of ova in the rat and rabbit following oral administration of 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-anisylethanol. Endocrinology 65:339–342
53. Barnes LE, Meyer RK (1962) Effects of ethamoxytriphetol, MRL-37, and clomiphene on reproduction in rats. Fertil Steril 13:472–480
54. Kistner RW, Smith OW (1959) Observations on the use of a nonsteroidal estrogen antagonist: MER 25. Surg Forum 10:725–729
55. Kistner RW, Smith OW (1961) Observations on the use of nonsteroidal estrogen antagonist: MER 25. Fertil Steril 12:121–141
56. Smith OW, Kistner RW (1963) Action of MER 25 and of clomiphene on the human ovary. JAMA 184:878–886
57. Holtkamp DE, Greslin JG, Root CA, Lerner LJ (1960) Gonadotrophin inhibiting and anti-fecundity effects of chloramiphene. Proc Soc Exp Biol Med 105:197–201
58. Davidson OW, Wada K, Segal SJ (1965) Effects of clomiphene at different stages of pregnancy in the rat. Implications regarding possible action mechanisms. Fertil Steril 16:195–201

Uncorrected Proof

Author's Proof

59. Lednicer D, Babcock JC, Marlatt PE et al (1965) Mammalian antifertility agents. I. Derivatives of 2,3-diphenylindenes. J Med Chem 8:52–57
60. Lednicer D, Lyster SC, Duncan GW (1967) Mammalian antifertility agents. IV. Basic 3,4-dihydronaphthalenes and 1,2,3,4-tetrahydro-1-naphthols. J Med Chem 10:78–84
61. Lednicer D, Lyster SC, Aspergren BD, Duncan GW (1966) Mammalian antifertility agents. 3. 1-Aryl-2-phenyl-1,2,3,4-tetrahydro-1-naphthols, 1-aryl-2-phenyl-3,4-dihydronaphthalenes, and their derivatives. J Med Chem 9:172–176
62. Ray S, Grover PK, Kamboj VP et al (1976) Antifertility agents. 12. Structure-activity relationship of 3,4-diphenylchromenes and -chromans. J Med Chem 19:276–279
63. Greenwald GS (1965) Effects of a dihydronaphthalene derivative (U-11100a) on pregnancy in the rat. Fertil Steril 16:185–194
64. Harper MJ, Walpole AL (1967) Mode of action of I.C.I. 46,474 in preventing implantation in rats. J Endocrinol 37:83–92
65. Emmens CW (1970) Postcoital contraception. Br Med Bull 26:45–51
66. Emmens CW, Humphrey K, Martin L, Owen WH (1967) Antifertility properties of two non-oestrogenic steroids and MRL 37. Steroids 9:235–243
67. Palopoli FP, Feil VJ, Allen RE et al (1967) Substituted aminoalkoxytriarylhaloethylenes. J Med Chem 10:84–86
68. DiPietro DL, Sanders FJ, Goss DA (1969) Effect of *cis* and *trans* isomers of clomiphene citrate on uterine hexokinase activity. Endocrinology 84:1404–1408
69. Schulz KD, Haselmayer B, Holzel F (1971) The influence of clomid and its isomers on dimethylbenzanthracene-induced rat mammary tumors. In: Hubinot PO, Leroy F, Galand P (eds) Basic action of sex steroids on target organs. Karger, Basel
70. Self LW, Holtkamp DE, Kuhn WL (1967) Pituitary gonad related effects of isomers of clomiphene citrate. Fed Proc Fed Am Soc Exp Biol 26:534
71. Jordan VC, Haldemann B, Allen KE (1981) Geometric isomers of substituted triphenylethylenes and antiestrogen action. Endocrinology 108:1353–1361
72. Harper MJ, Walpole AL (1966) Contrasting endocrine activities of *cis* and *trans* isomers in a series of substituted triphenylethylenes. Nature 212:87
73. Bedford GR, Richardson DN (1966) Preparation and identification of *cis* and *trans* isomers of a substituted triarylethylene. Nature (London) 212:733
74. Kilbourn BT, Mais RHB, Owston PG (1968) Identification of isomers of a substituted triarylethylene: the crystal structure of 1-p-(2-dimethylaminoethoxyphenyl)-1,2-*cis*-diphenyl but-1-ene hydrobromide. Chem Commun 1:291
75. Harper MJ, Walpole AL (1967) A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 13:101–119
76. Collins DJ, Hobbs JJ, Emmens CW (1971) Antiestrogenic and antifertility compounds. 4. 1,1,2-triarylalkan-1-ols and 1,1,2-triarylalk-1-enes containing basic ether groups. J Med Chem 14:952–957
77. Callantine MR, Humphrey RR, Lee SL et al (1966) Action of an estrogen antagonist on reproductive mechanisms in the rat. Endocrinology 79:153–167
78. Jensen EV, Jacobson HI, Smith S et al (1972) The use of estrogen antagonists in hormone receptor studies. Gynecol Invest 3:108–123
79. Arbogast LY, DeSombre ER (1975) Estrogen-dependent in vitro stimulation of RNA synthesis in hormone-dependent mammary tumors of the rat. J Natl Cancer Inst 54:483–485
80. Salzer W (1946) Uber neue synthetische hochwirksame oestrogene. Hoppe-Seyler's Z. Physiol Chem 274:39–47
81. Silverman M, Bogert MT (1946) The synthesis of some indene and dihydronaphthalene derivatives related to stilbestrol. J Org Chem 11:34–49
82. Clark ER, Jordan VC (1976) Oestrogenic, anti-oestrogenic and fertility effects of some triphenylethanes and triphenylethylenes related to ethamoxytriphetol (MER 25). Br J Pharmacol 57:487–493

Uncorrected Proof

Author's Proof

83. Abbott AC, Clark ER, Jordan VC (1976) Inhibition of oestradiol binding to oestrogen receptor proteins by a methyl-substituted analogue of tamoxifen. J Endocrinol 69:445–446
84. Robertson DW, Katzenellenbogen JA, Hayes JR, Katzenellenbogen BS (1982) Antiestrogen basicity–activity relationships: a comparison of the estrogen receptor binding and antiuterotrophic potencies of several analogues of (Z)-1,2-diphenyl-1-[4-[2-(dimethylamino)ethoxy] phenyl]-1-butene (tamoxifen, Nolvadex) having altered basicity. J Med Chem 25:167–171
85. Jones CD, Suarez T, Massey EH et al (1979) Synthesis and antiestrogenic activity of [3,4-dihydro-2-(4-methoxyphenyl)-1-naphthalenyl][4-[2-(1-pyrrolidinyl)ethoxy]-phe nyl] methanone, methanesulfonic acid salt. J Med Chem 22:962–966
86. Kamboj VP, Setty BS, Chandra H et al (1977) Biological profile of centchroman–a new postcoital contraceptive. Indian J Exp Biol 15:1144–1150
87. Mukerjee SS, Sethi N, Srivastava GN et al (1977) Chronic toxicity studies of centchroman in rats and rhesus monkeys. Indian J Exp Biol 15:1162–1166
88. Dwani SAA, Saxena R, Setty BS, Gupta RC, Koll PL, Ray S, Anand N (1979) Secooestradiols and some non-steroidal estrogens: structural correlates of oestrogenic action. J Steroid Biochem 11:67
89. Saeed A, Durani N, Durani S et al (1984) Cis isomer of centchroman–a selective ligand for the microsomal antiestrogen binding site. Biochem Biophys Res Commun 125:346–352
90. Roy S, Datta JK (1976) Nature of estrogenic and anti-estrogenic actions of centchroman on rat uterus. Contraception 13:597–604
91. Toft D, Gorski J (1966) A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc Natl Acad Sci U S A 55:1574–1581
92. Toft D, Shyamala G, Gorski J (1967) A receptor molecule for estrogens: studies using a cell-free system. Proc Natl Acad Sci U S A 57:1740–1743
93. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44:30–38
94. Korenman SG (1970) Relation between estrogen inhibitory activity and binding to cytosol of rabbit and human uterus. Endocrinology 87:1119–1123
95. Skidmore J, Walpole AL, Woodburn J (1972) Effect of some triphenylethylenes on oestradiol binding in vitro to macromolecules from uterus and anterior pituitary. J Endocrinol 52:289–298
96. Jordan VC, Collins MM, Rowsby L, Prestwich G (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305–316
97. Shiau AK, Barstad D, Loria PM et al (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937
98. Soule HD, Vazguez J, Long A et al (1973) A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51:1409–1416
99. Shafie SM (1980) Estrogen and the growth of breast cancer: new evidence suggests indirect action. Science 209:701–702
100. Lippman ME, Bolan G (1975) Oestrogen-responsive human breast cancer in long term tissue culture. Nature 256:592–593
101. Greene GL, Fitch FW, Jensen EV (1980) Monoclonal antibodies to estrophilin: probes for the study of estrogen receptors. Proc Natl Acad Sci U S A 77:157–161
102. Greene GL, Nolan C, Engler JP, Jensen EV (1980) Monoclonal antibodies to human estrogen receptor. Proc Natl Acad Sci U S A 77:5115–5119
103. Greene GL, Gilna P, Waterfield M et al (1986) Sequence and expression of human estrogen receptor complementary DNA. Science 231:1150–1154
104. Green S, Walter P, Kumar V et al (1986) Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature 320:134–139
105. Lieberman ME, Maurer RA, Gorski J (1978) Estrogen control of prolactin synthesis in vitro. Proc Natl Acad Sci U S A 75:5946–5949

Uncorrected Proof

Author's Proof

106. Lieberman ME, Jordan VC, Fritsch M et al (1983) Direct and reversible inhibition of estradiol-stimulated prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4734–4740
107. Jordan VC, Lieberman ME, Cormier E et al (1984) Structural requirements for the pharmacological activity of nonsteroidal antiestrogens in vitro. Mol Pharmacol 26:272–278
108. Jordan VC, Lieberman ME (1984) Estrogen-stimulated prolactin synthesis in vitro. Classification of agonist, partial agonist, and antagonist actions based on structure. Mol Pharmacol 26:279–285
109. Jordan VC, Koch R, Mittal S, Schneider MR (1986) Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. Br J Pharmacol 87:217–223
110. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48:812–815
111. Jordan VC, Koch R (1989) Regulation of prolactin synthesis in vitro by estrogenic and antiestrogenic derivatives of estradiol and estrone. Endocrinology 124:1717–1726
112. Jordan VC (1984) Biochemical pharmacology of antiestrogen action. Pharmacol Rev 36:245–276
113. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS (1986) Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A 83:2496–2500
114. Bindal RD, Carlson KE, Katzenellenbogen BS, Katzenellenbogen JA (1988) Lipophilic impurities, not phenolsulfonphthalein, account for the estrogenic activity in commercial preparations of phenol red. J Steroid Biochem 31:287–293
115. Bindal RD, Katzenellenbogen JA (1988) Bis(4-hydroxyphenyl)[2-(phenoxysulfonyl)phenyl] methane: isolation and structure elucidation of a novel estrogen from commercial preparations of phenol red (phenolsulfonphthalein). J Med Chem 31:1978–1983
116. Murphy CS, Langan-Fahey SM, McCague R, Jordan VC (1990) Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. Mol Pharmacol 38:737–743
117. Murphy CS, Parker CJ, McCague R, Jordan VC (1991) Structure-activity relationships of nonisomerizable derivatives of tamoxifen: importance of hydroxyl group and side chain positioning for biological activity. Mol Pharmacol 39:421–428
118. Jordan VC (1987) Laboratory models of breast cancer to aid the elucidation of antiestrogen action. J Lab Clin Med 109:267–277
119. Brzozowski AM, Pike AC, Dauter Z et al (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
120. Wolf DM, Jordan VC (1994) The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31:129–138
121. Jiang SY, Jordan VC (1992) Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. J Natl Cancer Inst 84:580–591
122. Schafer JI, Liu H, Tonetti DA, Jordan VC (1999) The interaction of raloxifene and the active metabolite of the antiestrogen EM-800 (SC 5705) with the human estrogen receptor. Cancer Res 59:4308–4313
123. MacGregor Schafer J, Liu H, Bentrem DJ et al (2000) Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine from aspartate at amino acid 351. Cancer Res 60:5097–5105
124. Liu H, Lee ES, Deb Los Reyes A et al (2001) Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor alpha complex. Cancer Res 61:3632–3639

Uncorrected Proof

Author's Proof

125. Liu H, Park WC, Bentrem DJ et al (2002) Structure-function relationships of the raloxifene-estrogen receptor-alpha complex for regulating transforming growth factor-alpha expression in breast cancer cells. J Biol Chem 277:9189–9198
126. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189
127. Lerner LJ, Holthaus FJ Jr, Thompson CR (1958) A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol. Endocrinology 63:295–318
128. Emmens CW, Cox RI, Martin L (1960) Oestrogen inhibition by steroids and other substances. J Endocrinol 20:198–209
129. Nicol T, Vernon-Roberts B, Quantock DC (1966) The effect of various anti-oestrogenic compounds on the reticulo-endothelial system and reproductive tract in the ovariectomized mouse. J Endocrinol 34:377–386
130. Shirley B, Wolinsky J, Schwartz NB (1968) Effects of a single injection of an estrogen antagonist on the estrous cycle of the rat. Endocrinology 82:959–968
131. Shelesnyak MC, Tic L (1963) Studies on the mechanism of decidualization. IV. Acta Endocrinol (Copenh) 42:465–472
132. Cutler A, Ober WB, Epstein JA, Kupperman HS (1961) The effect of 1-(p-2-diethylaminoethoxy-phenyl)-1-phenyl-2-panisyl ethanol upon rat pituitary and uterine responses to estrogen. Endocrinology 69:473–482
133. Jordan VC, Koerner S, Robison C (1975) Inhibition of oestrogen-stimulated prolactin release by anti-oestrogens. J Endocrinol 65:151–152
134. Wood JR, Wrenn TR, Bitman J (1968) Estrogenic and antiestrogenic effects of clomiphene, MER-25 and CN-55,945-27 on the rat uterus and vagina. Endocrinology 82:69–74
135. Harris DN, Lerner LJ, Hilf R (1968) The effects of progesterone and ethamoxytriphetol on estradiol-induced changes in the immature rat uterus. Trans N Y Acad Sci 30:774–782
136. Black LJ, Goode RL (1980) Uterine bioassay of tamoxifen, trioxifene and a new estrogen antagonist (LY117018) in rats and mice. Life Sci 26:1453–1458
137. Jordan VC, Rowsby L, Dix CJ, Prestwich G (1978) Dose-related effects of non-steroidal antioestrogens and oestrogens on the measurement of cytoplasmic oestrogen receptors in the rat and mouse uterus. J Endocrinol 78:71–81
138. Terenius L (1971) Structure-activity relationships of anti-oestrogens with regard to interaction with 17-beta-oestradiol in the mouse uterus and vagina. Acta Endocrinol (Copenh) 66:431–447
139. Terenius L (1970) Two modes of interaction between oestrogen and anti-oestrogen. Acta Endocrinol (Copenh) 64:47–58
140. Lee AE (1974) Effects of oestrogen antagonists on mitosis and (3H)oestradiol binding in the mouse uterus. J Endocrinol 60:167–174
141. Lee AE (1971) The limitations of using mouse uterine weight as an assay of oestrogen antagonism. Acta Endocrinol (Copenh) 67:345–352
142. Emmens CW (1971) Compounds exhibiting prolonged antioestrogenic and antifertility activity in mice and rats. J Reprod Fertil 26:175–182
143. Emmens CW, Carr WL (1973) Further studies of compounds exhibiting prolonged antioestrogenic and antifertility activity in the mouse. J Reprod Fertil 34:29–40
144. Martin L, Middleton E (1978) Prolonged oestrogenic and mitogenic activity of tamoxifen in the ovariectomized mouse. J Endocrinol 78:125–129
145. Martin L (1981) Effects of antiestrogens on cell proliferation in the rodent reproductive tract. In: Sutherland RL, Jordan VC (eds) Non-steroidal antiestrogens. Academic, Sydney, p 143
146. Kang YH, Anderson WA, DeSombre ER (1975) Modulation of uterine morphology and growth by estradiol-17beta and an estrogen antagonist. J Cell Biol 64:682–691
147. Jordan VC, Dix CJ (1979) Effect of oestradiol benzoate, tamoxifen and monohydroxy-tamoxifen on immature rat uterine progesterone receptor synthesis and endometrial cell division. J Steroid Biochem 11:285–291

Uncorrected Proof

Author's Proof

148. Clark JH, Hardin JW, Padykula HA, Cardasis CA (1978) Role of estrogen receptor binding and transcriptional activity in the stimulation of hyperestrogenism and nuclear bodies. Proc Natl Acad Sci U S A 75:2781–2784
149. Kaye AM, Sheratzky D, Lindner HR (1972) Kinetics of DNA synthesis in immature rat uterus: age dependence and estradiol stimulation. Biochim Biophys Acta 261:475–486
150. Greenblatt RB, Barfield WE, Jungck EC, Ray AW (1961) Induction of ovulation with MRL/41. Preliminary report. JAMA 178:101–104
151. Klopper A, Hall M (1971) New synthetic agent for the induction of ovulation: preliminary trials in women. Br Med J 1:152–154
152. Chen CL, Meites J (1960) Effects of estrogen and progesterone on serum and pituitary prolactin levels in ovariectomized rats. Endocrinology 66:96
153. Heuson JC, Waelbroeck C, Legros N et al (1971) Inhibition of DMBA-induced mammary carcinogenesis in the rat by 2-br- -ergocryptine (CB 154), an inhibitor of prolactin secretion, and by nafoxidine (U-11, 100 A), an estrogen antagonist. Gynecol Invest 2:130–137
154. Jordan VC, Koerner S (1976) Tamoxifen as an anti-tumour agent: role of oestradiol and prolactin. J Endocrinol 68:305–311
155. Jordan VC, Dix CJ, Allen KE (1981) Effects of antioestrogens in carcinogen-induced rat mammary cancer. In: Sutherland RL, Jordan VC (eds) Non-steroidal anti-oestrogens. Academic, Sydney, pp 261–280
156. de Quijada M, Timmermans HA, Lamberts SW (1980) Tamoxifen suppresses both the growth of prolactin-secreting pituitary tumours and normal prolactin synthesis in the rat. J Endocrinol 86:109–116
157. de Quijada M, Timmermans HA, Lamberts SW, MacLeod RM (1980) Tamoxifen enhances the sensitivity of dispersed prolactin-secreting pituitary tumor cells to dopamine and bromocriptine. Endocrinology 106:702–706
158. Labhsetwar AP (1970) Role of estrogens in ovulation: a study using the estrogen-antagonist, I.C.I. 46,474. Endocrinology 87:542–551
159. Bainbridge JG, Labhsetwar AP (1971) The role of oestrogens in spontaneous ovulation: location of site of action of positive feedback of oestrogen by intracranial implantation of the anti-oestrogen I.C.I. 46474. J Endocrinol 50:321–327
160. Billard R, McDonald PG (1973) Inhibition of ovulation in the rat by intrahypothalamic implants of an antioestrogen. J Endocrinol 56:585–590
161. Sheth AR, Vaidya RA, Arbatti NJ, Devi PK (1977) Effect of Centchroman on serum gonadotropins & prolactin in rats. Indian J Exp Biol 15:1191–1193
162. Dohler KDVSMA, Dohler U (1977) ICI46,474-lack of oestrogenic activity in the rat. ICRS Med Sci 5:185
163. Bartke A, Mason M, Dalterio S, Bex F (1978) Effects of tamoxifen on plasma concentrations of testosterone and gonadotrophins in the male rat. J Endocrinol 79:239–240
164. Hsueh AJ, Erickson GF, Yen SS (1978) Sensitisation of pituitary cells to luteinising hormone releasing hormone by clomiphene citrate in vitro. Nature 273:57–59
165. Miller WL, Huang ES (1981) Antiestrogens and ovine gonadotrophs: antagonism of estrogen-induced changes in gonadotropin secretions. Endocrinology 108:96–102
166. Segal SJ, Thompson CR (1956) Inhibition of estradiol-induced pituitary hypertrophy in rats. Proc Soc Exp Biol Med 91:623–625
167. Jordan VC, Schafer JM, Levenson AS et al (2001) Molecular classification of estrogens. Cancer Res 61:6619–6623
168. Bentrem D, Fox JE, Pearce ST et al (2003) Distinct molecular conformations of the estrogen receptor alpha complex exploited by environmental estrogens. Cancer Res 63:7490–7496
169. Aten RF, Weinberger MJ, Eisenfeld AJ (1980) Kinetics of in vivo nuclear translocation of the male and female rat liver estrogen receptors. Endocrinology 106:1127–1132
170. Aten RF, Dickson RB, Eisenfeld AJ (1979) Female and male green monkey liver estrogen receptor. Biochem Pharmacol 28:2445–2450

Uncorrected Proof

Author's Proof

171. Bichon M, Bayard F (1979) Dissociated effects of tamoxifen and oestradiol-17 beta on uterus and liver functions. J Steroid Biochem 10:105–107
172. Kneifel MA, Katzenellenbogen BS (1981) Comparative effects of estrogen and antiestrogen on plasma renin substrate levels and hepatic estrogen receptors in the rat. Endocrinology 108:545–552
173. Sakai F, Cheix F, Clavel M et al (1978) Increases in steroid binding globulins induced by tamoxifen in patients with carcinoma of the breast. J Endocrinol 76:219–226
174. Sutherland R, Mester J, Baulieu EE (1977) Tamoxifen is a potent "pure" anti-oestrogen in chick oviduct. Nature 267:434–435
175. Binart N, Catelli MG, Geynet C et al (1979) Monohydroxytamoxifen: an antioestrogen with high affinity for the chick oviduct oestrogen receptor. Biochem Biophys Res Commun 91:812–818
176. Lazier CB, Capony F, Williams DL (1981) Antioestrogen action in chick liver: effects on oestrogen receptors in oestrogen-induced proteins. In: Sutherland RL, Jordan VC (eds) Non-steroidal antioestrogens. Academic, Sydney, pp 215–230
177. Carter SB (1967) Effects of cytochalasins on mammalian cells. Nature 213:261–264
178. Welshons WV, Grady LH, Judy BM et al (1993) Subcellular compartmentalization of MCF-7 estrogen receptor synthesis and degradation. Mol Cell Endocrinol 94:183–194
179. Clark GM, McGuire WL, Hubay CA et al (1983) Progesterone receptors as a prognostic factor in stage II breast cancer. N Engl J Med 309:1343–1347
180. Horwitz KB, McGuire WL (1975) Predicting response to endocrine therapy in human breast cancer: a hypothesis. Science 189:726–727
181. McGuire WL, Carbone PP, Vollmer EP (1975) Estrogen receptors in human breast cancer. Raven, New York
182. Jensen EV, Jordan VC (2003) The estrogen receptor: a model for molecular medicine. Clin Cancer Res 9:1980–1989
183. Jordan VC (1995) Third annual William L. McGuire Memorial Lecture. "Studies on the estrogen receptor in breast cancer"–20 years as a target for the treatment and prevention of cancer. Breast Cancer Res Treat 36:267–285
184. Wolf DM, Jordan VC (1993) William L. McGuire Memorial Symposium. Drug resistance to tamoxifen during breast cancer therapy. Breast Cancer Res Treat 27:27–40
185. Jordan VC (2001) Selective estrogen receptor modulation: a personal perspective. Cancer Res 61:5683–5687

Uncorrected Proof

Author's Proof

Author Queries

Chapter No.: 1

Query Refs.	Details Required	Author's response
AU1	Please check if "Dihydroxyhexahydroxchrysene" should be changed to "Dihydroxyhexahydrochrysene".	
AU2	Please check sentence starting "High-dose synthetic. . ." for completeness.	
AU3	Please check if edit to sentence starting "A model of. . ." is okay.	
AU4	Please check if "site effects" should be changed to "side effects".	
AU5	Please check if inserted publisher location for Lerner (1981) is okay.	
AU6	Please check if inserted page range for Segal (1958), Korenman (1970), MacGregor Schafer (2000), Kaye et al. (1972) are okay.	

Uncorrected Proof

Author's Proof

Metadata of the chapter that will be visualized online

Chapter Title	Tamoxifen Goes Forward Alone	
Copyright Year	2013	
Copyright Holder	Springer Basel	
Corresponding Author	Family Name	**Maximov**
	Particle	
	Given Name	**Philipp Y.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**McDaniel**
	Particle	
	Given Name	**Russell E.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**Jordan**
	Particle	
	Given Name	**V. Craig**
	Suffix	
	Division	Lombardi Comprehensive Cancer Center
	Organization	Georgetown University Medical Center
	Address	Washington, DC, USA
Abstract	Tamoxifen (ICI 46,474), the *trans* isomer of a substituted triphenylethylene, was discovered in the fertility program at Imperial Chemical Industries, Pharmaceuticals Division, Cheshire, England. The plan was to use tamoxifen to regulate fertility, but this failed and interest refocused outside the company for applications to treat breast cancer. The initial application of the nonsteroidal antiestrogen was for the treatment of metastatic breast cancer in postmenopausal women and by the 1980s tamoxifen had replaced high-dose diethylstilbestrol therapy. Efficacy when compared with diethylstilbestrol was similar, but tamoxifen had fewer side effects. No other antiestrogens were developed by the pharmaceutical industry, as this was not considered a financially lucrative development strategy.	

Author's Proof

Chapter 2
Tamoxifen Goes Forward Alone

Abstract Tamoxifen (ICI 46,474), the *trans* isomer of a substituted triphenylethylene, was discovered in the fertility program at Imperial Chemical Industries, Pharmaceuticals Division, Cheshire, England. The plan was to use tamoxifen to regulate fertility, but this failed and interest refocused outside the company for applications to treat breast cancer. The initial application of the nonsteroidal antiestrogen was for the treatment of metastatic breast cancer in postmenopausal women and by the 1980s tamoxifen had replaced high-dose diethylstilbestrol therapy. Efficacy when compared with diethylstilbestrol was similar, but tamoxifen had fewer side effects. No other antiestrogens were developed by the pharmaceutical industry, as this was not considered a financially lucrative development strategy.

Introduction

History is lived forward but is written in retrospect. "We know the end before we consider the beginning and we can never wholly recapture what it was to know the beginning only" (C.V. Wedgewood, *William the Silent*). That is, unless one has lived through the evolving applications of tamoxifen.

Tamoxifen (ICI 46,474; Nolvadex), a nonsteroidal antiestrogen, started life as the endocrine treatment of choice for advanced breast cancer [1]. Adjuvant therapy with tamoxifen also proved to be effective [2] because a sustained survival advantage is noted for women with node-positive and node-negative disease. The Food and Drug Administration (FDA) approved the use of tamoxifen as an adjuvant therapy with chemotherapy (1986), as an adjuvant therapy alone (1988) in node-positive postmenopausal patients and pre- and postmenopausal node-negative patients with ER-positive disease (1990). Tamoxifen is used to treat breast cancer in men (1993). However, remarkably tamoxifen was also approved to reduce the risk of breast cancer in women at high risk (1998). Tamoxifen was also FDA approved for treatment of ductal carcinoma in situ (DCIS) (2000). No other cancer therapy is so widely approved and had so dramatic an impact on cancer care. Tamoxifen is,

Uncorrected Proof

P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
DOI 10.1007/978-3-0348-0664-0_2, © Springer Basel 2013

Author's Proof

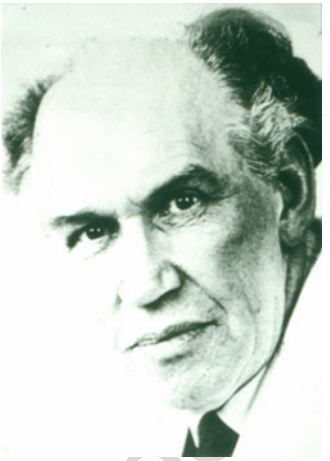


Fig. 2.1 Arthur Walpole who died suddenly on 2 July 1977. At the time of his death, he had retired as head of the Fertility Control Program at ICI's Pharmaceuticals Division at Alderley Park, near Macclesfield, Cheshire, but he had continued to work as a consultant on the joint research scheme between ICI and the Department of Pharmacology at the University of Leeds, England

however, one of those remarkable examples of a drug originally designed for one primary purpose that fails but is then steered by dedicated scientists toward a recognized secondary application where it becomes enormously successful.

The chief credit for the discovery of tamoxifen in 1962, and its subsequent application as an orphan drug treatment for metastatic breast cancer, must be given to Dr. Arthur L. Walpole (Fig. 2.1), then head of the fertility control program for Imperial Chemical Industries (ICI) Pharmaceuticals Division. Tamoxifen was identified as an effective postcoital contraceptive in rats [3–5] and there was a distinct possibility that antiestrogens could be developed as "morning-after" pills [6]. However, the basic pharmacology and physiology of ovulation and implantation are critically different in women and rats. When tamoxifen was tested in patients in preliminary clinical studies, it was found to induce ovulation rather than reduce fertility [7, 8] and so is marketed in some countries for the induction of ovulation in subfertile women [1].

The ovarian dependence of some breast cancers has long been recognized [9, 10] and the first antiestrogens [11, 12] were shown to be effective in their treatment, but the drugs then available were considered to be too toxic for chronic use [13–15] (Table 2.1). By the end of the 1960s, the direct role of estrogen in breast cancer growth was further substantiated with the description of ERs in breast tumors [18–20] and the subsequent clinical correlation with hormone dependency [21, 22]. However, clinical research with tamoxifen was not based on the ER but on proven antifertility activity as an antiestrogen in the rat. Walpole encouraged the clinical testing of the antiestrogen tamoxifen at the Christie Hospital and Holt Radium Institute in Manchester [16]. He had a long interest in cancer research [23] but also wanted to determine whether tamoxifen was an estrogen or an antiestrogen in humans because of the life between estrogens and breast cancer growth. A subsequent dose response was published by Dr. Harold Ward [17]. But in 1972, ICI Pharmaceutical Division

Uncorrected Proof

Author's Proof

Table 2.1 Comparison of the early chemical experience with antiestrogen as a treatment for metastatic breast cancer

Antiestrogen	Daily dose (mg)	Year	Response rate (%)	Toxicity
Ethamoxytriphetol	500–4,500	1960	25	Acute psychotic episodes
Clomiphene	100–300	1964–1974	34	Fear of cataracts
Nafoxidine	180–240	1976	31	Cataracts, ichthyosis, photophobia
Tamoxifen	20–40	1971–1973	31	Transient thrombocytopenia[a]

[a]"The particular advantage of this drug is the low incidence of troublesome side effects" [16]. "Side effects were usually trivial" [17]

chose to abandon clinical development because there would be no financial gain for the limited applications in the treatment of metastatic breast cancer where only one in three patients respond for, on average, 2 years [24].

This chapter will trace the "resurrection" and development of tamoxifen for the treatment of advanced breast cancer in postmenopausal patients and consider the unusual set of circumstances that set the stage for the subsequent success of tamoxifen as a long-term adjuvant therapy in patients with node-positive and node-negative disease. In 1990, the fashion was to change again with a plan to test the worth of tamoxifen as a preventive in women at risk for breast cancer [25–27]. Much of the basic laboratory work in animal models was conducted in the period 1974–1992. This produced a strong rationale to move forward with clinical trials and the meticulous evaluations of pharmacology of tamoxifen (Fig. 2.2). This is our story.

ICI 46,474: The Early Years

In 1958, Lerner and coworkers described the first nonsteroidal antiestrogen MER 25. The drug was tested in clinical trials but proved to be toxic at the high doses required [28]. A successor compound, clomiphene (also known as chloramiphene or MRL41) (Fig. 2.2), now known to be a mixture of two geometric isomers with opposing biological activities, was a postcoital contraceptive in rats but was developed only clinically as a fertility drug [29] (see Chap. 1).

To understand the obstacles that had to be overcome before the successful clinical development of tamoxifen, it is necessary to recapture the mood of the times in the 1950s/1960s. Coronary heart disease was a primary target for drug development and was proving to be a lucrative market. However, one product—triparanol (MER29) (Fig. 2.2)—was to become a cause *célèbre* and a major issue in the relationship between product safety and regulatory authorities. Indeed, this case was taught to Craig Jordan as an undergraduate at Leeds University, in the Pharmacology Department (1965–1969), to illustrate how drug development can go very wrong.

Uncorrected Proof

Author's Proof

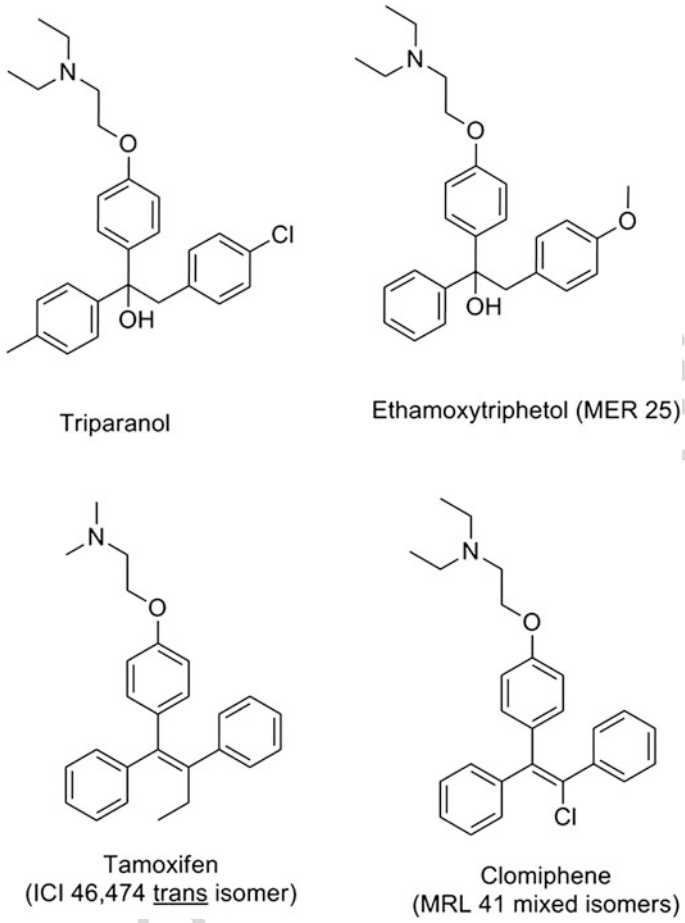


Fig. 2.2 Chemical structures of the first antiestrogens developed in the 1950s and 1960s, including tamoxifen

Triparanol was an orally active lipid-lowering agent developed by the Merrell Company during the 1960s [30]. Unfortunately, acute cataract formation was noted in young women treated with triparanol [31] and this ultimately led to the withdrawal of the medicine. The toxicity was linked to the accumulation of desmosterol as a consequence of the inhibition of cholesterol biosynthesis [32] (Fig. 2.3).

The punitive legal issues surrounding the withdrawal of triparanol forced the Merrell Company to avoid long-term treatments with any agents known to, or thought to, cause increases in the circulating levels of desmosterol. Triparanol [33], ethamoxytriphetol, and clomiphene [14] were all tested as treatments for breast cancer, but their potential to harm through cataract formation forced the Merrell Company to abandon work in the treatment of breast cancer. The administration of clomiphene for a few

Author's Proof

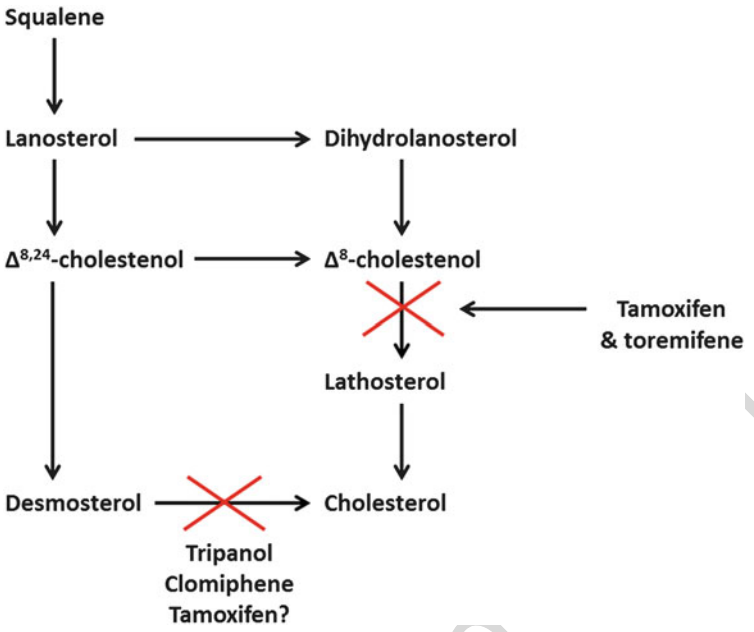


Fig. 2.3 The inhibition of cholesterol biosynthesis by triparanol, clomiphene, tamoxifen, and the chlorinated derivative of tamoxifen toremifene (see Chap. 3, Fig. 3.7)

days to induce ovulation was considered safe compared with the years of therapy necessary for breast cancer treatment.

Arthur Walpole, as head of the Fertility Control Program at ICI Pharmaceuticals Division Alderley Park, was already interested in the pharmacology of nonsteroidal estrogens and was asked to find a safer nonsteroidal antiestrogen in the early 1960s. Richardson was the synthetic organic chemist for the program (Fig. 2.4) and a young reproductive endocrinologist Michael J. K. Harper conducted the antifertility studies in the rat model (Fig. 2.4). The discovery of ICI 46,474 with reduced concerns about desmosterol accumulation was an advance.

From the time that tamoxifen was first available in clinical practice (1973) until the late 1980s, there were remarkably few concerns about the toxicity of tamoxifen, because the side effects from chemotherapy, by contrast, were so severe. Only with the extended use of tamoxifen as an adjuvant therapy in node-negative women, and the proposed use of tamoxifen as a chemopreventive, was there a return to an evaluation of the toxicity of tamoxifen, both by laboratory studies and by the analysis of randomized clinical trials. Despite the fact that tamoxifen was considered safe for long-term adjuvant therapy in women with breast cancer, analysis of the prevention trials organized and run by the National Surgical Adjuvant Bowel and Breast Project (NSABP) would demonstrate a small increase in cataracts and cataract operations for women without disease taking tamoxifen to reduce breast cancer incidence [34, 35].

Uncorrected

Author's Proof

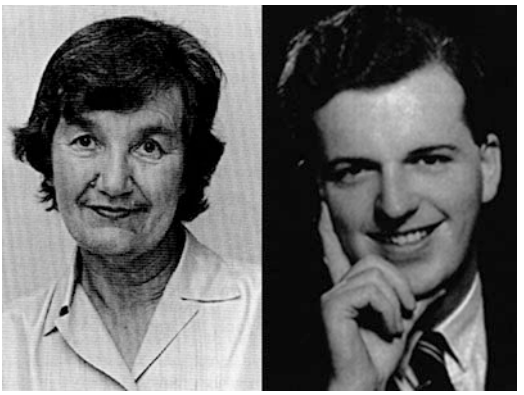


Fig. 2.4 (*Left*) Dora Richardson was a co-patent holder for ICI 46,474 and the organic chemist responsible for the synthesis of triphenylethylenes at ICI Pharmaceuticals. This photograph was taken on the occasion of her retirement in 1979. (*Right*) Mike Harper who discovered the opposing biological activities of the *cis* and *trans* isomers of substituted triphenylethylenes AU1

ICI 46,474 was first synthesized by Dr. Dora Richardson at ICI Ltd., Pharmaceuticals Division (Fig. 2.4), and was shown to be an antifertility agent in rodents [4, 5]. Dr. Michael Harper (Fig. 2.4) [3] made the discovery that the geometric isomers of substituted triphenylethylenes have opposing biological properties: the *cis* isomer ICI 47,699 is an estrogen, whereas the *trans* isomer ICI 46,474 has antiestrogenic activity. Thus the structure of the drug can program the cells for estrogenic or antiestrogenic properties [36–38]. Another observation made by Harper and Walpole was that ICI 46,474 exhibits species specificity; in short-term tests, the compound is an estrogen in the mouse and an antiestrogen in the rat [3, 4]. The triphenylethylene derivative blocks the binding of [^{3}H]estradiol to ERs derived from both rat and mouse target tissues [39–42], but no completely satisfactory subcellular mechanism for the species difference of ICI 46,474 has yet been established. In fact, the situation is probably more complex than may at first be appreciated. The long-term administration of tamoxifen to ovariectomized mice results in an initial estrogen-like effect in the vagina [40] and the uterus [43], but as treatment progresses both the uterus and vagina become refractory to the effects of exogenous estrogen, and ICI 46,474 becomes a complete antiestrogen in the vagina.

Preliminary clinical studies with ICI 46,474 to treat advanced breast cancer in postmenopausal women were conducted by Mary Cole and coworkers [16] at the Christie Hospital in Manchester. The confirmation that ICI 46,474 could be used successfully as palliative in advanced disease but produces few side effects [17, 44] acted as a catalyst to encourage the study of the mode of action of the drug in animal tumor models. Indeed the conversation between the laboratory and the clinic became the hallmark for the successful development of tamoxifen.

Animal studies were first started in 1973 at the Worcester Foundation for Experimental Biology Shrewsbury, Massachusetts [45–50]. The dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model, originally described a

Uncorrected Proof

Author's Proof

decade earlier by the Nobel Laureate Professor Charles Huggins [51], was used to study the efficacy and mode of action of ICI 46,474 under controlled laboratory conditions. The model was considered to be state of the art, because no other hormone-dependent models were then available for study. Rob Nicholson, then a graduate student at the Tenovus Institute for Cancer Research in Cardiff, Wales, also selected the DMBA-induced rat mammary carcinoma model for this study of the antitumor actions of ICI 46,474 and related compounds [52]. These parallel research ventures fully described the antitumor activity of the antiestrogen in vivo [41, 48–50, 53, 54] at a time when the efficacy of tamoxifen was being established widely in breast cancer clinical trials [55].

ICI 46,474 to Tamoxifen

In 1973, Nolvadex, the ICI brand of tamoxifen (as its citrate salt), was approved for the treatment of breast cancer by the Committee on the Safety of Medicines in the United Kingdom. Similar approval was given in the United States for the treatment of advanced disease in postmenopausal women by the Food and Drug Administration on 30 December 1977. Nolvadex is available in more than 110 countries as the first-line endocrine therapy for the treatment of breast cancer [1]. To mark this achievement, ICI Pharmaceutical Division was presented with the Queen's Award for Technological Achievement by the Lord Lieutenant of Cheshire, Viscount Leverhulme, on 6 July 1978. The remarkable success of tamoxifen encouraged a closer examination of its pharmacology with a view to further development and wider applications.

The metabolism of tamoxifen in animals and patients was first described by Fromson and coworkers [56, 57]. The major metabolic route to be described was hydroxylation to form 4-hydroxytamoxifen, which was subsequently shown to have high binding affinity for the estrogen receptor and to be a potent antiestrogen in its own right [58] with antitumor properties in the DMBA model [59]. Indeed it is an advantage for the tamoxifen to be metabolically activated to 4-hydroxytamoxifen [60], but this is not a prerequisite for antiestrogen action. The metabolite was subsequently shown to localize in target tissues after the administration of radioactive tamoxifen to rats [61]. Originally, 4-hydroxytamoxifen was believed to be the major metabolite in patients [57], but Hugh Adam [62] at ICI Pharmaceutical Division demonstrated that N-desmethyltamoxifen is the principal metabolite found in patients. There is usually a blood level ratio of 2:1 for N-desmethyltamoxifen that has twice the plasma half-life of tamoxifen (14 days vs. 7 days) [63]. The ubiquitous use of tamoxifen resulted in the publication of numerous methods to estimate tamoxifen and its metabolites in serum (reviewed in [64]). The metabolites that have been identified in patients are shown in Fig. 2.5. The minor metabolites, metabolite Y [65], metabolite Z [66], and 4-hydroxy-N-desmethyltamoxifen [67], all contribute to the antitumor actions of tamoxifen, because they are all antiestrogens which inhibit the binding of estradiol to the ER. The metabolism of tamoxifen will be considered in more detail in Chap. 3.

The next significant advance came with the availability of hormone-dependent human breast cancer cells to study antitumor mechanisms in the laboratory.

Uncorrected Proof

Author's Proof

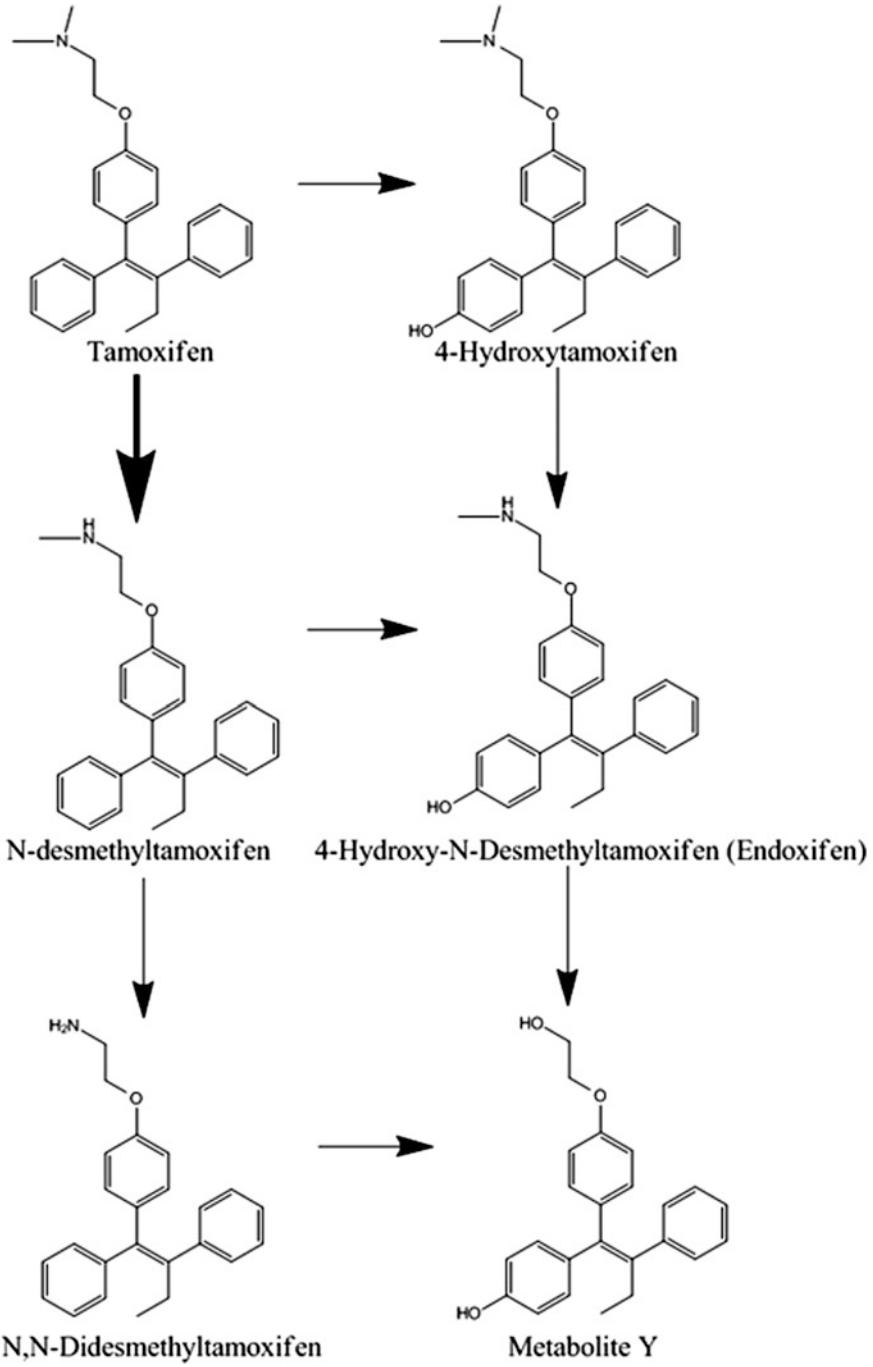


Fig. 2.5 The scheme of tamoxifen metabolism and the structures of its metabolites

Author's Proof

Marc Lippman [68] was the first to describe the ability of tamoxifen to inhibit the growth of MCF-7 ER-positive breast cancer cells [69] in culture and to demonstrate that the addition of estrogen could reverse the action of tamoxifen. Nearly a decade later, Kent Osborne [70] and Rob Sutherland [71] independently described the blockade by tamoxifen of breast cancer cells at the G_1 phase of the cell cycle.

Studies with the heterotransplantation of MCF-7 cells into athymic mice demonstrated that, unlike estradiol, tamoxifen does not support the growth of tumors [72]. Tamoxifen [73] and its metabolites [74] will block estrogen-stimulated tumor growth. However, very high circulatory levels (2,300 pg/ml) of estradiol in a low-tamoxifen environment (40 ng/ml) can partly reverse the inhibitory actions of tamoxifen for MCF-7 tumor growth [75]. Overall, these studies of the reversibility of tamoxifen action could have implications for its extended adjuvant use in premenopausal women.

These significant biological advances propelled tamoxifen forward to become the only nonsteroidal estrogen antagonist that would become the "gold standard" for the endocrine therapy of breast cancer for two decades. But none of this seemed possible in the 1970s when ICI Pharmaceutical Division was chauffeuring thousands of rats from Alderley Park to Leeds University. This investment in independent academic research would convert an orphan drug to be multibillion GBP blockbuster that saved millions of women's lives [76]. What is amazing is that the early work occurred without patent protection, but that changed.

Patenting Problems

Adequate patent protection is required to develop an innovation in a timely manner. In 1962, ICI Pharmaceuticals Division filed a broad patent in the United Kingdom (UK) (Application number GB19620034989 19620913). The application stated, "The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolaemic activity."

This was published in 1965 as UK Patent GB1013907, which described the innovation that different geometric isomers of substituted triphenylethylenes had either estrogenic or antiestrogenic properties [3]. Indeed, this observation was significant, because when scientists at Merrell subsequently described the biological activity of the separated isomers of their drug clomiphene, they inadvertently reversed the naming [77]. This was subsequently rectified [78].

Although tamoxifen was approved for the treatment of advanced breast cancer in postmenopausal women in 1977 in the United States (the year before ICI Pharmaceuticals Division received the Queen's Award for Technological Achievement in the UK), the patent situation was unclear. ICI Pharmaceuticals Division was repeatedly denied patent protection in the United States until the 1980s because of the perceived primacy of the earlier Merrell patents and because no advance (i.e., a safer, more

Uncorrected Proof

Author's Proof

specific drug) was recognized by the patent office in the United States. In other words, the clinical development advanced steadily for more than a decade in the United States without the assurance of exclusivity. This situation also illustrates how unlikely the usefulness of tamoxifen was considered to be by the medical advisors to the pharmaceutical industry in general. No other company chose to "steal" tamoxifen. Remarkably, when tamoxifen was hailed as the adjuvant endocrine treatment of choice for breast cancer by the National Cancer Institute in 1984 [79], the patent application, initially denied in 1984, was awarded through the court of appeals in 1985. This was granted with precedence to the patent dating back to 1965! So, at a time when worldwide patent protection was being lost, the patent protecting tamoxifen started a 17-year life in the United States. The unique and unusual legal situation did not go uncontested by generic companies, but AstraZeneca (as the ICI Pharmaceuticals Division is now called) rightly retained patent protection for their pioneering product, most notably, from Smalkin's decision in Baltimore, 1996 (Zeneca, Ltd. vs. Novopharm, Ltd.Civil Action No S95-163 United States District Court, D. Maryland, Northern Division, 14 March 1996).

Conclusion

The unprecedented advance of tamoxifen from the first unsure steps seems unbelievable but actually occurred. This situation was dependent on the correct prepared individuals being at the right place at the right time to advance a pioneering medicine that saves lives.

Postscript. In September 1972, at the time of the examination of my Ph.D. thesis by Dr. Arthur Walpole, I was unaware that the research director at ICI Pharmaceutical Division had ordered the termination of the clinical development of tamoxifen. This was a financial decision based on nonprofitability. My understanding is that all of the clinical research on tamoxifen (then ICI 46,474) had been reviewed in March 1972 at a symposium at Alderley Park [24].

The termination of tamoxifen's development toward registration and clinical use had resulted in Walpole requesting early retirement. Scientists at ICI Pharmaceutical Division did none of the laboratory work on tamoxifen as an antitumor agent; that was outsourced to me for a decade. But how did that happen?

I had already been recruited to the faculty as a lecturer in Pharmacology at Leeds, but first I was required to spend a couple of years in America to obtain my BTA (Been to America, a colloquial acronym as a prestigious research qualification). It had been arranged that I would go to the Worcester Foundation for Experimental Biology (the home of the oral contraceptive) to work with Mike Harper, who had left ICI Pharmaceutical Division some years earlier and now headed an Agency for International Development Program, to create a once a month contraceptive based on prostaglandins (the new research fashion!). I remember my conversation with Mike Harper on the telephone as I stood in the corridor on the phone in the old Medical School in Leeds. He asked three questions: "Could you start in September

Uncorrected Proof

Author's Proof

(1972)?" "Would $12,000 a year be acceptable?" and "Would you work on prostaglandins?" "Yes, yes, yes," I replied and headed off to the library to find out what prostaglandins were!

Walpole, my committee, and I met for my examination in the Department of Pharmacology at the Leeds University in early September 1972. This had become a matter of urgency as I had to complete the examination, drive to Southampton to board the QEII, and then travel from New York to Worcester, MA, to be a visiting scientist for 2 years at the Worcester Foundation for Experimental Biology.

When I arrived to the Worcester Foundation in September 1972—incidentally not knowing anything about prostaglandins—I discovered that Mike Harper had accepted a job with the World Health Organization in Geneva. My new boss Ed Klaiber said: "Next week give me a plan of research you propose to complete here in the next two years" and "You can do anything you like as long as some of it includes prostaglandins." Armed with a brand new Ph.D. in "failed contraceptives" (a topic not designed to equip me for a research career!), I immediately found myself as an independent investigator and planned my work on prostaglandins. However, my new circumstances would also allow me to explore my passion—to develop a drug to treat breast cancer.

A phone call to Walpole started the process of turning ICI 46,474 into tamoxifen, the gold standard for the endocrine treatment of breast cancer for the next 30 years. Walpole informed me the ICI Pharmaceuticals Division had just acquired Stuart Pharmaceuticals in Wilmington, Delaware, and they had created a new company ICI Americas. Lois Trench, the drug monitor for tamoxifen, would be the individual involved in the investment in my laboratory at the Worcester Foundation with an unrestricted research grant to determine how best to use tamoxifen in the clinic. But how to start? I was a pharmacologist with experience in "failed contraceptive" not a cancer research scientist. It seems that the way forward depends upon a clear plan, enthusiasm, and who you meet.

The National Cancer Act was passed in 1971 in the United States and the "war on cancer" began. The president of the Worcester Foundation Mahlon Hoagland realized that the research resources of the foundation in reproductive endocrinology could be steered toward endocrine-dependent cancers with the right advisor on the Scientific Advisory Board. Dr. Elwood Jensen, director of the Ben May Laboratory for Cancer Research at the University of Chicago, was a pioneer in the identification of the ER in estrogen target tissues in the rat and the application of this knowledge for the identification of estrogen-dependent breast tumors in women with metastatic breast cancer. The absence of ER in the tumor meant that there was no possibility of a response to endocrine ablation. Jensen spent a couple of days at the foundation in late 1972 and we spent time together going over my thesis work. I told him of my plans for tamoxifen and he generously agreed to have his staff (or rather Silvia Smith) teach me techniques of ER analysis and most importantly his colleague Dr. Gene DeSombre to teach me the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. My visit to Chicago to learn the techniques was a dream come true!

Uncorrected Proof

Author's Proof

Lois Trench arranged for me to receive a small collection of deep-frozen breast tumors so we started the program of translational research with the aid of Suzane Koerner, a superb technician. Lois insisted I became a consultant to ICI Americas to encourage clinicians in oncology groups to study tamoxifen in clinical trial. I lectured to the members of the Eastern Cooperative Oncology Group Breast Committee at their meetings in Miami and Jasper National Park in 1974. Too many adventures there to fit in the limited space here, I am afraid! Lois, then sponsored me to present the first study on tamoxifen as a preventive of mammary cancer in rats at the International Steroid Hormone Congress in Mexico City in September 1974 [45] (more adventures with my boss Ed Klaiber in Acapulco).

The idea of publishing my emerging data for the treatment and prevention of breast cancer did not occur immediately. Nobody in the scientific or clinical community really cared about the development of another (more expensive) endocrine therapy of limited effectiveness. However, that perspective was to change. Eliahu Caspi called me to his office one day in July 1974 and announced he had been charged with the responsibility of evaluating my CV and bibliography to explore the possibility of me staying at the foundation as a staff and not returning to Leeds University. He was rather frightening as an individual and stared at me across his desk. He reiterated that he had been told to interview me and evaluate my CV. He then said: "but you haven't got one as you have not published anything." After a stunned silence from me, I replied: "but I haven't discovered anything," to which he then gave me the best advice I had received about developing an academic career up to that point. "Tell them the story so far; each paper can be written within about 2 weeks and create a theme of interlocking research papers." I have followed his advice ever since.

I would like to recount an unanticipated honor that occurred by chance in 2002. At the commencement of the University of Massachusetts Medical School at the Mechanics Hall in Worcester in 2001, I was delivering my acceptance speech for an honorary Doctor of Science degree and told my Eliahu Caspi story about publication—emphasizing that if you don't publish, it never happened. A year later I was asked to deliver the inaugural Eliahu Caspi Memorial Lecture at the Worcester Foundation. It was then that I learned of the remarkable background of Dr. Caspi and had the pleasure of spending time with his accomplished family. As a young man in Poland, Caspi had survived a Russian prison camp, escaped to the emerging Israel, joined the Haganah (early Israeli Defense Forces), and then came to America to complete his Ph.D. at Clark University in Worcester. He then joined the Worcester Foundation having a distinguished career in glucocorticoid metabolism and synthesis until his death in May 2001.

