

Award Number: W81XWH-06-1-0590

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

PRINCIPAL INVESTIGATOR: Virgil Craig Jordan, Ph.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center  
Philadelphia, PA 19111

REPORT DATE: September 2007

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.**

<b>1. REPORT DATE (DD-MM-YYYY)</b> 01-09-2007		<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED (From - To)</b> 1 SEP 2006 - 31 AUG 2007	
<b>4. TITLE AND SUBTITLE</b>  A New Therapeutic Paradigm for Breast Cancer Exploiting Low Dose Estrogen-Induce Apoptosis				<b>5a. CONTRACT NUMBER</b>	
				<b>5b. GRANT NUMBER</b> W81XWH-06-1-0590	
				<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b> Virgil Craig Jordan, Ph.D.  E-Mail: V.Craig.Jordan@fccc.edu				<b>5d. PROJECT NUMBER</b>	
				<b>5e. TASK NUMBER</b>	
				<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  Fox Chase Cancer Center Philadelphia, PA 19111				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
				<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited					
<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> 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 resistance breast cancer. To achieve the goal, we have created an optimal collaborative network to study laboratory models of the regulation of estrogen-induced growth and apoptosis in breast cancer. The molecular mechanisms of estrogen action (ER) mediated regulation are being deciphered by the systematic distribution of processed tissues from the Fox Chase Cancer Center (FCCC) to Translational Genomics (TGen) for genomics (siRNA analysis, CGH, and Agilent gene array) and to Georgetown University (GU) for proteomic analysis. All derived data is being loaded on a secure website for analysis. A complimentary clinical trial is positioned to evaluate the antitumor effects of high dose estradiol (30 mg daily) in patients following the success and failure of two consecutive antihormonal therapies. We have already discovered new estrogen regulated growth mechanisms and a new secreted protein, CEACAM-6 in estrogen-deprived breast cancer cells that enhances tumor invasions.					
<b>15. SUBJECT TERMS</b> Antihormone Resistance; Estrogen; Apoptosis; Aromatase Inhibitors; Selective Estrogen Receptor Modulators; Tamoxifen; Raloxifene; Estrogen Receptor; Proteomics; Gene Array; Signal Transduction Pathways					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>	<b>18. NUMBER OF PAGES</b>	<b>19a. NAME OF RESPONSIBLE PERSON</b>
<b>a. REPORT</b>	<b>b. ABSTRACT</b>	<b>c. THIS PAGE</b>			<b>USAMRMC</b>
U	U	U	UU	302	<b>19b. TELEPHONE NUMBER (include area code)</b>

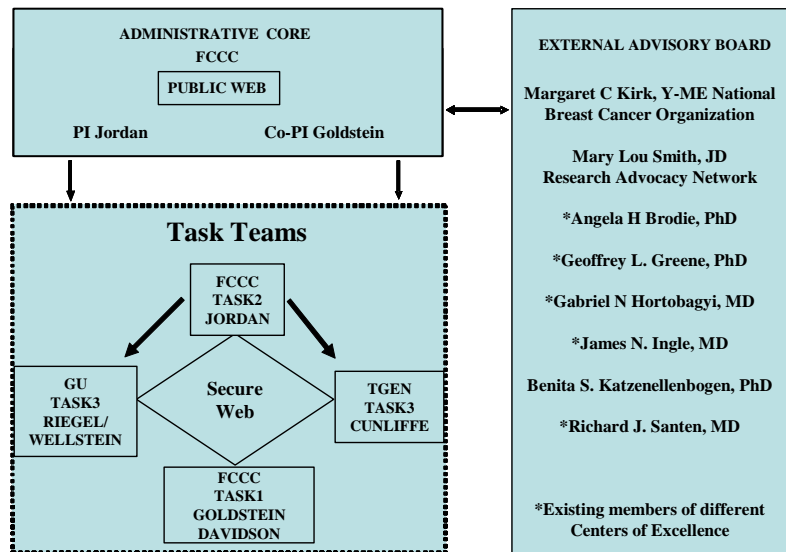
## Table of Contents

Introduction .....	4
Body .....	4
Key Research Accomplishments .....	52
Reportable Outcomes .....	53 & 58
Conclusion .....	55
References .....	55
Appendices .....	57 & 232

# BC050277 – “A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis”.

## Introduction

The Center of Excellence Grant will complete 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 have established an integrated organization 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**).

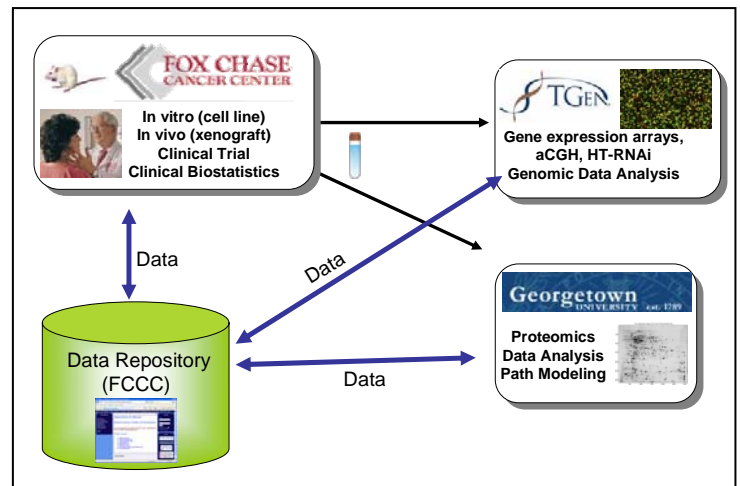


## Body

The body will report our exceptional progress starting with the Administrative Core and then reports from the four Task teams.

## Administration and data tracking

The COE is generating large quantities of data of diverse types (see figure). As a result, we created the Biostatistics and Bioinformatics Consortium Core (BBCC) to provide rigorous experimental design, data analysis and bioinformatics support to all COE investigators and physicians. The BBCC also provides tools for data access, management, annotation and publishing. The combination of this experienced group of biostatisticians, bioinformaticians and data management experts from FCCC (Dr. Ross, Dr. Litwin, Ms. Tchuvatkina, Mr. Collins), Georgetown (Drs. Wu, Seillier-Moiseiwitsch, Ransom, Hu, Huang) and TGen (Drs.



Bittner, Kim, Suh, Balagurunathan) will provide a cohesive core that effectively serves COE scientists and physicians. These individuals are knowledgeable about cancer biology, genetics, and epidemiology; and have broad experience in quantitative applications for clinical trials, pre-clinical studies, functional genomics, proteomics, translational investigations and cancer prevention and control research.

## Accomplishments:

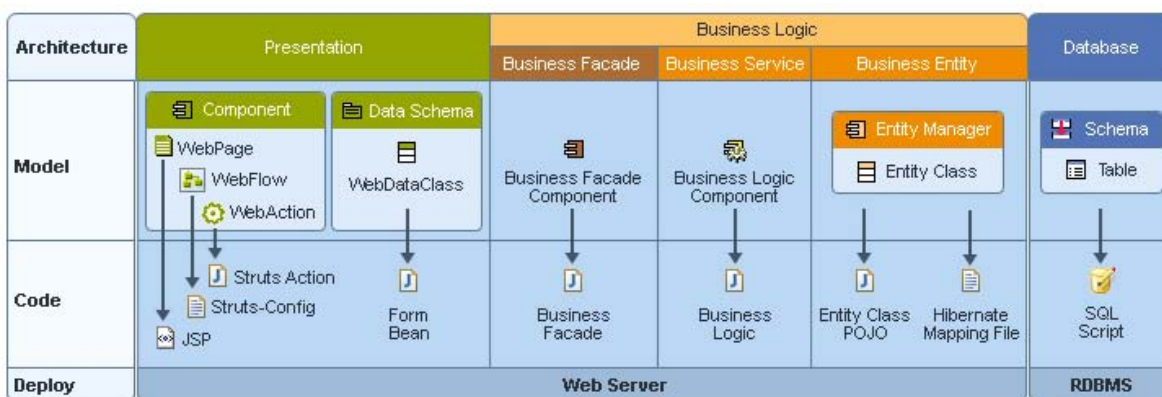
- Over the past 12 months Drs. Ross and Litwin completed statistical design for a clinical trial of Estrace (oral estradiol). The primary objective is to estimate the clinical response rate of treatment with estradiol in postmenopausal women with hormone receptor-positive metastatic breast cancer who have failed two or more sequential endocrine therapies. We will use an early stopping phase II trial design to achieve this end. We will test the null hypothesis that response rate is at most 10% versus the alternative that it is greater than 10%. Response is defined as either complete response (CR) or partial response (PR). We will initially recruit 40 evaluable patients. If no more than 4 patients respond we will terminate the study for lack of efficacy. If greater than 4 patients respond to treatment, we will continue recruitment to a total of 80 evaluable patients. If at least 12 of 80 patients respond, we will reject the null hypothesis, otherwise we will accept it. The design has 8.8% type I error and 86.2% power if the true response rate is 20%. Under the null hypothesis, the study has a 63.4% chance of early termination. Assuming 10% of recruited patients will be inevaluable we will need to recruit approximately 88 patients. To protect patient safety, rules were also developed by BBCC statisticians for early study termination due to excess toxicity. If 7 patients have a Dose Limiting Toxicity (DLT) at any point during treatment of the first 40, we will terminate the trial early as too toxic. If, at any point in the second cohort, the DLT total reaches 12 then treatment will likewise be terminated and declared too toxic. Data analysis plans were also finalized for the secondary objectives. In addition, BBCC statisticians completed the statistical design and data analysis plans for the follow-up clinical study which will evaluate efficacy at a reduced dose of estradiol. Core members developed a novel 3-stage phase II design for this follow-up clinical trial.

- The BBCC has established the COE web-portal ([coe.fccc.edu](http://coe.fccc.edu)) to enable the four geographically separate institutions to function in a more unified way by allowing timely sharing of research data and enhancing day-to-day communications among COE investigators (see figure). A Sun Microsystems Sun Fire T2000 Server with 8 gigabytes of DDR2 memory and 1.8 terabytes of disk space was purchased to support the various functions of the COE web-portal. The portal is maintained at FCCC and runs under the UNIX operating system. It has public and private components. The public component includes a description of each project/core and links to the participating organizations. Access to of the private component of the portal is controlled through a robust, role-based security system. Restrictions are applied to each user commensurate with their needs to access the data. As studies expand, these various privileges will be reviewed and modified as needed. The secure portion of the portal provides a number of critical capabilities to the COE. These include:

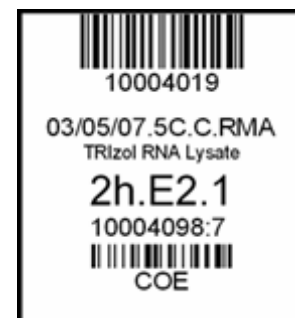


- Data repository to facilitate information collection/sharing and investigator collaboration. The website permits uploading and controlled access to tissue culture, high-throughput genomic and proteomic data generated by the COE. Genomic data (e.g., RNA expression microarray data) is generated at TGEN and uploaded to the COE Data Repository (COE-DR) at FCCC. Similarly, proteomics data generated at Georgetown University (GU) can be transmitted to FCCC (and retrieved) directly through the portal. In the future, the COE-DR will also include animal model and de-identified clinical data.
- Work Group Collaboration and Communication Tools: These portal features facilitate communications and cooperative work among geographically diverse COE participants. The content management system was implemented through tikiwiki (a PHP based open source software product ([tikiwiki.org](http://tikiwiki.org))). COE investigators are able to archive documents, data, presentation materials (e.g., PowerPoint), important references and research protocols through this system. Blogs and forums available through this portal, allow users to post questions, remarks and comments on experiments or relevant journal articles.

- The BBCC designed and developed an information system to support COE cell culture experiments. This J2EE multi-tier application accommodates the collection and storage of information generated by the cell culture experiments conducted at FCCC. This information includes sample availability, sample location, quality control measurements and information about sample shipping. The system also stores data about all sample transformations such as processing, creation of aliquots and pooling. The web interface provides screens to enter new, and review previously entered information. Web interface was also designed to be used in conjunction with a laser bar-code label scanner (see label below). This functionality improves data entry efficiency and quality control. A more complete description of this system can be found in the Task 2 section of this report. Strict quality control is enforced through this system. The architecture diagram of the system is displayed below:



- The BBCC collaborated with COE investigators in the design and development of a bar coded sample identification system. In this system, each sample has a unique identifier composed of the study name (COE) and a unique sample number. Both study and sample IDs are displayed in human readable form and as 1D-bar codes on a sample label. The generic sample identification system is being used for all biological samples collected through the COE project. Depending on a sample source (cell line experiment, clinical trial, xenograft tumor experiment) various additional fields are displayed on the label. For example, each cell line experiment label (see figure) includes: experiment code, sample cell line, assay name, sample preparation/treatment, harvesting time point, condition, replicate number, box identifier and sample location inside the box. Alternatively, samples collected through clinical investigations will present sample type (serum, plasma, frozen biopsy, biopsy formalin block), site number, patient sequence number, and date of collection. For easy distinction, labels for each biosample type have different background color.
- The BBCC collaborated with COE investigators in the preliminary design of an information system for the clinical trial of estradiol in postmenopausal women with hormone receptor-positive metastatic breast cancer. The resulting system will accommodate the collection and storage of information generated by the Clinical Trials Consortium studies including: enrollment logs, patient demographics, health history, physical exams, prior treatment, concomitant medications, drug compliance, adverse events/toxicities, clinical responses, clinical labs, quality of life, and optional biopsy/blood sample information. This web-based application will be built using J2EE technologies.
- The BBCC conducted monthly web-conferences. The purpose of these web-meetings (which began in March, 2007) was to build collaborations, exchange data analysis and data management capabilities, ensure coordination of biostatistical and bioinformatics efforts across the several COE institutions, and define requirements for the COE-DR. Each conference lasted 1 hour and included a formal presentation by one of the sites followed by group discussion. A commercial web-meeting software product (www.livemeeting.com) was used to augment voice conference calls via simultaneous video of the



presentation (e.g., software demonstration, PowerPoint slides) on their desktop computers via internet connections. Agendas and supporting materials are available on the secure portion of the COE portal.

**Task 1. To conduct exploratory clinical trials to determine the efficacy and dose response of proapoptotic effects of estrogen [diethylstilbestrol (DES)] in patients following the failure of two successful antihormonal therapies.**

**Task 1a.**

During the first year of funding, we have focused on building the clinical infrastructure for the conduct of this multi-institutional clinical trial associated with the award. During the first four months after being awarded the Department of Defense (DOD) COE award, we then sought and successfully secured funding for this investigator-initiated clinical trial as a non-restricted grant from Astra-Zeneca Pharmaceuticals to financially support the clinical trial operations. These funds will support the Fox Chase Cancer Center protocol support management office which will serve as the functional “central operations center” for the adverse event monitoring and regulatory surveillance and control, as well as quality assurance of the clinical trial. As such, the protocol support management team has created a portfolio of case report forms enabling reporting of adverse events, patient enrollment logs, pill diary forms, as well as recording measurement of response to treatment. Additionally, in collaboration with the Fox Chase Cancer Center Biostatistics department, over the past year, we have developed an electronic database for the clinical information acquisition including patient enrollment logs and demographics, health history, physical exams, prior treatment(s), concomitant medications, drug compliance, adverse events/toxicities, clinical responses, clinical labs and quality of life assessments.

The clinical trial which was originally reviewed and approved by the Fox Chase Cancer Center Institutional Review Board in June of 2006 has also subsequently been reviewed and approved by grant committee of Astra-Zeneca Pharmaceuticals (March 2007) in accordance with providing funding. Additionally, the clinical trial protocol has undergone initial review by the Department of Defense (September 2006 and July 2007) and is currently undergoing final review prior to activation of the clinical trial. It is anticipated that the Department of Defense final review will be completed within the subsequent thirty days. To ensure compliance with the Food and Drug Administration (FDA) as well as both Astra-Zeneca and the DOD, we have filed for an Investigational New Drug (IND) exemption for both of the therapeutic agents, anastrozole and Estrace, to be administered in the clinic trial. In July of 2007, we were granted an IND exemption for anastrozole by the FDA and the response for Estrace is pending at this time and also expected within the subsequent 30 days.

In conclusion, we believe that this first years’ accomplishments have provided the financial, regulatory and electronic infrastructure to successfully conduct the clinical trial examining a new therapeutic paradigm for breast cancer exploiting low dose estrogen to induce apoptosis and reverse resistance to anti-estrogen therapy.

**Task 2: To elucidate the molecular mechanism of E<sub>2</sub>-induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.**

**Task 2a (original goal)** Complete a series of experiments using sets of well defined breast cancer models of E<sub>2</sub>-induced survival and apoptosis *in vivo* and *in vitro* [at the Fox Chase Cancer Center (FCCC)]. For each **model module** we will generate samples for the analyses [carried out] under **Task 3** [at Georgetown University (GU)] and **Task 4** [at Translational Genomics Research Institute (TGen)]. Each **module** contains combinations of *in vitro* or *in vivo* models to create a biological resource linked to well defined survival (cell growth) or apoptotic end points. Only the effect of estrogen, a SERM, or no treatment will be investigated and compared.

Model Module 1	Model Module 2	Model Module 3
<b><i>In vitro</i></b> a) MCF-7 +/-E <sub>2</sub> b) MCF-7/5C +/-E <sub>2</sub>  <b><i>In vivo</i></b> c) MCF-7/5C +/-E <sub>2</sub>	<b><i>In vivo</i></b> a)MCF-7 +/- E <sub>2</sub> b)MCF-7/RAL1 +/- E <sub>2</sub> c)MCF-7/RAL2 +/- E <sub>2</sub>	<b><i>In vitro</i></b> a) MCF-7/2A +/- 2 concentrations of E <sub>2</sub>  <b><i>In vivo</i></b> a) MCF-7/TAMLT +/- E <sub>2</sub>
Year 1	Year 2	Year 3

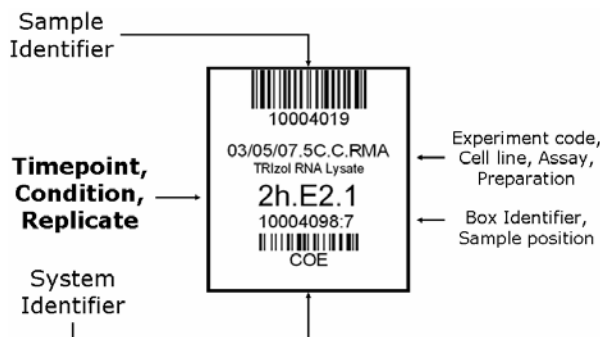
**Original projected timeline and milestones for Task 2a:**

Model modules of studies *in vitro* and *in vivo* from **Task 2** (FCCC) will be completed annually during years 1-3 to provide a firm database of estrogen-induced survival and apoptosis mechanisms linked to biology. Tissues from **Task 2** will be processed for protein analysis and RNA under **Tasks 3 (GU)** and **4 (TGen)** respectively. Coded samples will be posted on the secure net.

**WORK ACCOMPLISHED**

**Development of the COE Laboratory and Clinical Trial Data Capturing and Management Information System (cellWeb)**

Before we could begin generating large series of specimens and derivatives of specimen on the order of thousands of samples, we needed to establish a barcoded specimen tracking system which could be accessed via a web interface. The tracking system, termed the COE Laboratory and Clinical Trial Data Capturing and Management Information System or cellWeb, was developed in collaboration with Biostatistics and Bioinformatics Consortium Core (BBCC) (see BBCC section for additional technical information). The barcoded tracking system was designed to capture all essential information from an experiment and manage physical relationships between samples generated by the cell culture experiments, and in the future, animal tumor experiments, and the clinical trial. To track each individual sample, the labels incorporated a unique identification (ID) number as a barcode and in human readable form, and all pertinent information regarding the sample. An example of a barcoded label is shown in Fig. 2:1. Each line under the unique barcode ID lists specific information about the sample. Line 1 of the label indicates the data of the experiment (“03/05/07”), the cell line [“5C” (MCF-7/5C), as opposed to “WS8” (wild-type MCF-7/WS8), or “2A” (MCF-7/2A)], the tissue type (“C” indicating cell line as opposed to xenograft tumor “T”), the assay for which the sample is intended (“RMA” for RNA for microarray analysis, or “2DE” for proteomics). Line 2 indicates the preparation stage in the processing of the sample, for instance, “TRIZol RNA lysate” indicates a crude RNA preparation that requires further purification. Line 3 shows in a larger font the time point (2 h), the treatment [“E2” or “CON” (control)], and the replicate number (*e.g.*, replicate 1). Line 4 of the label lists the specific box’s ID number (*e.g.*, box 10001098) and position within the box (*e.g.*, position 7) that the sample is located in ultracold storage(-80° C). Line 5 of the label indicates the computer tracking system ID (“COE”).



**Figure 2:1. Barcode Label**

Multiple unique IDs are generated for each original sample representing a different stage in preparation of the sample for its ultimate purpose. For instance, cells for microarray studies are initially harvested in TRIzol reagent to rapidly disrupt cells, destroy nucleases, and stabilize RNA integrity. The TRIzol RNA lysates are then processed to isolate RNA,

which is then used to synthesize single-strand cDNA for real-time PCR assays for gene expression quality control studies (see below for quality control studies). The most efficient way to track at which step in the process and where a sample is stored was to create a new ID for each step that required transferring part of a sample to a new tube. Within the cellWeb informatics system, all derivative IDs corresponding to different preparation stages of a single sample are linked to the originating sample's ID or parent ID. For example, the TRIzol RNA lysate ID corresponds to a parent ID and is used in managing microarray data. If more RNA needs to be isolated from the original TRIzol lysate for follow-up confirmatory studies, new data can be easily linked to the original sample and its corresponding microarray dataset. The microarray studies conducted at TGen will employ the Agilent platform, which entails competitive hybridizations of a treated and untreated sample. To minimize variation, we will pool the 6 replicate control-treated samples and hybridize this pooled reference against each of 6 replicate E2-treated samples, with a different pooled reference for each time point. Hence, there will be 6 arrays per time point, corresponding to 6 separate IDs. The datasets linked to the 6 array IDs can then be collapsed into an average across replicate arrays, which will generate 1 ID per cell line and time point to correlate to parallel time points in the proteomic experiments by ID mapping. In proteomic applications, 12 protein lysate replicates per group are pooled into a single lysate, which is used for the downstream applications. Hence, for proteomic analyses, new IDs will be generated corresponding to pooled samples and linked to the replicate sample IDs which compose the pooled sample. Thus, the relationships among all sample IDs can be traced within the cellWeb informatics system, which also allows possible sources of variation to be tracked, to ensure reliable conclusions from the data.

An additional important purpose of the cellWeb informatics system is validation that the correct samples are processed. Before an experiment is executed, the samples to be generated by the experiment and corresponding IDs are planned using an Excel spreadsheet. The system administrator then uploads the planned experiment sample IDs into the cellWeb application. When the samples are harvested during an experiment or retrieved from ultracold storage for manipulation, the barcoded labels together with a handheld laser scanning gun allow a user to scan and login or logout samples into or out of inventory. However, before samples can be logged in or out, the user must navigate through the cellWeb application to the specific experiment and protocol to be carried out, allowing the cellWeb system to automatically generate the list of sample IDs required. The system then verifies that scanned samples are indeed the intended sample in a graphical layout to allow the user to readily recognize a potential error. After scanning samples, the user must review the samples logged by the system to confirm they are indeed correct (Figure 2:2).

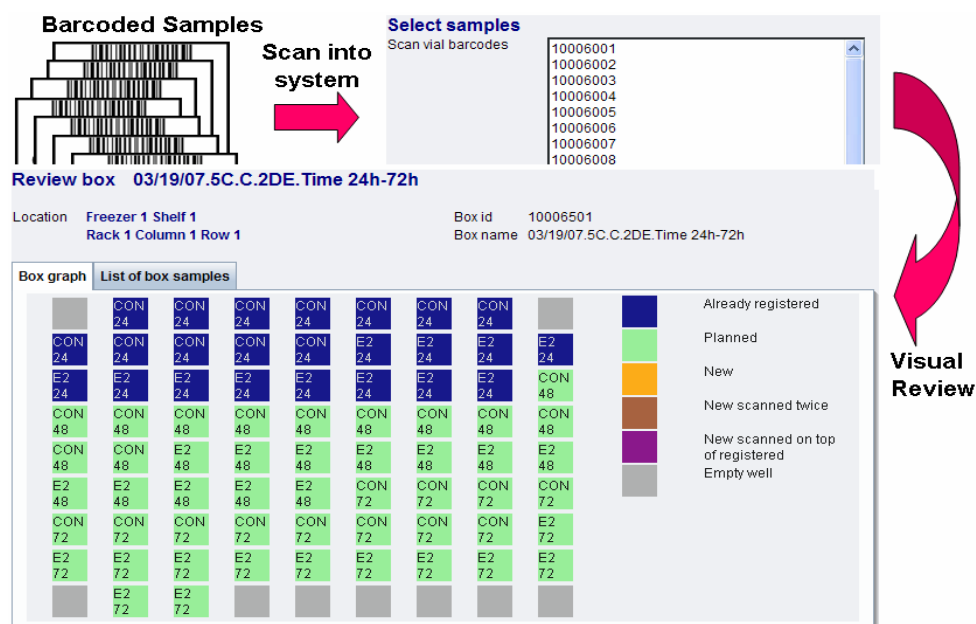


Figure 2:2. Composite screenshot of the cellWeb informatics system showing validation of correct sample usage by cross-verification of scanned sample barcode IDs and the pre-defined inventory list of sample IDs.

The cellWeb informatics system is also used as a central storage location of data generated for each sample ID. Following completion of harvesting samples from an experiment and logging samples into cellWeb, the samples are subjected to quality control testing using molecular markers to validate that the originating cells had been treated as planned with E2 or not, and whether the experiment as a whole responded with growth stimulation or inhibition as predicted in response to E2. These quality control measures are discussed in more detail below. Once quality control testing has been completed, the results are uploaded into cellWeb.

Once samples have been generated, processed, and completed quality control testing at FCCC, the samples are shipped to collaborators at TGen for microarray analysis or GU for proteomic analysis. To track shipping of samples to collaborators, samples are logged out of inventory in the cellWeb system, which validates that the correct samples are being shipped, and a weblink is created to the shipment carrier's website using a shipment tracking number allowing the shipment to be tracked and confirms receipt of delivery.

In summary, the cellWeb informatics system allows users to inventory collected samples, validate correct sample usage, capture essential experimental data, track physical relationships between parent samples and derivatives of parent samples resulting from different stages of sample preparation or pooling samples, and whether samples have shipped to a collaborator (Figure 2:3). However, the cellWeb informatics system is a work in progress and will continue to be developed to support the needs of the investigators as they evolve.

## **GENERATION OF CELL LINE SAMPLES FOR PROTEOMIC (UNDER TASK 3) AND MICROARRAY ANALYSES (UNDER TASK 4)**

### **Experiments Completed**

*Experiment 1)* Production of MCF-7/WS8 protein samples for proteomics.

*Experiment 2)* Production of MCF-7/5C protein samples for proteomics.

*Experiment 3)* Production of MCF-7/WS8 RNA samples for microarrays.

*Experiment 4)* Production of MCF-7/5C RNA samples for microarrays.

### **Experimental Design**

**RNA and Protein Amounts Required.** Initially, we had tried to prepare both microarray and proteomics samples in a single experiment, but these experiments proved too big to execute effectively and our quality control proliferation studies indicated poor growth responses. Therefore, we downsized experiments to only generate samples for either microarray or proteomics studies. Ideally, we would use the same time course for both microarray and proteomics studies. But proteomic studies required at least 4 mg and optimally 6 mg of pooled protein per treatment per time point. To meet this protein requirement, pilot studies indicated 12 replicate large 15 cm tissue culture dishes of cells per group were needed. Hence, we needed to limit the time points to just 3 (24 h, 48 h, and 72 h) to effectively execute the experiment. For microarray studies, 6 replicate chip hybridizations per group were needed to achieve sufficient statistical power. Since only 10 µg of RNA per sample was required for each array plus follow-up confirmatory real-time PCR studies, and this amount of RNA could be isolated from a single 15 cm dish of cells, additional time points could be added to the study. Also, since changes in gene expression would occur at the RNA level before protein levels were affected, earlier time points than used for proteomic studies were needed. Hence, RNA samples were collected at 2 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h. Therefore, proteomic and microarray samples were not generated in the same experiment, but all the proteomic time points have parallel microarray time points, though the time course of the microarray samples was more extensive.

## METHODS

### Cell Lines

The cell lines used to generate microarray and proteomics samples were wild-type MCF-7/WS8 cells, which were clonally selected from parental MCF-7 cells for sensitivity to growth stimulation by 17 $\beta$ -estradiol (E<sub>2</sub>) (1, 2), and MCF-7/5C cells, which were clonally selected from parental MCF-7 cells cultured long-term under estrogen-free conditions (1) and undergo E<sub>2</sub>-induced apoptosis (3). MCF-7/WS8 cells were maintained in fully estrogenized media (phenol red-containing RPMI-1640 and 10% whole fetal bovine serum (FBS), supplemented with 6 ng/ml insulin, 2 mM glutamine, 100  $\mu$ M non-essential amino acids, and 100 U of penicillin and streptomycin per ml). MCF-7/5C cells were maintained in estrogen-free media (phenol red-free RPMI-1640 and 10% dextran-coated charcoal-treated FBS plus the same supplements as for fully estrogenized media). Cells were maintained at 37° C in a humidified 5% CO<sub>2</sub> atmosphere. Three days prior to an experiment, MCF-7/WS8 cells were switched to estrogen-free media.

### Protocol for Proteomic Sample-producing Experiments

MCF-7/WS8 cells, which had been switched from fully estrogenized to estrogen-free media 3 days prior, were seeded at 2 million cells per 15 cm plate in estrogen-free media. Since MCF-7/5C cells undergo E<sub>2</sub>-induced apoptosis, MCF-7/5C cells were seeded at 4 million cells per 15 cm plate in a total of 72 plates corresponding to 12 replicate plates per each of 2 treatment groups per each of 3 time points. Treatments were control (CON) media or media containing 10<sup>-9</sup> M E<sub>2</sub>. Media were replenished at 48 h. Paired CON- and E<sub>2</sub>-treatment groups of cells were collected at 24 h, 48 h, and 72 h after beginning treatments and prepared as protein lysates.

### Protocol for Microarray Sample-producing Experiments

The protocol for producing samples for microarrays was the same as in producing samples for proteomics except that 6 replicate plates per treatment group (CON or E<sub>2</sub>) per each of 7 time points were used for a total of 84 plates of cells. Paired CON- and E<sub>2</sub>-treatment groups of cells were collected at 2 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h after beginning treatments and prepared as TRIzol lysates for RNA purification.

### Quality Control

Quality control tests were implemented to: 1) validate the expected growth response of the cells to 10<sup>-9</sup> M E<sub>2</sub>, either growth induction in MCF-7/WS8 cells or growth inhibition due to apoptosis in MCF-7/5C cells, 2) confirm that individual samples had been treated correctly with 10<sup>-9</sup> M E<sub>2</sub> or without E<sub>2</sub> by measuring RNA expression markers.

**Growth response quality control.** This was carried out in parallel in each experiment by measuring DNA content per well in 24-well dishes using the fluorescent dye Hoechst 33258. MCF-7/WS8 cells were seeded at 72,000 or 15,000 cells per well in the respective experiments to produce proteomic or microarray samples from these cells. MCF-7/5C cells were seeded at 20,000 cells per well. Cells were incubated with control (CON), 10<sup>-9</sup> M E<sub>2</sub>, and 10<sup>-6</sup> M fulvestrant media using 4 replicate wells per treatment. Cells were allowed to grow for 7 days, except the MCF-7/WS8 cells in the plate seeded at 72,000 cells per well were allowed to grow for 3 days. Media was changed on days 2, 4 and 6. On the last day, cells were washed with ice-cold phosphate-buffered saline and frozen. Lysates were generated by sonicating the frozen cells in hypotonic (0.1X) Hank's balanced salt solution. Cellular DNA content in the lysates were measured using the DNA Quantitation kit (BioRad) and compared to a standard curve of known calf thymus DNA amounts by linear regression analysis.

**RNA expression quality control.** This was accomplished using quantitative real-time polymerase chain reaction (PCR) assays to measure levels of two classical E<sub>2</sub>-responsive genes, c-myc which exhibits early kinetics of E<sub>2</sub>-induction, and pS2 which exhibits later kinetics of E<sub>2</sub>-induction. In experiments executed to produce protein samples for proteomics, 10% of the cells were removed from each 15 cm dish from which RNA was purified and cDNA synthesized. The remaining cells were used to generate protein lysates. In experiments

executed to produce RNA samples for microarrays, a portion of RNA isolated from each 15 cm dish was used to generate cDNA.

**Statistical Considerations for RNA expression quality control.** A sample failed RNA expression quality control if the endogenous normalization gene (18S rRNA or GAPDH) indicated that the RNA was degraded, defined as the normalization gene level for a given sample being significantly different than all of the samples in the entire experiment by 1-way ANOVA with a Bonferroni's Multiple Comparison Post Test. If the sample was a planned E2-treated sample, then it must also exhibit a higher level than at least one of the two marker E2-responsive genes (c-myc or pS2) by at least 2 standard deviations than the average of the entire paired CON-treated group to pass quality control. If the sample was a planned CON-treated sample, then it must also exhibit a lower RNA level than at least one of the two marker E2-responsive gene by at least 2 standard deviations than the average of the entire paired E2-treated group to pass quality control.

### **Protein Lysate Preparation**

Cells were washed with ice cold phosphate-buffered saline, and lysed in a solution of 7 M urea, 2 M thiourea, 32.5 mM CHAPS, and 20 mM dithiothreitol. Lysates were subjected to a freeze-thaw cycle, sonicated, and cleared by centrifugation at 14,000xg for 30 min at 4° C.

### **RNA Purification**

Cells were washed with ice cold phosphate-buffered saline, and lysed in TRIzol reagent (Invitrogen). TRIzol lysates were heated to 65° C for 30 minutes, and extracted with chloroform to form an aqueous phase solution, which was mixed 1:1 by volume with 70% ethanol. The resulting mixture was applied to RNeasy (Qiagen) anion-exchange columns and processed following the manufacturer's directions to elute purified total RNA.

### **cDNA Synthesis and Quantitative Real-time PCR Assays**

Single-strand cDNA was synthesized from RNA using random hexamers and oligodeoxynucleotide dT<sub>15</sub> as primers and an MuLV reverse transcriptase-based kit (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). Real-time PCR assays were carried out using 10 ng cDNA per well in a total volume of 25 ul and either the TaqMan Universal PCR Master Mix (Applied Biosystems) for dual fluorescently-labeled probe-based assays, or Power SYBR Green Master Mix (Applied Biosystems) for non-probe-based assays. Each cDNA sample was assayed in triplicate. PCR product accumulation was measured in real-time using an ABI 7700 Sequence Detection System (Applied Biosystems). To quantitate RNA levels, the threshold cycles of PCR product accumulation of unknown samples were compared against a standard curve consisting of 6 2-fold serial dilutions of reference MCF-7/WS8 cDNA. RNA levels of the target gene were normalized to either 18S or GAPDH RNA levels.

PCR primer sequences were as follows: 18S forward 5'-CGG CTA CCA CAT CCA AGG AA-3', 18S reverse 5'-GCT GGA ATT ACC GCG GCT-3, 18S probe 5'-[6FAM]-CAC CAG ACT TGC CCT C-[BHQ1]-3'; c-myc forward 5'-GCC ACG TCT CCA CAC ATC AG-3', c-myc reverse 5'-TCT TGG CAG CAG GAT AGT CCT T-3', c-myc probe 5'-[6FAM]-ACG CAG CGC CTC CCT CCA CTC-[BHQ1]-3'; ER $\alpha$  forward 5'-GGA GGG CAG GGG TGA A-3', ER $\alpha$  reverse 5'-GGC CAG GCT GTT CTT CTT AGA-3', ER $\alpha$  probe 5'-[6FAM]-GCC AAG CCC GCT CAT GAT CAA ACG C-[BHQ1]-3'; GAPDH forward 5'-AGC CGA GCC ACA TCG CT-3', GAPDH reverse 5'-ATG GCA ACA ATA TCC ACT TTA CCA GAG-3, GAPDH probe 5'-[6FAM]-CCC TGG TGA CCA GGC-[BHQ1]-3'; GPR30 forward 5'-CTG CCT AAA CCC CCT CAT CT-3', GPR30 reverse 5'-AAC CTC ACA TCC GAC TGC TC-3', GPR30 probe 5'-[6FAM]-CTG AAC CGC TTC TGT CAC GCT GCC C-[BHQ1]-3'; pS2 forward 5'-CAT CGA CGT CCC TCC AGA AGA G-3', pS2 reverse 5'-CTC TGG GAC TAA TCA CCG TGC TG-3'.

## RESULTS

### Experiment 1) Production of MCF-7/WS8 protein samples for proteomics.

The quality control DNA-based proliferation assay (Fig. 2:3) showed that the MCF-7/WS8 cells used to produce protein lysates for proteomic analysis responded to 72 h of 10-9 M E2 with a 2.0-fold increase in growth compared to CON-treated cells ( $P$ -value < 0.001). Cells treated with the complete antiestrogen fulvestrant (FUL), did not show decreased proliferation compared to the CON-treated cells at any time point, thus the media containing charcoal-stripped FBS was not contaminated with endogenous estrogens. Therefore, the MCF-7/WS8 cells exhibited E2-induced growth stimulation, indicating that the cells were suitable for producing proteomic samples.

The RNA expression quality control assays of c-myc and pS2 in MCF-7/WS8 cells are shown in Fig. 2:4 and 5, respectively. The RNA expression of the endogenous normalization gene 18S indicated that 4 samples contained degraded RNA (data not shown), and one sample was misplaced; these samples have been omitted from the graphs (Fig. 2:4 and 2:5). The E2-treated samples all showed induction of either c-myc or pS2. Replicate 12 in the 24 h CON group showed a high level of c-myc mRNA expression (Fig. 2:4), but the pS2 mRNA level in this replicate was not different than the remaining individual CON samples in the group (Fig. 2:5). Hence the relatively high level of c-myc in this CON sample may reflect biological variation. All of the remaining CON-treated samples showed low levels of c-myc and pS2 expression.

Therefore, 93% (67 out of 72) of the MCF-7/WS8 samples passed quality control. Protein lysates from these samples have been shipped to GU for proteomic studies under Task 3.

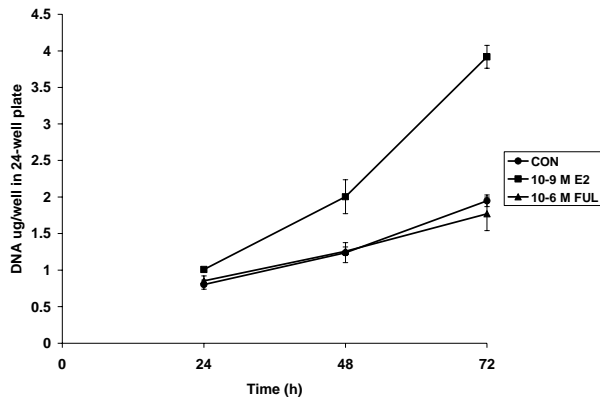


Figure 2:3. Growth over 3 Days of MCF-7/WS8 Cells Used to Produce Proteomic Samples.

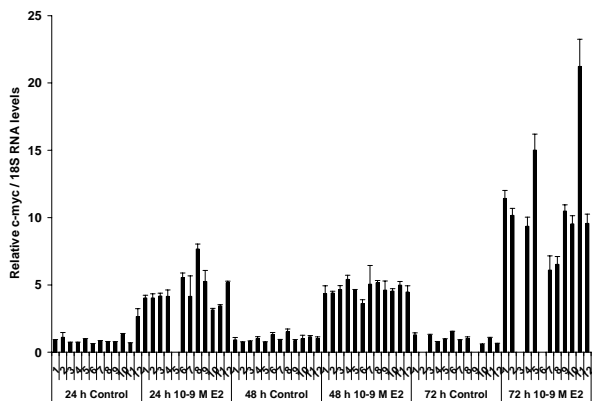
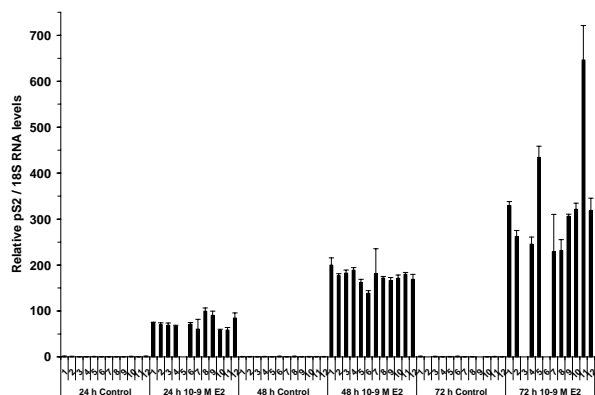


Figure 2:4. c-myc RNA Expression in Individual MCF-7/WS8 Proteomic Samples.



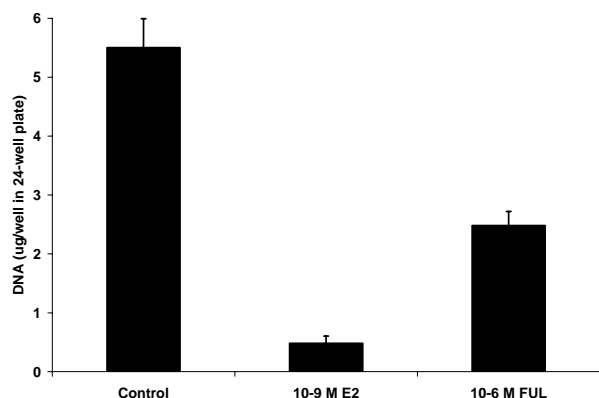
**Figure 2:5. pS2 RNA Expression in Individual MCF-7/WS8 Proteomic Samples.**

### **Experiment 2) Production of MCF-7/5C protein samples for proteomics.**

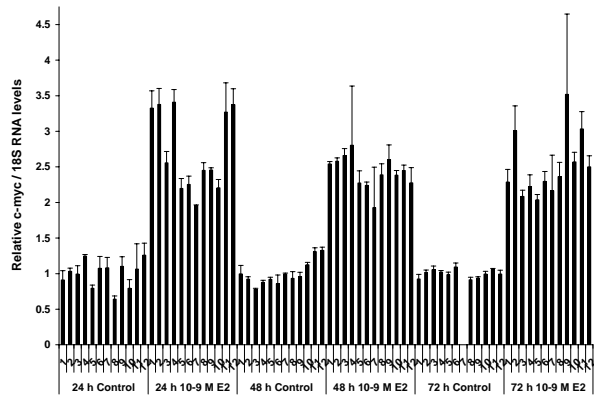
As shown in Fig. 2:6, MCF-7/5C cells used to produce protein lysates for proteomic analysis exhibited a 91.3% inhibition of growth in response to 7 days of  $10^{-9}$  M E2 treatment compared to CON treatment ( $P$ -value  $< 0.001$ ), likely as a result of apoptosis as previously reported (3). MCF-7/5C cells treated with  $10^{-6}$  M FUL also showed decreased growth by 54.9% compared to CON-treated cells ( $P$ -value  $< 0.001$ ), but significantly more growth than that of the E2-treated cells ( $P$ -value  $< 0.001$ ). Hence, MCF-7/5C cells exhibited partial resistance to FUL, as previously reported (3). Overall, the MCF-7/5C cells exhibited the expected growth responses to E2 and FUL, indicating that the cells were appropriate to use for producing proteomic samples.

The 18S rRNA levels in the MCF-7/5C cells indicated that replicate 7 in the 72 h CON contained degraded RNA (sample omitted from the graphs). All of the E2-treated cells showed induction of c-myc (Fig. 2:7) and pS2 RNA levels (Fig. 2:8), while low levels of the RNAs were observed in CON-treated cells.

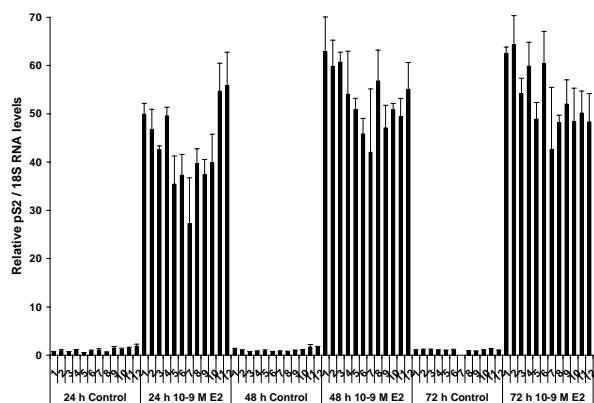
Therefore, 99% (71 out of 72) of the MCF-7/5C samples passed quality control. Protein lysates from these samples have been shipped to GU for proteomic studies under Task 3.



**Figure 2:3. Growth over 7 Days of MCF-7/5C Cells Used to Produce Proteomic Samples.**



**Figure 2:7. c-myc RNA Expression in Individual MCF-7/5C Proteomic Samples.**



**Figure 2:4. pS2 RNA Expression in Individual MCF-7/5C Proteomic Samples.**

### **Experiment 3) Production of MCF-7/WS8 RNA samples for microarrays.**

The proliferation of MCF-7/WS8 cells (Fig. 2:9) used to produce microarray samples showed a 3.75-fold increase over 7 days of 10-9 M E2 treatment compared to CON-treated cells ( $P$ -value < 0.001). Therefore, the MCF-7/WS8 cells exhibited the expected E2-induced growth stimulation, and indicated that they were acceptable for producing proteomic samples.

The RNA expression quality control assays of the endogenous normalization gene GAPDH indicated that none of samples contained degraded RNA (data not shown). However, replicates 2 and 3 in the 2 h CON group were mistakenly combined. RNA levels of c-myc (Fig. 2:10) and pS2 (Fig. 2:11) in the MCF-7/WS8 cells for microarrays indicated that every sample had been properly treated. The E2-treated samples all showed induction of either c-myc or pS2, while the CON-treated samples did not. Although 2 CON replicates had been combined, the CON-treated replicates in the same group will eventually be combined to serve as a chip hybridization reference RNA in the microarray experiments, hence these pre-maturely combined CON replicates were retained in the experiment.

Therefore, 100% (84 out of 84) of the MCF-7/WS8 RNA samples passed quality control and have been shipped to TGen for microarray studies under Task 4.

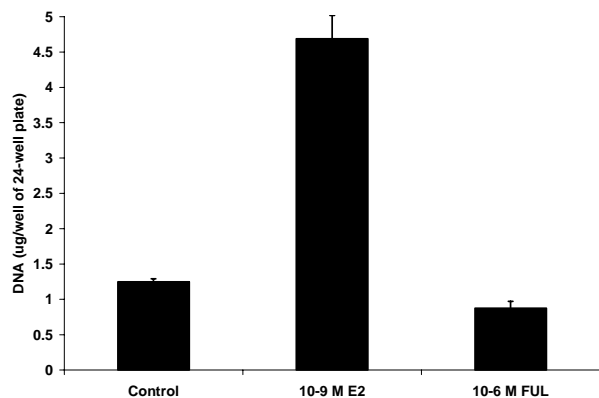


Figure 2:9. Growth over 7 Days of MCF-7/WS8 Cells Used to Produce Microarray Samples.

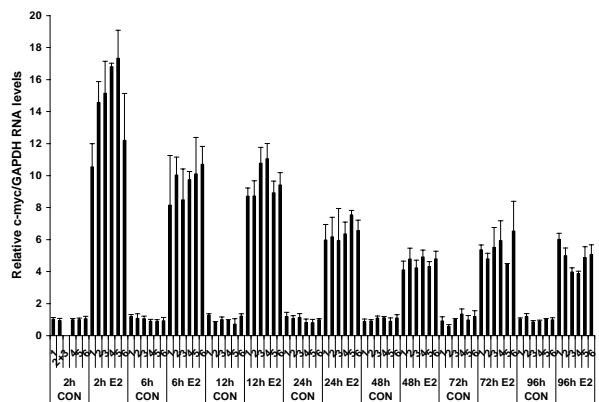


Figure 2:10. c-myc RNA Expression in Individual MCF-7/WS8 Microarray Samples.

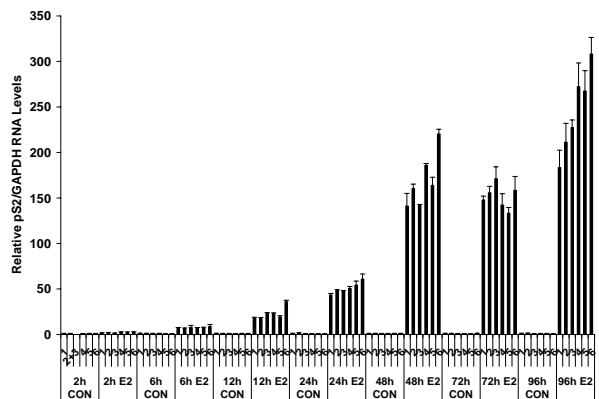


Figure 2:11. pS2 RNA Expression in Individual MCF-7/WS8 Microarray Samples.

#### Experiment 4) Production of MCF-7/5C RNA samples for microarrays.

The MCF-7/5C cells (Fig. 2:12) used to produce microarray samples showed 79.6% inhibition of growth after 7 days of 10-9 M E2 treatment compared to the CON-treated cells ( $P$ -value < 0.001), again likely due to E2-induced apoptosis. Therefore, the MCF-7/5C cells exhibited the expected growth in response to E2 treatment, and that they were suitable for microarray analysis.

The mRNA levels of GAPDH in the MCF-7/5C cells indicated that 5 of the samples contained degraded RNA (data not shown). The E2-treated MCF-7/5C cells for microarrays all exhibited induction of either c-myc (Fig. 2:13) or pS2 (Fig. 2:14), while the CON-treated samples did not. Hence the cells had been properly treated. The tube containing the 12 h CON replicate 4 cDNA sample had cracked and the cDNA sample was lost prior to determining the pS2 RNA level, but the RNA remained. Since this sample showed robust c-myc induction and the RNA was not lost, this sample passed quality control.

Therefore, 94% (79 out of 84) of the MCF-7/5C RNA samples passed quality control and have been shipped to TGen for microarray studies under Task 4.

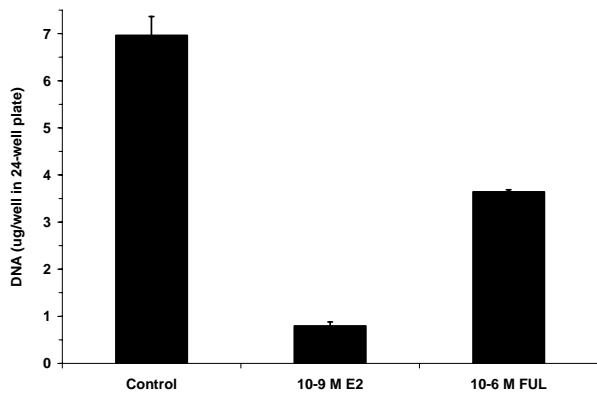


Figure 2:12. Growth of MCF-7/5C Used to Produce Microarray Samples.

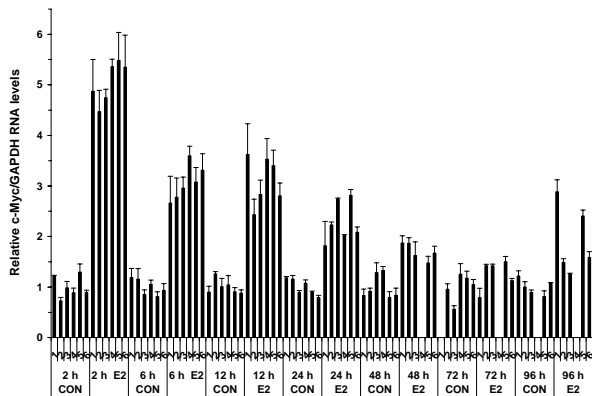


Figure 2:13. c-myc RNA Expression in Individual MCF-7/5C Microarray Samples.

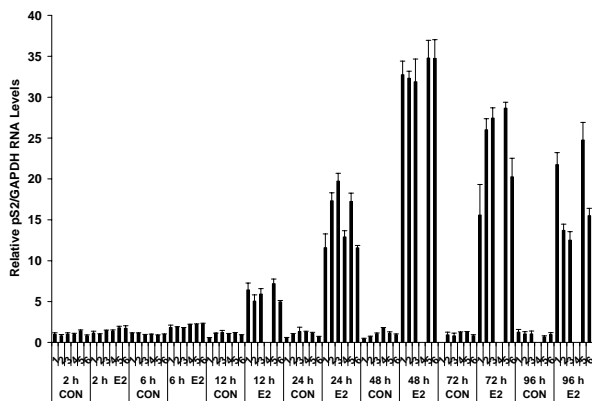


Figure 2:14. pS2 RNA Expression in Individual MCF-7/5C Microarray Samples.

**PRELIMINARY EXPERIMENTS: PRODUCTION OF XENOGRAFT TUMOR SAMPLES FOR MICROARRAY ANALYSES (UNDER TASK 4)**

The experiments involving growth of the *in vivo* xenograft tumor models are described in greater detail in the publication attached as an appendix by Ariazi et al., “Emerging principles for the development of resistance to antihormonal therapy: Implications for the clinical utility of fulvestrant” (J Steroid Biochem Mol Biol, 102: 128-138, 2006) (4).

## Experiments Completed

*Experiment 1*) Production of wild-type MCF-7/E2 tumor samples for microarrays (Fig. 2:15).

*Experiment 2*) Production of phase I-resistant MCF-7/RAL1 tumor samples for microarrays (Fig. 2:16).

*Experiment 3*) Production of phase II-resistant MCF-7/RAL2 tumor samples for microarrays (Fig. 2:17).

*Experiment 4*) Production of phase II-resistant MCF-7/TAM2 tumor samples for microarrays (Fig. 2:18).

*Experiment 5*) Production of estrogen withdrawal-resistant MCF-7/5C tumor samples for microarrays (Fig. 2:19).

## Methods

### Athymic Mice, Tumor Inoculation, and Tumor Tracking

All procedures involving animals have been approved by the Fox Chase Cancer Center's Internal Animal Care and Use Committee.

All animal studies employed female ovariectomized athymic BALB/c nude (*nu/nu*) mice (Taconic, Hudson, NY, USA) that were inoculated with tumor cells at 5-6 weeks of age. For experiments employing tumor models which were generated and serially propagated as xenografts (*in vivo*), 1 mm<sup>3</sup> tumor sections were bilaterally transplanted using a trocar into the axillary mammary fat pads. For studies using tumor models which were generated and maintained in tissue culture (*in vitro*), cells were suspended in phosphate-buffered saline and bilaterally injected into axillary mammary fat pads at 10<sup>7</sup> cells per site.

Tumor growth was tracked by weekly measurements of tumor length (*l*) and width (*w*) using Vernier calipers, from which the tumor cross-sectional area was calculated using the equation:  $(l/2) \times (w/2) \times \pi$ . Tumor growth curves are expressed as the average cross-sectional tumor area per treatment group  $\pm$  standard error (SE).

### Drug treatments

Mice were treated with estrogen by implanting a 0.3 cm E<sub>2</sub> silastic capsule subcutaneously into the intrascapular region on the back of the mouse at the time of tumor cell inoculation. The capsules were prepared by filling silicone tubing (0.078 inch inner diameter/ 0.125 inch outer diameter; Fisher) 0.3 cm in length with a 1:3 (*w/w*) mixture of E<sub>2</sub> (Sigma-Aldrich, St. Louis, MO, USA) and silastic elastomer (Dow Corning, Midland, MI, USA), and then sealing the ends with silicone adhesive (Dow Corning) and sterilized by gamma irradiation. Athymic mice implanted with these capsules achieve mean serum levels of 83.8 pg/ml (308 pM) E<sub>2</sub> (5), which approximates perimenopausal E<sub>2</sub> levels in women. RAL and TAM were orally administered by gastric intubation at 1.5 mg/day 5 days per week. Evista tablets (Eli Lilly Pharmaceuticals, Indianapolis, IN, USA; purchased from the Fox Chase Cancer Center's pharmacy), the clinically available form of RAL (60 mg/tablet), were initially dissolved in water, and then suspended at 10 mg/ml in 10% polyethylene glycol 400/Tween 80 (99.5% polyethylene glycol 400, 0.5% Tween 80) and 0.9% carboxymethyl cellulose. TAM (Sigma) was initially dissolved in ethanol (EtOH), and then suspended at 10 mg/ml in 10% polyethylene glycol 400/Tween 80 (99.5% polyethylene glycol 400, 0.5% Tween 80) and 0.9% carboxymethyl cellulose. The FUL administered was the clinical faslodex preparation, a 50 mg/ml proprietary solution of FUL in primarily EtOH supplemented with castor oil as a release rate modifier. FUL was administered as 2 mg *sc* injections in the scruff of the neck given 5 days per week. The clinical Faslodex preparation was purchased from the Fox Chase Cancer Center's pharmacy.

### Derivation of the MCF-7/E2 Xenograft Tumor Model

The MCF-7/E2 xenograft tumor model, representing the antihormonal-sensitive stage of breast cancer, was originally developed by bilateral injection of 10<sup>7</sup> MCF-7 cells, grown in tissue culture, into the axillary mammary fat pads of female ovariectomized athymic BALB/c *nu/nu* mice implanted with a 0.3 cm E<sub>2</sub> capsule (6). The resulting MCF-7/E2 tumors have been propagated *in vivo* by serial transplantation into likewise E<sub>2</sub>-treated ovariectomized athymic mice.

### Derivation of MCF-7/RAL1 Xenograft Tumor Model

The MCF-7/RAL1 (Phase I) SERM-resistant tumor model was derived by transplantation of MCF-7/E2 tumors into RAL-treated ovariectomized athymic mice. After extended RAL treatment, a small percentage of these tumors showed minimal but significant growth, and following re-transplantation into new RAL-treated ovariectomized athymic mice, these tumors then exhibited robust RAL-stimulated growth (7). MCF-7/RAL1 tumors have been propagated *in vivo* for over 3 years by serial transplantation into RAL-treated ovariectomized athymic mice.

### **Derivation of MCF-7/RAL2 Xenograft Tumor Model**

The MCF-7/RAL2 (Phase II) SERM-resistant tumor model was developed *in vitro* by tissue culture of MCF-7 cells in estrogen-free medium supplemented with 1  $\mu$ M RAL for over 1 year (8). MCF-7/RAL2 cells were maintained in culture in estrogen-free MEM plus 5% dextran-coated charcoal-treated calf serum, 2 mM glutamine, 6 ng/ml bovine insulin, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 1 $\times$  non-essential amino acids (8). MCF-7/RAL2 tumors were generated by growing these cells in culture, and then bilaterally injecting them into the axillary mammary fat pads of ovariectomized athymic mice at  $10^7$  cells per site.

### **Derivation of MCF-7/TAM2 Xenograft Tumor Model**

The MCF-7/TAM2 (Phase II) SERM-resistant tumor model was developed in a similar manner as the MCF-7/RAL1 tumor model, by initial transplantation of MCF-7/E2 tumor pieces into TAM-treated ovariectomized athymic mice, and repeated transplantation of minimally growing tumors into new TAM-treated mice until robust TAM-stimulated growth occurred. These MCF-7/TAM tumors passed through different phases of SERM resistance; they were initially stimulated to grow by both TAM and E<sub>2</sub> (9, 10), but have evolved over 5 years of serial propagation *in vivo* to a stage in which only TAM, but not E<sub>2</sub>, stimulates growth (11-13).

### **Derivation of Estrogen Deprivation-resistant MCF-7/5C Xenograft Tumor Model**

MCF-7/5C cells were clonally isolated from a population of MCF-7 cells grown under long-term estrogen-free conditions (1) and represent Phase II LTED resistance. The MCF-7/5C cells were grown in culture as described above, and then bilaterally injected into the axillary mammary fat pads of ovariectomized athymic mice at  $10^7$  cells per site.

### **Statistical Analyses for Tumor Models**

Tumor growth was analyzed longitudinally with two-factor analysis of variance (ANOVA) to determine significant differences in cross-sectional areas between all tumors in each treatment group in a time-dependent manner. All statistical tests were two-sided and calculated using SAS (SAS Institute, Cary, NC, USA).

## **RESULTS**

### ***Experiment 1) Production of wild-type MCF-7/E2 tumor samples for microarrays.***

MCF-7/E2 xenograft tumors are propagated *in vivo* by serial transplantation into 0.3 cm E<sub>2</sub> capsule-implanted ovariectomized athymic mice. MCF-7/E2 tumor cores were implanted into 15 ovariectomized athymic mice and separated into 3 groups of 5 mice each, or 10 tumors per group. The treatment groups were control (no treatment), 0.3 cm E<sub>2</sub> capsule *sc*, or 0.3 cm E<sub>2</sub> capsule *sc* plus FUL. The FUL formulation corresponded to the clinical Faslodex preparation, which is a proprietary solution of primarily EtOH and some castor oil as a slow release-rate modifier. The clinical Faslodex preparation was administered as a 2 mg *sc* injection given 5 times per week, totaling 10 mg/week.

The MCF-7/E2 tumors grew robustly when treated with the 0.3 cm E<sub>2</sub> capsule, but did not grow in the control group (Fig. 2:15, E<sub>2</sub> vs. Control,  $P < 0.0001$ ), demonstrating that these tumors were dependent on E<sub>2</sub>. The implanted capsules produce E<sub>2</sub> levels that are in the physiologic range observed in perimenopausal women. The cross-sectional areas of the MCF-7/E2 tumors treated with E<sub>2</sub> plus FUL was significantly smaller than those tumors treated with E<sub>2</sub> alone (Fig. 15,  $P$ -values  $< 0.0001$ ). Therefore, FUL inhibited E<sub>2</sub>-stimulated growth of MCF-7/E2 tumors.

For microarray analysis (under Task 4), only established MCF-7/E2 tumors grown in the presence of E2 were used. However, two weeks before the tumors were collected, the 0.3 cm E2 capsule was removed from 2 mice to generate 4 E2-withdrawn control tumors.

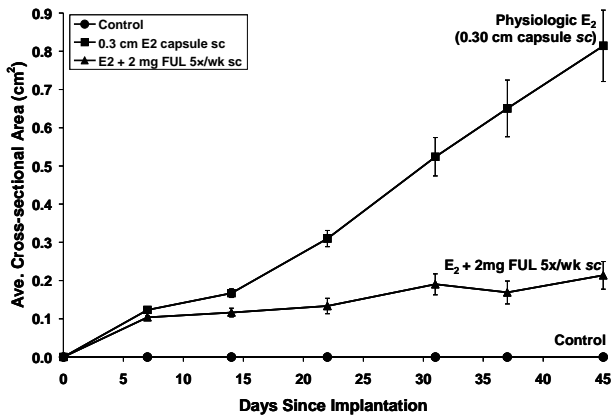


Figure 2:5. Preliminary Experiment with MCF-7/E2 Tumors Used for Microarray Analysis.

**Experiment 2) Production of phase I-resistant MCF-7/RAL1 tumor samples for microarrays.**

MCF-7/RAL1 tumors are maintained *in vivo* by serial transplantation into 1.5 mg/day RAL-treated ovariectomized athymic mice. To illustrate the phase of SERM resistance the MCF-7/RAL1 tumor should be categorized into, MCF-7/RAL1 tumor cores were implanted into 20 ovariectomized athymic mice and separated into 4 treatment groups of 5 mice each (10 tumors/group) corresponding 1.5 mg/day RAL *po*, 0.3 cm E2 capsule *sc*, 2 mg/day FUL *sc*, and control (no treatment). The MCF-7/RAL1 tumors were significantly stimulated to grow by RAL treatment ( $P < 0.0001$ ) and by E2 treatment ( $P < 0.0001$ ) compared to control treatment (Fig. 2:16). However, a modest amount of growth was observed in the control-treated group, indicating that these tumors are not absolutely dependent upon an ER ligand with partial agonist activity. We have previously shown that primary cultures of MCF-7/RAL1 tumors exhibit equivalent levels of estrogen response element (ERE)-regulated reporter gene activity in the absence of E2 as did primary cultures of MCF-7/E2 tumors when treated with E2 (7). Thus, the unliganded ER activity in MCF-7/RAL1 tumors is high and probably contributed to the modest growth of these tumors without the need of RAL or E2. FUL did not significantly effect the growth of MCF-7/RAL1 tumors (Fig. 2:16). Thus, either a SERM or E2, but not FUL, supports the growth of these MCF-7/RAL1 xenografts. Therefore, these tumors are categorized as Phase I SERM-resistant.

For microarray analysis (under Task 4), only established MCF-7/RAL1 tumors grown in the presence of RAL were used. However, two weeks before the tumors were collected, RAL treatment was removed from 2 mice to generate 4 RAL-withdrawn control tumors.

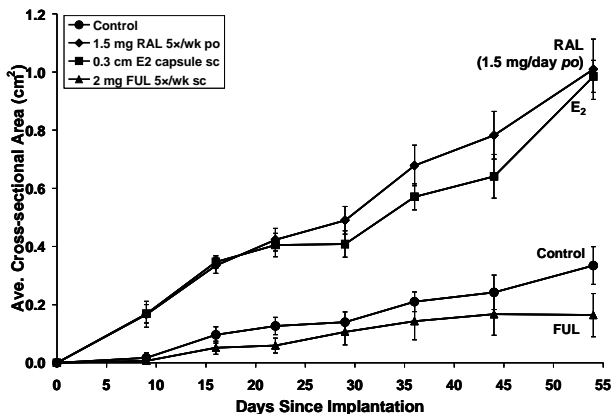


Figure 2:6. Preliminary Experiment with MCF-7/RAL1 Tumors Used for Microarray Analysis.

### Experiment 3) Production of phase II-resistant MCF-7/RAL2 tumor samples for microarrays.

MCF-7/RAL2 tumor cells are maintained *in vitro* by culture in media containing 1  $\mu$ M RAL. To study the growth properties of MCF-7/RAL2 cells *in vivo*, the cells were grown in culture and injected into 20 ovariectomized athymic mice, which were separated into 4 groups of 5 (10 tumors/group) and treated with 1.5 mg/day RAL *po*, 0.3 cm E<sub>2</sub> capsule *sc*, 2 mg/day FUL *sc*, or control (not treated). The MCF-7/RAL2 tumors only grew when treated with RAL (RAL vs. control,  $P < 0.0001$ ), and did not form any palpable tumors by day 42 when treated with E<sub>2</sub>, FUL or not treated (control) (Fig. 2:17). We have previously shown that when MCF-7/RAL2 tumors are allowed to grow by treating with TAM until they are established and then switching treatments to E<sub>2</sub>, E<sub>2</sub> causes tumor regression by inducing apoptosis as measured by TUNEL staining (8). Therefore, growth of the MCF-7/RAL2 tumors was dependent on RAL, but inhibited by E<sub>2</sub> and FUL, which categorizes these tumors as Phase II SERM-resistant.

For microarray analysis (under Task 4), only established MCF-7/RAL2 tumors grown in the presence of RAL were used. However, two weeks before the tumors were collected, RAL treatment was removed from 2 mice to generate 4 RAL-withdrawn control tumors.

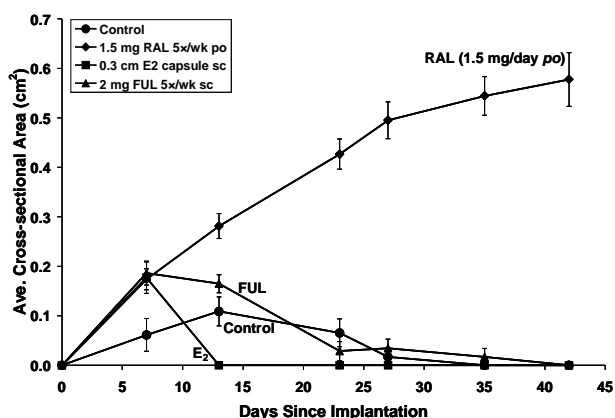


Figure 2:7. Preliminary Experiment with MCF-7/RAL2 Tumors Used for Microarray Analysis.

### Experiment 4) Production of phase II-resistant MCF-7/TAM2 tumor samples for microarrays.

MCF-7/TAM2 tumors are propagated *in vivo* by serial transplantation into 1.5 mg/day TAM-treated ovariectomized athymic mice. To characterize the growth properties of this tumor type, MCF-7/TAM2 tumor cores were implanted into 20 ovariectomized athymic mice, which were separated into 4 groups of 5 (10 tumors/group) and treated with 1.5 mg/day TAM *po*, 0.3 cm E<sub>2</sub> capsule *sc*, 2 mg/day FUL (Faslodex) *sc*, or not treated (control). MCF-7/TAM2 tumors were stimulated to grow by TAM compared to the control group (Fig. 2:4,  $P < 0.0001$ ). The control group did show a minimal amount of growth (Fig. 2:18), which is hypothesized to be due to substantial unliganded ER activity as in the MCF-7/RAL1 model. FUL did not significantly effect growth of the MCF-7/TAM2 tumors versus control treatment. Interestingly, E<sub>2</sub> did significantly inhibit tumor growth compared to the control group (Fig. 2:18,  $P = 0.0004$ ). As with the MCF-7/RAL2 tumors, we have previously demonstrated that E<sub>2</sub> treatment leads to regression of MCF-7/TAM2 tumors (11, 13) by inducing apoptosis as detected by TUNEL staining (12). Therefore, TAM stimulated growth, FUL did not support growth, and E<sub>2</sub> inhibited growth of MCF-7/TAM2 tumors, defining this model as Phase II SERM-resistant.

For microarray analysis (under Task 4), only established MCF-7/TAM2 tumors grown in the presence of TAM were used. However, two weeks before the tumors were collected, TAM treatment was removed from 2 mice to generate 4 TAM-withdrawn control tumors.

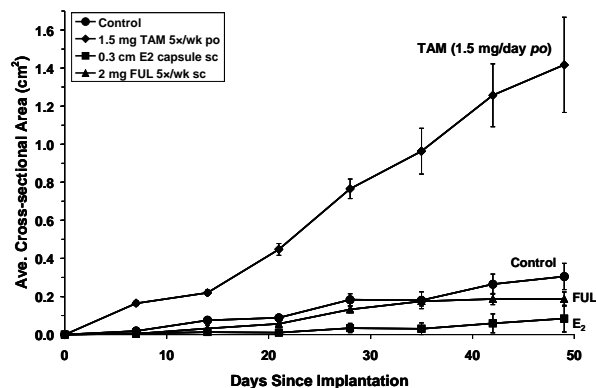


Figure 2:8. Preliminary Experiment with MCF-7/TAM2 Tumors Used for Microarray Analysis.

**Experiment 5) Production of estrogen withdrawal-resistant MCF-7/5C tumor samples for microarrays.**

We verified that the MCF-7/5C cells grown *in vitro* in cell culture behaved similarly *in vivo* as a xenograft tumor. MCF-7/5C cells were grown in culture and injected into 20 ovariectomized athymic mice. The animals were separated into 4 treatment groups of 5 mice each (10 tumors/group), corresponding to control (not treated), 0.3 cm E<sub>2</sub> capsule *sc*, 2 mg/day FUL *sc* (Faslodex), and 0.3 cm E<sub>2</sub> capsule *sc* plus 2 mg/day FUL *sc* (Faslodex). MCF-7/5C cells rapidly formed substantial tumors at every injection site (10 out of 10) in control-treated mice by 21 days after inoculation, but only 1 palpable tumor formed out of 10 injection sites in mice treated with E<sub>2</sub>, resulting in a highly significant difference in the average tumor cross-sectional area between the two treatment groups (Fig. 2:19,  $P < 0.0001$ ). In a prior report, we have shown that E<sub>2</sub> induces tumor regression and apoptosis in established MCF-7/5C xenograft tumors (3). Importantly, MCF-7/5C xenograft tumors showed robust growth in the presence of FUL or E<sub>2</sub> plus FUL, which was not significantly different than growth of the control (no treatment) group, but was significantly greater than in the E<sub>2</sub> treatment group (Fig. 2:19, FUL vs. E<sub>2</sub>,  $P < 0.0001$ ; E<sub>2</sub>+FUL vs. E<sub>2</sub>,  $P < 0.0001$ ). Hence, the MCF-7/5C xenograft tumor model was resistant to growth inhibition by FUL, and FUL treatment abrogated E<sub>2</sub>-mediated growth inhibition.

For microarray analysis (under Task 4), only established MCF-7/5C tumors grown in the absence of E<sub>2</sub> were used. However, two days before the tumors were collected, 3 mice were implanted with 0.3 cm E<sub>2</sub> capsules *sc* to generate 6 E<sub>2</sub>-treated tumors.

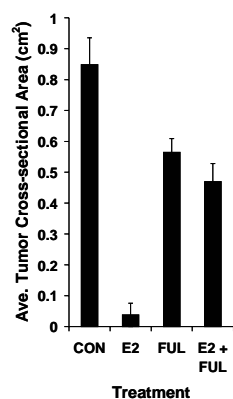
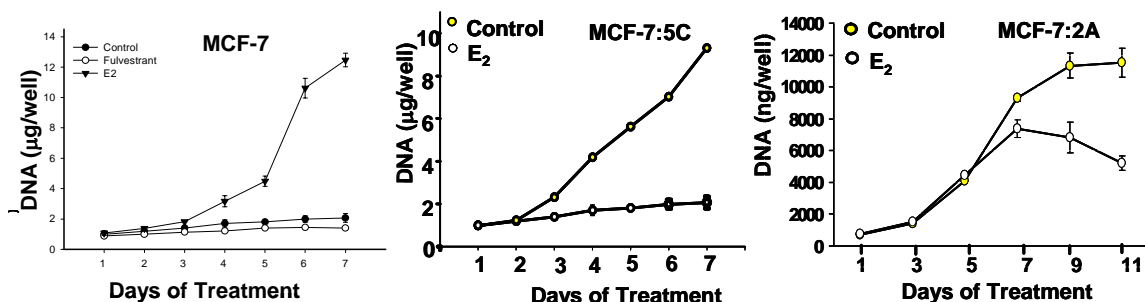


Figure 2:9. Preliminary Experiment with MCF-7/5C Tumors Used for Microarray Analysis.

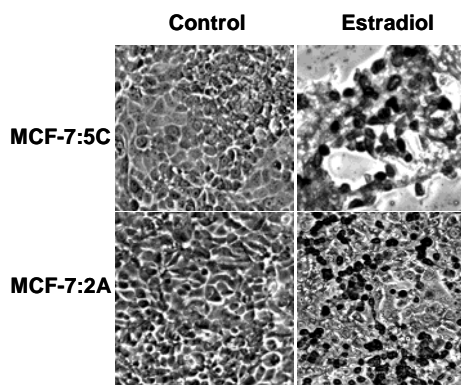
**Task 2. (Lewis-Wambi) To elucidate the molecular mechanism of estradiol (E<sub>2</sub>)-induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.**

**Task 2a. Complete a series of experiments using sets of well defined breast cancer models of E<sub>2</sub>-induced survival and apoptosis *in vivo* and *in vitro*.**

In order to address Task 2a, our laboratory has created a panel of MCF-7 breast cancer cells *in vitro* that have been E<sub>2</sub>-deprived (ED) for several years to replicate resistance to estrogen deprivation (i.e. resistance to aromatase inhibitors). **Model module 1** includes the parental (naïve) MCF-7 cell line which responds to physiologic concentrations of E<sub>2</sub> with growth and two estrogen-deprived breast cancer cell clones, MCF-7:5C and MCF-7:2A cells, both of which undergo apoptosis (programmed cell death) in the presence of E<sub>2</sub> (**Figure 2:20**). Interestingly, it appears that the apoptotic effect of E<sub>2</sub> in MCF-7:2A cells occurs after 7 days of treatment whereas in MCF-7:5C cells apoptosis occurs after 2 days of E<sub>2</sub> treatment (**Figure 2:20 & 2:21**).



**Figure 2:20. Effects of E<sub>2</sub> on the growth of parental MCF-7 cells and long-term E<sub>2</sub>-deprived MCF-7:5C and MCF-7:2A cells.** For growth assays, approximately  $2 \times 10^4$  MCF-7, MCF-7:2A, and MCF-7:5C cells were seeded in 24-well plates in estrogen-free RPMI medium and then treated with either E<sub>2</sub> ( $10^9$  M) or fulvestrant ( $10^6$  M) for 7 or 11 days. Note, for fulvestrant treatment of MCF-7 cells, growth assay was performed in phenol-red RPMI medium containing 10% fetal bovine serum (FBS). Cells were harvested at the indicated time point and total DNA ( $\mu\text{g}/\text{well}$  or  $\text{ng}/\text{well}$ ) was determined using a DNA fluorescence quantitation kit. Data shown is representative of four separate experiments with similar results and arrow bars indicate mean  $\pm$ SE of triplicate values.



**Figure 2:21 E<sub>2</sub> induces apoptosis in MCF-7:5C and MCF-7:2A cells.** For experiment, MCF-7:5C and MCF-7:2A cells ( $2 \times 10^5$ ) were seeded in 8-well chamber slides in estrogen-free RPMI medium and then treated with  $10^9$  M E<sub>2</sub> or ethanol vehicle (control) for 3 days (MCF-7:5C cells) or 7 days (MCF-7:2A cells). Apoptotic cells were identified by TdT-mediated dUTP nick end labeling (TUNEL) using the In Situ Cell Death Detection Kit, POD (Roche-Applied Science, cat# 11684817910). The In Situ Cell Death Detection Kit is designed as a precise, fast and simple, non-radioactive technique to detect and quantify apoptotic cell death at single cell level in cells and tissues. **Apoptotic cells are darkly stained.** Images were viewed and captured by an inverted Nikon TE300 objective microscope equipped with a Spot RT (Diagnostic Instruments) monochrome camera.

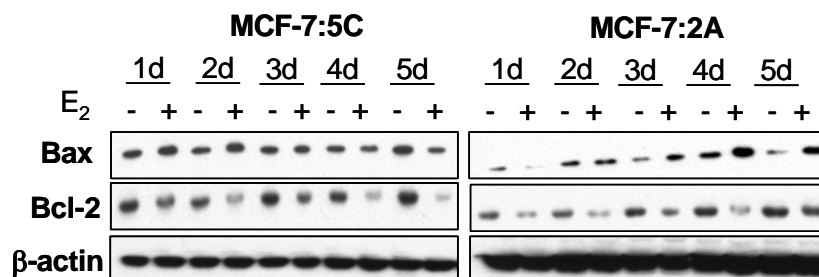
**Mechanism of E<sub>2</sub>-induced apoptosis in MCF-7:5C and MCF-7:2A cells.** The expression of genes that regulate apoptotic cell death plays an important role in determining the sensitivity of tumor cells to chemotherapy and other apoptotic drugs. Up-regulation of the antiapoptotic Bcl-2 family members in tumors has been associated with drug resistance (14-16). The functional blockade of Bcl-2 or other antiapoptotic proteins, such as Bcl-xL, could either induce apoptosis in cancer cells or sensitize these cells for chemotherapy (17-18). Thus, antiapoptotic members of the Bcl-2 family have attracted intensive interest in drug discovery to develop a new class of anticancer agents.

In order to determine the mechanism of E<sub>2</sub>-induced apoptosis in MCF-7:5C and MCF-7:2A cells, we performed microarray analysis on RNA samples isolated from cells treated with ethanol vehicle (control) or 1nM E<sub>2</sub> for 48

hours. Each treatment group had 5 replicate samples. Total RNA was isolated using Trizol Reagent (Invitrogen) followed by cleanup using the RNAeasy mini system (Qiagen) and the data was analyzed by TGen. **Table 2:1** shows a list of apoptotic genes that were significantly altered in MCF-7:5C and MCF-7:2A cells following E<sub>2</sub> treatment. In particular, we found that E<sub>2</sub> significantly increased the expression of proapoptotic Bax, Noxa, Bim, GADD45 $\beta$  genes and decreased the expression of antiapoptotic Bcl-2 and Bcl-xl genes. Western blot analysis were performed to determine whether E<sub>2</sub> treatment also increased proapoptotic protein expression in these cells. **Figure 2:22** shows that E<sub>2</sub> significantly increased the protein levels of proapoptotic Bax in a time-dependent manner in both MCF-7:5C and MCF-7:2A cells with maximum induction observed at 2 days in MCF-7:5C cells and 4 days in MCF-7:2A cells, which is consistent with our microarray data (**Table 2:1**), cell growth data (Figure 2:20), and our apoptosis assay (Figure 2:21).

**Table 2:1.** E<sub>2</sub> up-regulated (red) and down-regulated (green) genes 48 hours after E<sub>2</sub> treatment of apoptosis-susceptible MCF-7:5C cells and MCF-7:2A cells. Examples of genes involved in the malignant phenotype are shown. Microarray analysis of mRNA was carried out by TGen with samples provided by FCCC. The relative ratios of expression are given. Microarray data was normalized using affymetrics GCOS software, then imported into a Filemaker Pro relational database for preprocessing.

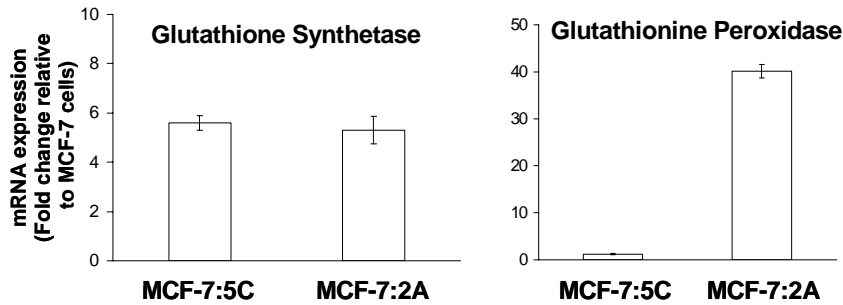
Gene	Function	MCF-7:5C		MCF-7:2A	
		ratio	+E <sub>2</sub> / -E <sub>2</sub>	ratio	+E <sub>2</sub> / -E <sub>2</sub>
BAX	Proapoptotic	6.0		3.0	
NOXA	Proapoptotic	5.5		2.1	
BIM	Proapoptotic	4.3		2.3	
GADD45 $\beta$	Proapoptotic	4.0		3.0	
BAD	Proapoptotic	2.0		2.5	
Bcl-2	Antiapoptotic	3.5		2.5	
Bclxl	Antiapoptotic	5.0		3.3	



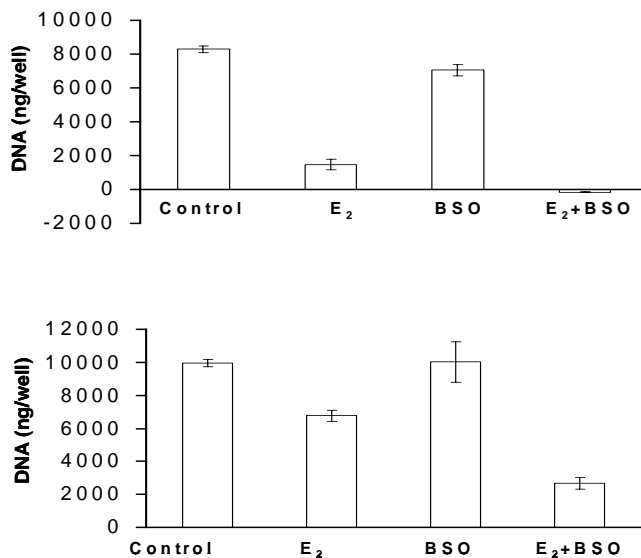
**Figure 2:22.** Western blot analysis for Bax and Bcl-2 protein expression in MCF-7:5C and MCF-7:2A cells following 1 to 5 days of treatment with ethanol vehicle (control) or 1 nM E<sub>2</sub>. Equal loading was confirmed by reprobing with an antibody against  $\beta$ -actin. The blots shown are representative of three independent experiments.

**Glutathione Depletion by Buthionine Sulfoximine (BSO) enhances the apoptotic effects of E<sub>2</sub> in MCF-7:5C and MCF-7:2A breast cancer cells.** Glutathione is the most abundant intracellular thiol, present at concentration of 0.1 to 10 mmol/L, and serves as a critical cellular antioxidant (19). Glutathione is required for the maintenance of protein sulfhydryl groups in a reduced state and for resistance to oxidative stress through detoxification of reactive oxygen species (ROS) (20). Glutathione can detoxify alkylating agents, including cisplatin, through the formation of glutathione adducts (21-22). It is known that depletion of glutathione by incubation with buthionine sulfoximine (BSO), a specific inhibitor of  $\gamma$ -glutamylcysteine synthetase, increases the cytotoxicity of alkylating drugs (23). Based on our gene array studies we found that glutathione synthetase (GSS) and glutathione peroxidase 2 (GPX2) genes were significantly upregulated in MCF-7:5C and MCF-7:2A cells compared to parental MCF-7 cells (**Figure 2:23**). Since GSS and GPX2 are known to regulate glutathione levels in cells, we examined whether depletion of glutathione by BSO might enhance the apoptotic effects of E<sub>2</sub> in MCF-7:5C and MCF-7:2A cells. **Figure 2:24** shows that BSO (100  $\mu$ M) significantly enhanced the apoptotic effect of E<sub>2</sub> (1 nM) in both MCF-7:5C and MCF-7:2A cells. In the presence of E<sub>2</sub> alone, approximately 80% of MCF-7:5C cells and 30% of MCF-7:2A cells died, however, when combined with BSO,

100% of MCF-7:5C cells and 75% of MCF-7:2A cells died. It should be noted that BSO by itself did not significantly inhibit the growth of either cell line.

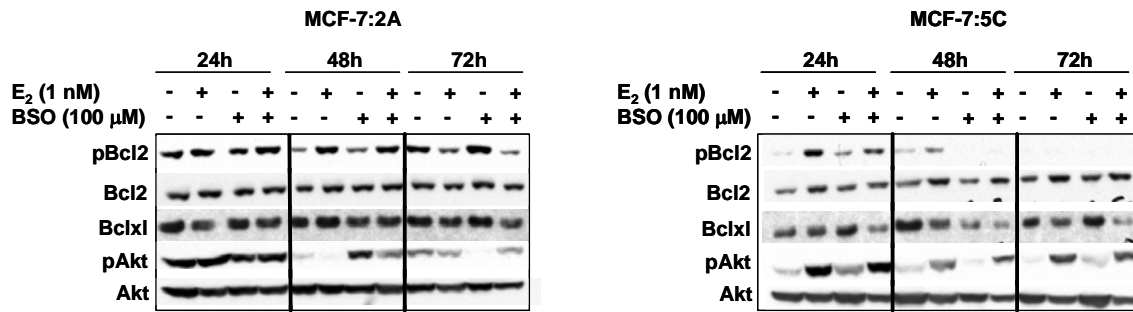


**Figure 2:23.** Microarray analysis of MCF-7:5C and MCF-7:2A cells showing overexpression of GSS and GPX2 genes. The fold change in gene expression is shown relative to parental MCF-7 cells. Microarray data was normalized using affymetrics GCOS software, then imported into a Filemaker Pro relational database for preprocessing.



**Figure 2:24.** Effect of BSO and E<sub>2</sub> on the growth of MCF-7:5C and MCF-7:2A cells. For growth assays, approximately 3 x 10<sup>4</sup> MCF-7:5C and MCF-7:2A cells were seeded in 24-well plates in estrogen-free RPMI medium and then treated with ethanol vehicle (control), E<sub>2</sub> (10<sup>-9</sup> M), 100 μM BSO, or E<sub>2</sub> (1 nM) + BSO (100 μM) for 7 days. Cells were harvested at the indicated time point and total DNA (ng/well) was determined using a DNA fluorescence quantitation kit. Data shown is representative of four separate experiments with similar results and arrow bars indicate mean ±SE of triplicate values.

**The role of Bcl-2 and Bcl-xl in the apoptotic action of E<sub>2</sub> and BSO in MCF-7:5C and MCF-7:2A cells.** It has been reported that the action of Bcl-2 is related to antioxidant protection against cellular damage (21). Previous studies have shown that Bcl-2 overexpression raises cellular glutathione levels (22). Since Bcl-2 appears to be an important target of E<sub>2</sub>-induced apoptosis in MCF-7:5C and MCF-7:2A cells (**Figure 2:22**), we examined whether BSO plus E<sub>2</sub> synergistically reduced the growth of MCF-7:5C and MCF-7:2A cells by targeting Bcl-2. Western blot analysis were performed on cells treated with E<sub>2</sub> alone, BSO alone, or E<sub>2</sub> + BSO for 24, 48, and 72 hours and Bcl-2, phosphorylated Bcl-2, and Bcl-xl protein were measured. **Figure 2:25** shows that E<sub>2</sub> + BSO significantly reduced phosphorylated Bcl-2 and Bcl-xl protein in both MCF-7:2A and MCF-7:5C cells in a time-dependent manner, however, Bcl-2 protein level did not significantly change with the combination treatment. It will be interesting to see whether suppression of Bcl-2 or Bcl-xl expression using small interfering RNA (siRNA) has the ability to enhance the apoptotic effects of E<sub>2</sub> alone or E<sub>2</sub> plus BSO. These experiments are currently being performed in our laboratory.



**Figure 2:25.** Western blot analysis for Bcl-2, phosphorylated Bcl-2 (pBcl2) and Bclxl protein in MCF-7:5C and MCF-7:2A cells following treatment with E<sub>2</sub>, BSO or E<sub>2</sub> + BSO for the above indicated time points. Equal loading was confirmed by reprobing with an antibody against Akt. The blots shown are representative of three independent experiments.

**In summary**, we have developed two estrogen receptor (ER)-positive breast cancer cell lines (MCF-7:5C and MCF-7:2A) that are resistant to estrogen deprivation but undergoes apoptosis in the presence of physiologic concentrations of E<sub>2</sub> in a time-dependent manner. The mechanism of the apoptotic action of E<sub>2</sub> in MCF-7:5C and MCF-7:2A appears to involve members of the Bcl-2 apoptotic family of proteins. Interestingly, we found that the apoptotic action of E<sub>2</sub> can be significantly enhanced by glutathione depletion using buthionine sulfoximine (BSO). A possible mechanism for this synergistic action of BSO plus E<sub>2</sub> might involve suppression of phosphorylated Bcl-2 and Bclxl expression in these cells.

**Long-term estrogen deprivation enhances the migratory and invasive potential of breast cancer cells.**

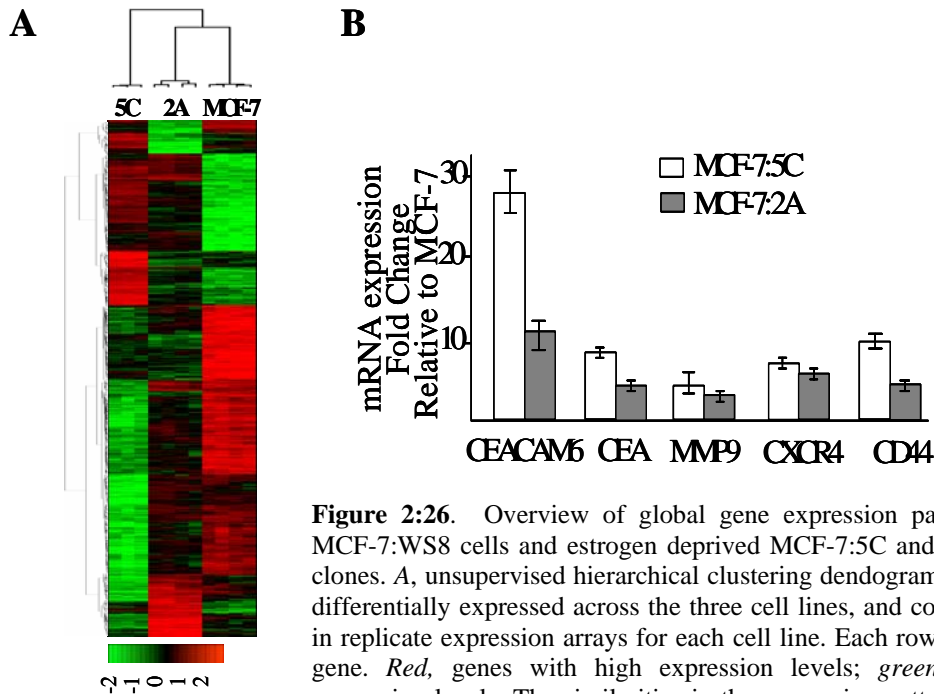
Invasion and metastasis are the hallmarks of cancer malignancy and they are the primary cause of patient mortality during breast cancer progression. Invasion refers to the ability of cancer cells to penetrate through the membranes that separate them from healthy tissues and blood vessels and metastasis refers to the spreading of cancer cells to other parts of the body (26). In order for a transformed cell to metastasize, it must first lose adhesion, penetrate and invade the surrounding extracellular matrix (ECM), enter the vascular system, and adhere to distant organs (26). These processes require extensive alterations in gene expression profiles, including the down-regulation of genes involved in cell anchorage and the up-regulation of genes involved in cell motility and matrix degradation (27-28) CEACAM6 is a glycosylphosphatidylinositol-anchored cell surface protein that functions as a homotypic intercellular adhesion molecule. It is overexpressed in a number of human malignancies including pancreatic cancer, gastrointestinal cancer, and breast cancers (29) and increased levels of CEACAM6 is associated with greater *in vivo* metastatic ability and increased invasiveness and migration (30-31). Based on our microarray studies, we found that several invasive genes including CEACAM6 was overexpressed in estrogen-deprived MCF-7:5C and MCF-7:2A cells compared to parental MCF-7 cells. Based on these observations, we investigated the role of CEACAM6 in cellular invasion and migration of long-term estrogen deprived MCF-7:5C and MCF-7:2A. We found a significant correlation between CEACAM6 expression and the ability of breast cancer cells to migrate and invade. In particular, we found that CEACAM6-overexpressing MCF-7:5C and MCF-7:2A cells were markedly more invasive and migratory than parental MCF-7 cells. Furthermore, we found that suppression of CEACAM6 expression using small interfering RNA (siRNA) completely reversed the invasive phenotype of MCF-7:5C and MCF-7:2A cells. Future studies will explore whether CEACAM6 interacts with other invasion genes and whether it affects tumor growth *in vivo* as well as metastasis in mice. **This work has recently been submitted to Cancer Research (manuscript number CAN-07-5107) and is under consideration for publication.**

A copy of this manuscript is included in the appendix.

**Global gene expression profiles associated with acquired resistance to long term estrogen deprivation.** To investigate whether aberrant gene expression patterns contributed to resistance to estrogen deprivation, we performed microarray analyses on wild-type MCF-7 cells and estrogen-deprived MCF-7:5C and MCF-7:2A

cells using the Affymetrix Human Genome U133 Plus 2.0 Array. The sample dendrogram showed that MCF-7:2A cells and wild-type MCF-7 cells clustered more closely, whereas MCF-7:5C cells clustered on a more distant branch, suggesting that MCF-7:2A cells are more similar to the parental MCF-7 cells than MCF-7:5C cells (**Figure 2:26A**). A graphical representation of some of the invasive genes overexpressed in estrogen deprived cells relative to parental MCF-7 cells is shown in **Figure 2:26B**.

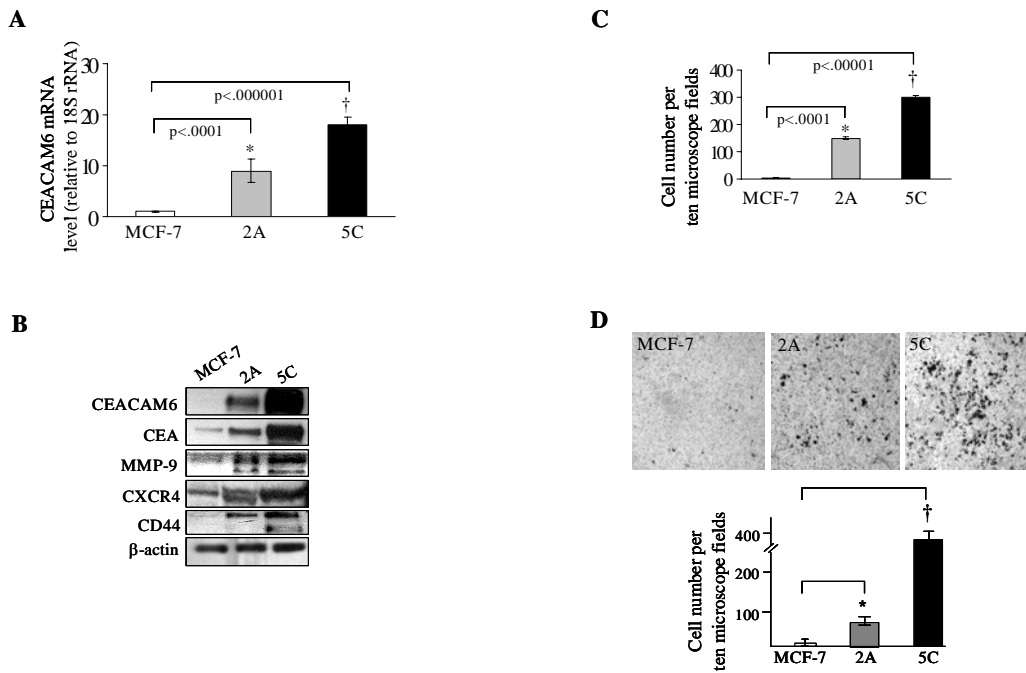
A complete list of differentially expressed genes between each cell line is provided in Supplemental Table 2:2.