References

1. Furr BJ, Jordan VC (1984) The pharmacology and clinical uses of tamoxifen. Pharmacol Ther 25:127–205
2. Group EBCTC (1992) Systematic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Lancet 339:1–15, and 71–85

Uncorrected Proof

Author's Proof

3. Harper MJ, Walpole AL (1966) Contrasting endocrine activities of *cis* and *trans* isomers in a series of substituted triphenylethylenes. Nature 212:87
4. Harper MJ, Walpole AL (1967) A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 13:101–119
5. Harper MJ, Walpole AL (1967) Mode of action of I.C.I. 46,474 in preventing implantation in rats. J Endocrinol 37:83–92
6. Emmens CW (1970) Postcoital contraception. Br Med Bull 26:45–51
7. Klopper A, Hall M (1971) New synthetic agent for the induction of ovulation: preliminary trials in women. Br Med J 1:152–154
8. Williamson JG, Ellis JD (1973) The induction of ovulation by tamoxifen. J Obstet Gynaecol Br Commonw 80:844–847
9. Beatson GT (1896) On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. Lancet 2:104–107
10. Boyd S (1900) On oophorectomy in cancer of the breast. Br Med J 2:1161–1167
11. Lerner LJ, Holthaus FJ Jr, Thompson CR (1958) A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol. Endocrinology 63:295–318
12. Holtkamp DE, Greslin JG, Root CA, Lerner LJ (1960) Gonadotrophin inhibiting and anti-fecundity effects of chloramiphene. Proc Soc Exp Biol Med 105:197–201
13. Kistner RW, Smith OW (1959) Observations on the use of nonsteroidal estrogen antagonist: MER 25. Surg Forum 10:725–729
14. Herbst AL, Griffiths CT, Kistner RW (1964) Clomiphene sitrate (Nsc-35770) in disseminated mammary carcinoma. Cancer Chemother Rep 43:39–41
15. Legha SS, Slavik M, Carter SK (1976) Nafoxidine–an antiestrogen for the treatment of breast cancer. Cancer 38:1535–1541
16. Cole MP, Jones CT, Todd ID (1971) A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer 25:270–275
17. Ward HW (1973) Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J 1:13–14
18. Sander S (1968) The in vitro uptake of oestradiol in biopsies from 25 breast cancer patients. Acta Pathol Microbiol Scand 74:301–302
19. Johansson H, Terenius L, Thoren L (1970) The binding of estradiol-17beta to human breast cancers and other tissues in vitro. Cancer Res 30:692–698
20. Korenman SG, Dukes BA (1970) Specific estrogen binding by the cytoplasm fof human breast carcinoma. J Clin Endocrinol Metab 30:639–645
21. Jensen EV, Block GE, Smith S et al (1971) Estrogen receptors and breast cancer response to adrenalectomy. Natl Cancer Inst Monogr 34:55–70
22. McGuire WLCP, Vollmer EP (1975) Estrogen receptors in human breast cancer. Raven, New York
23. Jordan VC (1988) The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. Breast Cancer Res Treat 11:197–209
24. Jordan VC (2006) Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 147(Suppl 1):S269–S276
25. Powles TJ, Hardy JR, Ashley SE et al (1989) A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. Br J Cancer 60:126–131
26. Powles TJ, Tillyer CR, Jones AL et al (1990) Prevention of breast cancer with tamoxifen–an update on the Royal Marsden Hospital pilot programme. Eur J Cancer 26:680–684
27. Fisher B (1992) The evolution of paradigms for the management of breast cancer: a personal perspective. Cancer Res 52:2371–2383
28. Lerner LJ (1981) The first nonsteroidal antioestrogen-MER 25. In: Sutherland RL, Jordan VC (eds) Non-steroidal antioestrogens: molecular pharmacology and antitumour activity. Academic, Sydney, pp 1–16
29. Clark JH, Markaverich BM (1982) The agonistic-antagonistic properties of clomiphene: a review. Pharmacol Ther 15:467–519

Uncorrected Proof

Author's Proof

30. Hollander W, Chobanian AV, Wilkins RW (1960) The effects of triparanol (MER-29) in subjects with and without coronary artery disease. JAMA 174:5–12
31. Laughlin RC, Carey TF (1962) Cataracts in patients treated with triparanol. JAMA 181:339–340
32. Avigan J, Steinberg D, Vroman HE et al (1960) Studies of cholesterol biosynthesis. I. The identification of desmosterol in serum and tissues of animals and man treated with MER-29. J Biol Chem 235:3123–3126
33. Kraft RO (1962) Triparanol in the treatment of disseminated mammary carcinoma. Cancer Chemother Rep 25:113–115
34. Fisher B, Costantino JP, Wickerham DL et al (2005) Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97:1652–1662
35. Fisher B, Costantino JP, Wickerham DL et al (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90:1371–1388
36. Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741–4745
37. Jordan VC, Koch R, Langan S, McCague R (1988) Ligand interaction at the estrogen receptor to program antiestrogen action: a study with nonsteroidal compounds in vitro. Endocrinology 122:1449–1454
38. Murphy CS, Langan-Fahey SM, McCague R, Jordan VC (1990) Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. Mol Pharmacol 38:737–743
39. Skidmore J, Walpole AL, Woodburn J (1972) Effect of some triphenylethylenes on oestradiol binding in vitro to macromolecules from uterus and anterior pituitary. J Endocrinol 52:289–298
40. Jordan VC (1975) Prolonged antioestrogenic activity of ICI 46, 474 in the ovariectomized mouse. J Reprod Fertil 42:251–258
41. Jordan VC, Jaspan T (1976) Tamoxifen as an anti-tumour agent: oestrogen binding as a predictive test for tumour response. J Endocrinol 68:453–460
42. Terenius L (1970) Two modes of interaction between oestrogen and anti-oestrogen. Acta Endocrinol (Copenh) 64:47–58
43. Jordan VC, Lababidi MK, Mirecki DM (1990) Anti-oestrogenic and anti-tumour properties of prolonged tamoxifen therapy in C3H/OUJ mice. Eur J Cancer 26:718–721
44. O'Halloran MJ, Maddock PG (1974) I.C.I. 46,474 in breast cancer. J Ir Med Assoc 67:38–39
45. Jordan VC (1974) Antitumor activity of the antiestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 5:354
46. Jordan VC (1974) The antiestrogen tamoxifen (ICI 46,474) as an antitumor agent. In: Proceedings of the Eastern cooperative oncology group, Miami
47. Jordan VC (1974) Tamoxifen: mechanism of antitumor activity in animals and man. In: Proceedings of the Eastern cooperative oncology group, Jasper
48. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
49. Jordan VC, Koerner S (1975) Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 11:205–206
50. Jordan VC, Koerner S (1976) Tamoxifen as an anti-tumour agent: role of oestradiol and prolactin. J Endocrinol 68:305–311
51. Huggins C, Grand LC, Brillantes FP (1961) Mammary cancer induced by a single feeding of polymucular hydrocarbons, and its suppression. Nature 189:204–207
52. Nicholson RI, Golder MP (1975) The effect of synthetic anti-oestrogens on the growth and biochemistry of rat mammary tumours. Eur J Cancer 11:571–579
53. Jordan VC, Dowse LJ (1976) Tamoxifen as an anti-tumour agent: effect on oestrogen binding. J Endocrinol 68:297–303

Uncorrected Proof

Author's Proof

54. Nicholson RI, Golder MP, Davies P, Griffiths K (1976) Effects of oestradiol – 17beta and tamoxifen on total and accessible cytoplasmic oestradiol – 17beta receptors in DMBA-induced rat mammary tumours. Eur J Cancer 12:711–717
55. Tamoxifen Workshop (1976) Proceedings of the annual spring meeting of the primary breast cancer therapy group, Key Biscayne, p 1409: 66
56. Fromson JM, Pearson S, Bramah S (1973) The metabolism of tamoxifen (I.C.I. 46,474). I. In laboratory animals. Xenobiotica 3:693–709
57. Fromson JM, Pearson S, Bramah S (1973) The metabolism of tamoxifen (I.C.I. 46,474). II. In female patients. Xenobiotica 3:711–714
58. Jordan VC, Collins MM, Rowsby L, Prestwich G (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305–316
59. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
60. Allen KE, Clark ER, Jordan VC (1980) Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 71:83–91
61. Borgna JL, Rochefort H (1981) Hydroxylated metabolites of tamoxifen are formed in vivo and bound to estrogen receptor in target tissues. J Biol Chem 256:859–868
62. Adam HK, Douglas EJ, Kemp JV (1979) The metabolism of tamoxifen in human. Biochem Pharmacol 28:145–147
63. Patterson JS, Settatree RS, Adam HK, Kemp JV (1980) Serum concentrations of tamoxifen and major metabolite during long-term nolvadex therapy, correlated with clinical response. Eur J Cancer 1:89–92
64. Jordan VC, Murphy CS (1990) Endocrine pharmacology of antiestrogens as antitumor agents. Endocr Rev 11:578–610
65. Jordan VC, Bain RR, Brown RR et al (1983) Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. Cancer Res 43:1446–1450
66. Kemp JV, Adam HK, Wakeling AE, Slater R (1983) Identification and biological activity of tamoxifen metabolites in human serum. Biochem Pharmacol 32:2045–2052
67. Lien EA, Solheim E, Lea OA et al (1989) Distribution of 4-hydroxy-N-desmethyltamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. Cancer Res 49:2175–2183
68. Lippman ME, Bolan G (1975) Oestrogen-responsive human breast cancer in long term tissue culture. Nature 256:592–593
69. Brooks SC, Locke ER, Soule HD (1973) Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J Biol Chem 248:6251–6253
70. Osborne CK, Boldt DH, Clark GM, Trent JM (1983) Effects of tamoxifen on human breast cancer cell cycle kinetics: accumulation of cells in early G1 phase. Cancer Res 43:3583–3585
71. Sutherland RL, Green MD, Hall RE et al (1983) Tamoxifen induces accumulation of MCF 7 human mammary carcinoma cells in the G0/G1 phase of the cell cycle. Eur J Cancer Clin Oncol 19:615–621
72. Shafie SM, Grantham FH (1981) Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. J Natl Cancer Inst 67:51–56
73. Osborne CK, Hobbs K, Clark GM (1985) Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res 45:584–590
74. Gottardis MM, Robinson SP, Jordan VC (1988) Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumoristatic action of tamoxifen. J Steroid Biochem 30:311–314
75. Iino Y, Wolf DM, Langan-Fahey SM et al (1991) Reversible control of oestradiol-stimulated growth of MCF-7 tumours by tamoxifen in the athymic mouse. Br J Cancer 64:1019–1024
76. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44: 30–38

Uncorrected Proof

Author's Proof

77. Palopoli FP, Feil VJ, Allen RE et al (1967) Substituted aminoalkoxytriarylhaloethylenes. J Med Chem 10:84–86
78. Ernst S, Hite G, Cantrell JS et al (1976) Stereochemistry of geometric isomers of clomiphene: a correction of the literature and a reexamination of structure-activity relationships. J Pharm Sci 65:148–150
79. Consensus conference (1985) Adjuvant chemotherapy for breast cancer. JAMA 254:3461–3463

Uncorrected Proof

Author's Proof

Author Queries

Chapter No.: 2

Query Refs.	Details Required	Author's response
AU1	Please check if edit to figure caption for Fig. 2.4 is okay.	
AU2	Please check if edit to sentence starting "There is usually...." is okay.	
AU3	Please check if edit to sentence starting "What is amazing...." is okay.	
AU4	Please check if all occurrences of "America" when referring to "the the United States" can be changed to "the United States" throughout the book for consistency.	
AU5	Please check if edit to sentence starting "Eliahu Caspi called. . ." is okay.	
AU6	Duplicate entry of Ref. [45] has been deleted. Please check if okay.	

Uncorrected Proof

Author's Proof

Metadata of the chapter that will be visualized online

Chapter Title	Metabolites of Tamoxifen as the Basis of Drug Development	
Copyright Year	2013	
Copyright Holder	Springer Basel	
Corresponding Author	Family Name	**Maximov**
	Particle	
	Given Name	**Philipp Y.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**McDaniel**
	Particle	
	Given Name	**Russell E.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**Jordan**
	Particle	
	Given Name	**V. Craig**
	Suffix	
	Division	Lombardi Comprehensive Cancer Center
	Organization	Georgetown University Medical Center
	Address	Washington, DC, USA
Abstract	By the early 1970s, a number of metabolites of tamoxifen had been identified in animals and following administration to a few patients. The hydroxylated metabolite of tamoxifen, 4-hydroxytamoxifen, proved to be the most interesting. The discovery of its high binding affinity for the estrogen receptor made it a new laboratory tool for all future in vitro studies of antiestrogen action and also provided the clue for all future structure-function relationships studies of new antiestrogens. These compounds would subsequently be developed as selective estrogen receptor modulators (SERMs). Tamoxifen is a prodrug but it is the metabolite 4-hydroxy-N-desmethyltamoxifen or endoxifen that has attracted pharmacogenetic interest. Mutations of the CYP2D6 gene control endoxifen production and have been associated with drug efficacy in some clinical trials.	

Author's Proof

Chapter 3
Metabolites of Tamoxifen as the Basis of Drug Development

Abstract By the early 1970s, a number of metabolites of tamoxifen had been identified in animals and following administration to a few patients. The hydroxylated metabolite of tamoxifen, 4-hydroxytamoxifen, proved to be the most interesting. The discovery of its high binding affinity for the estrogen receptor made it a new laboratory tool for all future in vitro studies of antiestrogen action and also provided the clue for all future structure-function relationships studies of new antiestrogens. These compounds would subsequently be developed as selective estrogen receptor modulators (SERMs). Tamoxifen is a prodrug but it is the metabolite 4-hydroxy-N-desmethyltamoxifen or endoxifen that has attracted pharmacogenetic interest. Mutations of the CYP2D6 gene control endoxifen production and have been associated with drug efficacy in some clinical trials.

Uncorrected Proof

Introduction

Tamoxifen is believed to be a prodrug and can be metabolically activated to 4-hydroxytamoxifen [1–4] or alternatively can be metabolically routed via N-desmethyltamoxifen to 4-hydroxy-N-desmethyltamoxifen [5, 6] (endoxifen) (Fig. 3.1). The hydroxylated metabolites of tamoxifen have a high binding affinity for the ER [1, 7]. The finding that the CYP2D6 subtype of cytochrome P450 activates tamoxifen to endoxifen [8] has implications for cancer therapeutics. It has been proposed that women with enzyme variants that cannot make endoxifen may not have as successful an outcome with tamoxifen therapy. Alternatively, women who have a wild-type enzyme may make high levels of the potent antiestrogen endoxifen and experience hot flashes. As a result, these women may take selective serotonin reuptake inhibitors (SSRIs) to ameliorate hot flashes but there are potential pharmacological consequences to this strategy. Some of the SSRIs are metabolically altered by the CYP2D6 enzyme [9]. It is therefore possible to envision a drug interaction whereby SSRIs block the metabolic activation of tamoxifen.

P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
DOI 10.1007/978-3-0348-0664-0_3, © Springer Basel 2013

Author's Proof

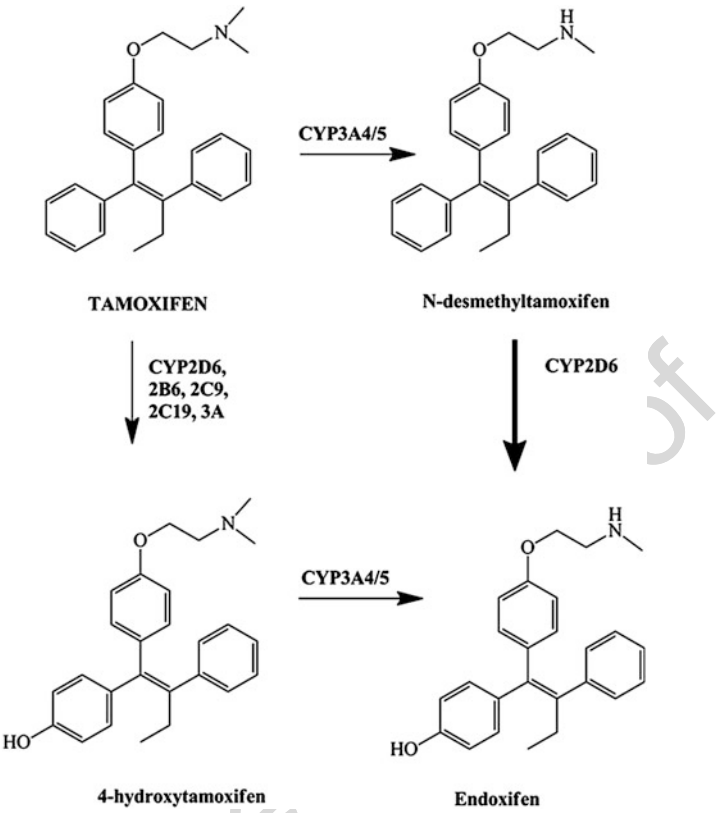


Fig. 3.1 The metabolic activation of tamoxifen to phenolic metabolites that have a high binding activity for the human ER. Both 4-hydroxytamoxifen and endoxifen are potent antiestrogens in vitro

This chapter will describe the scientific twists and turns that tamoxifen and its metabolites have taken over the past 30 years. The story is naturally dependent on the fashions in therapeutic research at the time. What seems obvious to us as a successful research strategy today, with millions of women taking tamoxifen, was not so 30 years ago at the beginning when the clinical community and pharmaceutical industry did not see "antihormones" as a priority at all for drug development [10]. In 1972, tamoxifen was declared an orphan drug with little prospect of successful clinical development [11].

Basic Mechanisms of Tamoxifen Metabolism

The original survey of the putative metabolites of tamoxifen was conducted in the laboratories of ICI Pharmaceuticals Division and published in 1973 [12]. A number of hydroxylated metabolites were noted (Fig. 3.2) following the administration of

Author's Proof

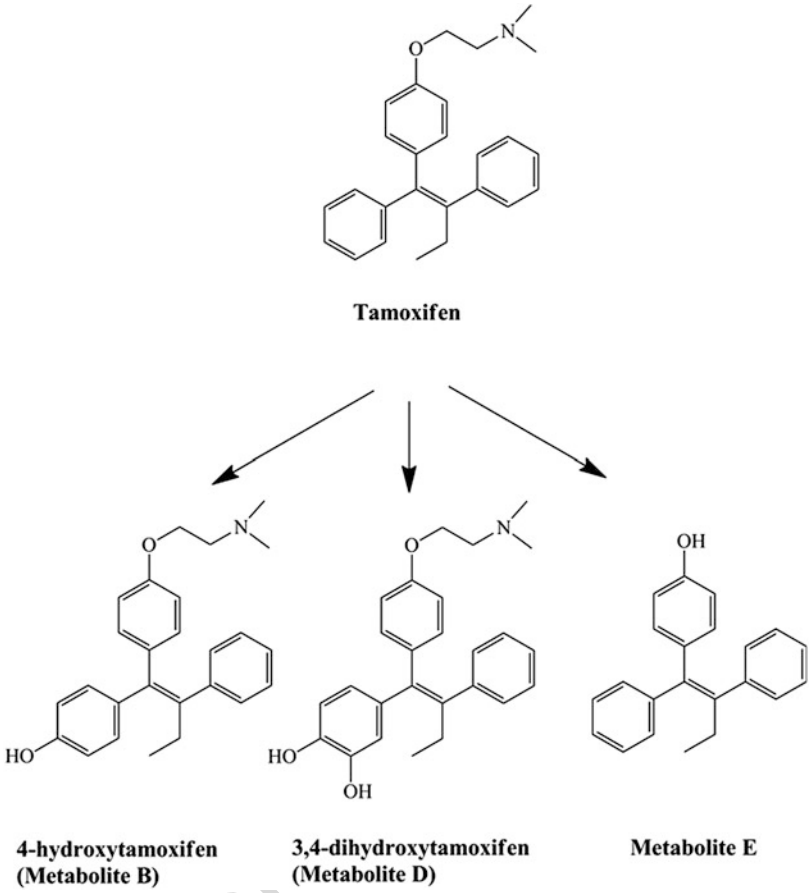


Fig. 3.2 The original hydroxylated metabolites of tamoxifen noted in animals by Fromson and coworkers [12]

^{14}C-labeled tamoxifen to various species (rat, mouse, monkey, and dog). The major route of excretion of radioactivity was in the feces. The rat and dog studies showed that up to 53 % of the radioactivity derived from tamoxifen was excreted via the bile and up to 69 % of this was reabsorbed via an enterohepatic recirculation until elimination eventually occurs [12]. The hydroxylated metabolites are excreted as glucuronides. However, no information about their biological activity was available until the finding that 4-hydroxytamoxifen had a binding affinity for the ER equivalent to 17β estradiol [1]. Similarly, 3, 4-dihydroxytamoxifen (Fig. 3.2) bound to the human ER but interestingly enough, 3, 4-dihydroxytamoxifen was not significantly estrogen-like in the rodent uterus despite being antiestrogenic [1, 4].

Additional studies on the metabolism of tamoxifen in four women [13] identified 4-hydroxytamoxifen as the primary metabolite using a thin layer chromatographic technique to identify ^{14}C-labeled metabolites. This assumption, coupled with the

Author's Proof

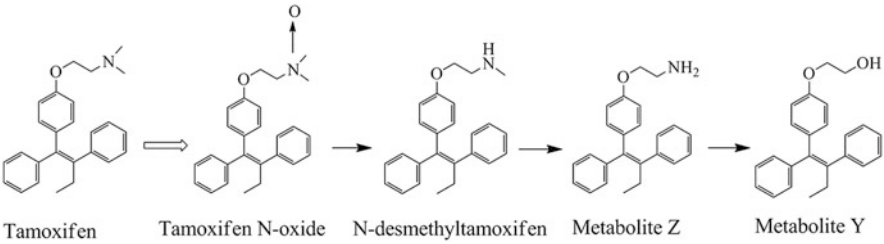


Fig. 3.3 The serial metabolic dimethylation and deamination of the antiestrogenic side chain of tamoxifen. Each of the metabolites is a weak antiestrogen with poor binding affinity for the ER

potent antiestrogenic actions of 4-hydroxytamoxifen [1] and the conclusion that it was an advantage, but not a requirement for tamoxifen to be metabolically activated [2, 14], seemed to confirm the idea that 4-hydroxytamoxifen was the active metabolite that bound in rat estrogen target tissues to block estrogen action [3]. However, the original analytical methods used to identify 4-hydroxytamoxifen as the major metabolite in humans were flawed [15] and subsequent studies identified N-desmethyltamoxifen (Fig. 3.3) as the major metabolite circulating in human serum [16]. The metabolite was found to be further demethylated to *N*-desdimethyltamoxifen (metabolite Z) [17] and then deaminated to metabolite Y, a glycol derivative of tamoxifen [18, 19]. The metabolites (Fig. 3.3) that are not hydroxylated at the 4 position of tamoxifen (equivalent to the three phenolic hydroxyl of estradiol) are all weak antiestrogens that would each contribute to the overall antitumor actions of tamoxifen at the ER based on their relative binding affinities for the ER and their actual concentrations locally.

At the end of the 1980s, the identification of another metabolite tamoxifen 4-hydroxy-N-desmethyltamoxifen in animals [20] and man [5, 6] was anticipated but viewed as obvious and uninteresting. The one exception that was of interest was metabolite E (Fig. 3.2 identified in the dog [12]. This phenolic metabolite without the dimethylaminoethyl side chain is a full estrogen [19, 21]. The dimethylaminoethoxy side chain of tamoxifen is necessary for antiestrogenic action [21].

It is not a simple task to study the actions of metabolites in vivo. Problems of pharmacokinetics, absorption, and subsequent metabolism all conspire to confuse the interpretation of data. Studies in vitro using cell systems of estrogen target tissues were defined and refined in the early 1980s to create an understanding of the actual structure-function relationships of tamoxifen metabolites. Systems were developed to study the regulation of the prolactin gene in primary cultures of immature rat pituitary gland cells [14, 22] or cell replication in ER-positive breast cancer cells [23–26]. Overall, these models were used to describe the importance of a phenolic hydroxyl to tether the triphenylethylenes appropriately in the ligand-binding domain of the ER and to establish the appropriate positioning of an "antiestrogenic" side chain in the "antiestrogen region" of the ER [22] to modulate gene activation and growth [14, 22, 27–30]. These structure-function studies that created hypothetical models of the ligand-ER complex were rapidly advanced with

Uncorrected Proof

Author's Proof

the first reports of the X-ray crystallography of the estrogen, 4-hydroxytamoxifen [31], or raloxifene ER ligand-binding domain [32] complexes. The ligand-receptor protein interaction was subsequently interrogated by examining the interaction of the specific amino acid asp 351 with the antiestrogenic side chain of the ligand [33]. A mutation was found as the dominant ER species in a tamoxifen-stimulated breast tumor grown in athymic mice [33, 34]. The structure-function relationships studies, that modulated estrogen action at a transforming growth factor-alpha gene target, demonstrated that the ligand shape would ultimately program the shape of the ER complex in a target tissue [35–39]. This concept is at the heart of metabolite pharmacology and is required to switch on and switch off target sites around the body. The other piece of the mechanism of the SERM puzzle that was eventually solved was the need for another player to partner with the ER complex. Coactivators [40] can enhance the estrogen-like effects of compounds at a target site [41]. However, in the early 1990s, the molecular and clinical use of this knowledge with the development and application of SERMs was in the future [42].

It is clear from this background about the early development of tamoxifen and the fact that tamoxifen was considered to be such a safe drug in comparison to other cytotoxic agents used in therapy during the 1970s and 1980s that there was little enthusiasm for in-depth studies of tamoxifen metabolism. However, this perspective was to change in the 1990s with the widespread use of tamoxifen as the gold standard for the treatment and prospect of clinical trials to evaluate the worth of tamoxifen for the prevention of breast cancer.

The urgent focus of translational research in the early1990s was to discover why tamoxifen was a complete carcinogen in rat liver [43, 44] and to determine whether there was a link between metabolism and the development of endometrial cancer noted in very small but significant numbers of postmenopausal women taking adjuvant tamoxifen [45, 46].

All interest in the metabolism of tamoxifen focused on the production of DNA adducts [47] that were responsible for rat liver carcinogenesis and, at the time, believed to be potentially responsible for carcinogenesis in humans [48]. Although many candidates were described [49–52], the metabolite found to be responsible for the initiation of rat liver carcinogenesis is α-hydroxytamoxifen [53–57] (Fig. 3.4). α-Hydroxytamoxifen has been resolved into R-(+) and S-(−)enantiomers. Metabolism by rat liver microsomes gave equal amounts of the two forms, but in hepatocytes the R form gave 8× the level of DNA adducts as the S form. As both had the same chemical reactivity toward DNA, Osborne et al. [58] suggested that the R form was a better sulfotransferase substrate. This enzyme is believed to catalyze DNA adduct formation. Subsequently, Osborne et al. [59] conducted studies with alpha-hydroxy-*N*-desmethyltamoxifen; the R-(+) gave 10× the level of adducts in rat hepatocytes as the S-(−).

There were reasonable concerns that the hepatocarcinogenicity of tamoxifen in rats would eventually translate to humans but fortunately this is now known to be untrue [60]. The demonstration of carcinogenesis in the rat liver appears to be related to poor DNA repair mechanisms in the inbred strains of rats. In contrast, it appears that the absence of liver carcinogenesis in women exposed to tamoxifen

Uncorrected Proof

Author's Proof

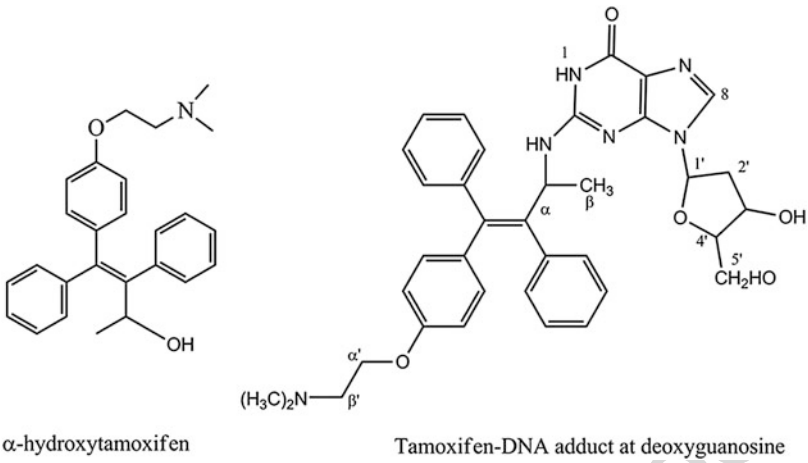


Fig. 3.4 The putative metabolite of tamoxifen, α- hydroxytamoxifen, that produces DNA adducts through covalent binding to deoxyguanosine in the rat liver

[61] is believed to result from the sophisticated mechanisms of DNA repair inherent in human cells. These concepts are described in more detail in Chap. 6.

The questions that next needed to be addressed were: Can improvements be made to the tamoxifen molecule? What happens to tamoxifen in patients?

Metabolic Mimicry

The demonstration [1, 2] that the class of compounds referred to as nonsteroidal antiestrogens were metabolically activated to compounds with high binding affinity for the ER created additional opportunities for the medicinal chemists within the pharmaceutical industry to develop new agents. An initial attempt was 3-hydroxytamoxifen (droloxifene) that was evaluated extensively in clinical trials. Those trials have been reviewed [62] but no advantages over tamoxifen were found. It is important to note that all studies used higher doses compared to tamoxifen. This emphasizes the principle that as droloxifene is a hydroxylated compound and is excreted more rapidly.

Drug discovery accelerated once the nonsteroidal antiestrogens [63] were recognized to be SERMs [64–66] and had applications not only for the treatment and prevention of breast cancer but also as potential agents to treat osteoporosis and coronary heart disease [67, 68]. The reader is referred to other recent review articles to obtain further details of new medicines under investigation [67, 68] but some current examples are worthy of note and will be mentioned briefly. Compounds of interest that have their structural origins from metabolites of nonsteroidal antiestrogens are summarized in Fig. 3.5.

Uncorrected Proof

Author's Proof

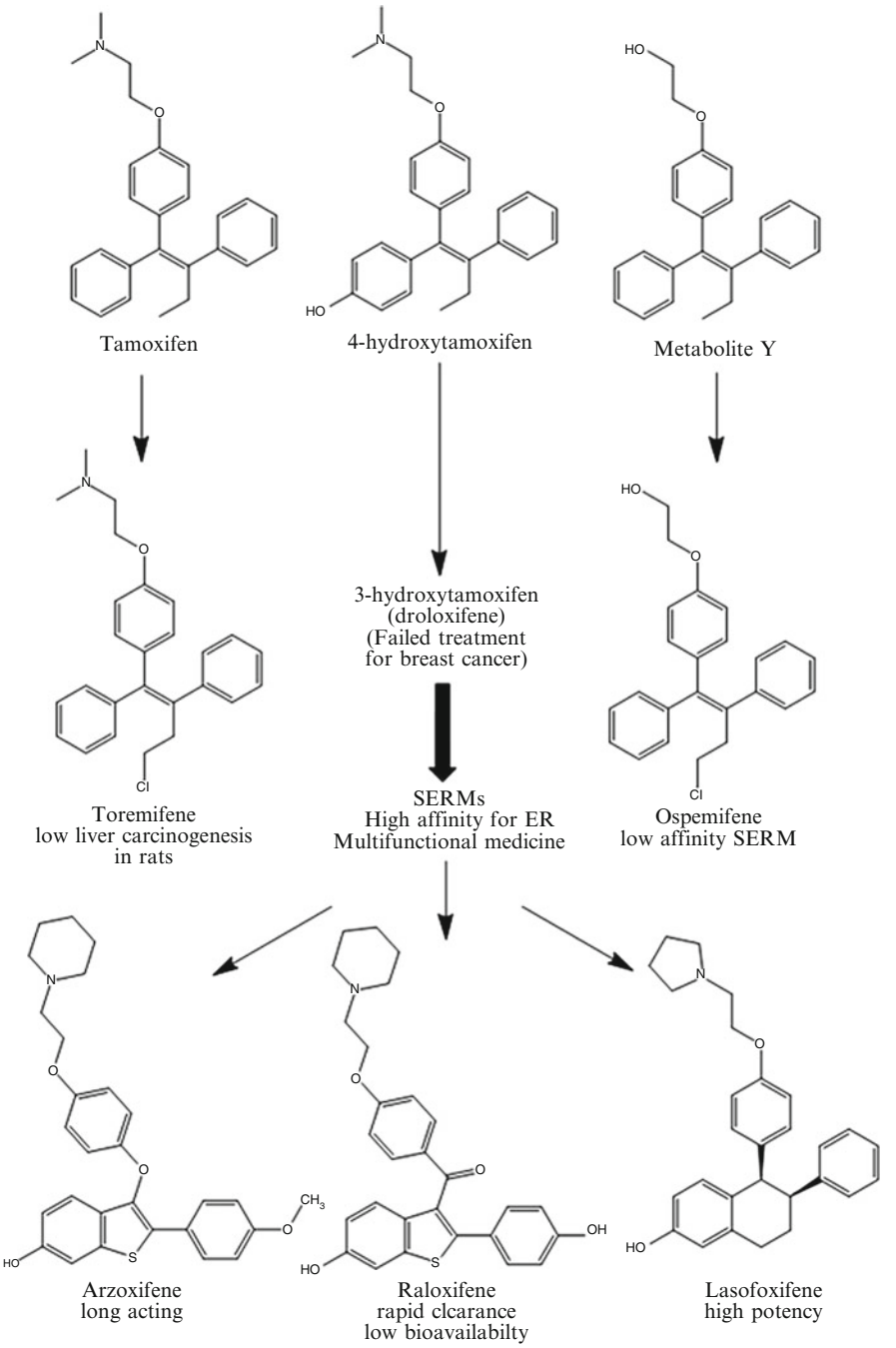


Fig. 3.5 The structures of new SERMs and their origins from other antiestrogens

Author's Proof

Raloxifene is an agent that originally was destined to be a drug to treat breast cancer but it failed in that application [69]. It appears that the pharmacokinetics and bioavailability of raloxifene are a challenge. Only about 2 % of administered raloxifene is bioavailable [70] but despite this, the drug is known to have a reasonable biological half-life of 27 h. The reason for this disparity is that raloxifene is a polyphenolic drug that can be glucuronidated and sulfated by bacteria in the gut so the drug cannot be absorbed [71, 72]. This phase II metabolism in turn controls enterohepatic recirculation and ultimately impairs the drug from reaching and interacting with receptors in the target. This concern has been addressed with the development of the long-acting raloxifene derivative arzoxifene that is known to be superior to raloxifene as a chemopreventive in rat mammary carcinogenesis [73]. One of the phenolic groups (Fig. 3.5) is methylated to provide protection from phase II metabolism.

Nevertheless, arzoxifene has not performed well as a treatment for breast cancer [74, 75]; higher doses are less effective than lower doses. These data imply that effective absorption is impaired by phase III metabolism. That being said, the results of trials evaluating the effects of arzoxifene as a drug to treat osteoporosis have been completed [76–78].

Unfortunately, the bioavailability of phenolic drugs is also dependent on phase II metabolism to inactive conjugates in the target tissue. 4-Hydroxytamoxifen [1] is only sulfated by three of seven sulfotransferase isoforms, whereas raloxifene is sulfated by all seven [79]. Maybe local phase II metabolism plays a role in neutralizing the antiestrogen action of raloxifene in the breast. Falany et al. [79] further report that SULT1E1, that sulfates raloxifene in the endometrium, is only expressed in the secretory phase. In contrast, 4- hydroxytamoxifen is sulfated at all stages of the uterine cycle.

Lasofoxifene is a diaryltetrahydronaphthalene derivative referred to as CP336156 [80] that has been reported to have high binding affinity for ER and have potent activity in preserving bone density in the rat [81, 82]. The structure of CP336156 is reminiscent of the putative antiestrogenic metabolic route for nafoxidine [83] (Chap. 1, Fig. 1.4) that failed to become a breast cancer drug because of unacceptable side effects [84]. CP336156 is the *l* enantiomer that has 20 times the binding affinity for the ER as the *d* enantiomer. Studies demonstrate that the *l* enantiomer had twice the bioavailability of the *d* enantiomer. The authors [80] ascribed the difference to enantioselective glucuronidation of the *d* isomer. An evaluation of CP336156 in the prevention and treatment of rat mammary tumors induced by *N*-nitroso-*N*-methylurea shows activity similar to that of tamoxifen [85].

Ospemifene or deaminohydroxytoremifene is related to metabolite Y formed by the deamination of tamoxifen [19]. Metabolite Y has a very low binding affinity for the ER [19, 86] and has weak antiestrogenic properties compared with tamoxifen. Ospemifene is a known metabolite of toremifene (4-chlorotoremifene) but unlike tamoxifen, there is little carcinogenic potential in animals [87]. It is possible that the large chlorine atom on the 4 position of toremifene and ospemifene reduces α-hydroxylation to the ultimate carcinogen related to α-hydroxytamoxifen (Fig. 3.5). Deaminohydroxytoremifene has very weak estrogenic and antiestrogenic

Uncorrected Proof

Author's Proof

properties in vivo [88] but demonstrates SERM activity in bone and lowers cholesterol. The compound is proposed to be used as a preventative for osteoporosis. Preliminary clinical data in healthy men and postmenopausal women demonstrate pharmacokinetics suitable for daily dosing between 25 and 200 mg [89]. Interestingly enough, unlike raloxifene, ospemifene has a strong estrogen-like action in the vagina but neither ospemifene nor raloxifene affects endometrial histology [90, 91].

Overall, the goal of developing a bone-specific agent is reasonable, but the key to commercial success will be the prospective demonstration of the prevention of breast and endometrial cancer as beneficial side effects. This remains a possibility based on prevention studies completed in the laboratory [92, 93].

Tamoxifen Metabolism Today

During the past decade, there has been considerable interest in the pharmacogenetics of tamoxifen-metabolizing enzymes in humans. The central hypothesis is that aberrant genes responsible for the metabolic activation of tamoxifen will influence therapeutics.

A comprehensive evaluation of the sequential biotransformation of tamoxifen has been completed by Desta and coworkers [8]. They used human liver microsomes and experiments with specifically expressed human cytochrome P450s to identify the prominent enzymes involved in phase I metabolism. Their results are summarized in Fig. 3.1 with the relevant CYP genes indicated for the metabolic transformations. The authors make a strong case that N-desmethyltamoxifen, the principal metabolite of tamoxifen that accumulates in the body, is converted to endoxifen by the enzyme variant CYP2D6. The CYP2D6 enzyme is also important to produce the potent primary metabolite 4-hydroxytamoxifen (this was first reported by David Kupfer at the Worcester Foundation 15 years ago! [94]), but the metabolite can also be formed by other enzymes: CYP2B6, CYP2C9, CY2C19, and CYP3A4.

The CYP2D6 phenotype is defined as the metabolic ratio (MR) by dividing the concentration of an unchanged probe drug, known to be metabolized by the CYP2D6 gene product, by the concentration of the relevant metabolite at a specific time. These measurements have resulted in the division of the CYP2D6 phenotype in four metabolic classes: poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM), and ultrarapid metabolizers (UM). Over 80 different single-nucleotide polymorphisms have been identified but there are inconsistencies in the precise definitions of ascribing a genotype to a phenotype [95, 96]. Bradford [96] and Raimundo and coworkers [97] have described the frequency of common alleles for CYP2D6. Pertinent to the current discussion of tamoxifen metabolism, the CYP2D6*4 allele [98] is estimated to have a frequency of12–23 % in Caucasians, 1.2–7 % in black Africans, and 0–2.8 % in Asians [95, 96]. A lower estimate of (<10 %) of the PM phenotype is presented by Bernard and coworkers [99].

Uncorrected Proof

Author's Proof

The molecular pharmacology of endoxifen has been reported [7, 100, 101]. Endoxifen and 4-hydroxytamoxifen were equally potent at inhibiting estrogen-stimulated growth of ER-positive breast cancer cells MCF-7, T47D, and BT474. Both metabolites are significantly superior in vitro to tamoxifen the parent drug. Additionally, the estrogen-responsive genes pS2 and progesterone receptor were both blocked to an equivalent degree by endoxifen and 4-hydroxytamoxifen [100, 101]. Lim and coworkers [101] have extended the comparison of endoxifen and 4-hydroxytamoxifen in MCF-7 cells by comparing and contrasting global gene regulation using the Affymetrix U133A Gene Chip Array. There were 4,062 total genes that were either up- or downregulated by estradiol, whereas, in the presence of estradiol, 4-hydroxytamoxifen or endoxifen affected 2,444 and 2,390 genes, respectively. Overall, the authors [101] demonstrated good correlation between RT-PCR and select genes from the microarray and concluded that the global effects of endoxifen and 4-hydroxytamoxifen were similar.

Stearns and coworkers [102]and Jin and coworkers [103] have confirmed and significantly extended Lien's original identification of endoxifen and observation [5, 6] that there are usually higher circulating levels of endoxifen than 4-hydroxytamoxifen in patients receiving adjuvant tamoxifen therapy. However, Flockhart's group [102] has advanced the pharmacogenomics and drug interactions surrounding tamoxifen therapy that should be a consideration in the antihormonal treatment of breast cancer.

The ubiquitous use of tamoxifen for the treatment of node-negative women [104] during the 1990s, the use of tamoxifen plus radiotherapy following lumpectomy for the treatment of ductal carcinoma in situ (DCIS) [105], as well as the option to use tamoxifen for chemoprevention in high-risk pre- and postmenopausal women [106] enhanced awareness of the menopausal side effects experienced by women when taking tamoxifen. Up to 45 % of women with hot flashes grade them as severe [106]; therefore, there have been efforts to improve quality of life. Treatments with the SSRIs are popular [102, 107, 108] (Fig. 3.6). The SSRIs are twice as effective as the "placebo" effect at reducing menopausal symptoms in randomized clinical trials [107–109], so there is naturally an increased usage of SSRIs with long-term tamoxifen treatment to maintain compliance. Unfortunately, the metabolism of tamoxifen to hydroxylated metabolites [94, 110, 111] and the metabolism of SSRIs [9, 112–115] both occur via the CYP2D6 gene product. Indeed Stearns and coworkers [102] showed that the paroxetine reduced the levels of endoxifen during adjuvant tamoxifen therapy and endoxifen levels decrease by 64 % in women with wild-type CYP2D6 enzyme. Patients were examined who were taking venlafaxine, sertraline, and paroxetine and compared with those women who were homozygotes for the CYP2D6*4/*4 inactive genotype. Patients with the wild-type gene who took the most potent inhibitor paroxetine had serum levels of endoxifen equivalent to the patients with the aberrant CYP2D6 gene. In fact, the clinical data were consistent with the inhibition constants for the inhibition of CYP2D6 by paroxetine (potent), fluoxetine, sertraline, citalopram (intermediate), and venlafaxine (weak) which are 0.05, 0.17, 1.5, 7, and 33 μmol/l, respectively.

Uncorrected Proof

Author's Proof

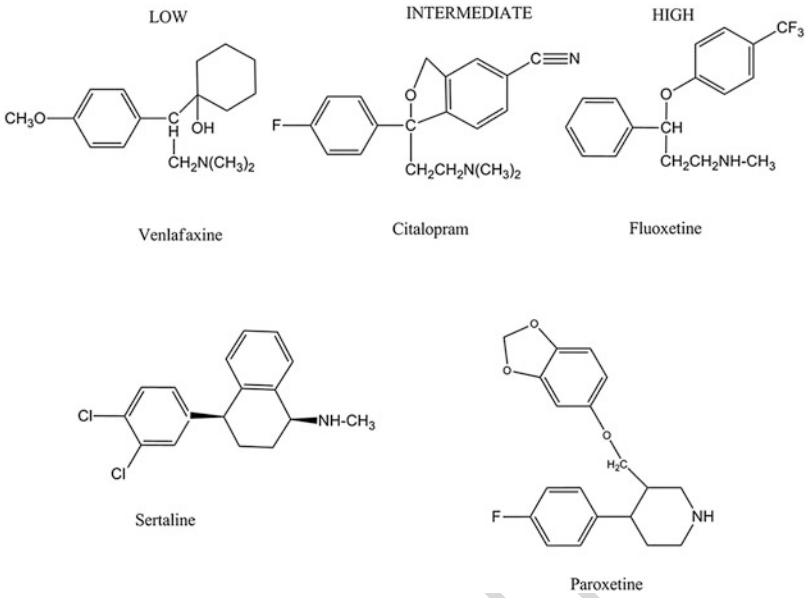


Fig. 3.6 The structures of selective serotonin reuptake inhibitors (SSRIs) that have low intermediate to high affinity for the CYP2D6 enzyme system. High affinity binders for CYP2D6 block the metabolic activation of tamoxifen to endoxifen (Fig. 3.1)

The CYP2D6 gene product that is fully functional (wild type) is classified as the CYP2D6*1. A large number of alleles are associated with no enzyme activity or reduced activity. Conversely, high metabolizers can have multiple copies of the CYP2D6 allele [116]. A recent study by Borges and coworkers [117] continues to expand our understanding of the detrimental effect of CYP2D6 variants plus concomitant administration of SSRIs on endoxifen levels. But, it is the clinical correlations with tumor responses and side effects that are of importance if pharmacogenomics is to be truly relevant in breast cancer therapy.

Clinical Correlations

The metabolic activation of tamoxifen to endoxifen by the CYP2D6 enzyme system still remains controversial to plan the treatment of patients with breast cancer. Since the discovery and description of the pharmacological properties of endoxifen, retrospective clinical trials were examined to determine the pharmacological relevance of endoxifen. The results of clinical trials, however, vary. Clinical investigation by Dieudonne and coworkers [118] have shown that patients with CYP2D6*4/*4 homozygous mutation, which reduces the levels of endoxifen in patients' plasma, are still responding to tamoxifen treatment and tamoxifen still has an effect

Author's Proof

on endometrial tissue and elevated the plasma levels of FSH and SHBG in those patients to the levels found in general tamoxifen-treated population. Study by Schroth and coworkers [119] have shown that there was an association of CYP2D6 genotype and clinical outcome for breast cancer patients, in particular the presence of two wild-type alleles correlated with better clinical outcomes and presence of mutant alleles with worse outcomes. Study by Kiyotani and coworkers [120] showed also that there is a significant correlation between the presence of risk alleles of CYP2D6, which are associated with lower plasma levels of endoxifen, and significantly lower recurrence-free survival in breast cancer patients that were taking tamoxifen as monotherapy. Study by Lammers and coworkers [121] also has demonstrated correlation between the overall survival of breast cancer patients that were taking tamoxifen 40 mg daily with poor metabolizer genotype, compared to patients with extensive metabolizer genotype. A study by Madlensky and coworkers [122] has shown that there is no association between breast cancer outcomes and the concentrations of 4-hydroxytamoxifen or endoxifen; however, they have demonstrated a threshold of endoxifen concentration, below which there is an increase in breast cancer recurrence rate and that about 80 % of patients are above that threshold. Interestingly, their threshold concentration of endoxifen is equivalent to concentrations found in patients with poor metabolizer genotype. In the study by Lash and coworkers [123], it was shown that there is virtually no correlation between recurrence rates of breast cancer in patients and the presence of one or two functional alleles. However, in 2012 results of studies from Arimidex, Tamoxifen, Alone or in Combination (ATAC) and Breast International Group (BIG) I-98 trails were published [124, 125]. The results concurrently showed no association between the recurrence rates and the genotypes of the postmenopausal patients taking tamoxifen alone or in combination with aromatase inhibitor. The results of the trials have sparked a controversy [126]. One thing that is certain is that endoxifen plasma levels do vary in patients taking tamoxifen depending on their metabolic genotype [127, 128]. It should be noted that in some of the trials the patients were postmenopausal or had previous chemotherapy. In 2012, we simulated the estrogen environment of postmenopausal women in vitro and test the antiestrogenic properties of tamoxifen and its metabolites in physiological concentrations on a panel of ER-positive human breast cancer cell lines (MCF-7, T47D, ZR-75-1, and BT474). The concentrations of estrogens (E1/E2) used to simulate postmenopausal women treated with tamoxifen were obtained from published studies [129, 130], as well as the concentrations of tamoxifen and its metabolites (N-desmethyltamoxifen, 4-hydroxytamoxifen, and endoxifen) based on the CYP2D6 genotype in postmenopausal breast cancer patients [128]. Our results demonstrate that irrespective of CYP2D6 genotype (extensive, intermediate, or poor metabolizers (EM, IM, and PM, respectively)), tamoxifen and its primary metabolites (N-desmethyltamoxifen and 4-hydroxytamoxifen) are able to inhibit completely the estrogen-stimulated growth of breast cancer cells in vitro. Additional endoxifen in any concentration corresponding to CYP2D6 genotype was not able to increase the antiestrogenic effect of tamoxifen and its primary metabolites. Moreover, we demonstrate that 4-hydroxytamoxifen is absolutely essential for

Uncorrected Proof

Author's Proof

inhibition of estrogen action. Based on our results, we can conclude that endoxifen is pharmacologically supportive but not essential for any genotype of CYP2D6 in a postmenopausal setting.

However, little is known on the role on the antiestrogenic impact of endoxifen in premenopausal women that are treated with tamoxifen. We have simulated premenopausal estrogen environment in vitro and used the same concentrations of tamoxifen and its metabolites found in different CYP2D6 genotypes. Our results show that tamoxifen and its primary metabolites are able to inhibit partially the estrogenic effect in the same panel of ER-positive human breast cancer cell lines; however, the addition of endoxifen, unlike in postmenopausal simulation, further inhibits the estrogens. Interestingly, the higher concentrations of endoxifen associated with EM and IM genotypes are inhibiting estrogens better, than at lower concentrations as found patients with PM genotype. It should be noted that addition of endoxifen at PM concentrations does not increase the antiestrogenic properties of tamoxifen and its primary metabolites in vitro. It was shown that the increase of tamoxifen dose in breast cancer patients increases the plasma levels of endoxifen [131–133]. In particular, it was shown by Irvin and coworkers [131] that the increase of tamoxifen dose to 40 mg daily administered by patients with IM and PM genotype increased the plasma levels of endoxifen. Using these levels of increased tamoxifen and its metabolites, we have simulated the average premenopausal estrogen setting in vitro and assessed the pharmacological impact of increased concentrations of endoxifen in IM and PM setting. Our results show that biologically there is no significant difference after treatments with tamoxifen primary metabolites and endoxifen at concentrations corresponding to 20 and 40 mg/daily. Interestingly, none of the tamoxifen treatments were able to fully inhibit the estrogen action in MCF-7 and T47D cells in the premenopausal setting; increasing the concentrations of endoxifen to levels higher than physiological was able to fully inhibit estrogen action. We conclude that endoxifen thus contributes to inhibition of estrogen action and growth of ER-positive breast cancer cells; however, endoxifen plays a supportive in a situation following chemotherapy in premenopausal patients.

Postscript. On my return from the United States to Leeds University in 1974, I was supported by the ICI Pharmaceutical Division Clinical Department and the Yorkshire Cancer Campaign. In 1975, I initially wished to study the hydroxylated metabolites of tamoxifen for two reasons: (1) would the metabolites be estrogens if low affinity was important for antiestrogenic activity or (2) would potent antiestrogen effects of the metabolites explain the potent antiestrogenic properties of tamoxifen in rats but the really weak antiestrogenic activity to block ER in vitro. Dora Richardson gave me their limited supply of the precious metabolites monohydroxytamoxifen (metabolite B) and dihydroxytamoxifen (metabolite D).

My students at Leeds University Clive Dix and Margaret Collins took the leading roles in discovering the pharmacological properties of 4-hydroxytamoxifen (the correct name of metabolite B). I recall telling Clive Dix to redo all ligand-binding experiments of 4-hydroxytamoxfien competition inhibiting the binding of

Uncorrected Proof

Author's Proof

[^{3}H]estradiol to rat uterine cytosolic ER. "Look Clive, there are no reports of a nonsteroidal antiestrogens binding to the ER with the same affinity as estradiol-learn to do your serial dilutions properly!" He was correct and it was an important discovery. When we discovered the potent antiestrogenic properties of 4-hydroxytamoxifen, I was informed by Sandy Todd at ICI Pharmaceutical Division that there were no patents for the metabolites. The scientists at ICI had clearly never believed the clinical development process would take off, as it did in the early 1970s with animal data to support clinical trials. A rule at ICI that all drug metabolites for a drug in active development and marketing had to be patented had been broken (remember the program was terminated in 1972). As a result, in 1976, I agreed to write up our paper, lodge it with ICI staff at ICI Pharmaceutical Division, and delay publishing until a patent was obtained for 4-hydroxytamoxifen. This occurred 1 year later. I also voluntarily agreed to not talk about our work in 1976 as it was important to get tamoxifen FDA approved in the United States. In 1976, I set off to Key Biscayne to the NSABP meeting to tell them all about tamoxifen [134].

What happened to 4-hydroxytamoxifen? It became the antiestrogen of choice for all laboratory studies in vitro for the next 30 years, but we also showed it was not the product to be developed instead of tamoxifen [135]. If tamoxifen was a prodrug, then 4-hydroxytamoxifen could be the active agent. The patent for 4-hydroxytamoxifen was sold to Besins International and a French physician Dr. Maurvais Jarvais, who advanced the proposal that breast cancer and breast pain could be resolved with a daily preparation rubbed on the breast [136]. Clinical trials have addressed this issue over the past 30 years.

I was subsequently awarded a Leeds University/ICI Pharmaceutical Division Joint Research scheme to evaluate the therapeutic potential of 6,7 alpha-substituted estradiol alkylated derivatives. We had discovered that substitution of the 6 and 7 positions of estradiol still retained significant binding affinity of the ligand for the receptor. The idea was to use the estradiol as the carrier molecule for an alkylating moiety to be delivered to the DNA precisely and kill ER-positive breast cancers. Alternatively, we could radiolabel the estradiol and subsequently discover the sites for estrogen-regulated genes. Neither of these ideas were successful. We published our findings [137] but did not follow up the 7-substituted estradiol with a $(CH_2)_{10}$ side chain. The further development of the idea was to result in the pure antiestrogen fulvestrant [138], but this was entirely the discovery of the Pharmaceutical Industry, with Alan Wakeling and his team.

We will find out what happened to the idea of estradiol with a long side chain at position 7 in Chap. 5.

References

1. Jordan VC, Collins MM, Rowsby L, Prestwich G (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305–316
2. Allen KE, Clark ER, Jordan VC (1980) Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 71:83–91

Uncorrected Proof

Author's Proof

3. Borgna JL, Rochefort H (1981) Hydroxylated metabolites of tamoxifen are formed in vivo and bound to estrogen receptor in target tissues. J Biol Chem 256:859–868
4. Jordan VC, Dix CJ, Naylor KE et al (1978) Nonsteroidal antiestrogens: their biological effects and potential mechanisms of action. J Toxicol Environ Health 4:363–390
5. Lien EA, Solheim E, Kvinnsland S, Ueland PM (1988) Identification of 4-hydroxy-N-desmethyltamoxifen as a metabolite of tamoxifen in human bile. Cancer Res 48:2304–2308
6. Lien EA, Solheim E, Lea OA et al (1989) Distribution of 4-hydroxy-N-desmethyltamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. Cancer Res 49:2175–2183
7. Johnson MD, Zuo H, Lee KH et al (2004) Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. Breast Cancer Res Treat 85:151–159
8. Desta Z, Ward BA, Soukhova NV, Flockhart DA (2004) Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: prominent roles for CYP3A and CYP2D6. J Pharmacol Exp Ther 310:1062–1075
9. Crewe HK, Lennard MS, Tucker GT et al (1992) The effect of selective serotonin re-uptake inhibitors on cytochrome P4502D6 (CYP2D6) activity in human liver microsomes. Br J Clin Pharmacol 34:262–265
10. Jordan VC, Brodie AM (2007) Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 72:7–25
11. Jordan VC (2003) Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2:205–213
12. Fromson JM, Pearson S, Bramah S (1973) The metabolism of tamoxifen (I.C.I. 46,474). I. In laboratory animals. Xenobiotica 3:693–709
13. Fromson JM, Pearson S, Bramah S (1973) The metabolism of tamoxifen (I.C.I. 46,474). II. In female patients. Xenobiotica 3:711–714
14. Lieberman ME, Jordan VC, Fritsch M et al (1983) Direct and reversible inhibition of estradiol-stimulated prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4734–4740
15. Adam HK, Gay MA, Moore RH (1980) Measurement of tamoxifen in serum by thin-layer densitometry. J Endocrinol 84:35–42
16. Adam HK, Douglas EJ, Kemp JV (1979) The metabolism of tamoxifen in humans. Biochem Pharmacol 27:145–147
17. Kemp JV, Adam HK, Wakeling AE, Slater R (1983) Identification and biological activity of tamoxifen metabolites in human serum. Biochem Pharmacol 32:2045–2052
18. Bain RR, Jordan VC (1983) Identification of a new metabolite of tamoxifen in patient serum during breast cancer therapy. Biochem Pharmacol 32:373–375
19. Jordan VC, Bain RR, Brown RR et al (1983) Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. Cancer Res 43:1446–1450
20. Robinson SP, Langan-Fahey SM, Jordan VC (1989) Implications of tamoxifen metabolism in the athymic mouse for the study of antitumor effects upon human breast cancer xenografts. Eur J Cancer Clin Oncol 25:1769–1776
21. Jordan VC, Gosden B (1982) Importance of the alkylaminoethoxy side-chain for the estrogenic and antiestrogenic actions of tamoxifen and trioxifene in the immature rat uterus. Mol Cell Endocrinol 27:291–306
22. Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741–4745
23. Katzenellenbogen JA, Carlson KE, Katzenellenbogen BS (1985) Facile geometric isomerization of phenolic non-steroidal estrogens and antiestrogens: limitations to the interpretation of experiments characterizing the activity of individual isomers. J Steroid Biochem 22:589–596
24. Katzenellenbogen BS, Norman MJ, Eckert RL et al (1984) Bioactivities, estrogen receptor interactions, and plasminogen activator-inducing activities of tamoxifen and hydroxytamoxifen isomers in MCF-7 human breast cancer cells. Cancer Res 44:112–119

Uncorrected Proof

Author's Proof

25. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS (1986) Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A 83:2496–2500
26. Murphy CS, Meisner LF, Wu SQ, Jordan VC (1989) Short- and long-term estrogen deprivation of T47D human breast cancer cells in culture. Eur J Cancer Clin Oncol 25:1777–1788
27. Jordan VC, Lieberman ME, Cormier E et al (1984) Structural requirements for the pharmacological activity of nonsteroidal antiestrogens in vitro. Mol Pharmacol 26:272–278
28. Jordan VC, Koch R, Mittal S, Schneider MR (1986) Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. Br J Pharmacol 87:217–223
29. Murphy CS, Langan-Fahey SM, McCague R, Jordan VC (1990) Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. Mol Pharmacol 38:737–743
30. Murphy CS, Parker CJ, McCague R, Jordan VC (1991) Structure-activity relationships of nonisomerizable derivatives of tamoxifen: importance of hydroxyl group and side chain positioning for biological activity. Mol Pharmacol 39:421–428
31. Shiau AK, Barstad D, Loria PM et al (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937
32. Brzozowski AM, Pike AC, Dauter Z et al (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
33. Wolf DM, Jordan VC (1994) The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31:129–138
34. Wolf DM, Jordan VC (1994) Characterization of tamoxifen stimulated MCF-7 tumor variants grown in athymic mice. Breast Cancer Res Treat 31:117–127
35. MacGregor Schafer J, Liu H, Bentrem DJ et al (2000) Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. Cancer Res 60:5097–5105
36. Levenson AS, Jordan VC (1998) The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. Cancer Res 58:1872–1875
37. Liu H, Lee ES, Deb Los Reyes A et al (2001) Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor alpha complex. Cancer Res 61:3632–3639
38. Levenson AS, Catherino WH, Jordan VC (1997) Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. J Steroid Biochem Mol Biol 60:261–268
39. Liu H, Park WC, Bentrem DJ et al (2002) Structure-function relationships of the raloxifene-estrogen receptor-alpha complex for regulating transforming growth factor-alpha expression in breast cancer cells. J Biol Chem 277:9189–9198
40. Onate SA, Tsai SY, Tsai MJ, O'Malley BW (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270:1354–1357
41. Jordan VC, O'Malley BW (2007) Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 25:5815–5824
42. Jordan VC, Gapstur S, Morrow M (2001) Selective estrogen receptor modulation and reduction in risk of breast cancer, osteoporosis, and coronary heart disease. J Natl Cancer Inst 93:1449–1457
43. Greaves P, Goonetilleke R, Nunn G et al (1993) Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. Cancer Res 53:3919–3924
44. Hard GC, Iatropoulos MJ, Jordan K et al (1993) Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. Cancer Res 53:4534–4541
45. Fornander T, Rutqvist LE, Cedermark B et al (1989) Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1:117–120

Uncorrected Proof

Author's Proof

46. Fisher B, Costantino JP, Redmond CK et al (1994) Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. J Natl Cancer Inst 86:527–537
47. Han XL, Liehr JG (1992) Induction of covalent DNA adducts in rodents by tamoxifen. Cancer Res 52:1360–1363
48. Rutqvist LE, Johansson H, Signomklao T et al (1995) Adjuvant tamoxifen therapy for early stage breast cancer and second primary malignancies. Stockholm Breast Cancer Study Group. J Natl Cancer Inst 87:645–651
49. Styles JA, Davies A, Lim CK et al (1994) Genotoxicity of tamoxifen, tamoxifen epoxide and toremifene in human lymphoblastoid cells containing human cytochrome P450s. Carcinogenesis 15:5–9
50. Lim CK, Yuan ZX, Lamb JH et al (1994) A comparative study of tamoxifen metabolism in female rat, mouse and human liver microsomes. Carcinogenesis 15:589–593
51. Moorthy B, Sriram P, Pathak DN et al (1996) Tamoxifen metabolic activation: comparison of DNA adducts formed by microsomal and chemical activation of tamoxifen and 4-hydroxytamoxifen with DNA adducts formed in vivo. Cancer Res 56:53–57
52. Pongracz K, Pathak DN, Nakamura T et al (1995) Activation of the tamoxifen derivative metabolite E to form DNA adducts: comparison with the adducts formed by microsomal activation of tamoxifen. Cancer Res 55:3012–3015
53. Potter GA, McCague R, Jarman M (1994) A mechanistic hypothesis for DNA adduct formation by tamoxifen following hepatic oxidative metabolism. Carcinogenesis 15:439–442
54. Phillips DH, Carmichael PL, Hewer A et al (1994) alpha-Hydroxytamoxifen, a metabolite of tamoxifen with exceptionally high DNA-binding activity in rat hepatocytes. Cancer Res 54:5518–5522
55. Phillips DH, Potter GA, Horton MN et al (1994) Reduced genotoxicity of [D5-ethyl]-tamoxifen implicates alpha-hydroxylation of the ethyl group as a major pathway of tamoxifen activation to a liver carcinogen. Carcinogenesis 15:1487–1492
56. Osborne MR, Hewer A, Hardcastle IR et al (1996) Identification of the major tamoxifen-deoxyguanosine adduct formed in the liver DNA of rats treated with tamoxifen. Cancer Res 56:66–71
57. Phillips DH, Carmichael PL, Hewer A et al (1996) Activation of tamoxifen and its metabolite alpha-hydroxytamoxifen to DNA-binding products: comparisons between human, rat and mouse hepatocytes. Carcinogenesis 17:89–94
58. Osborne MR, Hewer A, Phillips DH (2001) Resolution of alpha-hydroxytamoxifen; R-isomer forms more DNA adducts in rat liver cells. Chem Res Toxicol 14:888–893
59. Osborne MR, Hewer A, Phillips DH (2004) Stereoselective metabolic activation of alpha-hydroxy-N-desmethyltamoxifen: the R-isomer forms more DNA adducts in rat liver cells. Chem Res Toxicol 17:697–701
60. Phillips DH (2001) Understanding the genotoxicity of tamoxifen? Carcinogenesis 22:839–849
61. Jordan VC (1995) What if tamoxifen (ICI 46,474) had been found to produce rat liver tumors in 1973? A personal perspective. Ann Oncol 6:29–34
62. Jordan VC, Gradishar WJ (1997) Molecular mechanisms and future uses of antiestrogens. Mol Aspects Med 18:167–247
63. Jordan VC (1984) Biochemical pharmacology of antiestrogen action. Pharmacol Rev 36:245–276
64. Jordan VC (1988) Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 6:589–595
65. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189
66. Jordan VC (2001) Selective estrogen receptor modulation: a personal perspective. Cancer Res 61:5683–5687