**Figure 2:26.** Overview of global gene expression patterns in wild-type MCF-7:WS8 cells and estrogen deprived MCF-7:5C and MCF-7:2A variant clones. *A*, unsupervised hierarchical clustering dendrogram of 904 genes most differentially expressed across the three cell lines, and consistently regulated in replicate expression arrays for each cell line. Each row represents a single gene. *Red*, genes with high expression levels; *green*, genes with low expression levels. The similarities in the expression pattern among the three cell lines are presented as a "condition tree" on the top of the matrix. *B*, fold change in expression of invasion genes CEACAM6, CEACAM5/CEA, MMP-9, CXCR4, and CD44 in estrogen deprived MCF-7:5C and MCF-7:2A cells compared to parental MCF-7 cells, as identified by microarray gene analysis.

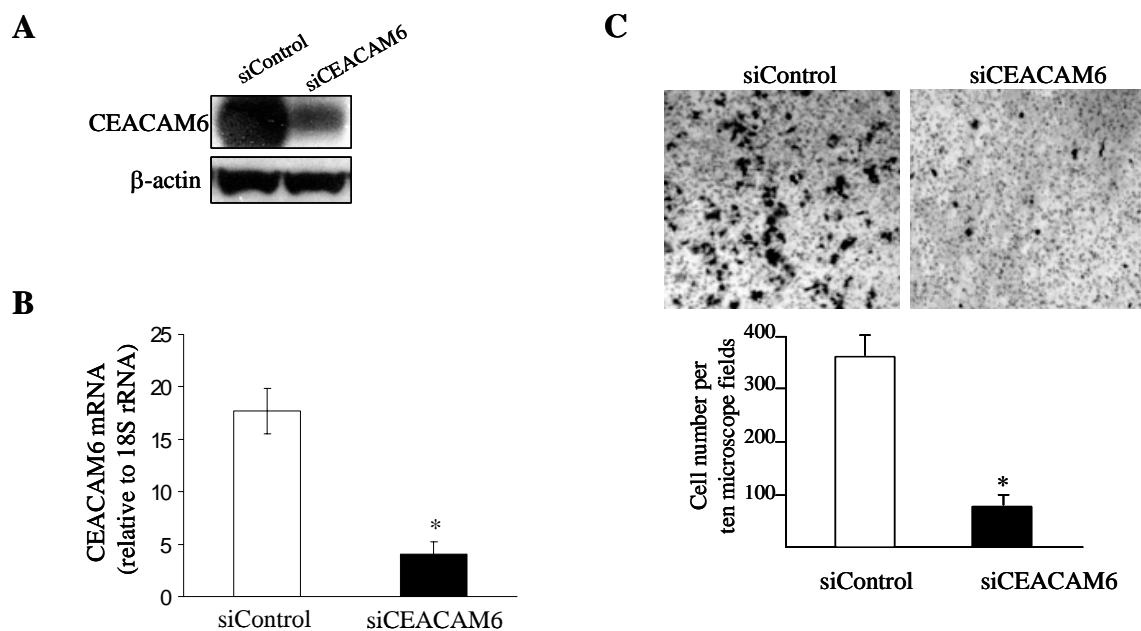
**Estrogen deprivation increases CEACAM6 expression and enhances migration and invasion of breast cancer cells.** Quantitative real-time PCR was performed on MCF-7, MCF-7:5C and MCF-7:2A cells to verify CEACAM6 expression in these cells. **Figure 2:27A** shows that CEACAM6 mRNA was upregulated 2-fold in MCF-7 cells, 17-fold in MCF-7:5C cells, and 10-fold in MCF-7:2A cells relative to the internal control 18S rRNA, thus validating the microarray results. Western blot analysis was performed to assess CEACAM6 protein expression. **Figure 2:27B**, CEACAM6 protein was significantly upregulated in MCF-7:5C and MCF-7:2A cells compared to wild-type MCF-7 cells which expressed minimal levels of the protein. CEACAM5 (CEA), MMP-9, CXCR4, and CD44 were also markedly elevated in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells.

Since the estrogen-deprived cells appeared to overexpress several genes that are linked to metastatic invasion, we assessed the migratory and invasive potential of these cells. Cell migration was measured using a modified Boyden chamber assay with 10% SFS as a chemoattractant. As shown in **Figure 8C**, both MCF-7:5C and MCF-7:2A cells had the highest numbers of migrating cells compared to MCF-7 cells; a phenotype that correlated with CEACAM6 expression. Similar results were obtained when the different cell lines were tested for their ability to invade through membranes coated with Matrigel. **Figure 8D** shows that MCF-7:5C and MCF-7:2A cells had the highest number of invading cells while MCF-7 cells were noninvasive. The overall ability of the cell lines to migrate and invade was as follows: MCF-7:5C > MCF-7:2A > MCF-7.



**Figure 2:27.** CEACAM6 promotes cell migration and invasion of long-term estrogen deprived breast cancer cells. *A*, CEACAM6 mRNA expression in MCF-7, MCF-7:5C (5C), and MCF-7:2A (2A) cells was determined by quantitative real-time PCR and normalized to 18S rRNA level. Error bars indicate SE. *p* values for comparisons are indicated. *B*, Western blot analysis of CEACAM6 and other invasion proteins in wild-type MCF-7 cells and estrogen-deprived MCF-7:5C and MCF-7:2A cells. *C*, the migration and invasiveness of estrogen-deprived breast cancer cells was compared with parental MCF-7 cells. For quantification of cells migrating across Transwell filters, cells were seeded onto the migration chamber ( $1 \times 10^4$  per chamber). After 24 h, chambers were stained and counted for migrated cells. *Columns*, average number of cells

**siRNA mediated CEACAM6 knockdown inhibits invasion and migration of MCF-7:5C cells.** To test the hypothesis that CEACAM6 is required for cell migration and invasion, we used siRNA to suppress CEACAM6 expression in MCF-7:5C cells. These cells were used because they expressed the highest level of CEACAM6 and were the most invasive. MCF-7:5C cells were transfected with CEACAM6-specific or control siRNA and Western blot analysis was performed 48 hours following siRNA exposure. **Figure 2:28A** shows that CEACAM6 protein was significantly suppressed in MCF-7:5C cells transfected with the CEACAM6-specific siRNA but not the control siRNA. Suppression of CEACAM6 expression by CEACAM6-specific siRNA was also confirmed at the transcript level using quantitative real-time PCR at 48 hours following transfection (**Figure 2:28B**). To clarify the role of CEACAM6 siRNA in cell invasion, MCF-7:5C cells were pretreated with CEACAM6 siRNA or control siRNA for 48 hours and invasiveness was measured over the subsequent 48 hours. **Figure 2:28C** shows that CEACAM6 siRNA almost completely reversed the invasiveness of MCF-7:5C cells whereas control siRNA did not affect cell invasion. The invasiveness of MCF-7:5C cells was inhibited by nearly 80% when CEACAM6 expression was suppressed. A similar trend was observed for cell migration (data not shown). Suppression of CEACAM6, however, did not alter the growth of MCF-7:5C cells. These results demonstrate that CEACAM6 expression is an important determinant of cell migration and invasion in estrogen-deprived breast cancer cells and therefore, modulation of CEACAM6 expression may be a potential therapeutic target for metastatic breast cancer.



**Figure 2:28.** CEACAM6 is critical for invasion of estrogen deprived MCF-7:5C breast cancer cells. *A*, siRNA-mediated gene knockdown of CEACAM6 in MCF-7:5C invasive breast cancer cells. Immunoblot analysis of MCF-7:5C cells transfected with siRNA against CEACAM6, siCEACAM6, and scrambled sequence control siRNA, siControl for 48 hours. Cell lysates were probed for CEACAM6 with the corresponding antibody and  $\beta$ -actin, as loading control. *B*, relative amounts of CEACAM6 mRNA expression normalized to 18S rRNA level was quantified in transfected cells by quantitative real-time PCR. Error bars indicate SE. \* $p < .0001$ . *C*, matrigel invasion assay of siControl and siCEACAM6-transfected MCF-7:5C cells. Transfected cells were seeded onto the invasion chamber ( $1 \times 10^4$  per chamber). After 48 h, cells that invaded through the Matrigel-coated transwells were fixed, stained, and photographed; each panel represents an example of three replicates. *Columns*, average number of cells invading per 10 microscope fields. Error bars indicate SE. \* $p < .0001$ .

### Task 3. To decipher cellular signaling pathways using proteomics.

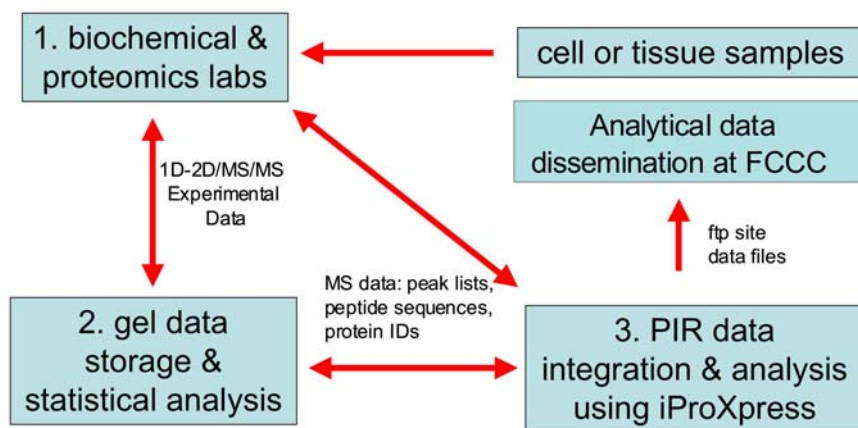
#### The SPECIFIC AIMS of the GU site within the COE are:

- To identify protein signatures that are hallmarks of estrogen induced apoptosis in breast cancer cells. Cell lines in which estrogen is a survival factor will be used for comparison.
- To validate the subcellular map of survival after melding data from Tasks 2, 3 and 4 and to deliver a single database of pathways for survival and apoptosis constructed by integrating gene array and proteomic data. Here we report work completed at the GU site during year 1 of this COE. The report is divided into three sections that reflect the contribution of different components (Fig. 3:1):

**1. Proteomics MS analysis.** We report on E2 effects in MCF-7 vs. MCF-7:5C .

**2. Analysis of protein gels.** We report on the analysis of gel images.

**3. Pathway analysis.** We report on data integration and pathway analysis.



**Figure 3:1:** Flow of samples and analytical data within the GU site (1, 2 and 3) and integration with the overall COE. The three components of the GU site of the COE as well as the interface with the national COE center at FCCC are depicted. **NOTE:** This report is organized along the three components of the GU COE site.

**1. Proteomics MS analysis.** Mass spectrometry (MS) analysis of the proteome from E2 treated MCF-7 (growth) versus MCF-7:5C cells (apoptosis) after fractionation by immunoprecipitations (IP).

**Cells and culture conditions.** As proposed, we initiated proteome analyses of MCF-7:5C cells that undergo apoptosis in response to E2 and compared this to MCF-7 (WT) that grow in response to E2. MCF-7 and MCF-7:5C cells were plated in 150 mm tissue culture dishes (each 150 mm dish yielded between 1-2 milligrams of total protein) and allowed to attach. Cells were then treated or not with estradiol for different times followed by lysis in NP40 lysis buffer. As our primary fractionation of the proteome of these cells to capture signaling complex subsets of proteins, we had proposed to use immunoprecipitation (IP) of signaling proteins from sample aliquots. These targets were the ER coactivator AIB1 to isolate proteins participating in the AIB1 signaling complex. In a separate series, IP for tyrosine phosphorylated proteins was proposed to isolate signaling complexes that contain tyrosine phosphoproteins. With these methods we capture distinct subsets of the signaling proteome. 2D gel electrophoresis of samples as a secondary fractionation was also initiated to evaluate whether major proteins might be altered.

**Immunoprecipitations.** For the primary analysis by immunoprecipitation, protein lysates were subjected to immunoprecipitation using gamma-bind G-Sepharose beads and an AIB1 monoclonal antibody we have evaluated before or a anti-phosphotyrosine monoclonal antibody (4G-10). The amount of protein extracts per set of experimental conditions were between 4 and 15 mg across a series of experiments. Some cell pellets were generated at the GU site, some were generated at the FCCC site to maximize the amount of material for the analyses. Examples for each are shown in Figs. 3:2, 3:3 & 3:4.

**Gel electrophoresis.** After extraction, samples were subjected to immunoprecipitation using the antibodies described above. The immunoprecipitated proteins were separated by denaturing SDS-PAGE on 4-12% Nu-PAGE gels (Invitrogen). After electrophoresis, gels were then stained with colloidal blue overnight then washed with ddH<sub>2</sub>O overnight to reduce background staining. Fig. 3:2, 3:3 and 3:4 show examples.

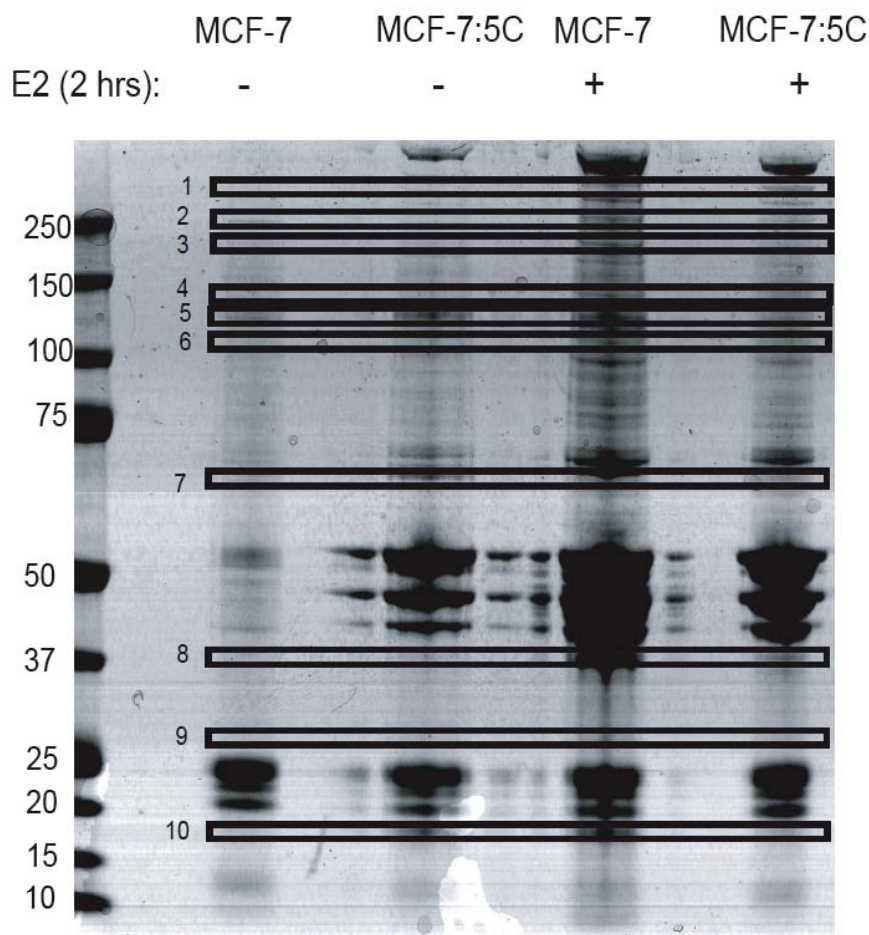
**Analysis of gels for distinctly regulated proteins.** The stained gels were imaged using a color scanner. These images were magnified and analyzed visually on a screen. They were also submitted as tiff files for storage, image and statistical analysis described below under the section “2. Analysis of protein gels”. From the latter analysis, a refined profile and quantitation of bands is obtained that guides selection of proteins from the gels. In brief, segments of the gels containing unique protein bands were identified by careful visual inspection and comparison of treatment sets (across and within cell lines). The statistical gel analysis allows for a quantitative (area under the curve) and qualitative (position of the bands plus identity of proteins from the MS – see below) cross-comparison across numerous gels.

**Isolation of distinctly regulated proteins.** After identification bands were cut from the gels and great care was taken to isolate the same segment of all lanes from the different treatments. Fig. 3:2 and 3:3 indicate the cut segments as rectangular boxes. A quantitative as well as qualitative read-out from the MS sequence analysis of proteins contained in each slice. As a general rule, more abundant proteins will show up in the MS analysis de novo of only one lane or will show a greater coverage by individual peptides assigned to the respective protein in a cross-lane comparison. This information was also cross-referenced with the data from the data obtained under “2. Analysis of protein gels”.

**Mass spectrometry analysis of isolated proteins.** Gel slices were subjected to tryptic digest and the digests run using MS and MS/MS. Figure 3:2 shows an example of a colloidal blue-stained gel with the segments cut for analysis indicated by the boxed areas.

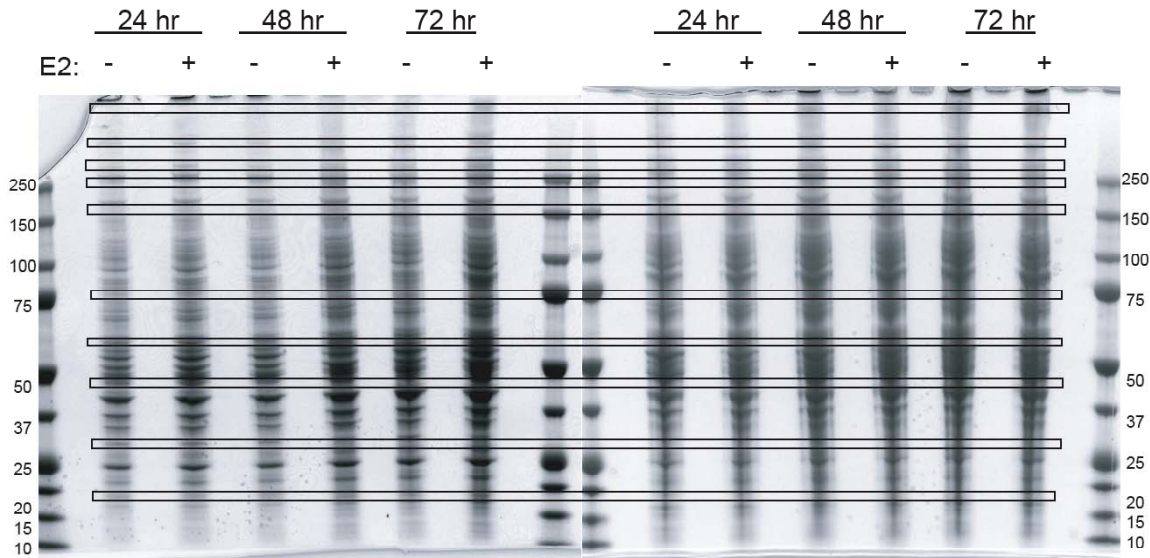
Proteins in the MS or MS/MS analysis were identified using the Mascot search engine database that integrates mass spectrometer readings and protein sequence analysis.

**Uploading of data to PIR for iProXpress analysis.** The protein lists from the MS analysis was provided for further pathway analysis as spreadsheet and subjected to the iProXpress analysis. This is described below under “3. Pathway analysis”. In addition to the spreadsheets derived from the Mascot search, raw MS data of peptide masses were uploaded to the PIR site. From an analysis of the latter the Mascot search can be verified and peptide modifications due to posttranslational modifications revealed. Obviously, this analysis requires the raw data and extensive exchange of information between the lab and PIR as indicated by the two-way arrows in the flow diagram of Fig. 3:1.



**Figure 3:2. Example of a colloidal-blue stained protein gel after IP anti-pY.** MCF-7 and MCF-7:5C cells were treated or not with E2 for 2 hours, proteins extracted and immunoprecipitated with an anti-phosphotyrosine antibody. Proteins separated by SDS-PAGE were stained and slices cut from the gel for each

segment that showed at least one distinctly regulated protein. The slice # is indicated ( 1 – 10). Also the molecular mass of the marker proteins is shown ( 10 – 250 kDa )



**Figure 3:3. Example of a colloidal-blue stained protein gels after IP anti-pY.** MCF-7 (left) and MCF-7:5C cells (right) were treated or not with E2 for 24, 48 and 72 hours. Proteins extracted and immunoprecipitated were separated by SDS-PAGE stained and slices cut from the gel for each segment that showed at least one distinctly regulated protein. Details in Fig. 3:2 (above).

In addition to the anti-pY IPs, we also ran IPs for AIB1. An example is shown in Fig. 3:4 (next section). High-resolution gel images will be posted at the COE website. Results from the experiments are discussed under section “**3. Pathway analysis**”

Overall, six separate experiments were run (3 repeats for IP anti-pY and 3 for anti-AIB1). Each experiment contained a head-to-head comparison of MCF-7:5C and MCF-7 without and with E2 treatment. So far 264 gel slices were cut from the gels based on differential stainings of proteins in the cross-comparisons. Fig. 3:2 and 3:3 provide some illustration from 2 of the experiments. Table 3:1 summarizes the experimental approach and numbers of gel slices harvested. The gel slices harvested were subjected to tryptic digests and MS/MS analysis of the eluates from the slices. One additional sample set is currently being processed by gel electrophoresis and sample capturing. **We will have close to 300 gel slices processed by the end of year 1.**

Typically, each of the gel slices with a visible protein stain will contain as many as 10 distinct proteins that are detectable by MS sequencing. Some of the gel slices will contain less detectable proteins or less amounts of given proteins. They serve as negative controls.

Cell Lines compared	IP	# of gel slices harvested
MCF-7, MCF-7:5C	pY	24 (12/lane)
MCF-7, MCF-7:5C	pY	40 (10/lane)
MCF-7, MCF-7:5C	AIB1	52 (13/lane)
MCF-7, MCF-7:5C	AIB1	48 (12/lane)
MCF-7, MCF-7:5C	pY	120 (10/lane)
MCF-7, MCF-7:5C	AIB1	ongoing

**Table 3:1. Overview of cell line comparisons by proteomics.** The different IP approaches and number (#) of distinct bands cut for MS analysis is shown. Fig. 3:2 and 3:3 depict the approach (rectangular boxes indicate the area of gels cut for analysis).

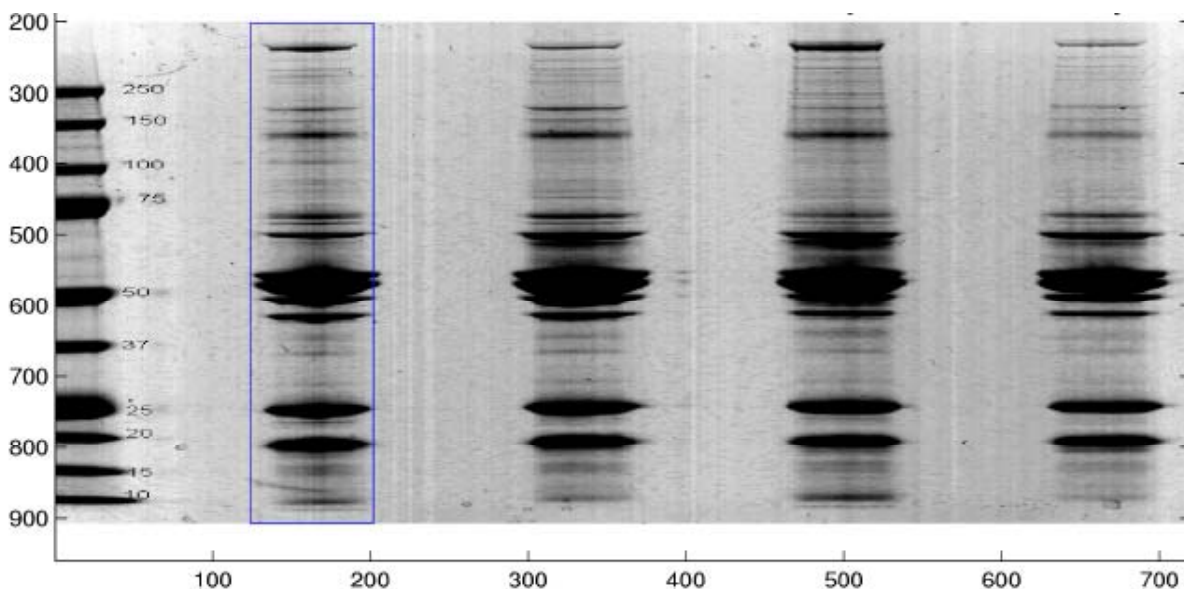
## Conclusions.

- We have successfully generated proteomics data from MCF-7 and MCF-7:5C cells under conditions of E2 induced growth or apoptosis respectively. Signaling complexes were isolated by immunoprecipitation (IP) with anti-pY and anti-AIB1 antibodies.
- By the end of year 1 we have isolated differentially regulated proteins in approximately 300 gel slices harvested from gel electrophoresis of the experimental samples. These are continuously analyzed by mass spectrometry and protein sequencing.
- We found that 24 hours after E2 treatment apoptosis is fully initiated as measured by the proteomics analysis. We thus expanded the analysis to earlier time points (2 hours) to identify the “trigger point” of signal initiation after E2.
- We found distinct proteomics signatures after 2 hours of E2 in both MCF-7 and MCF-7:5C that were apparent after IP with anti-pY and anti-AIB1 antibodies.

## 2. Analysis of protein gels.

Here we report on the analysis of multidimensional gel images developed to compare across experiments and support the isolation and quantitation of proteins subjected to MS.

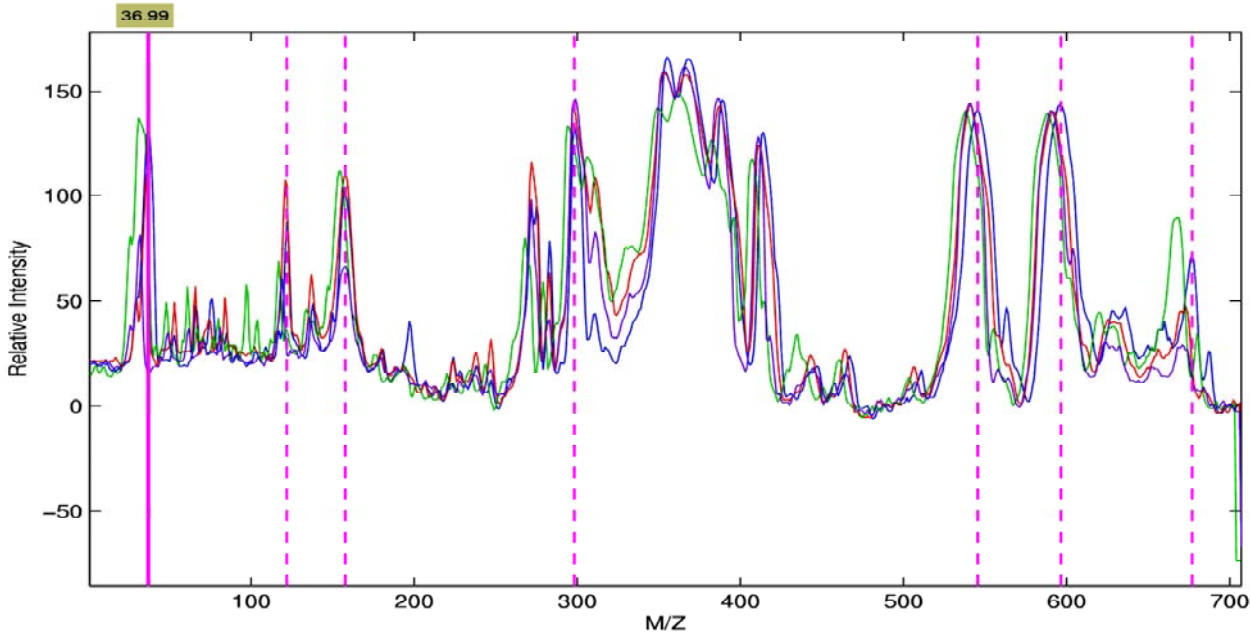
As proposed in the COE, the Bioinformatics group worked on data-analytical methodologies for one-dimensional and two-dimensional gels. Fig. 3:4 shows a gel containing 4 lanes with samples and a slane with mass standards (left-most). To extract information from each lane, we consider rectangular regions (indicated in Fig. 3:4) and calculate the median for each row in this region. This results in four spectra, one for each lane (Fig. 3:5).



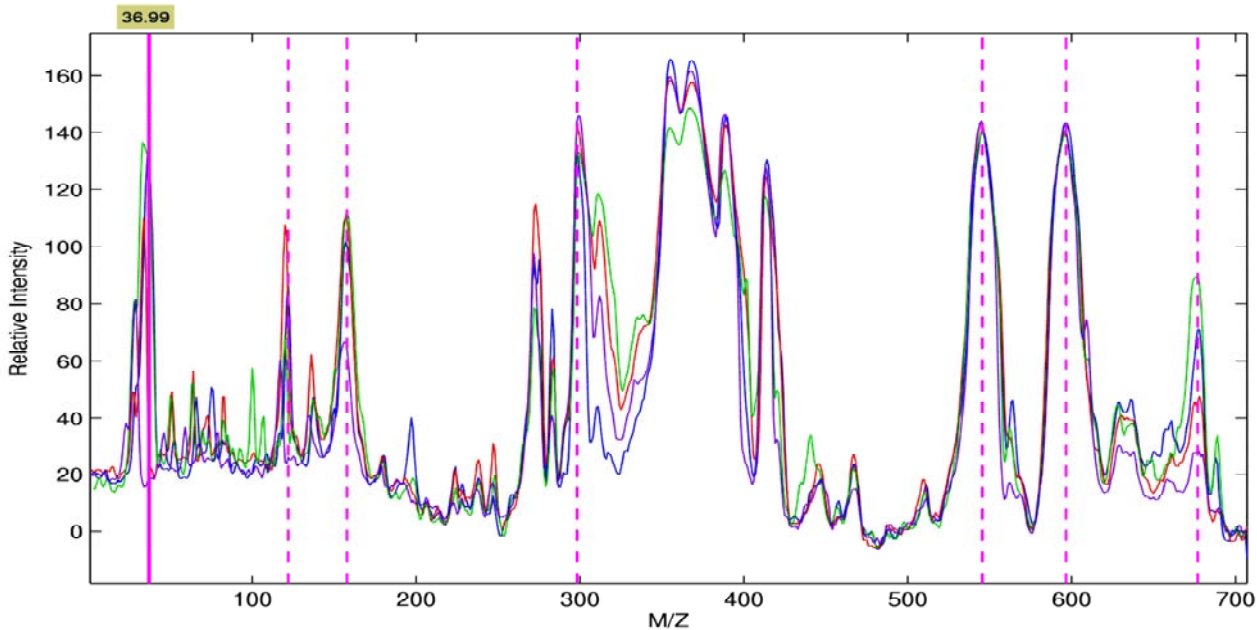
**Figure 3:4. TIFF image of a colloidal-blue stained gel.** This data set is from an IP anti-AIB1 experiment. The left-most lane contains the molecular mass standard proteins. The four experimental lanes have the same arrangement as in Fig. 3. Lane 1 = MCF-7 (-)E2, lane 2 = MCF-7:5C (-)E2, lane 3 = MCF-7 (+)E2 ; lane 4 = MCF-7:5C (+)E2. The TIFF image was provided by the proteomics laboratory (see section 1) via an upload of a high-density image to the COE at the GU Biostats server. The schematic rectangular region around the first lane shows an example used in the analysis shown in Fig. 3:5 and resulted in the spectrum indicated by the blue line.

We compared these data by superimposing them, and found that there are differences of experimental, biological, and geometric nature. The influence of the latter can be reduced by aligning the sequences' peaks. This is done by selecting markers (the reference peaks) on one of the samples and by using Matlab to align the corresponding peaks in the other bands. This is achieved by maximizing the cross-correlation between the spectra and the so-called synthetic spectrum. The results are shown in Figures 3:5 and 3:6. The spectra are aligned using the MSALIGN routine in Matlab's bioinformatics toolbox.

The alignment reveals that some regions that appeared to contain differently positioned peaks, i.e. proteins of different apparent mass now collapse to peaks with the same position but different amounts. This can be appreciated from a visual inspection of the region around 680 M/Z in Fig. 5 versus Fig. 6 by comparing the green vs blue and red spectrum with distinct peaks. After the alignment the green, blue and red peaks that appeared to run at a distinct position and apparent molecular mass actually run at the same position after alignment with the markers. However, the abundance of the respective protein(s) will be different based on the amount of staining by colloidal blue.

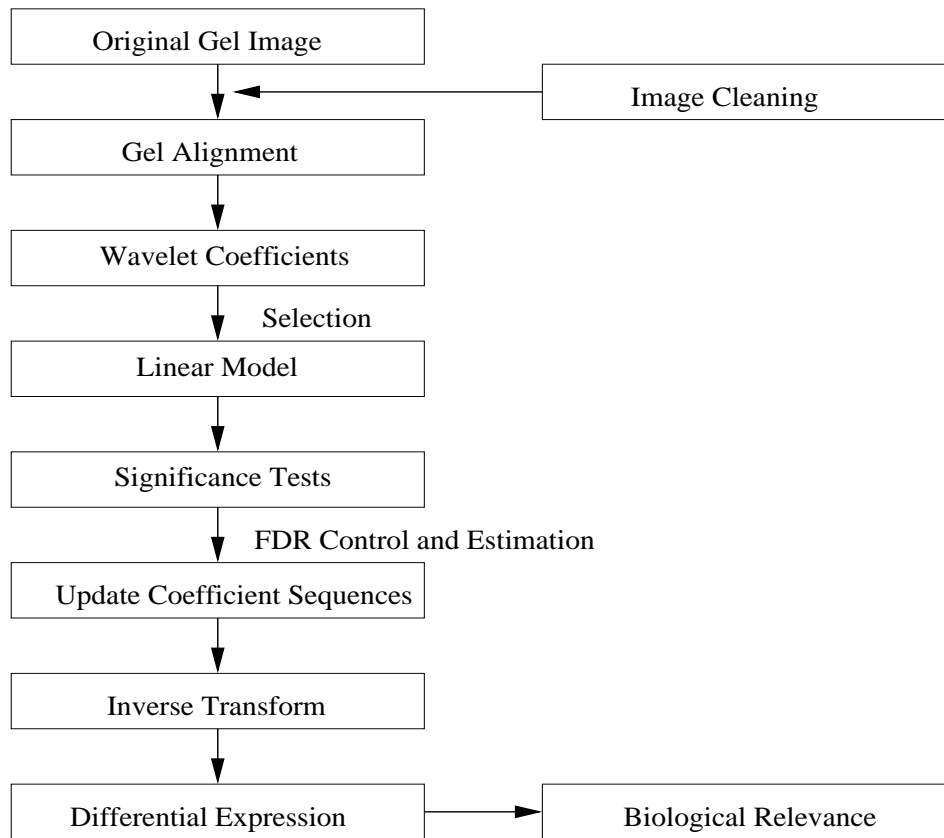


**Figure 3:5.** Raw data of spectra superimposed with selected markers. These data are derived from the protein gel in Fig.3:4.



**Figure 3:6.** Aligned spectra using the selected markers (see Fig. 3:5). These data are derived from the protein gel in Fig. 3:4.

We developed a novel analytical pipeline for 2D-PAGE images. Classical approaches segment images to obtain spot locations. Images are then aligned and spots are matched. The bottleneck and weakness of these approaches are mainly in the spot-matching step. It is time consuming, and it is generally recognized that the fewer and fewer spots can be efficiently matched as the number of gels increases. The validity of the spot detection algorithm depends on user-selected thresholds, which are determined by trial and error. To avoid the spot matching step, and base our inferences on mathematically and statistically sound methods, we have developed a novel approach that is summarized in Figure 3:7. Its main feature is that, by working with summaries of the images, it bypasses spot detection.



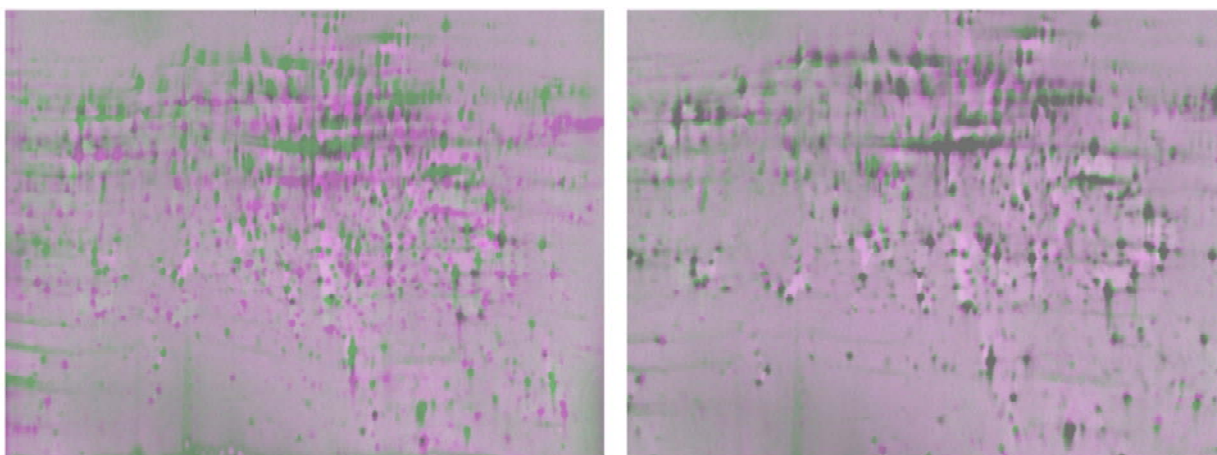
**Figure 3:7.** Flow diagram for analysis of 2D gel images.

“Gel cleaning” proceeds by first smoothing the images (using a level 1 wavelet approximation), removing spikes (by second-difference smoothing), and removing streaks and background (using a gradient method). For the latter, we take advantage of the gradient characteristics of spots and streaks. The algorithm consists of the following steps: (i) image enhancement via a power function (to safeguard faint spots), (ii) image segmentation with the watershed algorithm (to create regions containing at most one object), (iii) region classification based on gradients and other features (to remove regions not containing a spot), (iv) gradient thresholding (based on a value obtained from a regression model) and (v) spot filling.

For step (iii), the Classification and Regression Tree algorithm is used to classify regions as spot containing or not on the basis of regional attributes. These regional attributes include the intensity integrated over the region, the area, the ratio of the area to that of smallest rectangle containing the region, and the range, mean, median and standard deviation for the intensity for the horizontal first-order partial derivative, for the horizontal second-order partial derivative, for the vertical first-order partial derivative, for the vertical second-order partial derivative and for the horizontal followed by vertical second-order partial derivative. In addition, we compute, for each region, the Hellinger distances between various probability density functions (p.d.f.) and the uniform p.d.f. The p.d.f.'s we consider are those for: the original intensity, the first-order horizontal and vertical partial derivatives, the second-order horizontal and vertical partial derivatives.

For regions containing a spot, the optimal thresholds for the gradients are obtained via multiple regression analyses. The independent variables are the regional attributes listed above. Each spot is segmented using the corresponding estimated threshold. The thresholded images are then aligned and analyzed for differential expression.

To align gels, we set the problem into a quadratic-programming framework, and have recourse to an interior-point method, a state-of-the-art mathematical optimization technique to solve it. We select a few landmarks on each gel, and estimate cubic-spline transformations describing changes between corresponding landmarks. We impose restrictions on the transformed points not be too far away from the true location and on the transformation to be as close as possible to the identity transformation. We thus construct the objective function that is a sum of curvature of the transformation, distance of the transformation to the identity transformation, and the distance between the original and transformed gels. Minimizing the objective function is subject to inequality constraints: the set of inequalities describing the difference between landmarks and their transformations, difference between ideal landmarks and corresponding geometric centers, and rigidity of the transformations. The quadratic optimization problem thus constructed is solved using the interior-point method software MOSEK (see Fig. 3:8 for an illustration).



**Figure 3:8.** Superimposed gels before alignment (left) and after alignment (right).

Building model for each pixel is a formidable task. We therefore cast the statistical analysis of differential expression in the wavelet domain. For each wavelet coefficient, we consider a model such as the ANOVA model described above. As before, for small sample sizes, we also consider the LPE and empirical Bayes approaches. Again, to account for multiple testing, we control the false-discovery rate. We then reconstruct the images with the significant coefficients, only. These reconstructions only feature the locations of differentially expressed proteins. Finally, to confirm our inferences, we perform a statistical analysis on the actual volumes of the protein that are associated with statistically significant wavelet coefficients.

#### **Conclusion:**

- We have established a protocol for uploading and feed-back of gel analyses with the proteomics laboratory (see Fig. 1, two-way arrows between 1 and 2)
- We have established a quantitative analysis of the gel profiles to evaluate differences amongst gels and allow for cross-comparisons.
- We have established a refined analysis of 2D gels.

These tools are applied to the selection of proteins for MS analysis and for protein quantitation in selected gel slices.

### **3. Pathway analysis.**

The integration of the data management at GU and the connection to the national site of the COE at FCCC is illustrated in Figure 3:1 at the beginning of this report. Below, we summarize, in an overview, the steps taken to populate the data base and drive the analyses. Also, we show the analysis of two distinct data sets. The detailed data and experimental conditions are posted on the password-protected website that is constantly updated with raw data and analyses.

#### **COE proteomic and gene expression data integration and analysis:**

- COE large scale data:
  - Proteomic (GU)
  - Gene array (TGen)
  - siRNA (TGen)
  - Sample tracking (FCCC)
- COE Data integration
  - Common sample type (sample tracking)
  - Common data element (gene, protein, antibody, siRNA)
  - ID mapping to UniProtKB ID (siRNA as a special case)
    - Each siRNA in the library is associated with its intended targeted gene (gene IDs); this gene id will then be mapped to UniProtKB protein ID.
    - Cell phenotypes induced by the siRNA can be part of the NOTE field in the iProXpress system, or as a separate column with links to the original data sets..
- COE integrated database (Master Data Directory)
  - Design: Primary protein ID, experimental data groupings, notes for samples and experimental conditions; other information matrices;
- Functional analysis – iProXpress proteomic system
  - Protein information matrix; pathway data
  - Enhancement plan (separate document)

#### **Preliminary analyses of two proteomic data sets:**

**3.1. Antibody array** of MCF-7 and derivative cells lines with or without estrogen treatment (written report posted on the COE-wiki site; see below)

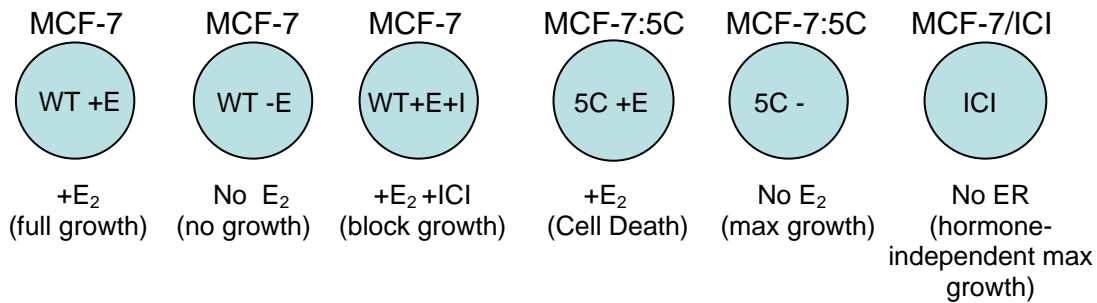
**3.2. 1D-Gel/MS study** of IP-pY and IP-AIB1 on MCF-7 and derivative cells. The figure below illustrates how 1D-MS proteomic data is analyzed using iProXpress.

#### **3.1. Antibody array. Data and pathway analysis via iProXpress.**

For the first application of the data integration and analysis system of this COE a data set from an antibody array survey of 389 defined signaling proteins (“Powerblot system”) generated before the establishment of the COE was used. MCF-7:5C cells that respond to E2 by apoptosis were compared to wild-type MCF-7 cells that respond to E2 by initiating growth (wild-type MCF-7) MCF-7 cells selected in the presence of an ER antagonist (ICI) to grow independent of E2 (MCF-7/ICI) were used as an additional model. Extracts from cells grown for 24 hours under conditions outlined in Fig. 3:9 were subjected to the analysis. This data set was provided by the FCCC site and had been generated during the preparation of the COE. We subjected the data to the iProXpress analysis to initiate the population of the data bank and evaluate the protocols for uploading of data as well as sample or experimental ID. The data are available [password protected] at:

<http://pir.georgetown.edu/iproxpress/> .

The original data sets were separated into 6 groups of cell lines and/or treatments:



**Figure 3:9: MCF-7 cells and treatments used in the initial iProXpress analysis of an antibody array survey (“Powerblot”).** The different MCF-7 cell lines were subjected to treatments indicated (24 hrs), and cell extracts analyzed by an antibody array.

The data from the experiment in Fig. 3:9 yields 15 possible comparisons amongst the 6 groups and these 15 comparisons were further divided into increase (+) or decrease (-) for each parameter, i.e. a total of 30 sets. The comparisons are shown in Table 3:2.

WT-E vs WT+E (-)	5C-E vs WT-E (-)	WT+E+I vs 5C+E (-)	ICI vs 5C+E (-)
WT-E vs WT+E (+)	5C-E vs WT-E (+)	WT+E+I vs 5C+E (+)	ICI vs 5C+E (+)
5C+E vs WT+E (-)	5C-E vs 5C+E (-)	WT+E+I vs 5C-E (-)	ICI vs 5C-E (-)
5C+E vs WT+E (+)	5C-E vs 5C+E (+)	WT+E+I vs 5C-E (+)	ICI vs 5C-E (+)
5C+E vs WT-E (-)	WT+E+I v WT+E (-)	ICI vs WT+E (-)	ICI vs WT+E+I (-)
5C+E vs WT-E (+)	WT+E+I v WT+E (+)	ICI vs WT+E (+)	ICI vs WT+E+I (+)
5C-E vs WT+E (-)	WT+E+I vs WT-E (-)	ICI vs WT-E (-)	
5C-E vs WT+E (+)	WT+E+I vs WT-E (+)	ICI vs WT-E (+)	

**Table 3:2: Group comparisons derived from the experiments under Figure 3:10.** In each set, protein levels in the first experimental condition or cell line relative to the second condition or cell line are compared and grouped for a decrease or an increase (e.g. WT-E vs WT+E (-), or WT-E vs. WT+E (+)) are decreased (minus) or increased (plus) for the respective protein. Protein profiles for each data set can thus be browsed and cross-compared.

### Results of the analysis

**General observations:** There were a total 389 UniProtKB protein entries mapped in the data sets. Protein profiles were generated for each of the 30 data sets, cross-data set comparison made based on the functional categories (GOs and KEGG pathway) and the results provided in the iProXpress system. Preliminary examination of the functional profiling identified several interesting functional changes from the data, which may be relevant to the study and can thus be further investigated.

**DNA replication (GO:0006260)** proteins are consistently decreased in WT-E, 5C+E and WT+E+I cells relative to WT+E cells. For example, in the WT-E vs WT+E (-) set, protein levels of proliferating cell nuclear antigen (PCNA) [P12004], DNA mismatch repair protein Msh2 [P43246], and DNA polymerase delta catalytic subunit [P28340] are decreased. Similarly in WT+E+I (E2 blocked) compared to 5C-E (no E2 with max growth), four DNA replication related proteins are also decreased. In contrast, three such proteins are increased in chronic ICI treated cells (grow independent of E2) relative to 5C+E (E2-induced cell death).

**Cell cycle (GO:0007049)** is another interesting category, where in 5C+E, 5C-E and ICI cells relative to WT+E, significantly more proteins of this category are decreased than those that are increased (e.g. in 5C+E vs WT+E sets, 10 proteins are increased and 3 decreased).

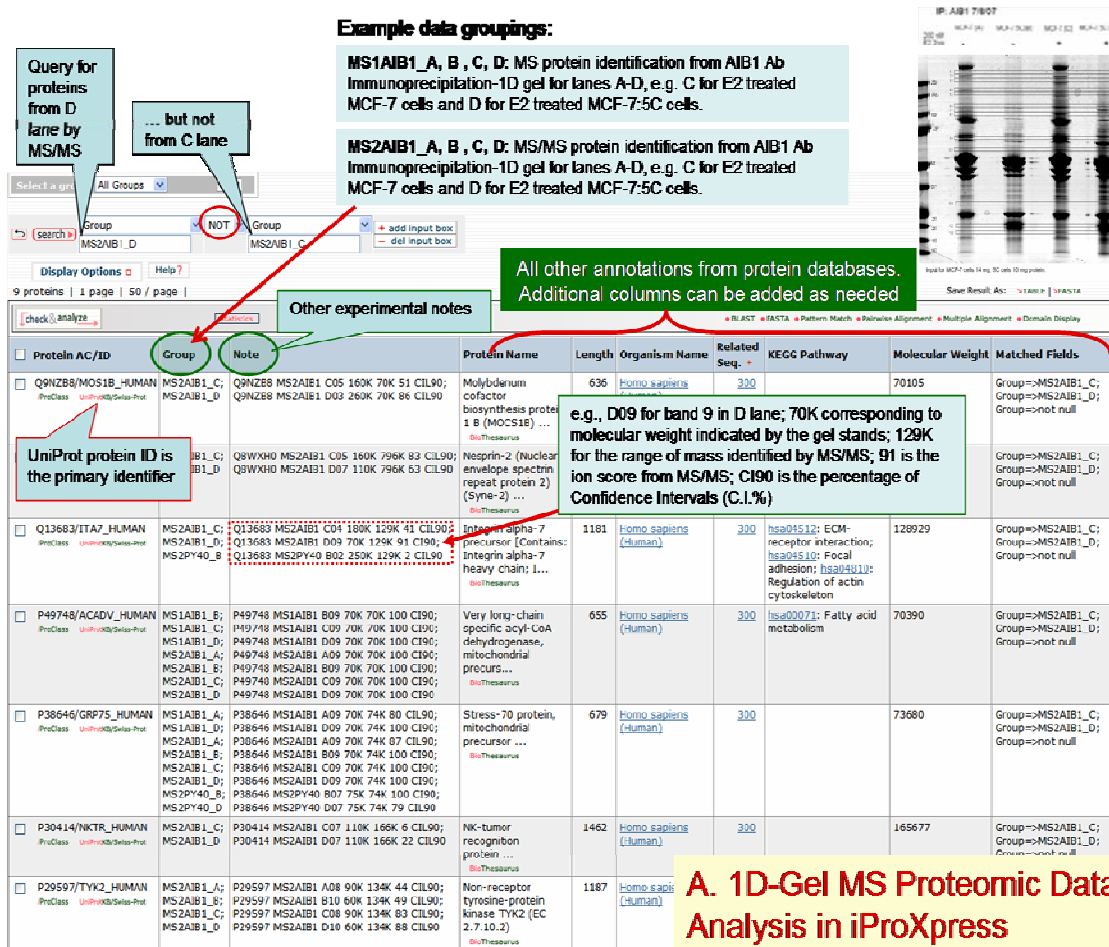
An interesting observation are the changes of proteins in the category of **cell death (GO:0008219)** in 5C+E and 5C-E cells. There are **7-9 cell death related proteins** that are decreased in 5C+E and 5C-E cells relative to the WT+E cell, while only one such protein is increased in both cases. Some detailed analyses will be required to confirm and to interpret such changes.

### Summary of the antibody array analysis

For pathway profiles, **cell cycle (KEGG PATH:hsa04110)** represents the most prevalent one (13 proteins) among this data set. It is interesting to note that depending on the data set, cell cycle protein may increase or decrease, or even in the same cells, some cell cycle protein may increase while others may decrease (e.g. in 5C-E vs WT-E sets). It is conceivable that depending on the location in a pathway, proteins could be changed in different directions because different pathways may intersect each other (pathways converge and diverge). In this case, superimposing proteins onto the pathway map to visualize the protein changes will facilitate the analysis.

### 3.2. Pathway analysis using 1D-Gel/MS study.

Mass spectrometry data from immunoprecipitations (IP) IP-pY and IP-AIB1 of MCF-7 and derivative cell extracts were provided by the proteomics laboratory (see section 1 for details). Figure 3:10 (below) illustrates how 1D-MS proteomic data is analyzed using iProXpress.



**Figure 3:10. Proteomic data presentation and analysis in iProXpress.** The different information sets included with the data in iProXpress are shown.

From the initial series of IppY and IP-AIB1 a total of 20 groups of data sets with 730 proteins were obtained in an unbiased analysis. 116 of these proteins were identified with over 90% C.I.

From that data set proteins differently regulated in the **cell death group are 15** and in the **proliferation group 8**. Further analyses are posted on the COE website as the analysis progresses.

**Verification of proteins and possible posttranslational modifications.** Also, the original peptide mass data are uploaded and analyzed to cross-check protein ID and to discover potential posttranslational modifications. This analysis is ongoing.

#### **Conclusions:**

- We have implemented and utilized the data integration and pathway analysis with data generated under this COE.
- The iProXpress system platform allows for the compilation of multidimensional data sets from diverse experiments and experimental design and is integrated with the pathway analysis.
- With the continuous addition of data sets generated under the COE (protein, mRNA and RNAi) we will obtain a refined identification of pathways that control estrogen-induced apoptosis.

#### **Task 4: To analyze E<sub>2</sub>-induced survival and apoptotic pathways using gene array and siRNAs**

**Overarching scheme of experiments in this task:** Array-based expression profiling of all *in vitro* and *in vivo* models generated under **Task 2** will be employed to identify genes and pathways associated with survival and apoptosis mechanisms.

**Task 4a. Catalogue the transcriptional response using array-based expression profiling.** Preliminary analysis of gene expression data from naïve MCF-7 and its long-term E<sub>2</sub>-deprived derivative MCF7:5C following 48 h E<sub>2</sub> treatment *in vitro* identified functional pathways with co-ordinately regulated sets of genes likely playing a role in a distinct mechanism of E<sub>2</sub>-mediated apoptosis. We will build on these initial successful analyses by transcriptionally profiling all breast cancer cell culture models and their xenograft derivatives as outlined in Task 2. In year 1, samples from a time course of E<sub>2</sub> treatment for MCF-7 and MCF-7:5C cells *in vitro* and from MCF-7:5C xenograft material will be sent to TGen for expression profiling using Human Genome U133 Plus 2.0 Arrays (Affymetrix).

**Task 4b. Identify regulatory networks for pathways indicative of differential responses to E<sub>2</sub>.** Gene-gene regulatory networks influenced by E<sub>2</sub> in each laboratory model will be investigated as modules as outlined in Task 2 (FCCC).

**Task 4c. Interrogate pathways of endocrine resistance using high throughput RNA interference (HT-RNAi)**

#### **WORK ACCOMPLISHED - Task 4a**

- 1). Gene expression Profiling and analysis of endocrine resistant xenograft tumor models.**
- 2). Gene expression Analysis of *in vitro* cell line models of endocrine resistance.**
- 3). Integration of array-based comparative genomic hybridization (aCGH).**

#### **1. Gene expression Profiling and analysis of endocrine resistant xenograft tumor models.**

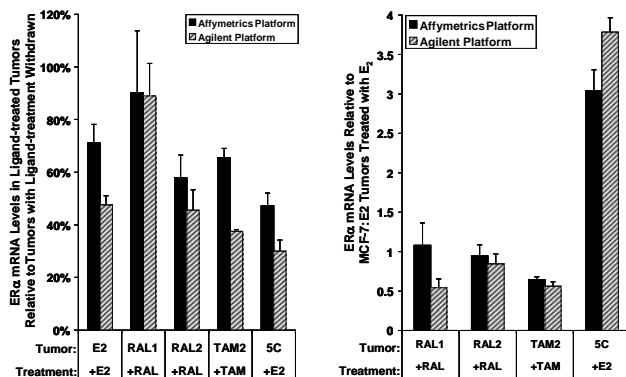
Gene expression profiling from xenograft material derived from endocrine resistant models has now been completed on two commercially available expression array platforms, Affymetrix Human U133 Plus2.0 arrays, and Agilent 22k Human 1A (V2) Oligo Microarrays. A summary of arrays performed is listed in Table 4:1.

Xenograft	treatment	Affymetrics	Facility	Agilent	Facility	Analysis
MCF7: E2	no ligand	yes	NWU	Yes	Tgen	TGen
MCF7: E2	Estrogen	yes	NWU	Yes	Tgen	TGen
MCF7: RAL1	no ligand	yes	TGen	Yes	Tgen	TGen
MCF7: RAL1	Raloxifene	yes	TGen	Yes	Tgen	TGen
MCF7: RAL2	no ligand	yes	NWU	Yes	Tgen	TGen
MCF7: RAL2	Raloxifene	yes	NWU	Yes	Tgen	TGen
MCF7: TAM2	no ligand	yes	TGen	Yes	Tgen	TGen
MCF7: TAM2	Tamoxifen	yes	TGen	Yes	Tgen	TGen
MCF7: 5C	no ligand	yes	NWU	Yes	Tgen	TGen
MCF7: 5C	Estrogen	yes	NWU	Yes	Tgen	TGen
Total arrays:		56		21		

**Table 4:1 Summary of Gene expression arrays performed on xenograft tumor models**

Affymetrics Arrays were performed by Northwestern University (NWU) microarray core facility and remaining hybridizations by TGen. 4 separate tumors pertaining to each tumor model were hybridized to Affymetrics arrays, while two replicates for each were hybridized to Agilent gene expression arrays. Specific RNA samples arrayed on each platform have been fully tracked for cross comparative purposes.

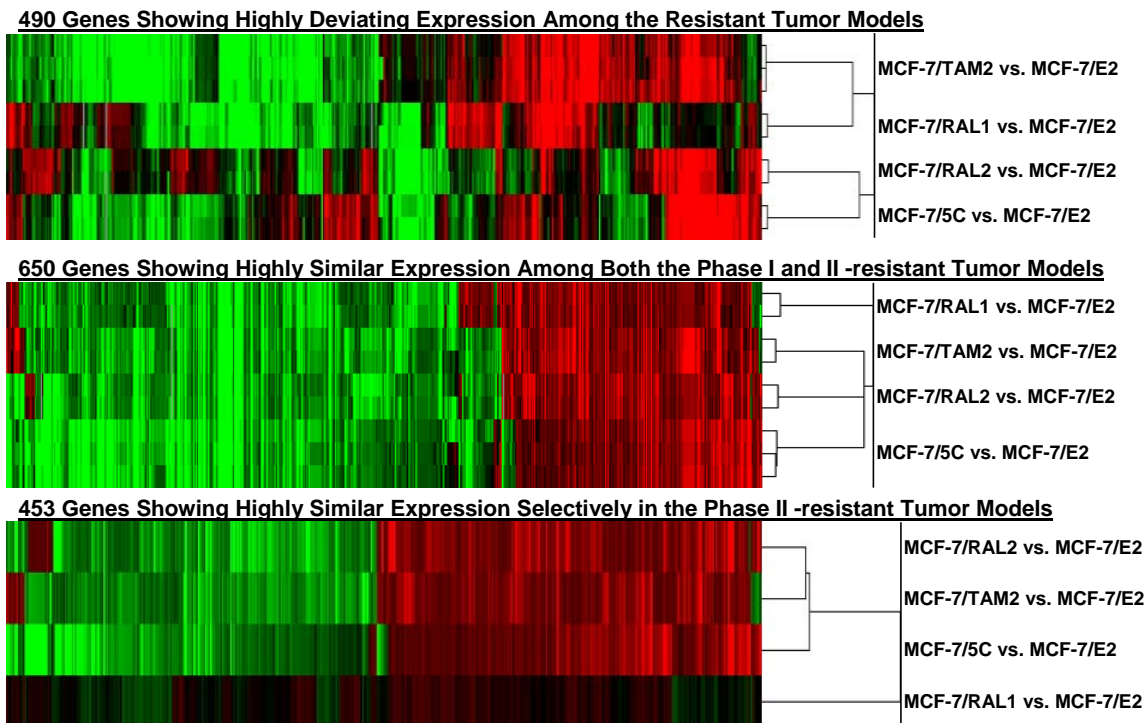
Unexpected variable quality was identified for a significant number of the Affymetrics hybridizations performed by the Northwestern microarray core Facility. This appeared to be due to DNA contamination of the RNA preparations from xenograft material. RNAs from xenograft material were re-extracted at TGen for the sub-optimal Affymetrics experiments and gene expression profiling on the Affymetrics platform was repeated. Replacement hybridizations for TAM2 and RAL1 tumor samples were used for subsequent data analysis. From previous experience at TGen, the Agilent expression array platform has traditionally outperformed Affymetrics-based expression analyses. At least two representative RNA samples from all tumor models were therefore labeled and hybridized to Agilent 22k Human 1A (V2) Oligo Microarrays for comparative analysis with the Affymetrics data. To determine which array dataset was optimal for analysis, a selection of genes whose transcripts are known to be highly regulated in this series of tumor models were amplified in the Jordan laboratory from the same RNA samples used for the microarray data, and their relative expression levels were compared to the Agilent and Affymetrics data readout. The data from the Agilent expression arrays was found to be more representative of the real time PCR expression data. Consequently, a decision was made by TGen and the FCCC to continue formal analysis with the Agilent dataset and to use the corresponding tumor profiling data generated on the Affymetrics platform as a validation series. Importantly, the relationship between tumor profiles for each model were found to be consistent in both platforms. As an example of cross-validation between the Affymetrics and Agilent platform, the mRNA expression of ER $\alpha$  is shown in Figure 4:1.



**Figure 4:1. ER $\alpha$  mRNA Expression in MCF-7-based Tumor Models by Microarray Analysis.**

## Global comparative analysis of endocrine-resistant tumor models

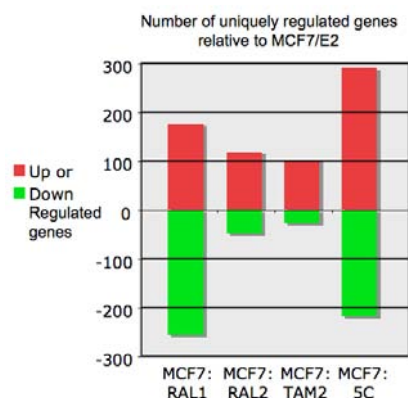
Analysis of genes that are differentially expressed between MCF-7/E2 and the MCF-7/RAL1, MCF-7/RAL2, MCF-7/TAM2 and MCF-7/5C xenograft tumor models are currently being performed.



**Figure 4:2. Hierarchical clustering of genes showing at least a 2-fold change in expression in the resistant tumor models compared to wild-type MCF-7/E2 tumors in the Agilent gene expression microarray dataset.** The common reference for each hybridization was MCF7:E2 with estrogen. SERM-resistant models are with corresponding SERM ligand. MCF75C cells were without ligand (All RNAs prepared from exponentially growing tumor xenografts). Filtering the dataset using a standard deviation of  $>0.4$  between tumor types reveals an overall relationship between endocrine-resistant tumor models.

We have identified overlapping and unique patterns of gene expression for each xenograft tumor model relative to MCF7:E2 (Figure 4:2). A similar sample dendrogram was derived from the Affymetrix dataset, although the replicate hybridization standard deviation was substantially higher (not shown).

MCF-7/5C cells grown in the absence of estrogen appear to be more related to the MCF-7/RAL2 model grown in the presence of raloxifene. The MCF-7/RAL1 and MCF-7/TAM2 models are distinctive on the sample dendrogram. Interestingly, we observed a small number of genes that are uniquely characteristic to each tumor model grown in the presence of ligand (or no ligand in the case of MCF-7/5C) relative to MCF-7/E2 (Figure 4:2). For example, MCF-7/RAL1 cells have unique downregulation of the BRCA1 gene; MCF-7/RAL2 cells have unique upregulation of ARF1 (ADP-ribosylation Factor 1, a member of the RAS family of small guanine nucleotide binding proteins). MCF-7/TAM2 cells have strong upregulation of SSX1, SSX3, SSX6 and SSX7, (Synovial sarcoma, x breakpoint proteins). Interestingly, these highly homologous proteins are capable of eliciting spontaneously humoral and cellular immune responses in cancer patients, and are potential useful targets in cancer vaccine-based immunotherapy.



**Figure 4:3. Number of genes uniquely regulated in each endocrine resistant tumor model relative to MCF-7/E2.** Genes that were differentially expressed in each tumor model relative to MCF-7/E2 up or down greater than two fold, and not in any other tumor model are plotted. To find potential pathways for mechanistic evaluation that may be involved in the etiology of each resistant tumor model, gene ontology analysis using the Meta Core Gene Go application was employed. The 2-fold changes in gene expression in each resistant model compared to the wild-type MCF-7/E2 tumors were used in the gene ontology analysis. Lists of the most significant gene ontologies for these particular comparisons are listed in Tables 4:2-4:5. Involvement of the small GTPase CDC42 pathway was indicated by the most significant p-value in the MCF-7/RAL1 (Table 4:2), MCF-7/TAM2 (Table 4:4), and MCF-7/5C models (Table 4:5), while focal adhesion kinase (FAK) signaling was likewise indicated in the MCF-7/RAL2 model (Table 4:3).

Folder   Map	Cell process	p-Valu e	Genes
CDC42 in cellular processes	small GTPase mediated signal transduction	4.49E-10	39 77
Role of Nek in cell cycle regulation	protein kinase cascade, cell cycle	2.64E-04	22 55
BRCA1 as transcription regulator	cell cycle, transcription	5.51E-04	14 30
Gap junctions	cell adhesion	1.07E-03	19 49
Keratin filaments	cell adhesion	1.69E-03	18 47
Endothelial cell contacts by non-junctional mechanisms	cell adhesion	1.77E-03	16 40
Cytoskeleton remodeling	cell adhesion	3.33E-03	49 177
RAC1 in cellular process	small GTPase mediated signal transduction	3.38E-03	14 35
Plasmin signaling	cell adhesion	4.52E-03	17 47
Integrin outside-in signaling	cell adhesion	5.26E-03	25 79
Role of tetraspanins in the integrin-mediated cell adhesion	cell adhesion	6.19E-03	19 56
Reverse signalling by ephrin B	cell adhesion	6.20E-03	26 84
Chemokines and adhesion	cytokine and chemokine mediated signaling pathway, cell adhesion	7.30E-03	42 153
77	cell adhesion	7.60E-03	24

**Table 4:2. MCF-7/RAL1 Ontology.** Metacore Gene Go Analysis of 4,077 Genes Showing a 2-fold Change in Expression vs. Wild-type MCF-7/E2 Tumors.

Folder   Map	Cell process	p-Value	Genes	
FAK signaling	protein kinase cascade, cell adhesion	5.34E-06	30	70
Chemokines and adhesion	cytokine and chemokine mediated signaling pathway, cell adhesion	1.23E-05	52	153
Non-genomic (rapid) action of Androgen Receptor	response to hormone stimulus	1.41E-04	27	70
Angiotensin signaling via PYK2	G-protein coupled receptor protein signaling pathway	4.09E-04	27	74
Role of tetraspanins in the integrin-mediated cell adhesion	cell adhesion	4.22E-04	22	56
PDGF activation of prostacyclin synthesis	intracellular receptor-mediated signaling pathway	6.80E-04	10	18
NTS activation of IL-8 in colonocytes	immune response	1.06E-03	23	63
RalA regulation pathway	small GTPase mediated signal transduction	1.20E-03	15	35
Integrin outside-in signaling	cell adhesion	1.30E-03	27	79
CXCR4 signaling via second messenger	G-protein coupled receptor protein signaling pathway	1.79E-03	20	54
VEGF signaling via VEGFR2 - generic cascades	intracellular receptor-mediated signaling pathway	2.29E-03	17	44
Angiotensin activation of ERK	G-protein coupled receptor protein signaling pathway	2.95E-03	20	56
Integrin inside-out signaling	cell adhesion	3.42E-03	24	72
Fibronectin-binding integrins in cell motility	cell adhesion	5.40E-03	18	51
Role of DAP12 receptors in NK cells	immune response	5.82E-03	20	59
IFN gamma signaling pathway	cytokine and chemokine mediated signaling pathway, immune response	5.96E-03	21	63
Regulation of G1/S transition (part 2)	cell cycle	6.03E-03	13	33
MIF-JAB1 signaling	immune response	6.93E-03	10	23
Cytoskeleton remodeling	cell adhesion	6.98E-03	48	177
FGFR signaling pathway	intracellular receptor-mediated signaling pathway	7.02E-03	19	56
EGF signaling pathway	intracellular receptor-mediated signaling pathway	7.29E-03	21	64
NGF signaling pathway	intracellular receptor-mediated signaling pathway	9.86E-03	10	24

**Table 4:3. MCF-7/RAL2 Ontology.** Metacore Gene Go Analysis of 3,853 Genes Showing a 2-fold Change in Expression vs. Wild-type MCF-7/E2 Tumors.

Folder   Map	Cell process	p-Value	Genes	
CDC42 in cellular processes	small GTPase mediated signal transduction	1.05E-09	38	77
Membrane-bound ESR1: interaction with growth factors signaling	response to hormone stimulus, transcription	6.95E-03	15	42
Regulation activity of EIF4F	translation	7.44E-03	20	62
Notch Signaling Pathway	response to extracellular stimulus	8.54E-03	14	39

**Table 4:4. MCF-7/TAM2 Ontology.** Metacore Gene Go Analysis of 3,620 Genes Showing a 2-fold Change in Expression vs. Wild-type MCF-7/E2 Tumors.

Folder   Map	Cell process	p-Value	Genes	
CDC42 in cellular processes	small GTPase mediated signal transduction	2.99E-07	38	77
Oncostatin M signaling via JAK-Stat in human cells	cytokine and chemokine mediated signaling pathway, immune response	1.02E-03	11	19
Chemokines and adhesion	cytokine and chemokine mediated signaling pathway, cell adhesion	1.02E-03	52	153
Formation of Sin3A and NuRD complexes	transcription	1.06E-03	20	45
Endothelial cell contacts by non-junctional mechanisms	cell adhesion	4.37E-03	17	40
Oncostatin M signaling via MAPK in human cells	cytokine and chemokine mediated signaling pathway, immune response	1.03E-02	17	43

**Table 4:5. MCF-7/5C Ontology.** Metacore Gene Go Analysis of 4,714 Genes Showing a 2-fold Change in Expression vs. Wild-type MCF-7/E2 Tumors.

## 2. Gene expression Analysis of *in vitro* cell line models of endocrine resistance.

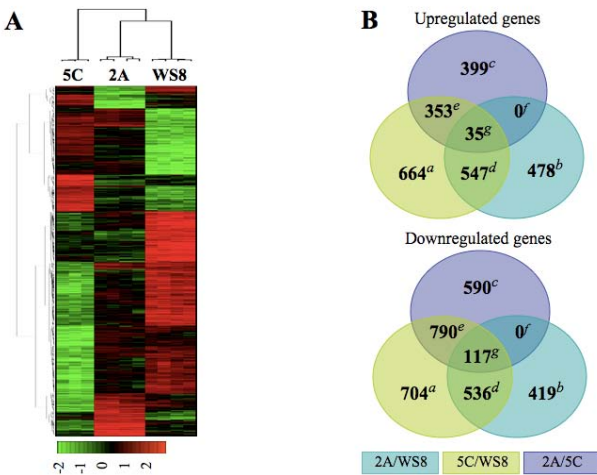
Wild type MCF7:WS8 breast cancer cells, and long-term estrogen deprived derivative cell lines MCF7:5C and MCF7:2A were treated or left untreated with estrogen as described in the preliminary data for this award. Gene expression arrays were performed by the Jordan laboratory as summarized in Table 4:2. All raw data files were sent to TGen for analysis.

Cell line	treatment	Affymetrics	Facility	Analysis
MCF7:WS8	no ligand	Yes	NWU	TGen
MCF7:WS8	E2	Yes	NWU	TGen
MCF7:WS8	ICI	Yes	NWU	TGen
MCF7:5C	no ligand	Yes	NWU	TGen
MCF7:5C	E2	Yes	NWU	TGen
MCF7:5C	ICI	Yes	NWU	TGen
MCF7:5C	E2 + ICI	Yes	NWU	Tgen
MCF7:2A	no ligand	Yes	NWU	TGen
MCF7:2A	E2	Yes	MWU	TGen
MCF7:2A	ICI	Yes	NWU	TGen
Total arrays		43		

**Table 4:6. Summary of Gene expression arrays performed on wild type MCF7 and long-term estrogen deprived derivative cell lines MCF7:5C and MCF7:2A**

All gene expression microarray data for the cell lines and treatments listed was generated by the Northwestern Microarray core using Affymetrics Human U133 Plus2.0 arrays. Treatment: E2, estrogen; ICI, ICI 182,780 (Fulvestrant). This data series met minimal quality metrics as recommended by Affymetrics Microarray Suite 5.0.

The first series of comparisons from the dataset listed in Table 4:6 above was an overview comparison of MCF-7 and the two derivative cell lines, without any added ligand (Figure 4:4). 2-dimensional hierarchical clustering of highly regulated genes was performed, identifying overlapping and distinct patterns of gene expression. CEACAM6 was identified as highly upregulated in the MCF7:2A and MCF7:5C cells, and mechanistically validated to play a key role in their enhanced malignant behavior relative to the parental cells.



**Figure 4:4. Overview of global gene expression patterns in wild-type MCF-7:WS8 cells and estrogen deprived MCF-7:5C and MCF-7:2A variant clones.** A, unsupervised hierarchical clustering dendrogram of 904 genes most differentially expressed across the three cell lines, and consistently regulated in replicate expression arrays for each cell line. Red, genes with high expression levels; green, genes with low expression levels. The similarities in the expression pattern among the three cell lines are presented as a "condition tree" on the top of the matrix. B, venn diagram representation of genes regulated greater than 2 fold between any of the three cell lines. Figure from Lewis-Wambi et al., "Overexpression of CEACAM6 promotes migration and invasion of long term estrogen deprived breast cancer cells" (manuscript submitted).

A comparison of genes commonly deregulated in the MCF7:5C and MCF7:2A cell lines relative to MCF7 were examined by gene ontology analysis to identify deregulated pathways consistent with long term deprivation to estrogen. A list of the most significant gene ontologies for this particular comparison is listed in Table 4:7. DNA-damage induced cell cycle-deregulation and enhanced mechanisms of cell survival (anti-apoptosis, NFκB and AKT signaling) are prominently featured.

Folder   Map	Cell process	p-Value	Genes	
Cell signaling/Cell cycle control	cell cycle	1.43E-16		
* <a href="#">Transition and termination of DNA replication</a>		4.06E-08	13	36
* <a href="#">ATM/ATR regulation of G1/S checkpoint</a>		1.59E-05	11	42
* <a href="#">ATM/ATR regulation of G2/M checkpoint</a>		2.14E-05	9	29
* <a href="#">Brca1 as transcription regulator</a>		2.90E-05	9	30
* <a href="#">Spindle assembly and chromosome separation</a>		6.01E-05	15	83
* <a href="#">Start of DNA replication in early S phase</a>		1.16E-04	10	43
* <a href="#">The metaphase checkpoint</a>		9.40E-04	8	37
* <a href="#">DNA-damages-induced responses</a>		1.04E-03	4	9
* <a href="#">Role of Brca1 and Brca2 in DNA repair</a>		1.61E-03	8	40
* <a href="#">NHEJ mechanisms of DSBs repair</a>		1.86E-03	6	24
* <a href="#">Chromosome condensation in prometaphase</a>		2.19E-03	7	33
* <a href="#">Role of NFB1 in DNA damage response</a>		3.57E-03	4	12
* <a href="#">Nucleocytoplasmic transport of CDK/Cyclins</a>		6.91E-03	5	22
* <a href="#">Role APC in cell cycle regulation</a>		9.69E-03	8	53
* <a href="#">Role 14-3-3 proteins in cell cycle regulation</a>		1.68E-02	5	27
* <a href="#">Role SCF complex in cell cycle regulation</a>		2.45E-02	6	40
* <a href="#">Cell cycle (generic schema)</a>		3.36E-02	4	22
* <a href="#">Sister chromatid cohesion</a>		4.65E-02	5	35
* <a href="#">Role of Nek in cell cycle regulation</a>		5.36E-02	6	48
* <a href="#">Regulation of G1/S transition (part1)</a>		6.15E-02	7	62
Cell signaling/Growth and differentiation/Growth and differentiation (common)	response to extracellular stimulus	5.23E-03		

<a href="#">* Hedgehog and PTH signaling pathways participation in bone acartilage</a>		8.16E-02	5	41
<a href="#">WNT signaling pathway. Part 2</a>		9.43E-02	8	82
<a href="#">TGF-beta receptor signaling</a>		1.04E-01	6	57
<a href="#">* TPO in cell process</a>		3.44E-01	4	52
<a href="#">Glucocorticoid receptor signaling</a>		4.00E-01	3	40
<a href="#">Estrogen receptor signaling pathway</a>		4.79E-01	2	28
<a href="#">WNT signaling pathway. Part 1. Degradation of beta-catenin iabsence WNT</a>		4.79E-01	2	28
<a href="#">* Leptin signaling via JAK/STAT and MAPK cascades</a>		4.98E-01	2	29
<a href="#">* IGF-RI signaling</a>		5.92E-01	4	72
<a href="#">Notch activating pathway for NF-kB</a>		6.00E-01	2	35
<a href="#">* PDGF activation of prostacyclin synthesis</a>		6.52E-01	1	18
<a href="#">* FGFR signaling pathway</a>		8.29E-01	2	55
<a href="#">* FGF-family signaling</a>		8.64E-01	1	34
<a href="#">Notch Signaling Pathway</a>		8.93E-01	1	38
<a href="#">* HGF signaling pathway</a>		9.20E-01	1	43
Cell signaling/Cell death/Apoptosis	apoptosis, cell death	1.03E-02		
<a href="#">* DNA-damages-induced apoptosis</a>		1.63E-04	6	16
<a href="#">* p53-dependent apoptosis</a>		2.19E-03	7	33
<a href="#">* Regulation of Apoptosis by Mitochondrial Proteins</a>		9.71E-02	4	31
<a href="#">* Cytoplasm/mitochondrial transport of proapoptotic proteins BidBmf and Bim</a>		1.36E-01	4	35
<a href="#">p53-independent apoptotic signaling</a>		1.40E-01	3	23
<a href="#">* Role of IAP-proteins in apoptosis</a>		1.46E-01	4	36
<a href="#">* Apoptotic TNF-family pathways</a>		1.91E-01	4	40
<a href="#">* Caspases cascade</a>		3.05E-01	3	34
<a href="#">* Role SUMO in p53 regulation</a>		3.17E-01	2	20
<a href="#">* Anti-apoptotic TNFs/NF-kB/Bcl-2 pathway</a>		4.31E-01	3	42
<a href="#">* FAS signaling cascades</a>		4.62E-01	3	44
<a href="#">* BAD phosphorylation</a>		5.92E-01	4	72
<a href="#">* TNFR1 signaling pathway</a>		7.57E-01	2	47
<a href="#">* Anti-apoptotic TNFs/NF-kB/IAP pathway</a>		8.38E-01	1	31
Cell signaling/Proteolysis	proteolysis	7.92E-02		
<a href="#">Role of Akt in hypoxia induced HIF1 activation</a>		1.54E-01	5	50
<a href="#">Putative SUMO-1 pathway</a>		2.12E-01	3	28

**Table 4:7. Metacore Gene Go analysis of genes deregulated at least 2 fold by both MCF7:5C and MCF7:2A relative to MCF7:WS8.** P-value represents the odds ratio between the number of differentially expressed genes (green) present in the list of genes associated with a given ontology node (red).

### 3). Integration of array-based comparative genomic hybridization (aCGH).

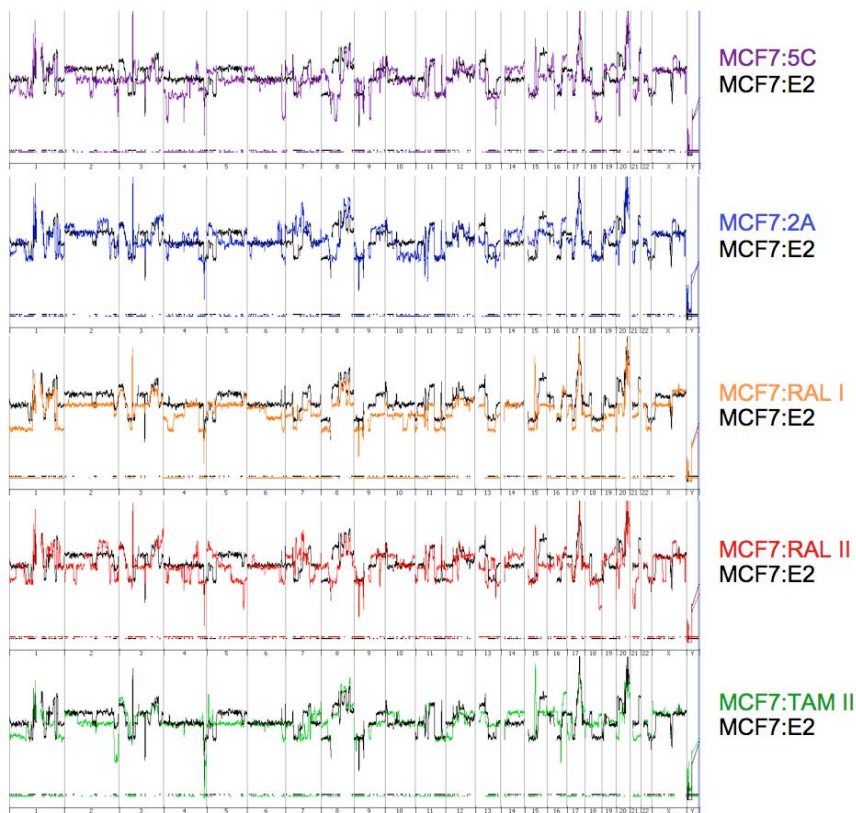
It is now understood that during tumor progression (including development of resistance to therapy), cancer genomes undergo extensive alteration and evolution. Important and well recognized mechanism leading to altered gene expression include DNA copy number gain (*e.g.* ERBB2, EGFR, IGF1R loci), loss (*e.g.* P16, p53, BRCA1) or rearrangement (a recent example: Recurrent Fusion of *TMPRSS2* and ETS Transcription Factor Genes observed in Prostate Cancer (32).

During preparation of this grant application, array-based comparative Genomic Hybridization (aCGH) was still in its infancy and optimization stages. In the last two years, this technology has rapidly advanced to where genomic evolution can now be viewed at exquisitely high resolution, allowing identification of precise chromosomal breakpoints between genes and even between exons, facilitating identification of regions of gain, loss, and predicted chromosomal rearrangement. Integration of genomic profiles with gene expression data has become very highly publicized, especially in the area of breast cancer.

It is estimated that approximately 40% of genomic gains and losses correspond to alterations in gene expression, making aCGH analysis of cancer genomes an important part of any genomic study. aCGH profiling is a technology highly implemented at Tgen. We have completed aCGH profiling from DNA isolated from all endocrine resistant tumor models and cell lines as part of the DoD study. An overview of the entire genome for each model relative to MCF7:E2 is shown in figure 4:5.

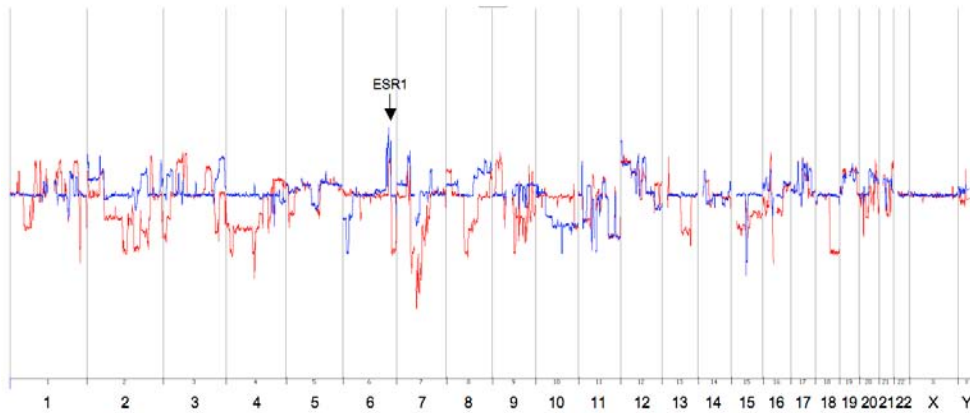
We noted large regions of chromosomal rearrangement for each cell line model, and noted several genes harboring intragenic chromosomal breaks. Our first extensive analysis was to compare the evolution of MCF7:2A and MCF7:5C cells, two clones derived following long-term deprivation of MCF7:WS8 cells as previously described. Figure 4:6 shows a hybridization of MCF7:5C against MCF7:WS8 (red) and MCF7:2A against MCF7:WS8. Regions of extensive rearrangement throughout the whole genome were observed. Interestingly, both cell lines had gained copies of the estrogen receptor gene (ESR1), the BRCA1 gene and CDK4, to name but a few. These three genes were also overexpressed in the MCF-7:2A and MCF-7:5C cells. This suggests much of the transcriptional response we are observing in the gene expression profiles for these tumor models is due to evolutionary chromosomal changes.

We are currently integrated genomic and gene expression information for MCF-7, MCF-7:5C and MCF-7:2A cells to identify consistent potential biomarkers of aromatase inhibitor resistance, as well as likely biological drivers of enhanced malignant behavior observed for the MCF-7:2A and MCF-7:5C cells.



**Figure 4:5. Whole genome aCGH view of DNA content for each model of endocrine resistance.** Probes were prepared from DNA isolated from each tumor xenograft model and hybridized to Agilent 244K CGH arrays along with normal diploid male genomic DNA (Promega). MCF7:E2 DNA is shown overlaid in black for comparison to each colored endocrine resistant cell model.

■ MCF7:5C v MCF7:WS8  
■ MCF7:2A v MCF7:WS8

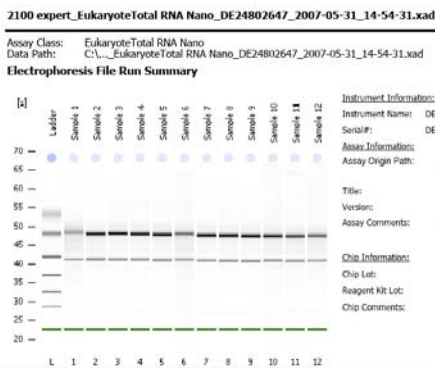


**Figure 4:6. Whole genome aCGH view of DNA content for MCF7:2A and MCF7:5C hybridized against MCF7:E2.**

**Task 4b. Identify regulatory networks for pathways indicative of differential responses to E2.** Gene-gene regulatory networks influenced by E2 in each laboratory model will be investigated as modules as outlined in Task 2 (FCCC).

**WORK ACCOMPLISHED – Task 4b**

Under Task 2, samples were prepared from model module 1. For microarray analysis, Trizol lysates were received by TGen from prepared from MCF7:WS8 and MCF7:5C cells treated or not treated with estrogen for 2, 6, 12, 24, 48, 72 and 96h.



**Figure 4:7. Total RNA electrophoresis (Agilent Bioanalyzer).** 1ul of total RNA electrophoresed for each sample (upper panel). RIN # metric between 9.0-10.0 is optimal for probe labeling according to Agilent recommended guidelines (lower panel).

RNA was extracted using protocols required for subsequent gene expression array analyses from from both cell lines, all time points. RNA integrity and purity were measured using a 2100 Bioanalyzer (Agilent Technologies; Palo Alto, CA) and a Nanodrop ND-1000 Spectrophotometer (Nanodrop; Wilmington, DE). Figure 4:7 shows an example of 12 total RNA samples extracted and electrophoresed for QC using the Agilent Bioanalyzer.

2ug of every RNA sample was reserved by TGen and the remaining RNA was returned to FCCC for RT-PCR-based quality assessment prior to array hybridization. Samples that did not meet FCCC Quality control (did not amplify) were flagged, and TGen was informed which samples not to be included in subsequent gene expression arrays.

Probe labeling for microarray analysis of all MCF7:5C time points has now been completed. The labeling efficiency summary is shown in table 4:8 below.

Pooled Ref. (Cy5)	ng pooled	Date Labeled	ng Labeled	dye pmol/ul	ng/ul	Sample (Cy3)	Concentration (ng/uL)	ng Labeled	Date Labeled	pmols/ul Dye	pmol cy/ug cRNA	ng/ul
5C 2hr Controls	200	8/31/11	500	3.95	173.98	5C 2hr - 1	191.00	250	8/31/13	2.68	16.68	160.7
	200					5C 2hr - 2	160.81	250	8/31/13	2.19	16.1	136.03
	200					5C 2hr - 3	195.53	250	8/31/13	2.28	15.47	147.35
	200					5C 2hr - 4	212.28	250	8/31/13	2.19	15.51	141.17
	200					5C 2hr - 5	173.72	250	8/31/13	1.38	12.02	114.8
	200					5C 2hr - 6	185.90	250	8/31/13	1.42	10.48	135.51
5C 6hr Controls	200	8/31/11	500	3.58	217.20	5C 6hr - 1	331.18	250	8/31/13	1.43	10.49	136.28
	200					5C 6hr - 2	266.68	250	8/31/13	2.09	12.98	161
	200					5C 6hr - 3	262.31	250	8/31/13	2.39	15.32	156.01
	200					5C 6hr - 4	277.83	250	8/31/13	2.3	15.02	153.15
	200					5C 6hr - 5	270.59	250	8/31/13	1.51	10.68	141.45
	200					5C 6hr - 6	211.93	250	8/31/13	0.74	6.71	110.35
5C 12hr Controls	200	8/31/11	500	2.22	221.20	5C 12hr - 1	255.26	250	8/31/13	1.52	14.04	108.3
	200					5C 12hr - 2	172.60	250	8/31/13	0.66	6.09	108.41
	200					5C 12hr - 3	261.97	250	8/31/13	0.67	5.26	127.3
	200					5C 12hr - 4	238.85	250	8/31/13	0.8	5.18	154.57
	200					5C 12hr - 5	230.38	250	8/31/13	2.6	13.7	189.8
	200					5C 12hr - 6	296.30	250	8/31/13	0.91	8.67	104.97
5C 24hr Controls	200	8/31/11	500	2.85	183.65	5C 24hr - 1	307.53	250	8/31/13	0.66	5.86	112.55
	200					5C 24hr - 2	292.66	250	8/31/13	1.31	10.41	125.81
	200					5C 24hr - 3	333.50	250	8/31/13	2.2	15.82	139.03
	200					5C 24hr - 4	297.92	250	8/31/13	2.08	13.09	158.85
	200					5C 24hr - 5	189.18	250	8/31/13	1.54	10.52	146.33
	200					5C 24hr - 6	337.35	250	8/31/13	2.33	13.94	167.1
5C 48hr Controls	200	8/31/11	500	3.56	230.79	5C 48hr - 1	390.3	250	8/31/13	0.77	5.8	132.76
	200					5C 48hr - 2	1194.50	250	8/31/13	0.68	9.98	68.17
	200					5C 48hr - 3	484.00	250	8/31/13	4	29.08	137.56
	200					5C 48hr - 4	1037.00	x	x	x	x	x
	200					5C 48hr - 5	526.50	250	8/31/13	1.86	16.43	113.22
	200					5C 48hr - 6	683.50	250	8/31/13	1.02	13.67	74.59
5C 72hr Controls	x	8/31/11	500	4.06	236.28	5C 72hr - 1	768.70	250	8/31/13	1.43	13.97	102.37
	200					5C 72hr - 2	506.50	250	8/31/13	2.25	11.02	204.15
	200					5C 72hr - 3	663.10	250	8/31/13	1.08	7.48	144.37
	200					5C 72hr - 4	690.75	250	8/31/13	4.76	29.81	159.66
	200					5C 72hr - 5	781.90	250	8/31/13	3.65	33.4	109.29
	200					5C 72hr - 6	777.75	250	8/31/13	1.8	14.04	128.25
5C 96hr Controls	200	8/31/11	500	2.07	135.11	5C 92hr - 1	682.30	250	8/31/13	1.47	8.65	169.86
	200					5C 92hr - 2	1061.90	250	8/31/13	1.36	13.33	102.01
	200					5C 92hr - 3	1221.60	250	8/31/13	1.28	11.52	111.12
	200					5C 92hr - 4	1064.85	250	8/31/13	1.27	11.6	109.52
	200					5C 92hr - 5	1390.75	250	8/31/13	0.77	11.59	66.44
	200					5C 92hr - 6	991.20	250	8/31/13	1.19	12.46	95.49

x = Do Not Hybe. Failed QC

**Table 4:8. Result of probe preparation for hybridization to Agilent gene expression arrays.**

All Samples passed labeling QC (pmol Cy dye per ug of cRNA >5%). X denotes samples that did not pass QC according to FCCC data.

**Task 4c. Interrogate pathways of endocrine resistance using high throughput RNA interference (HT-RNAi)**

**WORK ACCOMPLISHED – Task 4c**

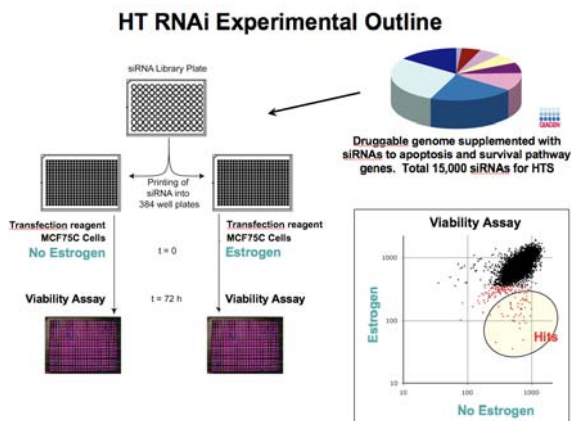
Cultures of MCF7:5C and MCF7:2A cells have been established at TGen. All serum was charcoal-dextran treated by FCCC and sent to Tgen for prepare estrogen-free growth media for these cell lines. Growth curves have been generated that are consistent with previous data generated for these cell lines by FCCC. We are now in the optimization stages for HT-RNAi. Table 4:9 shows the optimization procedures nearing completion prior to the RNAi screen.

Parameter	Key factors
Cell line for screening	transfection efficiency, growth rate, assay sensitivity
Cell growth media	should not interfere with drug activity, readout, or transfection efficiency
[siRNA]	concentration must produce effective silencing and limit off-target effects
Plate format	medium evaporation, machine readout, barcode
Positive control siRNA	previously identified; cell line-specific; proliferation-specific; general killers
Sensitizing positive control siRNA	provides measure of overall assay function
Negative control siRNA	should have no effect on cell growth or drug activity
Transfection reagent	should be effective in introducing siRNA and have low toxicity
Transfection reagent diluent	should not interfere with drug activity, readout, or transfection efficiency
Transfection reagent ratio	toxicity vs. efficiency
Transfection reagent incubation time	enough time to complex siRNA and transfection reagent
Mechanism for addition of transfectin reagent	minimize well-to-well, plate-to-plate variability
Complexing Time	enough time to complex siRNA and transfection reagent
Cell Volume Added	well-to-well, plate-to-plate variability
Cell Number Added	optimized to give greatest dynamic range at read out
Incubation time (before drug)	enough time for siRNA to silence transcripts
Incubation time (after drug)	enough time for drug action
Mechanism for addition of cells	minimize well-to-well, plate-to-plate variability
Drug	should have an effect on the screen cell line
Drug Diluent (Vehicle)	should readily solubilize drug
Drug volume	should be minimal
Drug stability	temperature, half-life, solution, medium
Final Drug concentration	should have desired effect at selected concentration
Mechanism for addition of Readout Reagent	minimize well-to-well, plate-to-plate variability
Cell viability measurement	sensitivity, accuracy, and cost
Added volume of Readout Reagent	should be minimal
Mechanism for addition of Readout Reagent	minimize well-to-well, plate-to-plate variability
Incubation time for Readout Reagent	optimized to give greatest dynamic range at read out
Readout method	sensitivity, accuracy

**Table 4:9. Key Parameters addressed in optimization prior to HT-RNAi screen.**

We are currently optimizing optimal estrogen concentration for estrogen-induced apoptosis of MCF7:C cells. We will use the same concentration of estrogen for both MCF75C cells and MCF7:2A cells. As outlined in the schema for HT-RNA in Figure 4:8, we hope that estrogen mediated apoptosis will be inhibited in the presence of a percentage of siRNAs in the library screen. These are what constitute “hits” as depicted in the graph in Figure 4:8. Conversely, we hope to induce estrogen-mediated apoptosis in the MCF7:2A cells, to look for potential genetic sensitizers of apoptosis reactivation to further evaluate. We anticipate both MCF-7:5C and MCF-7:2A cells will have HT-RNAi analysis completed by January/February of 2008.