Uncorrected Proof

Author's Proof

67. Jordan VC (2003) Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. J Med Chem 46:1081–1111
68. Ariazi EA, Ariazi JL, Cordera F, Jordan VC et al (2006) Estrogen receptors as therapeutic targets in breast cancer. Curr Top Med Chem 6:195–216
69. Buzdar AU, Marcus C, Holmes F et al (1988) Phase II evaluation of Ly156758 in metastatic breast cancer. Oncology 45:344–345
70. Snyder KR, Sparano N, Malinowski JM (2000) Raloxifene hydrochloride. Am J Health Syst Pharm 57:1669–1675, quiz 76–8
71. Kemp DC, Fan PW, Stevens JC (2002) Characterization of raloxifene glucuronidation in vitro: contribution of intestinal metabolism to presystemic clearance. Drug Metab Dispos 30:694–700
72. Jeong EJ, Liu Y, Lin H, Hu M (2005) Species- and disposition model-dependent metabolism of raloxifene in gut and liver: role of UGT1A10. Drug Metab Dispos 33:785–794
73. Suh N, Lamph WW, Glasebrook AL et al (2002) Prevention and treatment of experimental breast cancer with the combination of a new selective estrogen receptor modulator, arzoxifene, and a new rexinoid, LG 100268. Clin Cancer Res 8:3270–3275
74. Baselga J, Llombart-Cussac A, Bellet M et al (2003) Randomized, double-blind, multicenter trial comparing two doses of arzoxifene (LY353381) in hormone-sensitive advanced or metastatic breast cancer patients. Ann Oncol 14:1383–1390
75. Buzdar A, O'Shaughnessy JA, Booser DJ et al (2003) Phase II, randomized, double-blind study of two dose levels of arzoxifene in patients with locally advanced or metastatic breast cancer. J Clin Oncol 21:1007–1014
76. Bolognese M, Krege JH, Utian WH et al (2009) Effects of arzoxifene on bone mineral density and endometrium in postmenopausal women with normal or low bone mass. J Clin Endocrinol Metab 94:2284–2289
77. Downs RW Jr, Moffett AM, Ghosh A et al (2010) Effects of arzoxifene on bone, lipid markers, and safety parameters in postmenopausal women with low bone mass. Osteoporos Int 21:1215–1226
78. Kendler DL, Palacios S, Cox DA et al (2011) Arzoxifene versus raloxifene: effect on bone and safety parameters in postmenopausal women with osteoporosis. Osteoporos Int AU4
79. Falany JL, Pilloff DE, Leyh TS, Falany CN (2006) Sulfation of raloxifene and 4-hydroxytamoxifen by human cytosolic sulfotransferases. Drug Metab Dispos 34:361–368
80. Rosati RL, Da Silva JP, Cameron KO et al (1998) Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryltetrahydronaphthalene. J Med Chem 41:2928–2931
81. Ke HZ, Paralkar VM, Grasser WA et al (1998) Effects of CP-336,156, a new, nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. Endocrinology 139:2068–2076
82. Ke HZ, Qi H, Crawford DT et al (2000) Lasofoxifene (CP-336,156), a selective estrogen receptor modulator, prevents bone loss induced by aging and orchidectomy in the adult rat. Endocrinology 141:1338–1344
83. Tatee T, Carlson KE, Katzenellenbogen JA et al (1979) Antiestrogens and antiestrogen metabolites: preparation of tritium-labeled (+/−)-cis-3-[p-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phenoxyl]-1,2-propanediol (U-23469) and characterization and synthesis of a biologically important metabolite. J Med Chem 22:1509–1517
84. Legha SS, Slavik M, Carter SK (1976) Nafoxidine–an antiestrogen for the treatment of breast cancer. Cancer 38:1535–1541
85. Cohen LA, Pittman B, Wang CX et al (2001) LAS, a novel selective estrogen receptor modulator with chemopreventive and therapeutic activity in the N-nitroso-N-methylurea-induced rat mammary tumor model. Cancer Res 61:8683–8688
86. Robertson DW, Katzenellenbogen JA, Hayes JR, Katzenellenbogen BS (1982) Antiestrogen basicity–activity relationships: a comparison of the estrogen receptor binding and

Uncorrected Proof

Author's Proof

antiuterotrophic potencies of several analogues of (Z)-1,2-diphenyl-1-[4-[2-(dimethylamino) ethoxy]phenyl]-1-butene (tamoxifen, Nolvadex) having altered basicity. J Med Chem 25:167–171
87. Hellmann-Blumberg U, Taras TL, Wurz GT, DeGregorio MW et al (2000) Genotoxic effects of the novel mixed antiestrogen FC-1271a in comparison to tamoxifen and toremifene. Breast Cancer Res Treat 60:63–70
88. Qu Q, Zheng H, Dahllund J et al (2000) Selective estrogenic effects of a novel triphenylethylene compound, FC1271a, on bone, cholesterol level, and reproductive tissues in intact and ovariectomized rats. Endocrinology 141:809–820
89. DeGregorio MW, Wurz GT, Taras TL et al (2000) Pharmacokinetics of (deaminohydroxy) toremifene in humans: a new, selective estrogen-receptor modulator. Eur J Clin Pharmacol 56:469–475
90. Rutanen EM, Heikkinen J, Halonen K et al (2003) Effects of ospemifene, a novel SERM, on hormones, genital tract, climacteric symptoms, and quality of life in postmenopausal women: a double-blind, randomized trial. Menopause 10:433–439
91. Komi J, Lankinen KS, Harkonen P et al (2005) Effects of ospemifene and raloxifene on hormonal status, lipids, genital tract, and tolerability in postmenopausal women. Menopause 12:202–209
92. Namba R, Young LJ, Maglione JE et al (2005) Selective estrogen receptor modulators inhibit growth and progression of premalignant lesions in a mouse model of ductal carcinoma in situ. Breast Cancer Res 7:R881–R889
93. Wurz GT, Read KC, Marchisano-Karpman C et al (2005) Ospemifene inhibits the growth of dimethylbenzanthracene-induced mammary tumors in Sencar mice. J Steroid Biochem Mol Biol 97:230–240
94. Dehal SS, Kupfer D (1997) CYP2D6 catalyzes tamoxifen 4-hydroxylation in human liver. Cancer Res 57:3402–3406
95. Beverage JN, Sissung TM, Sion AM et al (2007) CYP2D6 polymorphisms and the impact on tamoxifen therapy. J Pharm Sci 96:2224–2231
96. Bradford LD (2002) CYP2D6 allele frequency in European Caucasians, Asians, Africans and their descendants. Pharmacogenomics 3:229–243
97. Raimundo S, Toscano C, Klein K et al (2004) A novel intronic mutation, 2988G>A, with high predictivity for impaired function of cytochrome P450 2D6 in white subjects. Clin Pharmacol Ther 76:128–138
98. Hanioka N, Kimura S, Meyer UA, Gonzalez FJ (1990) The human CYP2D locus associated with a common genetic defect in drug oxidation: a G1934—A base change in intron 3 of a mutant CYP2D6 allele results in an aberrant 3′ splice recognition site. Am J Hum Genet 47:994–1001
99. Bernard S, Neville KA, Nguyen AT, Flockhart DA (2006) Interethnic differences in genetic polymorphisms of CYP2D6 in the U.S. population: clinical implications. Oncologist 11:126–135
100. Lim YC, Desta Z, Flockhart DA, Skaar TC (2005) Endoxifen (4-hydroxy-N-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen. Cancer Chemother Pharmacol 55:471–478
101. Lim YC, Li L, Desta Z et al (2006) Endoxifen, a secondary metabolite of tamoxifen, and 4-OH-tamoxifen induce similar changes in global gene expression patterns in MCF-7 breast cancer cells. J Pharmacol Exp Ther 318:503–512
102. Stearns V, Johnson MD, Rae JM et al (2003) Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. J Natl Cancer Inst 95:1758–1764
103. Jin Y, Desta Z, Stearns V et al (2005) CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. J Natl Cancer Inst 97:30–39
104. Fisher B, Costantino J, Redmond C et al (1989) A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. N Engl J Med 320:479–484

Uncorrected Proof

Author's Proof

105. Fisher B, Dignam J, Wolmark N et al (1999) Tamoxifen in treatment of intraductal breast cancer: National Surgical Adjuvant Breast and Bowel Project B-24 randomised controlled trial. Lancet 353:1993–2000
106. Fisher B, Costantino JP, Wickerham DL et al (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90:1371–1388
107. Loprinzi CL, Kugler JW, Sloan JA et al (2000) Venlafaxine in management of hot flashes in survivors of breast cancer: a randomised controlled trial. Lancet 356:2059–2063
108. Loprinzi CL, Sloan JA, Perez EA et al (2002) Phase III evaluation of fluoxetine for treatment of hot flashes. J Clin Oncol 20:1578–1583
109. Stearns V, Beebe KL, Iyengar M, Dube E (2003) Paroxetine controlled release in the treatment of menopausal hot flashes: a randomized controlled trial. JAMA 289:2827–2834
110. Crewe HK, Ellis SW, Lennard MS, Tucker GT (1997) Variable contribution of cytochromes P450 2D6, 2C9 and 3A4 to the 4-hydroxylation of tamoxifen by human liver microsomes. Biochem Pharmacol 53:171–178
111. Coller JK, Krebsfaenger N, Klein K et al (2002) The influence of CYP2B6, CYP2C9 and CYP2D6 genotypes on the formation of the potent antioestrogen Z-4-hydroxy-tamoxifen in human liver. Br J Clin Pharmacol 54:157–167
112. Lessard E, Yessine MA, Hamelin BA et al (2001) Diphenhydramine alters the disposition of venlafaxine through inhibition of CYP2D6 activity in humans. J Clin Psychopharmacol 21:175–184
113. Albers LJ, Reist C, Vu RL et al (2000) Effect of venlafaxine on imipramine metabolism. Psychiatry Res 96:235–243
114. Yoon YR, Cha IJ, Shon JH et al (2000) Relationship of paroxetine disposition to metoprolol metabolic ratio and CYP2D6*10 genotype of Korean subjects. Clin Pharmacol Ther 67:567–576
115. Jeppesen U, Gram LF, Vistisen K et al (1996) Dose-dependent inhibition of CYP1A2, CYP2C19 and CYP2D6 by citalopram, fluoxetine, fluvoxamine and paroxetine. Eur J Clin Pharmacol 51:73–78
116. Andersson T, Flockhart DA, Goldstein DB et al (2005) Drug-metabolizing enzymes: evidence for clinical utility of pharmacogenomic tests. Clin Pharmacol Ther 78:559–581
117. Borges S, Desta Z, Li L et al (2006) Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. Clin Pharmacol Ther 80:61–74
118. Dieudonne AS, Lambrechts D, Claes B et al (2009) Prevalent breast cancer patients with a homozygous mutant status for CYP2D6*4: response and biomarkers in tamoxifen users. Breast Cancer Res Treat 118:531–538
119. Schroth W, Goetz MP, Hamann U et al (2009) Association between CYP2D6 polymorphisms and outcomes among women with early stage breast cancer treated with tamoxifen. JAMA 302:1429–1436
120. Kiyotani K, Mushiroda T, Imamura CK et al (2010) Significant effect of polymorphisms in CYP2D6 and ABCC2 on clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients. J Clin Oncol 28:1287–1293
121. Lammers LA, Mathijssen RH, van Gelder T et al (2010) The impact of CYP2D6-predicted phenotype on tamoxifen treatment outcome in patients with metastatic breast cancer. Br J Cancer 103:765–771
122. Madlensky L, Natarajan L, Tchu S et al (2011) Tamoxifen metabolite concentrations, CYP2D6 genotype, and breast cancer outcomes. Clin Pharmacol Ther 89:718–725
123. Lash TL, Cronin-Fenton D, Ahern TP et al (2011) CYP2D6 inhibition and breast cancer recurrence in a population-based study in Denmark. J Natl Cancer Inst 103:489–500
124. Regan MM, Leyland-Jones B, Bouzyk M et al (2012) CYP2D6 genotype and tamoxifen response in postmenopausal women with endocrine-responsive breast cancer: the breast international group 1–98 trial. J Natl Cancer Inst 104:441–451

Uncorrected Proof

Author's Proof

125. Rae JM, Drury S, Hayes DF et al (2012) CYP2D6 and UGT2B7 genotype and risk of recurrence in tamoxifen-treated breast cancer patients. J Natl Cancer Inst 104:452–460
126. Schroth W, Antoniadou L, Fritz P et al (2007) Breast cancer treatment outcome with adjuvant tamoxifen relative to patient CYP2D6 and CYP2C19 genotypes. J Clin Oncol 25:5187–5193
127. Lim JS, Chen XA, Singh O et al (2011) Impact of CYP2D6, CYP3A5, CYP2C9 and CYP2C19 polymorphisms on tamoxifen pharmacokinetics in Asian breast cancer patients. Br J Clin Pharmacol 71:737–750
128. Murdter TE, Schroth W, Bacchus-Gerybadze L et al (2011) Activity levels of tamoxifen metabolites at the estrogen receptor and the impact of genetic polymorphisms of phase I and II enzymes on their concentration levels in plasma. Clin Pharmacol Ther 89:708–717
129. Jordan VC, Fritz NF, Tormey DC (1987) Endocrine effects of adjuvant chemotherapy and long-term tamoxifen administration on node-positive patients with breast cancer. Cancer Res 47:624–630
130. Ravdin PM, Fritz NF, Tormey DC, Jordan VC (1988) Endocrine status of premenopausal node-positive breast cancer patients following adjuvant chemotherapy and long-term tamoxifen. Cancer Res 48:1026–1029
131. Irvin WJ Jr, Walko CM, Weck KE et al (2011) Genotype-guided tamoxifen dosing increases active metabolite exposure in women with reduced CYP2D6 metabolism: a multicenter study. J Clin Oncol 29:3232–3239
132. Barginear MF, Jaremko M, Peter I et al (2011) Increasing tamoxifen dose in breast cancer patients based on CYP2D6 genotypes and endoxifen levels: effect on active metabolite isomers and the antiestrogenic activity score. Clin Pharmacol Ther 90:605–611
133. Kiyotani K, Mushiroda T, Imamura CK et al (2012) Dose-adjustment study of tamoxifen based on CYP2D6 genotypes in Japanese breast cancer patients. Breast Cancer Res Treat 131:137–145
134. Jordan VC (1976) Antiestrogenic and antitumor properties of tamoxifen in laboratory animals. Cancer Treat Rep 60:1409–1419
135. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
136. Rouanet P, Linares-Cruz G, Dravet F et al (2005) Neoadjuvant percutaneous 4-hydroxytamoxifen decreases breast tumoral cell proliferation: a prospective controlled randomized study comparing three doses of 4-hydroxytamoxifen gel to oral tamoxifen. J Clin Oncol 23:2980–2987
137. Jordan VC, Fenuik L, Allen KE et al (1981) Structural derivatives of tamoxifen and oestradiol 3-methyl ether as potential alkylating antioestrogens. Eur J Cancer 17:193–200
138. Wakeling AE, Dukes M, Bowler J (1991) A potent specific pure antiestrogen with clinical potential. Cancer Res 51:3867–3873

Uncorrected Proof

Author's Proof

Author Queries

Chapter No.: 3

Query Refs.	Details Required	Author's response
AU1	Please check the usage of "the 4 position" and all other occurrences of "position" prefixed with a number throughout the book for correctness. There is also an occurrence of "a long side chain at position 7" in this chapter.	
AU2	Please provide closing parenthesis in the sentence starting "The one exception that was of interest. . .".	
AU3	Figure numbers have been renumbered from 3.5 to 3.6 in order to maintain the sequence order. Please check if this okay.	
AU4	Please provide volume number and page range for Ref. [78] if possible.	

Uncorrected Proof

Author's Proof

Metadata of the chapter that will be visualized online

Chapter Title	Adjuvant Therapy: The Breakthrough	
Copyright Year	2013	
Copyright Holder	Springer Basel	
Corresponding Author	Family Name	**Maximov**
	Particle	
	Given Name	**Philipp Y.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**McDaniel**
	Particle	
	Given Name	**Russell E.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**Jordan**
	Particle	
	Given Name	**V. Craig**
	Suffix	
	Division	Lombardi Comprehensive Cancer Center
	Organization	Georgetown University Medical Center
	Address	Washington, DC, USA
Abstract	The finding that long-term tamoxifen therapy of rats previously treated with a chemical carcinogen 7,12-dimethylbenzanthracene (DMBA) has a suppression of mammary tumorigenesis as long as treatment was continued to create a new strategy to save lives. These pivotal laboratory studies changed clinical practice. The initiation of numerous international randomized clinical trials of extended adjuvant tamoxifen therapy for patients with ER-positive tumors demonstrated longer was better to save lives. Recurrences were controlled by tamoxifen and mortality decreases by at least 30 %. Current indications are that 10 years of adjuvant tamoxifen is superior to 5 years of adjuvant tamoxifen.	

Author's Proof

Chapter 4
Adjuvant Therapy: The Breakthrough

Abstract The finding that long-term tamoxifen therapy of rats previously treated with a chemical carcinogen 7,12-dimethylbenzanthracene (DMBA) has a suppression of mammary tumorigenesis as long as treatment was continued to create a new strategy to save lives. These pivotal laboratory studies changed clinical practice. The initiation of numerous international randomized clinical trials of extended adjuvant tamoxifen therapy for patients with ER-positive tumors demonstrated longer was better to save lives. Recurrences were controlled by tamoxifen and mortality decreases by at least 30 %. Current indications are that 10 years of adjuvant tamoxifen is superior to 5 years of adjuvant tamoxifen.

Introduction

The initial success of adjuvant monotherapy with L-phenylalanine mustard [1] or combination chemotherapy [2] to delay the recurrence of node-positive breast cancer encouraged the investigation of other, perhaps less toxic, therapies. Most of the beneficial effects of adjuvant chemotherapy were noted in premenopausal women. In retrospect, this result was almost certainly a "chemical oophorectomy" produced by the cancer treatment. During the 1970s and 1980s, numerous reports [3, 4] described the changes in women's endocrinology as ovarian function is destroyed. Indeed, in the premenopausal women with breast cancer, combination cytotoxic chemotherapy can be considered to be endocrine therapy [5]. The low reported incidence of side effects noted with tamoxifen [6, 7] with modest efficacy naturally caused clinicians to consider adjuvant antiestrogen therapy. But the question to be addressed was "How long is long enough for adjuvant tamoxifen therapy?"

During the 1970s, at a time that tamoxifen was available in the United Kingdom for the treatment of metastatic breast cancer in postmenopausal women, and only being evaluated for that indication in the United States until approval by the FDA in

Uncorrected Proof

P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
DOI 10.1007/978-3-0348-0664-0_4, © Springer Basel 2013

Author's Proof

December 1977. The laboratory studies in the 1970s would encourage the testing of long-term adjuvant treatment, but the change in conservative clinical philosophy about using a "palliative" treatment of low efficacy would take a decade [8].

Laboratory studies using the DMBA-induced rat mammary carcinoma model were first used to explore whether tamoxifen would be an effective adjuvant therapy and whether the drug produces a tumoristatic or tumoricidal effect in vivo. Studies with estrogen receptor (ER) in positive MCF-7 breast cancer cells in vitro had previously indicated that tamoxifen could be a tumoricidal drug [9], but the results from the DMBA studies in vivo (first reported at a breast cancer symposium at King's College, Cambridge, England, in September 1977) (Fig. 4.1) demonstrated that a short course of tamoxifen therapy (1 month) given 1 month after the carcinogenic insult only delayed the appearance of mammary tumors; continuous therapy (for 6 months) resulted in 90 % of the animals remaining tumor free (Fig. 4.2) [12, 13]. Indeed if tamoxifen therapy is stopped, tumors appear [14]. Thus, tamoxifen was shown to have a tumoristatic component to its mode of action, and the laboratory results indicated that long-term (up to 5 years) or indefinite therapy might be the best clinical strategy for adjuvant treatment. Subsequent laboratory studies using DMBA- or N-nitrosomethylurea (NMU)-induced rat mammary tumors [15–17] or human breast cancer cell lines inoculated into athymic mice [18–20] have all supported the initial observation. However, most attention has naturally focused on the clinical evaluation of adjuvant tamoxifen therapy.

Adjuvant Therapy with Tamoxifen

Several trials of tamoxifen monotherapy as an adjuvant to mastectomy were initiated toward the end of the 1970s. The majority of clinical trial organizations selected a conservative course of 1 year of adjuvant tamoxifen [21–27]. This decision was, however, based on a number of reasonable concerns. Patients with advanced disease usually respond to tamoxifen for 1 year, and it was expected that ER-negative disease would be encouraged to grow prematurely during adjuvant therapy. If this growth was to occur, then the physician would have already used a valuable palliative drug and would have only combination chemotherapy to slow the relentless growth of recurrent disease. A related argument involved the changing strategy for the application of adjuvant combination chemotherapy. Recurrent treatment cycles (2 years) of cytotoxic chemotherapy were found to be of no long-term benefit for the patient. An aggressive course of short-term treatment (6 months) with the most active cytotoxic drugs could have the best chance to kill tumor cells before the premature development of drug resistance. The same argument provided an intuitive reluctance to use long-term tamoxifen therapy because it would lead to premature drug resistance: longer might not be better.

Finally, there were sincere concerns about the side effects of adjuvant therapy and the ethical issues of treating patients who might never have recurrent disease.

Uncorrected Proof

Author's Proof



Fig. 4.1 Breast cancer symposium at King's College, Cambridge, England, in September 1977. The concept of extended adjuvant tamoxifen treatment was first proposed at this meeting. Clinical studies of 1-year adjuvant tamoxifen were in place; regrettably, a decade later, this approach was shown to produce little survival benefit for patients. In the insets, (*top*) V. Craig Jordan, who presented the new concept, and (*bottom left*) Dr. Helen Stewart, who was a participant at the conference. She would initiate a pilot trial in 1978 and, led by Sir Patrick Forest, would later guide the full randomized Scottish trial of the 5-year adjuvant tamoxifen treatment versus control in the 1980s. Both clinical trials were later proven to produce survival advantages for patients. The concept of longer tamoxifen treatment producing more survival benefits for patients was eventually established indirectly by the Oxford Overview Analysis in 1992 [10] and directly by the Swedish group led by Dr. Lars Rutqvist [11]

Although this argument primarily focused on chemotherapy and node-negative patients, it is fair to say that few women in the mid-1970s had received extended therapy with tamoxifen, so that long-term side effects were largely unknown. The majority of tamoxifen-treated patients had received only about 2 years of treatment for advanced disease before drug resistance occurred. Potential side effects of thrombosis, osteoporosis, and so on were only of secondary importance. The use of tamoxifen in the disease-free patient would change that perspective.

Author's Proof

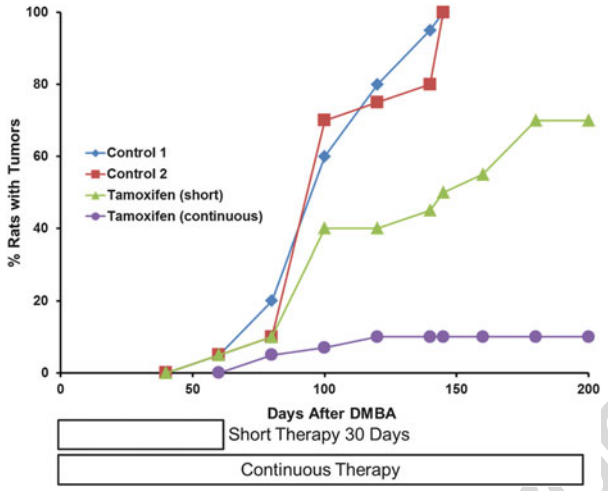


Fig. 4.2 The effectiveness of long-term tamoxifen treatment in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. The administration of 20-mg DMBA by gavage to 50-day-old female Sprague-Dawley rats in all animals developing mammary tumors 160 days later. The short-term (30 days) administration of different daily doses (12.5–800 μg) of tamoxifen between days 30 and 60 after DMBA results in a delay of tumor formation. However, not all animals are protected from the carcinogen. In contrast, the daily administration of a clinically relevant dose (50 μg daily = 0.25 mg/kg in rats or 20 mg daily to a 70 kg woman) of tamoxifen continuously, starting 30 days after DMBA, results in 90 % of animals remaining tumor free

In 1977, Dr. Douglass C. Tormey organized the first evaluation of long-term tamoxifen therapy in node-positive patients treated with combination chemotherapy plus tamoxifen [28, 29]. This pilot study was initiated to determine whether patients could tolerate 5 years of adjuvant tamoxifen therapy and whether metabolic tolerance would occur during long-term tamoxifen therapy. No unusual side effects of tamoxifen therapy were noted, and blood levels of tamoxifen and its metabolites N-desmethyltamoxifen and metabolite Y remained stable throughout the 5 years of treatment. Although this study was not a randomized trial, those patients who received long-term tamoxifen therapy continued to make excellent progress, and many patients took the drug for more than 14 years. We reported [30] that tamoxifen does not produce metabolic tolerance during 10 years of administration. Serum levels of tamoxifen and its metabolites are maintained.

The metabolic stability data and the DMBA-induced rat mammary carcinoma data [31] were used to support randomized Eastern Cooperative Oncology Group (ECOG) trials EST 4181 and 5181. An early analysis of EST 4181, which compares short-term tamoxifen with long-term tamoxifen (both with combination chemotherapy), demonstrated an increase in disease-free survival with long-term tamoxifen therapy [32]. In fact, the 5-year tamoxifen arm went through a second randomization either to stop the tamoxifen or to continue the antiestrogen indefinitely. The National Surgical Adjuvant Breast and Bowel Project (NSABP) clinical trial organization has conducted a registration study of 2 years of combination

Uncorrected Proof

Author's Proof

chemotherapy (L-PAM, 5-FU) plus tamoxifen with an additional year of tamoxifen alone [33] to build on the successes of the earlier trials that demonstrated the efficacy of tamoxifen in receptor-positive postmenopausal patients [34–36]. Overall, these investigators conclude that 3 years of tamoxifen confers a significant advantage for patients over 2 years of tamoxifen.

Although the 2-year adjuvant tamoxifen study that was conducted by the Nolvadex Adjuvant Trial Organization (NATO) was the first to demonstrate a survival advantage for women [37], subsequent clinical trials all evaluated a longer duration of tamoxifen therapy. A small, randomized clinical trial of 3 years of tamoxifen versus no treatment demonstrated a survival advantage for ER-positive patients who receive tamoxifen [38]. Similarly, the Scottish trial that evaluated 5 years of tamoxifen versus no treatment demonstrated a survival advantage for patients who take tamoxifen [39]. The Scottish trial is particularly interesting because it addresses the question of whether to administer tamoxifen early as an adjuvant or to save the drug until recurrence. This comparison was possible because most patients in the control arm received tamoxifen at recurrence. Early concerns that long-term adjuvant tamoxifen would result in premature drug resistance are unjustified, because the patients have a survival advantage on the adjuvant tamoxifen arm. Indeed, an analysis of non-cancer-related deaths in the Scottish trial demonstrated a significant decrease in fatal myocardial infarction for patients receiving adjuvant tamoxifen for 5 years [40]. A number of other studies also demonstrate a decrease in coronary heart disease with tamoxifen [41, 42] but there is no overall consensus on this point and the overview analysis of clinical trials does not support enhanced survival by reduced coronary heart disease in women taking tamoxifen.

Studies in Premenopausal Women

Tamoxifen was initially used in premenopausal women to treat menometrorrhagia [43] and to induce ovulation in infertile women [44, 45]. Subsequent evaluation of the endocrine effects of tamoxifen by Groom and Griffiths [46] revealed an increase in ovarian estrogen production.

Although concerns have been expressed about the potential for the reversal of tamoxifen's action in a high-estrogen environment, tamoxifen can effectively control the growth of advanced breast cancer in premenopausal patients [47–51], and small clinical trials have demonstrated that tamoxifen and oophorectomy [52, 53] have similar efficacy. Adjuvant monotherapy with tamoxifen has shown efficacy in node-positive premenopausal patients [54], but most experience has been derived from the study B_{14} of node-negative ER-positive premenopausal patients conducted by the NSABP [55]. Tamoxifen increases the disease-free survival and, perhaps most importantly, the antiestrogen is active in premenopausal women. The protocol used an initial treatment period of 5 years of adjuvant tamoxifen, continue tamoxifen for an additional 5-year period. No advantages were found for longer

Uncorrected Proof

Author's Proof

adjuvant therapy but there were more reported side effects [55]. However, this is a very small trial and the issue of extending tamoxifen therapy in the ATLAS (Adjuvant Tamoxifen: Longer Against Shorter) trial from 5 to 10 years is currently being addressed. The following questions have now been asked: (1) What are the advantages and disadvantages of 5 versus 10 years of adjuvant tamoxifen? (2) What are the improvements in mortality during and after 10 years of adjuvant tamoxifen? The initial results of the ATLAS trial with 12,984 women who have completed 5 years of adjuvant tamoxifen are randomized to stop or continue for a further 5 years. The report of 6,846 women with ER-positive disease is reported [56] and compared with the earlier analysis of no treatment versus 5 years of adjuvant tamoxifen [57]. These enormous data sets confirm that endometrial cancer is the only side effect of concern in postmenopausal women, but deaths from endometrial cancer do not offset the benefits of adjuvant tamoxifen with an enhanced 50 % decrease in mortality in the decade after 10 years of tamoxifen.

These data [56] will be compared with aTTom (adjuvant Tamoxifen Treatment—offer more?) in 2013 and regular follow-ups will occur with reporting over the next 2 years.

Tamoxifen is currently available to treat selected patients at each stage of breast cancer, but the overview analysis of randomized clinical trials has precisely described the worth of antiestrogen therapy. By way of an introduction, the overview analysis wonderfully demonstrated that "longer is better" for the effectiveness of different durations of adjuvant tamoxifen alone used to treat premenopausal women with ER-positive breast cancer. One year of adjuvant therapy is completely ineffective in improving either recurrence or survival (Fig. 4.3). Five years of tamoxifen produces 30 % decrease in mortality and a 50 % decrease in recurrence.

Overview of Clinical Trials

The first overview analysis of adjuvant therapy for breast cancer was conducted in 1984 by Richard Peto, Rory Collins, and Richard Gray leading the team for the Clinical Trials Unit of Oxford University. Analysis of clinical trials' results pertaining to tamoxifen demonstrated not only a decrease in recurrence-free survival for postmenopausal women receiving tamoxifen but also increase in overall survival. These data were refined, checked, and presented again at the National Cancer Institute Consensus Conference in Bethesda, Maryland, in 1985, where the panel concluded that adjuvant tamoxifen should be the standard of care for all postmenopausal women with ER-positive primary tumor and positive nodes [58].

As an aside, this was the year that ICI Pharmaceutical Division (Zeneca) was awarded the start of their "use patent" for tamoxifen as a treatment for breast cancer originally submitted and denied from 1965 onward (25 years!) (see Chap. 2). The patent would now extend into the twenty-first century creating the resources to advance chemoprevention and tamoxifen in the United States and the major clinical

Uncorrected Proof

Author's Proof

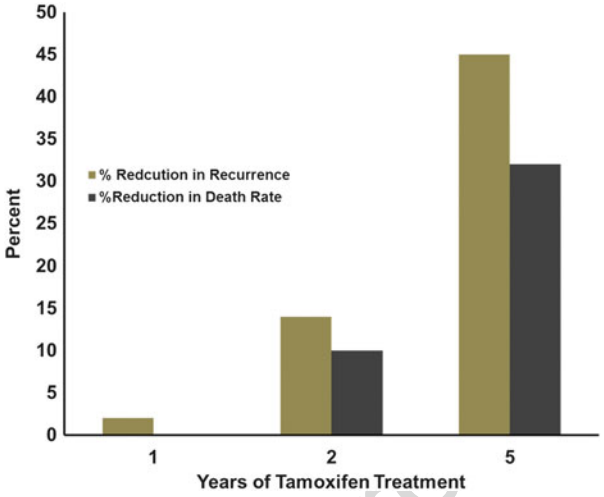


Fig. 4.3 The antitumorigenic action of tamoxifen in postmenopausal women. The results from the overview analysis have proven "the longer the better" concept for treatment with tamoxifen

trial of anastrozole, their aromatase inhibitor. Anastrozole versus tamoxifen and the combination (ATAC), then the single largest adjuvant endocrine clinical trial and became pivotal to lead progress with breast cancer therapy [59].

The overview of the clinical trials with tamoxifen was published in 1998 and 2005 [60, 61]. The 1998 and 2005 reports had three major therapeutic conclusions:

1. Tamoxifen was only effective as an adjuvant therapy in patients with an ER-positive breast tumor.
2. Longer was better than short adjuvant therapy in the treatment of ER-positive breast cancer. The power of this principle was best illustrated in premenopausal women receiving tamoxifen monotherapy: 1 year of adjuvant tamoxifen was completely ineffective at improving either recurrence rates or mortality but 5 years decreased recurrence by 50 % and mortality by 30 %. The scientific principles [8], published before any of the trials had started to recruit patients, were proven to have veracity.
3. The concern that the increased incidence of endometrial cancer during long-term adjuvant tamoxifen therapy might significantly reduce the value of tamoxifen as a cheap and effective life-saving medicine was calculated to be incorrect [60, 61].

We will now summarize the 2011 report of the relevance of breast cancer hormone receptors to the efficacy of adjuvant tamoxifen [57]. The meta-analysis of data was derived from 20 randomized clinical trials (n = 21,457) of adjuvant tamoxifen employing a 5-year treatment duration (80 % compliance). Again the continuing evaluation of adjuvant tamoxifen demonstrates the veracity of science in "the real world":

1. The ER positive disease (n = 10,645) tamoxifen reduced recurrence rates during the first 10 years but thereafter, there was no gain or loss out to 15 years.

Uncorrected Pro

Author's Proof

2. Marginal ER-positive disease (10–19 femtomoles/mg cytosol protein—from assays no longer used or quantitation employed) recurrence rates were substantial and significant.
3. Progesterone receptor was of no value to predict responsiveness to tamoxifen.
4. Breast cancer mortality was reduced by a third for the first 15 years.
5. All-cause mortality was substantially reduced despite small increases in thromboembolic and uterine cancer deaths (only women over 55 years of age) in women taking tamoxifen.

However, with the shift of the use of tamoxifen to aromatase inhibitors in postmenopausal patients, we felt it is appropriate to summarize the clinical trial to clarify the state of knowledge with the use of aromatase inhibitors versus tamoxifen.

Arrival of Aromatase Inhibitors as Adjuvant Therapy

The meta-analysis of the data from different trials (the Austrian Breast and Colorectal Cancer Study Group (ABCSG) XII trial, the Breast International Group (BIG) I-98/International Breast Cancer Study Group (IBCSG) 18–98 trial, and the ATAC trial) submitted to the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) was published in 2010 and described the comparison of the third-generation aromatase inhibitors (AI) against tamoxifen in breast cancer patients [62]. The patients were divided into two cohorts: cohort one comprised 9,856 patients that underwent treatment with AI immediately after surgery for 5 years and were compared to patients treated with tamoxifen; cohort two comprised 9,015 patients to assess the AI treatment with AI after 2–3 years of tamoxifen. The results of this analysis have shown that the administration of AI immediately after surgery for 5 years in the first cohort of patients has significantly reduced the recurrence of breast cancer by 23 % comparing to 5 years of tamoxifen. In the other cohort of patients, the efficacy of the switch to AI after 2–3 years of tamoxifen treatment was analyzed and it was shown that there was a 40 % reduction in risk of recurrence during the 3 following years after tamoxifen treatment. The authors of that study suggest that tamoxifen treatment after 3 years has sensitized the cancer cells to AI treatment; however, there is no experimental data supporting that. Also patients in both cohorts had follow-ups (5.8 years in cohort one and 3.9 years in cohort two) to assess the recurrence of the disease. The reduction of recurrence of breast cancer in both cohorts at 5 years after diagnosis was approximately 3 % and highly significant (2.9 %, SE = 0.7 % in cohort 1; and 3.1 %, SE = 0.6 % in cohort 2). The mortality rates in both cohorts were analogous at 5 years after diagnosis; however, there was a further decrease of mortality from breast cancers in the second cohort (AI after 2–3 years of tamoxifen). The authors concluded that AIs achieve "modest" improvements in breast cancer end points with significant reductions in recurrence in both cohorts of patients and specifically reduced mortality from breast cancer in

Uncorrected Proof

Author's Proof

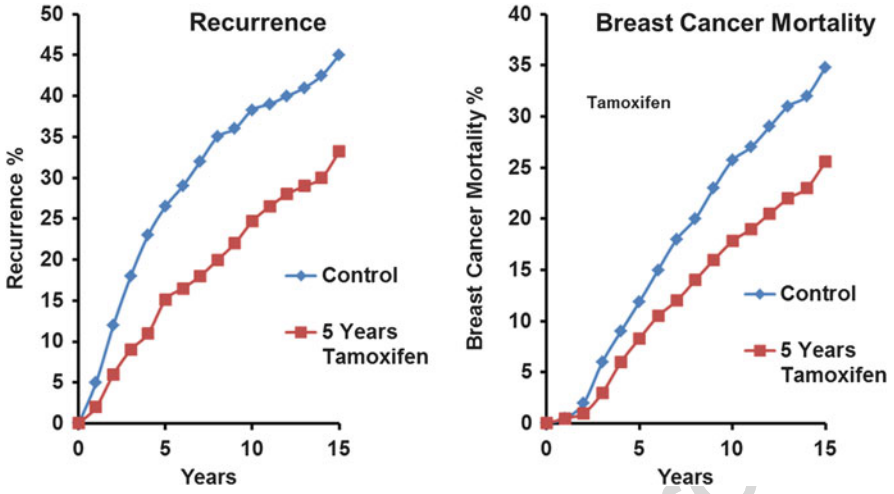


Fig. 4.4 The therapeutic action of tamoxifen after the treatment termination. The decrease of recurrence and mortality from breast cancer continues even 15 years after the treatment with tamoxifen stopped

the second cohort. However, it should be noted that AIs also have different side effects versus tamoxifen. AIs are associated with fewer endometrial cancers and thromboembolic events than tamoxifen but with increased incidents of arthralgia and bone fractures [63, 64].

Increasing Survivorship Following 5 Years of Adjuvant Tamoxifen

A significant mystery is why mortality continues to decrease following 5 years of adjuvant tamoxifen, i.e., after tamoxifen treatment has stopped [57, 61] (Fig. 4.4). Tamoxifen is a complete inhibitor of estrogen action at the tumor ER, so no drug would imply estrogen would bind to the unoccupied ER to cause tumor regrowth and increase mortality. But it does not!

However, a possible explanation occurred more by accident than design, through a study of acquired drug resistance to tamoxifen (Chap. 9). With the acceptance that long-term adjuvant tamoxifen was the appropriate strategy for the treatment of node-positive/node-negative breast cancer, in the late 1980s, it was imperative to develop a realistic model of acquired drug resistance to tamoxifen in the laboratory to determine mechanisms and diverse strategies for second-line therapy. The first transplantable model of acquired resistance was propagated in athymic mice. The ER-positive MCF-7 breast cancer cell line was used to develop the model [20]. AU5 Acquired resistance to tamoxifen developed within 2 years and once acquired either tamoxifen or physiologic estradiol utilizing the tumor ER to cause growth [65].

Uncorrected Proof

Author's Proof

However, the tumors could only be propagated in mice, and no successful transfer from tumor to tissue culture occurred. The model did not seem to replicate adjuvant therapy but rather metastatic breast cancer that fails tamoxifen treatments within 2 years. This seemed to be bad news but this became the good news as the unique tumor model could only be retained for study by routine propagation to tamoxifen-treated mice over years.

The finding that, following 5 years of retransplantation of tumors with acquired tamoxifen resistance into successive generations of tamoxifen-treated athymic mice, physiologic estrogen causes tumors to melt away was both mystifying and exciting. We will expand on this exciting new biology of estrogen-induced apoptosis in Chap. 9, but suffice to say it raised the possibility that acquired drug resistance to tamoxifen evolves and that the act of stopping tamoxifen after 5 years of adjuvant therapy causes the woman's own estrogen to seek out the appropriately reconfigured and sensitized breast cancer cells and triggers apoptosis. These data were first reported at the St. Gallen Breast Cancer Meeting with the hypothesis that the women's own estrogen caused the decrease in the patient mortality by killing appropriately sensitive microscopic foci of breast cancer cells [66].

In closing this chapter, it is important to stress that the hypothesis was not well received by the clinical community or the idea that physiologic estrogen administration might be of therapeutic significance. Despite the fact that no peer-reviewed funding was forthcoming, our research was sustained through philanthropy by the Lynn Sage Breast Cancer Foundation and the Robert H. Lurie Comprehensive Cancer Center at Northwestern University in Chicago, IL. Almost by chance, talented surgeons (Drs. Yao, Lee, England, and Bentrem) were looking for a project to exploit and this was it. They reproduced the Wolf data [66] over a 5-year period and it became clear that by year 5 of tamoxifen treatment, physiologic estrogen administration killed breast cancer cells with acquired resistance to tamoxifen. Estradiol killed the resistant cells but the remaining cells were again sensitive to antihormone therapy [67]. The process was cyclical (Fig. 4.5) and would eventually be tested in clinical trial and the molecular biology of estradiol-induced apoptosis clarified (Chap. 9). The concept was extended to the SERM raloxifene in an exceptionally long 10-year transplantation study of an MCF-7 study of acquired raloxifene resistance in athymic mice [68]. The original Wolf study and Balaburski study some 20 years apart are illustrated in Fig. 4.5.

Postscript. Perhaps the most important continuing support that ICI Pharmaceutical Division made to the development of tamoxifen (Nolvadex) was the hundreds of rats they chauffeured from Alderley Park nearby to Leeds University Medical School. Over the years (1974–1978), this strategy, instituted and paid for by Dr. Roy Cotton in the clinical department, was visionary. He was investing in a young enthusiastic pharmacologist who wanted to develop drugs to treat cancer. To a young faculty member in the Department of Pharmacology at Leeds University, armed with additional grants from the Yorkshire Cancer Research Campaign to purchase expensive ultracentrifuges (they were happy to invest in a BTA, Been to America), and ultimately an ICI/University of Leeds Joint Research Scheme

Uncorrected Proof

Author's Proof

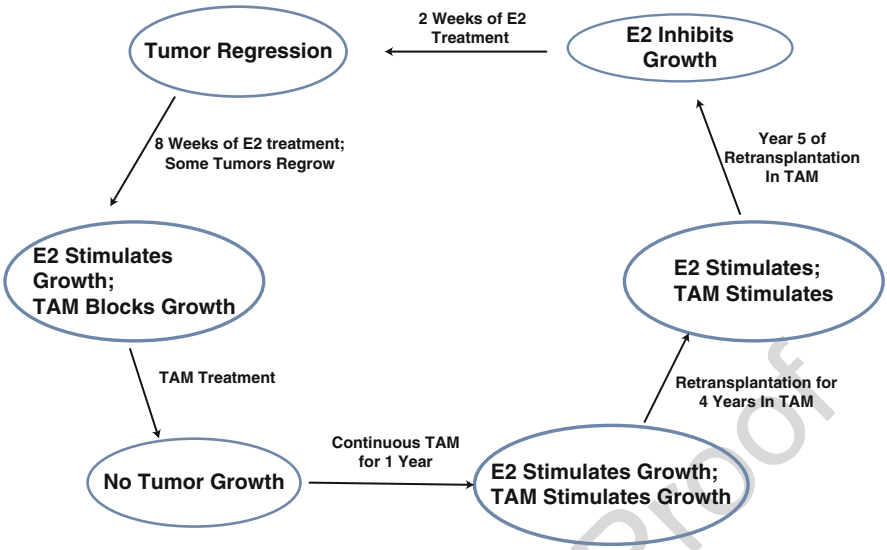


Fig. 4.5 The cyclical sensitivity and resistance of breast cancer cells to tamoxifen and estradiol. Estradiol is able to induce apoptosis in resistant cells; however, the remaining cells were again sensitive to tamoxifen treatment

co-headed by Walpole for ICI and I for Leeds to create 6,7-substituted alkylating estrogens, the unlimited animals were more valuable than gold. What did the investment yield? We did extensive studies on the mechanism of action of tamoxifen [69–75]; we were the first to discover the pharmacological properties of 4-hydroxytamoxifen [15, 17, 76–81], discovered the metabolic activation of tamoxifen, and most importantly created the strategy with animal models, to employ long-term adjuvant tamoxifen treatment for patients with ER-positive breast cancer [82–86].

We had two strategic goals with the studies of Karen Allen, an extremely talented technician who had trained in my group when she was an undergraduate in the Department of Pharmacology, and Clive Dix, an exceptional PhD student funded with an ICI Graduate Student Fellowship. Our first goal was to establish whether short-term high-dose tamoxifen administered to rats 30 days after the DMBA to induce mammary cancer for a short period of time (4 weeks which we considered to be equivalent to 1 year in a woman's life) would "cure the animals." It did not, but we realized that suppression of tumor development by tamoxifen was dose related, i.e., once the accumulated and slowly excreted tamoxifen was gone from the body, the tumors appeared. Clive demonstrated that continuous tamoxifen treatment was necessary to prevent tumorigenesis, almost completely, and was superior to oophorectomy [15]. Thus, long-term adjuvant therapy was going to be better to control the recurrence of ER-positive disease effectively after primary surgery.

Uncorrected Proof

Author's Proof

Our second goal was to determine whether 4-hydroxytamoxifen, a more potent antiestrogen than tamoxifen, was a more potent antitumor agent in the rat. It was not, although continuous therapy was effective at controlling tumor development [17]. We concluded that rapidly excreted hydroxylated antiestrogens were poor antitumor agents, a principle that was to recur with polyhydroxylated raloxifene [16] when used for the prevention of breast cancer [87] and proven over the next 30 years!

The opportunity to present our new concept for the adjuvant use of tamoxifen occurred in September 1977 at an ICI Pharmaceutical Division Breast Cancer Symposium at King's College, Cambridge, England. Michael Baum was the chair of my session and it was clear that plans were in place to increase the duration of adjuvant tamoxifen therapy from the standard 1 year to 2 years with the NATO trial (the acronym was based on the belief the Americans would read their subsequent papers and refer to them in their publications if they believed it was an American sponsored trial. The acronym actually stands for "Nolvadex Adjuvant Trial Organization") and the proposed 5 years for the Scottish trial. Each of the trialists considered their choice of trial design was arbitrary, but we already had the scientific basis in plan that would prove to be successful in their clinical trials.

The week following the King's College meeting I began a 3-month sabbatical at the University of Wisconsin Clinical Cancer Center, Madison, Wisconsin. There I proposed the "tamoxifen forever" clinical strategy as a forward thinking goal to accelerate tamoxifen's development and prevent disease recurrence. I should restate that tamoxifen at that time was not FDA approved in the United States even for the treatment of metastatic breast cancer. This would occur on 29 December 1977. Presentation of the strategy with compelling laboratory data to create potential survival advantages for patients with ER-positive breast cancer caught on with both the Eastern Cooperative Oncologic Group (ECOG) and the National Adjuvant Breast and Bowel Project (NSABP) as they advanced their adjuvant therapy trials from 2 to 5 years. This was a critical decision that saved hundreds of thousands of women's lives worldwide.

The good news for my career was that this 3-month sabbatical time in the Wisconsin Clinical Cancer Center in Madison resulted in a job offer because by this time I had lots of publications and Eliahu Caspi's lesson had been learned! (See Chap. 2.) After a year setting up the Ludwig Institute in Bern, Switzerland (1979–1980), and forging friendships that would last a career, I moved to Wisconsin to learn and recreate my Tamoxifen Team in America (Chap. 5).

Uncorrected Proof

References

1. Fisher B, Carbone P, Economou SG et al (1975) 1-Phenylalanine mustard (L-PAM) in the management of primary breast cancer. A report of early findings. N Engl J Med 292:117–122
2. Bonadonna G, Brusamolino E, Valagussa P et al (1976) Combination chemotherapy as an adjuvant treatment in operable breast cancer. N Engl J Med 294:405–410

Author's Proof

3. Jordan VC, Fritz NF, Tormey DC (1987) Endocrine effects of adjuvant chemotherapy and long-term tamoxifen administration on node-positive patients with breast cancer. Cancer Res 47:624–630
4. Rose DP, Davis TE (1980) Effects of adjuvant chemohormonal therapy on the ovarian and adrenal function of breast cancer patients. Cancer Res 40:4043–4047
5. Jordan VC (1998) Chemotherapy is antihormonal therapy–how much proof do oncologists need? Eur J Cancer 34:606–608
6. Cole MP, Jones CT, Todd ID (1971) A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer 25:270–275
7. Ward HW (1973) Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J 1:13–14
8. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44:30–38
9. Lippman ME, Bolan G (1975) Oestrogen-responsive human breast cancer in long term tissue culture. Nature 256:592–593
10. Early Breast Cancer Trialists' Collaborative Group (1992) Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Lancet 339:1–15
11. Swedish Breast Cancer Cooperative Group (1996) Randomized trial of two versus five years of adjuvant tamoxifen for postmenopausal early stage breast cancer. J Natl Cancer Inst 88:1543–1549
12. Jordan VC (1978) Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. Rev Endocr Relat Cancer:49–55 AU6
13. Jordan VC, Dix CJ, Allen KE (1979) The effectiveness of long-term treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In: Salmon SE, Jones SE (eds) AU7
14. Robinson SP, Mauel DA, Jordan VC (1989) Antitumor actions of toremifene in the 7,12-dimethylbenzanthracene (DMBA)-induced rat mammary tumor model. Eur J Cancer Clin Oncol 24:1817–1821
15. Jordan VC, Allen KE, Dix CJ (1980) Pharmacology of tamoxifen in laboratory animals. Cancer Treat Rep 64:745–759
16. Gottardis MM, Jordan VC (1987) Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 47:4020–4024
17. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
18. Osborne CK, Hobbs K, Clark GM (1985) Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res 45:584–590
19. Osborne CK, Coronado EB, Robinson JP (1987) Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. Eur J Cancer Clin Oncol 23:1189–1196
20. Gottardis MM, Robinson SP, Jordan VC (1988) Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumoristatic action of tamoxifen. J Steroid Biochem 30:311–314
21. Ribeiro G, Palmer MK (1983) Adjuvant tamoxifen for operable carcinoma of the breast: report of clinical trial by the Christie Hospital and Holt Radium Institute. Br Med J (Clin Res Ed) 286:827–830
22. Rose C, Thorpe SM, Andersen KW et al (1985) Beneficial effect of adjuvant tamoxifen therapy in primary breast cancer patients with high oestrogen receptor values. Lancet 1:16–19
23. Ribeiro G, Swindell R (1985) The Christie Hospital tamoxifen (Nolvadex) adjuvant trial for operable breast carcinoma–7-yr results. Eur J Cancer Clin Oncol 21:897–900
24. Cummings FJ, Gray R, Davis TE et al (1985) Adjuvant tamoxifen treatment of elderly women with stage II breast cancer. A double-blind comparison with placebo. Ann Intern Med 103:324–329

Uncorrected Proof

Author's Proof

25. Baum MaomotNATO (1985) Controlled trial of tamoxifen as single adjuvant agent in management of early breast cancer. Lancet 1:836–840
26. Pritchard KI, Meakin JW, Boyd NF, Ambus K, DeBoer G, Paterson AHG, Sutherland DJA, Wilkinson RH, Bassett AA, Evans WK, Beale FA, Clark RM, Keane TJ (1984) A randomized trial of adjuvant tamoxifen in postmenopausal women with axillary node positive breast cancer. In: Jones SE, Salmon SE (eds) Adjuvant therapy of breast cancer. Grune and Stratton, New York, pp 339–348
27. Wallgren A, Baral E, Castensen J, Friberg S, Glas U, Hjalmar JL, Kargas M, Nordenskjold B, Skoog L, There N-O, Wilking N (1984) Should adjuvant tamoxifen be given for several years in breast cancer. In: Jones SE, Salmon SE (eds) Adjuvant therapy of breast cancer. Grune and Stratton, New York, pp 331–338
28. Tormey DC, Jordan VC (1984) Long-term tamoxifen adjuvant therapy in node-positive breast cancer: a metabolic and pilot clinical study. Breast Cancer Res Treat 4:297–302
29. Tormey DC, Rasmussen P, Jordan VC (1987) Long-term adjuvant tamoxifen study: clinical update. Breast Cancer Res Treat 9:157–158
30. Langan-Fahey SM, Tormey DC, Jordan VC (1990) Tamoxifen metabolites in patients on long-term adjuvant therapy for breast cancer. Eur J Cancer 26:883–888
31. Jordan VC (1983) Laboratory studies to develop general principles for the adjuvant treatment of breast cancer with antiestrogens: problems and potential for future clinical applications. Breast Cancer Res Treat 3(Suppl):S73–S86
32. Falkson HC, Gray R, Wolberg WH et al (1990) Adjuvant trial of 12 cycles of CMFPT followed by observation or continuous tamoxifen versus four cycles of CMFPT in postmenopausal women with breast cancer: an Eastern Cooperative Oncology Group phase III study. J Clin Oncol 8:599–607
33. Fisher B, Brown A, Wolmark N et al (1987) Prolonging tamoxifen therapy for primary breast cancer. Findings from the National Surgical Adjuvant Breast and Bowel Project clinical trial. Ann Intern Med 106:649–654
34. Fisher B, Redmond C, Brown A et al (1981) Treatment of primary breast cancer with chemotherapy and tamoxifen. N Engl J Med 305:1–6
35. Fisher B, Redmond C, Brown A et al (1983) Influence of tumor estrogen and progesterone receptor levels on the response to tamoxifen and chemotherapy in primary breast cancer. J Clin Oncol 1:227–241
36. Fisher B, Redmond C, Brown A et al (1986) Adjuvant chemotherapy with and without tamoxifen in the treatment of primary breast cancer: 5-year results from the National Surgical Adjuvant Breast and Bowel Project Trial. J Clin Oncol 4:459–471
37. Baum M, Brinkley DM, Dossett JA et al (1983) Controlled trial of tamoxifen as adjuvant agent in management of Early Breast Cancer. Interim analysis at four years by the Nolvadex Adjuvant Trial Orginisation. Lancet 321:257–261
38. Delozier T, Julien JP, Juret P et al (1986) Adjuvant tamoxifen in postmenopausal breast cancer: preliminary results of a randomized trial. Breast Cancer Res Treat 7:105–109
39. Scottish Cancer Trials Office (MRC) E (1987) Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee. Lancet 2:171–175
40. McDonald CC, Stewart HJ (1991) Fatal myocardial infarction in the Scottish adjuvant tamoxifen trial. The Scottish Breast Cancer Committee. BMJ 303:435–437
41. Rutqvist LE, Mattsson A (1993) Cardiac and thromboembolic morbidity among postmenopausal women with early-stage breast cancer in a randomized trial of adjuvant tamoxifen. The Stockholm Breast Cancer Study Group. J Natl Cancer Inst 85:1398–1406
42. Hackshaw A, Roughton M, Forsyth S et al (2011) Long-term benefits of 5 years of tamoxifen: 10-year follow-up of a large randomized trial in women at least 50 years of age with early breast cancer. J Clin Oncol 29:1657–1663
43. el Sheikha Z, Klopper A, Beck JS (1972) Treatment of menometrorrhagia with an anti-oestrogen. Clin Endocrinol (Oxf) 1:275–282

Uncorrected Proof

Author's Proof

44. Klopper A, Hall M (1971) New synthetic agent for the induction of ovulation: preliminary trials in women. Br Med J 1:152–154
45. Williamson JG, Ellis JD (1973) The induction of ovulation by tamoxifen. J Obstet Gynaecol Br Commonw 80:844–847
46. Groom GV, Griffiths K (1976) Effect of the anti-oestrogen tamoxifen on plasma levels of luteinizing hormone, follicle-stimulating hormone, prolactin, oestradiol and progesterone in normal pre-menopausal women. J Endocrinol 70:421–428
47. Manni A, Trujillo JE, Marshall JS et al (1979) Antihormone treatment of stage IV breast cancer. Cancer 43:444–450
48. Pritchard KI, Thomson DB, Myers RE et al (1980) Tamoxifen therapy in premenopausal patients with metastatic breast cancer. Cancer Treat Rep 64:787–796
49. Kalman AM, Thompson T, Vogel CL (1982) Response to oophorectomy after tamoxifen failure in a premenopausal patient. Cancer Treat Rep 66:1867–1868
50. Planting AS, Alexieva-Figusch J, Blonk-vdWijst J, van Putten WL (1985) Tamoxifen therapy in premenopausal women with metastatic breast cancer. Cancer Treat Rep 69:363–368
51. Sawka CA, Pritchard KI, Paterson AH et al (1986) Role and mechanism of action of tamoxifen in premenopausal women with metastatic breast carcinoma. Cancer Res 46:3152–3156
52. Ingle JN, Krook JE, Green SJ et al (1986) Randomized trial of bilateral oophorectomy versus tamoxifen in premenopausal women with metastatic breast cancer. J Clin Oncol 4:178–185
53. Buchanan RB, Blamey RW, Durrant KR et al (1986) A randomized comparison of tamoxifen with surgical oophorectomy in premenopausal patients with advanced breast cancer. J Clin Oncol 4:1326–1330
54. Party CABTW (1988) Cyclophosphamide and tamoxifen as adjuvant therapies in the management of breast cancer. Br J Cancer 57:604–607
55. Fisher B, Costantino J, Redmond C et al (1989) A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. N Engl J Med 320:479–484
56. Davies C, Hongchao P, Godwin J et al (2012) Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomized trial. Lancet
57. Davies C, Godwin J, Gray R et al (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378:771–784
58. Consensus Conference (1985) Adjuvant chemotherapy for breast cancer. JAMA 254:3461–3463
59. Howell A, Cuzick J, Baum M et al (2005) Results of the ATAC (arimidex, tamoxifen, alone or in combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 365:60–62
60. EBCTCG (1998) Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 351:1451–1467
61. EBCTCG (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 365:1687–1717
62. Dowsett M, Cuzick J, Ingle J et al (2010) Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 28:509–518
63. Lin NU, Winer EP (2008) Advances in adjuvant endocrine therapy for postmenopausal women. J Clin Oncol 26:798–805
64. Lonning PE, Geisler J (2008) Indications and limitations of third-generation aromatase inhibitors. Expert Opin Investig Drugs 17:723–739
65. Gottardis MM, Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 48:5183–5187
66. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127:23–33

Uncorrected Proof

Author's Proof

67. Yao K, Lee ES, Bentrem DJ et al (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6:2028–2036
68. Balaburski GM, Dardes RC, Johnson M et al (2010) Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. Int J Oncol 37:387–398
69. Abbott AC, Clark ER, Jordan VC (1976) Inhibition of oestradiol binding to oestrogen receptor proteins by a methyl-substituted analogue of tamoxifen. J Endocrinol 69:445–446
70. Jordan VC (1976) Antiestrogenic and antitumor properties of tamoxifen in laboratory animals. Cancer Treat Rep 60:1409–1419
71. Jordan VC, Prestwich G (1977) Binding of [3H]tamoxifen in rat uterine cytosols: a comparison of swinging bucket and vertical tube rotor sucrose density gradient analysis. Mol Cell Endocrinol 8:179–188
72. Jordan VC, Dix CJ, Rowsby L, Prestwich G (1977) Studies on the mechanism of action of the nonsteroidal antioestrogen tamoxifen (I.C.I. 46,474) in the rat. Mol Cell Endocrinol 7:177–192
73. Jordan VC, Naylor KE (1979) The binding of [3H]-oestradiol-17 beta in the immature rat uterus during the sequential administration of non-steroidal anti-oestrogens. Br J Pharmacol 65:167–173
74. Jordan VC, Prestwich G (1978) Effect of non-steroidal anti-oestrogens on the concentration of rat uterine progesterone receptors. J Endocrinol 76:363–364
75. Jordan VC, Rowsby L, Dix CJ, Prestwich G (1978) Dose-related effects of non-steroidal antioestrogens and oestrogens on the measurement of cytoplasmic oestrogen receptors in the rat and mouse uterus. J Endocrinol 78:71–81
76. Jordan VC, Collins MM, Rowsby L, Prestwich G (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305–316
77. Jordan VC, Dix CJ, Naylor KE et al (1978) Nonsteroidal antiestrogens: their biological effects and potential mechanisms of action. J Toxicol Environ Health 4:363–390
78. Jordan VC, Dix CJ (1979) Effect of oestradiol benzoate, tamoxifen and monohydroxy-tamoxifen on immature rat uterine progesterone receptor synthesis and endometrial cell division. J Steroid Biochem 11:285–291
79. Dix CJ, Jordan VC (1980) Subcellular effects of monohydroxytamoxifen in the rat uterus: steroid receptors and mitosis. J Endocrinol 85:393–404
80. Allen KE, Clark ER, Jordan VC (1980) Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 71:83–91
81. Dix CJ, Jordan VC (1980) Modulation of rat uterine steroid hormone receptors by estrogen and antiestrogen. Endocrinology 107:2011–2020
82. Jordan VC, Dowse LJ (1976) Tamoxifen as an anti-tumour agent: effect on oestrogen binding. J Endocrinol 68:297–303
83. Jordan VC, Jaspan T (1976) Tamoxifen as an anti-tumour agent: oestrogen binding as a predictive test for tumour response. J Endocrinol 68:453–460
84. Jordan VC, Jaspan T (1976) Oestrogen binding as a predictive test for DMBA-induced tumour response to tamoxifen therapy. In: Hellman K, Conors TA (eds) Chemotherapy. Plenum, New York, pp 89–94 AU8
85. Jordan VC (1978) Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. Rev Endocr Relat Cancer:49–55 (October supplement)
86. Jordan VC, Dix CJ, Allen KE (1979) The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In: Salmon SE, Jones SE (eds) Adjuvant therapy of cancer II. Grune and Strattin, New York, pp 19–26
87. Vogel VG, Costantino JP, Wickerham DL et al (2010) Update of the National Surgical Adjuvant Breast and Bowel Project study of tamoxifen and raloxifene (STAR) P-2 trial: preventing breast cancer. Cancer Prev Res (Phila) 3:696–706

Uncorrected Proof

Author's Proof

Author Queries

Chapter No.: 4

Query Refs.	Details Required	Author's response
AU1	Please check sentence starting "During the 1970s..." for completeness.	
AU2	Please check sentence starting "The protocol used..." for completeness.	
AU3	Please check sentence starting "Anastrozole versus tamoxifen..." for completeness.	
AU4	Please check sentence starting "The ER positive disease...." for sense.	
AU5	Please check latter part of sentence starting "Acquired resistance to..." for completeness.	
AU6	Please provide volume number for Refs. [12, 13, 56, 85] if possible.	
AU7	Please provide book title, publisher name and publisher location for Ref. [13].	
AU8	Please check if inserted publisher name to Ref. [84] is okay.	

Uncorrected Proof

Author's Proof

Metadata of the chapter that will be visualized online

Chapter Title	The Wisconsin Story in the 1980s: Discovery of Target Site-Specific Estrogen Action	
Copyright Year	2013	
Copyright Holder	Springer Basel	
Corresponding Author	Family Name	**Maximov**
	Particle	
	Given Name	**Philipp Y.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**McDaniel**
	Particle	
	Given Name	**Russell E.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**Jordan**
	Particle	
	Given Name	**V. Craig**
	Suffix	
	Division	Lombardi Comprehensive Cancer Center
	Organization	Georgetown University Medical Center
	Address	Washington, DC, USA
Abstract	The idea that tamoxifen could potentially be employed to prevent breast cancer in populations of women with high risk naturally mandated an extensive laboratory and clinical investigation of potential toxicological concerns. It was reasoned that if estrogen was necessary to maintain bone density and protect women from coronary heart disease, then an “antiestrogen” might prevent breast cancer but increase the risks of osteoporosis and coronary heart disease. Laboratory results and translation to clinical trial proved the reverse was true, and the new drug group, selective estrogen receptor modulators (SERMs), was discovered. Tamoxifen (and raloxifene) paradoxically prevented bone loss in ovariectomized rats (estrogen-like) but prevented rat mammary carcinogenesis (antiestrogen-like). The same was true in patients with tamoxifen (and raloxifene) maintaining bone density but preventing breast cancer. Additionally, circulating cholesterol decreased (an estrogen-like effect) in patients. However, an estrogen-like effect of tamoxifen that became a concern was the discovery that in the laboratory, tamoxifen prevented breast cancer growth but enhanced the growth of endometrial cancer.	

Author's Proof

Chapter 5
The Wisconsin Story in the 1980s: Discovery of Target Site-Specific Estrogen Action

Abstract The idea that tamoxifen could potentially be employed to prevent breast cancer in populations of women with high risk naturally mandated an extensive laboratory and clinical investigation of potential toxicological concerns. It was reasoned that if estrogen was necessary to maintain bone density and protect women from coronary heart disease, then an "antiestrogen" might prevent breast cancer but increase the risks of osteoporosis and coronary heart disease. Laboratory results and translation to clinical trial proved the reverse was true, and the new drug group, selective estrogen receptor modulators (SERMs), was discovered. Tamoxifen (and raloxifene) paradoxically prevented bone loss in ovariectomized rats (estrogen-like) but prevented rat mammary carcinogenesis (antiestrogen-like). The same was true in patients with tamoxifen (and raloxifene) maintaining bone density but preventing breast cancer. Additionally, circulating cholesterol decreased (an estrogen-like effect) in patients. However, an estrogen-like effect of tamoxifen that became a concern was the discovery that in the laboratory, tamoxifen prevented breast cancer growth but enhanced the growth of endometrial cancer.

Introduction

In the early 1980s, Professor Trevor Powles, the head of the Breast Cancer Unit at the Royal Marsden hospital, took the bold step to initiate a pilot clinical trial of tamoxifen to treat healthy women with a high risk of breast cancer. The goals were to determine whether healthy women without disease would take tamoxifen for years, monitor side effects, and use the experience gained as a vanguard for a large placebo-controlled chemotherapeutics study of tamoxifen. The scientific rationale was based on two dominant facts: (1) In the laboratory, tamoxifen was known to prevent the initiation and promotion of mammary cancer by estrogen in the DMBA-induced rat mammary carcinoma model [1, 2]. (2) Tamoxifen, used as an adjuvant

Uncorrected Proof

P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
DOI 10.1007/978-3-0348-0664-0_5, © Springer Basel 2013

Author's Proof

therapy, was noted in a letter to the Lancet [3] to reduce the incidence of contralateral breast cancer.

In other words, tamoxifen inhibited rat mammary carcinogens in the standard laboratory model used in breast cancer research at the time, and tamoxifen actually inhibited the incidence of primary breast cancer.

At this time in the 1980s, tamoxifen was classified as a nonsteroidal antiestrogen [4] and clinical trials with long-term adjuvant therapy were reporting a good safety profile for the drug administered between 2 years and potentially indefinite therapy [5–7]. However, the idea of treating healthy women with an "antiestrogen" raised some important issues that had to be addressed. If estrogen was important to maintain bone density and, at the time, there was the conviction that estrogen protected women from coronary heart disease, then the administration of an "antiestrogen" might well prevent half a dozen breast cancers per year in a 1,000 high-risk women, but the antiestrogenic interaction would expose the majority of women to crushing osteoporosis and an increased risk of dying from coronary heart disease. The target site pharmacology needed to be investigated in the laboratory, and steps had to be taken to translate the findings to clinical practice.

Two approaches were addressed that were ultimately to change clinical perceptions about "nonsteroidal antiestrogens" and, more importantly, to change the application of these drugs in medicine. We will describe the developing set of laboratory studies that would result in a new understanding of the pharmacology of tamoxifen and raloxifene (then called keoxifene) and then describe the clinical studies that occurred simultaneously that opened the door to the descriptions of a new drug group—the selective estrogen receptive modulators or SERMs. This program was unique to the Wisconsin Comprehensive Cancer Center, so we will describe the associated information from others that confirmed or supported our research strategy during the 1980s.

Laboratory Studies on the Target Site-Specific Pharmacology of "Nonsteroidal Antiestrogens"

The early studies in the literature concerning ICI 46,474 (later tamoxifen) described its antifertility and antiestrogenic properties in the immature rat uterus and in ovariectomized rat Allen-Doisy tests [8, 9]. Paradoxically, tamoxifen was estrogenic in the mouse uterus [10–12]. Tamoxifen was also known to lower circulating cholesterol in the rat with no significant increase in circulating desmosterol [8]. In contrast, LY156758 (keoxifene to become raloxifene) and LY117018 were both antiestrogens in the rat and mouse uterus and blocked estrogen and tamoxifen induced increase in uterine weight [13–17]. There was initially no information about circulating cholesterol in animals, as all interest was then focused upon the use of keoxifene as a treatment for breast cancer, an indication for which it was eventually to fail, and work was discontinued at Eli Lilly in the late 1980s.

AU2

Uncorrected Proof

Author's Proof

There was interest in comparing and contrasting the actions of tamoxifen and keoxifene on the rodent uterus, rat bone density, and carcinogen-induced rat mammary cancers and human tumors (breast and endometrial) grown in athymic mice. The differential effects of tamoxifen in the athymic mouse uterus transplanted with a growing estrogen-stimulated ER-positive MCF-7 tumor was particularly interesting. Administration of estradiol caused an increase in uterine weight and the growth of the MCF-7 tumor. However, tamoxifen caused an increase in mouse uterine weight but did not cause MCF-7 tumor growth. In fact, tamoxifen blocked estrogen-stimulated growth. We analyzed the tamoxifen metabolites in both estrogen target organs and found they were comparable, so we concluded "that the drug can selectively stimulate or inhibit events in the target tissues of different species without metabolic intervention" [18]. It was realized, however, that the target site specificity had clinical relevance to the application of tamoxifen as a long-term adjuvant therapy and as a potential chemopreventive.

Dr. Satyaswaroop at Penn State Medical School in Hershey, Pennsylvania, had dedicated considerable efforts to establish human endometrial cancer that grew in athymic mice [19]. He also noted that tamoxifen would increase the growth of human endometrial cancers [20] but had not stated that these data could be translated to clinical practice. In a pioneering experiment that hereafter changed clinical practice, human endometrial cancer and an MCF-7 tumor were transplanted into athymic mice and treated with both physiologic estrogen and tamoxifen. The goal was to establish whether tamoxifen would stop the estrogen-stimulated growth of both human tumors in the same mouse. The results (Fig. 5.1) demonstrated that tamoxifen inhibited estrogen-stimulated tumor growth but enhanced the growth of the human endometrial tumor. It was concluded that "these findings suggest that the disparate pharmacology of TAM is a tissue-specific phenomenon" [21] and suggested that "Until the influence of TAM and other antiestrogens on endometrial cancers has been fully investigated, vigilance by physicians treating patients with these agents is needed to establish the clinical relevance (if any) of these observations." In other words, it was possible that tamoxifen could prevent the growth of breast cancer but enhance the growth of endometrial cancer. The clinical community was quick to replicate the same target tissue concept in patients treated with long-term adjuvant tamoxifen therapy [22] with tamoxifen decreasing contralateral breast cancer but increasing the incidence of endometrial cancer in postmenopausal women. It was clear that tamoxifen was enhancing the growth of some target tissues but blocking the growth of others, so tamoxifen may not be appropriate in postmenopausal women at high risk of breast cancer.

A new dimension was necessary. Chemoprevention was to be a reality with antiestrogens and that new dimension would be keoxifene (raloxifene). Raloxifene was compared with tamoxifen in rats to prevent mammary carcinogenesis [23] and endometrial cancer [24].

There was a concern about "nonsteroidal antiestrogens" inhibiting bone regeneration and causing osteoporosis during long-term adjuvant tamoxifen treatment or during the use of tamoxifen as a chemopreventive, so there was a focus on

Uncorrected Proof

Author's Proof

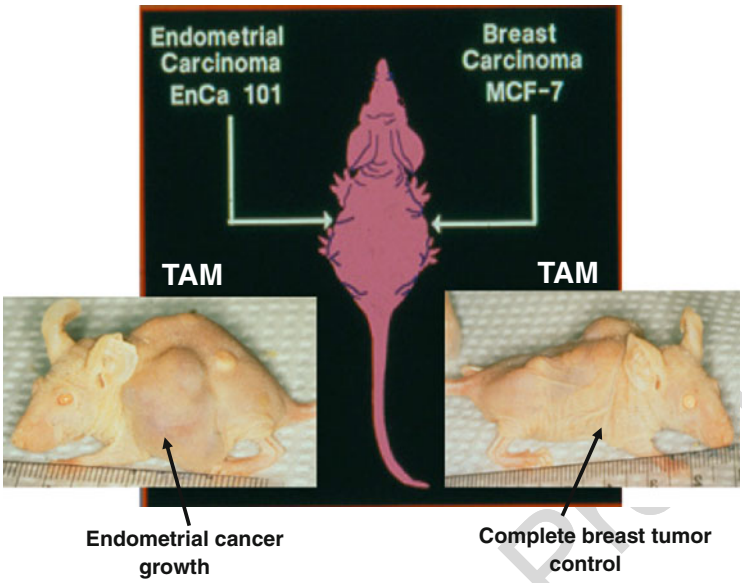


Fig. 5.1 The pioneering bitransplantation study by Gottardis with an ER-positive breast tumor (MCF-7) implanted in one axilla and an ER-positive endometrial tumor (EnCa 101) in the other axilla. Tamoxifen blocks estrogen-stimulated growth of the breast tumor, but tamoxifen encourages the growth of the endometrial tumor

measurements of rat bone following ovariectomy and antiestrogen treatment. Earlier, Beall and coworkers [25] had reported that clomiphene (a mixture of estrogenic and antiestrogenic geometric isomers) maintained bone density in ovariectomized retired breeder rats. However, since the administered drug was an impure mixture and not an antiestrogenic drug specifically, there was no proof that the estrogenic isomer had not caused an increase in bone density.

In contrast, the same model was used in the rat to determine the effect of the pure antiestrogenic isomer tamoxifen, and the results were compared with raloxifene, an antiestrogen with less estrogen-like actions than tamoxifen in the rat uterus and a fixed ring structures. Both antiestrogens maintained bone density, and in fact a combination of antiestrogens and estrogen was additive [26]. A study of tamoxifen and raloxifene to prevent rat mammary carcinogens demonstrated efficacy for both antiestrogens, but tamoxifen was shown to be superior and raloxifenes' effectiveness was found to be not long lasting [23]. More than 20 years later, these data were to be relevant in the STAR trial (Chap. 8) with tamoxifen having long-term and lasting actions to prevent breast tumor incidence, but raloxifene was not able to sustain the antitumor effect after treatment was stopped [27].

Finally, raloxifene was less effective at stimulating the growth of human endometrial cancer in laboratory models [24] and less effective at stimulating the growth of rodent uterine in vivo [15]. Taken together, these data generated in the same laboratory over a period of 2–3 years described the target site-specific actions of

Uncorrected Proof

Author's Proof

nonsteroidal antiestrogens to switch on and switch off estrogen target sites around the body. Those data lead to the proposal first stated at the First International Chemoprevention Conference in New York [28].

> . . .an extensive clinical investigation of available antioestrogens. Could analogs be developed to treat osteoporosis or even retard the development of atherosclerosis? Should the agent also retain anti-breast tumour actions then it might be expected to act as a chemosuppressive on all developing breast cancers.
>
> . . . . . .a bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century.

This vision became a reality and it led to the further clinical evaluation of tamoxifen in bone and then raloxifene as a selective estrogen/antiestrogen in target sites around a human's body. Tamoxifen was the drug of choice to study because it was approved clinically. The agent of choice by the clinical community to study chemoprevention in high-risk women was tamoxifen and raloxifene (aka keoxifene) that was unavailable for clinical testing. AU3

The Wisconsin Tamoxifen Study

A preliminary study of bone mineral density in women treated with adjuvant tamoxifen showed no detrimental effects at 2 years, i.e., the antiestrogenic actions of tamoxifen did not decrease bone density [29]. These data encouraged the establishment of a double-blind placebo-controlled trial of node-negative postmenopausal (no menses for 12 months) breast cancer patients with a diagnosis up to 10 years previously.

It is important to emphasize that in the late 1980s, adjuvant tamoxifen treatment was not the standard of care for the node-negative patient. Women were randomized to either tamoxifen or placebo for 2 years (Fig. 5.2) with evaluations for bone density, symptoms, and cardiovascular risk factors at baseline, 3, 6, 12, 18, and 24 months later.

The main results were reported in a series of publications in the early 1990s [30–32]. The changes in cardiovascular risk factors during tamoxifen treatment were encouraging for long-term safety of adjuvant tamoxifen and as a potential chemopreventive agent in high-risk women. Total cholesterol decreased by 12 % during the 2-year period and this remained statistically significant ($P < 0.001$). The main effect was driven by a specific decrease of 20 % in low-density lipoprotein (LDL) cholesterol ($P < 0.0001$) with stable high-density lipoprotein (HDL) cholesterol. Fibrinogen rapidly decreased by 20 % at 6 months ($P < 0.0003$) and a 7 % decrease in platelets with a significant decrease in antithrombin III was observed in tamoxifen-treated women.

The bone parameters were highly significant and established the idea that tamoxifen could maintain or build bone translated from the laboratory [26, 33–35] to clinical practice. The placebo group had a decrease in radius of

Uncorrected Proof

Author's Proof

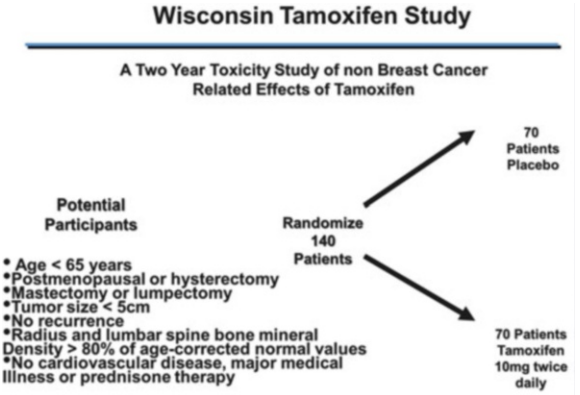


Fig. 5.2 The design of the Wisconsin Tamoxifen Study recruited 140 node-negative breast cancer patients to be randomized to either tamoxifen (20 mg/daily) or placebo. Bone mineral density was measured by dual-photon absorptiometry at regular intervals, and bloods were drawn to determine circulating lipids and clotting factors

1–1.292 % per year ($P < 0.0001$) and spine of −0.9967 % per year ($P < 0.0008$). Tamoxifen-treated women lost bone in the radius from baseline of −0.878 % per year ($P < 0.0002$) and lumbar spine a gain of 0.611 % per year ($P < 0.04$). A comparison of both lumbar spine linear rates was highly significant by 2 years ($P < 0.0001$). Symptoms were consistent with prior reports with only a modest rise in hot flashes compared with placebo. Gynecological symptoms increased modestly when vaginal discharge, vaginal dryness, bleeding, and genital pruritus were identified. Interestingly, there were fewer headaches.

In general, these data from the Wisconsin Tamoxifen Study were confirmed by other publications around this time [36–40].

Translational Research

The results with tamoxifen and raloxifene in the ovariectomized rat in the mid-1980s were subsequently confirmed by others, first for tamoxifen [33–35] and then eventually raloxifene [41–43]. The clinical research on tamoxifen was set to demonstrate that circulating cholesterol was reduced and postmenopausal bone density was maintained in contrast to placebo-treated controls. The links between tamoxifen and endometrial cancer (Chap. 6) and rat liver carcinogenesis were naturally of concern for the testing of tamoxifen as a chemopreventive (Chap. 6), but a new strategy was in place in the refereed literature when Leonard Lerner and I were awarded the Bruce F. Cain Award by the American Association for Cancer Research for laboratory research that resulted in a successful strategy to treat cancer [44]. Simply stated, the roadmap for pharmaceutical industry to follow was as follows:

> Is this the end of the possible applications for antioestrogens? Certainly not. We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered

Uncorrected Proof

Author's Proof

about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be post-menopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer.

Numerous companies followed the roadmap but not before keoxifene was renamed raloxifene and became the first SERM to treat and prevent osteoporosis and prevent breast cancer in postmenopausal women.

Postscript. During the 1980s, the nascent Breast Cancer Program led by Dr. Douglass Tormey, former head of the Breast Cancer Program at the National Cancer Institute, was building rapidly to create a multidisciplinary group able to conduct important translational research with the potential to "export" ideas to the Eastern Cooperative Oncology Group. Tormey had been the chief of the Breast Committee throughout the 1970s and was instrumental in recruiting me to Wisconsin. Lois Trench was also key, as the drug monitor for tamoxifen, and was the one to "get me started" at the Worcester Foundation for Experimental Biology (1972–1974). I was ICI America's first scientific consultant to use my laboratory results to facilitate clinical trials in America. I arrived in Madison, Wisconsin, in 1980 following a period establishing a breast cancer center for the Ludwig Institute for Cancer Research in Bern, Switzerland (1979–1980). My brief in Madison was to establish a major center for tamoxifen research and act as a link between basic science at the University of Wisconsin and clinical trials. Remember, tamoxifen was only approved to treat metastatic breast cancer by the FDA at the end of 1977, but we had plans! To achieve this, all my students had Dr. Jack Gorski on their Ph. D. thesis committee, and I recruited (with Jack's encouragement) numerous of his trainees to my laboratory at the Comprehensive Cancer Center. The late Mara Lieberman, Wade Welshons, and Mike Fritsch were all outstanding.

Another important scientist of note at the cancer center was Dr. David Rose who introduced me to a range of new antiestrogens (LY117018, trioxifene, LY156758) from Eli Lilly. David left Madison in the early 1980s, and it was decided that I should assume the responsibility for his staff, his laboratory space, and the ER clinical laboratory that served the hospitals in Southern Wisconsin. This was a frightening turn of events, so I called my mentor Bill McGuire in San Antonio to explain that I did not feel prepared for the task. He replied that I was looking at this incorrectly—"it's an opportunity" and so it was. In 1988, I was appointed as the director of the Breast Cancer Research and Treatment Program for the cancer center.

Wisconsin created the optimal environment to advance exciting translational science and create new careers. There we created an outstanding Tamoxifen Team in the Department of Human Oncology for 14 years; everyone was excellent, played their part, and contributed important skills and publications that changed medicine. It was a superb cancer center where young ambitions could be realized in a nurturing environment of an outstanding community focused on science. But from AU4

Uncorrected Proof

Author's Proof

the many in my Tamoxifen Team, several must be mentioned because they either changed medical practice, created new knowledge in tamoxifen pharmacology that would change the way we perceived mechanisms, or created new models that would be critical for future advances.

Anna Riegel (née Tate) demonstrated outstanding skills as an undergraduate student at Leeds University Department of Pharmacology where I was her tutor, and she received a first-class honors degree in pharmacology, a distinction in her master's degree in steroid endocrinology, and was awarded a Fulbright Hays Scholarship to study for a Ph.D. with me at the McArdle Laboratory for Cancer Research at the University of Wisconsin-Madison. She published a pivotal paper in cancer research with myself, Elwood Jensen, and Geoffrey Greene, on the shape of the estrogen and 4-hydroxytamoxifen ER complex conceived through as study of antibodies to the human ER [45]. This model complemented studies I was conducting with Jack Gorski [46] that presaged (rather accurately) the subsequent crystallization of the ligand-binding domain of the ER with estrogens and antiestrogens some 15 years later [47, 48]. Anna was also an important part of our team that contributed to the debate in the early 1980s about the localization of the ER within the cells of estrogen target tissues. The two-step hypothesis stated that estrogen diffuses into the cell, binds with high affinity to the cytoplasmic ER, and is translocated to the nucleus where it is transformed (activated) to initiate estrogen-specific gene transcription (protein synthesis and growth) [49]. However, McGuire's group in San Antonio and others had suggested that unoccupied ER was actually in the nucleus [50]. Two pieces of evidence swayed scientific opinion to create a new model of estrogen action: monoclonal antibodies demonstrated nuclear ER in breast cancer cells in an estrogen-free environment [51] and the Gorski group used cytochalasin B with GH3 rat pituitary cells to create nucleoplasts and cytoplasts to show ER only on the nucleoplasts [52]. It was strange to recall I had worked as a summer student with Steven Carter, at ICI Pharmaceuticals Division in the summer of 1967, who discovered the cell enucleation property of the natural product cytochalasin B [53].

In 1983, we reported to the Endocrine Society in San Antonio that tamoxifen analogs that could not be metabolically activated to 4-hydroxytamoxifen switched on growth of the immature rat uterus and induced progesterone synthesis but apparently without translocating the ER complex from the cytoplasm to the nucleus. The person responsible for these studies was the late Barbara Gosden whom Anna recommended for a job in my laboratory for 2 years. Anna and Barbara were students of the master's course in steroid endocrinology at Leeds in 1979 when Barbara completed her studies in vivo. She was concerned that she had the wrong answer—but she had made a discovery. This was exploited and confirmed using triphenylethylene estrogens that only weakly bound to the ER in the rat uterus. The uterus grew and progesterone receptors were made, but the ER "appeared" to remain in the cytosolic fraction (or cytoplasmic) and not in the nuclear fraction. We got the same result as the metabolically resistant tamoxifen analogs and proposed using this example of tamoxifen structural pharmacology to

Uncorrected Proof

Author's Proof

suggest that it was the technique of uterine cell disruption that caused the abnormal result inconsistent with the 2-step model—but nobody cared [54]!

Marco M. Gottardis was a superb experimentalist with animal models. I inherited him from David Rose in 1983, and he accepted my invitation to become a Ph.D. student on the Human Oncology Ph.D. Training Program in 1984. The publications from his Ph.D. changed medicine. Marco demonstrated the chemopreventive actions of tamoxifen and raloxifene in carcinogen-induced rat mammary carcinoma model [23]. He concluded that raloxifene in the long term would not be superior to tamoxifen. The update of the STAR trial (Chap. 8) was to prove his data correct some 20 years later [27]. In the mid-1980s, at a time when long-term adjuvant tamoxifen treatment was being tested, there was no knowledge about acquired drug resistance to tamoxifen. Marco established the first laboratory model of acquired tamoxifen resistance in athymic mice [55]. Tamoxifen resistance is unique in the transplantable model as it takes the form of tamoxifen-stimulated growth. He showed that estrogen withdrawal (a decade later this was equivalent to aromatase inhibitor treatment) and used the first pure antiestrogen [56] to demonstrate that these strategies were appropriate second-line therapies to be used in clinic.

Perhaps of greatest significance clinically was the superb experimental model of bitransplantation of a human endometrial and breast tumor in athymic mice. The tumors were both ER positive, but tamoxifen only blocked estrogen-stimulated growth of the breast tumor but enhanced the growth of the endometrial tumor. The clinical significance was clear. Women taking long-term tamoxifen needed to be checked for endometrial cancer growth. I presented a pivotal lecture in Italy during a celebration of the 900th anniversary of the University of Bologna, and this was noted by clinicians in the audience. Dr. Hardell from Sweden immediately reported about our laboratory finding in a letter to the Lancet and described several anecdotal cases he had observed of endometrial cancer in tamoxifen-treated patients. I replied [57] that we needed a placebo-controlled clinical evaluation to settle the matter once and for all. Fornander and colleagues [22] showed that 5 years of adjuvant tamoxifen would increase the detection of endometrial cancer by fivefold in postmenopausal women compared to placebo-treated women. The standard of care changed for women treated with tamoxifen with the introduction of routine gynecological examinations. This saved lives and is an excellent example of the potential for improvements in women's health with rapid clinical translation. The process from conceiving the laboratory study to publicizing and publishing the results in Cancer Research, followed by correspondence to the Lancet and the fast clinical publications in the Lancet, was 2–3 years.

Shun Yen Jiang came to my laboratory on a 4-year scholarship from Taiwan to learn molecular biology. However, she gave my Tamoxifen Team far more with a succession of firsts. She created two estrogen deprivation-resistant breast cancer cell lines from MCF-7 cells. These are MCF7:5C [58] that was to be so critical for our understanding of estrogen-induced apoptosis. These cells were waiting for Joan Lewis to "discover" in the deep freeze a decade later (Chap. 9). Shun Yen also created the MCF7:2A cells, the only breast cancer cells with a high molecular

Uncorrected Proof

Author's Proof

weight ER protein completely characterized by John Pink and found to be 6 and 7 exon repeats in the ligand-binding domain [59, 60]. John also documented the two different systems regulating ER synthesis in breast cancer [61] and with Cathy Murphy the first ER-positive to ER-negative transition in breast cancer cell lines during estrogen deprivation [62, 63]. Shun Yen Jiang subsequently reversed the process by creating the first stable transfectant of the ER gene into an ER-negative breast cancer cell line MDA-MB-231 [64]. This advance in cell biology was passed on to Bill Catherino who created a stable transfectant of a natural mutant ER asp351tyr (BC2) [65] from a tamoxifen-stimulated tumor developed by Doug Wolf [66] who discovered the mutant ER in one particular tumor cell line [66].

All my students start with multiple projects in the expectation that one would bear fruit. With Doug, all bore fruit but this was not clear at the time. But this is what good (and reliable) results in the laboratory really are. I gave Doug another couple of projects to address and to discover the mechanism of tamoxifen-stimulated growth. One hypothesis at the time in the early 1990s was that metabolic activation of tamoxifen to the 4-hydroxytamoxifen metabolite would also produce an estrogenic *cis* isomer to cause growth (Cathy Murphy demonstrated that this was not true as not all isomers were antiestrogenic [67]). Doug used a fixed ring tamoxifen analog that could not isomerize to prove that it was the actual drug not an isomer that caused growth [68]. All of this closely interconnected research passed from generations of students to the next as the optimal model for progress. Progress and knowledge to aid patients was achieved in the nested environment at UW-Madison. A big breakthrough for us at the UW-Madison was yet to come. In the early 1990s, growth factor pathways were the answer to cancer. I set Doug Wolf the problem of characterizing estrogen and tamoxifen-stimulated tumor growth through their growth factor pathways in Marco's model of tamoxifen resistance. However, when Doug addressed the question, all the physiologic estrogen-stimulated tumors derived from acquired tamoxifen-resistant tumors disappeared. He was embarrassed and very apologetic that he had repeated the experiment several times—tamoxifen-stimulated tumors grew just as Marco described 5 years earlier, but estrogen caused tumors to melt away. He believed he had failed to deliver the expected result from Marco's work, but he had made a discovery—estrogen-induced apoptosis [69]. This was confirmed at a new institution, the Robert Lurie Comprehensive Cancer Center at North Western University [70], and ultimately changed medicine through first providing us with data to be funded by the Department of Defense to study mechanisms that would be used to develop treatment for antihormone-resistant breast cancer [71] and the results of the WHI estrogen-alone study where there is a significant decrease in breast cancers and mortality [72].

But it did not end there with innovation of discovery by students. Mei Wei Jeng was a student from Taiwan, who had obtained a master's degree in Iowa. She made several important advances in cellular pharmacology. Using Shun Yen Jiang's stable transfectants of wild-type ER in MDA-MB-231 cells (S-10s, all my students named their own cell lines!), Mei Wei Jeng addressed what seemed the obvious hypothesis that the cause of estrogen action to stop growth of the S-10 cells was

Uncorrected Proof

Author's Proof

because it blocked TGF-α (a growth-stimulating hormone) production but increased TGF-β (a growth inhibitor) production. This was not true [73], it was the other way around, but new knowledge gave the Tamoxifen Team standard estrogen target gene TGF-α for all our subsequent work. Meey-Huey Jeng was also very keen to discover the role of the progestogens in the modulation of TGF-β. Instead, she discovered that 19-nortestosterone derivatives of the oral contraceptives were estrogens on MCF-7 cell growth [74], as was the antiprogentin RU486 at high doses [75].

So why did we ever do a bone study? Dr. Urban Lindgren, from the Karolinska Institute in Stockholm, was doing a sabbatical at the UW-Madison. He approached me to consider creating a rapidly developing osteoporosis model in ovariectomized rats. Nothing was really known about the effect of individual nonsteroidal antiestrogens on rat bone, so it seemed fairly simple as an experiment: antiestrogens would create bone loss in the ovariectomized rat. I obviously selected tamoxifen as there was really nothing known clinically about the action on bone, and it might aid the move to clinical testing of tamoxifen as a preventive for breast cancer. After Eli Lilly abandoned their anticancer program to create a rival to tamoxifen with keoxifene for breast cancer treatment, I was left with a large quantity of the nonsteroidal antiestrogen in the laboratory. I selected keoxifene as a competitor to tamoxifen. The reason was because keoxifene was less estrogenic in the uterus than tamoxifen [15]; this would probably make bone loss much worse. Lindgren taught Eric Phelps, an exchange student at UW-Madison, how to do the ash density study and then to our surprise another discovery! Tamoxifen was estrogen-like in bone as was keoxifene, and the combination with estradiol benzoate was additive [26]. These data were repeatedly rejected in "Bone" journals, so I wrote our results up for the refereed journal Breast Cancer Research and Treatment as I guessed correctly that the medical community would be interested in our findings. The results with tamoxifen were confirmed by others, and the Wisconsin Tamoxifen Study was propelled forward with other clinicians committed to the idea that tamoxifen would built bone [40]. Keoxifene became raloxifene, and funnily enough, the target site specificity of a combination of estrogen and a nonsteroidal antiestrogen being clinically valuable has now evolved into bazedoxifene and conjugated equine estrogen being used to control menopausal symptoms but with uterine and breast safety (Chap. 10)! A lot was initiated in Wisconsin that would change medical science with selective estrogen receptor modulators (SERMs).

All of this decade of discovery at the Wisconsin Comprehensive Cancer Center would provide a foundation for the subsequent interrogation of the modulation of the ER by selective ER modulator by Jennifer MacGregor-Schafer [76, 77] and Hong Liu [78, 79] at the Robert H. Laurie Cancer Center, Northwestern University, Chicago. The Wisconsin scientists would pass the baton of estrogen-induced apoptosis to the Northwestern Medical Scientists Kathy Yao, Dave Bentrem [70, 80], Clodio Osipo [81], Hong Liu [82], and Joan Lewis [83] (Chap. 9). It has always been a Tamoxifen Team effort from generation to generation.

Uncorrected Proof

Author's Proof

References

1. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
2. Allen KE, Clark ER, Jordan VC (1980) Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. Br J Pharmacol 71:83–91
3. Cuzick J, Baum M (1985) Tamoxifen and contralateral breast cancer. Lancet 2:282
4. Jordan VC (1984) Biochemical pharmacology of antiestrogen action. Pharmacol Rev 36:245–276
5. (1983) Controlled trial of tamoxifen as adjuvant agent in management of early breast cancer. Interim analysis at four years by Nolvadex Adjuvant Trial Organisation. Lancet 1:257–261
6. Tormey DC, Jordan VC (1984) Long-term tamoxifen adjuvant therapy in node-positive breast cancer: a metabolic and pilot clinical study. Breast Cancer Res Treat 4:297–302
7. Scottish Cancer Trials Office (MRC) E (1987) Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee. Lancet 2:171–175
8. Harper MJ, Walpole AL (1967) A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 13:101–119
9. Harper MJ, Walpole AL (1967) Mode of action of I.C.I. 46,474 in preventing implantation in rats. J Endocrinol 37:83–92
10. Harper MJ, Walpole AL (1966) Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. Nature 212:87
11. Terenius L (1970) Two modes of interaction between oestrogen and anti-oestrogen. Acta Endocrinol (Copenh) 64:47–58
12. Terenius L (1971) Structure-activity relationships of anti-oestrogens with regard to interaction with 17-beta-oestradiol in the mouse uterus and vagina. Acta Endocrinol (Copenh) 66:431–447
13. Black LJ, Goode RL (1980) Uterine bioassay of tamoxifen, trioxifene and a new estrogen antagonist (LY117018) in rats and mice. Life Sci 26:1453–1458
14. Black LJ, Goode RL (1981) Evidence for biological action of the antiestrogens LY117018 and tamoxifen by different mechanisms. Endocrinology 109:987–989
15. Black LJ, Jones CD, Falcone JF (1983) Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 32:1031–1036
16. Jordan VC, Gosden B (1983) Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 113:463–468
17. Jordan VC, Gosden B (1983) Differential antiestrogen action in the immature rat uterus: a comparison of hydroxylated antiestrogens with high affinity for the estrogen receptor. J Steroid Biochem 19:1249–1258
18. Jordan VC, Robinson SP (1987) Species-specific pharmacology of antiestrogens: role of metabolism. Fed Proc 46:1870–1874
19. Satyaswaroop PG, Zaino RJ, Mortel R (1983) Human endometrial adenocarcinoma transplanted into nude mice: growth regulation by estradiol. Science 219:58–60
20. Satyaswaroop PG, Zaino RJ, Mortel R (1984) Estrogen-like effects of tamoxifen on human endometrial carcinoma transplanted into nude mice. Cancer Res 44:4006–4010
21. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48:812–815
22. Fornander T, Rutqvist LE, Cedermark B et al (1989) Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1:117–120
23. Gottardis MM, Jordan VC (1987) Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 47:4020–4024

Uncorrected Proof

Author's Proof

24. Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC (1990) Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. Cancer Res 50:3189–3192
25. Beall PT, Misra LK, Young RL et al (1984) Clomiphene protects against osteoporosis in the mature ovariectomized rat. Calcif Tissue Int 36:123–125
26. Jordan VC, Phelps E, Lindgren JU (1987) Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10:31–35
27. Vogel VG, Costantino JP, Wickerham DL et al (2010) Update of the National Surgical Adjuvant Breast and Bowel Project study of tamoxifen and raloxifene (STAR) P-2 trial: preventing breast cancer. Cancer Prev Res (Phila) 3:696–706
28. Jordan VC (1988) Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 6:589–595
29. Love RR, Mazess RB, Tormey DC et al (1988) Bone mineral density in women with breast cancer treated with adjuvant tamoxifen for at least two years. Breast Cancer Res Treat 12:297–302
30. Love RR, Newcomb PA, Wiebe DA et al (1990) Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 82:1327–1332
31. Love RR, Wiebe DA, Newcomb PA et al (1991) Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann Intern Med 115:860–864
32. Love RR, Mazess RB, Barden HS et al (1992) Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 326:852–856
33. Turner RT, Wakley GK, Hannon KS, Bell NH (1987) Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. J Bone Miner Res 2:449–456
34. Turner RT, Wakley GK, Hannon KS, Bell NH (1988) Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. Endocrinology 122:1146–1150
35. Turner RT, Evans GL, Wakley GK (1993) Mechanism of action of estrogen on cancellous bone balance in tibiae of ovariectomized growing rats: inhibition of indices of formation and resorption. J Bone Miner Res 8:359–366
36. Jones AL, Powles TJ, Treleaven JG et al (1992) Haemostatic changes and thromboembolic risk during tamoxifen therapy in normal women. Br J Cancer 66:744–747
37. Kedar RP, Bourne TH, Powles TJ et al (1994) Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial. Lancet 343:1318–1321
38. Powles TJ, Jones AL, Ashley SE et al (1994) The Royal Marsden Hospital pilot tamoxifen chemoprevention trial. Breast Cancer Res Treat 31:73–82
39. Powles TJ, Hickish T, Kanis JA et al (1996) Effect of tamoxifen on bone mineral density measured by dual-energy x-ray absorptiometry in healthy premenopausal and postmenopausal women. J Clin Oncol 14:78–84
40. Turken S, Siris E, Seldin D et al (1989) Effects of tamoxifen on spinal bone density in women with breast cancer. J Natl Cancer Inst 81:1086–1088
41. Black LJ, Sato M, Rowley ER et al (1994) Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 93:63–69
42. Evans G, Bryant HU, Magee D et al (1994) The effects of raloxifene on tibia histomorphometry in ovariectomized rats. Endocrinology 134:2283–2288
43. Evans GL, Bryant HU, Magee DE, Turner RT (1996) Raloxifene inhibits bone turnover and prevents further cancellous bone loss in adult ovariectomized rats with established osteopenia. Endocrinology 137:4139–4144
44. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189

Uncorrected Proof

Author's Proof

45. Tate AC, Greene GL, DeSombre ER et al (1984) Differences between estrogen- and antiestrogen-estrogen receptor complexes from human breast tumors identified with an antibody raised against the estrogen receptor. Cancer Res 44:1012–1018
46. Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741–4745
47. Brzozowski AM, Pike AC, Dauter Z et al (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
48. Shiau AK, Barstad D, Loria PM et al (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937
49. Jensen EV, DeSombre ER (1973) Estrogen-receptor interaction. Science 182:126–134
50. Zava DT, McGuire WL (1977) Estrogen receptor. Unoccupied sites in nuclei of a breast tumor cell line. J Biol Chem 252:3703–3708
51. King WJ, Greene GL (1984) Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature 307:745–747
52. Welshons WV, Lieberman ME, Gorski J (1984) Nuclear localization of unoccupied oestrogen receptors. Nature 307:747–749
53. Carter SB (1967) Effects of cytochalasins on mammalian cells. Nature 213:261–264
54. Jordan VC, Tate AC, Lyman SD et al (1985) Rat uterine growth and induction of progesterone receptor without estrogen receptor translocation. Endocrinology 116:1845–1857
55. Gottardis MM, Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 48:5183–5187
56. Gottardis MM, Jiang SY, Jeng MH, Jordan VC (1989) Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res 49:4090–4093
57. Jordan VC (1989) Tamoxifen and endometrial cancer. Lancet 1:733–734
58. Jiang SY, Wolf DM, Yingling JM et al (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 90:77–86
59. Pink JJ, Jiang SY, Fritsch M, Jordan VC (1995) An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. Cancer Res 55:2583–2590
60. Pink JJ, Wu SQ, Wolf DM et al (1996) A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. Nucleic Acids Res 24:962–969
61. Pink JJ, Jordan VC (1996) Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. Cancer Res 56:2321–2330
62. Murphy CS, Meisner LF, Wu SQ, Jordan VC (1989) Short- and long-term estrogen deprivation of T47D human breast cancer cells in culture. Eur J Cancer Clin Oncol 25:1777–1788
63. Pink JJ, Bilimoria MM, Assikis J, Jordan VC (1996) Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. Br J Cancer 74:1227–1236
64. Jiang SY, Jordan VC (1992) Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. J Natl Cancer Inst 84:580–591
65. Catherino WH, Wolf DM, Jordan VC (1995) A naturally occurring estrogen receptor mutation results in increased estrogenicity of a tamoxifen analog. Mol Endocrinol 9:1053–1063
66. Wolf DM, Jordan VC (1994) The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31:129–138
67. Murphy CS, Langan-Fahey SM, McCague R, Jordan VC (1990) Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. Mol Pharmacol 38:737–743

Uncorrected Proof

Author's Proof

68. Wolf DM, Langan-Fahey SM, Parker CJ et al (1993) Investigation of the mechanism of tamoxifen-stimulated breast tumor growth with nonisomerizable analogues of tamoxifen and metabolites. J Natl Cancer Inst 85:806–812
69. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127:23–33
70. Yao K, Lee ES, Bentrem DJ et al (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6:2028–2036
71. Ellis MJ, Gao F, Dehdashti F et al (2009) Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 302:774–780
72. Anderson GL, Chlebowski RT, Aragaki AK et al (2012) Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. Lancet Oncol 13:476–486
73. Jeng MH, Jiang SY, Jordan VC (1994) Paradoxical regulation of estrogen-dependent growth factor gene expression in estrogen receptor (ER)-negative human breast cancer cells stably expressing ER. Cancer Lett 82:123–128
74. Jeng MH, Parker CJ, Jordan VC (1992) Estrogenic potential of progestins in oral contraceptives to stimulate human breast cancer cell proliferation. Cancer Res 52:6539–6546
75. Jeng MH, Langan-Fahey SM, Jordan VC (1993) Estrogenic actions of RU486 in hormone-responsive MCF-7 human breast cancer cells. Endocrinology 132:2622–2630
76. MacGregor Schafer J, Liu H, Bentrem DJ et al (2000) Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. Cancer Res 60:5097–5105
77. Schafer JI, Liu H, Tonetti DA, Jordan VC (1999) The interaction of raloxifene and the active metabolite of the antiestrogen EM-800 (SC 5705) with the human estrogen receptor. Cancer Res 59:4308–4313
78. Liu H, Lee ES, Deb Los Reyes A et al (2001) Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor alpha complex. Cancer Res 61:3632–3639
79. Liu H, Park WC, Bentrem DJ et al (2002) Structure-function relationships of the raloxifene-estrogen receptor-alpha complex for regulating transforming growth factor-alpha expression in breast cancer cells. J Biol Chem 277:9189–9198
80. Bentrem D, Fox JE, Pearce ST et al (2003) Distinct molecular conformations of the estrogen receptor alpha complex exploited by environmental estrogens. Cancer Res 63:7490–7496
81. Osipo C, Gajdos C, Liu H et al (2003) Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 95:1597–1608
82. Liu H, Lee ES, Gajdos C et al (2003) Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 95:1586–1597
83. Lewis JS, Meeke K, Osipo C et al (2005) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97:1746–1759

Uncorrected Proof

Author's Proof

Author Queries

Chapter No.: 5

Query Refs.	Details Required	Author's response
AU1	Please check if edit to sentence starting "The goals were...." is okay.	
AU2	Please check if edit to sentence starting "There was initially. . ." is okay.	
AU3	Please check if edit to sentence starting "The agent of choice...." is okay.	
AU4	Please check if edit to sentence starting "But from the. . ." is okay.	
AU5	Please check if "Meey-Huey Jeng" should be changed to "Mei Wei Jeng".	
AU6	Please check if "antiprogentin" should be changed to "antiprogestin".	
AU7	Duplicate entry of Ref. [1] has been deleted. Please check if okay.	
AU8	Please provide author name for Ref. [6].	

Uncorrected Proof

Author's Proof

Metadata of the chapter that will be visualized online

Chapter Title	Carcinogenesis and Tamoxifen	
Copyright Year	2013	
Copyright Holder	Springer Basel	
Corresponding Author	Family Name	**Maximov**
	Particle	
	Given Name	**Philipp Y.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**McDaniel**
	Particle	
	Given Name	**Russell E.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**Jordan**
	Particle	
	Given Name	**V. Craig**
	Suffix	
	Division	Lombardi Comprehensive Cancer Center
	Organization	Georgetown University Medical Center
	Address	Washington, DC, USA
Abstract	The laboratory study to show that tamoxifen was likely to increase the risk of endometrial cancer in women was initially rapidly confirmed by examination of an adjuvant clinical trials database. However, there was a concern raised that tamoxifen was producing high-grade endometrial cancer, but this claim turned out to be unsubstantiated. In general, the NSABP (P-1) study showed low-grade good prognosis disease with no deaths from endometrial cancer. In contrast, the laboratory finding in the early 1990s that select strains of rats were vulnerable to hepatocarcinoma following lifetime exposure to high daily doses of tamoxifen was of concern and caused labeling changes for tamoxifen. The concerns that there would be significant increases in fatal hepatocellular carcinomas were unfounded on examination of clinical trials data and subsequently ongoing monitoring of epidemiology databases.	

Author's Proof

Chapter 6
Carcinogenesis and Tamoxifen

Abstract The laboratory study to show that tamoxifen was likely to increase the risk of endometrial cancer in women was initially rapidly confirmed by examination of an adjuvant clinical trials database. However, there was a concern raised that tamoxifen was producing high-grade endometrial cancer, but this claim turned out to be unsubstantiated. In general, the NSABP (P-1) study showed low-grade good prognosis disease with no deaths from endometrial cancer. In contrast, the laboratory finding in the early 1990s that select strains of rats were vulnerable to hepatocarcinoma following lifetime exposure to high daily doses of tamoxifen was of concern and caused labeling changes for tamoxifen. The concerns that there would be significant increases in fatal hepatocellular carcinomas were unfounded on examination of clinical trials data and subsequently ongoing monitoring of epidemiology databases.

Introduction

The toxicological requirements to develop a drug as a breast cancer therapy contrast dramatically from the requirements necessary for approval for a drug to be used in well women. Metastatic breast cancer is fatal, so a small therapeutic index between toxicity and clinical benefit is appropriate. In contrast, drugs must be rigorously tested and demonstrate no toxicological issues in tests of mutagenesis and carcinogenesis in preclinical models prior to FDA approval for use in humans without disease.

Tamoxifen was launched as a treatment for metastatic breast cancer in postmenopausal women in the United Kingdom in 1973, and similar approvals occurred in the United States in December 1977. Toxicology was based on short-term tests in two species, and clinical data showed efficiency with a remarkable lack of side effects [1, 2]. However, the successful use of tamoxifen as an adjuvant therapy in node-positive breast cancer and the expanded use of tamoxifen as an adjuvant therapy in node-negative breast cancer, where the majority of patients are cured

Uncorrected Proof

P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
DOI 10.1007/978-3-0348-0664-0_6, © Springer Basel 2013

Author's Proof

by early surgery and radiation, enhanced enthusiasm to use tamoxifen to prevent breast cancer in high-risk populations of well women. The laboratory data supported the development of prospective clinical trials [3, 4], and it was already known that tamoxifen, used as an adjuvant therapy, reduced the incidence of contralateral breast cancer by 50 % [5]. The idea that tamoxifen would be used in well women therefore mandated a renewed evaluation of the toxicology of tamoxifen despite the fact that the drug had been successfully used ubiquitously in breast cancer therapy for 20 years.

A surprise was in store. Firstly was the finding that tamoxifen was target site specific and enhanced endometrial cancer growth but, at the same time, prevented estrogen-stimulated breast tumor growth [6] (Fig. 5.1, Chap. 5). Secondly was the findings of long-term carcinogenesis studies in the rat; tamoxifen was a liver carcinogen.

Tamoxifen and the Endometrial Carcinoma

The association between tamoxifen and endometrial carcinoma in humans is based upon clinical observations during the period 1988–1994. There is believed to be an increased incidence of endometrial carcinoma associated with breast cancer; therefore, physicians need to take extra precautions for the routine care of their patients. Tamoxifen is known to have estrogen-like properties in the uterus of some patients [7–9], so treatment would be expected to encourage the growth of preexisting disease, a principle which was first illustrated in the laboratory (Fig. 5.1, Chap. 5). When a breast tumor and endometrial carcinoma are co-transplanted into athymic mice, tamoxifen will block the estrogen-stimulated growth of the breast tumor while stimulating the endometrial carcinoma to grow [6, 10]. This is a demonstration of tamoxifen's target site specificity.

When evaluating reports of tamoxifen-induced endometrial carcinoma, it is important to appreciate that the incidence of occult endometrial tumors found in autopsy specimens is approximately five times the reported incidence in the general population [11]. The estrogen-like properties of tamoxifen can cause uterine hyperemia and proliferation, facilitating the growth of occult disease and leading to symptoms such as spotting and bleeding. Deaths from endometrial carcinoma occurred during tamoxifen therapy for breast cancer, initially raising the possibility that an aggressive form of the disease could be caused by tamoxifen. However, it should be remembered that only one-third of metastatic endometrial cancer is hormonally responsive, so tamoxifen would not be expected to control the majority of advanced endometrial cancer.

Uncorrected Proof

Author's Proof

Deaths from Endometrial Carcinoma

Magriples and coworkers [12] completed a computer search of the Yale New Haven Hospital tumor registry for the decade 1980–1990 and identified 53 patients with a history of breast cancer who subsequently developed endometrial cancer. Fifteen of these patients received tamoxifen and 38 did not. A total of 3,457 women were initially identified with breast cancer, but the proportion receiving tamoxifen was not stated. Interestingly enough, all of the tamoxifen-treated patients received 40-mg tamoxifen daily rather than the standard 20 mg daily. Five patients died of endometrial carcinoma during tamoxifen therapy, and the tumors from tamoxifen-treated patients were in general (67 %) poorly differentiated endometrial carcinomata (Table 6.1). The authors concluded "it appears that women receiving tamoxifen as treatment for breast cancer who subsequently develop uterine cancer are at risk for high-grade endometrial cancers that have a poor prognosis." Examination of the duration of tamoxifen therapy received by women before detection and subsequent death from endometrial carcinoma shows that three patients received tamoxifen for 12 months or less.

Deaths in women taking tamoxifen for relatively short time periods were also reported in the Stockholm study [13] (Table 6.2) and the NSABP study B14 [14] (Table 6.3). In the Stockholm study, 931 patients were randomized to receive either 2 or 5 years of tamoxifen 40 mg daily. Seventeen patients have been diagnosed with endometrial carcinoma; however, examination of patient records shows that each of the women received tamoxifen for less than 2 years, and the reported tumors were grades 1 and 2. One of the major conclusions of the study was that the probability of developing endometrial carcinoma was increased with duration of tamoxifen therapy [15]. However, examination of the 17 cases of endometrial carcinoma detected in the nearly 1,000 patients shows that 13 of the women who developed endometrial carcinoma received less than 2 years of tamoxifen treatment [13].

In the NSABP study [14], 1,419 patients were randomized to receive 20-mg tamoxifen daily for 5 years, and 1,220 patients were recruited and registered to receive at least 5 years of tamoxifen. Twenty-three women developed endometrial carcinoma with an average time of evaluation of 8 years and 5 years for randomized and registered patients, respectively. Six patients in the tamoxifen-treated arms died after a diagnosis of endometrial carcinoma (Table 6.3). Three of the six women took tamoxifen for less than 2 years, and one woman never took tamoxifen, although she was included in the analysis based on intention to treat. Overall, eight of the total of 23 women taking tamoxifen received the drug for less than 2 years.

Based on an analysis of current clinical trials data available in the 1990s, it was possible to address the question [12] of whether an aggressive high-grade disease develops during tamoxifen therapy.

Uncorrected Proof

Author's Proof

t1.1 **Table 6.1** Clinical and pathological features of tamoxifen-treated breast cancer patients who died of endometrial carcinoma in the Yale Haven Cancer Survey [12]

t1.2	Patient	Age	Months on tamoxifen	Endometrial histology	FIGO stage
t1.3	1	71	120	Adenosquamous FG3	NS
t1.4	2	85	96	Endometrial	IIIC
t1.5	3	60	12	Endometrioid FG3	NS
t1.6	4	71	12	MMT	IVB
t1.7	5	87	3	Papillary serous	NS

t1.8 *NS* not stated, *MMT* mixed Mullerian tumor

t2.1 **Table 6.2** Clinical and pathological features of tamoxifen-treated patients who died of endometrial carcinoma in the Stockholm trial [13]

t2.2	Age	Months on tamoxifen	Patient	Endometrial histology	FIGO stage
t2.3	68	24	1	NS grade I	I
t2.4	69	13	2	NS grade II	I
t2.5	70	11	3	NS grade II	IV

t3.1 **Table 6.3** Characteristics and pathological feature of tamoxifen-treated breast cancer patients who died of endometrial carcinoma (EC) in the NSABP B14 trial [14]

t3.2	Patient	Age	Months on tamoxifen	Off tamoxifen to diagnosis (months)	Histology	FIGO stage	Cause of death
t3.3	1	68	65	0	Papillary	IVG1	PE
t3.4	2	54	42	23	Carcinosarcoma	11BG3	EC
t3.5	3	58	22	73	Papillary	1BG3	EC
t3.6	4	68	5	0	Endometrioid	1A	CV disease
t3.7	5	63	9	0	Endometrioid	1BG2	EC
t3.8	6	66	0	0	Endometrioid	1BG1	EC

t3.9 *CV* cardiovascular, *PE* pulmonary embolus

106 Tamoxifen and the Stage of Endometrial Carcinoma

107 The discovery that high doses of tamoxifen will cause adduct formation in rat liver
108 DNA [16] occurred at the same time that Magriples and coworkers [12] reported
109 tamoxifen was associated with high-grade endometrial carcinoma. This naturally
110 lead to the possibility that tamoxifen may be causing progression of preexisting
111 disease. However, randomized clinical trial [14] and an epidemiology study [17]
112 did not support this proposition, although, in each case, the authors state that the
113 numbers are too low to draw any definite conclusions. Fisher and coworkers [14]
114 compared the stages of endometrial carcinoma and tumor grades found in their
115 study and in the Yale Tumor Registry Study and the Swedish Trail. An epidemiol-
116 ogy study from the Netherlands Cancer Institute is included for comparison [17]
117 (Table 6.4). It is difficult to make absolute comparisons of these data, but several
118 points can be made. The studies all found that the majority of tumors reported were
119 stage 1 endometrial carcinoma. The percentage of low-grade tumors was variable

Uncorrected Proof

Author's Proof

with 78 %, 33 %, 53 %, and 52 % for the NSABP, Yale, Swedish, and Netherlands studies, respectively. Additionally, for comparison purposes, a Gynecologic Oncology Group Study [18] of 222 patients found the distribution of cases to be 82 % low-grade cases (FIGO I and 2) and 18 % high-grade cases (FIGO 3). Overall, the Yale group stood alone having the largest proportion of high-grade tumors, with 67 %. However, the fact that the events were so low, and patients with already advanced endometrial carcinoma were being given tamoxifen to treat breast cancer, made this fact not unexpected. Based on this analysis of available data, there was insufficient evidence to support the statement that "women receiving tamoxifen as treatment for breast cancer who subsequently develop uterine cancer are at high risk for high-grade endometrial cancers that have a poor prognosis [12]." Nevertheless, the fact that there was an increase in the incidence of endometrial cancer was a major clue with the use of tamoxifen for both treatment and prevention in the 1990s. How bad was the fear of tamoxifen for some patients? In the mid-1990s, one patient said to me: "Thank God! I have been diagnosed with ER-negative breast cancer and I don't have to take tamoxifen."

Incidence of Endometrial Cancer with Tamoxifen

It is now possible to give a precise rate for the incidence of endometrial carcinoma in tamoxifen-treated patients. The results from the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) [19] have shown that tamoxifen increases the incidence of endometrial cancer and was strongly correlated with age. The risk of incidence or death from endometrial cancer was very little in the younger age group (<45, or 45–54 years) with only one death and 11 incidents of endometrial cancer in the <45 years group with ER-positive breast cancer and seven deaths and 71 incident cases of endometrial cancer in the 55–69 years group with ER-positive breast cancer (incidence 3.8 % in the tamoxifen group vs. 1.1 % in the control group; absolute increase 2.6 %, 95 % CI) [19].

Most importantly, the new knowledge about the small but significant increase in endometrial cancer in postmenopausal patients treated with long-term adjuvant tamoxifen therapy acted as a forewarning for the NCI NSABP P-1 prevention trial to remain vigilant for signs of spotting and bleeding on protocol. Accurate results for the detection of endometrial cancer in pre- and postmenopausal women at risk for breast cancer are documented in Table 6.4. There is a significant increase in endometrial cancer in postmenopausal population, but not in premenopausal women at risk for breast cancer [20, 21]. It is important to note that no patient died from endometrial cancer in the NCI/NSABP P-1 study, probably because of the meticulous surveillance practices during the study.

Finally, another clinical trial Study of Tamoxifen and Raloxifene (STAR), also known as NSABP P-2 trial, concluded, as well, that tamoxifen treatment increases the incidence of invasive uterine cancer in comparison to women with high risk of breast cancer treated with raloxifene [22]. Increase by 45 % (RR, 0.55; 95 % CI, AU1

Uncorrected Proof

Author's Proof

t4.1 **Table 6.4** Comparison of the uterine cancers in tamoxifen-treated and control patients [12–14, 17]

t4.2		NSABP		Yale tumor registry			Swedish trial				Netherlands cancer institute			
t4.3		Tamoxifen n = 25		Tamoxifen n = 15		No tamoxifen n = 38	Tamoxifen n = 17		No tamoxifen n = 5		Tamoxifen n = 23		No tamoxifen n = 75	
t4.4		Events	%	Events	%	Events %	Events	%	Events	%	Events	%	Events	%
t4.5	Stage													
t4.6	I	21	88	7	78	23 88	14	82	4	100	17	85	62	87
t4.7	II–IV	3	12	2	22	3 12	3	18	0	0	3	15	9	13
t4.8	Total no. staged	24		9		26	17		4		20		71	
t4.9	Histological grade													
t4.10	Low (good)	18	78	5	33	26 74	8	53	4	100	12	52[a]	24	32[a]
t4.11	High (poor)	5	22	10	67	9 26	7	47	0	0	11	48	51	68
t4.12	Total no. graded	23		15		35	15		4		23		75	

t4.13 [a]Calculated from a statement made by the authors in the discussion of the paper [17]. No breakdown of histological grade was presented in the results, although the morphological classification for users and nonusers of tamoxifen was in the same proportions. The proportion of well-differentiated tumors in the no tamoxifen group of this study is very low in comparison to all the studies and the survey in [18]

Uncorrected Proof

Author's Proof

0.36–0.83) for invasive uterine cancer, and 80 % higher incidence of endometrial cancer in tamoxifen-treated group than in raloxifene treated (RR, 0.19; 95 %CI, 0.12–0.29).

Tamoxifen and Rat Liver Carcinogenesis

It is now clear that if I had pursued the idea of giving high doses of tamoxifen to prevent rat mammary carcinogenesis [4], then rat liver carcinogenesis would have been discovered in 1973 [23], and there would have been no tamoxifen, and hundreds of thousands of women would now be dead of breast cancer. There would probably be no aromatase inhibitors or SERMs. The pharmacological industry would not have advanced a known carcinogen for long-term therapy (adjuvant therapy) or chemoprevention, so the "gold standard" would not have existed for others to beat.