Integration of siRNA data with temporal genomic data being prepared and analysed in parallel in 2007 will provide significant information to interrogate pathways endocrine resistance following long term estrogen deprivation.



**Figure 4:8. Experimental design for HT-RNAi screen.**

## **METHODS**

### **Gene expression and aCGH Microarrays.**

All protocols pertaining to RNA isolation, quantitation, qualification, labeling and hybridization were followed exactly as recommended by the manufacturers of the arrays.

### **Affymetrix data filtering.**

Assessment of data quality was conducted following default guidelines in the Affymetrix GeneChip® Expression Analysis Data Analysis Fundamentals Training Manual. Global scaling for average signal intensity for all arrays was set to 500. Four biological replicates from each of the three cell lines were arrayed to determine consistent and reproducible patterns of gene expression. To determine overlapping and distinct patterns of gene expression between each of the three cell lines, data was filtered as follows. Genes across all hybridizations with an expression intensity  $<70$  were removed. Genes with a present call (consistently within replicates) for at least one cell line were included. To eliminate genes with variable expression within a group of replicates (intra-set deviation), normalized gene intensity ratios (signal intensities divided by the median gene intensity all hybridizations) were derived, then the standard deviation of the  $\log_2$ -transformed normalized intensity ratios were calculated for each group of replicates. Genes with a with a standard deviation  $<0.15$  were excluded. Lastly, to filter for genes with variable expression between cell lines (inter-set deviation), genes were retained that showed a standard deviation of  $\log_2$ -transformed normalized intensity ratios  $>0.3$ .

### **Agilent 22k Human 1A (V2) Oligo Microarray data filtering.**

Arrays were washed in an ozone-controlled environment ( $<0.1$  ppb). Slides were scanned using the Agilent Microarray Scanner (model G2505B). Data was extracted, processed and normalized using Agilent's Feature Extraction software (v8.1.1.1). All arrays were quality controlled for a minimum median intensity of greater than 85 units and a maximum average background level of 50 units in each channel. The variance of the abundance measurements on a single chip was estimated by determining the median standard deviation of the  $\log_2$  ratios observed for 100 genes that have 10 identical oligonucleotide detectors each, printed randomly across the chip. In this experimental series the largest median standard deviation measured was 0.079. This variance is associated with a 99.9% confidence interval for the ratio fold change of 0.85 to 1.18. Our 2 fold cut off for significantly regulated genes is therefore highly significant on these extremely sensitive arrays.

## **Key Research Accomplishments**

- Established an effective multi-institutional collaborative research program to link the biology of estrogen action (growth or apoptosis) with proteomics and genomic changes. Data can now be catalogued, stored and evaluated electronically.
- Discovered that the invasion protein CEACAM-6 is elevated in cells that become resistant to estrogen withdrawal.
- Discovered that the MCF-7:5C and 2A have different time courses for their apoptotic response to estrogen. Further studies can now compare time courses in more detail and discover the reason for the potential resistance to estrogen.
- Conducted the first CGH analysis of cells resistant to estrogen withdrawal to identify gene aberrations for further study.

## Reportable Outcomes

### Publications:

1. Ariazi EA, Lewis-Wambi JS, Gill SD, Pyle JR, Ariazi JL, Kim HR, Sharma CG, Cordera F, Shupp HA, Li T, Jordan VC. Emerging principles for the development of resistance to antihormonal therapy: implications for the clinical utility of fulvestrant. *J Steroid Biochem Mol Biol* 2006;102(1-5):128-38.
  2. Jordan VC. Optimising endocrine approaches for the chemoprevention of breast cancer. *Beyond the Study of Tamoxifen and Raloxifene (STAR) Trial. European Journal of Cancer* 2006;42:2909-2913.
  3. Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. *Nature Reviews Cancer* 2007;7:46-53.
  4. Jordan VC. SERMs: meeting the promise of multifunctional medicines. (Commentary). *Journal of the National Cancer Institute* 2007;99:351-357.
  5. Jordan VC. Beyond raloxifene for the prevention of osteoporosis and breast cancer. *Br J Pharmacol.* 2007;150:3-4.
  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. Jordan VC, Gelber R. Problems with the progesterone receptor in practice? *J Clinical Oncology* 2007;25:1957-9.
  8. Patel RR, Sharma GN, Jordan VC. Optimizing the antihormonal treatment and prevention of breast cancer. *Breast Cancer* 2007; 14: 113-122.
  9. Jordan VC. Successful Translation Research with Selective Oestrogen Receptor Modulators to treat and prevent breast cancer. *German Journal of Obstetrics and Gynecology. Georg Thieme Verlag KG, Stuttgart, NY.* 2007;67:443-450.
  10. Jordan VC, Lewis-Wambi J, Kim H, Cunliffe H, Ariazi E, Sharma C, Shupp HA, Swaby R. Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients. *Breast* 2007;(in press).
  11. Jordan VC, O'Malley BM. Selective estrogen receptor modulators and antihormonal resistance in breast cancer. *Journal of Clinical Oncology* 2007;(accepted).
  12. Jordan, VC. Tamoxifen or Raloxifene: A tale of two choices. *Cancer Epidemiology Biomarkers and Prevention* 2007 (accepted).
  13. Swaby RF, Sharma CG, Jordan VC. SERMs for the treatment and prevention of breast cancer. *Rev Endocr Metab Disord* (online) April, 2007.
- Jordan VC. New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer. *Steroids* 2007;(in press).
14. Ariazi, EA, Leitao A, Oprea TI, Chen B, Louis T, Bertucci AM, Sharma GN, Gill SD, Kim HR, Shupp HA, Pyle JR, Madrack A, Donato AL, Cheng D, Paige J, Jordan, VC. Exemestane's 17-hydroxylated metabolite exerts biological effects as an androgen. *Molecular Cancer Therapeutics* 2007(in press).

### Abstracts

1. *Abstract submitted to AACR Advances in Breast Cancer Research, October 17<sup>th</sup>, 2007.*  
*Temporary Abstract ID number: 6348*

### **Comparative Global Gene Expression Profiling to Identify Unifying and Selective Pathways Involved in Tamoxifen, Raloxifene, and Aromatase Inhibitor-resistant Breast Cancer Xenograft Tumors**

Eric A. Ariazi, Heather E. Cunliffe, Amanda L. Willis, Catherine M. Mancini, Yoganand Balagurunathan, Shaun D. Gill, Jennifer R. Pyle, Heather A. Shupp, V. Craig Jordan

2. *Abstract submitted to AACR Advances in Breast Cancer Research, October 17<sup>th</sup>, 2007.*

**Long-term estrogen deprivation of breast cancer cells causes significant genomic evolution and development of enhanced malignant behavior.**

Catherine M. Mancini, Joan S. Lewis-Wambi, Eric A. Ariazi, Helen R. Kim, Amanda L. Willis, V. Craig Jordan and Heather Cunliffe

3. Abstract #5710 was selected for an oral presentation in a Minisymposium session at the 2007 AACR Annual Meeting in Los Angeles, CA and was published in the 2007 Proceedings of the American Association for Cancer Research.

**Overexpression of CEACAM6 promotes invasion and migration of aromatase inhibitor-resistant breast cancer cells.**

Joan S. Lewis-Wambi, Helen R. Kim, Heather Cunliffe, and V. Craig Jordan, Fox Chase Cancer Center, Philadelphia, PA and Translational Genomics Research Institute (TGen), Phoenix, AZ, USA.

**Presentations:**

**2006**

1. Successful Translational Research with SERMs, Frankfurt, Germany, October, 2006.
2. Hormone Therapies used in the treatment of Breast Cancer, Fox Chase Cancer Support Group, Fox Chase Cancer Center, Philadelphia, PA, November, 2006.
3. The role of estrogen in the life and death of breast cancer cells. Abramson Family Cancer Research Center, Institute/Hematology-Oncology Series, University of Pennsylvania, Philadelphia, PA. November, 2006.
4. The new biology of estrogen action: can new knowledge help patients?, City of Hope, California, November, 2006.
5. The role of estrogen in the life and death of breast cancer cells, Genetech, California, November, 2006.
6. Clinical Application and development of SERMs. San Antonio Breast Cancer Conference, December 2006.

**2007**

1. Update on Hormonal Therapies for the Prevention and Treatment of Breast Cancer, Northern California Breast Cancer Symposium, February, 2007.
2. Optimizing treatment and prevention of breast cancer: time for a change, Primary Therapy of Early Breast Cancer, 10th International Conference, St. Gallen, Switzerland, Anniversary Meeting, March, 2007.
3. A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis, Translational Science Lecture Series, Fox Chase Cancer Center, Philadelphia, PA, March, 2007.
4. What do we do about antihormonal resistance in breast cancer?, AACR Annual Meeting, April, 2007.
5. Search for a novel apoptotic target in cancer, AstraZeneca Presentation, April 10, 2007.
6. Hormones and Women's Health in the 21st Century, Joint Steering Committee to Public Policy, April, 2007
7. Oestrogen in the life and death of breast cancer, University of Leeds Symposium, May, 2007.
8. Targeted breast cancer therapy: A change to aromatase inhibitors, National Surgery Oncology Conference, Antalya, Turkey, May, 2007.
9. Successful Translational Research for the targeted treatment and prevention of breast cancer, Annual Retreat for Cancer Research, UMDNJ, New Jersey, May, 2007.
10. Optimizing treatment and prevention of breast cancer: time for a change, White Nights in St. Petersburg, Russia, June, 2007.

11. Selective estrogen receptor modulators for the treatment and prevention of breast cancer. Worcester Foundation for Biomedical Research, Pincus Award, June 14, 2007
12. Oestrogen is bad for patients with breast cancer ? Controversies in Breast Cancer, Edinburgh, September 3-4, 2007
13. What is New in Chemoprevention for breast cancer? Aultman Cancer Conference, Ohio, September 14-15, 2007
14. Background, scientific rationale and organization, Center of Excellence External Advisory Board meeting, September 18, 2007
15. Estrogens and antiestrogens in the Life and death of breast cancer cells: new treatment opportunities. Ohio State University Grand Rounds, September 21, 2007
16. Tamoxifen, the first targeted therapy for the treatment of breast cancer. Medical Oncology Fellows Conference, September 24, 2007
17. Practical Issues in Endocrine Chemoprevention, 9<sup>th</sup> Lynn Sage Meeting, Chicago, IL, September, 2007

## Grants

### Joan Lewis-Wambi, PhD

Award document number: KCA120051A

Grant title: The new biology of estrogen action in aromatase resistance breast cancer cells

Project Period begins: 8/87/2007

Project Period ends: 07/31/2012

## Conclusion

We have established an interconnected network that is evaluating the estrogen regulated signal transduction pathways for the initiation of growth or apoptosis in breast cancer cells. This will be accomplished by using unique antihormone resistant models developed in our laboratory over the past two decades. We are progressing on schedule with the systematic evaluation of cell lines at the FCCC (**Task 2**) and have completed an extensive pilot study of tumor model systems grown in athymic mice. An extensive quality control system is in place to ensure the correct allocation of tissues to treatment groups before the evaluation of gene array experiments (**T-Gen, Task 4**) or proteomics (**GU Task 3**). Our unique ability to compare and contrast breast cancer models of estrogen regulated cell growth, hormone independent growth, SERM stimulated growth, and estrogen-induced apoptosis is already creating a unique view of estrogen regulation in cells not previously appreciated. Already we are investigating specific proteins that can act as a mediator of estrogen action is, in fact, critical for estrogen-dependent growth **and** which are amplified in estrogen deprived cells. Additionally, based on gene array analysis of our unique estrogen deprived cell lines, we have identified dramatic elevations of the invasion protein, CEACAM6 compared with antihormone treatment naïve breast cancer cells. These examples illustrate the power of our integrated approach to deciphering the signal transduction pathways in our unique models using genomics and proteomics. Most importantly, in future years, the developing survival and apoptotic map will be interrogated using tissue samples from our clinical trials using estrogen to treat patients who have developed resistance to antihormonal therapy. We have successfully secured funding and approved by AstraZeneca to support a clinical trial of 12 weeks of high dose estradiol (30 mg daily) therapy for patients who have successfully been treated with an failed at least two successive antihormonal therapies. All documentation and IRB approvals are in place and await the completion of administrative approval by the DoD.

## References

1. 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.
2. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 1996;56:2321-30.

3. Lewis JS, 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;97:1746-59.
4. Ariazi EA, Lewis-Wambi JS, Gill SD, et al. Emerging principles for the development of resistance to antihormonal therapy: implications for the clinical utility of fulvestrant. *J Steroid Biochem Mol Biol* 2006;102:128-38.
5. O'Regan RM, Cisneros A, England GM, et al. Effects of the antiestrogens tamoxifen, toremifene, and ICI 182,780 on endometrial cancer growth. *J Natl Cancer Inst* 1998;90:1552-8.
6. Gottardis MM, Robinson SP, Jordan VC. Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumorigenic action of tamoxifen. *J Steroid Biochem* 1988;30:311-4.
7. O'Regan RM, Osipo C, Ariazi E, et al. Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. *Clin Cancer Res* 2006;12:2255-63.
8. Liu H, Lee E-S, Gajdos C, et al. Apoptotic Action of 17{beta}-Estradiol in Raloxifene-Resistant MCF-7 Cells *In Vitro* and *In Vivo*. *J Natl Cancer Inst* 2003;95:1586-97.
9. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 1988;48:5183-7.
10. 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;49:4765-9.
11. Yao K, Lee ES, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;6:2028-36.
12. 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.
13. Osipo C, Gajdos C, Cheng D, Jordan VC. Reversal of tamoxifen resistant breast cancer by low dose estrogen therapy. *J Steroid Biochem Mol Biol* 2005;93:249-56.
14. Willis S, Day CL, Hinds MG, Huang DC. The Bcl-2-regulated apoptotic pathway. *Journal of cell science* 2003;116:4053-6.
15. Del Bufalo D, Biroccio A, Leonetti C, Zupi G. Bcl-2 overexpression enhances the metastatic potential of a human breast cancer line. *Faseb J* 1997;11:947-53.
16. Kobayashi S, Iwase H, Ito Y, et al. Clinical significance of bcl-2 gene expression in human breast cancer tissues. *Breast cancer research and treatment* 1997;42:173-81.
17. Emi M, Kim R, Tanabe K, Uchida Y, Toge T. Targeted therapy against Bcl-2-related proteins in breast cancer cells. *Breast Cancer Res* 2005;7:R940-52.
18. Wang S, Yang D, Lippman ME. Targeting Bcl-2 and Bcl-XL with nonpeptidic small-molecule antagonists. *Seminars in oncology* 2003;30:133-42.
19. Estrela JM, Ortega A, Obrador E. Glutathione in cancer biology and therapy. *Critical reviews in clinical laboratory sciences* 2006;43:143-81.
20. Engel RH, Evens AM. Oxidative stress and apoptosis: a new treatment paradigm in cancer. *Front Biosci* 2006;11:300-12.
21. Chen G, Waxman DJ. Role of cellular glutathione and glutathione S-transferase in the expression of alkylating agent cytotoxicity in human breast cancer cells. *Biochemical pharmacology* 1994;47:1079-87.
22. Batist G, Schechter R, Woo A, Greene D, Lehnert S. Glutathione depletion in human and in rat multi-drug resistant breast cancer cell lines. *Biochemical pharmacology* 1991;41:631-5.
23. Rosi A, Grande S, Luciani AM, et al. Role of glutathione in apoptosis induced by radiation as determined by 1H MR spectra of cultured tumor cells. *Radiation research* 2007;167:268-82.
24. Voehringer DW. BCL-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity. *Free radical biology & medicine* 1999;27:945-50.
25. Celli A, Que FG, Gores GJ, LaRusso NF. Glutathione depletion is associated with decreased Bcl-2 expression and increased apoptosis in cholangiocytes. *The American journal of physiology* 1998;275:G749-57.
26. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* 2003;3:453-8.

27. van den Brule FA, Engel J, Stetler-Stevenson WG, Liu FT, Sobel ME, Castronovo V. Genes involved in tumor invasion and metastasis are differentially modulated by estradiol and progestin in human breast-cancer cells. *Int J Cancer* 1992;52:653-7.
28. Vleminckx K, Vakaet L, Jr., Mareel M, Fiers W, van Roy F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* 1991;66:107-19.
29. Blumenthal RD, Leon E, Hansen HJ, Goldenberg DM. Expression patterns of CEACAM5 and CEACAM6 in primary and metastatic cancers. *BMC Cancer* 2007;7:2.
30. Duxbury MS, Ito H, Benoit E, Ashley SW, Whang EE. CEACAM6 is a determinant of pancreatic adenocarcinoma cellular invasiveness. *British journal of cancer* 2004;91:1384-90.
31. Duxbury MS, Matros E, Clancy T, et al. CEACAM6 is a novel biomarker in pancreatic adenocarcinoma and PanIN lesions. *Ann Surg* 2005;241:491-6.
32. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science*. 2005; 310:644-8.

### **Appendices:**

- [1] Swaby, RF and Jordan VC. Low Dose Estrogen Therapy to Reverse Acquired Antihormonal Resistance in the Treatment of Breast Cancer. *Clinical Breast Cancer* 2007 (submitted).
- [2] Lewis-Wambi JS, Cunliffe HE, Kim HR, Willis AL, Jordan VC. Overexpression of CEACAM6 promotes migration and invasion of estrogen deprived breast cancer cells. *Cancer Research* 2007 (submitted).



Published in final edited form as:

*J Steroid Biochem Mol Biol.* 2006 December ; 102(1-5): 128–138.

## Emerging principles for the development of resistance to antihormonal therapy: Implications for the clinical utility of fulvestrant\*

Eric A. Ariazi, Joan S. Lewis-Wambi, Shaun D. Gill, Jennifer R. Pyle, Jennifer L. Ariazi, Helen R. Kim, Catherine G.N. Sharma, Fernando Cordera, Heather A. Shupp, Tianyu Li, and V. Craig Jordan<sup>†</sup>

Fox Chase Cancer Center, Philadelphia, PA 19111-2497, USA

### Abstract

We seek to evaluate the clinical consequences of resistance to antihormonal therapy by studying analogous animal xenograft models. Two approaches were taken. 1) MCF-7 tumors were serially transplanted into selective estrogen receptor modulator (SERM) -treated immunocompromised mice to mimic five years of SERM treatment. The studies *in vivo* were designed to replicate the development of acquired resistance to SERMs over years of clinical exposure. 2) MCF-7 cells were cultured long-term under SERM-treated or estrogen withdrawn conditions (to mimic aromatase inhibitors), and then injected into mice to generate endocrine-resistant xenografts. These tumor models have allowed us to define Phase I and Phase II antihormonal resistance according to their responses to E<sub>2</sub> and fulvestrant. Phase I SERM-resistant tumors were growth stimulated in response to estradiol (E<sub>2</sub>), but paradoxically, Phase II SERM and estrogen withdrawn-resistant tumors were growth inhibited by E<sub>2</sub>. Fulvestrant did not support growth of Phase I and II SERM-resistant tumors, but did allow growth of Phase II estrogen withdrawn-resistant tumors. Importantly, fulvestrant plus E<sub>2</sub> in Phase II antihormone-resistant tumors reversed the E<sub>2</sub>-induced inhibition and instead resulted in growth stimulation. These data have important clinical implications. Based on these and prior laboratory findings, we propose a clinical strategy for optimal third-line therapy: patients who have responded to and then failed at least two antihormonal treatments may respond favorably to short-term low-dose estrogen due to E<sub>2</sub>-induced apoptosis, followed by treatment with fulvestrant plus an aromatase inhibitor to maintain low tumor burden and avoid a negative interaction between physiologic E<sub>2</sub> and fulvestrant.

### Keywords

Breast Cancer; Estradiol; Tamoxifen; Raloxifene; Fulvestrant

---

\*Proceedings of the 17<sup>th</sup> International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, 'Recent Advances in Steroid Biochemistry and Molecular Biology' (Seefeld, Tyrol, Austria, 31 May - 3 June, 2006).

<sup>†</sup> Corresponding author: V. Craig Jordan, OBE, PhD, DSc, Vice President and Research Director for Medical Sciences, Alfred G. Knudson Chair of Cancer Research, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111-2497, USA. Tel.: +1 215 728 7410; Fax: +1 215 728 7034. *E-mail address:* v.craig.jordan@fccc.edu (V.C. Jordan).

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

## 1. Introduction

The target for endocrine therapy is the estrogen receptor (ER), and the translation of laboratory findings on the control of estrogen-regulated tumor growth has established the current treatment strategies which have been validated in clinical trials [1–7]. Tamoxifen (TAM), the prototype selective estrogen receptor modulator (SERM), is a current standard adjuvant treatment used for 5 years in all stages of ER-positive breast cancer [8–12]. However, aromatase inhibitors (AIs) are becoming the leading choice for antihormonal treatment of ER-positive breast cancer in postmenopausal patients. Still, there is a need to study the long-term therapeutic consequences of TAM because of its use in premenopausal ER-positive breast cancer [9,10], and as a chemopreventive agent to reduce the risk of breast cancer in high-risk women [13]. There is also considerable interest in the use of raloxifene (RAL), a related SERM, as a chemopreventive agent [14,15], since it has recently been shown in the STAR trial (Study of TAM and RAL) to exhibit equivalent efficacy as TAM in reducing the risk of breast cancer [16]. Additionally, RAL is used for the treatment and prevention of osteoporosis in postmenopausal women [17], has been noted to have endometrial safety [14,15,17], and reduces the risk of cardiovascular disease [18–20]. Since RAL may have to be given indefinitely to prevent osteoporosis, RAL-exposed breast cancer will almost certainly occur. Overall, there is a large and growing population of women at risk for developing endocrine therapy-resistant breast cancer.

It is important to emphasize that the successful treatment of patients with one endocrine agent and then failure, leads to exhaustive endocrine therapy with the succession of agents, each with decreasing efficacy. The failure of TAM as a first-line therapy forms the basis of the use of AIs or fulvestrant (ICI 182,780, Faslodex<sup>®</sup>) as second-line therapies for the treatment of breast cancer.

In postmenopausal women, the aromatase enzyme converts androgens to estrogens in peripheral tissues such as adipose tissue and in the breast cancer tissue itself [21,22]. AIs block activity of this enzyme and fall into two classes, steroidal and non-steroidal [23–25]. Exemestane (Aromasin<sup>®</sup>) [26,27], a steroidal AI, irreversibly binds aromatase at the catalytic site and inactivates the enzyme. Anastrozole (Arimidex<sup>®</sup>) [28,29] and letrozole (Femara<sup>®</sup>) [30,31], non-steroidal AIs, bind aromatase at a different site, a heme group, to reversibly inhibit the enzyme. AIs have been evaluated in advanced breast cancer and in the adjuvant setting [26–31]. In the largest adjuvant trial, the ATAC trial (Arimidex, TAM, Alone or in Combination), patients in the anastrozole arm versus the TAM arm showed significantly longer disease-free survival, reduced contralateral breast cancer, and reduced distant metastases [32,33]. Indeed, AIs are now recommended and may replace TAM as the standard first-line antihormonal adjuvant therapy in postmenopausal ER-positive breast cancer patients. Further, due to the success of this and of other trials evaluating AIs for extended adjuvant therapy, AIs are also indicated after 5 years [34] and even 2 years of TAM [26,29].

FUL is an analogue of E<sub>2</sub> and the first in a new class of drugs that are complete antiestrogens, that is, they display no agonist activity via AF-1 or AF-2 of the ER [35,36]. FUL also leads to potent downregulation of ER protein expression because FUL binding to ER induces an abnormal conformation that results in accelerated ubiquitylation and shuttling of the ER to the proteasome for degradation [35,37]. Two large Phase III clinical trials have been conducted to evaluate FUL versus the AI anastrozole in postmenopausal advanced ER-positive breast cancer patients who have failed TAM. Both of these trials showed that FUL was equally effective as anastrozole in terms of time to progression and objective response rates [38,39]. Hence, FUL has been approved as a second-line therapy. FUL is also currently being evaluated in combination with AIs [36].

Over the past two decades, we have developed unique MCF-7 breast cancer xenograft models of long-term SERM (TAM and RAL) treatment and models of long-term estrogen withdrawal that could reasonably mimic resistance to AIs. These tumor models were developed *in vivo* and *in vitro*. The *in vivo* tumor models were designed to mimic the selection process needed over years to develop acquired resistance in the clinic by serially implanting MCF-7 tumors into SERM-treated and ovariectomized immunodeficient mice also over a period of years [40–50]. The *in vitro* tumor models were developed by culturing MCF-7 cells in estrogen-free conditions, with or without SERM treatment if appropriate, for over 1 year to develop antihormone resistance, and then injecting these cells into ovariectomized athymic mice treated with the SERM, if appropriate, and allowing tumors to grow [51–53]. We now have in hand a panel of breast cancer xenograft and tissue culture models that have allowed us to define the evolution of resistance to antihormonal therapy into at least two phases, each of which exhibits distinct growth responses to E<sub>2</sub> and FUL. We found that the growth of Phase I SERM-resistant tumors is stimulated by E<sub>2</sub>, while growth of Phase II SERM or estrogen withdrawn-resistant tumors is, paradoxically, inhibited by E<sub>2</sub> treatment. Previous studies conducted by our group have shown that E<sub>2</sub> not only inhibits growth of Phase II SERM and estrogen withdrawn-resistant tumors; it also induces apoptosis, leading to tumor regression. However, a fraction of these Phase II tumors eventually re-grow after E<sub>2</sub>-induced regression occurs, but these tumors are again re-sensitized to antihormonal therapy. We also found that while FUL does not support the growth of Phase I and II SERM-resistant tumors, it does allow growth of Phase II estrogen withdrawn-resistant tumors. Further, we found that while E<sub>2</sub> blocked growth of Phase II antihormone-resistant tumors, the combination of E<sub>2</sub> plus FUL resulted in robust growth. Phase II antihormonal resistance has not yet been widely recognized, but could be exploited by developing a novel third-line treatment plan based on short-term low-dose estrogen to debulk patients' tumors who fail exhaustive endocrine therapy, followed by the combination of FUL plus an AI to maintain low tumor burden and avoid a negative interaction between physiologic E<sub>2</sub> and FUL.

## 2. Materials and methods

### 2.1. Athymic mice, tumor inoculation, and tumor tracking

All procedures involving animals have been approved by the Fox Chase Cancer Center's Internal Animal Care and Use Committee.

All animal studies employed female ovariectomized athymic BALB/c nude (*nu/nu*) mice (Taconic, Hudson, NY, USA) that were inoculated with tumor cells at 5–6 weeks of age. For experiments employing tumor models which were generated and serially propagated as xenografts (*in vivo*), 1 mm<sup>3</sup> tumor sections were bilaterally transplanted using a trochar into the axillary mammary fat pads. For studies using tumor models which were generated and maintained in tissue culture (*in vitro*), cells were suspended in phosphate-buffered saline and bilaterally injected into axillary mammary fat pads at 10<sup>7</sup> cells per site.

Tumor growth was tracked by weekly measurements of tumor length (*l*) and width (*w*) using Vernier calipers, from which the tumor cross-sectional area was calculated using the equation:  $(l/2) \times (w/2) \times \pi$ . Tumor growth curves are expressed as the average cross-sectional tumor area per treatment group  $\pm$  standard error (SE).

### 2.2. Drug treatments

Mice were treated with estrogen by implanting a 0.3 cm E<sub>2</sub> silastic capsule subcutaneously into the intrascapular region on the back of the mouse at the time of tumor cell inoculation. The capsules were prepared by filling silicone tubing (0.078 inch inner diameter/ 0.125 inch

outer diameter; Fisher) 0.3 cm in length with a 1:3 (w/w) mixture of E<sub>2</sub> (Sigma-Aldrich, St. Louis, MO, USA) and silastic elastomer (Dow Corning, Midland, MI, USA), and then sealing the ends with silicone adhesive (Dow Corning) and sterilized by gamma irradiation. Athymic mice implanted with these capsules achieve mean serum levels of 83.8 pg/ml (308 pM) E<sub>2</sub> [54], which approximates perimenopausal E<sub>2</sub> levels in women. RAL and TAM were orally administered by gastric intubation at 1.5 mg/day 5 days per week. Evista tablets (Eli Lilly Pharmaceuticals, Indianapolis, IN, USA; purchased from the Fox Chase Cancer Center's pharmacy), the clinically available form of RAL (60 mg/tablet), were initially dissolved in water, and then suspended at 10 mg/ml in 10% polyethylene glycol 400/Tween 80 (99.5% polyethylene glycol 400, 0.5% Tween 80) and 0.9% carboxymethyl cellulose. TAM (Sigma) was initially dissolved in ethanol (EtOH), and then suspended at 10 mg/ml in 10% polyethylene glycol 400/Tween 80 (99.5% polyethylene glycol 400, 0.5% Tween 80) and 0.9% carboxymethyl cellulose. FUL was administered by *sc* injection in the scruff of the neck at a total of 10 mg/week. For the experiment depicted in Figure 1, four different FUL formulations and dosing schedules were used: FUL was initially dissolved in 1) EtOH or 2) dimethylsulfoxide (DMSO), and then made into a suspension with peanut oil at 50 mg/ml and administered as a 5 mg injection twice per week; 3) FUL was dissolved in only DMSO at 50 mg/ml and administered as a 2 mg injection 5 days per week; or 4) the clinical faslodex preparation, a 50 mg/ml proprietary solution of FUL in primarily EtOH supplemented with castor oil as a release rate modifier, was administered as 2 mg injections 5 days per week. For all other experiments, only the clinical Faslodex preparation was used and administered as 2 mg injections 5 days per week. FUL powder was a kind gift of AstraZeneca (Macclesfield, United Kingdom), and the clinical Faslodex preparation was purchased from the Fox Chase Cancer Center's pharmacy.

### 2.3. Generation of MCF-7/E2 xenograft tumors

The MCF-7/E2 xenograft tumor model, representing the antihormonal-sensitive stage of breast cancer, was originally developed by bilateral injection of 10<sup>7</sup> MCF-7 cells, grown in tissue culture, into the axillary mammary fat pads of female ovariectomized athymic BALB/c *nu/nu* mice implanted with a 0.3 cm E<sub>2</sub> capsule [40]. The resulting MCF-7/E2 tumors have been propagated *in vivo* by serial transplantation into likewise E<sub>2</sub>-treated ovariectomized athymic mice.

### 2.4. Generation of MCF-7/RAL1 xenograft tumors

The MCF-7/RAL1 (Phase I) SERM-resistant tumor model was derived by transplantation of MCF-7/E2 tumors into RAL-treated ovariectomized athymic mice. After extended RAL treatment, a small percentage of these tumors showed minimal but significant growth, and following re-transplantation into new RAL-treated ovariectomized athymic mice, these tumors then exhibited robust RAL-stimulated growth [50]. MCF-7/RAL1 tumors have been propagated *in vivo* for over 3 years by serial transplantation into RAL-treated ovariectomized athymic mice.

### 2.5. Generation of MCF-7/RAL2 xenograft tumors

The MCF-7/RAL2 (Phase II) SERM-resistant tumor model was developed *in vitro* by tissue culture of MCF-7 cells in estrogen-free medium supplemented with 1 μM RAL for over 1 year [51]. For every experiment involving MCF-7/RAL2 tumors, cells were grown in culture, and then bilaterally injected into the axillary mammary fat pads of ovariectomized athymic mice at 10<sup>7</sup> cells per site. MCF-7/RAL2 cells were maintained in culture in estrogen-free MEM plus 5% dextran-coated charcoal-treated calf serum (DCC-CS), 2 mM glutamine, 6 ng/ml bovine

insulin, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1× non-essential amino acids (all media components from Invitrogen, Carlsbad, CA, USA).

## 2.6. Generation of MCF-7/TAM2 xenograft tumors

The MCF-7/TAM2 (Phase II) SERM-resistant tumor model was developed in a similar manner as the MCF-7/RAL1 tumor model, by initial transplantation of MCF-7/E2 tumor pieces into TAM-treated ovariectomized athymic mice, and repeated transplantation of minimally growing tumors into new TAM-treated mice until robust TAM-stimulated growth occurred. These MCF-7/TAM tumors passed through different phases of SERM resistance; they were initially stimulated to grow by both TAM and E<sub>2</sub> [41,55], but have evolved over 5 years of serial propagation *in vivo* to a stage in which only TAM, but not E<sub>2</sub>, stimulates growth [47–49].

## 2.7 Generation of MCF-7 long-term estrogen-deprived cell culture models

MCF-7:ED cells were derived by maintaining a population of MCF-7 cells under estrogen-deprived conditions for >1 year to mimic AI treatment, and represent Phase I resistance to long-term estrogen withdrawal. MCF-7:ED cells were originally selected in phenol red-free MEM plus 5% dextran-coated charcoal-treated calf serum, but have more recently been maintained (this report) in phenol red-free RPMI, 10% dextran-coated charcoal-treated fetal bovine serum, 2 mM glutamine, 6 ng/ml bovine insulin, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1× non-essential amino acids.

MCF-7/5C cells were clonally isolated from a population of MCF-7 cells grown under long-term estrogen-free conditions [56] and represent Phase II LTED resistance. MCF-7/5C cells were originally cultured in estrogen-free MEM plus 5% dextran-coated charcoal-treated calf serum, and under these conditions, MCF-7/5C cells exhibited estrogen and SERM independent growth [52,56]. However, we have observed that when MCF-7/5C cells are switched to estrogen-free RPMI plus 10% dextran-coated charcoal-treated calf serum, the cells undergo rapid apoptosis when treated with 1 nM E<sub>2</sub> [52,53]. In all experiments described in this report, MCF-7/5C cells were maintained under the latter media conditions (estrogen-free RPMI plus 10% dextran-coated charcoal-treated calf serum, 2 mM glutamine, 6 ng/ml bovine insulin, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1× non-essential amino acids).

## 2.8 Generation of MCF-7/5C estrogen-deprived xenograft tumors

The MCF-7/5C (Phase II) long-term estrogen withdrawn-resistant tumors were generated by bilateral injection of these cells grown in culture into the axillary mammary fat pads of ovariectomized athymic mice at 10<sup>7</sup> cells per site.

## 2.9 Cell proliferation assays

Wild-type MCF-7 cells were switched from phenol red-containing RPMI medium supplemented with 10% FBS to phenol red-free RPMI medium supplemented with 10% dextran-coated charcoal-treated fetal bovine serum for 4 days prior to beginning the proliferation assay. Since MCF-7/ED and MCF-7/5C cells are routinely maintained in estrogen-free media, no media switch was required. MCF-7, MCF-7/ED, and MCF-7/5C cells were seeded in estrogen-free RPMI containing 10% DCC-FBS at a density of 2×10<sup>4</sup> cells per well in 24-well plates. After 24 hours (Day 0), the medium was replaced with fresh estrogen-free RPMI medium and cells were treated with 0.1% ethanol vehicle (control), 1 nM E<sub>2</sub>, 10 nM FUL, or 1 nM E<sub>2</sub> + 10 nM FUL. Cells were retreated with the drugs on days 2, 4, and 6 and the experiment was stopped on day 7. The DNA content of the cells, a measure of proliferation, was determined using a Fluorescent DNA Quantitation kit (Bio-Rad

Laboratories, Hercules, CA). For each analysis, six replicate wells were used, and at least three independent experiments were performed. Proliferation of cell lines following 7 day of growth are shown as the mean DNA amount per well per treatment  $\pm$  standard deviation (SD).

## 2.10 Statistical analyses

In tumor growth experiments in which treatments were started at the time of tumor inoculation, tumors were analyzed longitudinally with two-factor analysis of variance (ANOVA) to determine significant differences in cross-sectional areas between all tumors in each treatment group in a time-dependent manner (data in Figures 1 through 4, and 6 through 7). In the tumor growth experiments in which treatments were started after the tumors were established, one-factor ANOVA was used to determine significant differences in cross-sectional areas between all tumors in each treatment group on the last day of the experiment (data in Fig. 8, day 52). All statistical tests were two-sided and calculated using SAS (SAS Institute, Cary, NC, USA).

## 3. Results

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

MCF-7/E2 xenograft tumors are propagated *in vivo* by serial transplantation into 0.3 cm E<sub>2</sub> capsule-implanted ovariectomized athymic mice. To explore the sensitivity of MCF-7/E2 tumors to FUL, MCF-7/E2 tumor cores were implanted into 30 ovariectomized athymic mice and separated into 6 groups of 5 mice each, or 10 tumors per group. The treatment groups were control (no treatment), 0.3 cm E<sub>2</sub> capsule *sc*, or 0.3 cm E<sub>2</sub> capsule *sc* plus 1 of 4 different formulations and dosing schedules of FUL totaling 10 mg/week *sc* (Fig. 1). Two of the FUL formulations were suspensions made with peanut oil, differing by whether FUL was initially dissolved in EtOH or in DMSO. These FUL (EtOH/peanut oil or DMSO/peanut oil) suspensions were administered as 5 mg *sc* injections given two days per week, totaling 10 mg/week. The third formulation was FUL dissolved in only DMSO, and was administered as a 2 mg *sc* injection given five days per week, totaling 10 mg/week. The fourth FUL formulation corresponded to the clinical Faslodex preparation, which is a proprietary solution of primarily EtOH and some castor oil as a slow release-rate modifier. The clinical Faslodex preparation was administered as a 2 mg *sc* injection given 5 times per week, totaling 10 mg/week.

The MCF-7/E2 tumors grew robustly when treated with the 0.3 cm E<sub>2</sub> capsule, but did not grow in the control group (Fig. 1, E<sub>2</sub> vs. Control,  $P < 0.0001$ ), demonstrating that these tumors were dependent on E<sub>2</sub>. The implanted capsules produce E<sub>2</sub> levels that are in the physiologic range observed in perimenopausal women. The cross-sectional areas of each of the four groups of MCF-7/E2 tumors treated with E<sub>2</sub> plus FUL was significantly smaller than those tumors treated with E<sub>2</sub> alone (Fig. 1, all  $P$ -values  $< 0.0001$ ). Therefore, FUL inhibited E<sub>2</sub>-stimulated growth of MCF-7/E2 tumors. However, the degree of growth inhibition varied depending upon the formulation. Comparing the FUL suspensions in peanut oil given 2 times per week, FUL initially dissolved in DMSO inhibited tumor growth significantly better than FUL initially dissolved in EtOH (Fig. 1,  $P = 0.001$ ). Comparing the FUL formulations given 5 times per week, FUL dissolved in only DMSO inhibited tumor growth significantly better than the clinical Faslodex preparation (Fig. 1,  $P = 0.004$ ). Although we did not measure circulating FUL levels, we hypothesize that circulating levels of FUL were higher when using DMSO-based formulations, leading to more potent inhibition of E<sub>2</sub>-stimulated tumor growth.

### 3.2 Growth of MCF-7/RAL1 tumors

MCF-7/RAL1 tumors are maintained *in vivo* by serial transplantation into 1.5 mg/day RAL-treated ovariectomized athymic mice. To illustrate the phase of SERM resistance the MCF-7/

RAL1 tumor should be categorized into, MCF-7/RAL1 tumor cores were implanted into 20 ovariectomized athymic mice and separated into 4 treatment groups of 5 mice each (10 tumors/group) corresponding 1.5 mg/day RAL *po*, 0.3 cm E<sub>2</sub> capsule *sc*, 2 mg/day FUL *sc* (Faslodex preparation), and control (no treatment). The MCF-7/RAL1 tumors were significantly stimulated to grow by RAL treatment ( $P < 0.0001$ ) and by E<sub>2</sub> treatment ( $P < 0.0001$ ) compared to control treatment (Fig. 2). However, a modest amount of growth was observed in the control-treated group, indicating that these tumors are not absolutely dependent upon an ER ligand with partial agonist activity. We have previously shown that primary cultures of MCF-7/RAL1 tumors exhibit equivalent levels of estrogen response element (ERE)-regulated reporter gene activity in the absence of E<sub>2</sub> as did primary cultures of MCF-7/E2 tumors when treated with E<sub>2</sub> [50]. Thus, the unliganded ER activity in MCF-7/RAL1 tumors is high and probably contributed to the modest growth of these tumors without the need of RAL or E<sub>2</sub>. FUL did not significantly effect the growth of MCF-7/RAL1 tumors (Fig. 2). Thus, either a SERM or E<sub>2</sub>, but not FUL, supports the growth of these MCF-7/RAL1 xenografts. Therefore, these tumors are categorized as Phase I SERM-resistant.

### 3.3 Growth of MCF-7/RAL2 tumors (Fig. 3)

MCF-7/RAL2 tumor cells are maintained *in vitro* by culture in media containing 1  $\mu$ M RAL. To study the growth properties of MCF-7/RAL2 cells *in vivo*, the cells were grown in culture and injected into 20 ovariectomized athymic mice, which were separated into 4 groups of 5 (10 tumors/group) and treated with 1.5 mg/day RAL *po*, 0.3 cm E<sub>2</sub> capsule *sc*, 2 mg/day FUL *sc* (Faslodex preparation), or control (not treated). The MCF-7/RAL2 tumors only grew when treated with RAL (RAL vs. control,  $P < 0.0001$ ), and did not form any palpable tumors by day 42 when treated with E<sub>2</sub>, FUL or not treated (control) (Fig. 3). We have previously shown that when MCF-7/RAL2 tumors are allowed to grow by treating with TAM until they are established and then switching treatments to E<sub>2</sub>, E<sub>2</sub> causes tumor regression by inducing apoptosis as measured by TUNEL staining [51]. Therefore, growth of the MCF-7/RAL2 tumors was dependent on RAL, but inhibited by E<sub>2</sub> and FUL, which categorizes these tumors as Phase II SERM-resistant.

### 3.4 Growth of MCF-7/TAM2 tumors

MCF-7/TAM2 tumors are propagated *in vivo* by serial transplantation into 1.5 mg/day TAM-treated ovariectomized athymic mice. To characterize the growth properties of this tumor type, MCF-7/TAM2 tumor cores were implanted into 20 ovariectomized athymic mice, which were separated into 4 groups of 5 (10 tumors/group) and treated with 1.5 mg/day TAM *po*, 0.3 cm E<sub>2</sub> capsule *sc*, 2 mg/day FUL (Faslodex) *sc*, or not treated (control). MCF-7/TAM2 tumors were stimulated to grow by TAM compared to the control group (Fig. 4,  $P < 0.0001$ ). The control group did show a minimal amount of growth (Fig. 4), which is hypothesized to be due to substantial unliganded ER activity as in the MCF-7/RAL1 model. FUL did not significantly effect growth of the MCF-7/TAM2 tumors versus control treatment. Interestingly, E<sub>2</sub> did significantly inhibit tumor growth compared to the control group (Fig. 4,  $P = 0.0004$ ). As with the MCF-7/RAL2 tumors, we have previously demonstrated that E<sub>2</sub> treatment leads to regression of MCF-7/TAM2 tumors [47,49] by inducing apoptosis as detected by TUNEL staining [48]. Therefore, TAM stimulated growth, FUL did not support growth, and E<sub>2</sub> inhibited growth of MCF-7/TAM2 tumors, defining this model as Phase II SERM-resistant.

### 3.5 Growth of long-term estrogen withdrawn-resistant models

Since having categorized each of the SERM-resistant tumor models as Phase I or II resistant, we characterized the growth properties of cells which have been cultured long-term under estrogen-free conditions to determine whether resistance to estrogen withdrawal (as a surrogate

for AI resistance) also evolves through distinct stages. Initially, we compared the proliferation of parental MCF-7 cells with two cell lines resistant to long-term estrogen withdrawal, MCF-7/ED (estrogen-deprived) and MCF-7/5C cells. MCF-7/ED cells were originally selected by culture of parental MCF-7 in estrogen-free medium for > 1 year, but were not cloned as a subline, rather they remain a population of cells. In a similar manner, MCF-7/5C cells were also derived from parental MCF-7 cells following long-term estrogen withdrawal, but were cloned as a subline [56]. Notably, MCF-7/ED and MCF-7/5C cells were generated independently in different studies, that is, MCF-7/5C cells were not subcloned from the MCF-7/ED cells.

Growth of parental MCF-7, MCF-7/ED and MCF-7/5C cells was determined by measuring DNA amounts after 7 days in culture. Before beginning the experiment, parental MCF-7 cells were cultured for 4 days in estrogen-free media, since they had been maintained in fully-estrogenized medium. The experiment was started by seeding each of the cell lines in 24-well plates in estrogen-free medium. The cells were treated every 2 days with EtOH (vehicle control), 1 nM E<sub>2</sub>, 10 nM FUL, and 1 nM E<sub>2</sub> plus 10 nM FUL. After 7 days, DNA quantities per well were determined using a fluorescence-based DNA assay. As expected in parental MCF-7 cells, E<sub>2</sub> induced growth by 6.9-fold (E<sub>2</sub> vs. control treatment), and this E<sub>2</sub>-stimulated proliferation was completely blocked by the addition of FUL (E<sub>2</sub> + FUL vs. control) (Fig. 5). Hence, E<sub>2</sub> stimulated proliferation of parental MCF-7 cells in an ER-dependent manner.

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

Finally, we evaluated MCF-7/5C cells, which were a clonal derivative of long-term estrogen-withdrawn cells. MCF-7/5C cells grew maximally under estrogen-free conditions (control treatment, 100% growth), but E<sub>2</sub> treatment almost completely blocked proliferation (9% of control) (Fig. 5). Interestingly, FUL-treated MCF-7/5C cells exhibited significant growth (52% of control) (Fig. 5). Further, MCF-7/5C cells treated with E<sub>2</sub> plus FUL showed still greater amounts of proliferation (85% of control) (Fig. 5). In prior studies, we have demonstrated that MCF-7/5C cells undergo apoptosis when treated with E<sub>2</sub>, and that co-treatment with FUL blocks this effect of E<sub>2</sub> [53]. Hence, MCF-7/5C cells required ER to be unliganded for maximal proliferation and survival, whereas E<sub>2</sub>-bound ER led to cytostasis and apoptosis. Further, FUL reversed the apoptotic signal of E<sub>2</sub> and promoted proliferation.

We next verified that the MCF-7/5C cells behaved similarly *in vivo* as a xenograft tumor as they did *in vitro* in cell culture. MCF-7/5C cells were grown in culture and injected into 20 ovariectomized athymic mice. The animals were separated into 4 treatment groups of 5 mice each (10 tumors/group), corresponding to control (not treated), 0.3 cm E<sub>2</sub> capsule *sc*, 2 mg/day FUL *sc* (Faslodex), and 0.3 cm E<sub>2</sub> capsule *sc* plus 2 mg/day FUL *sc* (Faslodex). MCF-7/5C cells rapidly formed substantial tumors at every injection site (10 out of 10) in control-treated mice by 21 days after inoculation, but only 1 palpable tumor formed out of 10 injection sites in mice treated with E<sub>2</sub>, resulting in a highly significant difference in the average tumor cross-sectional area between the two treatment groups (Fig. 6, *P* < 0.0001). In a prior report, we have shown that E<sub>2</sub> induces tumor regression and apoptosis in established MCF-7/5C xenograft tumors [53]. Importantly, MCF-7/5C xenograft tumors showed robust growth in the presence of FUL or E<sub>2</sub> plus FUL, which was not significantly different than growth of the control (no

treatment) group, but was significantly greater than in the E<sub>2</sub> treatment group (Fig. 6, FUL vs. E<sub>2</sub>,  $P < 0.0001$ ; E<sub>2</sub>+FUL vs. E<sub>2</sub>,  $P < 0.0001$ ). Hence, the MCF-7/5C xenograft tumor model was resistant to growth inhibition by FUL, and FUL treatment abrogated E<sub>2</sub>-mediated growth inhibition.

Considering these varied growth responses together, parental MCF-7 cells model the therapeutic stage of antihormonal therapy; MCF-7/ED cells represent Phase I resistance to estrogen withdrawal since they grew independent of E<sub>2</sub> yet remained sensitive to FUL; and MCF-7/5C tumors/cells were classified as Phase II resistant to estrogen withdrawal since E<sub>2</sub> inhibited their growth, but were resistant to growth inhibition by FUL or E<sub>2</sub> plus FUL.

### 3.6 Response of Phase II SERM-resistant tumor models to E<sub>2</sub> plus FUL (Fig. 7 and 8)

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

We then tested whether this interaction between physiologic E<sub>2</sub> and FUL also occurred in the MCF-7/TAM2 tumor model of Phase II SERM resistance. However, this experiment was designed to evaluate effects of different treatments on tumors once they are established by allowing tumors to grow in the presence of TAM until they were palpable, and then randomized to different treatment groups. MCF-7/TAM2 tumor cores were implanted into 25 ovariectomized athymic mice. All animals were treated with 1.5 mg/day TAM *po* until tumors grew to an average cross-sectional area of 0.24 cm<sup>2</sup>, at which time TAM treatment was withdrawn for 1 week to allow time for this drug to be completely metabolized and clear the animals' systems. Following the 1 week of TAM withdrawal, the average cross-sectional area of all tumors was 0.37 cm<sup>2</sup>, and the animals were randomized into 5 groups of 5 mice each (10 tumors/group) corresponding to continuing 1.5 mg/day TAM *po*, 0.3 cm E<sub>2</sub> capsule *sc*, 2 mg/day FUL *sc*, 0.3 cm E<sub>2</sub> capsule *sc* plus 2 mg/day FUL *sc*, and control (no treatment). As would be predicted from the MCF-7/TAM2 experiment depicted in Figure 4, TAM treatment significantly stimulated growth ( $P = 0.0026$ ) and E<sub>2</sub> significantly inhibited growth ( $P = 0.0098$ ) compared to the control group on day 52 (Fig. 8). The size of FUL treated tumors was not significantly different than the control group. In contrast, we noted that tumors treated with the combination of E<sub>2</sub> + FUL did exhibit significantly greater growth than the control group (Fig. 8,  $P = 0.018$ ). Thus, in a second model of Phase II SERM resistance, growth inhibition by E<sub>2</sub> alone was negated in the presence of FUL, leading to growth stimulation.

## 4. Discussion

We sought to discover unifying principles involved in the development of antihormone resistance by systematically studying the growth properties of a panel of antihormonally resistant MCF-7-based breast cancer xenograft tumor models. We have confirmed and extended prior observations [40–53] that have allowed the categorization of these tumor models as either Phase I or Phase II antihormone resistant. Phase I SERM resistance was characterized by growth stimulation in response to either a SERM or E<sub>2</sub> (MCF-7/RAL1 tumors, Figure 2), while in Phase II SERM resistance, only the SERM stimulated growth and E<sub>2</sub> inhibited growth (MCF-7/RAL2 and MCF-7/TAM2, Fig. 3 and 4, respectively). Phase I long-term estrogen withdrawn (AI) -resistant cells in culture grew independently of E<sub>2</sub> (MCF-7/ED cells, Figure

5), but Phase II resistant tumors were growth inhibited by E<sub>2</sub> (MCF-7/5C tumors, Figure 6). Hence, long-term blockade of ER activity by either SERMs or estrogen withdrawal can lead to selection of cells in which E<sub>2</sub> signals no longer proliferation, but rather inhibition, of growth, and as we have previously reported, apoptosis [48,51,53].

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

Noteworthy, the concentrations of FUL used in the cell culture proliferation experiments was 10 nM. We chose this concentration of FUL to reflect the circulating levels that are achieved clinically. In the clinic, FUL is not administered orally because of low bioavailability; rather it is given intra muscularly (as a single 250 mg dose once per month) to achieve slow constant release of the drug. In two independent multi-national Phase III clinical efficacy trials evaluating FUL in advanced breast cancer patients, the steady state circulating concentrations of FUL were determined to be approximately 6 – 7 µg/L (9.9 – 11.5 nM) in the European trial and 9 µg/L (14.8 nM) in the North American trial [57]. Hence, we used FUL at 10 nM in cell culture, reflecting the circulating concentrations of FUL achieved in women, but this concentration was much lower than the 100 nM to 1 µM FUL concentrations used in most cell culture studies. We hypothesize that the low circulating concentrations of FUL in patients may contribute to the lower than expected response rates in the clinic as would be predicted by the effectiveness of FUL in cell culture. In support of this hypothesis, we have found that while MCF-7/5C cells proliferate in the presence of 10 nM FUL, they do not in 1 µM FUL (unpublished, JS Lewis-Wambi and VC Jordan).

The distinct growth responses of the tumor and cell culture models studied here illustrate that resistance to hormonal blockade therapy continually evolves but can be separated into at least two phases (Fig. 9). Antihormonal resistance develops from selection of specific cell types that survive and proliferate when the ER is bound by a partial antiestrogen (Phase I SERM resistance) or unliganded (Phase I estrogen withdrawn-resistance). Prolonged hormonal blockade therapy maintains selective pressure, such that Phase I resistant cells continue to evolve to a Phase II resistant phenotype, and likely undefined additional phases. However, the study of Phase II antihormonal resistance has revealed a new biology of E<sub>2</sub> action involved in apoptosis that could be exploited to benefit breast cancer patients who have been exhaustively treated with SERMs and AIs. Moreover, the finding that FUL in a background of physiologic E<sub>2</sub> stimulated growth of Phase II resistant cells has important clinical implications. This knowledge can be implemented to optimize the application of third-line antihormonal therapy (Fig. 10). We propose that patients who have responded and then failed two antihormone therapies may exhibit Phase II resistant characteristics, and therefore respond to low-dose short-term estrogen therapy. The estrogen therapy would lead to apoptosis in the Phase II resistant cells and thereby debulk the tumor. Prior laboratory studies indicate that cells which remain are re-sensitized to first-line or second-line antihormonal therapy [47,49]. Hence, the low-dose short-term estrogen therapy would be followed by FUL plus an AI, to avoid the possible selection of cells that could grow in response to FUL plus physiologic E<sub>2</sub>.

#### Acknowledgements

This work is supported by the Department of Defense Breast Program under award number BC050277 Center of Excellence (V.C.J.) (Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army

or the Department of Defense), SPORE in Breast Cancer CA89018 (V.C.J.), R01 GM067156 (V.C.J.), the Avon Foundation (V.C.J.), the Weg Fund (Fox Chase Cancer Center), and NIH P30 CA006927 (Fox Chase Cancer Center).

## References

- Jensen EV, Block GE, Smith S, Kyser K, DeSombre ER. Estrogen receptors and breast cancer response to adrenalectomy. *Natl Cancer Inst Monogr* 1971;34:55–70. [PubMed: 5140877]
- McGuire, WL.; Carbone, PP.; Vollmer, EP. Oestrogen receptors in human breast cancer: An overview. In: McGuire, WL.; Carbone, PP.; Vollmer, EP., editors. *Oestrogen receptors in human breast cancer*. 6. Raven Press; New York: 1975.
- Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth cain memorial award lecture. *Cancer Res* 1990;50:4177–4189. [PubMed: 2194650]
- Jordan VC. Fourteenth gaddum memorial lecture. A current view of tamoxifen for the treatment and prevention of breast cancer. *Br J Pharmacol* 1993;110:507–517. [PubMed: 8242225]
- 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. [PubMed: 15894097]
- Jensen EV, Jordan VC. The estrogen receptor: A model for molecular medicine. *Clin Cancer Res* 2003;9:1980–1989. [PubMed: 12796359]
- Ariazi EA, Ariazi JL, Cordera F, Jordan VC. Estrogen receptors as therapeutic targets in breast cancer. *Curr Top Med Chem* 2006;6:181–202. [PubMed: 16515478]
- Strasser-Weippl K, Goss PE. Advances in adjuvant hormonal therapy for postmenopausal women. *J Clin Oncol* 2005;23:1751–1759. [PubMed: 15755983]
- 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. [PubMed: 15894097]
- Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: An overview of the randomised trials. *Lancet* 1998;351:1451–1467. [PubMed: 9605801]
- Fisher B, Dignam J, Bryant J, Wolmark N. Five versus more than five years of tamoxifen for lymph node-negative breast cancer: Updated findings from the national surgical adjuvant breast and bowel project b-14 randomized trial. *J Natl Cancer Inst* 2001;93:684–690. [PubMed: 11333290]
- Smith I. Breast cancer: Adjuvant treatment for early breast cancer. *Ann Oncol* 2005;16:ii182–187. [PubMed: 15958454]
- Cuzick J, Powles T, Veronesi U, Forbes J, Edwards R, Ashley S, Boyle P. Overview of the main outcomes in breast-cancer prevention trials. *Lancet* 2003;361:296–300. [PubMed: 12559863]
- 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. The effect of raloxifene on risk of breast cancer in postmenopausal women: Results from the more randomized trial. *JAMA* 1999;281:2189–2197. [PubMed: 10376571]
- Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, Secrest RJ, Cummings SR. Continuing outcomes relevant to evista: Breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J Natl Cancer Inst* 2004;96:1751–1761. [PubMed: 15572757]
- Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER Jr, Wade JL 3rd, Robidoux A, Margolese RG, James J, Lippman SM, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N. 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;295:2727–2741. [PubMed: 16754727]
- Cranney A, Tugwell P, Zytaruk N, Robinson V, Weaver B, Adachi J, Wells G, Shea B, Guyatt G Iv. Meta-analysis of raloxifene for the prevention and treatment of postmenopausal osteoporosis. *Endocr Rev* 2002;23:524–528. [PubMed: 12202467]

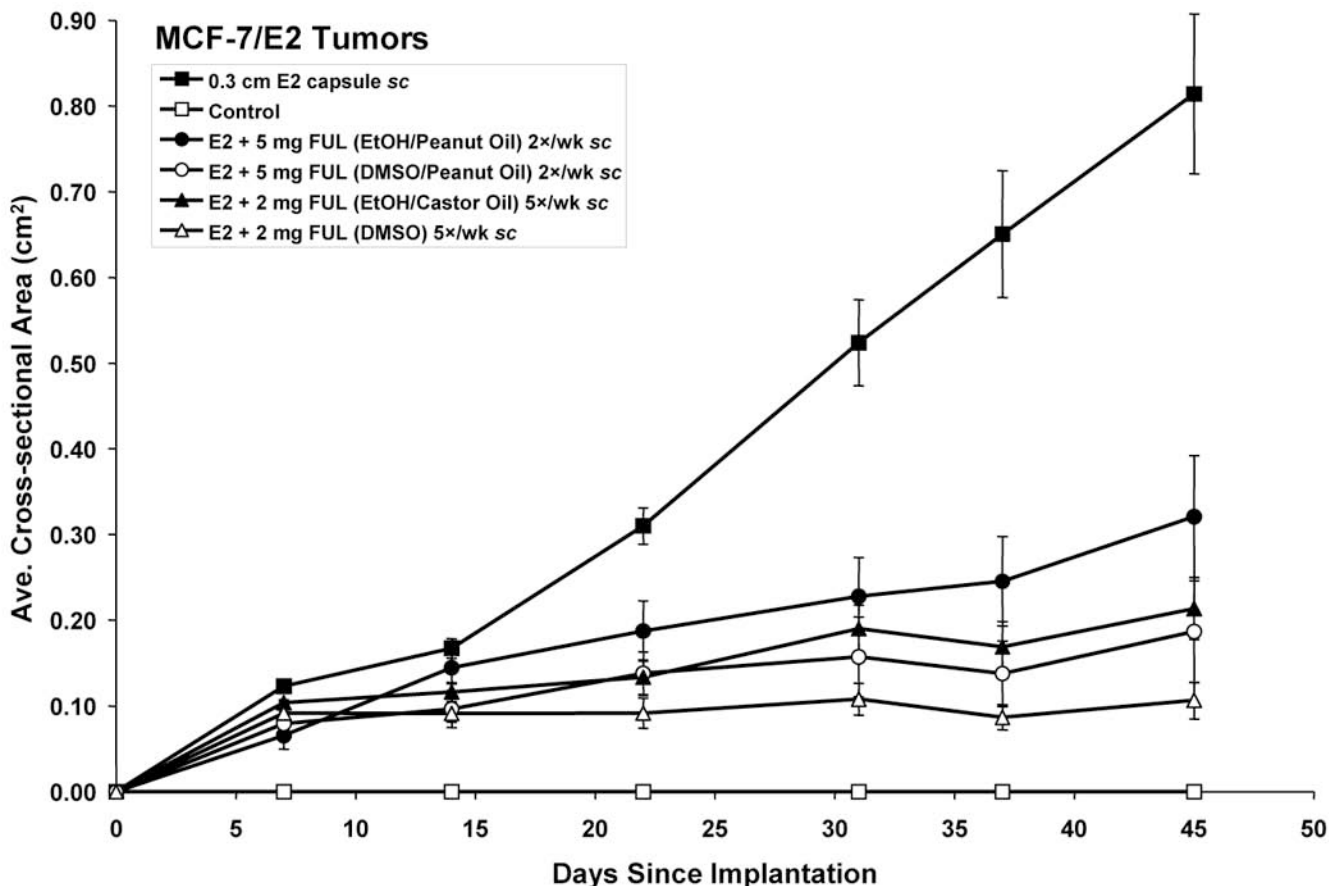
18. Barrett-Connor E, Cox DA, Anderson PW. The potential of serms for reducing the risk of coronary heart disease. *Trends in Endocrinology and Metabolism* 1999;10:320–325. [PubMed: 10481163]
19. Wenger NK, Barrett-Connor E, Collins P, Grady D, Kornitzer M, Mosca L, Sashegyi A, Baygani SK, Anderson PW, Moscarelli E. Baseline characteristics of participants in the raloxifene use for the heart (RUTH) trial. *The American Journal of Cardiology* 2002;90:1204–1210. [PubMed: 12450599]
20. Mosca L, Barrett-Connor E, Wenger NK, Collins P, Grady D, Kornitzer M, Moscarelli E, Paul S, Wright TJ, Helterbrand JD, Anderson PW. Design and methods of the raloxifene use for the heart (RUTH) study. *The American Journal of Cardiology* 2001;88:392–395. [PubMed: 11545760]
21. Evans CT, Ledesma DB, Schulz TZ, Simpson ER, Mendelson CR. Isolation and characterization of a complementary DNA specific for human aromatase-system cytochrome p-450 mRNA. *Proc Natl Acad Sci U S A* 1986;83:6387–6391. [PubMed: 3018730]
22. Nelson LR, Bulun SE. Estrogen production and action. *Journal of the American Academy of Dermatology* 2001;45:S116–S124. [PubMed: 11511861]
23. Smith IE, Dowsett M. Aromatase inhibitors in breast cancer. *N Engl J Med* 2003;348:2431–2442. [PubMed: 12802030]
24. Joensuu H, Ejlertsen B, Lonning PE, Rutqvist LE. Aromatase inhibitors in the treatment of early and advanced breast cancer. *Acta Oncol* 2005;44:23–31. [PubMed: 15848903]
25. Geisler J, Lonning PE. Aromatase inhibition: Translation into a successful therapeutic approach. *Clin Cancer Res* 2005;11:2809–2821. [PubMed: 15837728]
26. Coombes RC, Hall E, Gibson LJ, Paridaens R, Jassem J, Delozier T, Jones SE, Alvarez I, Bertelli G, Ortmann O, Coates AS, Bajetta E, Dodwell D, Coleman RE, Fallowfield LJ, Mickiewicz E, Andersen J, Lonning PE, Cocconi G, Stewart A, Stuart N, Snowdon CF, Carpentieri M, Massimini G, Bliss JM. the Intergroup Exemestane Study. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. *N Engl J Med* 2004;350:1081–1092. [PubMed: 15014181]
27. Mouridsen HT. Exemestane following tamoxifen in postmenopausal women with primary breast cancer. *J Clin Oncol* 2004;22:3833–3834. [PubMed: 15365083]
28. Bonnetterre J, Thurlimann B, Robertson JFR, Krzakowski M, Mauriac L, Koralewski P, Vergote I, Webster A, Steinberg M, von Euler M. Anastrozole versus tamoxifen as first-line therapy for advanced breast cancer in 668 postmenopausal women: Results of the tamoxifen or arimidex randomized group efficacy and tolerability study. *J Clin Oncol* 2000;18:3748–3757. [PubMed: 11078487]
29. Boccardo F, Rubagotti A, Puntoni M, Guglielmini P, Amoroso D, Fini A, Paladini G, Mesiti M, Romeo D, Rinaldini M, Scali S, Porpiglia M, Benedetto C, Restuccia N, Buzzi F, Franchi R, Massidda B, Distante V, Amadori D, Sismondi P. Switching to anastrozole versus continued tamoxifen treatment of early breast cancer: Preliminary results of the Italian tamoxifen anastrozole trial. *J Clin Oncol* 2005;23:5138–5147. [PubMed: 16009955]
30. Mouridsen H, Gershanovich M, Sun Y, Perez-Carrion R, Boni C, Monnier A, Apffelstaedt J, Smith R, Sleeboom HP, Jaenicke F, Pluzanska A, Dank M, Becquart D, Bapsy PP, Salminen E, Snyder R, Chaudri-Ross H, Lang R, Wyld P, Bhatnagar A. Phase iii study of letrozole versus tamoxifen as first-line therapy of advanced breast cancer in postmenopausal women: Analysis of survival and update of efficacy from the international letrozole breast cancer group. *J Clin Oncol* 2003;21:2101–2109. [PubMed: 12775735]
31. Mouridsen H, Gershanovich M, Sun Y, Perez-Carrion R, Boni C, Monnier A, Apffelstaedt J, Smith R, Sleeboom HP, Janicke F, Pluzanska A, Dank M, Becquart D, Bapsy PP, Salminen E, Snyder R, Lassus M, Verbeek JA, Staffler B, Chaudri-Ross HA, Dugan M. Superior efficacy of letrozole versus tamoxifen as first-line therapy for postmenopausal women with advanced breast cancer: Results of a phase III study of the international letrozole breast cancer group. *J Clin Oncol* 2001;19:2596–2606. [PubMed: 11352951]
32. Baum M, Budzar AU, Cuzick J, Forbes J, Houghton JH, Klijn JG, Sahmoud T. 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;359:2131–2139. [PubMed: 12090977]

33. Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, Forbes JF, Hochtin-Boes G, Houghton J, Locker GY, Tobias JS. Results of the ATAC (arimidex, tamoxifen, alone or in combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* 2005;365:60–62. [PubMed: 15639680]
34. Goss PE, Ingle JN, Martino S, Robert NJ, Muss HB, Piccart MJ, Castiglione M, Tu D, Shepherd LE, Pritchard KI, Livingston RB, Davidson NE, Norton L, Perez EA, Abrams JS, Cameron DA, Palmer MJ, Pater JL. 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* 2005;97:1262–1271. [PubMed: 16145047]
35. Carlson RW. The history and mechanism of action of fulvestrant. *Clin Breast Cancer* 6 Suppl 2005;1:S5–8.
36. Gradishar WJ, Sahmoud T. Current and future perspectives on fulvestrant. *Clin Breast Cancer* 2005;6 (Suppl 1):S23–29. [PubMed: 15865847]
37. Pike AC, Brzozowski AM, Walton J, Hubbard RE, Thorsell AG, Li YL, Gustafsson JA, Carlquist M. Structural insights into the mode of action of a pure antiestrogen. *Structure (Camb)* 2001;9:145–153. [PubMed: 11250199]
38. Howell A, Robertson JFR, Quaresma Albano J, Aschermannova A, Mauriac L, Kleeberg UR, Vergote I, Erikstein B, Webster A, Morris C. 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* 2002;20:3396–3403. [PubMed: 12177099]
39. Osborne CK, Pippen J, Jones SE, Parker LM, Ellis M, Come S, Gertler SZ, May JT, Burton G, Dimery I, Webster A, Morris C, Elledge R, Buzdar A. 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* 2002;20:3386–3395. [PubMed: 12177098]
40. Gottardis MM, Robinson SP, Jordan VC. Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: A model to study the tumoristatic action of tamoxifen. *J Steroid Biochem* 1988;30:311–314. [PubMed: 3386259]
41. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 1988;48:5183–5187. [PubMed: 3409244]
42. Gottardis MM, Jiang SY, Jeng MH, 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. [PubMed: 2743303]
43. Wolf DM, 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. [PubMed: 8502820]
44. Wolf DM, Jordan VC, William L. Mcguire Memorial Symposium. Drug resistance to tamoxifen during breast cancer therapy. *Breast Cancer Res Treat* 1993;27:27–40. [PubMed: 7505127]
45. Wolf DM, Jordan VC. Characterization of tamoxifen stimulated MCF-7 tumor variants grown in athymic mice. *Breast Cancer Res Treat* 1994;31:117–127. [PubMed: 7981452]
46. Wolf DM, Langan-Fahey SM, Parker CJ, McCague R, Jordan VC. Investigation of the mechanism of tamoxifen-stimulated breast tumor growth with nonisomerizable analogues of tamoxifen and metabolites. *J Natl Cancer Inst* 1993;85:806–812. [PubMed: 8487325]
47. Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, Jordan VC. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;6:2028–2036. [PubMed: 10815929]
48. 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;95:1597–1608. [PubMed: 14600092]
49. Osipo C, Gajdos C, Cheng D, Jordan VC. Reversal of tamoxifen resistant breast cancer by low dose estrogen therapy. *J Steroid Biochem Mol Biol* 2005;93:249–256. [PubMed: 15860267]

50. O'Regan RM, Osipo C, Ariazi E, Lee ES, Meeke K, Morris C, Bertucci A, Sarker MA, Grigg R, Jordan VC. Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. *Clin Cancer Res* 2006;12:2255–2263. [PubMed: 16609042]
51. Liu H, Lee E-S, Gajdos C, Pearce ST, Chen B, Osipo C, Loweth J, McKian K, De Los Reyes A, Wing L, Jordan VC. Apoptotic action of 17{beta}-estradiol in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*. *J Natl Cancer Inst* 2003;95:1586–1597. [PubMed: 14600091]
52. 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;94:131–141. [PubMed: 15862958]
53. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, Jordan VC. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 2005;97:1746–1759. [PubMed: 16333030]
54. O'Regan RM, Cisneros A, England GM, MacGregor JI, Muenzner HD, Assikis VJ, Bilimoria MM, Piette M, Dragan YP, Pitot HC, Chatterton R, Jordan VC. Effects of the antiestrogens tamoxifen, toremifene, and ICI 182,780 on endometrial cancer growth. *J Natl Cancer Inst* 1998;90:1552–1558. [PubMed: 9790548]
55. 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. [PubMed: 2758410]
56. 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. [PubMed: 1301400]
57. Robertson JF, Erikstein B, Osborne KC, Pippen J, Come SE, Parker LM, Gertler S, Harrison MP, Clarke DA. Pharmacokinetic profile of intramuscular fulvestrant in advanced breast cancer. *Clin Pharmacokinet* 2004;43:529–538. [PubMed: 15170367]

## Abbreviations

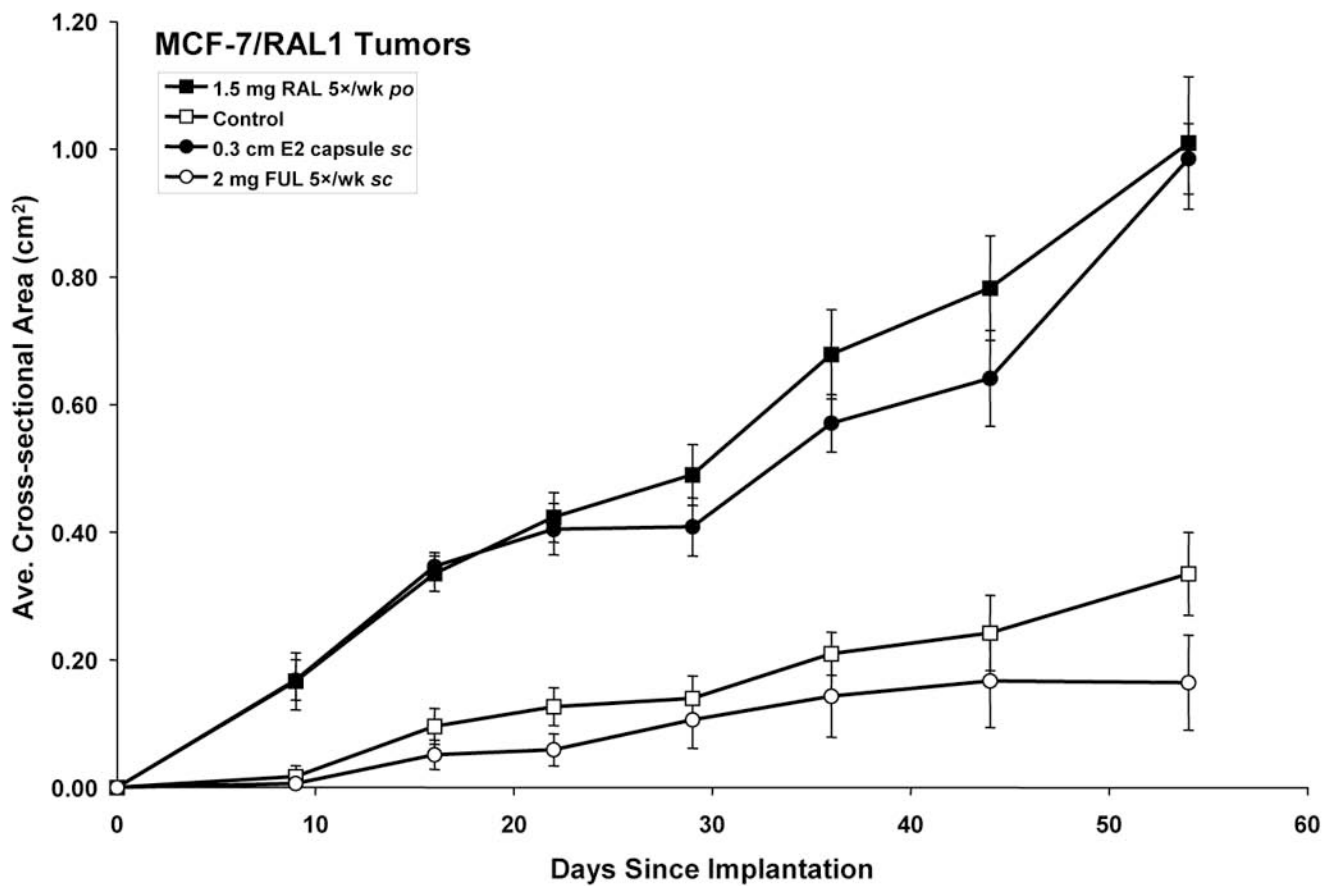
<b>AI</b>	aromatase inhibitor
<b>ER</b>	estrogen receptor
<b>E<sub>2</sub></b>	17β -estradiol
<b>FUL</b>	fulvestrant
<b>RAL</b>	raloxifene
<b>SERM</b>	selective estrogen receptor modulator



**Fig 1.**

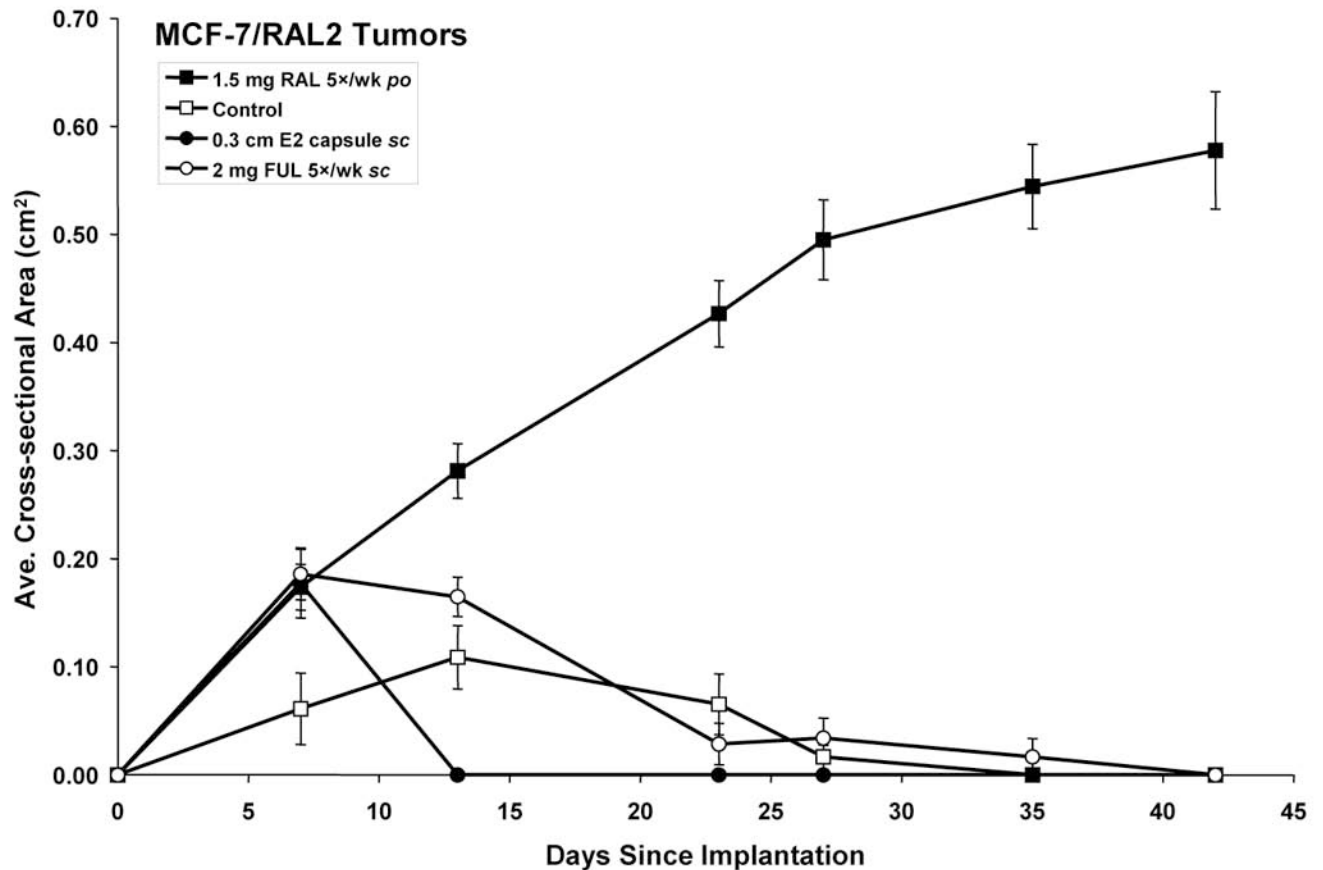
Growth inhibition of MCF-7/E2 tumors in response to different FUL formulations and dosing schedules. Thirty ovariectomized athymic nude mice were bitransplanted in the axillary mammary fat pads with MCF-7/E2 tumor pieces  $1 \text{ mm}^3$  in size. At the time of tumor implantation, the mice were separated into 6 treatment groups of 5 mice each, or 10 tumors per group. The treatment groups were Control (no treatment), 0.3 cm E<sub>2</sub> silastic capsule implanted *sc*, and 4 groups of different formulations/dosing schedules of 10 mg total FUL per week plus the 0.3 cm E<sub>2</sub> capsule *sc*. The 4 FUL formulations/dosing schedules corresponded to: 1) a 50 mg/ml suspension of FUL dissolved first in EtOH and then mixed with peanut oil, and administered 2 times per week as a 5 mg *sc* injection; 2) the clinically used Faslodex preparation consisting of a 50 mg/ml solution of FUL in EtOH and castor oil, and administered 5 times per week as a 2 mg *sc* injection; 3) a 50 mg/ml suspension of FUL dissolved first in DMSO and then mixed with peanut oil, and administered 2 times per week as a 5 mg *sc* injection; or 4) a 50 mg/ml solution of FUL in 100% DMSO, and administered daily 5 times per week as a 2 mg *sc* injection. Tumor growth was tracked by weekly measurements using Vernier calipers and calculating the tumor cross-sectional area according to the formula:  $(\text{length}/2 \times \text{width}/2 \times \pi)$ . The data shown represent the average tumor cross-sectional area ( $\text{cm}^2$ ) per group  $\pm$  SE. The cross-sectional area of E<sub>2</sub>-treated tumors was statistically different from that of each of the four E<sub>2</sub> + FUL groups (all  $P$ -values  $< 0.0001$ ). Also, the cross-sectional area of tumors in the E<sub>2</sub> + 5 mg FUL (EtOH/Peanut Oil suspension given 2 days per week) was statistically different from those in the E<sub>2</sub> + 5 mg FUL (DMSO/Peanut Oil suspension given 2 days per week) group ( $P = 0.0013$ ). Likewise, the cross-sectional area of tumors in the E<sub>2</sub> + 2 mg FUL (EtOH/Castor Oil solution given 5 days per week) group was statistically

different from that of the E<sub>2</sub> + 2 mg FUL (100% DMSO solution given 5 days per week) group ( $P = 0.0038$ ).



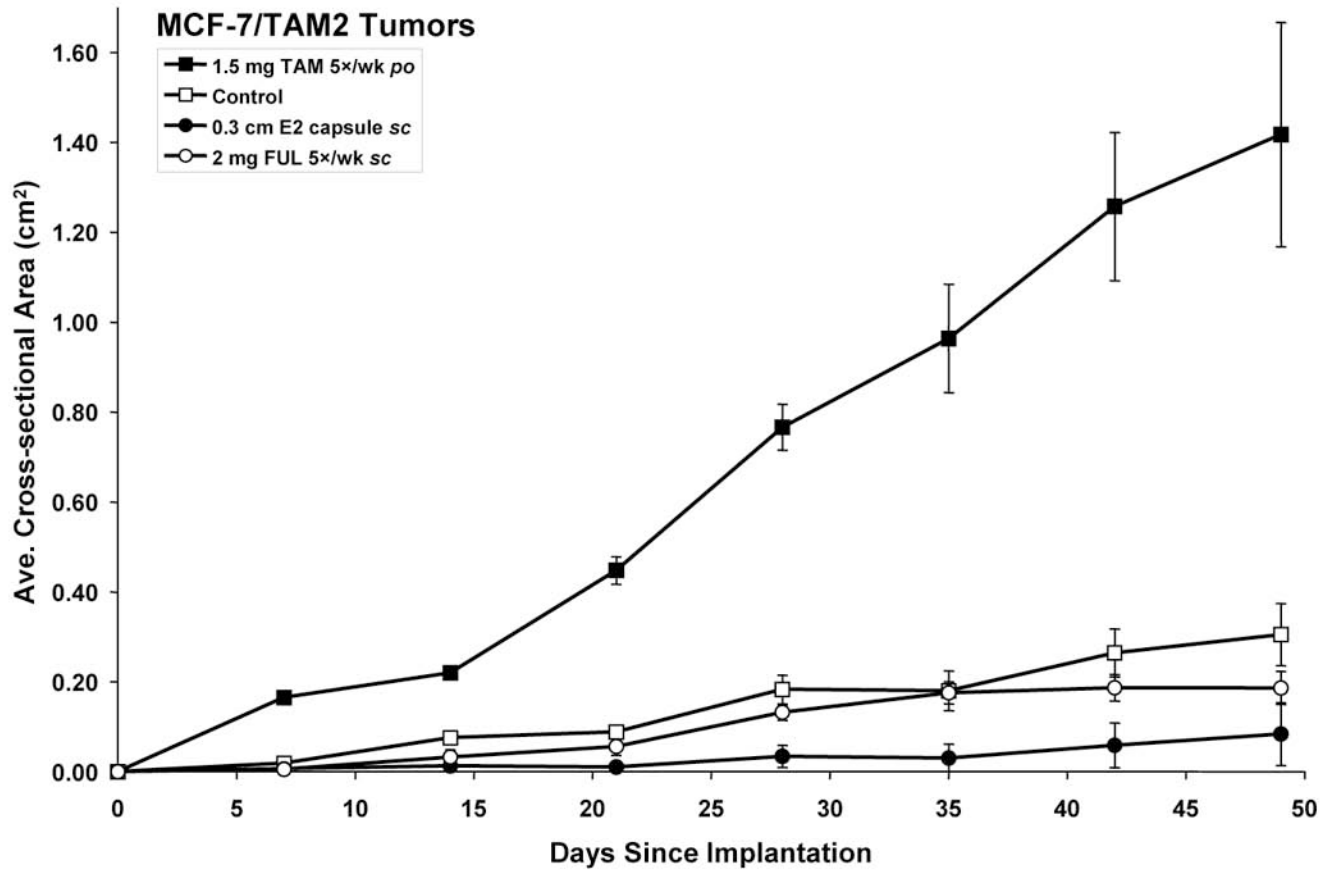
**Fig 2.**

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



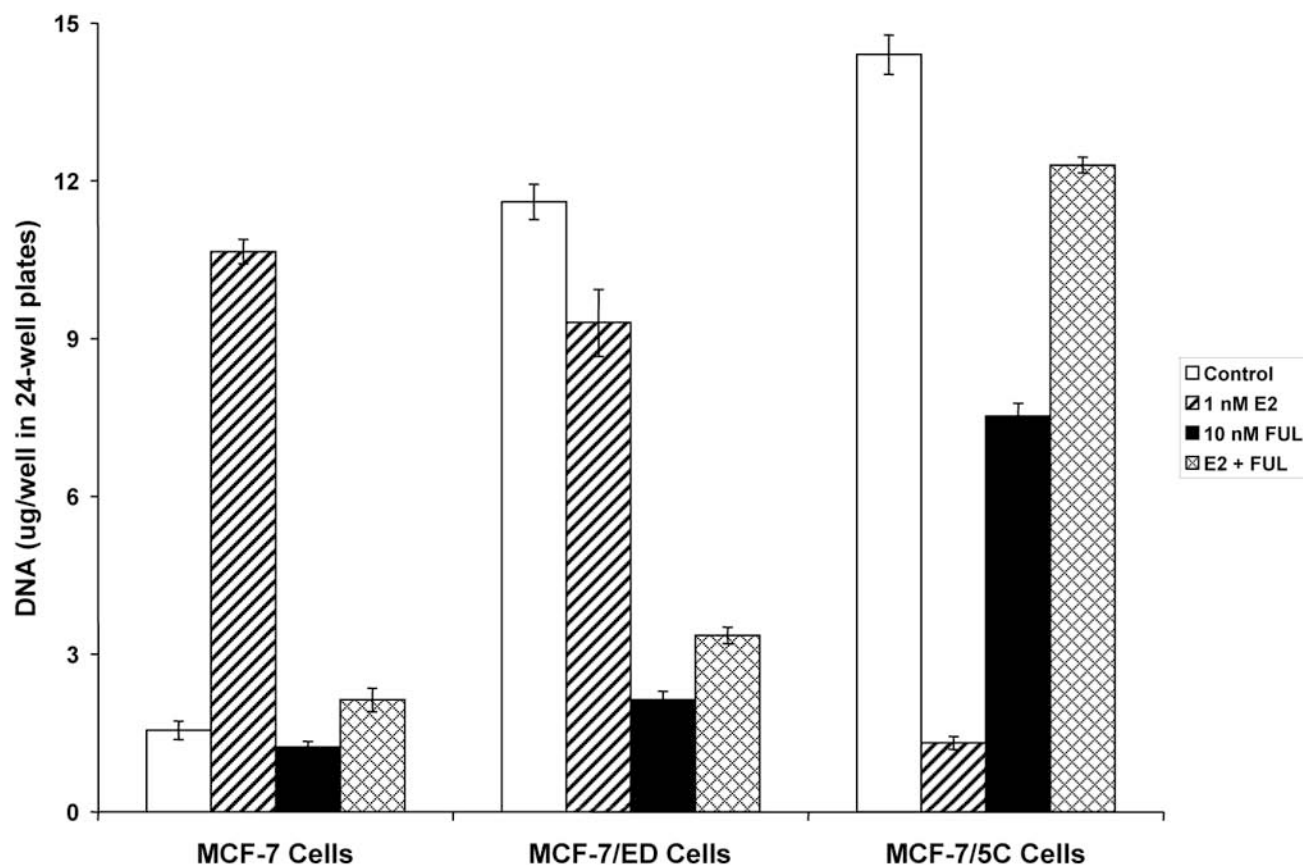
**Fig 3.**

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



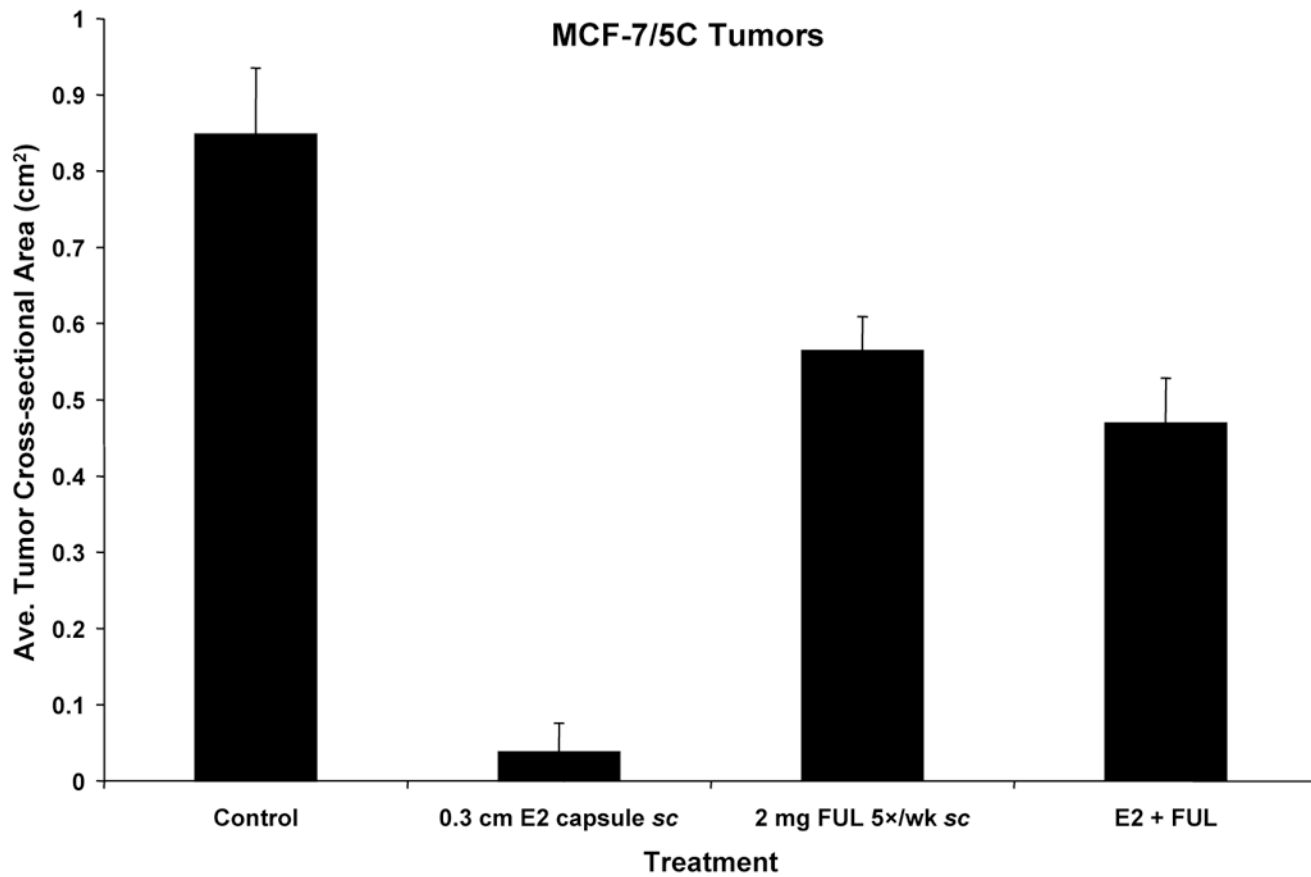
**Fig 4.**

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



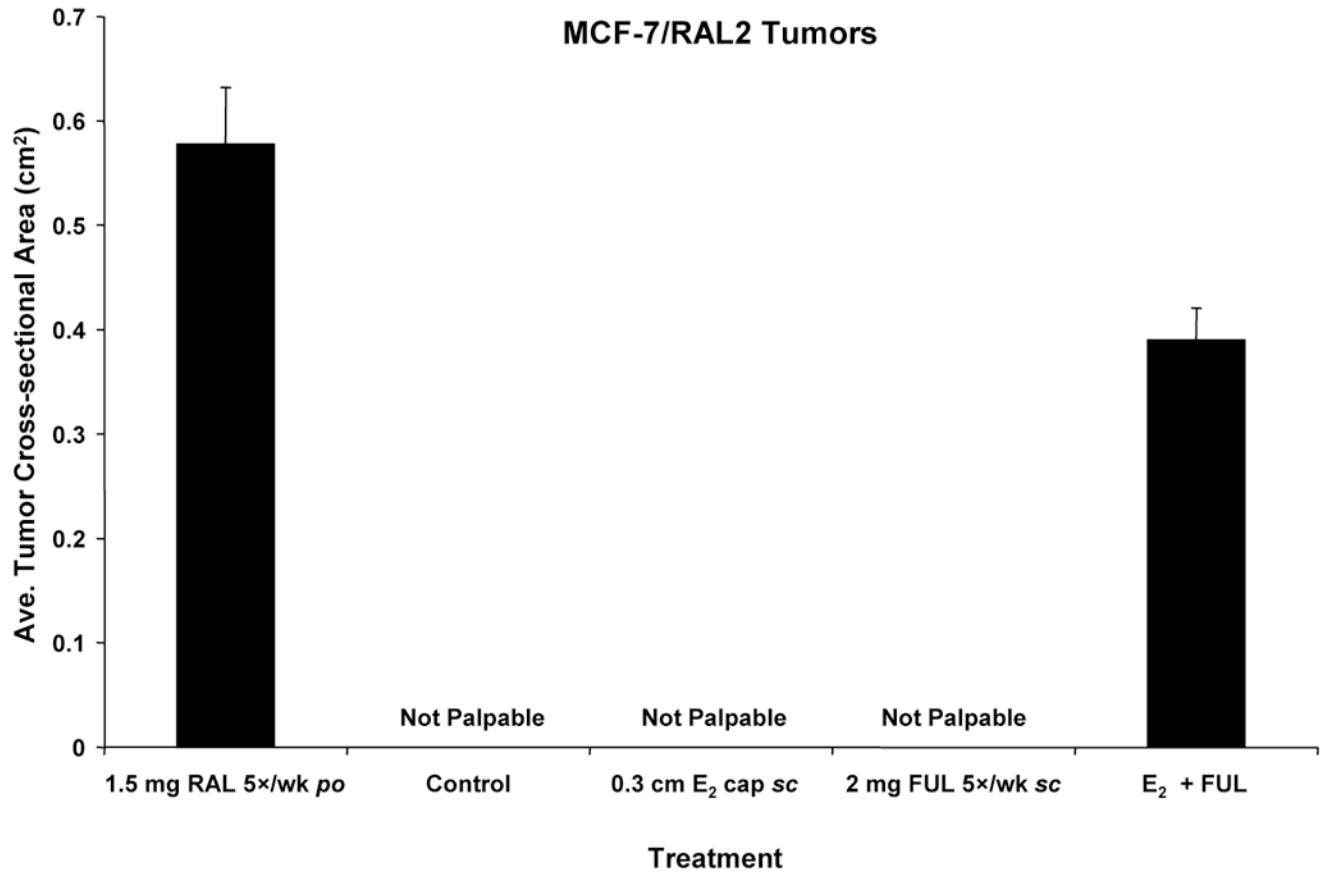
**Fig 5.**

Differential proliferation of MCF-7 long-term estrogen withdrawn cell culture models in response to E<sub>2</sub>, FUL, and E<sub>2</sub> plus FUL for 7 days. Cells were cultured under estrogen-free conditions for 4 days, and then seeded at  $2 \times 10^4$  cells per well in a 24-well plate. Beginning 24 hours after seeding (day 0) and every 2 days thereafter up to 6 days (days 2, 4, and 6), the cells were treated with 1 nM E<sub>2</sub>, 10 nM FUL, 1 nM E<sub>2</sub> + 10 nM FUL, or Control (0.1% EtOH)-treated. The experiment was stopped on day 7. As a measure of proliferation, the amount of DNA per well was determined using a fluorescence-based DNA quantitation assay. Data are shown as the mean of 6 replicate wells per group  $\pm$  SD. The experiment was performed 3 times independently, and one representative experiment is shown.