High daily doses of tamoxifen will produce hepatocellular carcinoma in the rat (Table 6.5) if administered for up to half the animal's lifetime. This is particularly true at a 45.2 mg/kg dose, when tumors are formed within 6 months in 29 % of the animals [24]. There is general agreement that high daily doses of tamoxifen result in the premature death of rats. In the study by Greaves and coworkers [25], 50 % of control female rats were alive and well at about 104 weeks (2 years), but treatment with 35 mg/kg tamoxifen daily produced 50 % deaths by 42 weeks. Interestingly, the low dose of 5 mg/kg/day increases the survival of male and female rats at 2 years (males, 30 % deaths in treated vs. 70 % deaths in controls; females, 25 % deaths in treated vs. 50 % deaths in controls). The authors note [25] that their low tamoxifen dose (5 mg/kg/day) completely inhibited the incidence of adenomas in the pituitary gland and adenocarcinomas of the mammary gland in female rats and almost completely inhibited adenomas of the pituitary gland and parathyroid gland in male rats.

The published studies indicate that there is a threshold level for liver carcinogenicity, which is approximately 3 mg/kg/day [24]. However, the study by Dragan and coworkers [27], using a different rat strain and experimental design, observed no hepatocellular carcinomata after 15 months of treatment. The design of the study divided carcinogenesis into initiation and promotion. Carcinogenesis was initiated with diethylnitrosamine (DEN 10 mg/kg oral) in partially hepatectomized Fischer F344 rats, and promotion to carcinogenesis was completed with tamoxifen in the feed at 250 ppm. Blood levels of tamoxifen were 230 $\pm$ 30 ng/ml (i.e., in the range of clinical experience [27]). It can be estimated that a 200-g rat consumes 10 g of food containing 2.5 mg tamoxifen per day, so a rat received a daily dose of 12.5 mg/kg, which is within the 10–30 mg/kg/day dosing regimens of other studies [24]. No hepatocellular carcinomata were observed if DEN, the initiator, was omitted, but tumors were seen if DEN was given with tamoxifen, leading the authors to conclude that tamoxifen is a promoter of hepatocellular carcinoma in the Fischer rat. However, all the other studies, mainly using Sprague-Dawley strains of rats and bolus administration of drug by lavage, suggest that tamoxifen is a complete carcinogen at high doses.

Uncorrected Proof

Author's Proof

t5.1 **Table 6.5** The occurrence of hepatocellular carcinoma in various rat strains during long-term tamoxifen treatment

t5.2 Strain of rat	Daily dose (mg/kg)	*n*	Duration (months)	Hepatocellular carcinoma %	(*n*)	Reference
t5.5 1. Sprague-Dawley (Crl:CD(BR))	2.8	57	15	0	(22)	[24]
t5.6	11.3	57	15	45	(11)	
t5.7	45.2	57	12	75	(4)	
t5.8 2. Wistar (Alpk: ApfSD)	5	52	24	16	(51)	[25]
t5.9	20	52	24	64	(51)	
t5.10	35	52	24	64	(51)	
t5.11 3. Sprague-Dawley (Crl:CD(BR))	11.3	84	12	44	(36)	[26]
t5.12	22.6	75	12	100	(24)	
t5.13 4. Fischer F344	12.5[a]	20	15	0	(8)	[27]

AU2

204 Tamoxifen and DNA Adduct Formation

205 Carcinogenesis requires genotoxicity, so it is important to correlate the formation of
206 DNA adducts with the formation of tumors in a particular organ for a sensitive
207 species. Mani and Kupfer [28] first showed that in human and rat liver microsome
208 systems in vitro [^{14}C], tamoxifen was metabolized by an NADPH-dependent
209 cytochrome P450-mediated activation system to intermediate(s) which covalently
210 bound to microsomal proteins. Han and Liehr [16] subsequently showed that the
211 administration intraperitoneally (i.p.) of tamoxifen (20 mg/kg/day) to Sprague-
212 Dawley rats resulted in two DNA adducts after only 1 day and up to six adducts
213 after 6 consecutive days of treatment. A similar result was observed by Hard and
214 associates [26] using 48 mg/kg/day tamoxifen for 7 days in Sprague-Dawley rats.
215 It is clear that large doses of tamoxifen can produce DNA adducts, but White and
216 coworkers [29] have investigated the dose adduct relationship in rats. Seven days of
217 dosing with between 5 and 45 mg tamoxifen/kg/day produced an almost linear
218 dose-dependent increase in DNA adducts in the Fischer 344 rat. At doses of less
219 than 5 mg/kg/day, tamoxifen did not alter the chromatograph from ^{32}P post-labeled
220 DNA from treated rats. It would appear, therefore, that there is a threshold for the
221 appearance of adducts with tamoxifen and the induction of liver tumors. The
222 metabolite α-hydroxytamoxifen was subsequently found to be responsible for
223 DNA adducts in rats (see Chap. 3).
224 White and colleagues [29] also examined whether adduct formation occurs in the
225 mouse, which does not produce liver tumors in response to tamoxifen. There is
226 DNA adduct formation in both C57B1/6 and DBA/2 mice; however, this is approx-
227 imately 30 % of that observed with a similar dosing schedule in the Fischer rats
228 [29], raising questions about the correlation between adduct formation and clini-
229 cally evident tumors.

Uncorrected Proof

Author's Proof

In humans, DNA adducts were not observed in the livers of tamoxifen-treated women; however, only limited samples were screened [30]. A study in vitro [31] demonstrated the ability to form DNA adducts with human and rat liver microsomes using 100-μM tamoxifen. Although the levels of DNA adducts are low and in the range of the studies in vivo with mice, the human liver was two to three times more effective at producing DNA adducts than the rat. The Sprague-Dawley rat livers used in the studies in vitro [31] are from a strain that is extremely sensitive to the carcinogenic actions of tamoxifen in vivo. Adduct formation in vitro can be dramatically altered by adding different cofactors [31], and the level of DNA adduct formation that is required for carcinogenesis may be dose related, as in the rat in vivo [29]. The level of adducts, 1–3 $\times$ 10^8 nucleotides, observed in the study of rat liver microsomes in vitro [31] is not in the carcinogenic range in vivo [29], although caution must be used when comparing in vivo and in vitro studies.

Overall, these data demonstrated that DNA adducts could be formed in vitro and in vivo, but the level of adduct formation seems critical for carcinogenesis. Adduct formation using human microsomes is very low, but this can be enhanced into the mouse range using cumene hydroperoxide as a cofactor [31]. However, mice do not produce liver tumors after long-term treatment. Thus, the most important issues in the 1990s were the species differences, the correlation between liver carcinogenesis and DNA adduct formation, the effect of the rate of repair of DNA in different species, and the relative doses used to demonstrate the carcinogenic effects of tamoxifen. However, the epidemiology of human liver cancer did not support patient risk evaluations in women taking tamoxifen. No correlation has been noted to this day, but in the 1990s, the concern was justified with the move to prevention and the possibility that the liver carcinogenesis could occur decades after taking the drug.

Doses of Tamoxifen in Animals and Man

A key argument made regarding rat liver carcinogenesis studies was that since the serum concentrations of tamoxifen obtained in the rat (Table 6.6) were within the range of serum concentrations achieved during the treatment of breast cancer, then the results are clinically relevant. It is generally believed that toxicology testing should be conducted to mimic human pharmacokinetics. However, the rat and mouse clear tamoxifen from the body at a much faster rate than the human so that higher doses must be administered to maintain the blood level in the human range used for treatment. Examination of the relative dosage regimens in different species and the resulting serum levels of tamoxifen illustrate the point. Serum levels of tamoxifen during the treatment of breast cancer with 10 mg twice daily (approximately 285 μg/kg daily for a 70-kg postmenopausal woman) are usually between 100 and 200 ng/ml [32]. In contrast, the administration of 50- or 100-μg tamoxifen

Uncorrected Proof

Author's Proof

daily to ovariectomized mature mice (approximately 2.5 mg/kg for a 20-g mouse) or immature rats (approximately 3 mg/kg for a 35-g rat) for 7–10 days results in pharmacological effects but produces serum levels of tamoxifen often below the level of detection by high performance liquid chromatography [33]. Only by giving high doses of tamoxifen (200 mg/kg) to animals can one adequately study circulating levels of drug [33]. We studied the circulating levels of tamoxifen in patients receiving high daily doses of tamoxifen. Increasing the daily dose to the limits of toxicity (10 mg/kg) [35] in humans reaches the dose range (5–35 mg/kg) used to treat rats in the liver carcinogenesis studies (Table 6.6). However, the blood levels are tenfold higher in the human. Comparable serum levels in the rat and human during tamoxifen treatment can only be produced by treating rats with high doses of tamoxifen. The schedules that are used to demonstrate liver carcinogenesis in the rat (5–40 mg/kg) are 20 times greater than the standard treatment regimen in women (20 mg daily or 285 μg/kg).

Testing at Comparable Therapeutic Levels

Tamoxifen, at a daily dose of 50 μg (250 μg/kg), inhibits the growth and development of dimethylbenzanthracene-induced rat mammary tumors [36]. This is equivalent to the therapeutic dose used to treat metastatic breast cancer and as an adjuvant therapy in node-positive and node-negative disease. The duration of therapy for the treatment of breast cancer can be indefinite in some clinical trials [37, 38], but most treatment plans use 5 years of adjuvant tamoxifen at a dose of 20 mg daily. With the life expectancy of most women being 80 years of age, this translates into about 6 % of a woman's lifetime, and most women are treated during their postmenopausal years. In contrast, studies of rat liver carcinogenesis employ a test system that starts at 6 weeks of age (just post-puberty) and treats daily with approximately 20 times the human dose for the rest of the animals' life. At a dose of 11.3 mg/kg, approximately half the rats develop liver tumors within a year [26] (Table 6.7).

It is important to state that the general need for carcinogenic testing is to establish whether an agent is carcinogenic per se not just at the level of therapeutic value. To achieve this, animals are tested with a high dose, with lower doses approaching the therapeutic range. A positive result in the animal test does not mean that human therapeutic levels will be carcinogenic but provides a warning of such a possibility. A treatment regimen of tamoxifen, 0.25 mg/kg daily, for 2–3 months during the second year of the rats' life would be an equivalent bioassay. This approach would give a realistic view of the toxicological risks observed in patients. Since the doses to be used are far below the level that causes adduct formation [29] and repair mechanisms occur after the cessation of therapy, there is little probability that animals will develop liver tumors, thus duplicating clinical experience.

Uncorrected Proof

Author's Proof

Table 6.6 Circulating serum levels obtained with different dosage regimens in the rat, mouse, and human (70-kg postmenopausal women) t6.1

Species	Dosage per day (mg/kg)	Duration	Tamoxifen concentration	Reference	
Human	0.28	>2 years	148	[32]	t6.3
Rat	3	7 days	<1	[33]	t6.4
Rat	200	7 days	1,000	[33]	t6.5
Mouse	2.5	7 days	<10	[33]	t6.6
Mouse	200	10 days	300	[33]	t6.7
Human	4.9	1 year	1,300	[33, 34]	t6.8
Human	Approx. 10	11 days	1,855	[35]	t6.9

Table 6.7 The levels of circulating tamoxifen achieved with the dosing regimens used in rats during carcinogenesis experiments t7.1

Rats	Dosage regimen (mg/kg)	Tamoxifen concentration (ng/ml)	Liver tumors	Reference	
1. Mature Wistar	5	166	Yes	[24]	t7.3
	20	644			t7.4
	35	636			t7.5
2. Mature Sprague-Dawley	11.3	138 ± 41	Yes	[25]	t7.6
	22.6	172 ± 103			t7.7
3. Mature Fischer	12.5[a]	230 ± 30	Yes	[27]	t7.8

[a]Based on estimate of daily food intake of 10 g per day of 250-mg tamoxifen/kg feed t7.9

Toxicological testing of new drugs in development to reduce the risks to patients is crucial, but tamoxifen has received extensive clinical testing over the past 40 years without producing major toxicities. Although it is argued that a decade is required for iatrogenic carcinogenesis in patients [39], there is currently little or no information to demonstrate that tamoxifen is a significant liver carcinogen in the human, as has been demonstrated for the rat [24]. The divergence of effects in rats and women is because of differences in the dose, duration and timing of tamoxifen treatment, differential metabolism, rapid repair responses in humans, and the susceptibility of some inbred strains of rat to hepatocellular carcinogens.

Conclusion

Overall, the effective translational research on the link between tamoxifen and the growth of endometrial cancer with the important step of taking our laboratory finding [6] to the clinical community [15] resulted in lives saved and put in place new gynecologic procedures that remain to this day. It was specifically stated: "Until the influence of TAM and other antiestrogens on endometrial cancers has been fully investigated, vigilance by physicians treating patients with these agents is needed to establish the clinical relevance (if any) of these observations." However, the other toxicological issue, rat liver carcinogenesis was not to evaporate so

Uncorrected Proof

Author's Proof

easily and is a lasting example of those observations in laboratory that do not necessarily translate to the clinic. However, Zeneca (originally ICI pharmaceuticals division) formally required a "black box" designation to comply with the toxicological findings. A lesson learned, but not unlike the fact the tamoxifen was a superb antifertility agent in the laboratory but did exactly the opposite in clinical practice!

Postscript. The results of the pioneering experiment by my Ph.D. student Marco Gottardis [6] on the target site specificity in breast cancer, and endometrial cancer was used by us to appeal to the clinical community to monitor their adjuvant clinical trials. This story is told in the Postscript to Chap. 5. Marco and I traveled to ICI pharmaceutical division in 1987, and he presented his work at Alderley Park for their staff. The staff at ICI took immediate action and contacted the Stockholm adjuvant clinical trial group to look at their database with different durations of tamoxifen [15, 40]. The results of their data collection process replicated our laboratory study, fewer contralateral breast cancers and more endometrial cancers with tamoxifen. It is interesting to observe that an examination of their paper published in 1989 shows that the axis for the duration of patient monitoring of their adjuvant tamoxifen trail for endometrial cancer extends for 10.5 years. In other words, they already had the data by the year 1987 when we first talked about our animal studies of human disease in 1987. The NSABP followed up with their evaluation of tamoxifen and endometrial cancer in 1991 [41]. All of these translational research successes were essential to prepare the clinical trials community for monitoring the proposed chemoprevention trials in women without breast cancer for endometrial cancer.

Wisconsin with its two cancer centers: the Wisconsin Comprehensive Cancer Center and McArdle Laboratory for Cancer Research had significant researchers in carcinogenesis. Henry Pitot, a former director of the McArdle Laboratory, was a world authority on hepatocarcinogenesis. Here was a superb opportunity to join forces on a "hot topic" in toxicology—rat carcinogenesis with nonsteroidal antiestrogens. Henry had a talented, keen, and enthusiastic postdoc. . .who successfully wrote up our proposal, and Henry generously resulted that I was the principal investigator as this was a "topical tamoxifen issue," and I was better positioned to be successful. He was correct and numerous publications subsequently followed. This is what cancer centers are all about—collaboration to aid and understanding of topics that will affect the well-being of patients, in this case the concern was about liver cancer with tamoxifen use. It also provided me with the opportunity to participate in the debate about the safety of tamoxifen at the national level and especially at hearings in the state of California. All through the development of tamoxifen, I had a philosophy of looking at "the good, the bad, and the ugly" of tamoxifen. Patient safety and patient mortality was always the goal.

Uncorrected Proof

Author's Proof

References

1. Cole MP, Jones CT, Todd ID (1971) A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer 25:270–275
2. EBCTCG (1988) Effects of adjuvant tamoxifen and of cytotoxic therapy on mortality in early breast cancer. An overview of 61 randomized trials among 28,896 women. N Engl J Med 319:1681–1692
3. Jordan VC (1974) Antitumour activity of the antiestrogen ICI 46,474 (Tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 5:354
4. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
5. Cuzick J, Baum M (1985) Tamoxifen and contralateral breast cancer. Lancet 2:282
6. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48:812–815
7. Cross SS, Ismail SM (1990) Endometrial hyperplasia in an oophorectomized woman receiving tamoxifen therapy. Case report. Br J Obstet Gynaecol 97:190–192
8. Gal D, Kopel S, Bashevkin M et al (1991) Oncogenic potential of tamoxifen on endometria of postmenopausal women with breast cancer–preliminary report. Gynecol Oncol 42:120–123
9. De Muylder X, Neven P, De Somer M et al (1991) Endometrial lesions in patients undergoing tamoxifen therapy. Int J Gynaecol Obstet 36:127–130
10. Satyaswaroop PG, Zaino RJ, Mortel R (1984) Estrogen-like effects of tamoxifen on human endometrial carcinoma transplanted into nude mice. Cancer Res 44:4006–4010
11. Horwitz RI, Feinstein AR (1986) Estrogens and endometrial cancer. Responses to arguments and current status of an epidemiologic controversy. Am J Med 81:503–507
12. Magriples U, Naftolin F, Schwartz PE, Carcangiu ML (1993) High-grade endometrial carcinoma in tamoxifen-treated breast cancer patients. J Clin Oncol 11:485–490
13. Fornander T, Hellstrom AC, Moberger B (1993) Descriptive clinicopathologic study of 17 patients with endometrial cancer during or after adjuvant tamoxifen in early breast cancer. J Natl Cancer Inst 85:1850–1855
14. Fisher B, Costantino JP, Redmond CK et al (1994) Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. J Natl Cancer Inst 86:527–537
15. Fornander T, Rutqvist LE, Cedermark B et al (1989) Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1:117–120
16. Han XL, Liehr JG (1992) Induction of covalent DNA adducts in rodents by tamoxifen. Cancer Res 52:1360–1363
17. van Leeuwen FE, Benraadt J, Coebergh JW et al (1994) Risk of endometrial cancer after tamoxifen treatment of breast cancer. Lancet 343:448–452
18. Boronow RC, Morrow CP, Creasman WT et al (1984) Surgical staging in endometrial cancer: clinical-pathologic findings of a prospective study. Obstet Gynecol 63:825–832
19. Davies C, Godwin J, Gray R et al (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378:771–784
20. Fisher B, Costantino JP, Wickerham DL et al (2005) Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97:1652–1662
21. Fisher B, Costantino JP, Wickerham DL et al (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90:1371–1388

Uncorrected Proof

Author's Proof

22. Runowicz CD, Costantino JP, Wickerham DL et al (2011) Gynecologic conditions in participants in the NSABP breast cancer prevention study of tamoxifen and raloxifene (STAR). Am J Obstet Gynecol 205:535 e1–535 e5
23. Jordan VC (1995) What if tamoxifen (ICI 46,474) had been found to produce rat liver tumors in 1973? A personal perspective. Ann Oncol 6:29–34
24. Williams GM, Iatropoulos MJ, Djordjevic MV, Kaltenberg OP (1993) The triphenylethylene drug tamoxifen is a strong liver carcinogen in the rat. Carcinogenesis 14:315–317
25. Greaves P, Goonetilleke R, Nunn G et al (1993) Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. Cancer Res 53:3919–3924
26. Hard GC, Iatropoulos MJ, Jordan K et al (1993) Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. Cancer Res 53:4534–4541
27. Dragan YP, Fahey S, Street K et al (1994) Studies of tamoxifen as a promoter of hepatocarcinogenesis in female Fischer F344 rats. Breast Cancer Res Treat 31:11–25
28. Mani C, Kupfer D (1991) Cytochrome P-450-mediated activation and irreversible binding of the antiestrogen tamoxifen to proteins in rat and human liver: possible involvement of flavin-containing monooxygenases in tamoxifen activation. Cancer Res 51:6052–6058
29. White IN, de Matteis F, Davies A et al (1992) Genotoxic potential of tamoxifen and analogues in female Fischer F344/n rats, DBA/2 and C57BL/6 mice and in human MCL-5 cells. Carcinogenesis 13:2197–2203
30. Martin EA, Rich KJ, White IN et al (1995) 32P-postlabelled DNA adducts in liver obtained from women treated with tamoxifen. Carcinogenesis 16:1651–1654
31. Pathak DN, Bodell WJ (1994) DNA adduct formation by tamoxifen with rat and human liver microsomal activation systems. Carcinogenesis 15:529–532
32. Langan-Fahey SM, Tormey DC, Jordan VC (1990) Tamoxifen metabolites in patients on long-term adjuvant therapy for breast cancer. Eur J Cancer 26:883–888
33. Robinson SP, Langan-Fahey SM, Johnson DA, Jordan VC (1991) Metabolites, pharmacodynamics, and pharmacokinetics of tamoxifen in rats and mice compared to the breast cancer patient. Drug Metab Dispos 19:36–43
34. Jordan VC, Bain RR, Brown RR et al (1983) Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. Cancer Res 43:1446–1450
35. Trump DL, Smith DC, Ellis PG et al (1992) High-dose oral tamoxifen, a potential multidrug-resistance-reversal agent: phase I trial in combination with vinblastine. J Natl Cancer Inst 84:1811–1816
36. Jordan VC (1983) Laboratory studies to develop general principles for the adjuvant treatment of breast cancer with antiestrogens: problems and potential for future clinical applications. Breast Cancer Res Treat 3(Suppl):S73–S86
37. Falkson HC, Gray R, Wolberg WH et al (1990) Adjuvant trial of 12 cycles of CMFPT followed by observation or continuous tamoxifen versus four cycles of CMFPT in postmenopausal women with breast cancer: an Eastern Cooperative Oncology Group phase III study. J Clin Oncol 8:599–607
38. Tormey DC, Gray R, Abeloff MD et al (1992) Adjuvant therapy with a doxorubicin regimen and long-term tamoxifen in premenopausal breast cancer patients: an Eastern Cooperative Oncology Group trial. J Clin Oncol 10:1848–1856
39. Jordan VC, Lababidi MK, Mirecki DM (1990) Anti-oestrogenic and anti-tumour properties of prolonged tamoxifen therapy in C3H/OUJ mice. Eur J Cancer 26:718–721
40. Fornander T, Rutqvist LE (1989) Adjuvant tamoxifen and second cancers. Lancet 1:616
41. Fisher B, Redmond C (1991) New perspective on cancer of the contralateral breast: a marker for assessing tamoxifen as a preventive agent. J Natl Cancer Inst 83:1278–1280

Uncorrected Proof

Author's Proof

Author Queries

Chapter No.: 6

Query Refs.	Details Required	Author's response
AU1	Please check sentence starting "Increase by 45%..." for completeness.	
AU2	Please provide explanation for "a", in Table 6.5.	

Uncorrected Proof

Author's Proof

Metadata of the chapter that will be visualized online

Chapter Title	Chemoprevention: Cinderella Waiting for the Ball	
Copyright Year	2013	
Copyright Holder	Springer Basel	
Corresponding Author	Family Name	**Maximov**
	Particle	
	Given Name	**Philipp Y.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**McDaniel**
	Particle	
	Given Name	**Russell E.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**Jordan**
	Particle	
	Given Name	**V. Craig**
	Suffix	
	Division	Lombardi Comprehensive Cancer Center
	Organization	Georgetown University Medical Center
	Address	Washington, DC, USA
Abstract	Tamoxifen was first shown to prevent the initiation and promotion of rat mammary carcinogenesis in the 1970s. During the 1990s, numerous trials were initiated to test the worth of tamoxifen to decrease the incidence of breast cancer in otherwise healthy women. The Royal Marsden study was first with a vanguard study in the 1980s followed by the National Surgical Breast and Bowel Project (NSABP) P-1 trial, the Italian Study of women not at risk, and the International Breast Cancer Study Group (IBIS). Multiple subsequent analyses all showed some efficacy to reduce breast cancer incidence, but the NSABP study was the strongest powered clinical trial uniformly demonstrating a 50 % decrease in incidence for both pre- and postmenopausal women at risk. As predicted, endometrial cancer was the most troublesome side effect, but only in postmenopausal women taking tamoxifen.	

Author's Proof

Chapter 7
Chemoprevention: Cinderella Waiting for the Ball

Abstract Tamoxifen was first shown to prevent the initiation and promotion of rat mammary carcinogenesis in the 1970s. During the 1990s, numerous trials were initiated to test the worth of tamoxifen to decrease the incidence of breast cancer in otherwise healthy women. The Royal Marsden study was first with a vanguard study in the 1980s followed by the National Surgical Breast and Bowel Project (NSABP) P-1 trial, the Italian Study of women not at risk, and the International Breast Cancer Study Group (IBIS). Multiple subsequent analyses all showed some efficacy to reduce breast cancer incidence, but the NSABP study was the strongest powered clinical trial uniformly demonstrating a 50 % decrease in incidence for both pre- and postmenopausal women at risk. As predicted, endometrial cancer was the most troublesome side effect, but only in postmenopausal women taking tamoxifen.

Uncorrected Proof

Introduction

The idea of the prevention of breast cancer is not new, but significant practical progress has been made, through translational research, to make the idea feasible in some women. It is now possible to reduce the incidence of breast cancer through the inhibition of estrogen action.

Professor Antoine Lacassagne [1] stated a vision for the prevention of breast cancer at the annual meeting of the American Association of Cancer Research in Boston in 1936.

> If one accepts the consideration of adenocarcinoma of the breast as the consequence of a special hereditary sensibility to the proliferative actions of estrone, one is led to imagine a therapeutic preventative for subjects predisposed by their heredity to this cancer. It would consist – perhaps in the very near future when the knowledge and use of hormones will be better understood – in the suitable use of a hormone antagonistic or excretory, to prevent the stagnation of estrone in the ducts of the breast.

But no agent that was "antagonistic to prevent the stagnation of oestrone in the breast" was available to the clinician for clinical trial until tamoxifen [2, 3]. AU1

P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
DOI 10.1007/978-3-0348-0664-0_7, © Springer Basel 2013

Author's Proof

Tamoxifen became the "antiestrogen" of choice because of the following (a) There was a large body of basic biological evidence that this was a valid hypothesis to test (b) Tamoxifen was noted to reduce the incidence of contralateral breast cancer when used as an adjuvant therapy to treat micrometastases from the original primary tumor. (c) There was a huge and expanding clinical experience with tamoxifen as a long-term treatment for node-positive and node-negative breast cancer. The later point was important as the majority of patients with estrogen receptor (ER)-positive node-negative breast cancers are cured by surgery (plus radiation) alone, so 5 years of adjuvant tamoxifen was essentially already being used in the majority of these cured "well women" [4, 5].

In this chapter, the changing fashions in endocrine chemoprevention will be described. The change in fashion occurred because of significant advances in our understanding of the pharmacology of the drug group called the "nonsteroidal antiestrogens" [6] that underwent a metamorphosis in the mid-1980s [7] to become the new drug group called the selective ER modulators (SERMs) [8, 9]. See Chap. 5.

AU2

The Link Between Estrogen and Breast Cancer

The topic has recently been reviewed [10] in the refereed research literature so only essential facts will be considered here. The link between estrogen action for breast cancer growth of the original tumor, ER, and 5 years of adjuvant tamoxifen therapy to block tumor growth is compelling and proven in randomized clinical trials [11]. The findings can be simply summarized: breast tumors that are ER negative do not respond to tamoxifen treatment, tamoxifen dramatically reduces recurrence and mortality during 5 years of treatment for patients with ER-positive breast cancer, and this is maintained for at least 15 years following completion of therapy (see Chap. 4). Tamoxifen reduces the incidence of contralateral breast cancer by 50 % and this is sustained, but tamoxifen also increases the incidence of endometrial cancer in postmenopausal women (and mortality). The negative actions of adjuvant tamoxifen, such as deaths from endometrial cancer or thromboembolic disease, do not affect the overall benefit of treatment [11], but do impact on the use of tamoxifen for chemoprevention. Profound target site-specific actions of tamoxifen on the uterus in the recent overview [11] recapitulate and confirm the translational research with tamoxifen completed in the 1980s [12, 13] with the recognition of a small but significant increase in the incidence of endometrial cancer in postmenopausal women treated with tamoxifen. This finding eventually resulted in the paradigm shift away from tamoxifen to new opportunities, but this advances our story too quickly. In the 1980s, tamoxifen was the only medicine available for testing therapeutic and chemopreventive strategies with SERMs in the 1990s. The clinical community advanced with a responsibility to weigh risks and benefits in clinical trials to ensure the safety and long-term health of women at risk for breast cancer.

The treatment trials database and translational research were essential to address the hypothesis that tamoxifen, a nonsteroidal antiestrogen, could effectively block

Uncorrected Proof

Author's Proof

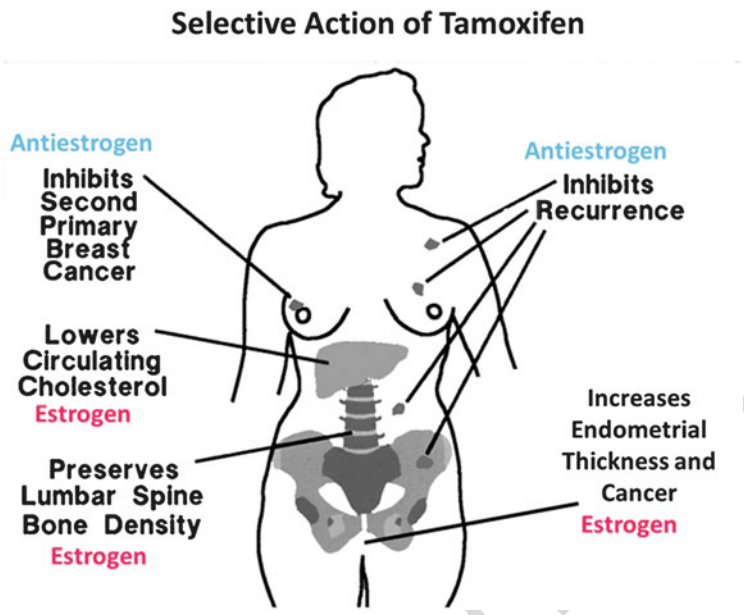


Fig. 7.1 Selective action of tamoxifen in target tissues. Tamoxifen is a SERM and has antiestrogenic action in the breast, but estrogenic properties in the bone, endometrium

the genesis and growth of ER-positive breast cancer but would be ineffective against the growth of ER-negative disease. Nevertheless in the 1980s, estrogen was also considered to be an essential component of women's health by maintaining bone density and preventing coronary heart disease. Thus, if tamoxifen, an antiestrogen, prevented the development and growth of ER-positive breast cancer in half a dozen high-risk women per year per thousand [14], hundreds of other women in the selected population might subsequently develop osteoporosis and coronary heart disease. The intervention with tamoxifen would be detrimental to public health. The good news was tamoxifen was not an antiestrogen everywhere; it was the lead compound of the drug group that selectively modulated ER target tissues around the body (Fig. 7.1). The original work (described in Chap. 5) to investigate the target site pharmacology of tamoxifen in the laboratory was to provide a database with which to predict clinical outcomes and safety for future chemoprevention trials. This discovery ultimately facilitated the development of a new strategy for the utilization of new SERMs as chemopreventives in breast cancer.

Prevention of Mammary Cancer in Rodents

The expanding literature on the prevention of rodent mammary cancer was used to support the clinical use of tamoxifen to prevent breast cancer. As mentioned earlier, Lacassagne predicted that a therapeutic intervention could be developed that would

Author's Proof

"prevent or antagonize the congestion of estrone in the breast." Unfortunately, no therapeutic agent was available and all his predictions were based upon the known effect of early oophorectomy on the development of mammary cancer in high-incidence strains of mice [1]. Clearly, the indiscriminate oophorectomy of young women would be an inappropriate intervention. The animal studies with tamoxifen were undertaken for two reasons: first, to establish the efficacy of tamoxifen in well-described models of carcinogenesis and, second, to discover whether tamoxifen would always be an inhibitor or whether the drug would ever exacerbate tumorigenesis. Two animal model systems were used extensively: the carcinogen-induced rat mammary carcinoma model and mouse mammary tumor virus (MMTV)-infected strains of mice.

The mammary carcinogens 7,12-dimethylbenz[α]anthracene (DMBA) [15] and N-nitrosomethylurea (NMU) [16] induce tumors in young female rats. The timing of the carcinogenic insult is very important, because as the animals age they become resistant to the mammary carcinogens. Tumorigenesis does not occur in oophorectomized animals, and the sooner oophorectomy is performed after the carcinogenic insult, the more effective it is in preventing the development of tumors [17].

The administration of tamoxifen to carcinogen-treated rats prevents the initiation of carcinogenesis, and animals remain tumor-free [18, 19]. The short-term administration of tamoxifen at different times after the carcinogenic insult is effective in reducing the number of tumors that develop [20, 21], although most animals develop at least one tumor after therapy is stopped.

Continuous tamoxifen therapy that is started at 1 month after the administration of carcinogens completely inhibits the appearance of mammary tumors [22, 23]. Under these circumstances, tamoxifen is preventing promotion and suppressing the appearance of occult disease. In fact, if treatment is stopped prematurely (i.e., a 3–4-month duration of therapy), the microfoci of transformed cells grow into palpable tumors. Because the timing of initiation in human breast cancer is unknown, and unlike the laboratory model not all women will develop tumors, tamoxifen will be given to target populations to suppress, and there is expectation that this will reverse the promotional effects of estrogen during carcinogenesis. Lacassagne performed his pioneering mammary tumor experiments linking estrogen with carcinogens in the high-risk mouse [24], so this was another model to use.

Until 1989, there was a paucity of information about the efficacy of tamoxifen to inhibit mouse mammary tumorigenesis. This was true in part because tamoxifen is estrogenic in short-term tests in oophorectomized [25] and immature mice [26]. However, the finding that long-term tamoxifen therapy renders the oophorectomized mouse vagina [27] and athymic mouse uterus [12] refractory to estrogenic stimuli prompted a reconsideration of the value of tamoxifen as a preventive in mouse mammary tumor models.

High-incidence strains of mice that develop mammary tumors are infected with MMTV, which is transferred to the offspring in the mothers' milk [28]. Tumorigenesis appears to be ovarian dependent, because the highest incidence of tumors appears in females, and tumorigenesis can be delayed or prevented depending upon the age at

Uncorrected Proof

Author's Proof

oophorectomy [29]. Steroid hormones activate the pro-viral MMTV [30], which in turn can initiate an increase in growth factors from the viral integration site Int. 2 [31]. Promotion of the initiated cells with steroid hormones and prolactin then completes tumorigenesis.

Long-term tamoxifen therapy, after an early cycle of pregnancy and weaning to facilitate early tumorigenesis, is equivalent to an ovariectomy performed at 4 months in reducing tumorigenesis to 50 % at 14 months of age. However, tamoxifen is superior to oophorectomy, even after therapy is stopped, because ovariectomized animals continue to develop tumors, whereas animals previously treated with tamoxifen do not develop any more tumors [32].

We followed up on initial observations with an investigation of tumorigenesis in virgin mice. In this study design, mice develop mammary tumors during their second year of life. Again, long-term tamoxifen therapy started at 3 months of age is superior to oophorectomy at 3 months. Fifty percent of the oophorectomized animals develop tumors by the third year of life, whereas 90 % of tamoxifen-treated mice remain tumor-free [33]. These studies are illustrated in Fig. 7.2.

Overall, the results of the studies in the mouse model are particularly interesting because they changed our view of the interspecies pharmacology of tamoxifen. Long-term treatment with tamoxifen results in an initial classification of tamoxifen as an estrogen, but within a few weeks the pharmacology changes and tamoxifen becomes an antiestrogen. An understanding of this process was seen to have important implications for the long-term use of tamoxifen as an adjuvant therapy and a preventive.

Uncorrected Proof

Tamoxifen: The First SERM for the Prevention of Breast Cancer in High-Risk Populations

Forty years ago, tamoxifen was shown to prevent the induction [18] and promotion [20] of carcinogen-induced mammary cancer in rats. Similarly, tamoxifen was also shown to prevent the development of mammary cancer induced by ionizing radiation in rats [34]. These laboratory observations, coupled with the emerging preliminary clinical observation that adjuvant tamoxifen could prevent contralateral breast cancer in women [35], provided a rationale for Dr. Trevor Powles, who, in 1986, established the vanguard study at the Royal Marsden Hospital in England to test whether tamoxifen could prevent breast cancer in high-risk women [36].

During the 1990s, much progress was achieved to answer the question: "Does tamoxifen have worth in the prevention of breast cancer in select high-risk women?" The results of four international trials that address this question—the Royal Marsden study, the NSABP/NCI study, the Italian study, and the IBIS trial—have been reported. These data will be presented in detail as well as their subsequent updates in the past decade. A summary of trial characteristics and findings are presented in Table 7.1.

Author's Proof

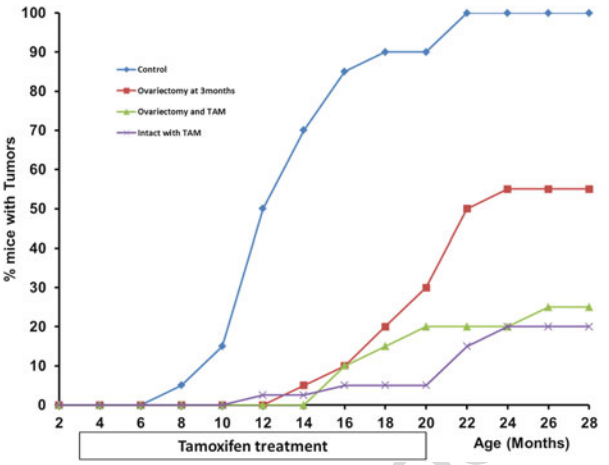


Fig. 7.2 The ability of long-term tamoxifen treatment or ovariectomy on the development of the mammary tumors in virgin C3H/OUJ mice. Long-term tamoxifen therapy is more effective as a chemopreventive than ovariectomy

t1.1 **Table 7.1** Comparison of the characteristics of various breast cancer treatment trials

t1.2 Characteristic	NSABP	Royal Marsden	Italian	IBIS
t1.3 Sample size	13,388	2,471	5,408	7,152
t1.4 Women years of follow-up	46,858	12,355	5,408	29,800
t1.5 Participants <50	40 %	62 %	36 %	52 %
t1.6 Breast cancer incidence per 1,000				
t1.7 Placebo	6.7	5.5	2.3	6.7
t1.8 Tamoxifen	3.4	4.7	2.1	4.7

Uncorrected Proof

176 Royal Marsden Study

177 Powles and coworkers recruited 2,484 women aged 30–70 to a placebo-controlled
178 trial using 20 mg of tamoxifen daily for up to 8 years. Women were eligible if their
179 risk of breast cancer was increased due to family history. Each participant had at
180 least one first-degree relative with breast cancer under age 50; or a first-degree
181 relative affected at any age, plus an additional affected first- or second-degree
182 relative; or a first-degree relative with bilateral breast cancer. Women with a history
183 of benign breast biopsy and an affected first-degree relative of any age were also
184 eligible. Women with a history of venous thrombosis, any previous malignancy, or
185 an estimated life expectancy of fewer than 10 years were excluded [37, 38]. A total
186 of 2,494 women consented to participate in the study, and 23 were excluded from
187 final analysis due to the presence of preexisting ductal carcinoma in situ (DCIS) or
188 invasive breast carcinoma [38]. The trial was undertaken to evaluate the problems
189 of accrual, acute symptomatic toxicity, compliance, and safety as a basis for
190 subsequent large national, multicenter trials designed to test whether tamoxifen
191 can prevent breast cancer. However, the trial has also been analyzed for breast
192 cancer incidence [38].

Author's Proof

Acute symptomatic toxicity was low for participants on tamoxifen or placebo, and compliance remained correspondingly high: 77 % of women on tamoxifen and 82 % of women on placebo remained on medication at 5 years, as predicted. There was a significant increase in hot flashes (34 % vs. 20 %), mostly in premenopausal women ($P < 0.005$); vaginal discharge (16 % vs. 4 %; $P < 0.005$); and menstrual irregularities (14 % vs. 9 %; $P < 0.005$), respectively. At the most recent follow-up, 320 women had discontinued tamoxifen and 176 had discontinued placebo prior to the study's completion ($P < 0.005$).

Until their report in 1994 [37], the Marsden group observed no thromboembolic episodes; a detailed analysis of other coagulation parameters in a sequential subset of women also found no significant changes in protein S, protein C, or cross-linked fibrinogen degradation products. At 70 months, no significant difference in the incidence of deep vein thrombosis or pulmonary embolism was observed between groups. A significant fall in total plasma cholesterol occurred within 3 months and was sustained over 5 years of treatment [39–41]. The decrease affected low-density lipoprotein, with no change in apolipoproteins A and B or high-density lipoprotein cholesterol.

In contrast, tamoxifen exerted antiestrogenic or estrogenic effects on bone density, depending on menopausal status. In premenopausal women, early findings demonstrated a small but significant ($P < 0.05$) loss of bone in both the lumbar spine and hip at 3 years [41]. In contrast, postmenopausal women had increased bone mineral density in the spine ($P < 0.005$) and hip ($P < 0.001$) compared to nontreated women.

Finally, the Marsden group made an extensive study of gynecological complications associated with tamoxifen treatment in healthy women. Since ovarian and uterine assessment by transvaginal ultrasound became available sometime after the trial's start, many subjects did not have a baseline evaluation. Ovarian screening demonstrated a significantly increased risk ($P < 0.005$) of detecting benign ovarian cysts in premenopausal women who had received tamoxifen for more than 3 months compared to controls. There were no changes in ovarian appearance in postmenopausal women [37]. A careful examination of the uterus with transvaginal ultrasonography using color Doppler imaging in women taking tamoxifen showed that the organ was usually larger; moreover, women with histological abnormalities had significantly thicker endometria [42]. Of particular interest in this regard was the observation that 20 mg of tamoxifen daily exerted a time-dependent proliferation of the endometrium in premenopausal and early postmenopausal women. This effect appeared to be mediated by the stromal component, since no cases of cancer or even epithelial hyperplasia were observed among the tamoxifen-treated group in the Italian study with 33 women [43].

Although the vanguard study has provided invaluable information about the biological effects of tamoxifen in healthy women, the trial was not designed to answer the question of whether tamoxifen prevents breast cancer. In spite of this, an analysis of breast cancer incidence was reported at a median follow-up of 70 months, when 42 % of the participants had completed therapy or withdrawn [38]. During the study, 336 women on tamoxifen and 305 on placebo received hormone-replacement

Uncorrected Proof

Author's Proof

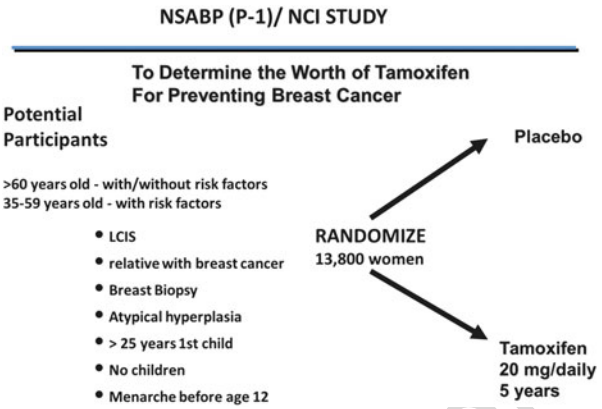


Fig. 7.3 The study design for the NSABP/NCI P-1 trial. On the *left* are the risk factors, according to the Gail model of breast cancer risk assessment based on which the participants of the study were selected

therapy. No difference in the incidence of breast cancer was observed between the groups. There were 34 carcinomas in the tamoxifen group and 36 in the placebo group-a relative risk of 1.06. Of the 70 cancers, only 8 were ductal carcinoma in situ. An analysis of the subset of women on hormone-replacement therapy did not demonstrate an interaction with tamoxifen treatment.

NSABP/NCI P-1 Study

This study opened in the United States and Canada in May of 1992 with an accrual goal of 16,000 women to be recruited at 100 North American sites. It closed after accruing 13,338 in 1997 due to the high-risk status of the participants. The study design is illustrated in Fig. 7.3. Those eligible for entry included any woman over the age of 60 or women between the ages of 35 and 59 whose 5-year risk of developing breast cancer, as predicted by the Gail model [14], was equal to that of a 60-year-old woman. Additionally, any woman over age 35 with a diagnosis of lobular carcinoma in situ (LCIS) treated by biopsy alone was eligible for entry to the study. In the absence of LCIS, the risk factors necessary to enter the study varied with age, such that a 35-year-old woman must have had a relative risk (RR) of 5.07, whereas the required RR for a 45-year-old woman was 1.79. Routine endometrial biopsies to evaluate the incidence of endometrial carcinoma in both arms of the study were also performed.

The breast cancer risk of women enrolled in the study was extremely high, with no age group having an RR of less than 4—including the over-60s group. Recruitment was also balanced, with about one-third younger than 50 years, one-third between 50 and 60 years, and one-third older than 60 years. Secondary end points of the study included the effect of tamoxifen on the incidence of fractures and cardiovascular deaths. Most importantly, the study planned to provide the first information about the role of genetic markers in the etiology of breast cancer. It was hoped to establish whether tamoxifen has a role to play in the treatment of

Uncorrected Proof

Author's Proof

women who are found to carry somatic mutations in the BRCA-1 gene. This did not occur as the number of patients with BRCA-1/2 mutations was not significant in the population [44].

The first results of the NSABP study were reported in September 1998, after a mean follow-up of 47.7 months [45]. There were a total of 368 invasive and noninvasive breast cancers in the participants: 124 in the tamoxifen group and 224 in the placebo group. A 49 % reduction in the risk of invasive breast cancer was seen in the tamoxifen group, and a 50 % reduction in the risk of noninvasive breast cancer was observed. A subset analysis of women at risk due to a diagnosis of LCIS demonstrated a 56 % reduction in this group. The most dramatic reduction was seen in women at risk due to atypical hyperplasia, where risk was reduced by 86 %.

The benefits of tamoxifen were observed in all age groups, with a relative risk of breast cancer ranging from 0.45 in women aged 60 and older to 0.49 for those in the 50- through 59-year-old age group, and 0.56 for women aged 49 and younger. A benefit for tamoxifen was also observed for women with all levels of breast cancer risk within the study, indicating that the benefits of tamoxifen are not confined to a particular lower risk or higher risk subset. Benefits were observed in women at risk on the basis of family history and those whose risk was due to other factors.

As expected, the effect of tamoxifen occurred on the incidence of ER-positive tumors, which were reduced by 69 % per year. The rate of ER-negative tumors in the tamoxifen group (1.46 per 1,000 women) did not significantly differ from the placebo group (1.20 per 1,000 women). Tamoxifen reduced the rate of invasive cancers of all sizes, but the greatest difference between the groups was the incidence of tumors 2.0 cm or less. Tamoxifen also reduced the incidence of both node-positive and node-negative breast cancer. The beneficial effects of tamoxifen were observed for each year of follow-up in the study. After year 1, the risk was reduced by 33 % and, in year 5, by 69 %.

Tamoxifen also reduced the incidence of osteoporotic fractures of the hip, spine, and radius by 19 %. However, the difference approached, but did not reach, statistical significance. This reduction was greatest in women aged 50 and older at study entry. No difference in the risk of myocardial infarction, angina, coronary artery bypass grafting, or angioplasty was noted between groups.

The study confirmed the association between tamoxifen and endometrial carcinoma (Figs. 7.4 and 7.5). The relative risk of endometrial cancer in the tamoxifen group was 2.5. The increased risk was seen in women aged 50 and older, whose relative risk was 4.01 (Fig. 7.5). There was no significance in the incidence of endometrial carcinoma in tamoxifen- or placebo-treated premenopausal women. All endometrial cancers in the tamoxifen group were grade 1 and none of the women on tamoxifen died of endometrial cancer. There was 1 endometrial cancer death in the placebo group. Although there is no doubt that tamoxifen increases the risk of endometrial cancer, it is important to recognize that this increase translates to an incidence of 2.3 women per 1,000 per year who develop endometrial carcinoma. More women in the tamoxifen group developed deep vein thrombosis (DVT) than in the placebo group (Fig. 7.6). Again, this excess risk was confined to women

Uncorrected Proof

Author's Proof

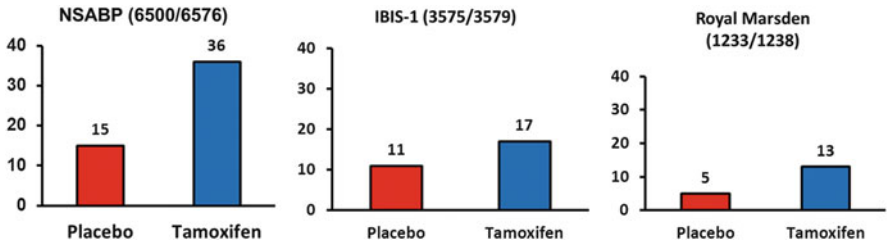


Fig. 7.4 The correlation between the increase in endometrial carcinoma incidence and tamoxifen treatment. In all three clinical trials show an increase in the incidence of endometrial carcinomas in tamoxifen-treated cohorts

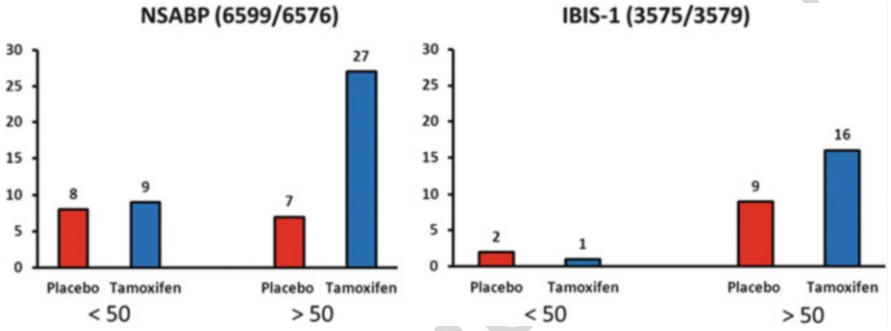


Fig. 7.5 The correlation between increase of endometrial carcinoma incidence and the age of the patient. The results of all three clinical trials showed that the increase in endometrial carcinoma significant incidence occurred in postmenopausal patients (>50 years of age) treated with tamoxifen in comparison to premenopausal patients (<50 years of age)

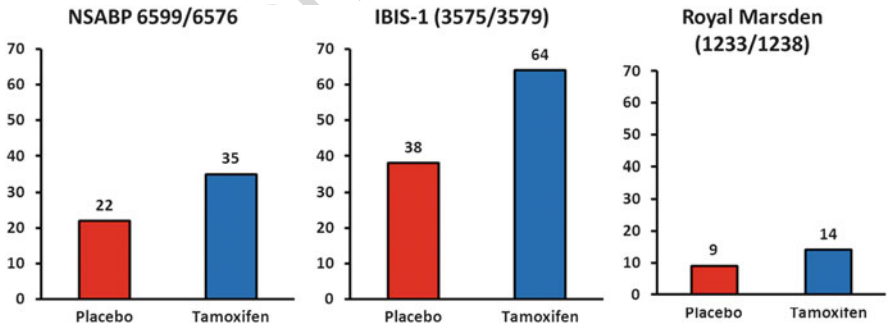


Fig. 7.6 Incidence of deep vein thrombosis (*DVT*) is significantly increased in tamoxifen-treated patients

aged 50 and older. The relative risk of DVT in the older age group was 1.71. (95 % CI 0.85–3.58). An increase in pulmonary emboli was also seen in the older women taking tamoxifen, with a relative risk of approximately 3 (Fig. 7.7). Three deaths

Author's Proof

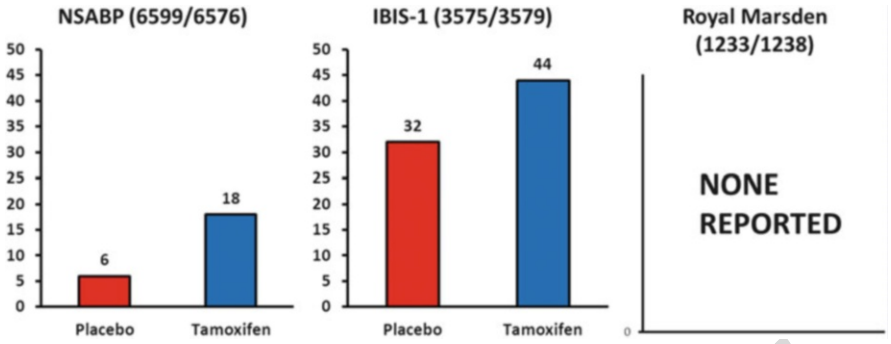


Fig. 7.7 Incidence of pulmonary embolism is increased in tamoxifen-treated patients. Observed in NSABP/P-1 and IBIS trials, but none were reported in the Royal Marsden trials

from pulmonary emboli occurred in the tamoxifen arm, but all were in women with significant comorbidities. An increased incidence of stroke (RR 1.75) was also seen in the tamoxifen group, but this did not reach statistical significance.

An assessment of the incidence of cataract formation was made using patient self-report. A small increase in cataracts was noted in the tamoxifen group—a rate of 24.8 women per 1,000 compared to 21.7 in the placebo group. There was also an increased risk of cataract surgery in the women on tamoxifen. These differences were marginally, statistically significant, and observed in the older patients in the study. This finding emphasizes the ocular safety of tamoxifen first predicted by Harper and Walpole in the 1960s [25], but as will be seen in Chap. 8, raloxifene does not have this effect. These findings emphasize the need to assess the patient's overall health status before making a decision to use tamoxifen for breast cancer risk reduction.

An assessment of quality of life showed no difference in depression scores between groups [46]. Hot flashes were noted in 81 % of the women on tamoxifen compared to 69 % of the placebo group, and the tamoxifen-associated hot flashes appeared to be of greater severity than those in the placebo group. Moderately bothersome or severe vaginal discharge was reported by 29 % of the women in tamoxifen group and 13 % in the placebo group [47]. No differences in the occurrence of irregular menses, nausea, fluid retention, skin changes, or weight gain or loss were reported.

Italian Study

The third tamoxifen prevention study, performed in Italy, began in October 1992 and randomized 5,408 women aged 35–70 to 20 mg of tamoxifen daily for 5 years [48]. Women were required to have had a hysterectomy for a nonneoplastic condition to obviate concerns about an increased risk of endometrial carcinoma.

Uncorrected Proof

Author's Proof

There was no requirement that participants be at risk for breast cancer development, and in fact, those who underwent premenopausal oophorectomy with hysterectomy actually had a slightly reduced risk of breast cancer development. Women with endometriosis, cardiac disease, and deep venous thrombosis were excluded from the study. Although 5,408 women were randomized into this study, 1,422 withdrew and only 149 completed 5 years of treatment.

The incidence of breast cancer did not differ between groups, with 19 cases in the tamoxifen group and 22 in the placebo group. Tumor characteristics, including size, grade, lymph node status, and receptor status, also did not differ between groups.

The incidence of thrombophlebitis was increased in the tamoxifen group. A total of 64 events were reported, 38 in the tamoxifen group and 18 in the placebo group ($P = 0.0053$). However, 42 of these were superficial phlebitis.

No differences in the incidence of cerebrovascular ischemic events were observed [48].

In 2003, a brief communication was published on the Italian Study that also compared the effectiveness of tamoxifen in cohorts of women who were using hormone-replacement therapy (HRT) or not. The results showed no significant difference between women taking tamoxifen or placebo in women who never used HRT and were in low-risk group ($P = 0.44$), and among women in the same cohort but in the high-risk group, there was a nonsignificant difference in favor of tamoxifen ($P = 0.099$). In the cohort of women that have used HRT during the trial and were in the low-risk group, there was also no statistically significant difference in women taking tamoxifen or placebo ($P = 0.31$); however, in the high-risk group there was a significant difference in favor of tamoxifen ($P = 0.009$).

The International Breast Cancer Intervention Study (IBIS-I)

The IBIS-I trial was a double-blind placebo-controlled randomized trial of tamoxifen [49]. Women at high risk (7,152) of breast cancer, between ages of 35 and 7 years were randomized into two groups. Women were randomized either into the placebo group (3,574) and women treated with 20 mg daily tamoxifen group (3,578). A total of 13 patients were excluded from the study, and the remaining were followed up for 5 years. The primary outcome measure was the incidence of breast cancer. After a median 50-month follow-up, 69 breast cancer cases were reported in the 5,378 women group treated with tamoxifen, and 101 cases in the 3,566 women placebo group, thus indicating a 32 % reduction ($P = 0.013$). Endometrial cancer was increased not significantly (11 vs. 2, $P = 0.2$) (Fig. 7.4), and thromboembolic events were significantly increased in the tamoxifen-treated group (43 vs. 17, $P = 0.001$) (Fig. 7.7). Based on these results, the authors concluded that preventive administration of tamoxifen is contradicted in women at high risk of thromboembolic disease. Tamoxifen should be stopped as an antithrombotic

AU3

Uncorrected Proof

Author's Proof

measure after surgeries or immobilization. However, tamoxifen does reduce the incidence of breast cancer by about a third, and non-breast-cancer causes of death are not increased by tamoxifen [49].

Follow-Up of Chemoprevention Studies with Tamoxifen

The main result from all the studies is that once tamoxifen is stopped, the antitumor effects sustained, systemic symptomology disappears, but the major side effect of an increase in endometrial cancer continues to accumulate in postmenopausal women [50–52]. Again it is tempting to speculate that the nascent breast cancer have been altered to survive in an environment of continuous tamoxifen, acquired resistance evolves, and then a woman's own estrogen causes apoptosis and tumoricidal actions in the "prepared" breast cancer cells after tamoxifen is stopped. This concept is discussed in detail in Chap. 9.

Tamoxifen again became a pioneering medicine but this time as the first drug to be FDA approved to reduce the risk of developing cancer, specifically ER-positive breast cancer. However, the translational research on endometrial cancer risk with tamoxifen [12, 53] demanded a safer solution to chemoprevention with SERMs. A strategy was already in place (Chap. 5) to move forward the first SERM to prevent osteoporosis and prevent breast cancer at the same time without the risk of endometrial cancer being increased. Keoxifene, the failed breast cancer drug, became raloxifene.

Two Approaches to the Chemoprevention of Breast Cancer

The successful clinical completion of the chemoprevention studies in women at high risk of developing breast cancer during the late 1990s resulted in FDA approval of tamoxifen for risk reduction in pre- and postmenopausal women in 1998. Despite reservations about tamoxifen and its toxicology (Chap. 6) for chemoprevention, the drug remains a cheap and lifesaving drug for the treatment of breast cancer worldwide. The data of endometrial cancer, deep vein thrombosis, and pulmonary embolism appear mainly in postmenopausal women [50]. However, the drug has both efficacy and an excellent safety profile in premenopausal women.

A recent review of the literature [54] concluded that "the risk of endometrial cancer, deep vein thrombosis and pulmonary embolism is low in women <50 years who take tamoxifen for breast cancer prevention. The risk decreases from the active to follow-up phase of treatment. Education and counseling are the cornerstones of breast cancer chemoprevention."

Nevertheless, despite the safety issues being low in premenopausal women, no other country has approved tamoxifen for chemoprevention in women with a high risk of developing breast cancer.

Uncorrected Proof

Author's Proof

Chemoprevention of breast cancer did, however, expanded dramatically throughout the 1990s based upon the laboratory work conducted with the discovery of selective estrogenic and antiestrogenic actions of estrogen target sites around the body. This work at the Wisconsin Clinical Cancer Center (Chap. 5) would subsequently be known in the literature as selective ER modulation. The strategic view described earlier (Chap. 5) was further refined to create a roadmap for drug development by the pharmaceutical industry. Simply stated, the proposal was to develop multifunctional medicines to aim at reducing the morbidity and mortality of a major disease affecting millions of women after menopause but, at the same time, reducing the risk of breast cancer. In 1990, this proposal was published in Cancer Research, the flagship journal of the American Association for Cancer Research [7]. This was the B.F. Cain Memorial Lecture for laboratory advances in cancer research that were having therapeutic impact in clinical applications and refined the original SERM concept (Chap. 5).

> We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines, and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids; it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high-risk group to prevent breast cancer.

Raloxifene: Abandoned and Resurrected

Raloxifene, originally called keoxifene, was first reported by scientists at Eli Lilly, Indianapolis, to be an antiestrogen with a high affinity for the estrogen receptor (ER) [55]. Much like its earlier analog, LY117018, raloxifene has only mild estrogen-like properties in the uterus [56].In fact, at very high doses, LY117018 can even block the antiuterotropic effects of a variety of steroidal and nonsteroidal compounds in the rat [57]. The drug has antitumor effects in the rat, but is less potent than tamoxifen [23, 58]. Although the original direction for raloxifene's clinical development was breast cancer therapy, Eli Lilly chose to abandon this approach toward the end of the 1980s. However, the discovery that raloxifene might prevent osteoporosis, [59] prevent breast cancer, [23] and, at the same time, have minor estrogen-like effects in the uterus laid the foundation for the subsequent confirmation of bone data in animals [56]. These discoveries also led to the completion of clinical trials that demonstrated maintenance of bone density in postmenopausal women at risk for osteoporosis [60].

As part of a safety profile for any estrogen-like drug for the prevention of osteoporosis, raloxifene had to be evaluated for breast safety. To this end, Eli Lilly organized an independent oncology advisory committee to adjudicate all breast cancers diagnosed in the randomized, placebo-controlled trials for the

Uncorrected Proof

Author's Proof

Table 7.2 The Raloxifene Oncology Advisory Committee formed by Eli Lilly t2.1

The Raloxifene Oncology Advisory Committee[a] t2.2
Alberto Costa, M.D.—European Institute for Oncology, Milan (Breast Surgeon, Co-PI Italian Tamoxifen Prevention Trial) t2.3
V. Craig Jordan, Ph.D., D.Sc.—Northwestern University Medical School, Chicago (Committee Chairperson) t2.4
Marc E. Lippman, M.D.—Georgetown University Medical School, Washington DC (Director, Lombardi Comprehensive Cancer Center) t2.5
Monica Morrow, M.D.—Northwestern University Medical School, Chicago (Breast Surgeon, Director, Lynn Sage Breast Cancer Program) t2.6
Larry Norton, M.D.—Memorial Sloan-Kettering Cancer Center, New York (Head, Division of Oncology) t2.7
Trevor J. Powels, FRCP, Ph.D.—Royal Marsden Hospital, London (Medical Oncologist, PI Royal Marsden, Tamoxifen Prevention Study) t2.8

[a]Responsible for the evaluation and adjudication of breast cancer cases in the 10,533 patients participating in randomized, placebo-controlled trials to prevent osteoporosis t2.9

prevention of osteoporosis. The committee (Table 7.2) was assembled to provide expertise in diagnosis, breast cancer prevention, and breast medical oncology. Committee members met every 6 months to review pathology, mammograms, and patient records to determine whether disease was preexisting at the time of entry to the trial and whether the cancer was invasive or noninvasive. All patients who developed breast cancer in all trials were adjudicated blind, and the results were then collated and analyzed by Biostatistician Steven Eckert of Eli Lilly.

The pivotal registration trial to establish the efficacy and value of raloxifene for the treatment and prevention of osteoporosis was called Multiple Outcomes of Raloxifene Evaluation (MORE) [61]. The MORE trial was a randomized double-blind trial that recruited 7,705 postmenopausal women (mean age 66.5 years) with osteoporosis defined as prior vertebral fractures or femoral neck or a spine T score 2.5SD or more below that of non-osteoporotic women. Participants were randomized to placebo or two raloxifene treatment groups: 60 or 120 mg daily.

Based on the positive results from the MORE trial, raloxifene is currently FDA approved for the prevention of osteoporosis. Raloxifene, 60 mg daily, produces a 1–2 % increase in postmenopausal bone density—an increase equivalent to that noted with tamoxifen. Raloxifene also reduces fractures by about 30–40 %. In addition, raloxifene is also approved to prevent osteoporosis in Europe and in more than a dozen other countries.

As part of the evaluation of osteoporosis in the MORE trial, there were several preplanned additional outcomes measures: histologically confirmed breast cancer, transvaginal ultrasonography to evaluate uterine effects of raloxifene in 1,781 randomly chosen participants, and an assessment of DVT and pulmonary embolism by chart review.

The MORE trial, analyzed at 3 years of follow-up, documented 27 cases of breast cancer in the control (2,576 women) but only a total of 13 cases in these

Uncorrected Proof

Author's Proof

treated with raloxifene (5,129 women). In other words, 126 women would need to be treated to prevent osteoporosis to prevent one case of breast cancer: the original hypothesis and roadmap [7, 62] was valid [63]!

Most importantly, the decrease in the risk of breast cancer was confined to ER-positive disease; there was a 90 % decrease in ER-positive breast cancer but no change in ER-negative breast cancer. Unlike previous experience with tamoxifen in postmenopausal women, there was no increase in the risk of endometrial cancer during raloxifene treatment. However, there was a threefold increase in venous thrombotic disease equivalent to that reported for both tamoxifen and estrogen in postmenopausal women. It is recommended that raloxifene, tamoxifen, or estrogen replacement is not taken by women with a history of thromboembolic disorders. The analysis of the MORE trail for breast cancer incidences at 3 years was confirmed with a 4 years reanalysis [64], demonstrating a 72 % decrease in the incidence of invasive breast cancer compared to placebo. The decision was made to revise and extend the MORE trial with Continuing Outcomes Relevant to Evista (CORE) trial.

During the 8 years of the MORE/CORE trials, the incidence of invasive breast cancer and ER-positive breast cancer was reduced by 66 % and 76 % respectively with no increase in the risk of endometrial cancer ($P < 0$), no endometrial hyperplasia ($P > 0.99$), and no vaginal bleeding ($P = 0.087$).

However, the fact that raloxifene was proven to reduce the risk of breast cancer but not increase the risk of endometrial cancer mandated that tamoxifen (the FDA-approved standard of care) and raloxifene must be tested head to head in postmenopausal women at high risk for the prevention of breast cancer.

The scene was now set for the NCI/NSAP P-2 study to go forward in high-risk postmenopausal women that would put tamoxifen versus raloxifene with a primary end point: the prevention of breast cancer. No placebo arm was recruited as it was considered unethical not to use tamoxifen, the approved drug of choice known to reduce the risk of breast cancer by 50 %.

However, wisely, the MORE trial was simultaneously extended out to 8 years of raloxifene treatment for women at risk for osteoporosis. All women who volunteered to continue on raloxifene (60 mg daily) had previously taken either 60 or 120 mg raloxifene. A total of 3,510 women were in the raloxifene are compared to 1,703 women in placebo arm [65]. During the CORE trial invasive breast cancer was decreased by 59 % and ER-positive breast cancer by 66 % compared to placebo. Overall, for the continued MORE/CORE trial, invasive breast cancer was reduced by 66 % and ER-positive breast cancer by 76 %.

Although the study of long-term raloxifene in the MORE/CORE trail was necessary because the treatment and prevention of osteoporosis requires continuous treatment (no drug benefit), the data was to be important once the results of the STAR trail were evaluated (Chap. 8).

Uncorrected Proof

Author's Proof

Conclusion

In the 20 years between the 1990s and 2010, not one but two agents were shown to reduce the incidence of invasive breast cancer in postmenopausal women at high risk to develop the disease. Raloxifene was approved for the prevention of osteoporosis in high-risk women with a dramatic reduction in the incidence of breast cancer as a beneficial side effect. The side effect of endometrial cancer with tamoxifen was solved. Overall, a triumph for translational research, the creation of a roadmap to follow and a new drug group called the SERMs.

Postscript. The first study that I ever completed and presented at the International Steroid Hormone Congress in Mexico City in 1974 was on the prevention of rat mammary carcinogenesis with tamoxifen. Arthur Walpole and I had previously discussed the results and we both appreciated the significance of the data for women's health. But the idea and these data were 20 years too soon! Tamoxifen was not even FDA approved for the treatment of breast cancer until December 1977, and this was for metastatic breast cancer. There was a long way to go before the NCI would fund Dr. Fisher's NSABP trial and it would start in 1992. Over the years our Tamoxifen Teams provided most of the translational information about safety (endometrial cancer), strategies with long-term therapy and bone safety. The story of "who did what" in the laboratory at Wisconsin to "set the scene" for the exploitation of SERMs has been told in the Postscript to Chap. 5.

Uncorrected Proof

References

1. Lacassagne A (1936) Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer 27:217–225
2. Jordan VC (2003) Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2:205–213
3. Jordan VC (2006) Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 147(Suppl 1):S269–S276
4. Scottish Cancer Trials Office (MRC), Edinburgh (1987) Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee. Lancet 2:171–175 AU4
5. Fisher B, Costantino J, Redmond C et al (1989) A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. N Engl J Med 320:479–484
6. Jordan VC (1984) Biochemical pharmacology of antiestrogen action. Pharmacol Rev 36:245–276
7. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189
8. Jordan VC (2006) The science of selective estrogen receptor modulators: concept to clinical practice. Clin Cancer Res 12:5010–5013
9. Jordan VC (2007) Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nat Rev Cancer 7:46–53

Author's Proof

10. Jordan VC (2009) A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 69:1243–1254
11. EBCTG (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378:771–784
12. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48:812–815
13. Fornander T, Rutqvist LE, Cedermark B et al (1989) Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1:117–120
14. Gail MH, Brinton LA, Byar DP et al (1989) Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. J Natl Cancer Inst 81:1879–1886
15. Huggins C, Grand LC, Brillantes FP (1961) Mammary cancer induced by a single feeding of polymucular hydrocarbons, and its suppression. Nature 189:204–207
16. Gullino PM, Pettigrew HM, Grantham FH (1975) N-nitrosomethylurea as mammary gland carcinogen in rats. J Natl Cancer Inst 54:401–414
17. Dao TL (1962) The role of ovarian hormones in initiating the induction of mammary cancer in rats by polynuclear hydrocarbons. Cancer Res 22:973–981
18. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
19. Turcot-Lemay L, Kelly PA (1980) Characterization of estradiol, progesterone, and prolactin receptors in nitrosomethylurea-induced mammary tumors and effect of antiestrogen treatment on the development and growth of these tumors. Cancer Res 40:3232–3240
20. Jordan VC, Allen KE, Dix CJ (1980) Pharmacology of tamoxifen in laboratory animals. Cancer Treat Rep 64:745–759
21. Wilson AJ, Tehrani F, Baum M (1982) Adjuvant tamoxifen therapy for early breast cancer: an experimental study with reference to oestrogen and progesterone receptors. Br J Surg 69:121–125
22. Jordan VC (1983) Laboratory studies to develop general principles for the adjuvant treatment of breast cancer with antiestrogens: problems and potential for future clinical applications. Breast Cancer Res Treat 3(Suppl):S73–S86
23. Gottardis MM, Jordan VC (1987) Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 47:4020–4024
24. Lacassagne A (1955) Endocrine factors concerned in the genesis of experimental mammary carcinoma. J Endocrinol 13:ix–xviii
25. Harper MJ, Walpole AL (1967) A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J Reprod Fertil 13:101–119
26. Terenius L (1971) Structure-activity relationships of anti-oestrogens with regard to interaction with 17-beta-oestradiol in the mouse uterus and vagina. Acta Endocrinol (Copenh) 66:431–447
27. Jordan VC (1975) Prolonged antioestrogenic activity of ICI 46, 474 in the ovariectomized mouse. J Reprod Fertil 42:251–258
28. Bittner JJ (1939) Relation of nursing to the extra-chromosomal theory of breast cancer in mice. Am J Cancer 35:90–97
29. Lathrop AE, Loeb L (1916) Further investigations on the origin of tumors in mice. III. On the part played by internal secretion in the spontaneous development of tumors. J Cancer Res 1:1–19
30. Hynes NE, Groner B, Michalides R (1984) Mouse mammary tumor virus: transcriptional control and involvement in tumorigenesis. Adv Cancer Res 41:155–184
31. Peters G, Brookes S, Placzek M et al (1989) A putative int domain for mouse mammary tumor virus on mouse chromosome 7 is a 5′ extension of int-2. J Virol 63:1448–1450

Uncorrected Proof

Author's Proof

32. Jordan VC, Lababidi MK, Mirecki DM (1990) Anti-oestrogenic and anti-tumour properties of prolonged tamoxifen therapy in C3H/OUJ mice. Eur J Cancer 26:718–721
33. Jordan VC, Lababidi MK, Langan-Fahey S (1991) Suppression of mouse mammary tumorigenesis by long-term tamoxifen therapy. J Natl Cancer Inst 83:492–496
34. Welsch CW, Goodrich-Smith M, Brown CK, Miglorie N, Clifton KH (1981) Effect of an estrogen antagonist (tamoxifen) on the initiation and progression of gamma-irradiation-induced mammary tumors in female Sprague–Dawley rats. Eur J Cancer Clin Oncol 17:1255–1258
35. Cuzick J, Baum M (1985) Tamoxifen and contralateral breast cancer. Lancet 2:282
36. Powles TJ, Hardy JR, Ashley SE et al (1989) A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. Br J Cancer 60:126–131
37. Powles TJ, Jones AL, Ashley SE et al (1994) The Royal Marsden Hospital pilot tamoxifen chemoprevention trial. Breast Cancer Res Treat 31:73–82
38. Powles T, Eeles R, Ashley S et al (1998) Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomised chemoprevention trial. Lancet 352:98–101
39. Jones AL, Powles TJ, Treleaven JG et al (1992) Haemostatic changes and thromboembolic risk during tamoxifen therapy in normal women. Br J Cancer 66:744–747
40. Powles TJ, Tillyer CR, Jones AL et al (1990) Prevention of breast cancer with tamoxifen—an update on the Royal Marsden Hospital pilot programme. Eur J Cancer 26:680–684
41. Powles TJ, Hickish T, Kanis JA, Tidy A, Ashley S (1996) Effect of tamoxifen on bone mineral density measured by dual-energy x-ray absorptiometry in healthy premenopausal and post-menopausal women. J Clin Oncol 14:78–84
42. Kedar RP, Bourne TH, Powles TJ et al (1994) Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial. Lancet 343:1318–1321
43. Decensi A, Fontana V, Bruno S et al (1996) Effect of tamoxifen on endometrial proliferation. J Clin Oncol 14:434–440
44. King MC, Wieand S, Hale K et al (2001) Tamoxifen and breast cancer incidence among women with inherited mutations in BRCA1 and BRCA2: National Surgical Adjuvant Breast and Bowel Project (NSABP-P1) Breast Cancer Prevention Trial. JAMA 286:2251–2256
45. Fisher B, Costantino JP, Wickerham DL et al (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90:1371–1388
46. Ganz PA (2001) Impact of tamoxifen adjuvant therapy on symptoms, functioning, and quality of life. J Natl Cancer Inst Monogr 30:130–134
47. Day R, Ganz PA, Costantino JP et al (1999) Health-related quality of life and tamoxifen in breast cancer prevention: a report from the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Clin Oncol 17:2659–2669
48. Veronesi U, Maisonneuve P, Costa A et al (1998) Prevention of breast cancer with tamoxifen: preliminary findings from the Italian randomised trial among hysterectomised women. Italian Tamoxifen Prevention Study. Lancet 352:93–97
49. Cuzick J, Forbes J, Edwards R et al (2002) First results from the International Breast Cancer Intervention Study (IBIS-I): a randomised prevention trial. Lancet 360:817–824
50. Fisher B, Costantino JP, Wickerham DL et al (2005) Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97:1652–1662
51. Cuzick J, Forbes JF, Howell A (2006) Re: Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 98:643, author reply 643–644
52. Powles TJ (1999) Re: Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 91:730
53. Jordan VC, Morrow M (1994) Should clinicians be concerned about the carcinogenic potential of tamoxifen? Eur J Cancer 30A:1714–1721

Uncorrected Proof

Author's Proof

54. Iqbal J, Ginsburg OM, Wijeratne TD et al (2012) Endometrial cancer and venous thromboembolism in women under age 50 who take tamoxifen for prevention of breast cancer: a systematic review. Cancer Treat Rev 38:318–328
55. Black LJ, Jones CD, Falcone JF (1983) Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 32:1031–1036
56. Black LJ, Sato M, Rowley ER et al (1994) Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 93:63–69
57. Jordan VC, Gosden B (1983) Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 113:463–468
58. Clemens JA, Bennett DR, Black LJ, Jones CD (1983) Effects of a new antiestrogen, keoxifene (LY156758), on growth of carcinogen-induced mammary tumors and on LH and prolactin levels. Life Sci 32:2869–2875
59. Jordan VC, Phelps E, Lindgren JU (1987) Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10:31–35
60. Delmas PD, Bjarnason NH, Mitlak BH et al (1997) Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N Engl J Med 337:1641–1647
61. Ettinger B, Black DM, Mitlak BH et al (1999) Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 282:637–645
62. Jordan VC (1988) Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. Cancer Invest 6:589–595
63. Cummings SR, Eckert S, Krueger KA et al (1999) The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA 281:2189–2197
64. Cauley JA, Norton L, Lippman ME et al (2001) Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial. Multiple outcomes of raloxifene evaluation. Breast Cancer Res Treat 65:125–134
65. Martino S, Cauley JA, Barrett-Connor E et al (2004) Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 96:1751–1761

Uncorrected Proof

Author's Proof

Author Queries

Chapter No.: 7

Query Refs.	Details Required	Author's response
AU1	Please check sentence starting "Tamoxifen became...." for completeness.	
AU2	Please check if edit to sentence starting "The change in. . ." is okay.	
AU3	Please check sentence starting "Women were randomized. . ." for clarity.	
AU4	Please check Ref. [4] for correctness.	

Uncorrected Proof

Author's Proof

Metadata of the chapter that will be visualized online

Chapter Title	Tamoxifen and Raloxifene Head to Head: The STAR Trial	
Copyright Year	2013	
Copyright Holder	Springer Basel	
Corresponding Author	Family Name	**Maximov**
	Particle	
	Given Name	**Philipp Y.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**McDaniel**
	Particle	
	Given Name	**Russell E.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**Jordan**
	Particle	
	Given Name	**V. Craig**
	Suffix	
	Division	Lombardi Comprehensive Cancer Center
	Organization	Georgetown University Medical Center
	Address	Washington, DC, USA
Abstract	The toxicological concern with the potential of tamoxifen to increase the incidence of endometrial cancer or hepatocellular carcinoma mandated a new approach to chemoprevention. The SERM raloxifene does not have the toxicological concern of tamoxifen and is approved for the treatment and prevention of osteoporosis but at the same time reduces breast cancer incidence. The Study of Tamoxifen and Raloxifene (STAR) demonstrated that the two SERMs were equivalent in reducing breast cancer incidence but raloxifene had a better safety profile. However, tamoxifen can reduce breast cancer incidence during therapy for 5 years, and this is maintained for at least a decade after treatment. In contrast, raloxifene must be given continuously.	

Author's Proof

Chapter 8
Tamoxifen and Raloxifene Head to Head: The STAR Trial

Abstract The toxicological concern with the potential of tamoxifen to increase the incidence of endometrial cancer or hepatocellular carcinoma mandated a new approach to chemoprevention. The SERM raloxifene does not have the toxicological concern of tamoxifen and is approved for the treatment and prevention of osteoporosis but at the same time reduces breast cancer incidence. The Study of Tamoxifen and Raloxifene (STAR) demonstrated that the two SERMs were equivalent in reducing breast cancer incidence but raloxifene had a better safety profile. However, tamoxifen can reduce breast cancer incidence during therapy for 5 years, and this is maintained for at least a decade after treatment. In contrast, raloxifene must be given continuously.

Uncorrected Proof

The STAR trial recruitment and evaluation was unprecedented in the history of clinical cancer trials (Fig. 8.1). The STAR trial was a phase III, double-blind trial that screened 184,480 postmenopausal women (mean age 58.5 years) for a full year with breast cancer risk over 1.65 %, and 19,747 were subsequently randomized to receive either tamoxifen (20 mg daily) or raloxifene (60 mg daily) for 5 years (Fig. 8.2). The primary aim of the trial was to assess the occurrence of invasive breast cancer in postmenopausal high-risk women with raloxifene and compare the preventive efficacy with, by then an established drug, tamoxifen. The secondary aim was to establish the efficacy of raloxifene treatment, such as cardiovascular, bone density, and general toxicities. Three groups of women were eligible: postmenopausal women over 60, irrespective of their risk of breast cancer; postmenopausal women who were diagnosed previously with lobular carcinoma in situ (LCIS); and postmenopausal women between the ages of 35 and 59, who have a high risk of developing breast cancer based on the presence of a combination of risk factors. The risk factors were assessed by using a modified Gail model that was used in the NSABP/P-1 trial. The main risk factors included age; number of first-degree relatives who have been diagnosed with breast cancer; whether the woman has had any children and the age of the first delivery; history of biopsies, especially if the results have shown atypical hyperplasia; and the age of the woman's first menstrual period.

P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
DOI 10.1007/978-3-0348-0664-0_8, © Springer Basel 2013

Author's Proof

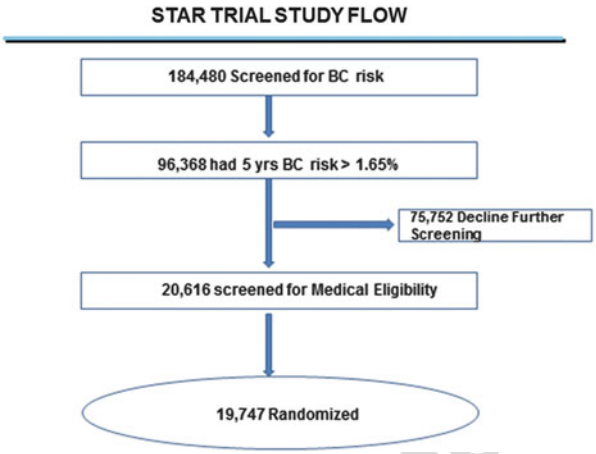


Fig. 8.1 STAR trial recruitment scheme. A total of 19,747 postmenopausal women were selected based on their eligibility to participate in the study

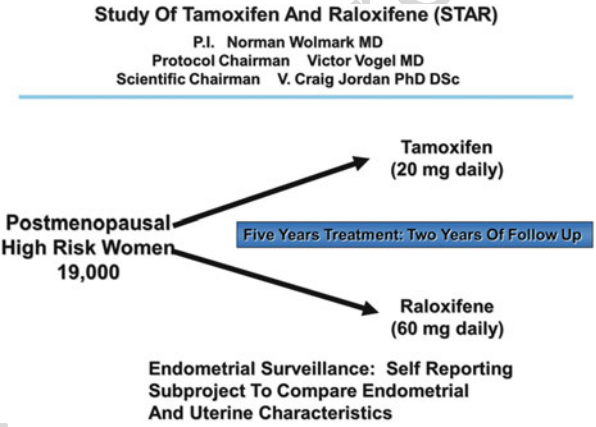


Fig. 8.2 STAR trial randomization scheme. A total of 19,747 selected women were randomized to be treated with either 20 mg of tamoxifen daily or 60 mg of raloxifene daily

A preplanned analysis was triggered when a total of 327 incidents of invasive breast cancers occurred. The trial was conducted beginning 1 July 1999 and was assessed at a cutoff date of 31 December 2005. The data reported initially were 6 years and 5 months after the STAR trial initiated recruitment [1].

There were a total of 168 invasive breast cancers in the raloxifene-treated group and 163 invasive breast cancers in the tamoxifen-treated group (Fig. 8.3). A control arm was not considered to be appropriate as tamoxifen was the FDA-approved medicine and the standard of care, but an estimate of invasive breast cancer in a hypothetical control arm based on the level of risk in an equivalent number of women not treated with a SERM was estimated at 312 (Fig. 8.3). Thus, both tamoxifen and raloxifene are producing about a 50 % decrease in breast cancer incidence. There were however fewer noninvasive breast cancer (57 cases) in the tamoxifen-treated group compared with the raloxifene-treated group (80 cases), but this was barely statistically significant ($P = 0.052$) (Fig. 8.4). However, a later

Author's Proof

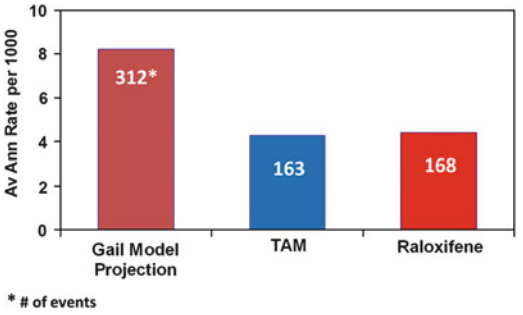


Fig. 8.3 The results of invasive breast cancer reduction in STAR trial. Raloxifene virtually was equivalent to tamoxifen in reducing the incidence of invasive breast cancer by 50 %, as compared to the projected untreated control. It was considered unethical to use untreated control as an approved breast cancer treatment with tamoxifen already was available at the time

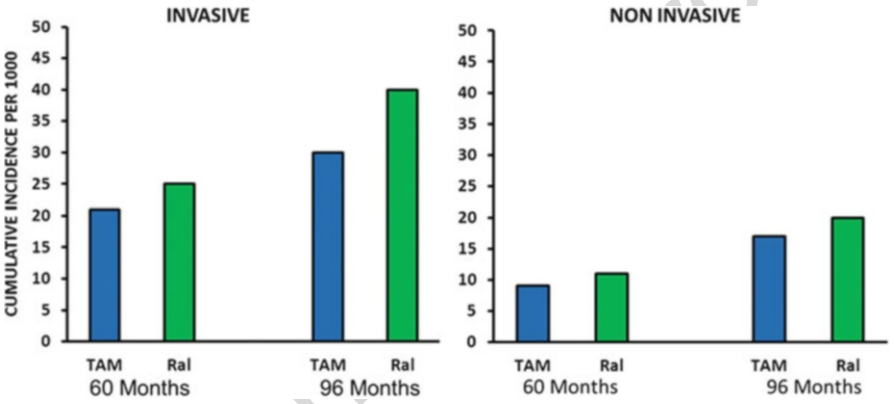


Fig. 8.4 Cumulative incidence of invasive breast cancer and noninvasive breast cancer in women treated with tamoxifen and raloxifene and followed up at 60 and 96 months post randomization

statistical study was initiated to assess the actual benefit/risk for breast cancer prevention for postmenopausal women [2]. The data were pooled from the Women's Health Initiative, STAR trial, and End Results Program and were used to develop a benefit/risk assessment index, which could be used for assessing the chemoprevention benefits with either raloxifene or tamoxifen. The results of the statistical analysis demonstrated that benefit/risk index was dependent on age, race, and history of hysterectomy. Postmenopausal women with no hysterectomy treated with raloxifene generally have better index than those treated with tamoxifen and so do premenopausal women with prior hysterectomy.

In contrast, there were fewer endometrial cancer (23 cases) in patients treated with raloxifene then those treated with tamoxifen (36 cases), though this does not reveal statistical significance ($P < 0.07$) (Fig. 8.5). However, this is deceptive as tamoxifen has a fundamentally different effect on the uterus than raloxifene.

Author's Proof

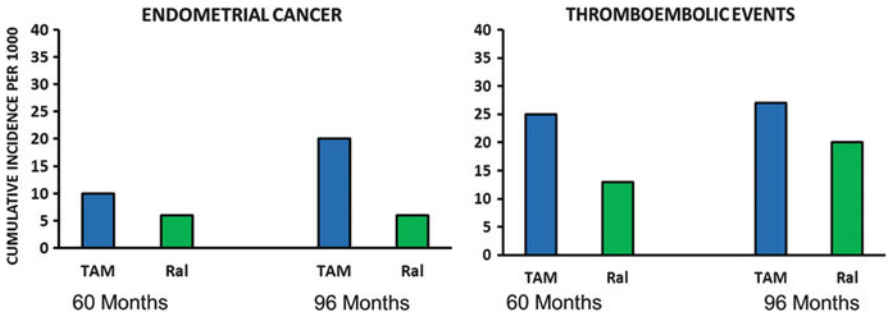


Fig. 8.5 Cumulative incidence of uterine cancers and thromboembolic events in women treated with either tamoxifen or raloxifene and followed up at 60 and 96 months post randomization

t1.1 **Table 8.1** The rates of developed cataracts and cataract surgeries during STAR trial. A total of 8,341 women were treated with tamoxifen and 8,336 were treated with raloxifene

t1.2		Events		Rate per 1,000	
t1.3	Cataracts and cataract surgery	Tamoxifen	Raloxifene	Tamoxifen	Raloxifene
t1.4	Developed cataracts	739	603	14.58	11.69
t1.5	Cataracts followed by surgery	575	462	11.18	8.85

Women elected to have 244 hysterectomies on tamoxifen can be compared to 111 hysterectomies in patients taking raloxifene. Similarly, there were fewer thrombotic events ($P = 0.01$) (Fig. 8.5), cataracts ($P = 0.002$), and cataract surgeries ($P = 0.03$) in women being treated with raloxifene (Table 8.1).

Therefore, overall, tamoxifen and raloxifene are equivalent during the treatment phase, for reducing the risk of breast cancer in high-risk postmenopausal women, but raloxifene appears to have a better safety profile than tamoxifen during treatment. However, this is where the pharmacology becomes interesting.

A subsequent analysis of the STAR trial at 90 months after initiating recruitment was reported [3]. Interestingly, the efficacy of raloxifene and tamoxifen did not remain equivalent in the post treatment phase of the study. The 5-year "pulse" of tamoxifen treatment seemed to have changed the breast cancer tissue or changed the tumor environment so that even 5 years after the therapy cessation, the occurrence of contralateral breast cancer was still prevented [4]. Similar results were shown in animals, where the number of breast tumors in tamoxifen-treated rats never reached the same levels as in control animals [5]. The efficacy of raloxifene to reduce the incidence of invasive breast cancer decreases so that within 2–3 years after treatment, raloxifene only retain 76 % of the ability of tamoxifen to prevent the occurrence of invasive breast cancer in post treatment period. However, based on the Martino study [6], raloxifene should be consulted as a continuous therapy and should not be stopped at 5 years.

Concerning safety with raloxifene, there was now a significant decrease in the incidence of endometrial cancers ($P = 0.003$), thrombotic events ($P = 0.007$),

Uncorrected Proof

Author's Proof

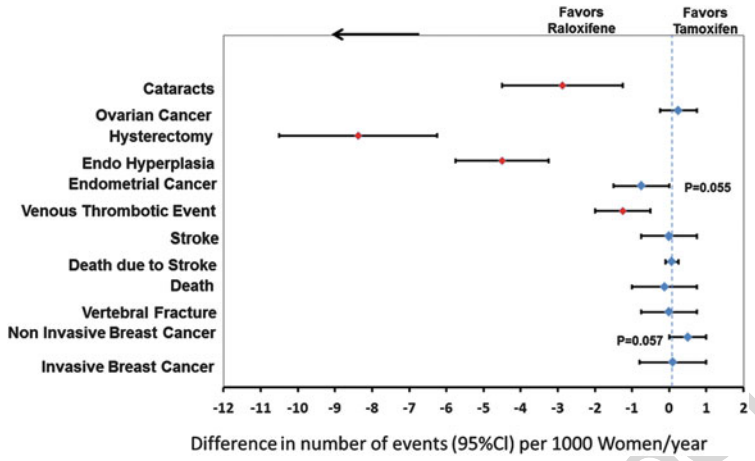


Fig. 8.6 Efficacy and important outcomes in favor of raloxifene and tamoxifen in the STAR trial

(Fig. 8.6) cataracts, and cataract operations (RR = 0.80; 95 % CI, 0.72–0.89 and RR = 0.79; 95 % CI, 0.70–0.90, respectively) (Fig. 8.6). As a summary of the efficacy and important outcomes of the STAR trial, Fig. 8.6 addresses outcomes in favor of either raloxifene or tamoxifen.

Raloxifene was approved by the FDA for the reductions of incidence of breast cancer in high-risk postmenopausal women and breast cancer in postmenopausal women with osteoporosis on 24 July 2007.

Postscript. During the past 40 years, the idea of preventing breast cancer in women at high risk for developing the disease has advanced from just that, an idea [7] to become a practical reality with not one but two FDA-approved medicines. Forty years ago, there was no tamoxifen, only ICI 46,474, a failed "morning-after pill" that was reinvented as an antiestrogen throughout the 1970s, and a strategy was established to enable progress to move forward in the clinic for both chemoprevention and long-term adjuvant therapy with an antihormonal agent [8]. Indeed, at that time, the word "chemoprevention" was not even in the English language. It was still for Michael Sporn to invent the idea of using chemicals to prevent cancer [9, 10] and establish the word chemoprevention. Raloxifene the failed "breast cancer drug" was conceptually reinvented as the first SERM to treat osteoporosis and prevent breast cancer at the University of Wisconsin Comprehensive Cancer Center.

At the start of the STAR trial, Dr. Norman Wolmark, principal investigator of the NCI grant and of the NSABP, appointed me as the scientific chair for the clinical trial. His goal was to recruit a qualified scientist to address unanticipated issues of importance to our patients, should they arise. My expertise was translational research on both SERMs. Fortunately, and remarkably, no cases arose during the tenure of the STAR trial (in contrast to the NCI/NSABP P-1 trail).

Uncorrected Proof

Author's Proof

The question was asked to me more than once: "How will you feel if raloxifene proves to be superior to tamoxifen in the STAR trial?" "Delighted" would be my reply as the scientific foundations for the applications of both SERMs (and the concept of the new SERM drug group—Chap. 5) had both emerged from my laboratory. Firstly with the reinvention of tamoxifen, a failed contraceptive to a potential chemopreventive at the Worcester Foundations and as a long-term adjuvant therapy at the University of Leeds in the early 1970s [11, 12]. Raloxifene was a failed breast cancer drug originally called keoxifene that was abandoned in the late 1980s by the pharmaceutical industry but reinvented in my laboratory at the Wisconsin Clinical Cancer Center as a potential candidate medicine with the goal: "prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer" [13]. Our laboratory data was subsequently confirmed [14], and clinical testing for raloxifene to treat and prevent osteoporosis advanced from about 1992 through clinical testing by the pharmaceutical industry. AU1

The evaluation of the use of two SERMs with different characteristics in the STAR trial taught important lessons in translational research. In the laboratory during the 1980s, it was clear that the structurally related polyhydroxylated compounds LY117,018 and LY156,758 were short acting and rapidly excreted drugs compared to tamoxifen [15–17]. Much higher daily doses of rapidly excreted antiestrogens were necessary for effective antitumor action [5, 17], and the antiestrogenic effects of the polyhydroxylated compounds disappeared rapidly once the drug is stopped. In contrast, tamoxifen accumulates. Thus, large daily doses of raloxifene are necessary to achieve the same efficacy as tamoxifen, and this is true with raloxifene (tamoxifen is used at a standard dose of 20 mg daily; the MORE trial used 60 vs. 120 mg daily of raloxifene). Additionally, the relative clearance rate of raloxifene and tamoxifen would have implications for a correlation between compliance and the actions of the SERMs to be chemopreventive agents in breast cancer. Tamoxifen accumulates but the drug can still be detected in the circulations 6 weeks after the last dose. Raloxifene is cleared rapidly within a few days of the last dose. Thus, missing a few tamoxifen tablets is of little consequence to the efficacy of the drug, but regularly missing raloxifene doses exposes the patient to estrogen-induced proliferations of nascent breast tumors. There is also another important aspect of tamoxifen's pharmacology that is only recently being understood. Long-term adjuvant tamoxifen therapy [18, 19] and chemoprevention [20–22] retain antitumor actions long after tamoxifen is stopped. This was noted in the NSABP P-1 trail [21] and in the NSABP/STAR P-2 trial [3]. In contrast, raloxifene was unable to maintain long-term antitumor effects in the STAR trial [3], confirming the earlier laboratory data [5, 17]. It was this finding that prompted the conclusion of recommendations from the NSABP: "It is unlikely that the optimal durations of raloxifene for chemoprevention will be evaluated in a breast cancer prevention setting; however, the use of raloxifene in treating and preventing osteoporosis is approved for an indefinite period time. Therefore, continuing raloxifene therapy beyond 5 years might be an approach that would preserve its full chemopreventive activity" [3].

Uncorrected Proof

Author's Proof

References 154

1. Vogel VG, Costantino JP, Wickerham DL et al (2006) Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 295:2727–2741
2. Freedman AN, Yu B, Gail MH et al (2011) Benefit/risk assessment for breast cancer chemoprevention with raloxifene or tamoxifen for women age 50 years or older. J Clin Oncol 29:2327–2333
3. Vogel VG, Costantino JP, Wickerham DL et al (2010) Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: preventing breast cancer. Cancer Prev Res (Phila) 3:696–706
4. Early Breast Cancer Trialists' Collaborative Group (1998) Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 351:1451–1467
5. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
6. Martino S, Cauley JA, Barrett-Connor E et al (2004) Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. J Natl Cancer Inst 96:1751–1761
7. Lacassagne A (1936) Hormonal pathogenesis of adenocarcinoma of the breast. Am J Cancer 27:217–225
8. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44:30–38
9. Sporn MB, Dunlop NM, Newton DL, Smith JM (1976) Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). Fed Proc 35:1332–1338
10. Sporn MB (1976) Approaches to prevention of epithelial cancer during the preneoplastic period. Cancer Res 36:2699–2702
11. Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
12. Jordan VC (1974) Antitumour activity of the antiestrogen ICI 46,474 (Tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. J Steroid Biochem 5:354
13. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189
14. Black LJ, Sato M, Rowley ER et al (1994) Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 93:63–69
15. Jordan VC, Gosden B (1983) Inhibition of the uterotropic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. Endocrinology 113:463–468
16. Jordan VC, Gosden B (1983) Differential antiestrogen action in the immature rat uterus: a comparison of hydroxylated antiestrogens with high affinity for the estrogen receptor. J Steroid Biochem 19:1249–1258
17. Gottardis MM, Jordan VC (1987) Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 47:4020–4024
18. EBCTCG (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 365:1687–1717
19. Davies C, Godwin J, Gray R et al (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378:771–784
20. Cuzick J, Forbes JF, Sestak I et al (2007) Long-term results of tamoxifen prophylaxis for breast cancer – 96-month follow-up of the randomized IBIS-I trial. J Natl Cancer Inst 99:272–282

Uncorrected Proof

Author's Proof

21. Fisher B, Costantino JP, Wickerham DL et al (2005) Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97:1652–1662
22. Powles TJ, Ashley S, Tidy A et al (2007) Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. J Natl Cancer Inst 99:283–290

Uncorrected Proof

Author's Proof

Author Queries

Chapter No.: 8

Query Refs.	Details Required	Author's response
AU1	Please check sentence starting "Firstly with the...." for completeness.	

Uncorrected Proof

Author's Proof

Metadata of the chapter that will be visualized online

Chapter Title	Acquired Resistance to Tamoxifen: Back to the Beginning	
Copyright Year	2013	
Copyright Holder	Springer Basel	
Corresponding Author	Family Name	**Maximov**
	Particle	
	Given Name	**Philipp Y.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**McDaniel**
	Particle	
	Given Name	**Russell E.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**Jordan**
	Particle	
	Given Name	**V. Craig**
	Suffix	
	Division	Lombardi Comprehensive Cancer Center
	Organization	Georgetown University Medical Center
	Address	Washington, DC, USA
Abstract	The clinical acceptance and validation of the therapeutic strategy of long-term adjuvant tamoxifen treatment mandated an examination of acquired drug resistance under laboratory conditions. The first model in vivo of acquired resistance of ER-positive breast cancer cells transplanted into immune deficient mice demonstrated tamoxifen-stimulated tumor growth after about 2 years of continuous treatment. When tamoxifen was stopped, tumors also grew with physiologic estradiol. The model showed that no estrogen (similar to the use of aromatase inhibitors) or a pure antiestrogen to destroy ER (fulvestrant) presaged this therapeutic approach in clinical trials a decade later. However, the long-term retransplantation of breast tumors with acquired tamoxifen resistance for at least 5 years demonstrated a vulnerability of these tumors. Tamoxifen-stimulated tumor growth but physiologic estrogen now caused tumor regression and apoptosis. The new biology of estrogen-induced apoptosis now is used to explain the decrease in mortality after adjuvant tamoxifen is stopped in patients and also the value of conjugated equine estrogens to reduce breast cancer incidence in women treated in their 60s.	

Author's Proof

Chapter 9
Acquired Resistance to Tamoxifen: Back to the Beginning

Abstract The clinical acceptance and validation of the therapeutic strategy of long-term adjuvant tamoxifen treatment mandated an examination of acquired drug resistance under laboratory conditions. The first model in vivo of acquired resistance of ER-positive breast cancer cells transplanted into immune deficient mice demonstrated tamoxifen-stimulated tumor growth after about 2 years of continuous treatment. When tamoxifen was stopped, tumors also grew with physiologic estradiol. The model showed that no estrogen (similar to the use of aromatase inhibitors) or a pure antiestrogen to destroy ER (fulvestrant) presaged this therapeutic approach in clinical trials a decade later. However, the long-term retransplantation of breast tumors with acquired tamoxifen resistance for at least 5 years demonstrated a vulnerability of these tumors. Tamoxifen-stimulated tumor growth but physiologic estrogen now caused tumor regression and apoptosis. The new biology of estrogen-induced apoptosis now is used to explain the decrease in mortality after adjuvant tamoxifen is stopped in patients and also the value of conjugated equine estrogens to reduce breast cancer incidence in women treated in their 60s.

Uncorrected Proof

Introduction

The idea that the determination of the estrogen receptor (ER) content in the breast tumor of a patient with metastatic breast cancer would predict response to ablative endocrine surgery (oophorectomy, adrenalectomy, hypophysectomy) became a clinical reality and requirement for each breast cancer patient in the mid-1970s [1]. This was established on a National Cancer Institute sponsored meeting in Bethesda, Maryland, at the Holiday Inn in 1974. The rationale was that if the ER was not present in the tumor, then the patient should not have ablative surgery. This would be 30 % of all patients and there would be no response. There would be a response of about 60 % in patients with an ER-positive tumor. Tamoxifen, however, was not considered in these deliberations as it was not yet in clinical trial in America.

P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
DOI 10.1007/978-3-0348-0664-0_9, © Springer Basel 2013

Author's Proof

Tamoxifen was Food and Drug Administration (FDA) approved and available to treat metastatic breast cancer in December 1977. By then, the ER had evolved into the target for tamoxifen action [2] and has subsequently become a drug to be used as a potential long-term adjuvant therapy, and there was laboratory data to indicate there was potential for tamoxifen as the first chemopreventive for breast cancer.

The fact that all metastatic breast tumors with ER did not respond to tamoxifen treatment, and those tumors that do respond, do so for about 1–2 years [3], created a classification of intrinsic resistance where treatment fails to control tumor growth at the 2-month evaluation point and acquired resistance where the tumor eventually escapes from estrogen blockade of the ER by tamoxifen and grows autonomously.

The prevalent theory in the 1970s for acquired resistance to endocrine therapy was that tumors were heterogeneous and those cells containing ER were controlled and died out, and the ER-negative tumor cells overgrew and become dominant. Thus, a tumor would evolve from ER positive to become ER negative. However, this was inconsistent with clinical experience by the medical oncology community. Select breast cancer could respond to, the then standard of care, high-dose diethylstilbestrol (DES) fail therapy evidenced by tumor regrowth, have a withdrawal response by stopping (DES) treatment, once the tumor regrew the clinician would try high-dose androgen or progestin therapy. This whole process of alternating endocrine therapies is called the endocrine treatment cascade and is used successfully to this day in selected patients before using cytotoxic combination chemotherapy. The practice of medicine therefore was not consistent with the theory that resistance occurred with a trend to ER-negative cell populations: the theory must therefore be incorrect. The solution to the problem was to come from studies utilizing athymic mice to grow human breast cancer cell lines and to study acquired resistance to tamoxifen.

AU1

The MCF-7 Breast Cancer Cell Line

The important cell line created by Soule and coworkers [4]at the Michigan Cancer Foundation (MCF) was from a pleural effusion from a nun, Sister Catherine Frances, initially treated with high-dose DES (tamoxifen was not available at that time). The cell line is ER positive [5] and became the "work horse" in the laboratory for the study of hormone-dependent breast cancer. Tamoxifen blocked spontaneous growth of MCF-7 cells in culture [6] and estradiol reversed the tamoxifen blockade. Estrogen did not however enhance growth in culture but it did in athymic mice [7] leading to the idea that a second factor in vivo was required. Within the decade of the 1980s, the Katzenellenbogen laboratory would discover that the ubiquitous phenol red indicator used in culture media contained a potent estrogenic contaminant [8, 9] (Fig. 1.7, Chap. 1).

Nevertheless, it was probably fortunate that the ER-positive MCF-7 cells were always grown in an estrogen-containing environment to maintain their hormone-responsive characteristics. Without estrogen in the media, the estrogen-responsive cells die [10].

Uncorrected Proof

Author's Proof

The value of the MCF-7 cell line to breast cancer research has been reviewed previously [11]. Throughout the 1980s and 1990s, numerous reports of tamoxifen resistance or resistance to raloxifene-like molecules or pure antiestrogens were published using MCF-7 cells in vitro or with other ER-positive cells [12–23]. We will, however, focus here on the importance of the transplantation of MCF-7 cells into athymic mice to create a breakthrough in deciphering acquired drug resistance. Shafie and Granthan [24] first showed that MCF-7 tumors grow in athymic mice with estradiol treatment, but not with tamoxifen treatment. Osborne [25] demonstrated that tamoxifen would control estrogen-stimulated MCF-7 growth in athymic mice but eventually the MCF-7 derived tumors would grow despite tamoxifen treatment [26]. Osborne concluded that part of the action of tamoxifen was tumoristatic; therefore, long-term treatment was necessary[26] . This was consistent with original data generated in carcinogen-induced rat mammary cancer models [27]. However, the finding that MCF-7 tumors with acquired resistance to tamoxifen grew *because* of estrogen or tamoxifen treatment not despite the tamoxifen was a discovery [28]. The tumors were transplantable and had adapted a mechanism for the tamoxifen-ER complex to cause growth somewhat similar to estrogen. Additionally, the resistance (tamoxifen-stimulated growth) is not athymic mouse specific, i.e., some strange metabolic difference or difference in NK cells. Tamoxifen-stimulated MCF-7 tumor growth occurs in either athymic rats or beige (NK deficient) mice [29]. Unfortunately (or as it turned out fortunately!), cell lines of tamoxifen-resistant tumors could not be treated and retain the tamoxifen-resistant growth phenotype. However, to explain the estrogen-like action of tamoxifen, an interesting hypothesis emerged in the early 1990s to explain tamoxifen-stimulated growth via the generation of estrogenic isomers of tamoxifen metabolites. This will be described briefly, as an interesting clinical approach was used to address the proposed molecular mechanism of acquired tamoxifen resistance. It is an excellent example of how basic structure-function relationship can resolve important clinical questions in the laboratory.

Uncorrected Proof

Tamoxifen Metabolism Hypothesis

Tamoxifen is metabolized to numerous hydroxylated compounds, some with estrogen-like actions and others with antiestrogenic actions (Chap. 3). The metabolism hypothesis with subsequent geometric isomerization to putative estrogens was based on the known estrogenic and antiestrogenic properties of the cis (ICI 47,699) and trans (ICI 46,474) isomers of tamoxifen [30–33]. It was noted that there is less tamoxifen in tumors with acquired resistance [31] and an increase in (E) 4-hydroxytamoxifen as a putative estrogen. Similar findings were made in patients failing tamoxifen therapy, i.e., lower levels of tamoxifen in the tumor and the ratio of E to Z isomers of 4-hydroxytamoxifen were higher [32]. Additionally [33], metabolite E, a weak estrogen, was identified in patients with tamoxifen refractory tumors. To evaluate the hypothesis a series of non-isomerizable fixed ring

Author's Proof

derivatives and isomers of 4-hydroxytamoxifen and metabolite E were synthesized. The E isomer of 4-hydroxytamoxifen is actually a very weak antiestrogen (not a full estrogen) [34, 35]. The Z isomer of metabolite E is only a very weak estrogen (Fig. 3.2, Chap. 3).

To address the tamoxifen metabolism hypothesis, further in vivo, a fixed ring version of tamoxifen was synthesized so that any metabolite that we produced could not isomerize to potent estrogens. Fixed ring tamoxifen is equally able to stimulate the acquired tamoxifen treatment tumors as the parent drug [36]. Thus, other resistance mechanisms, based on growth factor driven tumor growth have now become most useful for developing future therapeutic strategies either as second time treatments or if given with tamoxifen initially may prevent resistance occurring in the first place.

Growth Factor-Driven Acquired and Intrinsic Resistance

There is compelling evidence that HER 2/neu can subvert hormone-responsive growth completely in ER-positive cells. Stable transfection of MCF-7 cells with the HER 2/neu gene results in tumor growth in athymic mice not regulated by tamoxifen [37].

Over the past 20 years the Osborne Group in Texas have refined the growth factor driven MCF-7 model in vivo and defined precisely the ways of blocking either the receptors or their tyrosine kinases singly or together to create long-term responses or "cures" in vivo while applying either tamoxifen or estrogen deprivations equivalent to aromatase inhibitors treatment strategies.

In recent years antibodies targeting HER2, or tyrosine kinase inhibitors that target the HER family (1–4) have become available for clinical evaluation. Among anti-HER2 monoclonal antibodies tested, trastuzumab is now a well-known and approved drug that is established though clinical trials as an important component of the first-line treatment of patients with HER2 amplified metastatic breast cancer [38–40]. When trastuzumab is administered in a preoperative setting, this strategy increases the pathological complete response rate [38]. In a large phase III trial investigating adjuvant therapy in HER2 positive early breast cancer, the addition of trastuzumab to chemotherapy increases both the disease-free and overall survival [41]. Aside from trastuzumab there is also pertuzumab, which is a monoclonal antibody against HER2 that blocks dimerization with HER1 and HER3 [42]. Although pertuzumab has therapeutic activity in HER2 positive breast cancer patients, combination therapy with trastuzumab has proven to be more effective [43]. Ertumaxomab is another monoclonal antibody against HER2 that has demonstrated strong immunological responses in HER2 positive breast cancer patients in phase I clinical trial [44].

Aside from monoclonal anti-HER2 antibodies, the use of tyrosine kinase inhibitors to target HER2 is proving to be effective. In particular, lapatinib, a dual tyrosine kinase inhibitor of both HER1 and HER2 and of Akt and mitogen-activated AU2

Uncorrected Proof

Author's Proof

protein kinase (MAPK), has been demonstrated to inhibit cell growth and induce apoptosis in several breast cancer cell lines [45]. Results from phase I and II clinical trials have shown that lapatinib has therapeutic value in a number of tumors, in particular in breast cancer patients [46–48]. In a xenograft mouse model, lapatinib is able to prevent tamoxifen resistance [49], showing the role of increased growth factor signaling pathways in resistance [50] and the potential benefit of targeting the increased growth factor signaling to reverse tamoxifen resistance in the clinic. A systemic review of the databases from clinical trials (including phase III) demonstrates that combination therapy of HER2-positive HR-positive metastatic breast cancers in postmenopausal women with lapatinib and anastrozole is superior to lapatinib monotherapy and superior to tamoxifen treatment [51].

Thus, it is now clear that exogenous inhibitors of the HER-signaling network and other mitogenic pathways can abrogate or improve the response rate of breast cancer with acquired resistance [52].

An Evolving Model of Acquired Resistance to SERMs and Aromatase Inhibitors

The transplantable model of acquired tamoxifen resistance in ER-positive breast cancer cell lines develops within about a year [28, 53]. This is the same time that resistance to tamoxifen treatment occurs in ER-positive metastatic breast cancer. Thus, the model recapitulates acquired resistance to tamoxifen in metastatic breast cancer, and therapeutic studies in the mice mimic the second-line responses of tumors in aromatase inhibitors or fulvestrant after tamoxifen failure in clinical trial [54, 55]. The laboratory-derived tumor of acquired resistance to tamoxifen does not grow without physiologic estrogen action. This is induced by the fact that no estrogen treatment maybe like aromatase inhibitor treatment or a pure antiestrogen ICI 164,384 (the lead compound that resulted in fulvestrant [56, 57]) blocks tumor growth [58].

At this time, in the mid-1980s, it was clear that long-term adjuvant tamoxifen therapy for years or indefinitely [59–61] was showing promise for enhanced survival, and there was no early recurrence of micrometastatic disease as a result of the development of early acquired resistance. Something was conceptually wrong with the link between the endocrine treatment of metastatic breast cancer with its greater bulk and the responsiveness of micrometastatic disease that is undetectable during adjuvant therapy.

One plausible explanation was proposed in the early 1990s based on the transplantable model of acquired resistance to tamoxifen maintained through serial transplantations into further generations of tamoxifen-treated year after year athymic mice. After about 5 years of retransplantations, the tamoxifen-stimulated MCF-7 tumors changed their survival characteristics and responsiveness to estrogen. Physiologic estrogen was no longer a survival signal causing tumor growth in

Uncorrected Proof

Author's Proof

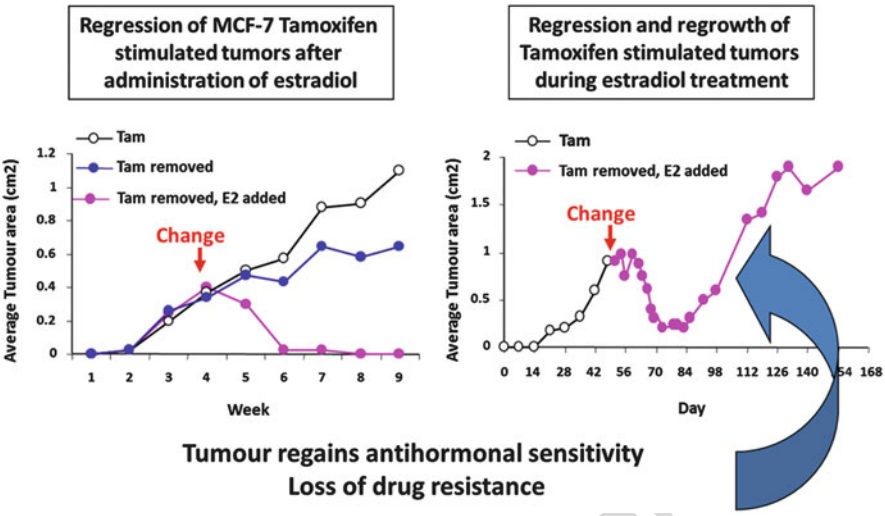


Fig. 9.1 Cyclic changes in sensitivity and resistance of breast tumors grown in vivo after treatments with tamoxifen and estradiol

the animals [28] but rather an inhibitor of tumor growth causing small tamoxifen-stimulated tumors to just melt away [62] (Fig. 9.1). It was suggested that following long-term adjuvant tamoxifen therapy, it was actually the act of stopping tamoxifen that reinforced and enhanced patient survival [63]. A woman's own estrogen was now killing the prepared and sensitized micrometastasis. Further study expanded the hypothesis to become a cyclical event with physiologic estrogen causing the destruction of a novel form of acquired antiestrogen resistance, but then the tumor was again responsive to antihormonal therapies such as aromatase inhibitors or indeed tamoxifen treatment (Fig. 9.1). It was suggested that physiologic estrogen could be used as a salvage therapy [64]. However, this was extremely controversial. The suggestion that administering estradiol to breast cancer patients after failing repeated antihormone therapies for breast cancer was unacceptable to IRBs in the 1990s and especially so to women's advocate groups. Nevertheless, with the therapeutic drift from tamoxifen to the aromatase inhibitors during the first decade of the twenty-first century, acquired resistance to estrogen deprivation became an important scientific issue.

In the 1970s and 1980s, Richard Santen [65, 66] had systematically and rigorously examined the clinical endocrine pharmacology of aminoglutethimide as an inhibitor of estrogen production, but the drug was not specific from the aromatase enzyme and glucocorticoids had to be coadministered. Angela Brodie had pioneered the practical applications of developing specific drugs to destroy the aromatase enzyme first in the 1970s at the Worcester Foundation and subsequently at the University of Maryland. Her discovery of the properties of 4-hydroxyandrostenedione went from the laboratory to clinical trial with approval in Europe [67–70]. The new aromatase inhibitors started to become the AU4

Uncorrected Pr

Author's Proof

antihormonal standard of care as long-term adjuvant therapy attention turned to acquired drug resistance. Santen's group reported that long-term estrogen-deprived (LTED) cells from the MCF-7 line would respond to estrogen in vitro initially claimed to have "acquired hypersensitivity" to minute amounts of estrogen in the environment to accomplish an apparent "estrogen-independent growth response" [71, 72]. Santen would subsequently show that LTED cells would respond to estrogen with apoptosis [73]. This was an explanation of Haddow's original chemical therapy for breast cancer, i.e., high-dose estrogen to treat postmenopausal patients with metastatic breast cancer [74, 75]. However, the new twist was that the antihormone therapies has now sensitized breast cancer cells to low doses of estrogen therapy, perhaps in the physiologic range. This concept could be used in the clinic as a salvage therapy and to explain the paradoxal new data with the estrogen replacement (CEE) in the Women's Health Initiative (WHI) of hysterectomized women [76]. There were fewer breast cancers!

Back to the Beginning

Paul Ehrlich created the first chemical therapy (chemotherapy) when he discovered Salvarsan for the treatment of syphilis in the later part of the nineteenth century. In the early years of the twentieth century, he turned his attentions to treating cancer and chose to develop animal models to facilitate drug testing. He had created this successful translational research process with his work on syphilis, so why not build on success? The year before his death in 1915, Ehrlich conceded defeat stating, "I have wasted 15 years of my life on experimental cancer research."

Sir Alexander Haddow accepted the challenge in the 1940s when he found that carcinogenic polycyclic hydrocarbons cause tumor regression in animal models. Clearly, it was not going to be possible to use these same hydrocarbons in patients, but he reasoned that the new synthetic estrogens diethylstilbestrol and the triphenylethylenes had multiple phenyl rings, so he tested them. Tumor regressions occurred in the animals, so high-dose estrogen therapy was tested in patients and produced therapeutic effect in about 30 % of metastatic breast cancer in postmenopausal women over 60 [74]. High-dose estrogen therapy remained the palliative treatment of choice until tamoxifen become the standard of care, and because it was safer and therefore more versatile, its applications extended to long-term adjuvant therapy and chemoprevention during the 1980s [3, 77].

Returning to high-dose estrogen therapy pre-tamoxifen, Haddow was the inaugural Karnofsky Memorial Lecturer at ASCO [78, 79]. In his lecture, Haddow expressed his concern about progress in cancer therapeutics. He did not believe there would ever be a cancer-specific target as Ehrlich had proposed; cancer was self, Haddow reasoned, unlike the story of antibiotics that could be tested in the laboratory to determine the correct antibiotic for the appropriate treatment of the actual disease. The crude cancer therapies were nonspecific and tried on the patient as the only way to determine whether the tumor was sensitive or not. He stated:

Uncorrected Proof

Author's Proof

t1.1 **Table 9.1** Objective response rates in postmenopausal women with metastatic breast cancer undergoing high dose estrogen therapy. The patients were divided based on years after menopause (Basil Stoll. Breast Cancer Management Early and Late. William Herman Medical Books Ltd., London pp. 133–146)

t1.2	Age since menopause	Patient #'s	% Regression
t1.3	Postmenopausal 0–5 years	63	9 %
t1.4	Postmenopausal > 5 years	344	35 %

260 the need exists for some method of prior screening to indicate the optimal choice (of
261 chemotherapy) in particular cases . . . efforts thus far have been disappointing.

262 He also stated:

263 . . . the extraordinary extent of tumour regression observed in perhaps 1 % of post-
264 menopausal cases (with oestrogen) has always been regarded as of major theoretical
265 importance, and it is a matter for some disappointment that so much of the underlying
266 mechanisms continues to elude us . . . [79]

267 The one bright glimmer of hope reason was the fact that high-dose DES was
268 extremely effective in some breast cancers. Haddow, it should be noted, also used
269 his preliminary data [74] to conduct a multicentric clinical trial through the Royal
270 Society of Medicine. He had a discovery:

271 When the various reports were assembled at the end of that time, it was fascinating to
272 discover that rather general impression, not sufficiently strong from the relatively small
273 numbers in any single group, became reinforced to the point of certainty; namely, the
274 beneficial responses were three times more frequent in women over the age of 60 years than
275 in those under that age; that oestrogens may, on the contrary, accelerate the course of
276 mammary cancer in younger women, and that their therapeutic use should be restricted to
277 cases 5 years beyond the menopause. Here was an early and satisfying example of the
278 advantages which may accrue from cooperative clinical trial.

279 This observation in clinical practice was supported by Dr. Basil Stoll whose
280 personal experience with high-dose DES for the treatment of metastatic breast
281 cancer in postmenopausal women replicated Haddow's observations (Table 9.1).
282 Thus, estrogen deprivation is the key to success for estrogen therapy, both for the
283 clinical use of high-dose therapy and for the interpretation of the CEE trial alone in
284 the WHI [76]. The women in the trial were an average 68 years of age! But can we
285 now seek a mechanism for the chain of events that causes the estrogen-ER complex
286 to trigger apoptosis?

287 Mechanisms of Estrogen-Induced Apoptosis

288 Studies of the molecular mechanisms of estradiol-induced apoptosis have occurred
289 only during the last decade. The study by Santen's group showed that estrogen
290 increases Fas ligand in LTED MCF-7 [73] cells but, by contrast, estradiol increases
291 Fas receptor in apoptotic long-term tamoxifen-resistant (phase II) MCF-7 tumors [80],

Uncorrected Proof

Author's Proof

both pointing to an extrinsic mechanism through "death receptors." However, these early studies were not time dependent but only snapshots of the apoptosis process at random times.

During the early 1990s, a couple of important estrogen-deprived cell lines were cloned from a cell population of MCF-7:WS8s following long-term (>1 year) estrogen deprivation in phenol-red-free media containing triple charcoal stripped serum. The two cell lines MCF-7:5C [81] and MCF-7:2A [82] were created in the anticipation of eventually being able to elucidate resistance to aromatase inhibitors, but they were placed in liquid nitrogen and stored for that day.

Lewis and coworkers [83] focused efforts in vitro on the MCF-7:5C cell line to describe the development of early apoptotic responses to estradiol. Rapid apoptotic events occurred at the intrinsic mitochondrial level with release of cytochrome C and a rise in proapoptotic gene products (BAX, BIM, and NOXA). Apoptosis was completely blocked by both fulvestrant (that destroys the cellular ER) and 4-hydroxytamoxifen, though the latter SERM did not affect the cell cycle in MCF-7:5C cells (i.e., these cells are resistant to SERMs). Flow cytometry was used to confirm the development of estrogen-induced apoptosis with increased annexin V and DAPI staining was used to confirm apoptosis by microscopy.