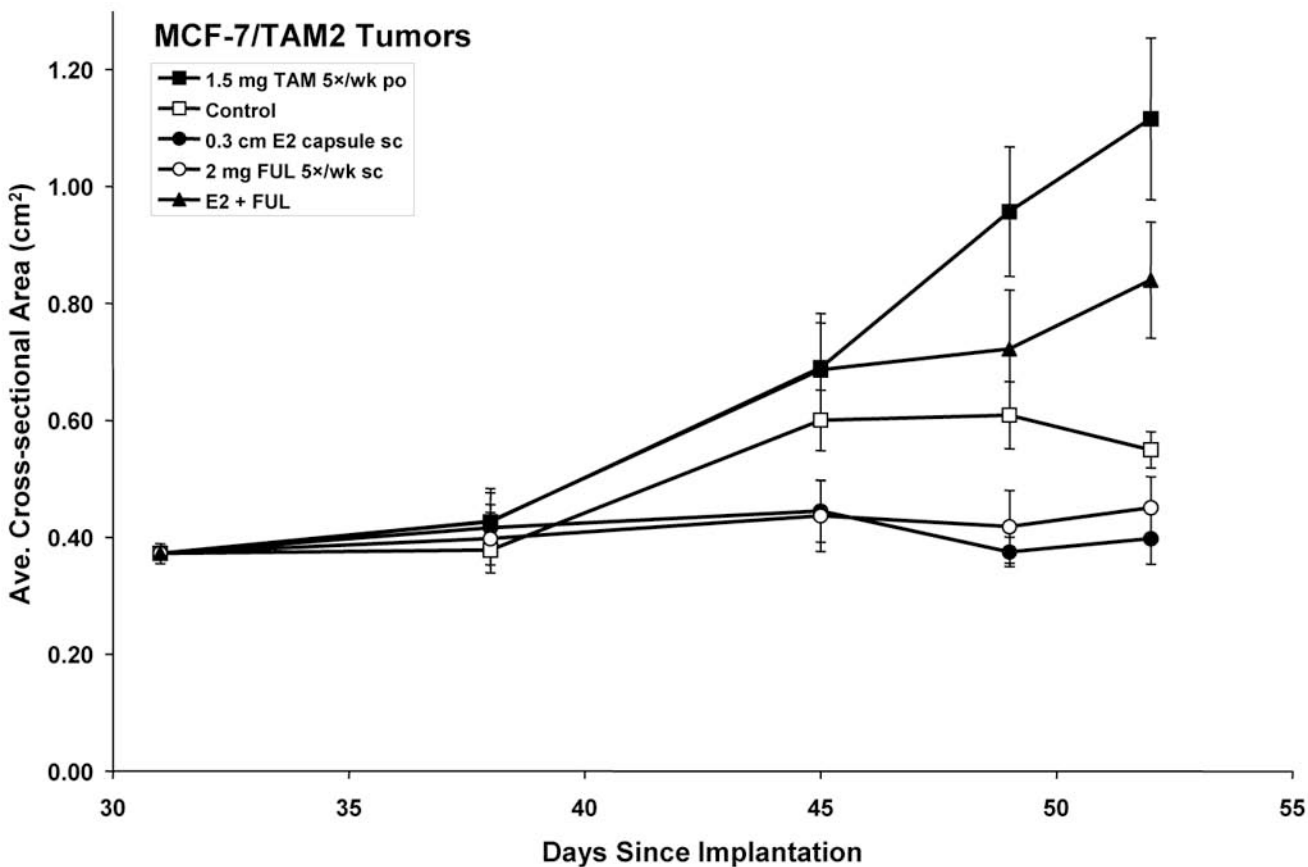


**Fig 6.**

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



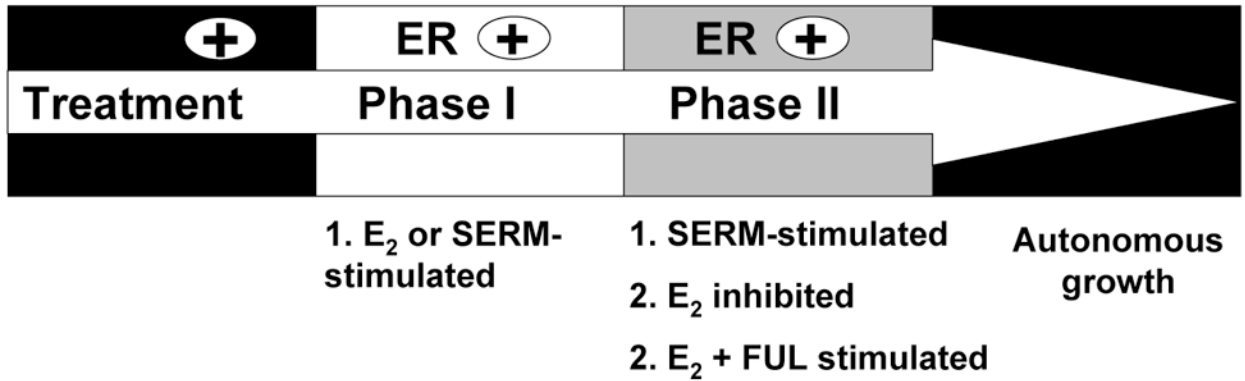
**Fig 7.** E<sub>2</sub> plus FUL-stimulated growth of MCF-7/RAL2 tumors. Data are from Figure 3 on day 42 and shown as a histogram, but supplemented with the additional group of 5 ovariectomized athymic mice (10 tumors) treated with 0.3 cm E<sub>2</sub> capsule *sc* plus 2 mg/day FUL *sc*. The cross-sectional area of E<sub>2</sub> plus FUL-treated MCF-7/RAL2 tumors was significantly different from that of Control-treated, E<sub>2</sub>-treated, and FUL-treated MCF-7/RAL2 tumors (all *P*-values < 0.0001).



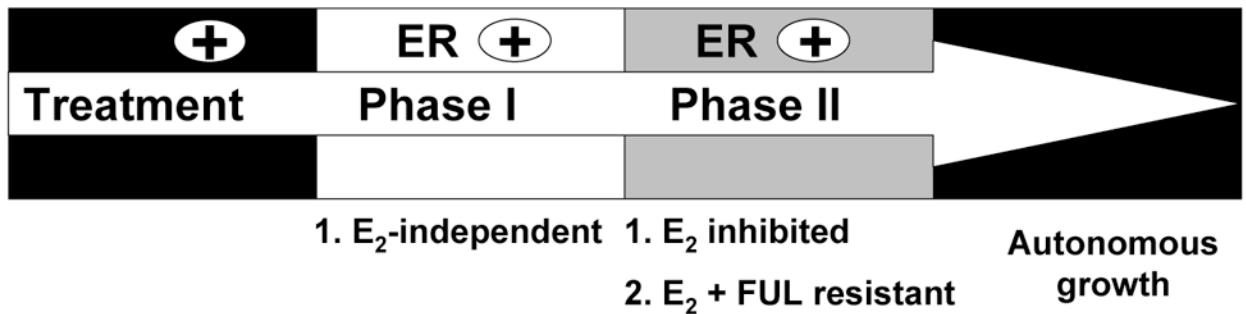
**Fig 8.**

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

## EVOLUTION OF RESISTANCE TO SERMs



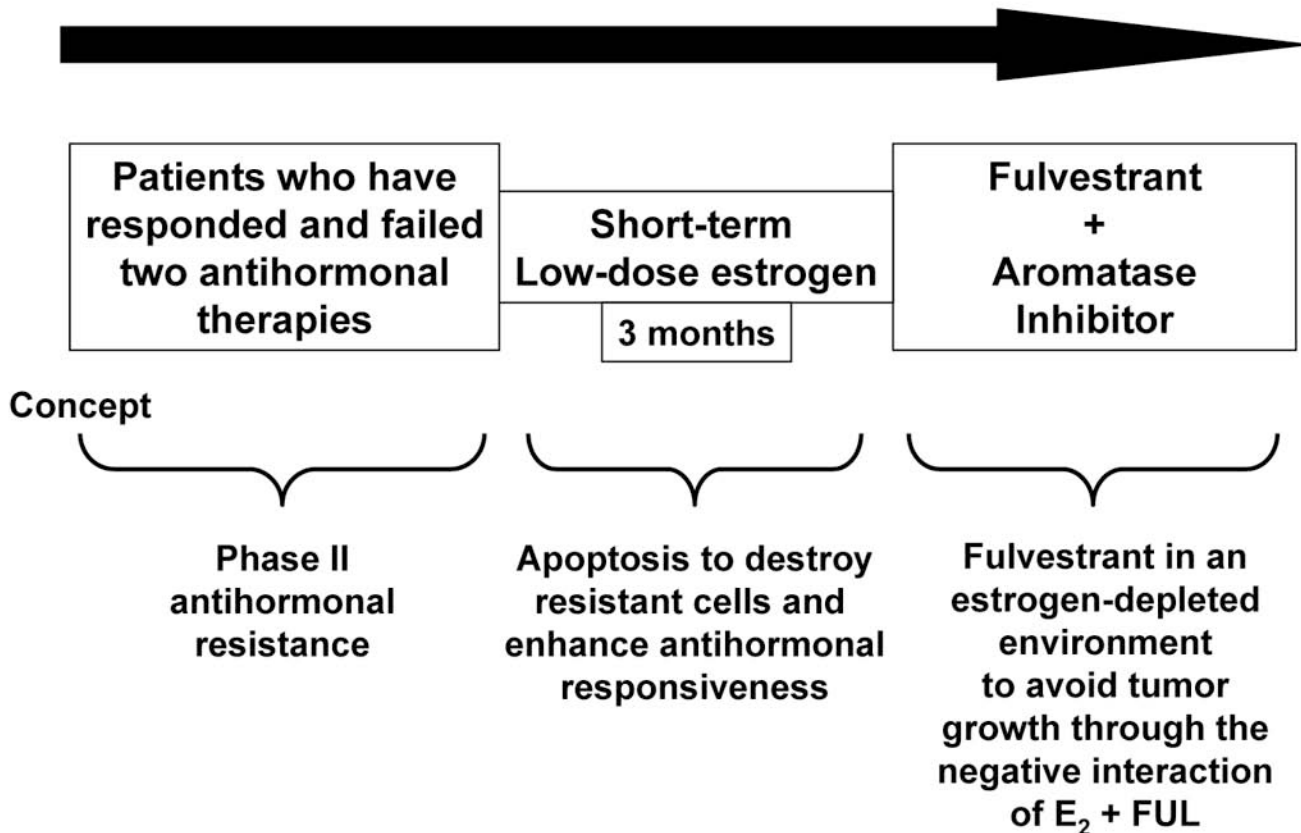
## EVOLUTION OF RESISTANCE TO ESTROGEN WITHDRAWAL



**Fig 9.**

Evolution of antihormonal resistance in laboratory models of breast cancer. Long-term antihormonal therapy leads to selection of resistant cells that are stimulated to grow by a SERM or grow in an estrogen-depleted environment (AI resistance). Based on prior laboratory studies (4–10, 12, 13) and data presented here, the progression of antihormonal resistance can be separated into at least 2 phases defined by different growth responses to E<sub>2</sub> and FUL. In Phase I resistant disease, tumor cells are either growth stimulated by E<sub>2</sub> (as in SERM resistance) or grow independently of E<sub>2</sub> (as in estrogen withdrawn/AI resistance). However, these Phase I resistant cells remain dependent on ER since they are sensitive to growth inhibition by FUL. Selection of tumor cells continues during exhaustive antihormonal therapy until Phase II resistance develops, which is characterized by a new biology of E<sub>2</sub> action. Both Phase II SERM and estrogen withdrawn-resistant tumors respond to E<sub>2</sub> with growth inhibition and apoptosis. FUL still inhibits growth of Phase II SERM-resistant tumors, but not of Phase II estrogen withdrawn-resistant tumors. Moreover, FUL interacts with E<sub>2</sub> at physiologic concentrations to promote growth of both Phase II SERM and estrogen withdrawn-resistant disease. These emerging concepts on the evolution of antihormonal resistance based on laboratory studies have important implications for the utility of estrogen and FUL in the clinic.

## TREATMENT PLAN FOR THIRD-LINE ANTIHORMONAL THERAPY



**Fig 10.**

A proposed clinical strategy for the optimal application of third-line antihormonal therapy. We propose that patients who have initially responded to, and then failed, two previous antihormonal therapies may exhibit Phase II antihormonal resistance, and would then benefit from short-term low-dose estrogen, which would induce apoptosis in the Phase II resistant cells and debulk the tumor. Prior laboratory evidence indicates that the small percentage of Phase II tumors which revert to an estrogen-stimulated stage after estrogen-induced regression, are also re-sensitized to antihormonal therapy [47,49]. A low tumor burden would be maintained by FUL in an estrogen-depleted environment, *i.e.* FUL plus an aromatase inhibitor, to avoid the possible emergence of tumor growth through a negative interaction between FUL and physiologic estrogen.

available at [www.sciencedirect.com](http://www.sciencedirect.com)journal homepage: [www.ejconline.com](http://www.ejconline.com)

## Current Perspective

# Optimising endocrine approaches for the chemoprevention of breast cancer

## Beyond the Study of Tamoxifen and Raloxifene (STAR) Trial ☆

V. Craig Jordan\*

Fox Chase Cancer Centre, 333 Cottman Avenue, Philadelphia, PA 19111, USA

### ARTICLE INFO

#### Article history:

Received 25 September 2006

Accepted 28 September 2006

Available online 24 October 2006

#### Keyword:

STAR

### ABSTRACT

The completion of the Study of Tamoxifen and Raloxifene (STAR) [Vogel VG, Costantino JP, Wickerham DL, et al. The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA* 2006;295:2727-2741.] and the ongoing studies with aromatase inhibitors [Goss PE. Breast cancer prevention—clinical trials strategies involving aromatase inhibitors. *J Steroid Biochem Mol Biol* 2003;86(3-5):487-93.] to assess their worth for the chemoprevention of breast cancer, creates an opportunity to consider the realistic future for chemoprevention as an option for women's healthcare and to identify strategies for future progress in an era of targeted therapeutics, managed healthcare and soaring costs.

© 2006 Published by Elsevier Ltd.

### What did the STAR Trial achieve?

Nineteen thousand seven hundred and forty seven postmenopausal women (mean age 58.8 years) with an increased 5 year Gail risk (mean 4.03) were randomised to receive either tamoxifen 20 mg or raloxifene 60 mg for 5 years. A final analysis was initiated after at least 327 invasive breast cancers were diagnosed. There were 163 and 168 invasive breast cancers observed in women assigned to tamoxifen and raloxifene respectively. Thus, the conclusion of the study was that raloxifene was equivalent to tamoxifen at reducing the risks of breast cancer in postmenopausal women at high risk. However, the side effect profile favoured raloxifene. There were 36 and 23 cases of uterine cancer with tamoxifen and raloxifene respectively, fewer hysterectomies with raloxifene,

and fewer thromboembolic events occurred with raloxifene when compared with tamoxifen. Similarly, there were fewer cataracts and cataract surgeries noted in women taking raloxifene but there was the same number of osteoporotic fractures in both groups. Thus, raloxifene, a drug that has been extensively investigated and used for the treatment and prevention of osteoporosis for the past 7 years, has now been shown to be a useful agent with reduced side effects to prevent breast cancer in high risk postmenopausal women.

To achieve the goal of practical progress in the chemoprevention of breast cancer which can truly enhance public health, three interdependent issues must be addressed satisfactorily: whom to treat, what agent to use and is the process affordable?

☆ This paper was presented at a meeting at the Royal College of Surgeons, Edinburgh entitled "Controversies in Breast Cancer" September 4, 2006 and formed the basis of the American Cancer Society Award Lecture presented at the American Society of Clinical Oncology on June 5, 2006 in Atlanta, Georgia USA.

\* Tel.: +1 215 728 7410; fax: +1 215 728 7034.

E-mail address: [v.craig.jordan@fccc.edu](mailto:v.craig.jordan@fccc.edu).

0959-8049/\$ - see front matter © 2006 Published by Elsevier Ltd.

doi:10.1016/j.ejca.2006.09.012

## Whom to treat?

The Gail model<sup>3</sup> has been used satisfactorily to select pre and postmenopausal women for inclusion in the NSABP P-1 Trial which compared tamoxifen versus placebo<sup>4</sup> and subsequently used to recruit high risk women into the STAR P-2 Trial.<sup>1</sup> However, it is interesting to note that in the Nurses Health Study<sup>5</sup> where 81,209 women have now been followed for several decades, that of the 1354 breast cancers that have been recorded, 44% of these tumours occur in women with the Gail risk of  $\geq 1.67$  a cut off for high risk but 54% of the cancers occur in women who would not be considered to have a risk for breast cancer. Clearly strategies need to be developed that are cost effective both for high risk and normal risk women.

To this end, in this era of targeted therapies for the treatment of cancer, it seems only reasonable to target specific populations with chemoprevention of breast cancer so we can enhance the value to public health. By simple analogy, it would be considered of no value to treat breast cancer patients with antihormone therapy if their tumour did not have the oestrogen receptor. Patients would then be treated when there was no possibility of any benefit. The BRCA1 and 2 genes are used as markers for familial breast cancer but unfortunately there are no equivalent markers to pre-select for spontaneous breast cancer. Therefore, either the high risk group should be considered for chemoprevention specifically or for breast chemoprevention where the strategy embraces the idea of additional benefits for public health.

Let us place this concept into perspective by examining the best agent that could be used as a chemopreventive. The link between oestrogen and breast cancer has been known for more than a century therefore creating 'a no oestrogen at all' state in postmenopausal women by using an aromatase inhibitor seems an entirely logical approach to provide the best results to prevent breast cancer. The idea is not new as Lacassagne proposed this general prevention strategy in 1936.<sup>6</sup> The evidence for choosing aromatase inhibitors as the agent of choice is readily available from the clinical trials literature that has used either tamoxifen or an aromatase inhibitor as adjuvant therapies for the treatment of oestrogen receptor positive breast cancer. In all cases, tamoxifen has performed well, producing an estimated 50% decrease in contralateral breast cancer,<sup>7</sup> (the same decrease noted in the P-1 trial.<sup>4</sup>) Nevertheless, aromatase inhibitors all produce greater decreases in contralateral breast cancer<sup>8–11</sup> and it can be stated that these agents are undoubtedly superior to tamoxifen.

Can the concept of using the best agent available to prevent breast cancer be put into practice? If Gail high risk postmenopausal women are selected for treatment under circumstances similar to the STAR trial,<sup>1</sup> it would be anticipated that with no treatment, 8 per 1000 women per year would develop breast cancer and the aromatase inhibitors would perform optimally preventing three out of four of the breast cancers. Unfortunately, looking at this projection another way, it means that to prevent six breast cancers in 1000 women, 992 additional women must be treated for the year. With apologies to Winston Churchill, 'Never in the field of cancer therapeutics has so much been given to so many to benefit so few'.

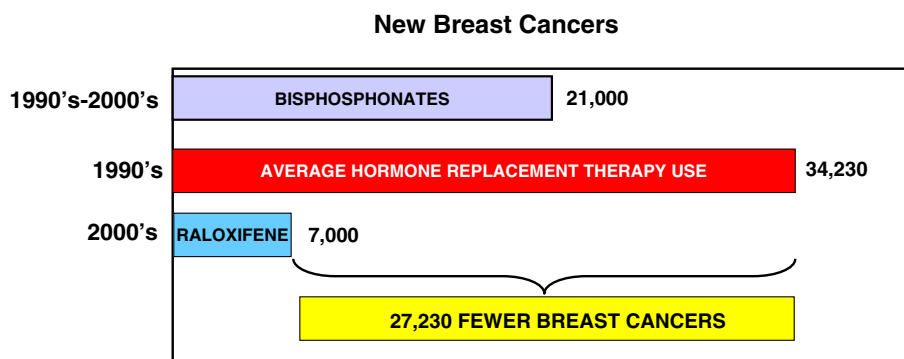
An alternative approach is to use a selective oestrogen receptor modulator and use it selectively in appropriate patient populations to provide the best results for the right women.

## What to use?

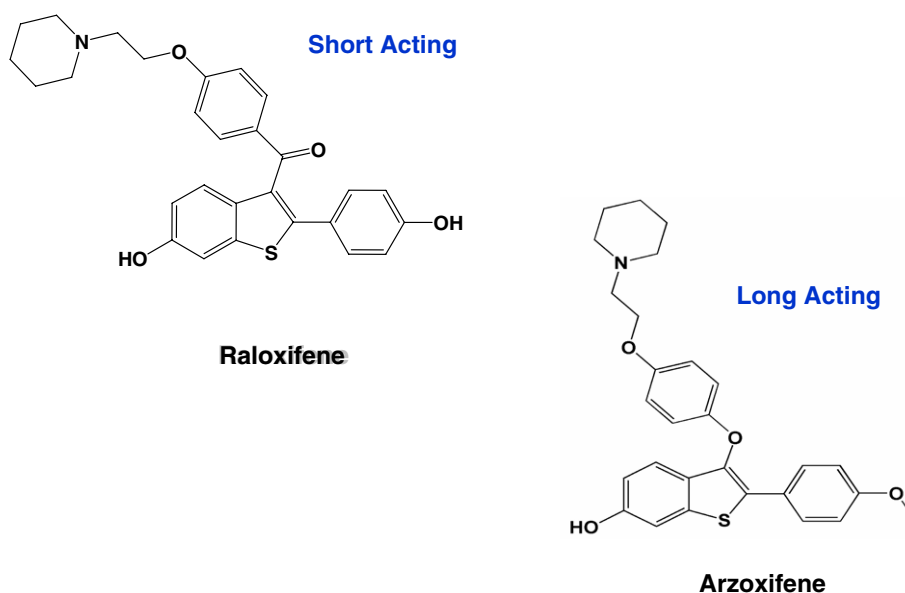
Selective oestrogen receptor modulation was first recognised in the late 1980s<sup>12</sup> when it was realised that the then named nonsteroidal antioestrogens, tamoxifen and raloxifene (then known as keoxifene) would not only prevent mammary cancer in rats but would also maintain bone density.<sup>13,14</sup> The class of drugs were antioestrogenic at some sites (breast, uterus) but oestrogenic at other sites (bone, circulating cholesterol). These laboratory findings subsequently translated to the clinic.<sup>15,16</sup> However, it was immediately clear that the idea of selective oestrogen receptor modulation could be applied to advance the chemoprevention of breast cancer.<sup>17</sup> The initial strategy<sup>17</sup> was subsequently refined, and defined as follows 'important clues have been garnered about the effects of tamoxifen on bone lipids so it is possible that derivatives could find targeted applications to retard osteoporosis and atherosclerosis. The ubiquitous application of novel compounds to prevent disease associated with the progressive changes after menopause, may, as a side effect, significantly prevent breast cancer'.<sup>18</sup>

It is now possible to test this evidenced based<sup>17,18</sup> hypothesis by examining clinical studies of raloxifene used to treat osteoporosis while monitoring the impact of breast cancer incidence at the same time. The first proof of principle was noted by Cummings and coworkers<sup>19</sup> but the initial 4 year osteoporosis study with raloxifene has now been extended out to 8 years in the Continuing Outcomes Relative to Evista (CORE). Martino and coworkers<sup>20</sup> found that in the placebo arm (2576 osteoporotic women) that there were 4.2 cases of breast cancer per 1000 women per year. In contrast, in the women treated with raloxifene (5129 osteoporotic women), there were only 1.4 cases of breast cancer per 1000 women per year. It is now possible, using these figures, to evaluate the progress made in chemoprevention over the last 20 years by calculating the approximate incidence of breast cancer in women being treated for osteoporosis (Fig. 1). It is estimated that 500,000 women are taking raloxifene worldwide<sup>1</sup> so this is a reasonable starting point to discover the potential impact on public health. If half a million women were using bisphosphonates to treat osteoporosis over a 10 year period then 21,000 women would develop breast cancer. If these same women had been appropriately treated for osteoporosis during the 1990s, using hormone replacement therapy (HRT), there would be a significant increase in the incidence of breast cancer based upon the Women's Health Initiative<sup>21</sup> and the Million Women's Study.<sup>22</sup> On average 500,000 women would develop 34,230 breast cancers over a 10 year period. In contrast, if those same women now take raloxifene for 10 years, there would only be 7000 women who will develop breast cancer; a net decrease of 27,320 breast cancers from those women that would have been taking HRT during the 1990s.

There is between a 75% and 65% decrease in breast cancer in women when raloxifene is used for the prevention of osteoporosis<sup>19,20</sup> However, by reference to the STAR trial<sup>1</sup> there appears to be only a 50% decrease in the incidence<sup>4</sup> of breast



**Fig. 1** – An estimation of breast cancer incidence in a population of 500,000 postmenopausal women with the same risk for osteoporotic fractures as participants in the CORE trial<sup>20</sup> treated for a 10 year period with a bisphosphonate, hormone replacement therapy (HRT) based on the average breast cancer risk between the Women's Health Initiative<sup>21</sup> and the Million Women's Study<sup>22</sup> or currently with raloxifene. The overall change in prescribing practices from the former practice of HRT would be the standard treatment to the current practice of prescribing raloxifene will produce a net decrease of 27,230 breast cancers.



**Fig. 2** – The formula of the short acting SERM raloxifene and its long acting derivative arzoxifene.

cancer. This is an assumption because there was no control group in STAR,<sup>1</sup> however, tamoxifen is known to produce a 50% decrease in breast cancer incidence and raloxifene was equivalent to tamoxifen in STAR. What could account for these differences? One possible explanation could be that raloxifene performs very well in the low oestrogen environment noted in osteoporotic women. In contrast, healthy women not suffering from osteoporosis undoubtedly have higher circulating levels of oestrogen. It has been known for about 20 years that drugs of the raloxifene class are very short acting and have very poor bioavailability.<sup>13,23,24</sup> Indeed, raloxifene is only 2% bioavailable in women and is rapidly excreted.<sup>25</sup> Clearly, compliance will be essential to maintain the antitumour actions of raloxifene. If no raloxifene is present, oestrogen will cause breast carcinogenesis. Thus, in the STAR trial, the actions of raloxifene as a chemopreventive could become undermined by poor compliance which would explain the inability of raloxifene to control invasive and noninvasive breast cancer optimally.<sup>1</sup> Clearly,

new long acting selective oestrogen receptor modulators (SERMs) need to be developed and tested in the clinic. One such compound arzoxifene is completing clinical studies as a preventive for osteoporosis and has already been shown to be superior to raloxifene in the prevention of rat mammary carcinogenesis (see Fig. 2).<sup>26</sup> Finally, it must be asked how cost effective will chemopreventive interventions be if they are to improve the standards of healthcare.

### Affordable?

A strategy to create a public health policy that will reduce the incidence of breast cancer can now be examined based on the application of different agents targeted to specific populations. In the same examination, it is appropriate to consider which agent is targeting which population and then attempt to create an approximate cost to prevent a standard number of breast cancers. There are three appropriate applications

**Table 1 – Appropriate populations for SERMs use as chemopreventives for breast cancer**

Agent	Targeted group	Alternative?
Tamoxifen	Very high risk <sup>a</sup> premenopausal women <sup>27</sup>	Not aromatase inhibitors
Raloxifene	Osteoporotic women	Not aromatase inhibitors
New long acting SERMs or raloxifene	Very high risk postmenopausal women	Aromatase inhibitors

The only alternative of aromatase inhibitor is also stated.  
a Atypical hyperplasia, LCIS 2 > 1st degree relatives or GAIL risk > 5.<sup>29</sup>

**Table 2 – The relative monthly costs per person of antihormonal agents potentially useful as chemopreventive agents in select groups of high risk women**

Medicine	Monthly cost <sup>a</sup>	5 year course cost <sup>a</sup>	Cost to prevent 300 breast cancers <sup>a</sup>
Anastrozole	68.56	4114	41,140,000
Exemestane	88.80	5328	53,280,000
Letrozole	83.16	4989	49,890,000 + bisphosphonate costs (approx. 3,600,00)
Tamoxifen pre	2.39	143.40	2,145,000 Approx. 750,000 bulk buying
Raloxifene post	19.86	1193.60	17,910,000 Bonus: prevents fractures
Raloxifene osteo	19.86	1193.60	23,880,000 Bonus: prevents fractures

Based on the cost of a 5 year course, the actual cost of preventing 300 breast cancers during that time period is estimated based on incidence of breast cancer. The high risk postmenopausal or premenopausal women is calculated at 8 breast cancers per 1,000 women per year. The risk for osteoporotic women was estimated based on the article by Martino and coworkers.<sup>20</sup>  
a NHS cost in GB pounds.

where a SERM can be targeted to reduce the incidence of breast cancer (Table 1). Each of these populations have been evaluated in clinical trial so this fact minimises speculation. Firstly, the best risk benefit ratio for tamoxifen is to target very high risk premenopausal women.<sup>27</sup> Tamoxifen is an effective chemopreventive but endometrial cancer and blood clots are not increased in premenopausal women.<sup>4</sup> In a recent evaluation<sup>28</sup> of potential mortality outcomes, populations of Gail Risk >3 are calculated to the best benefit but only in countries with cheap tamoxifen. It should be noted that tamoxifen is the only agent available for this application as raloxifene and aromatase inhibitors cannot be used in premenopausal women. Raloxifene has not been tested in this application and the manufacturer recommends against this application. Aromatase inhibitors are ineffective in premenopausal women with an intact hypothalamo-pituitary-ovarian axis. Secondly, raloxifene when it is used specifically to prevent osteoporosis in postmenopausal women effectively prevents breast cancer as a beneficial side effect.<sup>20</sup> Bisphosphonates do not alter the incidence of breast cancer and aromatase inhibitors are inappropriate for use in this application. Finally, the case for the use of raloxifene in very high risk postmenopausal women is complex. Raloxifene provides a significant benefit<sup>1</sup> but as noted in the previous section, it seems appropriate that new long acting SERMs are developed to provide optimal chemoprevention. Aromatase inhibitors will clearly be beneficial in this category but are they affordable?

Table 2 summarises costs to the National Health Service in GB pounds of the three leading aromatase inhibitors, anastrozole, exemestane and letrozole that are currently being evaluated as chemopreventives for breast cancers.<sup>2</sup> Table 2 illustrates the monthly costs and the cost of an appropriate 5 year course for each individual choosing this strategy for chemoprevention. To standardise a comparison of the cost

to the health service in prevention, an approximate calculation has been made to prevent 300 breast cancers for the appropriate risk group i.e. eight breast cancers per 1000 women per year. The costs are very large ranging between 40 and 50 million pounds but there is an additional hidden cost for the supply of bisphosphonates to approximately 1/3 of women who may develop osteopenia and osteoporosis during the 5 year course of an aromatase inhibitor. In contrast, tamoxifen is exceptionally cheap with the cost of preventing 300 breast cancers in high risk premenopausal women of around 2 million pounds. That being said, this cost drops in many Trusts because of the bulk buying of generic drugs. Thus tamoxifen is extremely affordable in this targeted application as has been noted previously.<sup>28,29</sup> The other SERM, raloxifene, when used as a chemopreventive in high risk postmenopausal women is less than half the cost noted for the aromatase inhibitors when preventing the 300 breast cancers. However, the hidden bonus is that a significant number of women will not be developing osteopenia or osteoporosis so there will be fewer fractures in this high risk breast cancer group. Finally, when raloxifene is used to prevent osteoporosis, although the cost to the health service is higher because more women will need to be treated to prevent the same standard 300 breast cancers (i.e. low risk group), the significant bonus to public health will be an approximate 30% decrease in fractures in this patient population.

## Conclusions

Clinical trials data are providing strong evidence that SERMs are affordable and cost effective when applied to targeted patient populations of the highest risk for developing breast cancer. Again, it is clear from their clinical applications that SERMs are more versatile than aromatase inhibitors for pre-

venting breast cancer. Despite the fact that raloxifene is now known to be ineffective in reducing the risk of coronary heart disease<sup>30</sup> the SERMs are progressing appropriately in fulfilling their promise to prevent multiple diseases.<sup>17,18</sup> The best evidence for this is the application of SERMs to prevent osteoporosis but at the same time preventing breast and endometrial cancer.<sup>20</sup>

### Conflict of interest statement

None declared.

### Acknowledgements

Dr. Jordan is supported by the Department of Defence Breast Programme under award number BC050277 Centre of Excellence (views and opinions of, and endorsements by, the author(s) do not reflect those of the US Army or the Department of Defence), SPOR in Breast Cancer CA 89018, R01 GM067156, FCCC Core Grant NIH P30 CA006927, the Avon Foundation and the Weg Fund of the Fox Chase Cancer Centre. I would like to thank Professor Alan Roberts and Dr. Stan Dobrzanski of the Bradford Teaching Hospitals NHS Foundation Trust for their invaluable assistance with UK drug pricing.

### REFERENCES

- Vogel VG, Costantino JP, Wickerham DL, et al. The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA* 2006;**295**:2727–41.
- Goss PE. Breast cancer prevention—clinical trials strategies involving aromatase inhibitors. *J Steroid Biochem Mol Biol* 2003;**86**(3–5):487–93.
- Gail MH, Brinton LA, Byar DP, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst* 1989;**81**(24):1879–86.
- 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.
- Rockhill B, Spiegelman D, Byrne C, Hunter DJ, Colditz GA. Validation of the Gail et al. model of breast cancer risk prediction and implications for chemoprevention. *J Natl Cancer Inst* 2001;**93**(5):358–66.
- Lacassagne A. Hormonal pathogenesis of adenocarcinoma of the breast. *Am J Cancer* 1936;**27**:217–25.
- EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;**354**:1451–67.
- Howell A, Cuzick J, Baum M, 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;**365**:60–2.
- Thurlimann B, Keshaviah A, Coates AS, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med*. 2005;**353**:2747–57.
- Goss PE, Ingle JN, Martino S, et al. 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* 2005;**97**:1262–71.
- Coombes RC, Hall E, Gibson LJ, et al. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. *N Engl J Med* 2004;**350**(11):1081–92.
- Jordan VC. Selective estrogen receptor modulation: a personal perspective. *Cancer Res* 2001;**61**:5683–7.
- Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea- induced rat mammary carcinoma model. *Cancer Res* 1987;**47**(15):4020–4.
- Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 1987;**10**(1):31–5.
- Love RR, Wiebe DA, Newcomb PA, et al. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Ann Intern Med* 1991;**115**(11):860–4.
- 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**(13):852–6.
- Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 1988;**6**(5):589–95.
- Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res* 1990;**50**:4177–89.
- 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.
- Martino S, Cauley JA, Barrett-Connor E, et al. For the CORE investigators 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.
- Writing Group for the Women's Health Initiative Investigators. Rossouw JE, Prentice RL, LaCroix AZ, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women. *JAMA* 2002;**288**(3):321–32.
- Million Women Study Collaborators. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet* 2003;**362**:419–427.
- Jordan VC. 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* 1983;**3**(Suppl):S73–86.
- Jordan VC, Gosden B. Inhibition of the uterotrophic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. *Endocrinology* 1983;**113**(2):463–8.
- Snyder KR, Sparano N, Malinowski JM. Raloxifene hydrochloride. *Am J Health Syst Pharm* 2000;**57**(18):1669–75. quiz 1676–8.
- Suh N, Lamph WW, Glasebrook AL, et al. 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* 2002;**8**(10):3270–5.
- Gail MH, Costantino JP, Bryant J, et al. Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J Natl Cancer Inst* 1999;**91**(21):1829–46.
- Melnikow J, Kuenneth C, Helms LJ, et al. Chemoprevention: Drug pricing and mortality: the case of tamoxifen. *Cancer* 2006.
- Hershman D, Sundararajan V, Jacobson JS, Heitjan DF, Neugut VR, Grann VR. Outcomes of tamoxifen chemoprevention for breast cancer in very high-risk women: a cost-effectiveness analysis. *J Clin Oncol* 2002;**20**(1):9–16.
- 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**(2):125–37.

## OPINION

### Chemoprevention of breast cancer with selective oestrogen-receptor modulators

V. Craig Jordan

**Abstract** | Twenty years ago, a new therapeutic dimension was conceived that not only had the potential to treat and prevent osteoporosis, but to prevent breast and endometrial cancer at the same time. As osteoporosis was known to be caused by oestrogen withdrawal after menopause, whereas breast and endometrial cancer are caused by unopposed oestrogen action, the new tissue-selective oestrogens and anti-oestrogens, or selective oestrogen-receptor modulators (SERMs), had to recruit new networks to activate or suppress target tissues selectively. New medicines now promise to provide chemoprevention strategies for women at risk for the development of many diseases.

Despite recent advances in the targeted treatment of cancer, there is clearly a benefit to preventing the development of cancer in the first place. Fewer patients being treated for cancers would reduce the burden on health-care systems, and enable increasing numbers of people to live healthier more productive lives. Less than 10 years ago, the promise of chemoprevention became a clinical reality. Tamoxifen, a compound originally called a nonsteroidal anti-oestrogen but now referred to as a selective oestrogen-receptor modulator (SERM)<sup>1</sup>, was shown to reduce the incidence of breast cancer in both pre and postmenopausal women at high risk<sup>2</sup>. In this case, high risk is defined by a validated computer model that assesses the reproductive and family history of individuals to estimate the likelihood of developing breast cancer during the next 5 years or as a lifetime risk<sup>3,4</sup>. Tamoxifen, which was already a successful breast cancer drug targeted to block the oestrogen receptor (ER) and prevent oestrogen-stimulated breast tumour growth<sup>1</sup>, became the first drug available for use in the United States to reduce breast cancer incidence in high-risk premenopausal and postmenopausal women. However, the recognition that

SERMs are oestrogen-like in bone but anti-oestrogenic in the breast created the possibility that both osteoporosis and breast cancer risk could be reduced<sup>5,6</sup>. This strategy of preventing osteoporosis in postmenopausal women at the same time as reducing breast cancer risk is now clinically validated. Raloxifene is the first SERM approved for the treatment and prevention of osteoporosis that also reduces breast cancer incidence<sup>7</sup>. However, the recent completion of the Study of Tamoxifen and Raloxifene (STAR)<sup>8</sup> that evaluated the benefits and side effects of these two SERMs for the reduction of breast cancer incidence in high-risk postmenopausal women now provides an opportunity to assess the molecular biology and pharmacology of SERMs as multifunctional medicines. New initiatives in the understanding of female physiology and disease will enable the correct SERM to be used for the appropriate at-risk women and facilitate the development of new and improved agents for further applications in health care.

The recognition of selective modulation of the ER in the laboratory<sup>9</sup> created a new drug group with several therapeutic possibilities (BOX 1). The evidence that

supported translational research and the initiation of clinical investigations was based on a re-examination of the drug group referred to as non-steroidal anti-oestrogens. Tamoxifen, the pioneering agent<sup>1</sup>, was described as both a partial oestrogen agonist and antagonist in the rat uterus, but was in fact classified as a full oestrogen in the mouse uterus and vagina. These were important biological facts with which to get a clearer picture of the target-site-specific actions of SERMs. Four main pieces of laboratory evidence converged to establish that the SERM concept was a class effect: first, ER-positive breast cancer cells inoculated into athymic (immune deficient) mice grew into tumours in response to oestradiol but not in response to tamoxifen, despite the fact that oestradiol and tamoxifen increased mouse uterine weight<sup>10</sup>; second, raloxifene (then known as LY156758 or keoxifene) was less oestrogenic than tamoxifen in the rodent uterus<sup>11</sup>, but both raloxifene and tamoxifen maintained bone density in ovariectomized rats<sup>12</sup> (oestrogenic action) and prevented rat mammary carcinogenesis (anti-oestrogenic action)<sup>13</sup>; third, tamoxifen blocked oestradiol-induced ER-positive breast tumour growth in athymic mice, but ER-positive endometrial carcinomas grew rapidly<sup>14</sup>; and fourth, raloxifene was less effective than tamoxifen at promoting endometrial cancer growth<sup>15</sup>.

The general conclusion was that tamoxifen and raloxifene could selectively switch on or switch off the ER at sites around the body depending on the tissue. These laboratory conclusions not only translated to patients, but also provided a strategy for the development of new tissue-selective drugs to simultaneously prevent osteoporosis and reduce the risk of breast cancer<sup>5,6</sup>.

#### Clinical evaluation of SERMs

Tamoxifen, the prototypical SERM, is available in the United States for the reduction of breast cancer incidence in high-risk premenopausal and postmenopausal women. Tamoxifen produces about a 50% decrease in breast cancer incidence<sup>2,16</sup>, which is consistent with the 50% decrease in contralateral breast cancer noted in adjuvant therapy

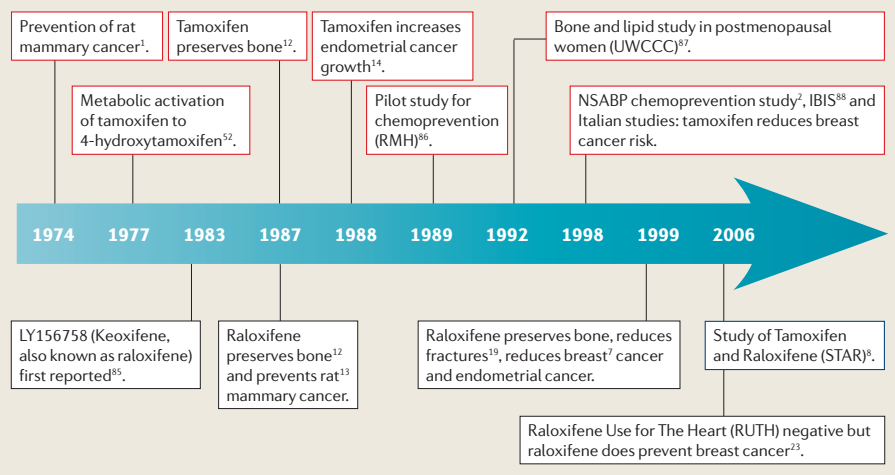
studies<sup>17</sup>. Nevertheless, the main concern about using tamoxifen is an increase in blood clots and endometrial cancer (BOX 2). Both of these side effects of tamoxifen are linked to its oestrogen-like properties in postmenopausal women, who have a low-oestrogen environment. Unfortunately, what is not emphasized is the favourable risk–benefit ratio for tamoxifen in premenopausal women because there is no significant increase in either blood clots or endometrial cancer compared with placebo controls<sup>2,4,16</sup>. Indeed, in countries where tamoxifen is cheap there is potentially a benefit to using tamoxifen as a chemopreventive in very high-risk premenopausal women<sup>18</sup> as a health-care policy. In the United States, the Gail score<sup>3</sup> is a population-based model to estimate the 5 year lifetime risk of breast cancer, and defines high risk as 1.67 or above. Very high risk would be a Gail score of 3.5 or above<sup>18</sup>.

Raloxifene must now be considered to be the first multifunctional SERM. The medicine has successfully been tested for the treatment and prevention of osteoporosis<sup>19</sup>, and is available in many countries for that indication. An evaluation of breast cancer incidence in women treated with raloxifene for the prevention of osteoporosis<sup>7</sup> shows a 75% decrease in invasive breast cancer, and, as with tamoxifen, only the ER-positive disease is reduced. These data emphasize the fact that SERMs target the ER-mediated growth mechanism<sup>20</sup>. However, because the use of any medicine to prevent osteoporosis requires years of treatment, the original 4 year trial, referred to as the Multiple Outcomes for Raloxifene Evaluation (MORE)<sup>7,19</sup>, was extended to 8 years as the Continuing Outcomes Relative to Evista (CORE) trial. The breast cancer endpoint remained at about 65% inhibition of invasive breast cancer<sup>21</sup>. These data validate the original hypothesis that a non-steroidal anti-oestrogen in the same class as tamoxifen could be used not only to prevent osteoporosis but also to prevent breast cancer as a beneficial side effect<sup>5,6</sup>.

The MORE trial data provided the rationale for the National Surgical Adjuvant Breast and Bowel Project (NSABP) to test raloxifene (60 mg a day) against tamoxifen (20 mg a day) to reduce breast cancer incidence in high-risk postmenopausal women in the STAR trial. In addition, on the basis of the ability of raloxifene to lower circulating cholesterol (that is, low density lipoprotein cholesterol)<sup>22</sup>, the study Raloxifene use for the Heart (RUTH) was initiated as a placebo-controlled trial to determine

### Box 1 | Development of tamoxifen and raloxifene for chemoprevention

The recognition and development of selective oestrogen-receptor modulators culminated in the Study of Tamoxifen and Raloxifene (STAR)<sup>8</sup> clinical trial. This journey has taken about 30 years. The laboratory finding that tamoxifen prevented rat mammary carcinogenesis and was metabolically activated to 4-hydroxytamoxifen<sup>52</sup> (FIG. 2) provided an important lead for renewed structure activity relationship studies<sup>1</sup> that resulted in the description of raloxifene (LY156758, originally called keoxifene)<sup>85</sup>. The ‘anti-oestrogen’ failed its application as a breast cancer drug, but through the recognition of the bone-preserving properties of both tamoxifen and LY156758 (REF. 12), keoxifene was reinvented as raloxifene in the 1990s and successfully tested as a treatment and preventive for osteoporosis in postmenopausal women. The evolution of tamoxifen from a successful treatment for breast cancer to become the first agent to be used to prevent the development of cancer occurred throughout the 1980s and 1990s. The selective oestrogenic and anti-oestrogenic actions of tamoxifen in bone<sup>12</sup> and endometrial cancer<sup>14</sup> translated from the laboratory in the 1980s to clinical practice in the 1990s<sup>84</sup>. Pilot chemoprevention studies occurred first at the Royal Marsden Hospital (RMH), UK<sup>86</sup>, and studies on human bone density<sup>87</sup> at the University of Wisconsin Clinical Cancer Center (UWCCC), US. It is known that tamoxifen and raloxifene are both equivalent for preventing fractures and reducing the incidence of breast cancer<sup>8</sup>, but raloxifene does not increase the risk of endometrial cancer<sup>7,23</sup>, which tamoxifen was found to do in the National Surgical Adjuvant Breast and Bowel Project (NSABP)<sup>2</sup> and the International Breast Intervention Study (IBIS)<sup>88</sup>. Red outline denotes tamoxifen, black outline raloxifene, and blue outline both tamoxifen and raloxifene.



whether the incidence of coronary heart disease could be reduced.

The STAR trial showed equivalence between tamoxifen and raloxifene for reducing the risk of invasive breast cancer in high-risk postmenopausal women<sup>8</sup>. There were fewer cases of thromboembolic disorders, cataracts, cataract surgery, endometrial cancer and hysterectomy in patients who took raloxifene compared with tamoxifen. However, there seemed to be fewer cases of non-invasive breast cancer (ductal carcinoma *in situ* and lobular carcinoma *in situ*)<sup>8</sup> in the tamoxifen-treated group than the raloxifene-treated group, although the difference did not reach statistical significance. This might be associated with the reduced biological half life of raloxifene compared with tamoxifen (see below). Nevertheless, raloxifene is a safer agent than tamoxifen for use as a chemopreventive in high-risk postmenopausal women.

The RUTH trial failed to show a benefit in reducing deaths or hospitalizations

for coronary heart disease<sup>23</sup>. Clearly, the presumed benefit from raloxifene based on lipid lowering is incorrect in practice. However, the placebo-controlled trial did again show that raloxifene could significantly reduce the incidence of invasive breast cancer and not increase the risk of endometrial cancer<sup>23</sup>.