The MCF-7:2A cells only slowly go through apoptosis during the second week of estradiol treatment but this can be accelerated by using buthionine sulfoximine (BSO) to prevent glutathione synthesis [84]. The reduction of mechanisms to protect cells from reactive oxygen species is clearly an important protective measure to ensure survival of aromatase resistant cells.

The unique cell lines that are so sensitive either to estradiol-induced growth MCF-7:WS8 or rapidly apoptotic MCF-7:5C cells and slowly apoptotic MCF-7:2A cells have formed the foundations for an extensive study of the mechanistic studies of basal gene levels of activations between estrogen-responsive and estrogen-independent cell growth and the timed gene responses of all those cell lines over a 96-h period and the rate of gene activation of the MCF-7:2A cells over the second week of estrogen exposure [85].

Eric Ariazi at the Fox Chase Cancer Center working with Heather Cunliffe at Translational Genomics in Arizona created a superb Agilent gene array database for a "movie" of pathway analysis in the life and death of breast cancer cells. Essentially the study [85] creates a sequenced cooperative enrichment analysis of inflammatory responses, ER signaling, inflammation, and folding protein responsiveness in the endoplasmic reticulum during the timed move to full apoptosis. Ping Fan has described AP-1 synthesis and activation to initiate apoptosis through the accumulation of reactive oxygen species (ROS), all of which can be blocked by 4-hydroxytamoxifen or paradoxically a cSrc inhibitor [86]. But with the cascade of caspases created by estrogen action in MCF-7:5C cells and its modulations by arachidonic acid [85], the question must be asked: "What is it about the ER that triggers apoptosis in the correctly conditioned estrogen-deprived cells?" To address the question and find an answer, one must first examine the relationship between the ligand, the ER and the actual shape of the ER complex. It is this interrogation that exposed the mechanism of the "Haddow paradox" [79].

Uncorrected Proof

Author's Proof

A New Classification of Estrogens

The crystallization of the human ER ligand-binding domain with estradiol, raloxifene [87] diethylstilbestrol and 4-hydroxytamoxifen [88] precisely revealed the nature of the structural changes in the ER complex to create a mechanism of estrogen action that neatly dovetailed with the structure-activity relationships first described for modulation with the prolactin gene by the ER complex [35, 89–92] and the studies of the modulation of the transforming growth factor-alpha (TGF-α) gene by mutant ER-α in the 1990s [93, 94] and the 2000s [95–98]. Simply summarized, these studies defined the interaction of antiestrogenic side chains, correctly positioned to interact, neutralize, or shield the exposed amino acid 351 once the activation function-2 (AF-2) binding site for coactivators on helix 12 has been pushed open like the jaws of a crocodile. Pharmacologically, the angular triphenylethylenes that form the backbone of the SERMs only become antiestrogenic at appropriate target sites like the breast or uterus with a correctly positioned side chain.

But it was Geoffrey Greene [88] who used the phrase "the bulky antiestrogenic side chain" that created our next conceptual advance, as the antiestrogenic side chain was a finger like alkylaminoethoxy side chain, a trivial amount of molecular "bulk." However, Greene was including the nonplanar phenyl ring! We hypothesized that the planar and angular nonsteroidal estrogens would fit the ER ligand-binding domain differently. All estrogens were not equal. A precise biological assay of two different cell lines derived using the ER-negative MDA-MB-231 cell line either stably transfected with wild-type ER or the asp 351gly mutant. Planar (class I) estrogens such as DES and estradiol and nonplanar (class II) triphenylethylene estrogens were compared and contrasted to switch on or off the TGF-α gene. The results were a simple yes/no answer. A planar estrogen (class I) would easily fit in the binding pocket of the LBD to activate AF 2 coactivator binding formed from a closed helix 12 sealing the ligand inside. Both cell lines would activate TGF-α. In contrast, the estrogen-like activity of 4-hydroxytamoxifen with a short antiestrogenic side chain results from the negatively charged aspartate 351 communicating with AF-1 to cause estrogen action (weak as it is) and activation of the TGF-α gene. In the cells with the asp351gly mutation, there would be no activation of TGF-α [99]. A triphenylethylene estrogen had some estrogen action with wild-type ER and an exposed asp 351, but with the asp 351 gly mutant with no charge, there was none [99]. It was proof that the shape of the ER complex with a triphenylethylene had a pushed back helix 12. Simply stated, crocodile jaws closed for a class I estrogen, jaws open for a class II estrogen. In the paper, it was stated that the authors had no idea what this would mean in biology [99] but there was a claim that it could be important. We showed the effect was reproducible by classifying the estrogen-like contaminant of the nonsteroidal didesmethyl methoxychlor (DDM) as a class 2 estrogen [100].

However, the fact that 4-hydroxytamoxifen completely blocked the action of estradiol to cause apoptosis in MCF-7:5C cells opened the door to prove that shape

Uncorrected Proof

Author's Proof

mattered for the estrogen-ER complex to trigger apoptosis. Did the "jaws of the crocodile" need to be closed to trigger apoptosis?

The first clue that the hypothesis was going to prove to be correct and control apoptosis was the triphenylethylene estrogen-ER complexes were shown not to be down regulated in MCF-7 cells like estradiol, but to accumulate like 4-hydroxytamoxifen. The ER complex for these nonplanar estrogens was like an antiestrogen! The shape of the ER with different types of estrogen did, in fact, control an important biological process—estrogen-induced apoptosis! Further studies exhaustively demonstrated that the triphenylethylenes stimulated the growth of MCF-7 cells just like estradiol, but with less potency, and confirmed and massively expanded earlier studies that triphenylethylene estrogens did block apoptosis. Triphenylethylene complex with ER did not bind the coactivator SRC-3 as avidly at the promoter regions of estrogen-responsive genes [101], and these data beautifully confirmed the observation in complimentary studies that SRC-3 was important for estrogen-induced apoptosis [102]. By studying SRC3-interacting proteins, one could decipher the early events in estrogen-induced apoptosis in vitro [102] and in vivo (...). AU6

However, during this conversation with nature to decipher the mechanism of estrogen-induced apoptosis, very important one fact was inconsistent. If estrogenic triphenylethylenes block estrogen-induced apoptosis in a cell like MCF-7:5C in the laboratory, then why did Haddow observe his best responses with estrogen-induced tumor regress with estrogenic triphenylethylenes used for the treatment of metastatic breast cancer in late postmenopausal women [74]? A clinical reality with tumor regression with estrogen trumps a laboratory study every time! This inconsistency was solved with that the triphenylethylenes kill the cells in culture in 2 weeks. The time course is extended with class II angular estrogens so the triggering process is only occurring slowly. In the patient the long-term retention and storage of triphenylethylenes in a woman's body fat provides a continuous high estrogen environment to produce optimal antitumor actions. A conversation with nature does work!

Uncorrected Proof

Final Thoughts on Four Decades of Discovery to Advance the Value of the ER Target in Breast Cancer

We begin and end our story with the actions of synthetic estrogens to kill breast cancer cells that have been prepared for sacrifice through estrogen deprivations. The best current example of the value of this knowledge in women's health are the results of the Women's Health Initiative with conjugated equine estrogens alone in hysterectomized women to reduce breast cancer incidence and mortality for women in their mid-60s [76]. The 40 years starting with the development of tamoxifen from a failed contraceptive to being the gold standard that saved the lives of millions of women through the prudent application of the laboratory principle of long-term adjuvant therapy [2] resulted in the mandatory laboratory study of acquired drug

Author's Proof

resistance to long-term tamoxifen therapy. Acquired resistance would surely occur, but no one could have predicted the development of tamoxifen-stimulated breast cancer growth or the evolution of acquired resistance to expose a fatal vulnerability in breast cancer so that physiologic estrogen triggered apoptosis. Each discovery was in the hands of young scientists as generations of Tamoxifen Teams that turned ideas into lives saved. Progress occurred through their outstanding skill in the laboratory and the philosophy that if Nature gives us the "wrong answer" to our question, Nature does not lie. The answer is the true answer to the question that must be considered as the solution to the problem to be solved.

Postscript. During his Ph.D. training, Doug Wolf discovered multiple valuable clues to understand SERMs, drug resistance, and estrogen-induced apoptosis, but at the time all of this was speculative with no real basis in scientific fact. The two discoveries that Doug contributed were both serendipity. In fact, all advances are serendipity in basic science, but it is the recognition of the new knowledge that becomes the key to discovery. One spots the clue and expands on the observation because it is a "conversation with Nature." The unimaginative scientist throws the clue away as it does not fit the model of what is correct or incorrect in their mind at the time.

In a search for mechanisms to explain acquired tamoxifen resistance, Doug was focused in two directions in his Ph.D. thesis. The two main questions were as follows: "Is acquired drug resistance to tamoxifen because a mutation of the estrogen receptor occurs to change the pharmacology of tamoxifen from an antiestrogen to an estrogen?" and secondly "What growth factor receptors and receptor signal transduction pathways are responsible for estradiol-stimulated growth of tumors with acquired tamoxifen resistance and does tamoxifen use the same pathways as estradiol?"

To address the issue of a mutation of the ER enhancing the estrogen-like effects of tamoxifen, Doug created a number of tamoxifen-stimulated tumor lines and screened them for ER mutations [62]. All tumor lines had wild-type ER except one with a large proportion of an ER with an asp 351 tyr mutation [62, 103]. We had a no idea at the time what this was going to mean for understanding the mechanics of SERM action but it was destined to be profound. Bill Catherino, an M.D., Ph.D. student in my laboratory at Wisconsin, subsequently created the BC-2 stably transfected cell line in MDA-MB-231 cells using a cDNA for the mutant receptor [104]. Anna Levenson, a postdoctoral fellow and then a research assistant professor at Northwestern used a transforming growth factor (TGF)-α target (discovered by Mei Huey Jeng) [105] to compare and contrast the estrogenic and antiestrogenic action of tamoxifen and raloxifene. The Asp351Tyr ER turned out to be the first and, to date, the only natural mutation of the human ER to change the pharmacology of a nonsteroidal antiestrogen from a complete antiestrogen to an estrogen [93, 106]. We were mystified why a mutation buried in the ligand-binding domain (LBD) of the ER could influence the pharmacology of raloxifene, but the reason became clear with the subsequent publications of the crystal structure of the raloxifene-ER LBD [87]. However, if one examines the X-ray crystallography in the papers it is almost impossible to interpret in "the real world" of protein-protein

Uncorrected Proof

Author's Proof

interactions. That is for the outside! The fact that we realized that Asp 351 was a surface amino acid on the ER complex was the key to finding the "antiestrogen region" was had predicted in paper published 15 years earlier [90, 107]. But the discovery was by chance and this chance created opportunities for a productive scientific collaboration. I had been invited to Signal Pharmaceuticals in California to discuss a new SERM, but as I was waiting for my taxi to take me to my hotel, I started to wander the corridors and struck up a conversation with a young man Jim Zapf who was "playing" on his computer. "What do you do?" says I. Jim replied, "I do docking of ligands with the ER ligand binding domain." "OK," I said. "How good is your program? Can you show me the outside of the ER complex dimer—this is what other proteins see?" "No problem," Jim replies. "Let me ask you this. Color in where helix 12 is with the estradiol or raloxifene ER complex?" In a second or two I exclaim, "It really is the crocodile model of estrogen and antiestrogen action." We had proposed this 15 years earlier [90]. "OK so where is aspartate 351 in the estradiol ER complex?" I inquired. Jim replies, "It's here under helix 12 on the surface of the complex but it does not play a role." As we switch to the raloxifene-ER complex, the significance of aspartate 351 was clear through its interaction with the "antiestrogenic side chain" of raloxifene. The pyrolidine ring shields and neutralized the aspartate producing a complete antiestrogen, but tamoxifen has a side chain that is a few Å shorter and cannot do the job completely and is promiscuous with estrogen-like actions. This chance meeting resulted in collaboration and a half a dozen publications of ER modulation. We subsequently interrogated the ligand asp 351 interactions (Chap. 5, Postscript) and this was reviewed by Levenson [108].

One of the Doug's other tasks was to utilize the Marco Gottardis athymic mouse model of acquired resistance to tamoxifen [28] to discover the growth factor pathways, responsible for estradiol or tamoxifen-stimulated tumor growth. At this time, in the early 1990s, growth factor signaling was the fashion [109] and primarily spearheaded by Dr. Marc E. Lippman who had just become the director of the Lombardi Cancer Center in Washington, DC. He had moved, with all his staff, from the National Cancer Institute in Bethesda where he was the head of the Breast program. Doug's project was simple. Grow up some of the tamoxifen-stimulated tumors with tamoxifen in athymic mice and then switch to either tamoxifen or physiologic estrogen released from subcutaneous capsules. Then, harvest growing tumors and measure all known growth factors and their receptors to answer the question: "Is estrogen or tamoxifen induced-growth stimulated by the same or different growth factors?" The tumors did not grow with physiologic estradiol; they disappeared—they just melted away in a few weeks! I suggested that the long-term tamoxifen exposure had somehow accelerated a natural sensitivity to estrogen-induced tumor cell death. It was the explanations of Haddow's landmark observation in patients 50 years before [74, 78]! These data at Wisconsin [63] were presented at the St. Gallen Breast Cancer Conference in 1992 and were replicated at Northwestern by a superb team of resident surgeons Kathy Yao, Eun-Sook Lee, Dave Bertram, Gale England, a medical oncology fellow Ruth O'Regan, and my Ph.D. student Jennifer MacGregor Shafer [64]. Their data showed that Doug's work

Uncorrected Proof

Author's Proof

was reproducible and the phenomenon occurred over a 5-year period (i.e., in the last 2 years of transplantation in tamoxifen-treated mice). Gale England showed this beautifully in her notebook and Dave Bentram stepped in to perform biotransplant tumor experiments requested by the referees for Kathy Yao's paper [64]. They thought the animals had changed, not the tumor. Dave showed that it was the tumor, not the animals. These data opened a new door of discovery for the next decade with the exploitation of the principle of successfully treating patients with acquired antihormone therapy with low doses of estradiol [110], the study of mechanisms [73, 80, 83, 85, 111] that answered Haddow's statement of dismay in his 1971 Karnofsky lecture:

> ... the extraordinary extent of tumour regression observed in perhaps 1 % of post-menopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us ...

I was thrilled to be selected as the 38th winner of the Karnofsky Memorial Lecture and selected as my title, "The paradoxical actions of estrogen in breast cancer: survival or death?" As Haddow and I were (are) British and my Tamoxifen Team have, through serendipity, now discovered the molecular mechanism of estrogen-induced apoptosis, this seemed to me to be the appropriate tribute to his pioneering advance in chemical therapy.

Our subsequent work also provided the basis for the explanation of the antitumor effects of physiologic estrogen when used as estrogen replacement therapy [76]. A valuable conversation with nature that could have been so easily abandoned in 1993 with the "wrong answer" from Doug's experiment that could not reproduce estrogen response in Marco's Model. But another twist was necessary to advance our Tamoxifen Team tale.

It is worth emphasizing the significant role that Dr. Joan Lewis-Wambi played in this story, our knowledge of estrogen-induced apoptosis and the cell model she breathed life into—by chance. Dr. Shun-Yuan Jiang created both the MCF-7:5C [81] cells and MCF-7:2A cells [82]. Both cell lines were cloned out of populations that were estrogen deprived for almost a year. The majority of cells died but some survived and grew under estrogen-free conditions. The MCF-7:5C [81] are ER positive and PgR negative, and we reported they did not respond to estrogen or antiestrogens. Joan Lewis almost 10 years later was given the task of studying these cells at a time that it was clear that the aromatase inhibitors would be an essential treatment option for breast cancer patients in the medical oncologists' armamentarium. What did she do? She charged the serum conditions to grow our MCF-7:5C cells and did not follow the essential tradition of repeating exactly what Shun-Yuan had done in her paper [81]. Amazingly, the MCF-7:5C cells grew spontaneously, but apoptosis occurred rapidly with physiologic estrogen in vitro and in vivo. We had never had a cell line that responded to estrogen as a cidal stimulus—and now we did [112]. She created a pivotal paper on the intrinsic mechanism of apoptosis [83] and followed this up with a super description of the delayed apoptosis (estrogen took a week longer to cause cell death in the MCF-7:2A cells) observed

Uncorrected Proof

Author's Proof

in the 2A cells that could be advanced to immediate cell death with estrogen if glutathione synthesis was blocked [84]. I should document that her husband Dr. Chris Wambi was conducting research on the redox role of glutathione using buthionine sulfoximine (BSO) to stop glutathione synthesis, and it was this husband and wife team that found our mechanism of cell survival that could be neutralized so that estrogen now caused rapid cell death. This is a good husband/wife synergy in science.

Without these cell models, our expanding experience and publications with acquired tamoxifen and raloxifene resistance in vivo, we could not have successfully competed for our Department of Defense Center of Excellence Grant. These studies and models passed on one to another over decades by my trainees significantly advanced women health and helped families stay together longer through the increased survival of women either with breast cancer or at risk of breast cancer.

References

1. McGuire WL, Carbone PP, Vollmer EP, United States. National Cancer Institute. Breast Cancer Treatment Committee (1975) Estrogen receptors in human breast cancer. Raven Press, New York, xiv, 284 pp
2. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44:30–38
3. Ingle JN, Ahmann DL, Green SJ et al (1981) Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. N Engl J Med 304:16–21
4. Soule HD, Vazguez J, Long A et al (1973) A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51:1409–1416
5. Brooks SC, Locke ER, Soule HD (1973) Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J Biol Chem 248:6251–6253
6. Lippman ME, Bolan G (1975) Oestrogen-responsive human breast cancer in long term tissue culture. Nature 256:592–593
7. Shafie SM (1980) Estrogen and the growth of breast cancer: new evidence suggests indirect action. Science 209:701–702
8. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS (1986) Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A 83:2496–2500
9. Bindal RD, Katzenellenbogen JA (1988) Bis(4-hydroxyphenyl)[2-(phenoxysulfonyl)phenyl] methane: isolation and structure elucidation of a novel estrogen from commercial preparations of phenol red (phenolsulfonphthalein). J Med Chem 31:1978–1983
10. Welshons WV, Jordan VC (1987) Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. Eur J Cancer Clin Oncol 23:1935–1939
11. Levenson AS, Jordan VC (1997) MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res 57:3071–3078
12. Nawata H, Bronzert D, Lippman ME (1981) Isolation and characterization of a tamoxifen-resistant cell line derived from MCF-7 human breast cancer cells. J Biol Chem 256:5016–5021
13. Miller MA, Lippman ME, Katzenellenbogen BS (1984) Antiestrogen binding in antiestrogen growth-resistant estrogen-responsive clonal variants of MCF-7 human breast cancer cells. Cancer Res 44:5038–5045

Uncorrected Proof

Author's Proof

14. Bronzert DA, Greene GL, Lippman ME (1985) Selection and characterization of a breast cancer cell line resistant to the antiestrogen LY 117018. Endocrinology 117:1409–1417
15. van den Berg HW, Lynch M, Martin J et al (1989) Characterisation of a tamoxifen-resistant variant of the ZR-75-1 human breast cancer cell line (ZR-75-9a1) and ability of the resistant phenotype. Br J Cancer 59:522–526
16. Wiseman LR, Johnson MD, Wakeling AE et al (1993) Type I IGF receptor and acquired tamoxifen resistance in oestrogen-responsive human breast cancer cells. Eur J Cancer 29A:2256–2264
17. Brunner N, Frandsen TL, Holst-Hansen C et al (1993) MCF7/LCC2: a 4-hydroxytamoxifen resistant human breast cancer variant that retains sensitivity to the steroidal antiestrogen ICI 182,780. Cancer Res 53:3229–3232
18. Lykkesfeldt AE, Madsen MW, Briand P (1994) Altered expression of estrogen-regulated genes in a tamoxifen-resistant and ICI 164,384 and ICI 182,780 sensitive human breast cancer cell line, MCF-7/TAMR-1. Cancer Res 54:1587–1595
19. Borras M, Jin L, Bouhoute A et al (1994) Evaluation of estrogen receptor, antiestrogen binding sites and calmodulin for antiestrogen resistance of two clones derived from the MCF-7 breast cancer cell line. Biochem Pharmacol 48:2015–2024
20. Brunner N, Boysen B, Jirus S et al (1997) MCF7/LCC9: an antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen. Cancer Res 57:3486–3493
21. Larsen SS, Madsen MW, Jensen BL, Lykkesfeldt AE (1997) Resistance of human breast-cancer cells to the pure steroidal anti-estrogen ICI 182,780 is not associated with a general loss of estrogen-receptor expression or lack of estrogen responsiveness. Int J Cancer 72:1129–1136
22. Parisot JP, Leeding KS, Hu XF et al (1999) Induction of insulin-like growth factor binding protein expression by ICI 182,780 in a tamoxifen-resistant human breast cancer cell line. Breast Cancer Res Treat 55:231–242
23. Chan CM, Lykkesfeldt AE, Parker MG, Dowsett M (1999) Expression of nuclear receptor interacting proteins TIF-1, SUG-1, receptor interacting protein 140, and corepressor SMRT in tamoxifen-resistant breast cancer. Clin Cancer Res 5:3460–3467
24. Shafie SM, Grantham FH (1981) Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. J Natl Cancer Inst 67:51–56
25. Osborne CK, Hobbs K, Clark GM (1985) Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res 45:584–590
26. Osborne CK, Coronado EB, Robinson JP (1987) Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. Eur J Cancer Clin Oncol 23:1189–1196
27. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
28. Gottardis MM, Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 48:5183–5187
29. Gottardis MM, Wagner RJ, Borden EC, Jordan VC (1989) Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro. Cancer Res 49:4765–4769
30. Harper MJ, Walpole AL (1966) Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. Nature 212:87
31. Osborne CK, Coronado E, Allred DC et al (1991) Acquired tamoxifen resistance: correlation with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxytamoxifen. J Natl Cancer Inst 83:1477–1482

Uncorrected Proof

Author's Proof

32. Osborne CK, Wiebe VJ, McGuire WL et al (1992) Tamoxifen and the isomers of 4-hydroxytamoxifen in tamoxifen-resistant tumors from breast cancer patients. J Clin Oncol 10:304–310
33. Wiebe VJ, Osborne CK, McGuire WL, DeGregorio MW (1992) Identification of estrogenic tamoxifen metabolite(s) in tamoxifen-resistant human breast tumors. J Clin Oncol 10:990–994
34. Murphy CS, Langan-Fahey SM, McCague R, Jordan VC (1990) Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. Mol Pharmacol 38:737–743
35. Jordan VC, Koch R, Langan S, McCague R (1988) Ligand interaction at the estrogen receptor to program antiestrogen action: a study with nonsteroidal compounds in vitro. Endocrinology 122:1449–1454
36. Wolf DM, Langan-Fahey SM, Parker CJ et al (1993) Investigation of the mechanism of tamoxifen-stimulated breast tumor growth with nonisomerizable analogues of tamoxifen and metabolites. J Natl Cancer Inst 85:806–812
37. Benz CC, Scott GK, Sarup JC et al (1992) Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res Treat 24:85–95
38. Slamon DJ, Leyland-Jones B, Shak S et al (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344:783–792
39. Marty M, Cognetti F, Maraninchi D et al (2005) Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. J Clin Oncol 23:4265–4274
40. Gasparini G, Gion M, Mariani L et al (2007) Randomized Phase II Trial of weekly paclitaxel alone versus trastuzumab plus weekly paclitaxel as first-line therapy of patients with Her-2 positive advanced breast cancer. Breast Cancer Res Treat 101:355–365
41. Robert N, Leyland-Jones B, Asmar L et al (2006) Randomized phase III study of trastuzumab, paclitaxel, and carboplatin compared with trastuzumab and paclitaxel in women with HER-2-overexpressing metastatic breast cancer. J Clin Oncol 24:2786–2792
42. Bernard-Marty C, Lebrun F, Awada A, Piccart MJ (2006) Monoclonal antibody-based targeted therapy in breast cancer: current status and future directions. Drugs 66:1577–1591
43. Cortes J, Fumoleau P, Bianchi GV et al (2012) Pertuzumab monotherapy after trastuzumab-based treatment and subsequent reintroduction of trastuzumab: activity and tolerability in patients with advanced human epidermal growth factor receptor 2-positive breast cancer. J Clin Oncol 30:1594–1600
44. Kiewe P, Hasmuller S, Kahlert S et al (2006) Phase I trial of the trifunctional anti-HER2 x anti-CD3 antibody ertumaxomab in metastatic breast cancer. Clin Cancer Res 12:3085–3091
45. Konecny GE, Pegram MD, Venkatesan N et al (2006) Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. Cancer Res 66:1630–1639
46. Gomez HL, Doval DC, Chavez MA et al (2008) Efficacy and safety of lapatinib as first-line therapy for ErbB2-amplified locally advanced or metastatic breast cancer. J Clin Oncol 26:2999–3005
47. Nelson MH, Dolder CR (2006) Lapatinib: a novel dual tyrosine kinase inhibitor with activity in solid tumors. Ann Pharmacother 40:261–269
48. Cameron D, Casey M, Press M et al (2008) A phase III randomized comparison of lapatinib plus capecitabine versus capecitabine alone in women with advanced breast cancer that has progressed on trastuzumab: updated efficacy and biomarker analyses. Breast Cancer Res Treat 112:533–543
49. Xia W, Bacus S, Hegde P et al (2006) A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. Proc Natl Acad Sci U S A 103:7795–7800

Uncorrected Proof

Author's Proof

50. Shou J, Massarweh S, Osborne CK et al (2004) Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst 96:926–935
51. Riemsma R, Forbes CA, Amonkar MM et al (2012) Systematic review of lapatinib in combination with letrozole compared with other first-line treatments for hormone receptor positive(HR+) and HER2+ advanced or metastatic breast cancer (MBC). Curr Med Res Opin 28(8):1263–1279
52. Maximov PY, Lewis-Wambi JS, Jordan VC (2009) The paradox of oestradiol-induced breast cancer cell growth and apoptosis. Curr Signal Transduct Ther 4:88–102
53. Schafer JM, Lee ES, O'Regan RM et al (2000) Rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice. Clin Cancer Res 6:4373–4380
54. Osborne CK, Pippen J, Jones SE et al (2002) Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. J Clin Oncol 20:3386–3395
55. Howell A, Robertson JF, Quaresma Albano J et al (2002) Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. J Clin Oncol 20:3396–3403
56. Wakeling AE, Bowler J (1987) Steroidal pure antioestrogens. J Endocrinol 112:R7–R10
57. Wakeling AE, Dukes M, Bowler J (1991) A potent specific pure antiestrogen with clinical potential. Cancer Res 51:3867–3873
58. Gottardis MM, Jiang SY, Jeng MH, Jordan VC (1989) Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res 49:4090–4093
59. Tormey DC, Jordan VC (1984) Long-term tamoxifen adjuvant therapy in node-positive breast cancer: a metabolic and pilot clinical study. Breast Cancer Res Treat 4:297–302
60. Scottish Cancer Trials Office (MRC), Edinburgh (1987) Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee. Lancet 2:171–175
61. Fisher B, Brown A, Wolmark N et al (1987) Prolonging tamoxifen therapy for primary breast cancer. Findings from the National Surgical Adjuvant Breast and Bowel Project clinical trial. Ann Intern Med 106:649–654
62. Wolf DM, Jordan VC (1994) Characterization of tamoxifen stimulated MCF-7 tumor variants grown in athymic mice. Breast Cancer Res Treat 31:117–127
63. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127:23–33
64. Yao K, Lee ES, Bentrem DJ et al (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6:2028–2036
65. Santen RJ, Samojlik E, Wells SA (1980) Resistance of the ovary to blockade of aromatization with aminoglutethimide. J Clin Endocrinol Metab 51:473–477
66. Santen RJ, Worgul TJ, Samojlik E et al (1981) A randomized trial comparing surgical adrenalectomy with aminoglutethimide plus hydrocortisone in women with advanced breast cancer. N Engl J Med 305:545–551
67. Brodie AM, Schwarzel WC, Brodie HJ (1976) Studies on the mechanism of estrogen biosynthesis in the rat ovary–I. J Steroid Biochem 7:787–793
68. Brodie AM, Schwarzel WC, Shaikh AA, Brodie HJ (1977) The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. Endocrinology 100:1684–1695
69. Brodie AM, Garrett WM, Hendrickson JR et al (1981) Inactivation of aromatase in vitro by 4-hydroxy-4-androstene-3,17-dione and 4-acetoxy-4-androstene-3,17-dione and sustained effects in vivo. Steroids 38:693–702
70. Coombes RC, Goss P, Dowsett M et al (1984) 4-hydroxyandrostenedione in treatment of postmenopausal patients with advanced breast cancer. Lancet 2:1237–1239

Uncorrected Proof

Author's Proof

71. Masamura S, Santner SJ, Heitjan DF, Santen RJ (1995) Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. J Clin Endocrinol Metab 80:2918–2925
72. Shim WS, Conaway M, Masamura S et al (2000) Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. Endocrinology 141:396–405
73. Song RX, Mor G, Naftolin F et al (2001) Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 93:1714–1723
74. Haddow A, Watkinson JM, Paterson E, Koller PC (1944) Influence of synthetic oestrogens on advanced malignant disease. Br Med J 2:393–398
75. Kennedy BJ, Nathanson IT (1953) Effects of intensive sex steroid hormone therapy in advanced breast cancer. J Am Med Assoc 152:1135–1141
76. Anderson GL, Chlebowski RT, Aragaki AK et al (2012) Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. Lancet Oncol 13:476–486
77. Jordan VC (2003) Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2:205–213
78. Haddow A (1971) Cancer research: the great debate. N Engl J Med 285:24–28
79. Haddow A, David A (1970) Karnofsky memorial lecture. Thoughts on chemical therapy. Cancer 26:737–754
80. Osipo C, Gajdos C, Liu H et al (2003) Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. J Natl Cancer Inst 95:1597–1608
81. Jiang SY, Wolf DM, Yingling JM et al (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. Mol Cell Endocrinol 90:77–86
82. Pink JJ, Jiang SY, Fritsch M, Jordan VC (1995) An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. Cancer Res 55:2583–2590
83. Lewis JS, Meeke K, Osipo C et al (2005) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97:1746–1759
84. Lewis-Wambi JS, Kim HR, Wambi C et al (2008) Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. Breast Cancer Res 10:R104
85. Ariazi EA, Cunliffe HE, Lewis-Wambi JS et al (2011) Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci U S A 108:18879–18886
86. Fan P, McDaniel RE, Kim HR et al (2012) Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines. Eur J Cancer 48(18):3488–3498
87. Brzozowski AM, Pike AC, Dauter Z et al (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
88. Shiau AK, Barstad D, Loria PM et al (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937
89. Jordan VC, Koch R, Mittal S, Schneider MR (1986) Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. Br J Pharmacol 87:217–223
90. Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741–4745
91. Lieberman ME, Jordan VC, Fritsch M et al (1983) Direct and reversible inhibition of estradiol-stimulated prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4734–4740
92. Jordan VC, Lieberman ME (1984) Estrogen-stimulated prolactin synthesis in vitro. Classification of agonist, partial agonist, and antagonist actions based on structure. Mol Pharmacol 26:279–285

Uncorrected Proof

Author's Proof

93. Levenson AS, Catherino WH, Jordan VC (1997) Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. J Steroid Biochem Mol Biol 60:261–268
94. Levenson AS, Tonetti DA, Jordan VC (1998) The oestrogen-like effect of 4-hydroxytamoxifen on induction of transforming growth factor alpha mRNA in MDA-MB-231 breast cancer cells stably expressing the oestrogen receptor. Br J Cancer 77:1812–1819
95. Bentrem D, Dardes R, Liu H et al (2001) Molecular mechanism of action at estrogen receptor alpha of a new clinically relevant antiestrogen (GW7604) related to tamoxifen. Endocrinology 142:838–846
96. MacGregor Schafer J, Liu H, Bentrem DJ et al (2000) Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. Cancer Res 60:5097–5105
97. Liu H, Park WC, Bentrem DJ et al (2002) Structure-function relationships of the raloxifene-estrogen receptor-alpha complex for regulating transforming growth factor-alpha expression in breast cancer cells. J Biol Chem 277:9189–9198
98. Liu H, Lee ES, Deb Los Reyes A et al (2001) Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor alpha complex. Cancer Res 61:3632–3639
99. Jordan VC, Schafer JM, Levenson AS et al (2001) Molecular classification of estrogens. Cancer Res 61:6619–6623
100. Bentrem D, Fox JE, Pearce ST et al (2003) Distinct molecular conformations of the estrogen receptor alpha complex exploited by environmental estrogens. Cancer Res 63:7490–7496
101. Maximov P, Sengupta S, Lewis-Wambi JS et al (2011) The conformation of the estrogen receptor directs estrogen-induced apoptosis in breast cancer: A hypothesis. Horm Mol Biol Clin Investig 5:27–34
102. Hu ZZ, Kagan BL, Ariazi EA et al (2011) Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. PLoS One 6:e20410
103. Wolf DM, Jordan VC (1994) The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31:129–138
104. Catherino WH, Wolf DM, Jordan VC (1995) A naturally occurring estrogen receptor mutation results in increased estrogenicity of a tamoxifen analog. Mol Endocrinol 9:1053–1063
105. Jeng MH, Jiang SY, Jordan VC (1994) Paradoxical regulation of estrogen-dependent growth factor gene expression in estrogen receptor (ER)-negative human breast cancer cells stably expressing ER. Cancer Lett 82:123–128
106. Levenson AS, Jordan VC (1998) The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. Cancer Res 58:1872–1875
107. Jordan VC (1984) Biochemical pharmacology of antiestrogen action. Pharmacol Rev 36:245–276
108. Levenson AS, MacGregor Schafer JI, Bentrem DJ et al (2001) Control of the estrogen-like actions of the tamoxifen-estrogen receptor complex by the surface amino acid at position 351. J Steroid Biochem Mol Biol 76:61–70
109. Dickson RB, Lippman ME (1995) Growth factors in breast cancer. Endocr Rev 16:559–589
110. Ellis MJ, Gao F, Dehdashti F et al (2009) Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. JAMA 302:774–780
111. Liu H, Lee ES, Gajdos C et al (2003) Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 95:1586–1597
112. Lewis JS, Osipo C, Meeke K, Jordan VC (2005) Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. J Steroid Biochem Mol Biol 94:131–141

Uncorrected Proof

Author's Proof

Author Queries

Chapter No.: 9

Query Refs.	Details Required	Author's response
AU1	Please check sentence starting "Select breast cancer could...." for completeness.	
AU2	Please check if edit to sentence starting "In particular, lapatinib...." is okay.	
AU3	Please check if edit to sentence starting "Thus, the model. . ." is okay.	
AU4	Please check if edit to sentence starting "As new aromatase. . ." is okay.	
AU5	Please check if edit to sentence starting "He did not. . ." is okay.	
AU6	Please provide appropriate citation reference.	

Uncorrected Proof

Author's Proof

Metadata of the chapter that will be visualized online

Chapter Title	The Legacy of Tamoxifen	
Copyright Year	2013	
Copyright Holder	Springer Basel	
Corresponding Author	Family Name	**Maximov**
	Particle	
	Given Name	**Philipp Y.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**McDaniel**
	Particle	
	Given Name	**Russell E.**
	Suffix	
	Organization	Georgetown University Medical Center
	Address	Washington District of Columbia, USA
Author	Family Name	**Jordan**
	Particle	
	Given Name	**V. Craig**
	Suffix	
	Division	Lombardi Comprehensive Cancer Center
	Organization	Georgetown University Medical Center
	Address	Washington, DC, USA
Abstract	Tamoxifen, the first targeted therapy to treat breast cancer, has dramatically changed medicine. Study of the pharmacology of tamoxifen created a successful adjuvant treatment strategy to save lives, created the first chemopreventive to prevent any cancer in humans, and was the pioneering selective estrogen receptor modulator (SERM) that resulted in the new drug group, the SERMs. New agents such as lasofoxifene and bazedoxifene show a promise in the range of beneficial effects they demonstrate in clinical trial to treat multiple diseases in women. Additionally, new agents and approaches with conjugated equine estrogen are being explored to prevent hot flashes, thereby enhancing the likelihood that compliance with SERMs improves.	

Author's Proof

Chapter 10
The Legacy of Tamoxifen

Abstract Tamoxifen, the first targeted therapy to treat breast cancer, has dramatically changed medicine. Study of the pharmacology of tamoxifen created a successful adjuvant treatment strategy to save lives, created the first chemopreventive to prevent any cancer in humans, and was the pioneering selective estrogen receptor modulator (SERM) that resulted in the new drug group, the SERMs. New agents such as lasofoxifene and bazedoxifene show a promise in the range of beneficial effects they demonstrate in clinical trial to treat multiple diseases in women. Additionally, new agents and approaches with conjugated equine estrogen are being explored to prevent hot flashes, thereby enhancing the likelihood that compliance with SERMs improves.

Introduction

During the 1970s and 1980s, the pharmaceutical industry worked diligently to study the structure-activity relationships of nonsteroidal antiestrogens to find a competitor for tamoxifen. The list includes droloxifene (3-hydroxytamoxifen), trioxifene, LY117,018, toremifene, and idoxifene [1]. Clinical trials were, in the main, unable to show any significant advantages over tamoxifen. The bench mark to predict success was less uterotrophic activity and LY117,018, which as a result evolved to become raloxifene via LY156,758. Toremifene was registered for the treatment of metastatic breast cancer but is not appropriate for adjuvant therapy in the United States. There has been interest in the use of toremifene for the treatment of prostate cancer [2, 3]. Tamoxifen, uniquely, remained the sole agent of choice as an adjuvant therapy for about 20 years.

ICI Pharmaceutical Division chose another direction to solve the "estrogenic tickle" of tamoxifen with a plan for the development of fulvestrant as an injectable pure antiestrogen.

Uncorrected Proof

P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
DOI 10.1007/978-3-0348-0664-0_10, © Springer Basel 2013

Author's Proof

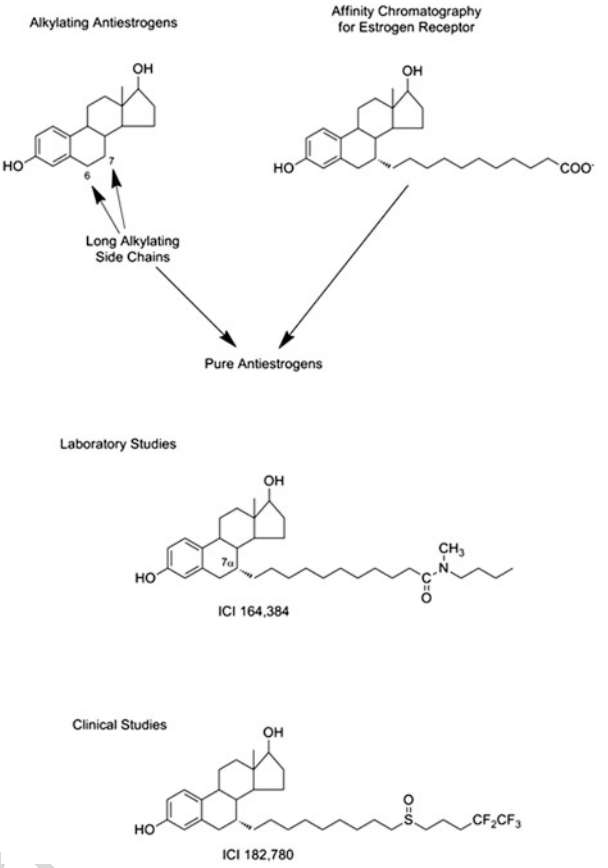


Fig. 10.1 The progress of two unrelated ideas coming together to create a new drug group: the pure antiestrogens. Estradiol derivatives substituted at 6 and 7 positions were created to deliver an alkylating agent via the ER to DNA. In contrast, estradiol was attached to long hydrocarbon chains on the Sephadex column to purify the ER. Both aspects of estradiol chemistry came together to create the pure antiestrogens at ICI Pharmaceuticals Division in the early 1980s

Pure Antiestrogens

The possibility that a pure antiestrogen could be developed with high binding affinity for the ER combines the observation that MER 25, the first antiestrogen, has virtually no estrogenic properties in any animal species [4], with the knowledge that binding affinity and biological activity are separate functions of the same molecule [5]. The antiestrogens ICI164,384 and ICI182,780 are derivatives of estradiol with an optimal binding affinity for the ER, but these structural analogs are unique because they do not have any estrogenic properties and they have a novel subcellular mechanism of action [6] (Fig. 10.1). The serendipitous discovery of pure antiestrogens occurred through two essentially unsuccessful research endeavors that converged thus providing the optimal intellectual environment for new drug discovery. Derivatives of estradiol or estrone substituted in the 6 and 7 positions were being evaluated as potential alkylating antiestrogens in the late 1970s through an ICI-Leeds University joint research scheme [7]. Independently, scientists in France were attempting to purify the ER using estradiol linked at the 7 position through a ten-membered carbon side chain to

Author's Proof

Sephadex columns [8]. Dr. Alan Wakeling brought both of these independent ideas together to discover the structure-function relationships of a new class of compounds that have no estrogenic properties in any test system [9–11]. The pure antiestrogen ICI 164,384 has been used extensively in laboratory studies [6], but the more potent ICI182,780 [11] is currently approved for the treatment of breast cancer in postmenopausal women metastatic breast cancer.

The compound is used as a 250-mg injectable 1-month sustained release preparation with therapeutic equivalence to anastrozole following failure of tamoxifen therapy [12, 13]. However, the endocrine option of fulvestrant has never achieved "first-line" status and as such never been evaluated as an adjuvant therapy. Nevertheless, 2-week strategies deserve mention.

The idea of combining an aromatase inhibitor with fulvestrant versus an aromatase inhibitor alone has merit from laboratory studies but has produced one result which was an improvement for the combination versus the aromatase inhibitor alone. By contrast, a second trial using the same treatment [14] protocol showed no difference for the combination versus the aromatase inhibitor alone [15]. It seems that the trial that showed no improvement for the combination [15] had a higher population of patients who had been exposed to tamoxifen treatment previously. Another issue is dosage. The pharmacokinetics of fulvestrant from the 250-mg depot injection is poor with low circulating levels [16]. To address this critically important issue, the CONFIRM trial has compared 250 versus 500 mg monthly injections [17]. The higher dose provides a superior response so this should now be considered to be the dosage of choice.

Angela Brodie's dedicated and pioneering work [18–20] was essential as proof of principle that a selective aromatase inhibitor could be discovered with clinical efficacy. The problem with her discovery, 4-hydroxyandrostenedione, was that it was an injectable rather than a more convenient oral preparation. However, the fact that the failed "morning-after pill" ICI46,474 was transformed successfully into the "gold standard" tamoxifen for the adjuvant treatment of breast cancer provided a new target (the aromatase enzyme) to improve antihormonal therapy in breast cancer. With profits expanding from sales of tamoxifen in the United States after 1990, the key issue for the successful drug development of an aromatase inhibitor would be satisfied: profits. The patent from tamoxifen would be running out in America by 2000, and aromatase inhibitors would be substituted, but only for the postmenopausal patients. Three orally active third-generation aromatase inhibitors were subsequently successfully developed for adjuvant therapy: anastrozole, letrozole, and exemestane. Each was demonstrated to have a small but consistent improvement over 5 years of tamoxifen alone whether given instead of tamoxifen in postmenopausal patients, after 5 years of tamoxifen, or switching after a couple of years of tamoxifen [22–29]. There has even been a successful trial of exemestane as a prevention in postmenopausal high-risk women [30]. However, it is hard to see how this approach would be superior to a sophisticated third-generation SERM functioning as a multifunctional medicine in women's health.

The advantages of aromatase inhibitors for postmenopausal patients are clear in large population trials and for healthcare systems. Patents for aromatase inhibitors are now running out or have run out and cheap generics are becoming available.

Uncorrected Proof

Author's Proof

The aromatase inhibitors were initially priced extremely high compared to tamoxifen to compensate for each only securing about 1/3 of the original tamoxifen market. A disease-free survival advantage is noted for adding an aromatase inhibitor to the treatment plan compared to tamoxifen alone [31] and concerns about endometrial cancer and blood clots are diminished. Current clinical studies to improve endocrine response rates seek to exploit emerging knowledge about the molecular mechanisms of antihormone resistance to aromatase inhibitors [32]. Combinations of letrozole and lapatinib, an inhibitor of the HER2 pathway, show some advantages over letrozole alone in ER-positive and HER-positive metastatic breast cancer [33]. A similar improvement in responsiveness to aromatase inhibitors is noted with a combination with the mTor inhibitor everolimus [34–36]. None of this would have come about but for 20 years of endocrine therapy using tamoxifen as the pioneer. AU1

SERM Successes

A failed "morning-after pill," ICI46,474, becomes tamoxifen and a failed "breast cancer drug," LY156,758, becomes raloxifene to give us the science of selective estrogen receptor modulators (SERMs). There two "wrongs" gave women's health a path that was the "right" research track. As a result the lives of millions of women were improved worldwide. The women who survived through tamoxifen treatment provided strength and support for their families, and the drug continues to fulfill that role in society. Grandmothers now see their grandchildren grow up and mothers see their children married to have families of their own. Women who use raloxifene to prevent osteoporosis have fewer breast cancers, perhaps 20,000 fewer breast cancers if the half a million women taking the drug continue to do so for a decade. Less morbidity occurs with the treatment of cancer and possibly less deaths from breast cancer in the long run. AU2

What is perhaps unique is that without tamoxifen there would be no raloxifene as there had to be a leader to beat. What is unusual is that the pharmacological basis for the development of two orphan drugs from two separate drug companies in separate continents should spring from the same laboratory. The Tamoxifen Team laboratory chose to move, after being talent spotted from Leeds University, to Switzerland and then Wisconsin. These were the opportunities presented and seized upon to be in the right place at exactly the right time, trained and ready to exploit the stream of scientific discoveries that charged medicine twice by the 1990s.

SERMs did not end with raloxifene and the principle created successes and failures over the years. We will close with the pharmacological success of SERMs despite the unsuccessful struggle in this harsh economic climate to create a viable economic model for new compounds. But that initially was the stages of both tamoxifen and raloxifene; the key to success was first to market with tamoxifen earning billions over the past 40 years after being abandoned as being financially unviable and raloxifene earning billions too after being totally abandoned for clinical development for half a decade!

Uncorrected Proof

Author's Proof

The market for the prevention of osteoporosis is much bigger than breast cancer so considerable effort has gone into the development of SERMs for this indication. We will not consider arzoxifene as it has been tested unsuccessfully as a breast cancer therapy and its effectiveness in osteoporosis is proven, and there are consistent decreases in breast cancer incidence. Development is terminated because of toxicity. We will consider ospemifene and lasofoxifene as agents modeled on earlier antiestrogens and bazedoxifene as a SERM with an interesting twist—combination with conjugated equine estrogen.

Lasofoxifene (CP-336156, Fablyn)

Lasofoxifene is interesting as its structure has its origins to the early days of the 1960s when Lednicer and coworkers [37–39] were seeking the optimal postcoital contraceptive (Fig. 10.2). Nafoxidine was the result that then evolved into a potential breast cancer drug that failed [40]. The search for SERMs defined and refined the possible structural components necessary for the new target—osteoporosis. The discovery and preliminary preclinical pharmacology of CP-33156 were first reported in 1998 and since then there has been a steady stream of important publications about this interesting compound. David Thompson's group has contributed most of the new knowledge describing the actions of CP-33156 in the rat with a particular focus on bone, circulating cholesterol, and the uterus [41–44]. The crystallography of lasofoxifene with the ligand-binding domain (LBD) of the ER is resolved [45]. The conformations of the complex is consistent with prior structure of 4-hydroxytamoxifen [46] and raloxifene [47] which adopt the antagonist conformation with helix 12 pushed back and unable to seal the lasofoxifene into the LBD.

An extremely interesting aspect of the pharmacology of lasofoxifene is the enhanced bioavailability of the levorotatory (l-) enantiomer being more potent in terms of ER binding affinity as well as enhanced bioavailability compound of the dextrorotatory (d-) enantiomer [48]. The potency in vivo is enhanced because the (l) isomer lasofoxifene is a poor substrate for glucuronidation.

A whole range of clinical trials with lasofoxifene have been completed for the prevention of osteoporosis [49, 50] with beneficial effects of significantly reducing strokes, coronary heart disease, and breast cancer [51] without increasing endometrial cancer. These are all the properties originally proposed for the potential of SERMs [52].

Finally one interesting aspect of lasofoxifene is the enhanced improvement in vaginal atrophy observed with treatment and increased vaginal lubrication [53]. However, despite the fact that lasofoxifene is approved for the treatment and prevention of osteoporosis in the European Union at doses 1/100 of those used for raloxifene, the SERM still is unable to control hot flashes. This is a serious barrier to compliance and quality of life. However, the road to development of the SERM bazedoxifene has produced an interesting solution.

Uncorrected Proof

Author's Proof

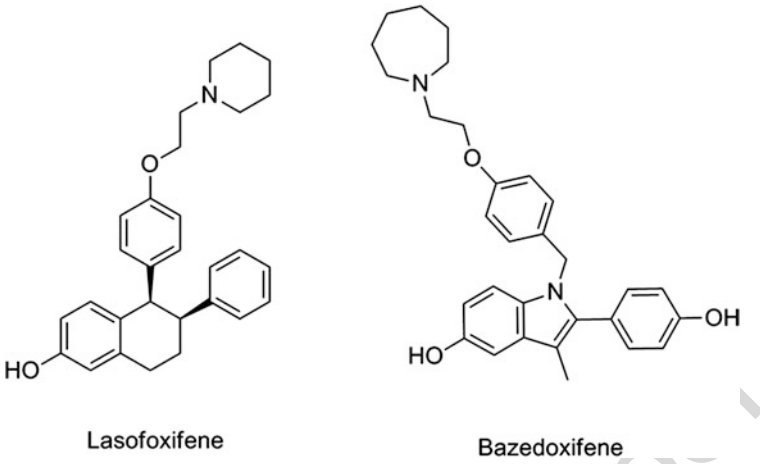


Fig. 10.2 Chemical structures of new SERMs lasofoxifene and bazedoxifene

Bazedoxifene (TSE-424, WAY-140424)

Bazedoxifene is an indole derivative, almost obviously developed from the earlier compound zindoxifene (Fig. 10.2) by attaching an alkylaminoethoxy phenyl side chain in the appropriate "antiestrogen" position of the molecule. The original metabolites of zindoxifene were actually estrogenic in laboratory tests [54] and zindoxifene was without activity for the treatment of breast cancer [55]. Initial laboratory studies with bazedoxifene showed activity as an antiestrogen in MCF-7 breast cancer cells but also was effective in causing cell death [56] in aromatase-resistant breast cancer cells derived from the MCF-7 cell line [57]. Bazedoxifene is a typical SERM which maintains bone density in the ovariectomized rat [58] and the cynomolgus monkey over an 18-month treatment period [59]. Clinical studies demonstrate the value of bazedoxifene from the treatment and potential of osteoporosis. But it is the pairing of bazedoxifene with conjugated equine estrogen (CEE) that enhances effects of lowering lipids and improving bone density while reducing vasomotor effects [60]. In fact, a comparison with lasofoxifene and raloxifene suggests a unique gene profiling for bazedoxifene and CEE on breast cancer cells [61].

Ospemifene (FC-1271a)

This triphenylethylene is a metabolite of toremifene with a unique glycol side chain. This transformation by deamination of the side chain of a nonsteroidal antiestrogen was first noted with tamoxifen when metabolite Y was first discovered [62, 63]. The same transformation occurs with toremifene. Ospemifene is a typical SERM in the rat [64]. Lowering cholesterol, building bone, and blocking estrogen stimulated uterine weight. A range of studies have demonstrated a lack of

Uncorrected Proof

Author's Proof

genotoxicity [65] significant antitumor actions in mice [66] and ability to block the growth of premalignant lesions in a mouse model of DCIS [67]. Indeed the pharmacological effects of ospemifene have been documented in rhesus macaque monkeys [68] as well as humans.

Refining the SERM Concept Further

The fact that there are two ERs, ERα and ERβ [69], naturally has caused a search for ER-specific subtype drugs. Most of our knowledge of the role of each ER subtype has come from a study of knockout mice for one or the other ER [70]. Pharmacologically the main difference between the ER seems to be that AF-1 region [71]. The ligand-binding pockets of ERα and ERβ are very similar with two amino acids Leu and Met in ERα replaced by Met and Leu in ERβ [72].

Despite the difficulties that need to be advanced for subtype-specific agents in very similar proteins, the quest for new medicines has been a priority; changing the antiestrogenic dimethylaminoethoxy side chain to an acrylic side chain creates ERα-specific activity in stimulating endometrial cancer cells [73]. The ERβ-specific agonist SERBA-1 caused involution of the mouse prostate with no effects on ventral prostrate or testicular weight [74]. The Wyeth ERβ-specific agonist ERβ-041 has a dramatic effect in preclinical models of adjuvant-induced arthritis [75]. Most importantly numerous pharmaceutical companies are addressing the issue of controlling hot flashes for a more acceptable SERM. Both Eli Lilly and Johnson & Johnson [76, 77] have compounds shown to control changes in skin temperature in the morphine-dependent rat models.

However, the SERM principle has now been applied to all members of the nuclear receptor superfamily to create selective nuclear receptor modulation to treat diseases with greater specificity not previously believed to be possible. There are now selective androgen receptor modulators (SARMs) [78], selective progesterone receptor modulators (SPRMs) [79], selective glucocorticoid receptor modulators (SGRMs) [80], selective mineralocorticoid receptor modulators (SMRMs) [81], selective thyroid receptor modulators (STRMs) [82], and selective peroxisome proliferator-activated receptor modulators (SPPARMs) [83]. The idea of switching on and off target sites around the body to improve human health and survival is very appealing as we increase longevity.

However, as we bring our story to a close, it is perhaps ironic to reflect that all this progress in a new pharmacology of receptor action was made possible by a potent postcoital contraceptive in the rat, originally designed to prevent life. Much good came from that failed contraceptive tamoxifen that has dramatically enhanced life expectancy, prevented breast cancers, created the SERMs, and dramatically enhanced the prospects of a longer healthier life.

Postscript. Breast cancer is the most prevalent cancer of women and death rates are only secondary to lung cancer. However, lung cancer has a known cause, smoking; the targeting of women by the advertising industry in the 1980s to encourage

Uncorrected Proof

Author's Proof

smoking as a positive lifestyle advance has had consequences with a rising mortality. Breast cancer has no such cause and effect solution to prevent the disease. Yet despite the huge problem of "where to start" in treatment and prevention of breast cancer, the last four decades of research has heralded a new era in personalized medicine for cancer in general, in large part because of the breakthroughs in breast cancer treatment.

The understanding of the links between hormones and breast cancer was to mature for over a century [84] but was, as with all breakthroughs, dependent on the fashions in research. Change occurred in 1971 with passing of the National Cancer Act. This important political step was to articulate a plan to sponsor research and translate the profound breakthroughs that would result into improved patient care. This would be achieved through a nationwide system of clinical cancer centers where laboratory scientists and clinical scientists would interact daily to decrease the mortality from cancer. I have had the privilege of either directing breast cancer programs (University of Wisconsin Comprehensive Cancer Center at Madison, Wisconsin, with Monica Morrow, M.D., perhaps the most accomplished breast cancer surgeon in the world; Robert H. Luire Comprehensive Cancer Center, Northwestern University, Chicago) or as the vice president of Medical Science (Fox Chase Cancer Center, Philadelphia) or as the scientific director (Georgetown University Lombardi Comprehensive Cancer Center, Washington, DC). But it was the experience of the first cancer center I experienced at the University of Wisconsin (Madison) that was critical for my development as a cancer scientist. The opportunity to be recruited was the reason I went to America. This was a wonderful place to learn and develop my ideas. I had the pleasure of working with Director Paul Carbone, Lasker Prize winner (for the development of MOPP and the treatment of Hodgkin's disease) and also the head of the Eastern Cooperative Group. I was talent spotted because of what I could achieve if given the chance to develop tamoxifen to its full potential. This clearly was a success and the wonderful environment of talented scientific colleagues and first-rate graduate students gave medicine SERMs. But it is my interaction with Harold Rusch the inaugural and then former director of the clinical cancer center that I cherish the most. Harold had his office next to mine and we talked every day. He taught me valuable lessons in scientific leadership and the requirement to advance the career development of one's staff. To this day I answer my phone with "How can I help you?" His book is a "must read." Something attempted, something done." He was also the first director of the McArdle Laboratory and built it to be a world-class center of excellence in cancer research. Through the tragedy of his daughter's death from breast cancer, he became one of this nation's strongest advocates for clinical cancer centers to take ideas to the clinic to save lives. There had to be a path to clinical trials and patient care, and he was strategically situated on the President's Cancer Panel to advocate change. He became the first director of the Wisconsin Clinical Cancer Center and then recruited Paul Carbone to continue the task. I was honored when Harold told me that on his death he would like me to speak at his memorial service. He had been diagnosed with prostate cancer and had but a short time to live. To me it was important to obtain a letter of gratitude for all Dr. Rusch had achieved for cancer

AU3

Uncorrected Proof

Author's Proof

research in the United States. I went to the president of the United States. This letter was received just in time at a ceremony at Dr. Rusch's home with the letter read and presented by Donna Shala, then chancellor of the University of Wisconsin-Madison. At Harold's memorial service, I read that same letter as my mark of respect for a great yet humble man, who thought of his staff and colleagues always before himself.

With regard to hormones and cancer, the "epicenter" for positive change I believe was the Worcester Foundation for Experimental Biology in Shrewsbury, Massachusetts. This was the home of the oral contraceptive and the founding Director Gregory Pincus [85] created a world-renowned research institution with a principal theme of reproduction research. It was at the foundation that Pincus turned the dream of oral contraception into a practical reality. His drive and commitment accelerated clinical testing with a progestin which was the culmination of a decade of laboratory investigations. But luck takes control, as often as not. I partially like the story of the first trials with a synthetic progestin that were found to contain an impurity. The progestin was purified and less effective as a contraceptive. The impurity was an estrogen so the combined oral contraceptives "so to speak" conceived. But by the late 1960s, fashions in research were changing and cancer research was to move center stage. With the passing of the National Cancer Act in 1971 came opportunity for funding. For one of us (VCJ), who was a visiting scientist at the foundation (1972–1974), from the University of Leeds, England, this was an important time and valuable to learn and exchange ideas. But the opportunities from the environment of the foundation catalyzed the conversion of ICI46,474 to tamoxifen (with a *big* push from Lois Trench).

The philosophy of the foundation was to advance new ideas and concepts. The first systemic studies with tamoxifen as a breast cancer drug were started [86] but remarkably, in a laboratory not more than 100 yards away from mine, Angela and Harvey Brodie were taking the first steps to create 4-hydroxyandronestedione [18] as the first specific aromatase inhibitor successfully tested in patients [21]. Angela's tenacity and vision was critical for the future development of new aromatase inhibitors. The subsequent pharmaceutical development of tamoxifen as the first long-term adjuvant endocrine therapy targeted to the ER and chemopreventive made the improvements with aromatase inhibitors certain. Tamoxifen and the aromatase inhibitors all continue to reduce mortality. These are the therapeutic cornerstones of the modern era of targeted treatments for breast cancer. All the successes in hormones and breast cancer started at the foundation to be a practical approach to the treatment and prevention.

Furthermore, it is remarkable to note that the scientists at the Worcester Foundation had already changed the world with the oral contraceptive and M. C. Chang had conducted seminal studies on in vitro fertilization with the discovery of sperm capacitation within the uterus. This immediately was used first in animals; I liked the stories I heard that Chang had taken sperm and egg from a mink and a stoat that normally would never mate to create a *stink*! The animal work was necessary to set the stage for the birth of Louise Brown at 11:47 p.m., 25 July 1978 (coincidentally my birthday). By 8 June 1980, health authorities in Virginia announced the first US-built

Uncorrected Proof

Author's Proof

clinic using the Edwards-Steptoe's method. Society has much to be grateful for from the research on hormones initiated in the confines of a couple of acres of land in Massachusetts and the vision of Gregory Pincus. There are four major advances in women's health: the oral contraceptive, in vitro fertilization, a clinical plan for tamoxifen, and the first specific aromatase inhibitor!

References

1. Gradishar WJ, Jordan VC (1997) Clinical potential of new antiestrogens. J Clin Oncol 15:840–852
2. Stein S, Zoltick B, Peacock T et al (2001) Phase II trial of toremifene in androgen-independent prostate cancer: a Penn cancer clinical trials group trial. Am J Clin Oncol 24:283–285
3. Raghow S, Hooshdaran MZ, Katiyar S, Steiner MS (2002) Toremifene prevents prostate cancer in the transgenic adenocarcinoma of mouse prostate model. Cancer Res 62:1370–1376
4. Lerner LJ, Holthaus FJ Jr, Thompson CR (1958) A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol. Endocrinology 63:295–318
5. Jordan VC, Collins MM, Rowsby L, Prestwich G (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305–316
6. Wakeling AE (1994) A new approach to breast cancer therapy-total estrogen ablation with pure antiestrogens. In: Jordan VC (ed) Long-term tamoxifen treatment for breast cancer. University of Wisconsin Press, Madison, pp 219–234
7. Jordan VC, Fenuik L, Allen KE et al (1981) Structural derivatives of tamoxifen and oestradiol 3-methyl ether as potential alkylating antioestrogens. Eur J Cancer 17:193–200
8. Bucourt R, Vignau M, Torelli V (1978) New biospecific adsorbents for the purification of estradiol receptor. J Biol Chem 253:8221–8228
9. Wakeling AE, Bowler J (1987) Steroidal pure antioestrogens. J Endocrinol 112:R7–R10
10. Bowler J, Lilley TJ, Pittam JD, Wakeling AE (1989) Novel steroidal pure antiestrogens. Steroids 54:71–99
11. Wakeling AE, Dukes M, Bowler J (1991) A potent specific pure antiestrogen with clinical potential. Cancer Res 51:3867–3873
12. Osborne CK, Pippen J, Jones SE et al (2002) Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. J Clin Oncol 20:3386–3395
13. Howell A, Robertson JF, Quaresma Albano J et al (2002) Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. J Clin Oncol 20:3396–3403
14. Mehta RS, Barlow WE, Albain KS et al (2012) Combination anastrozole and fulvestrant in metastatic breast cancer. N Engl J Med 367:435–444
15. Bergh J, Jonsson PE, Lidbrink EK et al (2012) FACT: an open-label randomized phase III study of fulvestrant and anastrozole in combination compared with anastrozole alone as first-line therapy for patients with receptor-positive postmenopausal breast cancer. J Clin Oncol 30:1919–1925
16. Howell A, DeFriend DJ, Robertson JF et al (1996) Pharmacokinetics, pharmacological and anti-tumour effects of the specific anti-oestrogen ICI 182780 in women with advanced breast cancer. Br J Cancer 74:300–308
17. Di Leo A, Jerusalem G, Petruzelka L et al (2010) Results of the CONFIRM phase III trial comparing fulvestrant 250 mg with fulvestrant 500 mg in postmenopausal women with estrogen receptor-positive advanced breast cancer. J Clin Oncol 28:4594–4600
18. Brodie AM, Schwarzel WC, Shaikh AA, Brodie HJ (1977) The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. Endocrinology 100:1684–1695

Uncorrected Proof

Author's Proof

19. Brodie AM, Longcope C (1980) Inhibition of peripheral aromatization by aromatase inhibitors, 4-hydroxy- and 4-acetoxy-androstene-3,17-dione. Endocrinology 106:19–21
20. Brodie AM, Marsh D, Brodie HJ (1979) Aromatase inhibitors – IV. Regression of hormone-dependent, mammary tumors in the rat with 4-acetoxy-4-androstene-3,17-dione. J Steroid Biochem 10:423–429
21. Coombes RC, Goss P, Dowsett M et al (1984) 4-hydroxyandrostenedione in treatment of postmenopausal patients with advanced breast cancer. Lancet 2:1237–1239
22. Baum M, Budzar AU, Cuzick J et al (2002) Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. Lancet 359:2131–2139
23. Howell A, Cuzick J, Baum M et al (2005) Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 365:60–62
24. Thurlimann B, Keshaviah A, Coates AS et al (2005) A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. N Engl J Med 353:2747–2757
25. Coates AS, Keshaviah A, Thurlimann B et al (2007) Five years of letrozole compared with tamoxifen as initial adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer: update of study BIG 1-98. J Clin Oncol 25:486–492
26. Coombes RC, Hall E, Gibson LJ et al (2004) A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. N Engl J Med 350:1081–1092
27. Boccardo F, Rubagotti A, Puntoni M et al (2005) Switching to anastrozole versus continued tamoxifen treatment of early breast cancer: preliminary results of the Italian Tamoxifen Anastrozole Trial. J Clin Oncol 23:5138–5147
28. Goss PE, Ingle JN, Martino S et al (2003) A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. N Engl J Med 349:1793–1802
29. Goss PE, Ingle JN, Martino S et al (2005) Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17. J Natl Cancer Inst 97:1262–1271
30. Goss PE, Ingle JN, Ales-Martinez JE et al (2011) Exemestane for breast-cancer prevention in postmenopausal women. N Engl J Med 364:2381–2391
31. Dowsett M, Cuzick J, Ingle J et al (2010) Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 28:509–518
32. Roop RP, Ma CX (2012) Endocrine resistance in breast cancer: molecular pathways and rational development of targeted therapies. Future Oncol 8:273–292
33. Riemsma R, Forbes CA, Amonkar MM et al (2012) Systematic review of lapatinib in combination with letrozole compared with other first-line treatments for hormone receptor positive(HR+) and HER2+ advanced or metastatic breast cancer(MBC). Curr Med Res Opin 28:1263–1279
34. Bachelot T, Bourgier C, Cropet C et al (2012) Randomized phase II trial of everolimus in combination with tamoxifen in patients with hormone receptor-positive, human epidermal growth factor receptor 2-negative metastatic breast sancer with prior exposure to aromatase inhibitors: A GINECO study. J Clin Oncol 30:2718–2724
35. Baselga J, Campone M, Piccart M et al (2012) Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med 366:520–529
36. Baselga J, Semiglazov V, van Dam P et al (2009) Phase II randomized study of neoadjuvant everolimus plus letrozole compared with placebo plus letrozole in patients with estrogen receptor-positive breast cancer. J Clin Oncol 27:2630–2637
37. Lednicer D, Babcock JC, Marlatt PE et al (1965) Mammalian antifertility agents. I. Derivatives of 2,3-diphenylindenes. J Med Chem 8:52–57

Uncorrected Proof

Author's Proof

38. Lednicer D, Lyster SC, Aspergren BD, Duncan GW (1966) Mammalian antifertility agents. 3. 1-aryl-2-phenyl-1,2,3,4-tetrahydro-1-naphthols, 1-aryl-2-phenyl-3,4-dihydronaphthalenes, and their derivatives. J Med Chem 9:172–176
39. Lednicer D, Lyster SC, Duncan GW (1967) Mammalian antifertility agents. IV. Basic 3,4-dihydronaphthalenes and 1,2,3,4-tetrahydro-1-naphthols. J Med Chem 10:78–84
40. Legha SS, Slavik M, Carter SK (1976) Nafoxidine – an antiestrogen for the treatment of breast cancer. Cancer 38:1535–1541
41. Ke HZ, Paralkar VM, Grasser WA et al (1998) Effects of CP-336,156, a new, nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. Endocrinology 139:2068–2076
42. Ke HZ, Qi H, Crawford DT et al (2000) Lasofoxifene (CP-336,156), a selective estrogen receptor modulator, prevents bone loss induced by aging and orchidectomy in the adult rat. Endocrinology 141:1338–1344
43. Ke HZ, Qi H, Chidsey-Frink KL et al (2001) Lasofoxifene (CP-336,156) protects against the age-related changes in bone mass, bone strength, and total serum cholesterol in intact aged male rats. J Bone Miner Res 16:765–773
44. Ke HZ, Foley GL, Simmons HA et al (2004) Long-term treatment of lasofoxifene preserves bone mass and bone strength and does not adversely affect the uterus in ovariectomized rats. Endocrinology 145:1996–2005
45. Vajdos FF, Hoth LR, Geoghegan KF et al (2007) The 2.0 A crystal structure of the ERalpha ligand-binding domain complexed with lasofoxifene. Protein Sci 16:897–905
46. Shiau AK, Barstad D, Loria PM et al (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937
47. Brzozowski AM, Pike AC, Dauter Z et al (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
48. Rosati RL, Da Silva Jardine P, Cameron KO et al (1998) Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryltetrahydronaphthalene. J Med Chem 41:2928–2931
49. Cummings SR, Ensrud K, Delmas PD et al (2010) Lasofoxifene in postmenopausal women with osteoporosis. N Engl J Med 362:686–696
50. Moffett A, Ettinger M, Bolognese M et al (2004) Lasofoxifene, a next generation SERM, is effective in preventing loss of BMD and reducing LDL-C in postmenopausal women. J Bone Miner Res 19:S96
51. LaCroix AZ, Powles T, Osborne CK et al (2010) Breast cancer incidence in the randomized PEARL trial of lasofoxifene in postmenopausal osteoporotic women. J Natl Cancer Inst 102: 1706–1715
52. Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. Cancer Res 50:4177–4189
53. Gennari L (2009) Lasofoxifene, a new selective estrogen receptor modulator for the treatment of osteoporosis and vaginal atrophy. Expert Opin Pharmacother 10:2209–2220
54. Robinson SP, Jordan VC (1987) Reversal of the antitumor effects of tamoxifen by progesterone in the 7,12-dimethylbenzanthracene-induced rat mammary carcinoma model. Cancer Res 47: 5386–5390
55. Jabara AG, Toyne PH, Harcourt AG (1973) Effects of time and duration of progesterone administration on mammary tumours induced by 7,12-dimethylbenz(a)anthracene in Sprague–Dawley rats. Br J Cancer 27:63–71
56. Chan O, Inouye K, Akirav E et al (2005) Insulin alone increases hypothalamo-pituitary-adrenal activity, and diabetes lowers peak stress responses. Endocrinology 146:1382–1390
57. Lewis-Wambi JS, Kim H, Curpan R et al (2011) The selective estrogen receptor modulator bazedoxifene inhibits hormone-independent breast cancer cell growth and down-regulates estrogen receptor alpha and cyclin D1. Mol Pharmacol 80:610–620

Uncorrected Proof

Author's Proof

58. Kanasaki H, Bedecarrats GY, Kam KY et al (2005) Gonadotropin-releasing hormone pulse frequency-dependent activation of extracellular signal-regulated kinase pathways in perifused LbetaT2 cells. Endocrinology 146:5503–5513
59. Smith S, Minck D, Jolette J et al (2005) Bazedoxifene prevents ovariectomy-induced bone loss in the Cynomolgus monkey. J Bone Miner Res 20:S174
60. Kharode Y, Bodine PV, Miller CP et al (2008) The pairing of a selective estrogen receptor modulator, bazedoxifene, with conjugated estrogens as a new paradigm for the treatment of menopausal symptoms and osteoporosis prevention. Endocrinology 149:6084–6091
61. Chang KC, Wang Y, Bodine PV et al (2010) Gene expression profiling studies of three SERMs and their conjugated estrogen combinations in human breast cancer cells: insights into the unique antagonistic effects of bazedoxifene on conjugated estrogens. J Steroid Biochem Mol Biol 118:117–124
62. Bain RR, Jordan VC (1983) Identification of a new metabolite of tamoxifen in patient serum during breast cancer therapy. Biochem Pharmacol 32:373–375
63. Jordan VC, Bain RR, Brown RR et al (1983) Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. Cancer Res 43:1446–1450
64. Qu Q, Zheng H, Dahllund J et al (2000) Selective estrogenic effects of a novel triphenylethylene compound, FC1271a, on bone, cholesterol level, and reproductive tissues in intact and ovariectomized rats. Endocrinology 141:809–820
65. Hellmann-Blumberg U, Taras TL, Wurz GT, DeGregorio MW (2000) Genotoxic effects of the novel mixed antiestrogen FC-1271a in comparison to tamoxifen and toremifene. Breast Cancer Res Treat 60:63–70
66. Wurz GT, Read KC, Marchisano-Karpman C et al (2005) Ospemifene inhibits the growth of dimethylbenzanthracene-induced mammary tumors in Sencar mice. J Steroid Biochem Mol Biol 97:230–240
67. Namba R, Young LJ, Maglione JE et al (2005) Selective estrogen receptor modulators inhibit growth and progression of premalignant lesions in a mouse model of ductal carcinoma in situ. Breast Cancer Res 7:R881–R889
68. Wurz GT, Hellmann-Blumberg U, DeGregorio MW (2008) Pharmacologic effects of ospemifene in rhesus macaques: a pilot study. Basic Clin Pharmacol Toxicol 102:552–558
69. Nilsson S, Gustafsson JA (2011) Estrogen receptors: therapies targeted to receptor subtypes. Clin Pharmacol Ther 89:44–55
70. Nilsson S, Makela S, Treuter E et al (2001) Mechanisms of estrogen action. Physiol Rev 81: 1535–1565
71. McInerney EM, Weis KE, Sun J et al (1998) Transcription activation by the human estrogen receptor subtype beta (ER beta) studied with ER beta and ER alpha receptor chimeras. Endocrinology 139:4513–4522
72. Pike AC, Brzozowski AM, Hubbard RE et al (1999) Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. EMBO J 18:4608–4618
73. Hoekstra WJ, Patel HS, Liang X et al (2005) Discovery of novel quinoline-based estrogen receptor ligands using peptide interaction profiling. J Med Chem 48:2243–2247
74. Norman BH, Dodge JA, Richardson TI et al (2006) Benzopyrans are selective estrogen receptor beta agonists with novel activity in models of benign prostatic hyperplasia. J Med Chem 49:6155–6157
75. Follettie MT, Pinard M, Keith JC Jr et al (2006) Organ messenger ribonucleic acid and plasma proteome changes in the adjuvant-induced arthritis model: responses to disease induction and therapy with the estrogen receptor-beta selective agonist ERB-041. Endocrinology 147: 714–723
76. Jain N, Xu J, Kanojia RM et al (2009) Identification and structure-activity relationships of chromene-derived selective estrogen receptor modulators for treatment of postmenopausal symptoms. J Med Chem 52:7544–7569

Uncorrected Proof

Author's Proof

77. Wallace OB, Lauwers KS, Dodge JA et al (2006) A selective estrogen receptor modulator for the treatment of hot flushes. J Med Chem 49:843–846
78. Mohler ML, Bohl CE, Jones A et al (2009) Nonsteroidal selective androgen receptor modulators (SARMs): dissociating the anabolic and androgenic activities of the androgen receptor for therapeutic benefit. J Med Chem 52:3597–3617
79. Chabbert-Buffet N, Meduri G, Bouchard P, Spitz IM (2005) Selective progesterone receptor modulators and progesterone antagonists: mechanisms of action and clinical applications. Hum Reprod Update 11:293–307
80. Hudson AR, Roach SL, Higuchi RI (2008) Recent developments in the discovery of selective glucocorticoid receptor modulators (SGRMs). Curr Top Med Chem 8:750–765
81. Baxter JD, Funder JW, Apriletti JW, Webb P (2004) Towards selectively modulating mineralocorticoid receptor function: lessons from other systems. Mol Cell Endocrinol 217:151–165
82. Yoshihara HA, Scanlan TS (2003) Selective thyroid hormone receptor modulators. Curr Top Med Chem 3:1601–1616
83. Berger JP, Petro AE, Macnaul KL et al (2003) Distinct properties and advantages of a novel peroxisome proliferator-activated protein [gamma] selective modulator. Mol Endocrinol 17: 662–676
84. Jordan VC (2009) A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 69:1243–1254
85. Speroff L (2009) A good man: Gregory Goodwin Pincus: the man, his story, the birth control pill. Arnica Pub., Portland, xxi, 359 pp
86. Jordan VC (2006) Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 147(Suppl 1):S269–S276

Uncorrected Proof

Author's Proof

Author Queries

Chapter No.: 10

Query Refs.	Details Required	Author's response
AU1	Please check sentence starting "None of this. . ." for sense.	
AU2	Please check if edit to sentence starting "Women who use. . ." is okay.	
AU3	Please provide appropriate opening quotes for sentence starting ". . . something done."	
AU4	Please check if edit to sentence starting "I partially like the...." is okay.	
AU5	Usage of all caps for emphasis has been changed to italics. Please check if okay.	
AU6	Please check if edit to sentence starting "This immediately was. . ." is okay.	

Uncorrected Proof

Author's Proof

Appendix A: Four Decades of Discovery in Breast Cancer Research and Treatment: An Interview with V. Craig Jordan

Marc Poirot

The past is never dead. It is not even the past.—William Faulkner

Uncorrected Proof

Abstract

V. Craig Jordan is a pioneer in the molecular pharmacology and therapeutics of breast cancer. As a teenager, he wanted to develop drugs to treat cancer, but at the time in the 1960s, this was unfashionable. Nevertheless, he saw an opportunity and, through his mentors, trained himself to reinvent a failed "morning-after pill" to become tamoxifen, the gold standard for the treatment and prevention of breast cancer. It is estimated that at least a million women worldwide are alive today because of the clinical application of Jordan's laboratory research. Throughout his career, he has always looked at "the good, the bad, and the ugly" of tamoxifen. He was the first to raise concerns about the possibility of tamoxifen increasing endometrial cancer. He described selective estrogen receptor modulation (SERM), and he was the first to describe both the bone protective effects and the breast chemopreventive effects of raloxifene. Raloxifene did not increase endometrial cancer and is now used to prevent breast cancer and osteoporosis. The scientific strategy he introduced of using long-term therapy for treatment and prevention caused him to study acquired drug resistance to SERMs. He made the paradoxical discovery that physiological estrogen can be used to treat and to prevent breast cancer once exhaustive antihormone resistance develops. His philosophy for his four decades of discovery has been to use the conversation between the laboratory and the clinic to improve women's health.

M. Poirot (✉)
Sterol Metabolism and Therapeutic Innovations in Oncology, INSERM UMR 1037, University of Toulouse III, Cancer Research Center of Toulouse, Institut Claudius Regaud, 20, rue du pont Saint Pierre, Toulouse Cedex 31052, France
e-mail: marc.poirot@inserm.fr

P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
DOI 10.1007/978-3-0348-0664-0, © Springer Basel 2013

Author's Proof

Abbreviations. AACR, American Association for Cancer Research; ASCO, American Society of Clinical Oncology; CEE, Conjugated equine estrogen, DES, Diethylstilbestrol; DMBA, Dimethylbenzanthracene; EBCTCG, Early Breast Cancer Trialists' Collaborative Group; ECOG, Eastern Cooperative Oncology Group; FDA, Food and Drug Administration; ICI, Imperial Chemical Industries; SERM, Selective estrogen receptor modulator; STAR, Study of Tamoxifen and Raloxifene; TGF-α, Transforming growth factor-alpha; WFEB, Worcester Foundation for Experimental Biology; WHI, Women's Health Initiative.

Tamoxifen, originally classified as a nonsteroidal antiestrogen but now known as the first selective estrogen receptor modulator (SERM), is a pioneering medicine that for more than 20 years was the gold standard for the adjuvant treatment of breast cancer in pre- and postmenopausal patients with ER-positive tumors [1]. Millions of women continue to live longer and healthier lives because of tamoxifen treatment. Tamoxifen is also a pioneering medicine, as it is the first drug to be approved in the United States of America by the Food and Drug Administration (FDA) for the reduction of the incidence of breast cancer in high-risk pre- and postmenopausal women [2].

Craig Jordan grew up with a passion for chemistry, but was specifically intrigued by the prospect of using organic chemistry to design drugs to treat cancer. At the age of 13, his mother allowed him to convert his bedroom into a chemistry laboratory, where he often got into difficulties during his experiments, either setting the curtains on fire as a rather overreactive experiment was being thrown out of the window or destroying the lawn outside. However, he did convince his mother that by using the chemistry of fertilizers, he could regrow the lawn again, but when he did, it came out an interesting shade of blue! Craig had a passion for teaching, and the chemistry and biology teachers at his school, Moseley Hall Grammar School in Cheadle, Cheshire, England, allowed him to have a laboratory to teach biochemistry. It was these same teachers who convinced his parents that he should apply to university. By contrast, Craig was more content with the idea of becoming an organic chemistry technician at the research laboratories of Imperial Chemical Industries (ICI) near where he lived.

Craig was given an opportunity for interview at only one university (Leeds University, West Yorkshire, England), but he succeeded in convincing the two faculty interviewers, Dr. Ronnie Kaye and Dr. Edward Clark, that he should have a chance in the Pharmacology Department. Years later, Craig found out that the reason he was given an interview was that they had been intrigued at the Headmaster's letter, which stated the candidate was "an unusual young man" and then repeated the statement in capitals. On July 18, 2001, Craig received the first honorary Doctor of Medicine degree from the University of Leeds for humanitarian research that has changed healthcare. The citation, presented by the Chancellor Lord Melvyn Bragg, starts: "Craig Jordan is one of the most distinguished medical scientists of the last one hundred years." He was delighted to be able to invite

Uncorrected Proof

Author's Proof

Drs. Clark and Kaye to the luncheon and the ceremony (Fig. A.1). These were the two individuals who talent-spotted Craig; Dr. Kaye was his tutor for his 4 years as an undergraduate, and Dr. Clark persuaded him to become a graduate student armed with the last available Medical Research Council studentship in the United Kingdom for the year 1969 (Fig. A.2). Someone had declined their studentship, thus allowing Craig to do a Ph.D.! Dr. Clark's project, which Craig found so attractive, was the prospect of extracting the ER from the rodent uterus, purifying it and then crystallizing the ER protein with an estrogen and a nonsteroidal antiestrogen. The x-ray crystallography would be completed at the Astbury Department of Biophysics at the University of Leeds, and all the work was estimated to take the 3 years of the scholarship. At that time, the nonsteroidal antiestrogens had failed to fulfill their promise in the pharmaceutical industry as "morning-after pills"; they were perfect in rats, but in women they did exactly the opposite and enhanced fertility by inducing ovulation.

The project in crystallizing the ER did not go as planned, so he rapidly changed his topic with a new title: "A study of the oestrogenic and anti-oestrogenic activities of some substituted triphenylethylenes and triphenylethanes" (Fig. A.3). This was a good strategic research choice, as no one has yet succeeded in crystallizing the whole ER with either an estrogen or antiestrogen. But further difficulties were to arise in Craig's journey to a career in cancer research.

As a Ph.D. student, Craig was talent spotted for an immediate tenure track faculty position because of his skill as a lecturer. He had no publications and his Ph.D. topic was going nowhere. No one was recommending careers in failed contraceptives! During the interview with the University Committee charged with making the appointment, he was told that he would have to go to America to get his BTA (been to America) before he could start the job. First, however, he had to get a Ph.D., and to do that, it had to be examined. However, the university could find no one in the country qualified for the task. Sir Charles Dodds, the discoverer of the synthetic estrogen, diethylstilbestrol (DES), declined with regrets as he had not kept up with the literature for the past 20 years! But here is where luck and chance take control. He was in the right place at the right time and, by meeting the right people, changed medicine.

Dr. Arthur Walpole was head of the Fertility Control Program at ICI's Pharmaceuticals Division and a personal friend of the chairman of Craig's Pharmacology Department. The university reluctantly accepted Dr. Walpole (despite the fact that he was from industry!) to be Craig's examiner, and he was also able to organize a 2-year visit to the Worcester Foundation for Experimental Biology (WFEB) in Shrewsbury, Massachusetts, to study with Dr. Michael Harper on new methods of contraception. Harper and Walpole had completed all the early work on ICI 46,474 as a contraceptive at ICI Pharmaceuticals in the early 1960s. Craig vividly remembers the transatlantic telephone call with Dr. Harper: "Can you come in September?" "Will $12,000 a year be enough?" "Will you work on prostaglandins?" "Yes, yes, yes," he replied and went off to the library to find out what prostaglandins were! But when he got to the WFEB in September 1972, he was told that Dr. Harper had gone to Geneva to be head of Contraception Research

Uncorrected Proof

Author's Proof



Fig. A.1 Photograph before the ceremony for the degree of Doctor of Medicine *honoris causa* at Leeds University on 18 July 2001. Dr. Edward R. Clark, my Ph.D. supervisor (1969–1972) (*left*), and Dr. Ronnie Kaye, head of my degree course (1965–1969) (*center*), formally from the Department of Pharmacology, University of Leeds, England. I am on the *right side* with my signature glass of Burgundy



Fig. A.2 I always love dressing up. The University of Leeds is my *alma mater*, and I have attended four ceremonies there: (**a**) Bachelor of Science, First Class Honours, 1969; (**b**) Doctor of Philosophy, 1973; (**c**) Doctor of Science, earned by examination. A select committee evaluated my refereed publications to establish a contribution to science, 1985; (**d**) Honorary Doctor of Medicine for Humanitarian Research, 2001

Author's Proof

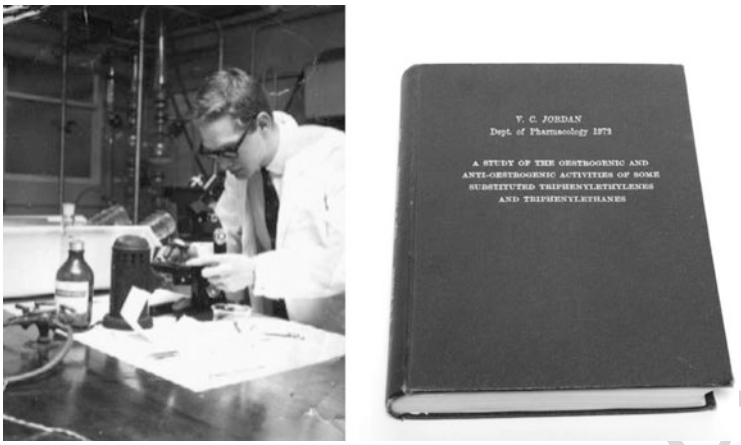


Fig. A.3 My first publicity photograph during the time that I was a Ph.D. student at the Department of Pharmacology, University of Leeds, England, 1969–1972. It was necessary as I had been selected as the Medical Research Council's student representative to the Nobel Prize Winner's meeting in Lindau, Germany, in 1972. I am examining cells from mouse vaginal smears; big science. Also shown is my Ph.D. that nobody wanted to examine

at the World Health Organization. Craig was told to sit down, write up what he would do for the next 2 years, and organize his own laboratory. He was now an independent investigator.

A phone call to Dr. Walpole explained his dilemma at the WFEB, but he felt that there was an opportunity for the failed morning-after pill, ICI 46,474, to be used for the treatment of breast cancer. This call was rewarded by Dr. Walpole arranging for funding and contacts with Ms. Lois Trench at ICI America for Craig to conduct the translational research on the drug that would become tamoxifen. As an independent investigator, the research funding from ICI was an unrestricted research grant, but as Craig was not a cancer research scientist and he was at WFEB, the home of the oral contraceptive, what was the first step to be? Again, it is who you meet. After the National Cancer Act in 1971, the WFEB director had made the decision to bring a cancer research specialist onto the Board of Scientific Advisors to help with future funding opportunities in hormones and cancer research. Dr. Elwood Jensen was the director of the Ben May Laboratory for Cancer Research in Chicago, Illinois, and was credited with the translational research where he described the ER in immature rat estrogen target tissues and then used this knowledge to propose a test for the hormone dependency of metastatic breast cancers. Simply stated, if the ER is absent in the tumor, the patient was unlikely to respond to endocrine ablation (oophorectomy, adrenalectomy, or hypophysectomy), but if the tumor was ER positive, there was a high probability that the tumor would respond to estrogen withdrawal. It was a practical test to avoid morbidity from unnecessary operations that require hospitalization.

Craig spent the day with Dr. Elwood Jensen in November 1972 and told him what he wanted to do with ICI 46,474. Craig subsequently traveled to the Ben May

Uncorrected Proof

Author's Proof

Laboratory for Cancer Research to be taught techniques of ER analysis and to learn all about the dimethylbenzanthracene (DMBA) rat mammary carcinoma model and then to Dr. Bill McGuire's laboratory in San Antonio, Texas, to learn complementary analytical methods for the ER. Armed with these techniques and resources from ICI throughout the 1970s (his first decade of discovery), he created the laboratory principles of targeting the tumor ER and advocating the use of long-term adjuvant tamoxifen therapy as the appropriate clinical strategy to save lives (Fig. A.4) [3, 4]. This proposition by Craig was not at all popular, as throughout the 1970s and 1980s in the United Kingdom, it was strongly believed there was no correlation between tamoxifen use and the presence of the ER in breast tumors. Additionally, nobody was interested in a new antihormone therapy, as combination cytotoxic chemotherapy was king. It was going to cure cancer. However, Craig persevered and had the courage of his convictions that his laboratory research would save lives. As it turned out, tamoxifen has probably saved more lives than any other cancer therapeutic drug.

Craig also learned an important lesson at the WFEB around the time he was to leave and return to Leeds. A senior scientist at the WFEB, Dr. Eliahu Caspi, invited Craig to his office for an interview to explore the possibility of Craig staying at the WFEB. Craig recalls this was a very frightening experience, for Dr. Caspi had a no-nonsense personality, judged people, and said what he thought. He stated that he had been asked to evaluate my CV, as everybody was of the opinion that I would be a useful asset at the WFEB. He stared at Craig across the desk and said, "You don't have a CV, as you have no publications." After the initial shock, Craig responded, "But I haven't discovered anything yet." The advice Craig received was some of the best advice he had received thus far in his career. He was told "to tell them the story so far and link together several related publications to create a theme." Craig has done this ever since, creating the theme of tamoxifen. In 1998, with the release of the successful chemoprevention trial with tamoxifen, Craig was referred to as the "Father of Tamoxifen" by the *Chicago Tribune,* a title that has stuck to this day.

Although many people published using tamoxifen in their studies as a laboratory tool or used it in the 1960s in reproduction research, Craig's focus from the outset was clear; the goal was to develop a medicine for the treatment and prevention of breast cancer (he conducted the first chemopreventive study in the laboratory in 1974 [7], 3 years before the drug was approved by the FDA for the treatment of metastatic breast cancer in postmenopausal women). Craig stresses that but for the unrestricted support from ICI, meeting the right people and his uncompromising determination (many referred to this at the time as poor career judgment), tamoxifen would probably not have happened. Scientists at ICI did not conduct any studies with the drug as an antitumor agent. Indeed, in late 1972, all of the data with ICI 46,474 was reviewed and the research director terminated clinical trials and stopped the development project. The Marketing Department had decided that a treatment for metastatic breast cancer was not going to generate sufficient revenue.

Arthur Walpole was toward the end of his career and chose to take early retirement, but only agreed to remain an employee if funds could be given to a young man he had met, Craig Jordan, who (as he did) wanted to turn ICI 46,474 into

Uncorrected Proof

Author's Proof



Fig. A.4 The I.C.I. Pharmaceuticals at King's College, Cambridge, Meeting in the summer of 1977. The goal of the meeting was physician education about research being done with tamoxifen. This was the first time I presented in public my ideas about targeting the tumor ER and using long-term treatment with tamoxifen as the best strategy to be applied to adjuvant therapy [5]. *Reviews on Endocrine-related Cancer* (49–55). However, the major presentation that made everything change clinically was in Arizona in 1979 [6]. In the above picture, Michael Baum (*right*), was the Chair of the session at King's College and stated that they had plans to use 2 years of tamoxifen as an adjuvant therapy (*on a hunch*). Helen Stewart (*left*) was considering starting a pilot trial in Scotland using 5 years of adjuvant tamoxifen for the treatment of patients. For the placebo arm, patients would be treated with tamoxifen at first recurrence. If toxicity was acceptable, they would

Author's Proof

a drug to treat breast cancer. Walpole and Craig subsequently worked together on an ICI/University joint research scheme when Craig returned as lecturer in the Department of Pharmacology at the University of Leeds in September 1974. Earlier in his career, Dr. Walpole was an accomplished cancer research scientist, but had not been allowed to work in this area by ICI because fertility control was considered to be potentially more lucrative [8]. Dr. Walpole died suddenly on July 2, 1977, before he could witness the success of Craig's laboratory strategy for the treatment and prevention of breast cancer.

The clinical development of tamoxifen was very progressive and validated all your assumptions. Could you tell us how you were involved in the clinical evaluation and how you convinced the company to invest in what may have been very challenging trials?

I think it's fair to say that this was not the real story, but the real story is unbelievable. I have always considered my research as being a conversation between the laboratory and the clinic, and I had the privilege of first introducing tamoxifen to clinical trials' organizations in America. My objective was to provide a scientific rationale for the clinical studies in treatment and prevention. My research and qualifications were required to obtain approval for tamoxifen as a medicine in both Japan and Germany, and I was delighted to be the only person invited from outside of ICI Pharmaceuticals to attend a celebration in 1977, of the Queen's Award for Technological Achievement for tamoxifen. The surprising part about the tamoxifen story is that although patents for the drug were obtained by ICI Pharmaceuticals around the world, in the mid-1960s, these same patents were denied in the United States of America. Thus, all of the work I was completing on the antitumor actions of tamoxifen in the United States was done without patent protection for ICI. Looked at another way, it was clear that all the other pharmaceutical companies had no interest in the clinical development of tamoxifen, because either the drug was not going to work very well or not generate enough revenue. But it was my clinical strategy of long-term adjuvant therapy that saved lives and made revenues [9]. Clinical testing went ahead and when the patents expired in the rest of the world, ICI was awarded the patent for the use of tamoxifen in the treatment of breast cancer in 1985, but backdated to the original patent application in 1965. Now, extended adjuvant therapy was the practical solution for effective treatment. Thus, for the next 20 years, ICI was able to generate

Uncorrected Proof

Fig. A.4 (continued) move forward to test the idea of early long-term treatment or late treatment at first recurrence. Both trials showed survival advantages for long-term adjuvant tamoxifen. The week after the King's College Meeting, I was at the University of Wisconsin at their Comprehensive Cancer Center to convince clinicians of the Eastern Cooperative Oncology Group (ECOG) that longer was going to be better. At the time, tamoxifen was not on the market in America but I was talent spotted by Paul Carbone, the Head of ECOG and the director of the Comprehensive Cancer Center, to be recruited to the University of Wisconsin, Department of Human Oncology. Eventually, I would be the director of their Breast Cancer Research and Treatment Program

Author's Proof

enormous revenues in the United States, as tamoxifen was the standard of care for long-term adjuvant tamoxifen therapy and the only game in town. This money catalyzed the advent of ICI marketing antiandrogens for prostate cancer and the aromatase inhibitors for breast cancer.

Watching your scientific activity since the beginning, you always seem fascinated by the development of small molecules since their conception up to their development. Is that what gives you much fun in your work?

I absolutely love experiments involving the structure-function relationships of the antiestrogens. My basic scientific research has been to create models of gene modulation or replication to determine the structure of the ER antiestrogen complex that subsequently could be interrogated. This passion resulted in a whole series of publications focused on the modulation of the prolactin gene [10–12] which then went through a metamorphosis to study the modulation of the SERM ER complex and the way that the ligand can interact with specific amino acids, thereby switching on or switching off the complex at target genes [13]. We actually found the only natural mutation of the human ER in a laboratory model of tamoxifen-stimulated tumor growth. We engineered the mutant ER into ER-negative breast cancer cells and found it would make the antiestrogen, raloxifene, an estrogen at the transforming growth factor-alpha (TGF-α) target gene. For me, this was important as one amino acid in the ER could change the pharmacology of raloxifene. In other words, this provided a fascinating insight into the relationship of the antiestrogenic side chain and a specific amino acid at the surface of the ER protein [14–17].

Do you think that a drug may have a commercial future in the chemoprevention of cancer?

As you know, we have made enormous progress with advancing the failed breast cancer drug, raloxifene, and millions of women are now benefiting from its use for the treatment of osteoporosis, but with a reduction in breast cancer incidence at the same time. This is the practical reality of our early translational research completed at the University of Wisconsin in the second decade of discovery (1980s). The "Tamoxifen Team" discovered selective estrogen receptor modulation and tamoxifen and raloxifene were both now classified as SERMs [18]. But the realization that tamoxifen could not possibly have widespread use because it increases the risk (though this is very small) of endometrial cancer in postmenopausal women [19], naturally guided us to our new SERM strategy in the late 1980s. We discovered that SERMs maintain bone density [20] and therefore could potentially prevent osteoporosis with the beneficial antiestrogenic side effect of preventing breast cancer [21]. We had solid translational research, as we had found that tamoxifen built bone both in the laboratory [20] and in clinical trial [22]. Raloxifene has a better safety profile and does not increase the risk of endometrial cancer [23], but it does not reduce the risk of coronary heart disease. I think the new SERM, lasofoxifene [24], is very good, as it prevents osteoporosis, breast cancer, coronary heart disease, and strokes, but without an increase of endometrial cancer. The problem is how to

Uncorrected Proof

Author's Proof

advance in a crowded market with low budgets for marketing. Lasofoxifene is approved but not marketed in the European Union.

No molecule targeting estrogen receptor has, to date, proved to be more efficient than tamoxifen in patients despite the development of a number of promising compounds. How do you explain that? Was it a choice of the pharmaceutical industry because of the cost of the development of such compound?

The issue with tamoxifen is unique. It was clearly lucky that tamoxifen had an acceptable toxicology profile for the treatment of cancer. It came onto the market at a time when the standard of care was combination cytotoxic chemotherapy, so tamoxifen looked good to patients. Tamoxifen was not supposed to succeed but advanced from strength to strength for 20 years. However, things change very rapidly in the arena of patient preference. In the early 1990s, when tamoxifen was being considered for testing as a chemopreventive and the specter of endometrial cancer translated from the laboratory [19] to clinical practice, this was clearly not good news for well women. Worse still, tamoxifen was found to produce DNA adducts in rat liver and initiate rat liver hepatocarcinogenesis [25]. Although liver tumors did not translate to clinical practice, this did not lessen concern, as the drug ended up with a black box label as a human carcinogen. Timing is everything with discovery and competitors could never catch up with clinical testing, despite the fact they may have been safer. We will never know.

To demonstrate that natural or synthetic molecules can prevent the occurrence of cancer is long and expensive. This raises the question of the life of the patents but also the natural molecules, which may not be patentable. Do you think there may be solutions to these problems?

I think it's currently impossible to find a solution to this dilemma. Clearly, the pharmaceutical industry will never advance with 20 year studies because the patents will run out. But here is a controversial point: the success of healthcare has now created the situation of increased longevity, so that drugs that enhance survival through prevention can only make matters worse. What is society to do? How does society find the resources to support an aging population?

You have developed recently a very provocative approach using estrogens for the treatment of breast cancers. This can be considered as a paradoxical use of estrogens? Could you explain us a little bit about that.

The third and fourth decades have been a wonderful surprise in our journey of discovery. We posed the question (based upon the clinical acceptance of long-term antihormonal therapy [9] as the most appropriate adjuvant treatment for breast cancer), what would be the mechanism and the timeframe for acquired antihormone resistance? Our first model clearly showed something unique as far as drug resistance is concerned—SERM-stimulated growth, something that is not seen with any other drug in cancer therapy [26]. This form of resistance occurred within a year or two and was consistent with the development of acquired resistance to tamoxifen in

Uncorrected Proof

Author's Proof

metastatic breast cancer. However, here was the dilemma: this model did not replicate the outstanding success observed with 5 years of adjuvant tamoxifen treatment [27]. In fact, 5 years of treatment continues to enhance decreases in mortality for more than a decade once tamoxifen is stopped. By a series of lucky accidents, one of my students (Doug Wolf) discovered that physiologic estrogen could cause dramatic tumor regression after 5 years of tamoxifen treatment, i.e., serial transplantation of tamoxifen-resistant tumors into generations of tamoxifen-treated mice [28]. This discovery reminded me of the words of Sir Alexander Haddow, FRS in 1970 during the Inaugural Karnofsky Lecture at the American Society of Clinical Oncology (ASCO): "... the extraordinary extent of tumour regression observed in perhaps 1 % of post-menopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us ..." [29]. It is now clear that aggressive estrogen deprivation with aromatase inhibitors or SERMs can rapidly reconfigure breast cancer cells through an evolution of drug resistance, which exposes a vulnerability that could not be anticipated—physiological estrogen-induced apoptosis [30, 31]. When Haddow did his original work using high-dose DES for the treatment of metastatic breast cancer in women during their late 60s and 70s, the best therapeutic results occurred the further away the patient was from the menopause. Antihormone therapy accelerates all of that in breast cancer, so physiologic estrogen can initiate the same triggering mechanism. Indeed, this is possibly the same mechanism that is occurring in the Women's Health Initiative (WHI) by conjugated equine estrogen (CEE) alone actually produces a decrease in the incidence of breast cancer in hysterectomized postmenopausal women [32]. What is particularly interesting about these data is the 6 years of monitoring after CEE is stopped, there is a continued reduction in the incidence of breast cancer, i.e., the estrogen has destroyed the nascent breast cancer cells in the ducts [33]. Our current laboratory work is focused entirely on deciphering the molecular mechanism of estrogen-induced apoptosis [34]. In this way, we may find the vulnerability triggered by the ER estrogen complex for cellular destruction; that vulnerable site in the cancer cell may be the next target for a new class of selective anticancer agents applicable to sites other than breast cancer.

Your contributions to medicine have received a lot of recognition but how does one become the "Diana, Princess of Wales Professor of Cancer Research"?

Life is all about chance meetings. In the mid-1990s, I was invited to organize a Breast Cancer Symposium in Chicago, and Diana was my keynote speaker (Fig. A.5). She came on a 3-day visit to Northwestern University and the Robert H. Lurie Comprehensive Cancer Center. Naturally, it was a very special time and when she left to return to London, we agreed to correspond and I sent her copies of my books on tamoxifen. There was even talk of a return trip for either her or Prince William or Prince Harry to open one of our new research buildings. Regrettably, everything changed with her untimely death in a tragic car accident in Paris on August 31, 1997. An anonymous donation was subsequently made to the Robert

Uncorrected Proof

Author's Proof

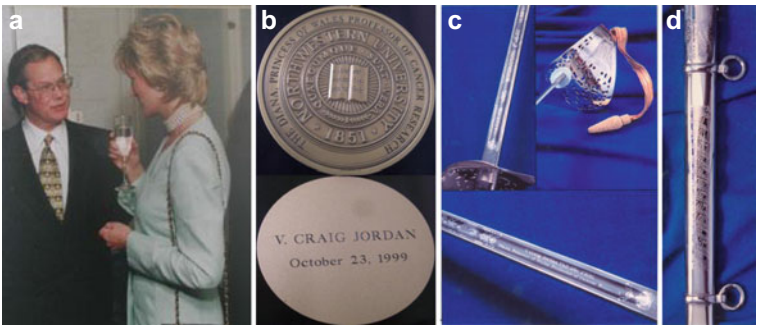


Fig. A.5 The Diana, Princess of Wales Chair of Cancer Research. In June 1996, Diana, the Princess of Wales visited Chicago for 3 days and we first met (**a**) at the evening reception at the home of the President of Northwestern University, Henry Bienen. The Chair was anonymously endowed at the Robert H. Lurie Comprehensive Cancer Center after Diana's untimely death on 31 August 1997. I was inaugurated on 23 October 1999, being presented with a unique Professorial medal (**b**) with copies being sent to her sons Prince William and Harry and also kept by my daughters, Helen and Alexandra. My students presented me with an engraved sword (**c**) to commemorate the event and their names, and the dates of the award of their Ph.D. degrees are engraved on the scabbard (**d**)

H. Lurie Comprehensive Cancer Center, and with letters from Lady Sarah McCorquodale (her sister) and the Earl Spencer (her brother), it was agreed that I would hold a professorship at Northwestern University in her name. Essentially, it was my British citizenship, a British medicine (tamoxifen), and our meeting and correspondence that was important to the family. On October 23, 1999, the Professorship was conferred on me by Henry Bienen, the president of Northwestern University, and over a 2-day period, there was a symposium in my honor by my former Ph.D. students, and during the celebration dinner, attended by representatives from the British Embassy, Barry Furr (the Chief Scientist from ICI), family, friends, and colleagues, my students presented me with an engraved sword (Fig. A.5) with each of the dates of their Ph.D. engraved on the scabbard as battle honors—very moving!

You have contributed more than 600 research and review papers to the literature with more than 23,000 citations and an h-index of 80. If you had to select ten of your research papers and three reviews, which would they be and why?

- Jordan V. C. (1976). *Eur J Cancer* 12: 419–424. Literally my first cancer research paper with tamoxifen that was rejected in 1974, but with kind and generous comments from one of the reviewers. I persevered and eventually this was one of the papers from my work used to justify the chemoprevention trials.
- Jordan V. C. and Allen K. E. (1980). *Eur J Cancer* 16: 239–251. The paper makes three points: this is the first refereed article that longer treatment is going to be better than shorter treatment; our discovery of 4-hydroxytamoxifen's

Uncorrected Proof

Author's Proof

pharmacology as a potent antiestrogen with a binding affinity for ER equivalent to estradiols [35] naturally made us think that this would be a more powerful anticancer agent—not true, cleared too quickly—and finally, we stated that antiestrogen treatment followed by estrogen deprivation would be a good strategy for people—true.

- Gottardis M. M., et al. (1988). *Cancer Res* 48: 812–815. This was the paper that warned the clinical community that tamoxifen could potentially increase the incidence of endometrial cancer in patients—true.
- Gottardis M. M. and Jordan V. C. (1988). *Cancer Res* 48: 5183–5187. This was the first report that acquired drug resistance with tamoxifen was unique and stimulated by SERMs—true.
- Love R. R., et al. (1992). *New Engl J Med* 326: 852–856. This was the randomized clinical trial based on our laboratory evidence and subsequently those of others that tamoxifen would maintain bone density in people. This paper opened the door to raloxifene.
- Levenson A. S. and Jordan V. C. (1998). *Cancer Res* 58: 1872–1875. A clean demonstration that a mutant ER found in a tamoxifen-stimulated tumor by a previous Ph.D. student (Doug Wolf) could change an antiestrogen to an estrogen. This could be done by a natural process.
- Cummings S. R., et al. (1999). *JAMA* 281: 2189–2197. Proof of principle that the concept we first articulated back in the late 1980s that you could develop a SERM to prevent osteoporosis and prevent breast cancer at the same time—true.
- Yao K., et al. (2000). *Clin Cancer Res* 6: 2028–2036. The first refereed publication to demonstrate that drug resistance to tamoxifen evolves and exposes a vulnerability to permit physiologic estrogen to cause tumor regression. Subsequently translated to the clinic—true.
- Vogel V. G., et al. (2006). The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA*. 295: 2727–2741. Two discarded drugs from the pharmaceutical industry that were reinvented in the same pharmacology laboratory to become the pioneering chemopreventive agents and FDA-approved—true.
- Vogel V. G., et al. (2010). *Cancer Prev Res* 3: 696–706. A follow-up of the trial several years after stopping SERM treatment, confirmed the predictions of one of my Ph.D. students (Marco Gottardis) in 1987 that tamoxifen would be the better chemopreventive in the long term.

I've always viewed an invitation to write a review article from a journal as a wonderful opportunity to project your personality, express your views, and, most importantly, reach out to young scientists and graduate students as theirs is the future. Here are my three choices:

- Jordan V. C. (1984). *Pharm Rev* 36: 245–276. This was my first major review when I first came to America. No one had really treated the topic as an issue in pharmacology, as all of the previous reviews in the 1960s and 1970s were about the control of fertility. I wanted a summary of the mechanisms of action of antiestrogens. It was all of our knowledge up to that point (423 citations).

Uncorrected Proof

Author's Proof

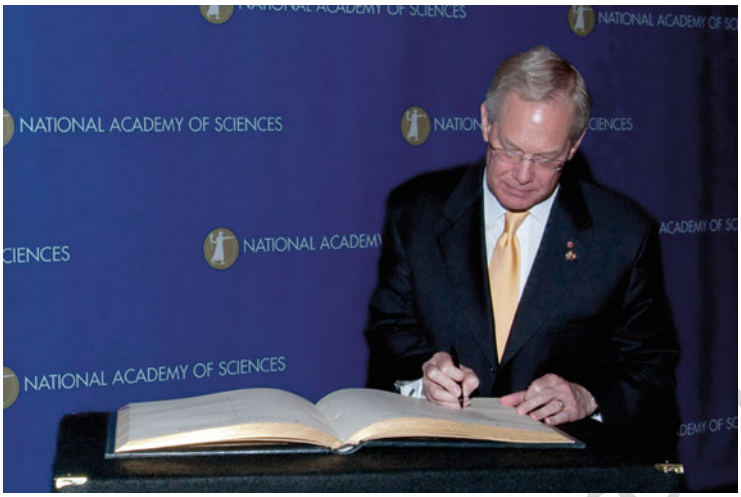


Fig. A.6 Signing the "Great Book" of Members of the National Academy of the Sciences USA during the Induction Ceremony on April 24, 2010

- Jordan V. C. (2006). *Br J Pharmacol* 147: S269–S276. I was thrilled to be asked by the British Pharmacological Society to write the story of my research in a Special Issue of our Journal. I got wonderful feedback from students.
- Jordan V. C. (2009). *Cancer Res*. 69: 1243–1254. I was proud to be asked by the American Association for Cancer Research (AACR) to contribute a review of progress in hormone dependent tumors as a part of a series to celebrate the 100th anniversary of AACR.

I see that you received the David A. Karnofsky Award in 2008 from ASCO, but it is stated in the regulations for the Award that it is given in "recognition of innovative clinical research and developments that have changed the way oncologists think about the general practice of oncology." You are a laboratory scientist and not a clinician; didn't this surprise you?

When I received the telephone call from the chair of the Awards Committee, Gabriel Hortobagyi, I was absolutely dumbfounded, because naturally, I knew I was not a clinician! All previous recipients were clinicians. This is ASCO's highest award, and I was being asked to join the legends of clinical practice. For the first 15 min of my conversation with Gabriel, I examined with him every reason why I should not be their recipient. After 15 min, he became exasperated and said, "Is this a 'Yes, I accept'?" I accepted the honor. Apparently, I learned that the reason the committee selected my work was because as a laboratory scientist and a pharmacologist, I had always been present at clinical breast cancer meetings over the decades, putting forward my point of view in cancer treatment with SERMs. For me, the promise of life was the most important goal. But safety was essential. The involvement I had every day with the clinical evaluation of tamoxifen [22], AU1

Uncorrected Proof

Author's Proof



Fig. A.7 Honorary Fellowship of the Royal Society of Medicine awarded by Professor Ilora Finlay, Baroness Finlay of Llandaff, president of the Royal Society of Medicine (2008). This honor is awarded to individuals of international standing who have eminently distinguished themselves in the service of medicine and the fields which influence it. The Society permits, at most, 100 people into this elite group at any one time. In 2008, there were only 89 Honorary Fellows worldwide. In 2009, I received the Jephcott Medal from the Royal Society of Medicine, and in 2010, I was elected as the president of the Royal Society of Medicine Foundation in North America

followed by leadership positions for the evaluation of raloxifene [23], and then as the scientific chair of the Study of Tamoxifen and Raloxifene (STAR) [36, 37] allowed me to deploy the knowledge generated by my "Tamoxifen Team" over decades to save lives and advance women's health [38]. Please remember that when I started this improbable and unlikely journey at the beginning of the 1970s, cancer therapeutics with a targeted agent, chemoprevention, and the drug group, SERMs (or even tamoxifen for that matter!) did not exist. Cancer research was not recommended as a career for the pharmacologist and the pharmacologist would not knowingly venture into women's health. All of the revenues in the pharmaceutical industry were derived from heart drugs and drugs that affected the central nervous system (e.g., tranquilizers) (Fig. A.6).

Author's Proof

When I was starting the research for my Ph.D. at Leeds University, Sir Alexander Haddow, FRS in the Inaugural Karnofsky Lecture [29], was dismayed at the prospect for cancer therapeutics. Unlike the success noted with antibiotics for the treatment of different infectious diseases, there were no laboratory tests to establish whether chemotherapy would be effective or not. The physician just had to give it to the patient and see if it worked! Haddow was also not convinced that a cancer-specific drug could be developed because cancer was self. In Haddow's Karnofsky Lecture publication, there was one glimmer of hope: Haddow had used the first chemical therapy to treat any cancer, i.e., high-dose estrogen to treat metastatic breast cancer in women in their late 60s and 70s. He observed that some of the responses just melted the tumors away. But he was dismayed that the mechanisms had remained elusive. I am pleased to say that we have now solved the question surrounding the mechanism of estrogen-induced apoptosis [34] (Fig. A.7).

It is fair to say that the work that has evolved and developed on the treatment and prevention of breast cancer over the past four decades has changed our outlook and replaced pessimism with hope. The first decade of discovery was essential to move forward in the field [9]. It has not only been possible to create change in medical practice, but the laboratory principles all translated to patient care to save or at least extend lives. That is what pharmacology is.

In closing, I must end where we began. I have thanked Drs. Kaye and Clark (Fig. A.1) many times for the opportunity they gave me with a place at Leeds University. The reply I received was usually "we were only doing our job." Good words to remember and live by.

Uncorrected Proof

References

AU3

1. Jordan VC (2003) Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2:205–213
2. Jordan VC (2007) Chemoprevention of breast cancer with selective oestrogen receptor modulators. Nat Rev Cancer 7:46–53
3. Jordan VC, Koerner S (1975) Tamoxifen (ICI46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 11:205–206
4. Jordan VC, Allen KE (1980) Evaluation of the antitumour activity of the nonsteroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 16:239–251
5. Jordan VC (1978) Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. Rev Endocr Relat Cancer Suppl:49–55
6. Jordan VC, Dix CJ, Allen KE (1979) The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In: Salmon SE, Jones SE (eds) Adjuvant therapy of cancer II. Grune & Stratton, Philadelphia, pp 19–26
7. Jordan VC (1976) Effect of tamoxifen (ICI46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. Eur J Cancer 12:419–424
8. Jordan VC (1988) The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. Breast Cancer Res Treat 11:197–209

Author's Proof

9. Jordan VC (2008) Tamoxifen: catalyst for the change to targeted therapy. Eur J Cancer 44:30–38
10. Lieberman ME, Jordan VC, Fritsch M, Santos MA, Gorski J (1983) Direct and reversible inhibition of estradiol-stimulated prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4734–4740
11. Lieberman ME, Gorski J, Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. J Biol Chem 258:4741–4745
12. Jordan VC, Lieberman ME (1984) Estrogen-stimulated prolactin synthesis in vitro classification of agonists, partial agonist and antagonist actions based on structure. Mol Pharm 26:279–285
13. Wolf DM, Jordan VC (1994) The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31:129–138
14. Levenson AS, Jordan VC (1998) The key to the antiestrogenic mechanism of raloxifene is Amino Acid 351 (Asp) in the estrogen receptor. Cancer Res 58:1872–1875
15. Macgregor-Schafer JI, Liu H, Bentrem D, Zapf J, Jordan VC (2000) Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex by substituting glycine for aspartate at amino acid 351. Cancer Res 60:5097–5105
16. Liu H, Lee ES, De Los Reyes A, Zapf JW, Jordan VC (2001) Silencing and reactivation of the selective estrogen receptor modulator (SERM)-ER alpha complex. Cancer Res 61:3632–3639
17. Liu H, Park W, Bentrem DJ, McKian KP, De Los Reyes A, Macgregor-Schafer J, Zapf JW, Jordan VC (2002) Structure-function relationships of the raloxifene-estrogen receptor alpha complex for regulating transforming growth factor-alpha expression in breast cancer cells. J Biol Chem 277:9189–9198
18. Jordan VC (2001) Selective estrogen receptor modulation: a personal perspective. (Perspectives in Cancer Research). Cancer Res 61:5683–5687
19. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48:812–815
20. Jordan VC, Phelps E, Lindgren JU (1987) Effects of antiestrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10:31–35
21. Gottardis MM, Jordan VC (1987) The antitumor actions of keoxifene (raloxifene) and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. Cancer Res 47:4020–4024
22. Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, Carbone PP, Demets DL (1992) Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Eng J Med 326:852–856
23. Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, Norton L, Nickelsen T, Bjarnason NH, Morrow M, Lippman ME, Black D, Glusman JE, Costa A, Jordan VC (1999) The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. JAMA 281:2189–2197
24. Cummings SR, Ensrud K, Delmas PD, Reid DM, Goldstein S, Sriram U, Lee A, Thompson J, Armstrong RA, Thompson DD, Powles T, Zanchetta J, Kendler D, Neven P, Eastell R (2010) Lasofoxifene in postmenopausal women with osteoporosis. N Engl J Med 362:686–696
25. Jordan VC (1995) What if tamoxifen (ICI46,474) had been found to produce liver tumors in rats in 1973? Ann Oncol 6:29–43
26. Gottardis MM, Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 48:5183–5187

Uncorrected Proof

Author's Proof

27. Early Breast Cancer Trialists' Collaborative Group (EBCTCG) (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378:771–784
28. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 127:23–33
29. Haddow A, David A (1970) Karnofsky memorial lecture. Thoughts on chemical therapy. Cancer 26:737–754
30. Yao K, Lee ES, Bentrem DJ, England G, Schafer JIM, O'Regan R, Jordan VC (2000) Antitumor action of physiologic estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. Clin Cancer Res 6:2028–2036
31. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li Y, Bell E, Chandel NS, Jordan VC (2005) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97:1746–1759
32. la Croix AZ, Chlebowski RT, Manson JE, Aragaki AK, Johnson KC, Martin L, Margolis KL, Stefanick ML, Brzyski R, Curb JD, Howard BV, Lewis CE, Wactawski-Wende J (2011) Health outcomes after stopping conjugated equines estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. JAMA 205:1305–1314
33. Jordan VC, Ford LS (2011) Paradoxical clinical effect of estrogen on breast cancer risk: A "new" biology of estrogen-induced apoptosis. Cancer Prev Res 4:633–637
34. Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, Tapia C, Kim HR, Yerrum S, Sharma CGN, Nicolas E, Balagurunathan Y, Ross EA, Jordan VC (2011) Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer via stress responses as identified by global gene expression across time. Proc Natl Acad Sci U S A 108 (47):18879–18886 AU4
35. Jordan VC, Collins MM, Rowsby L, Prestwich G (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305–316
36. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER, Wade JL, Robidoux A, Margolese RG, James J, Lippman SM, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N (2006) The Study of Tamoxifen and Raloxifene (STAR): report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. JAMA 295:2727–2741
37. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER, Wade JL, Robidoux A, Margolese RG, James J, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N (2010) Update of the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: preventing breast cancer. Cancer Prev Res 3:696–706
38. Jordan VC (2008) The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer–survival or death? J Clin Oncol 26:3073–3082
39. Jordan VC (1984) Biochemical pharmacology of antiestrogen action. Pharm Rev 36:245–276
40. Jordan VC (2006) 75th Anniversary Edition British Journal of Pharmacology Special Issue. Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 147:S269–S276
41. Jordan VC (2009) A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. Cancer Res 69:1243–1254

Uncorrected Proof

Author's Proof

Appendix B: Selected Awards That Recognize the Contribution of Tamoxifen and Raloxifene to Medicine

Uncorrected Proof

2012	ASPET Goodman and Gilman Award	586
2011	St. Gallen International Breast Cancer Award	587
2009	Elected to National Academy of Sciences (USA)	588
2009	Fellow of the Academy of Medical Sciences (UK)	589
2008	Honorary Fellowship of the Royal Society of Medicine	590
2008	American Society of Clinical Oncology 38th David A. Karnofsky Award	591
2007	Gregory G. Pincus Award and Medal. Worcester Foundation for Biomedical Research	592
2006	American Society Award for Chemoprevention, American Society of Clinical Oncology	593
2005–2009	Endowed Chair: Alfred G Knudson Chair in Basic Science, Fox Chase Comprehensive Cancer Center	594
2005	Honorary Doctor of Science Degree. University of Bradford, England	595
2003	The Charles F. Kettering Prize of the General Motors Cancer Research Foundation.	596
2002	Officer of the Most Excellent Order of the British Empire (OBE)	597
2002	American Cancer Society Medal of Honor (Basic Research Award).	598
2002	Inaugural Dorothy P. Landon American Association for Cancer Research (AACR) Prize in Translational Research.	599
2001	Bristol Myers Squibb Award and Medal for Distinguished Achievement in Cancer Research	600
2001	Honorary Doctor of Medicine Degree. University of Leeds, England	601
2001	Honorary Doctor of Science Degree. University of Massachusetts	602
2000	Strang Award. Cornell Medical School	603
2000	Honorary Fellowship Award and Medal. Faculty of Medicine of University Colllege, Dublin	604
1999–2004	Endowed Chair: Diana, Princess of Wales Professor of Cancer Research, Robert H. Lurie Cancer Center, Northwestern University Feinberg School of Medicine	605
1994	William L. McGuire Memorial Award. San Antonio Breast Cancer Symposium	606
1993	Cameron Prize Award. University of Edinburgh	607
1993	American Society for Pharmacology and Experimental Therapeutics Award for Experimental Therapeutics. Contributions to research on human disease	608
1993	Gaddum Memorial Award for research contributions to pharmacology, British Pharmacological Society	609

(continued)

P.Y. Maximov et al., *Tamoxifen*, Milestones in Drug Therapy,
DOI 10.1007/978-3-0348-0664-0, © Springer Basel 2013

Author's Proof

610

611 1992	Inaugural Brinker International Breast Cancer Award for Basic Science. Susan G. Komen Foundation
612 1989	Eighth Bruce F. Cain Memorial Award American Association for Cancer Research

Uncorrected Proof

Author's Proof

Author Queries

Chapter No.:

Query Refs.	Details Required	Author's response
AU1	Please check if "Is this a 'Yes, I accept'?" is okay as edited.	
AU2	Please check if inserted citations for Figs. A.6 and A.7 in text are okay.	
AU3	Please cite Refs. [39, 40, 41] in text.	
AU4	Please check if update Ref. [34] is okay.	

Uncorrected Proof