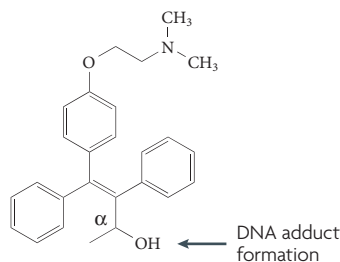
Therefore, the clinical evaluation of SERMs as chemopreventives for ER-positive breast cancer has shown that the concept is valid. However, the clinical use of these agents naturally aroused curiosity about how a simple model of oestrogen and anti-oestrogen action could explain the SERM phenomenon. The generally accepted principle in the 1980s was that oestrogens bound to the ER to initiate the transcription of genes and increase cell division and/or reduce cell death. A non-steroidal anti-oestrogen blocked the ER and some or all of the actions of oestradiol. The old model clearly could not cope with the new biology of the SERMs.

## Box 2 | Endometrial cancer and SERMs

The laboratory finding that tamoxifen encourages the growth of human endometrial cancer but blocks oestrogen-stimulated growth of breast cancer transplanted to the same athymic mouse<sup>14</sup> translated to clinical practice<sup>84,89</sup>. It is estimated that tamoxifen causes a 4–5-fold increase in the detection of endometrial cancers in postmenopausal women, but not in premenopausal women. The risk of developing endometrial cancer and dying is small compared with the lives saved during the treatment of breast cancer, but the concern in high-risk populations of the possibility of developing endometrial cancer during tamoxifen treatment is a significant factor in women declining chemoprevention. Raloxifene is less oestrogenic in the rodent uterus than tamoxifen, and does not increase the risk of endometrial cancer in postmenopausal women<sup>7,8,23</sup>.

**Mechanisms**

Unopposed oestrogen treatment is associated with a higher incidence of endometrial cancer in postmenopausal women. Recent studies suggest an association between oestrogen, endometrial cancer and the *SULT1A1\*2* allele that impairs sulphation so that oestrogen undergoes reduced local phase II metabolism<sup>90</sup> (for an explanation of phase II metabolism, see the Molecular Pharmacology of SERMs (selective oestrogen-receptor modulators) section of this Perspective). Tamoxifen can induce growth in endometrial cancer cells under laboratory conditions<sup>14</sup>, and as such could increase the detection of growing pre-existing disease in postmenopausal women<sup>91</sup>. The increase in gynaecological symptoms (such as bleeding) caused by tamoxifen might, in fact, lead to increased detection of pre-existing disease because patients are examined more rigorously. Tamoxifen-stimulated growth seems to result from the gene *PAX2* (paired box gene 2) becoming hypomethylated in endometrial cancer, and both oestrogen and tamoxifen can increase the proliferation and growth of endometrial tumours through an oestrogen receptor- $\alpha$  and *PAX2*-mediated mechanism<sup>92</sup>. Nevertheless, based on the fact that tamoxifen is a complete carcinogen in the rat liver, owing to the formation of DNA adducts by  $\alpha$ -hydroxytamoxifen (see figure), considerable effort has focused on identifying DNA adducts in humans<sup>93,94</sup>. No unifying molecular mechanism of causation is universally accepted. However, recent reports about the direct carcinogenic activity of oestrogen for the causation of breast cancer<sup>95,96</sup> might have some relevance to events in the uterus.



known, and co-repressor molecules also exist to prevent gene transcription by unliganded receptors<sup>37</sup>.

Naturally, the finding that there are two ERs has resulted in the synthesis of a range of receptor-specific ligands to switch a particular receptor on or off<sup>38</sup>. But, it is the external shape of the resulting complex that becomes the catalyst for changing the response to a SERM in a tissue target. Kraichely and co-workers<sup>39</sup> used new agonists for ER $\alpha$  and ER $\beta$  to detect subtle quantitative differences in their interaction with members of the SRC family (SRC 1, 2 and 3), but the molecular biology of SERM action is far more complex.

It is reasonable to ask how does the ligand programme the receptor complex to interact with other proteins? X-ray crystallography of the ligand-binding domains of the ER bound to either oestrogens or anti-oestrogens shows the potential of ligands to promote co-activator binding or prevent co-activator binding on the basis of the shape of the oestrogen or anti-oestrogen receptor complex<sup>40,41</sup>. Evidence has accumulated that the broad spectrum of ligands that bind to the OER can create a broad range of OER complexes that are either fully oestrogenic or anti-oestrogenic at a particular target site<sup>42</sup>. Therefore, a mechanistic model of oestrogen action and anti-oestrogen action (FIG. 1) has emerged based on the shape of the ligand that programmes the complex to adopt a particular shape that ultimately interacts with co-activators or co-repressors in target cells to determine the oestrogenic or anti-oestrogenic response, respectively. But how the response is initiated?

Not surprisingly, the co-activator model of steroid hormone action has increased in complexity, thereby amplifying the molecular mechanisms of modulation. It seems that co-activators are not simply protein partners that connect one site to another in a complex<sup>43</sup>. The co-activators actively participate in modifying the activity of the complex. The post translational modification of co-activators through many kinase pathways initiated by cell surface growth factor receptors (for example, 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 co-activator (such as *SRC3*) (FIG. 1) first recruits a specific set of co-co-activators (such as *p300* and ubiquitin-conjugating ligases) under the direction of many protein remodellers (for example, the peptidyl-prolyl isomerase *PIN1*, heat shock

**Molecular mechanisms of SERM action**

There are two ERs, referred to as ER $\alpha$  and ER $\beta$ <sup>24,26</sup>. The receptor proteins are encoded on different chromosomes and have homology as members of the steroid receptor superfamily, but there are distinct patterns of distribution and distinct and subtle differences in structure and ligand binding affinity<sup>27</sup>. An additional dimension that might be significant for tissue modulation is the ratio of ER $\alpha$  to ER $\beta$  at a target site. A high ER $\alpha$ –ER $\beta$  ratio correlates well with very high levels of cellular proliferation, whereas the predominance of functional ER $\beta$  over ER $\alpha$  correlates with low levels of proliferation<sup>28–31</sup>. The ratio of ERs in normal and neoplastic breast tissue might be an important factor for the long-term success of chemoprevention with SERMs.

There are functional differences between ER $\alpha$  and ER $\beta$  that can be traced to the differences in the activating function 1 (AF1) domain located in the N terminus of the ER. The amino-acid homology of AF1 is poorly conserved (only 20%). By contrast, the AF2 region located at the C terminus of the ligand-binding domain differs by only

one amino acid — D545 in ER $\alpha$  and N496 in ER $\beta$ . As the AF1 and AF2 regions are crucial for interaction with other co-regulatory proteins and gene transcription, the structural differences between them provides a clue about the potential functional differences between ER $\alpha$  and ER $\beta$ . Studies that used chimeras of ER $\alpha$  and ER $\beta$  by switching the AF1 regions showed that this region contributes to the cell-specific and promoter-specific differences in transcriptional activity. In general, SERMs can partially activate engineered genes regulated by an oestrogen-response element through ER $\alpha$  but not ER $\beta$ <sup>32–34</sup>. By contrast, 4-hydroxytamoxifen and raloxifene can stimulate activating protein-1 (*AP1*)-regulated reporter genes with both ER $\alpha$  and ER $\beta$  in a cell-dependent fashion<sup>35</sup>.

The simple model for oestrogen action, with either ER $\alpha$  or ER $\beta$  controlling oestrogen-regulated events, has now evolved into a fascinating mix of protein partners that have the potential to modulate gene transcription (FIG. 1). It is more than a decade since the first steroid receptor co-activator protein (*SRC1*) was first described<sup>36</sup>. Now dozens of co-activator molecules are

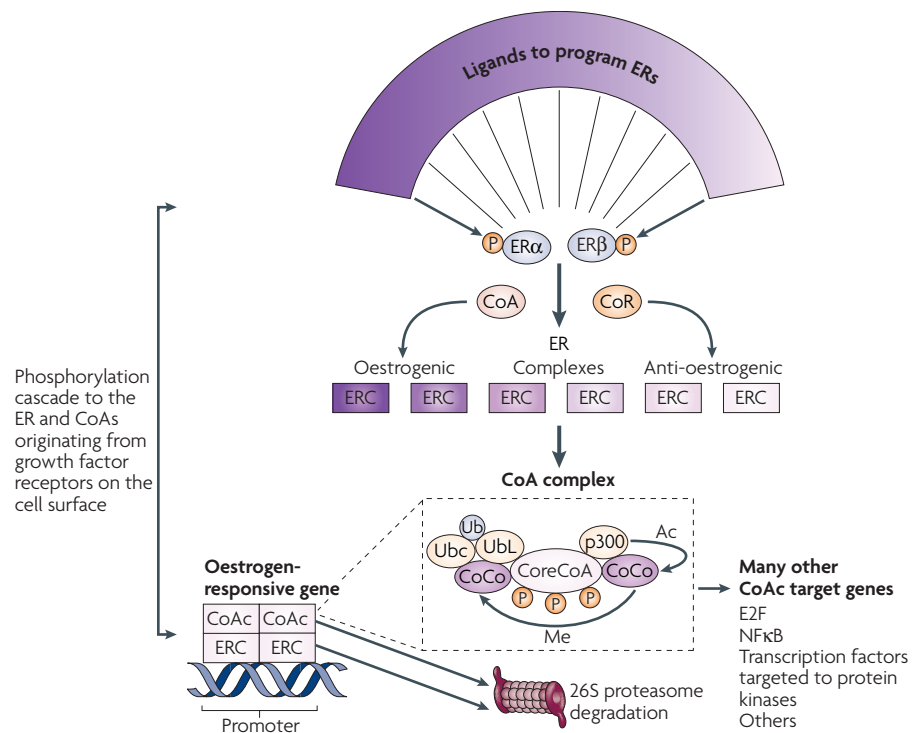
proteins and proteasome ATPases) to form a multi-protein co-activator complex that interacts with the phosphorylated ER at the specific gene-promoter site<sup>43</sup>. Most importantly, the proteins assembled by the core co-activator as the core co-activated complex have individual enzymatic activities to acetylate or methylate adjacent proteins. This results in the dissociation of the complex and simultaneous tagging with activated ubiquitin. The activated ubiquitin is transferred to the ubiquitin-conjugating enzyme that interacts with the ubiquitin ligase, which has already identified its protein target. Several cycles of the reaction can polyubiquitylate a substrate, that is, ER or a co-activator, to either be activated further (Lys63 linkage) or degraded by the 26S proteasome (Lys48 linkage)<sup>44</sup>, depending on the ubiquitin-ubiquitin linkage.

Therefore, for effective gene transcription programmed and targeted by the shape and phosphorylation status of the ER and co-activators, a dynamic and cyclic process of remodelling capacity is required for transcriptional assembly<sup>45</sup> that is immediately followed by the routine destruction of transcription complexes by the proteasome. Oestrogen and SERM receptor complexes have different accumulation patterns in the target cell nucleus<sup>46,47</sup>, primarily because the relative rates of destruction of the complexes are different<sup>48</sup>.

These fundamental mechanisms<sup>43,49</sup> can also be applied to the potential development of drug resistance to tamoxifen in breast cancer. Model systems have shown the conversion of the tamoxifen-ER complex from an anti-oestrogenic to an oestrogenic signal in an environment of increased phosphorylation caused by the overexpression of the ERBB2 cell-surface receptor and an increase in the SRC3 (AIB1) co-activation pool<sup>50,51</sup>. SRC3 probably increases independent of ERBB2 increases. However, the increased level of this co-activator and its increased phosphorylation state derived from an activated ERBB2 phosphorylation pattern will increase the oestrogen-like activity of tamoxifen at the ER. Clearly, issues of SERM action at target tissues and the eventual development of drug resistance in breast cancer will converge as the duration of SERM use extends from a few years to at least a decade.

### Molecular pharmacology of SERMs

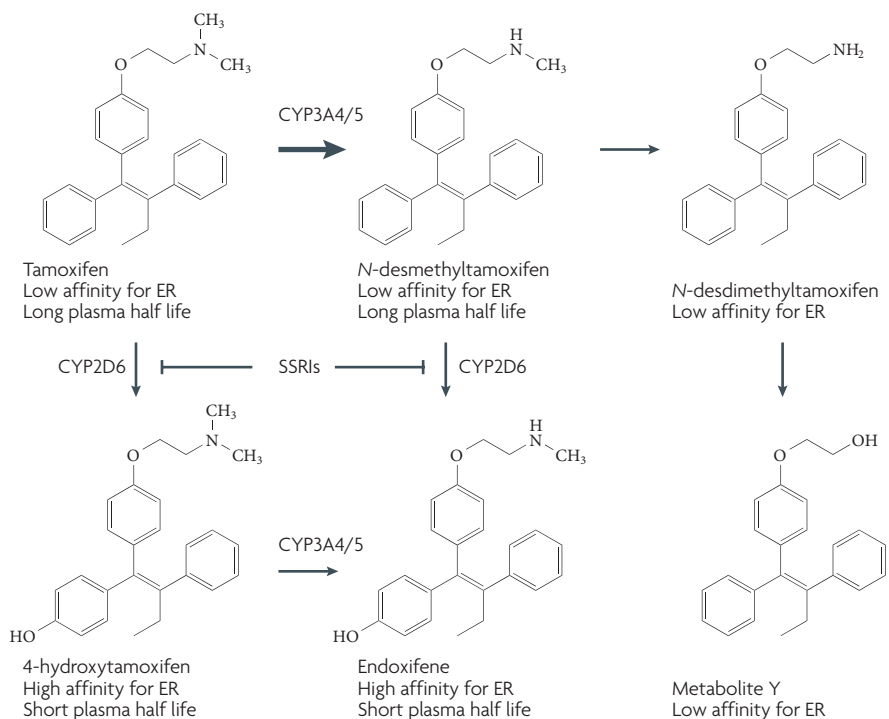
The metabolism, pharmacogenomics and pharmacokinetics of SERMs continue to present challenges. Just when everything



**Figure 1 | Molecular networks potentially influence the expression of SERM action in a target tissue.** The shape of the ligands that bind to the oestrogen receptors (ERs)α and β programmes the complex to become an oestrogenic or anti-oestrogenic signal. The context of the ER complex (ERC) can influence the expression of the response through the numbers of co-repressors (CoR) or co-activators (CoA). In simple terms, a site with few CoAs or high levels of CoRs might be a dominant anti-oestrogenic site. However, the expression of oestrogenic action is not simply the binding of the receptor complex to the promoter of the oestrogen-responsive gene, but a dynamic process of CoA complex assembly and destruction<sup>43</sup>. 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 oestrogen-responsive gene promoter to switch on transcription. The CoCo proteins then perform methylation (Me) and acetylation (Ac) to activate dissociation of the complex. Simultaneously, ubiquitinylation 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<sup>43</sup>. However, the co-activator, specifically SRC3, has ubiquitous action and can further modulate or amplify the ligand-activated trigger through many modulating genes<sup>101</sup> 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 oestrogen action and anti-oestrogen action on the basis of the shape of the ligand and the sophistication of the tissue-modulating network. NFκB, nuclear factor κB.

seems to be straightforward, old drugs create unanticipated surprises. Initially, there was little pharmacological information or interest in the metabolism of tamoxifen in animals or man; this was not a main requirement to register a drug to treat advanced breast cancer in the 1970s<sup>1</sup>. The situation remained the same during the 1980s, a time when tamoxifen was about to become the standard of care as the adjuvant anti-hormonal treatment of ER-positive breast cancer, and studies were planned to evaluate the worth of

tamoxifen to prevent breast cancer in high-risk women<sup>1</sup>. At that time, it was accepted that tamoxifen was either metabolically activated to 4-hydroxytamoxifen<sup>52,53</sup>, a minor metabolite with high binding affinity for the ER but with a short half life<sup>54</sup>, or demethylated to *N*-desmethyltamoxifen, a compound with low binding affinity for the ER but a long biological half life (FIG. 2). *N*-Desmethyltamoxifen was thought to be further demethylated to desdimethyltamoxifen, and subsequently deaminated to the weakly anti-oestrogenic glycol derivative



**Figure 2 | The metabolism of tamoxifen in humans.** Circulating drug levels of tamoxifen (20 mg a day) reach a steady state in about 3–4 weeks. The principal route of tamoxifen metabolism is through CYP3A4/5 (cytochrome P450, family 3, subfamily A, polypeptide 4/5) demethylation to *N*-desmethyltamoxifen, which has twice the serum half life as tamoxifen, and levels are usually 50–100% higher than those of tamoxifen. *N*-Desmethyltamoxifen is further demethylated to desdimethyltamoxifen and then deaminated to metabolite Y, a glycol derivative. Tamoxifen and *N*-desmethyltamoxifen are both para-hydroxylated by CYP2D6 to form 4-hydroxytamoxifen and endoxifene, respectively. Levels of endoxifene are twice those seen for 4-hydroxytamoxifen, which in turn are about 5% of those observed for tamoxifen. Specific selective serotonin reuptake inhibitors (SSRIs) (BOX 3) bind to CYP2D6 to prevent the hydroxylation of tamoxifen and *N*-desmethyltamoxifen. This prevents the metabolic activation of tamoxifen. ER, oestrogen receptor.

of tamoxifen referred to as metabolite Y<sup>55</sup>. These anti-oestrogenic metabolites deactivate the ER, but based on concentrations in SERM and affinity, all were considered to have a role in blocking oestrogen action.

The ubiquitous application of tamoxifen as a long-term, well-tolerated treatment for breast cancer during the past decade, and its use as a preventive in high-risk women, resulted in the close examination of symptom management, especially hot flashes, to increase compliance. Selective serotonin reuptake inhibitors (SSRIs) (BOX 3) are effective at controlling the hot flashes experienced by up to 45% of patients treated with tamoxifen. However, the recent identification and characterization<sup>56–58</sup> of the high-affinity metabolite of tamoxifen, 4-hydroxy-*N*-desmethyltamoxifen (endoxifene), and the finding that endoxifene levels are reduced by the co-administration of SSRIs<sup>59–61</sup>, is an important observation that has potential

therapeutic implications. It follows that as SSRIs block CYP2D6 (cytochrome P450, family 2, subfamily D, polypeptide 6), therefore inhibiting the metabolism of tamoxifen to endoxifene, then the efficacy of tamoxifen as an anticancer agent (treatment or chemopreventive) 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 endoxifene. Preliminary evidence suggests that this might be the case<sup>61,62</sup>.

Knowledge of the metabolic activation of tamoxifen to hydroxylated metabolites with high affinity for the ER created an opportunity to design the high affinity SERMs, raloxifene, basedoxifene and lasofoxifene (BOX 4). However, the pharmacokinetics and pharmacodynamics of these polyphenolic compounds make it difficult to deliver sufficient concentrations to the breast tissue of healthy women. Raloxifene

and other SERM members that are benzothiophene derivatives are short acting<sup>13,63,64</sup>. However, raloxifene has a plasma elimination half-life of about 27 hours, which apparently results from reversible phase II metabolism, which conjugates the polyphenolic drugs before their excretion as sulphates and glucuronides.

There seem to be two aspects to be considered for a polyphenolic SERM to be an effective chemopreventive for breast cancer. First, raloxifene is conjugated by the human intestinal enzymes UDP-glucuronosyltransferase 1A8 (UGT1A8) and UGT1A10 (REF. 65), but it is the dynamic relationship between absorption, phase II metabolism and excretion in the intestine<sup>66</sup> that controls the 2% bioavailability of raloxifene<sup>64</sup>. The second aspect for consideration is the retention of raloxifene in the target tissue. This depends on local sulphation, which inactivates the SERM before diffusion out of the tissue. Here again, there are disparities in the efficacy of multiple-sulphation enzymes (sulphotransferases, SULTs) to terminate the bioactivity of raloxifene in a target site. For example, 4-hydroxytamoxifen<sup>52</sup> is only sulphated by three of seven SULT isoforms, whereas raloxifene is sulphated by all seven<sup>67</sup>. In addition, SULT1E1, which sulphates raloxifene in endometrial tissue, is only expressed in the secretory phase<sup>67</sup> of the menstrual cycle after ovulation. An alternative to designing phase II metabolism-insensitive SERMs is to create a long-acting SERM with protected phenolic groups that require metabolic activation. Arzoxifene (BOX 4) is a methoxy derivative of raloxifene<sup>68</sup> that is superior to raloxifene as a chemopreventive for rat mammary carcinoma<sup>68</sup>. The SERM shows activity for the treatment of ER-positive metastatic breast cancer, but in two trials a low dose (20 mg a day) was superior to a high dose (50 mg a day)<sup>69,70</sup>. Arzoxifene is completing evaluation as a treatment and preventive for osteoporosis, and trials to determine its efficacy for the prevention of breast and endometrial cancer are eagerly awaited.

#### Other chemoprevention strategies

If oestrogen is responsible for the development and growth of breast cancer, it is only natural to consider that a 'no oestrogen' state would be the ultimate chemopreventive for breast cancer. The current agents of choice to prevent oestrogen synthesis in postmenopausal women are referred to as aromatase inhibitors. These drugs block the conversion of steroidal precursors, androstenedione and testosterone, to oestrogens by the CYP19 aromatase enzyme in a woman's body fat,

as well as breast epithelial and stromal cells. Several different aromatase inhibitors are available (for example, anastrozole, letrozole and exemestane), and have shown increased superiority against tamoxifen for the treatment of breast cancer. Aromatase inhibitors have few side effects, specifically blood clots or endometrial cancer, when studies<sup>71–75</sup> have compared and contrasted their efficacy and safety against tamoxifen for the adjuvant treatment of breast cancer in postmenopausal women. Nevertheless, the fact that it is not possible to determine precisely whom to select for chemoprevention means that many postmenopausal women who will never develop breast cancer will be treated with aromatase inhibitors to benefit a few. In other words, based on the population of the STAR trial<sup>8</sup>, if 1,000 very high-risk postmenopausal women were selected that might develop 10 breast cancers (8 of these would be ER-positive and receptive to therapy) during a year, then 992 women would be treated unnecessarily to prevent 8 ER-positive tumours. This is clearly inappropriate as a public-health policy.

Nevertheless, the use of aromatase inhibitors as chemopreventive agents in postmenopausal women is currently being evaluated in clinical trials. The scientific rationale is valid on the basis of the reduced incidence of contralateral breast cancer noted in trials of adjuvant therapy<sup>71–75</sup>. A trial by the NSABP is currently comparing raloxifene with the aromatase inhibitor letrozole, the International Breast Intervention Study 2 (IBIS-2) is comparing anastrozole with placebo<sup>76</sup>, and the National Cancer Institute of Canada is comparing exemestane with placebo (MAP3)<sup>77</sup>. Apart from the inability to appropriately target high-risk women, there is a need to monitor bone density and institute supplemental treatment, where necessary, with bisphosphonates. Bisphosphonates are analogues of pyrophosphate that have their central oxygen atom replaced by carbon. They form a three-dimensional structure that chelates calcium in bone and prevents resorption<sup>78</sup>. At present, the high cost of aromatase inhibitors with associated monitoring might be too expensive for a routine government-sponsored health-care intervention.

To avoid the concerns about a general decrease in circulating oestrogen produced by current aromatase inhibitors, there is interest in determining whether breast-specific inhibitors could be developed. The idea is to create a selective aromatase inhibitor to exploit the observation that several tissue-specific promoter regions have been identi-

### Box 3 | SSRIs

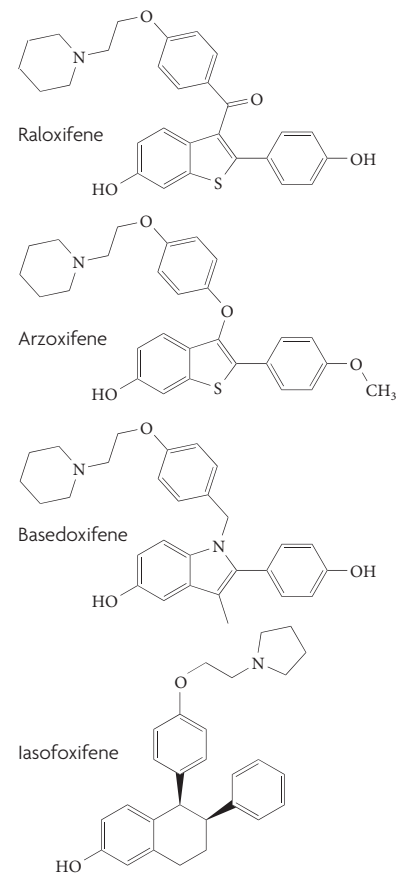
Selective serotonin reuptake inhibitors (SSRIs) can be used to treat depression associated with general medical illness, but more specifically hot flashes and associated menopausal symptoms. The SSRIs specifically target serotonin-containing neurons. Serotonin is synthesized from L-tryptophan by hydroxylation, and subsequently deaminated to 5-hydroxytryptamine (5HT) or serotonin. The release of 5HT causes an interaction with a wide range of 5HT receptors that are both autoinhibitory and stimulatory. The long-term use of SSRIs prevents the reuptake of 5HT from the synapse into the presynaptic serotonin neuron, where secondary deamination and inactivation occurs through monoamine oxidase. The inability to remove and destroy 5HT causes neuronal desensitization. Most importantly, some of the SSRIs (fluoxetine and paroxetine) are potent inhibitors of the CYP2D6 (cytochrome P450, family 2, subfamily D, polypeptide 6) enzyme, that along with CYP1A2 mediates hydroxylation of the aromatic ring on tamoxifen. Ongoing studies show that potent inhibitors of CYP2D6 in the SSRI family significantly decrease levels of the tamoxifen metabolite endoxifene<sup>60,61</sup>. Evidence is available that venlafaxine (Effexor) does not lower endoxifene levels, but these conclusions<sup>61</sup> need to be confirmed with a larger study.

fied upstream of the *CYP19* gene<sup>79,80</sup>. As an example of progress with exploiting the possibility of tissue selectivity, the orphan nuclear receptor liver receptor homolog-1 (LRH1) is a specific transcription activator of aromatase in breast pre-adipocytes<sup>81</sup>. Clearly LRH1 would be an interesting therapeutic target. It is known that prostaglandin E<sub>2</sub> is an important regulator of aromatase expression in breast cancer, and

the non-steroidal anti-inflammatory drugs that inhibit COX1 and COX2 also suppress aromatase activity<sup>82</sup>. The authors of the study noted<sup>82</sup> that a COX2 inhibitor was particularly active. However, recently a new series of sulphonilide analogues has been reported that suppress aromatase activity independent of COX2 (REF. 83) in breast cancer cells. Clearly, there is enormous potential for drug development.

### Box 4 | New SERMs

Several new selective oestrogen-receptor modulators (SERMs) are being evaluated at present in clinical trials for the prevention of osteoporosis. Arzoxifene<sup>68</sup> and basedoxifene<sup>97,98</sup> bear a striking resemblance to the structure of raloxifene. By contrast, lasofoxifene<sup>99,100</sup> is a derivative of the non-steroidal anti-oestrogen nafoxidine that was tested as a breast cancer drug in the 1970s but was not pursued because most patients experienced light sensitivity<sup>1</sup>. The new molecule is the L-enantiomer that has 20 times the binding affinity for the oestrogen receptor (ER) as the D-enantiomer, and the L-enantiomer has twice the bioavailability of the D-enantiomer<sup>99</sup>. The difference is believed to be the result of enantioselective glucuronidation of the L-enantiomer. The fact that lasofoxifene and basedoxifene are polyphenolic compounds that are both susceptible to phase II metabolism might make the transition from treatment for osteoporosis to a widely used chemopreventive more challenging. If the SERMs are poorly bioavailable because of first-pass metabolism in the liver, then the medicine might still perform well as an anti-tumour agent in the low-oestrogen environment observed in osteoporotic postmenopausal women. By contrast, if the SERMs are used to prevent breast cancer in healthy postmenopausal women with high levels of body fat, this will create an environment of high circulating oestrogen levels. As a result, the low bioavailability will be unable to constantly block the breast from oestrogen action.



**Chemoprevention today**

It is now possible to select women who will benefit from taking SERMs to reduce the risk of breast cancer. It should be obvious that as a group, the SERMs are more flexible than other drug classes such as the aromatase inhibitors, which are restricted to breast cancer in postmenopausal women. The reason for this is that constitutive oestrogen synthesis occurs in peripheral body fat in postmenopausal women, and this can be blocked by aromatase inhibitors. By contrast, oestrogen synthesis in the ovaries of premenopausal women is regulated by gonadotrophins during the menstrual cycle. Decreases in circulating oestrogen that could occur with an aromatase inhibitor are immediately increased through a compensatory increase of gonadotrophins. Decreases in circulating oestrogen are detected at the hypothalamo–pituitary axis, thereby activating the oestrogen-regulated negative feed–back loop for the increased secretion of gonadotrophins. Therefore, the action of a competitive inhibitor of aromatase is reversed. A future question is whether the efficacy of a suicide inhibitor such as exemestane would be superior to a SERM.

Knowledge of the beneficial risk–benefit ratio of tamoxifen in premenopausal women<sup>4</sup> must now be melded with knowledge that tamoxifen must be given for at least 5 years for effective chemoprevention in premenopausal women. This is the current standard of care. However, the increased incidence of side effects, such as menopausal symptoms of hot flashes, often becomes a real challenge to maintaining patient compliance. If the drug is stopped there will be no benefit. Unfortunately, if an inappropriate SSRI is prescribed to reduce hot flashes the efficacy of the chemoprevention strategy will be undermined, as the SSRI blocks endoxifene production by CYP2D6 (REFS 61,62) (BOX 3, FIG. 2). In addition, if a woman has a non-enzymatic variant of CYP2D6, then there is potentially no value to tamoxifen treatment. Advances in our knowledge of drug interactions and pharmacogenetics are proving to be valuable for targeting tamoxifen to the appropriate premenopausal woman.

Raloxifene now seems to offer real benefits in reducing the incidence of breast cancer in high-risk postmenopausal populations<sup>8</sup> or in women already receiving raloxifene for the prevention of osteoporosis<sup>21</sup>. Although aromatase inhibitors might, at a later date, offer advantages over raloxifene as a breast cancer preventive, the additional monitoring of patient bone density and intervention with

a bisphosphonate will require additional medical resources.

Finally, it is important to stress that SERMs are not static, but as a drug group continue to evolve as new members are tested and introduced into clinical practice (BOX 4). Furthermore, the principles used<sup>5,6</sup> to create SERMs are now being applied to other members of the steroid receptor superfamily<sup>37</sup> so that the required target site effect of a particular hormone can be switched on or off. This advance holds the promise of an expanded menu of medicines to address the selective treatment and prevention of many diseases once thought to be impossible.

V. Craig Jordan is at the Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, Pennsylvania 19111-2497, USA. e-mail: v.craig.jordan@fccc.edu

doi:10.1038/nrc2048

- Jordan, V. C. Tamoxifen: a most unlikely pioneering medicine. *Nature Rev. Drug Discovery* **2**, 205–213 (2003).
- Fisher, B. *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.* **90**, 1371–1388 (1998).
- Gail, M. H. *et al.* Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J. Natl Cancer Inst.* **81**, 1879–1886 (1989).
- Gail, M. H. *et al.* Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J. Natl Cancer Inst.* **91**, 1829–1846 (1999).
- Jordan, V. C. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest.* **6**, 589–595 (1988).
- Lerner, L. J. & Jordan, V. C. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res.* **50**, 4177–4189 (1990).
- Cummings, S. R. *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* **281**, 2189–2197 (1999).
- Vogel, V. G. *et al.* The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA* **295**, 2727–2741 (2006).
- Jordan, V. C. Selective estrogen receptor modulation: a personal perspective. *Cancer Res.* **61**, 5683–5687 (2001).
- Jordan, V. C. & Robinson, S. P. Species-specific pharmacology of antiestrogens: role of metabolism. *Fed. Proc.* **46**, 1870–1874 (1987).
- Black, L. J., Jones, C. D. & Falcone, J. F. Antagonism of estrogen action with a new benzothioephene derived antiestrogen. *Life Sci.* **32**, 1031–1036 (1983).
- Jordan, V. C., Phelps, E. & Lindgren, J. U. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res. Treat.* **10**, 31–35 (1987).
- Gottardis, M. M. & Jordan, V. C. Antitumor actions of keoxifen and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res.* **47**, 4020–4024 (1987).
- Gottardis, M. M., Robinson, S. P., Satyasarwoop, P. G. & Jordan, V. C. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res.* **48**, 812–815 (1988).
- Gottardis, M. M., Ricchio, M. E., Satyasarwoop, P. G. & Jordan, V. C. 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 (1990).
- Fisher, B. *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.* **97**, 1652–1662 (2005).
- 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* **365**, 1687–1717 (2005).
- Melnikow, J. *et al.* Chemoprevention: drug pricing and mortality: the case of tamoxifen. *Cancer* **107**, 950–958 (2006).
- Ettinger, B. *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* **282**, 637–645 (1999).
- Jensen, E. V. & Jordan, V. C. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. *Clin. Cancer Res.* **9**, 1980–1989 (2003).
- Martino, S. *et al.* For the CORE investigators. 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 (2004).
- Walsh, B. W. *et al.* Effects of raloxifene on serum lipids and coagulation factors in healthy postmenopausal women. *JAMA* **279**, 1445–1451 (1998).
- Barrett-Connor, E. *et al.* Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N. Engl J. Med.* **355**, 125–137 (2006).
- Jensen, E. V. & Jacobson, H. I. Basic guides to the mechanism of estrogen action. *Recent Progr. Hormone Res.* **18**, 387–414 (1962).
- Greene, G. L. *et al.* Sequence and expression of human estrogen receptor complementary DNA. *Science* **231**, 1150–1154 (1986).
- Kuiper, G. G., Enmark, E., Peltö-Huikko, M., Nilsson, S. & Gustafsson, J. A. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl Acad. Sci. USA* **93**, 5925–5930 (1996).
- Kuiper, G. G. *et al.* Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138**, 863–870 (1997).
- Roger, P. *et al.* Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. *Cancer Res.* **61**, 2537–2541 (2001).
- Shaaban, A. M. *et al.* Declining estrogen receptor-beta expression defines malignant progression of human breast neoplasia. *Am. J. Surg. Pathol.* **27**, 1502–1512 (2003).
- Paruthiyil, S. *et al.* Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. *Cancer Res.* **64**, 425–428 (2004).
- Aconcia, F. *et al.* Survival versus apoptotic 17beta-estradiol effect: role of ER alpha and ER beta activated non-genomic signaling. *J. Cell Physiol.* **203**, 193–201 (2005).
- McInerney, E. M., Weis, K. E., Sun, J., Mosselman, S. & Katzenellenbogen, B. S. Transcription activation by the human estrogen receptor subtype beta (ER beta) studied with ER beta and ER alpha receptor chimeras. *Endocrinology* **139**, 4513–4522 (1998).
- Barkhem, T. *et al.* Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. *Mol. Pharmacol.* **54**, 105–112 (1998).
- Hall, J. M. & McDonnell, D. P. 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* **140**, 5566–5578 (1999).
- Paech, K. *et al.* Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* **277**, 1508–1510 (1997).
- Onate, S. A., Tsai, S. Y., Tsai, M. J. & O'Malley, B. W. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270**, 1354–1357 (1995).
- Smith, C. L. & O'Malley, B. W. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocrine Rev.* **25**, 45–71 (2004).
- Jordan, V. C. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *J. Med. Chem.* **46**, 1081–1111 (2003).
- Kraichely, D. M., Sun, J., Katzenellenbogen, J. A. & Katzenellenbogen, B. S. 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* **141**, 3534–3545 (2000).
40. Shiau, A. K. *et al.* The structural basis of estrogen receptor/co-activator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**, 927–937 (1998).
  41. Brzozowski, A. M. *et al.* Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389**, 753–758 (1997).
  42. Wijayaratne, A. L. *et al.* Comparative analyses of mechanistic differences among antiestrogens. *Endocrinology* **140**, 5828–5840 (1999).
  43. Lonard, D. M. & O'Malley, B. W. The expanding cosmos of nuclear receptor coactivators. *Cell* **125**, 411–414 (2006).
  44. Sun, Y. E3 Ubiquitin Ligases as Cancer Targets and Biomarkers. *Neoplasia* **8**, 645–654 (2006).
  45. Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A. & Brown, M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* **103**, 843–852 (2000).
  46. Jordan, V. C., Dix, C. J., Rowsby, L. & Prestwich, G. Studies on the mechanism of action of the nonsteroidal antiestrogen tamoxifen (I. C. I. **46**, 474) in the rat. *Mol. Cell. Endocrinol.* **7**, 177–192 (1977).
  47. Horwitz, K. B. & McGuire, W. L. Nuclear mechanisms of estrogen action. Effects of estradiol and antiestrogens on estrogen receptors and nuclear receptor processing. *J. Biol. Chem.* **253**, 8185–8191 (1978).
  48. Wijayaratne, A. L. & McDonnell, D. P. 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.* **276**, 35684–35692 (2001).
  49. Wu, R. C., Smith, C. L. & O'Malley, B. W. Transcriptional regulation by steroid receptor coactivator phosphorylation. *Endocrine Rev.* **26**, 393–399 (2006).
  50. Osborne, C. K. *et al.* Role of the estrogen receptor coactivator AIB1 (SRC3) and HER2/neu in tamoxifen resistance in breast cancer. *J. Natl Cancer Inst.* **95**, 353–361 (2003).
  51. Shou, J. *et al.* Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J. Natl Cancer Inst.* **96**, 926–935 (2004).
  52. Jordan, V. C., Collins, M. M., Rowsby, L. & Prestwich, G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J. Endocrinol.* **75**, 305–316 (1977).
  53. Allen, K. E., Clark, E. R. & Jordan, V. C. Evidence for the metabolic activation of non-steroidal antiestrogens: a study of structure-activity relationships. *Br. J. Pharmacol.* **71**, 83–91 (1980).
  54. Jordan, V. C. & Allen, K. E. Evaluation of the antitumour activity of the non-steroidal antiestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur. J. Cancer* **16**, 239–251 (1980).
  55. Jordan, V. C. *et al.* Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient during therapy for advanced breast cancer. *Cancer Res.* **43**, 1446–1450 (1983).
  56. Johnson, M. D. *et al.* Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. *Breast Cancer Res. Treat.* **85**, 151–159 (2004).
  57. Lim, Y. C., Desta, Z., Flockhart, D. A. & Skaar, T. C. 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 (2005).
  58. Lim, Y. C. *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.* **318**, 503–512 (2006).
  59. Stearns, V. *et al.* Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. *J. Natl Cancer Inst.* **95**, 1758–1764 (2003).
  60. Jin, Y. *et al.* CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. *J. Natl Cancer Inst.* **97**, 30–39 (2005).
  61. Borges, S. *et al.* Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. *Clin. Pharmacol. Ther.* **80**, 61–74 (2006).
  62. Goetz, M. P. *et al.* Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. *J. Clin. Oncol.* **23**, 9312–9318 (2005).
  63. Jordan, V. C. & Gosden, B. Inhibition of the uterotrophic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. *Endocrinology* **113**, 463–468 (1983).
  64. Snyder, K. R., Sparano, N. & Malinowski, J. M. Raloxifene hydrochloride. *Am. J. Health Syst. Pharm.* **57**, 1669–1675 (2000).
  65. Kemp, D. C., Fan, P. W. & Stevens, J. C. Characterization of raloxifene glucuronidation *in vitro*: contribution of intestinal metabolism to presystemic clearance. *Drug Metab. Dispos.* **30**, 694–700 (2002).
  66. Jeong, E. J., Lin, H. & Hu, M. Disposition mechanisms of raloxifene in the human intestinal Caco-2 model. *J. Pharmacol. Exp. Ther.* **310**, 376–385 (2004).
  67. Falany, J. L., Pilloff, D. E., Leyh, T. S. & Falany, C. N. Sulfation of raloxifene and 4-hydroxytamoxifen by human cytosolic sulfotransferases. *Drug Metab. Dispos.* **34**, 361–368 (2006).
  68. Suh, N. *et al.* Arzoxifene, a new selective estrogen receptor modulator for chemoprevention of experimental breast cancer. *Cancer Res.* **61**, 8412–8415 (2001).
  69. Baselga, J. *et al.* 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 (2003).
  70. Buzdar, A. *et al.* 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 (2003).
  71. ATAC Trialists' Group. 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 randomized trial. *Lancet* **359**, 2134–3139 (2002).
  72. Goss, P. E. *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.* **349**, 1–10 (2003).
  73. Howell, A. *et al.* 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 (2005).
  74. Coombes, R. C. *et al.* 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 (2004).
  75. Thurlimann, B. *et al.* A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N. Engl. J. Med.* **353**, 2747–2757 (2005).
  76. Goss, P. E. Breast cancer prevention—clinical trials strategies involving aromatase inhibitors. *J. Steroid Biochem. Mol. Biol.* **86**, 487–493 (2003).
  77. Goss, P. E. & Strasser-Weippl, K. Prevention strategies with aromatase inhibitors. *Clin. Cancer Res.* **10**, 3725–3795 (2004).
  78. Licata, A. A. Discovery, clinical development, and therapeutic uses of bisphosphonates. *Ann. Pharmacother.* **39**, 668–677 (2005).
  79. Simpson, E. R. *et al.* Tissue-specific promoters regulate aromatase cytochrome P450 expression. *J. Steroid Biochem. Mol. Biol.* **44**, 321–330 (1993).
  80. Simpson, E., Clyne, C., Rubin, G. *et al.* Aromatase—a brief overview. *Annu. Rev. Physiol.* **64**, 93–127 (2002).
  81. Safi, R. *et al.* Coactivation of liver receptor homologue-1 by peroxisome proliferator-activated receptor gamma coactivator-1 $\alpha$  on aromatase promoter II and its inhibition by activated retinoid X receptor suggest a novel target for breast-specific antiestrogen therapy. *Cancer Res.* **65**, 11762–11770 (2005).
  82. Diaz-Cruz, E. S., Shapiro, C. L. & Brueggemeier, R. W. Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells. *J. Clin. Endocrinol. Metab.* **90**, 2563–2570 (2005).
  83. Su, B., Diaz-Cr, E. S., Landini, S. & Brueggemeier, R. W. Novel sulfonanilide analogues suppress aromatase expression and activity in breast cancer cells independent of COX-2 inhibition. *J. Med. Chem.* **49**, 1413–1419 (2006).
  84. Fornander, T. *et al.* Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* **1**, 117–120 (1989).
  85. Black, L. J., Jones, C. D. & Falcone, J. F. Antagonism of estrogen action with a new benzothioephene derived antiestrogen. *Life Sci.* **32**, 1031–1036 (1983).
  86. Powles, T. J. *et al.* A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *Br. J. Cancer* **60**, 126–131 (1989).
  87. Love, R. R. *et al.* Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N. Engl. J. Med.* **326**, 852–856 (1992).
  88. Cuzick, J. *et al.* First results from the International Breast Cancer Intervention Study (IBIS-1): a randomised prevention trial. *Lancet* **360**, 817–824 (2002).
  89. Fisher, B. *et al.* 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 (1994).
  90. Rebbeck, T. R. *et al.* Estrogen sulfation genes, hormone replacement therapy, and endometrial cancer risk. *J. Natl Cancer Inst.* **98**, 1311–1320 (2006).
  91. Horwitz, R. I., Feinstein, A. R., Horwitz, S. M. & Robboy, S. J. Necropsy diagnosis of endometrial cancer and detection-bias in case/control studies. *Lancet* **2**, 66–68 (1981).
  92. Wu, H. *et al.* Hypomethylation-linked activation of PAX2 mediates tamoxifen-stimulated endometrial carcinogenesis. *Nature* **438**, 981–987 (2005).
  93. Phillips, D. H., Hewer, A., Grover, P. L., Poon, G. K. & Carmichael, P. L. Tamoxifen does not form detectable DNA adducts in white blood cells of breast cancer patients. *Carcinogenesis* **17**, 1149–1152 (1996).
  94. Phillips, D. H. Understanding the genotoxicity of tamoxifen? *Carcinogenesis* **22**, 839–849 (2001).
  95. Cavalieri, E. *et al.* Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. *Biochim. Biophys. Acta* **1766**, 63–78 (2006).
  96. Russo, J. *et al.* 17 $\beta$ -Estradiol induces transformation and tumorigenesis in human breast epithelial cells. *Faseb J.* **20**, 1622–1634 (2006).
  97. Miller, C. P. *et al.* Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. *J. Med. Chem.* **44**, 1654–1657 (2001).
  98. Greenberger, L. M. *et al.* A new antiestrogen, 2-[4-(4-hydroxy-phenyl)-3-methyl-1-[4-(2-piperidin-1-yl-ethoxy)-benzyl]-1H-in dol-5-ol hydrochloride (ERA-923), inhibits the growth of tamoxifen-sensitive and-resistant tumors and is devoid of uterotrophic effects in mice and rats. *Clin. Cancer Res.* **7**, 3166–3177 (2001).
  99. Rosati, R. L. *et al.* Discovery and preclinical pharmacology of a novel, potent, nonsteroidal estrogen receptor agonist/antagonist, CP-336156, a diaryl-tetrahydronaphthalene. *J. Med. Chem.* **41**, 2928–2931 (1998).
  100. Cohen, L. A. *et al.* Lasofoxifene, 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 (2001).
  101. O'Malley, B. W. Little molecules with big goals. *Science* **313**, 1749–1750 (2006).

#### Acknowledgements

V.C.J. is supported by the Department of Defense Breast Program (views and opinions of, and endorsements by the author do not reflect those of the US Army or the Department of Defense), SPORE in Breast Cancer, Fox Chase Cancer Center (FCCC) Core Grant, the Avon Foundation and the Weg Fund, and the Alfred G. Knudson Chair in Cancer Research of the FCCC.

#### Competing interests statement

The author declares no competing financial interests.

#### DATABASES

The following terms in this article are linked online to: Entrez Gene: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>  
 AP1 | COX1 | COX2 | CYP2D6 | CYP19 | ERBB2 | ER $\alpha$  | ER $\beta$  | p300 | PIN1 | SRC1 | SRC3  
 Access to this interactive links box is free online.

## SERMs: Meeting the Promise of Multifunctional Medicines

V. Craig Jordan

The successful development and clinical evaluation of the selective estrogen receptor modulators in the Study of Tamoxifen and Raloxifene trial provides an occasion to reflect on the milestone that has been achieved and the potential for further progress in the chemoprevention of breast cancer. The evolution of tamoxifen from a successful treatment for breast cancer to the first chemopreventive for any cancer took two decades. Clinicians gained an enormous amount of experience with the use of tamoxifen as a treatment, and, as a result, there were few surprises in terms of efficacy or the side effect profile when the medicine was used to prevent breast cancer in high-risk women. In contrast, raloxifene emerged via the novel path of the evidence-based hypothesis that a drug targeted at one disease, osteoporosis, could also prevent breast cancer. Changes in health care strategies to implement chemoprevention take time, but the evidence now suggests that chemoprevention has become a reality in clinical practice.

J Natl Cancer Inst 2007;99:351–7

With declining investment in cancer research and reluctance by the pharmaceutical industry to address prevention, why must the chemoprevention of breast cancer remain a priority? The disease has a high incidence, with an estimated 1 million women worldwide diagnosed with breast cancer annually. Solid tumors are difficult to control, but it can be argued that substantial progress is being made with targeted therapies for breast cancer that save lives. Treatments targeting the tumor estrogen receptor (ER) (1) or the growth factor receptor HER2/neu (2) confer statistically significant survival advantages in clinical trials (3–7). In the case of tamoxifen, there is evidence that the drug has contributed to the reduction in national death rates from breast cancer (4). With the experience gained from the successful treatment of breast cancer with targeted antiestrogens, it should be obvious to the casual observer that the application of the same principle—in the right women at the right time—to prevent the development of the disease would provide much needed relief for a society burdened with an overextended and overexpensive health care system.

However, it would be naive to expect that the medical science community can prevent breast cancer at a single stroke. The lessons of the Study of Tamoxifen and Raloxifene (STAR) (8) trial show that progress is slow, often unpredictable, and, at least in the case of STAR trial, dependent on three factors: good ideas that translate from the laboratory to the clinic, fashions in research, and the development of a patenting strategy that ensures exclusivity for a company during clinical testing.

The practical application of molecular theory to the prevention of cancer requires collaborative teams from multiple disciplines to translate a concept into lives saved. Unfortunately, society has erected an artificial barrier to achieving success in chemoprevention. This is because in assessing the successfulness of a new treatment for breast cancer, the benchmark is “lives saved”. In chemoprevention, it may take a generation to quantify “lives saved”, but if the medicine is safe and there is a dramatic reduction in the incidence of breast cancer, it follows that the treatment must ultimately reduce the death rate from breast cancer. Most importantly, the medicine used for chemoprevention should have

minimal side effects to ensure compliance. To achieve the goal of chemoprevention, prospective clinical trials must demonstrate advantages over current approved therapies or the traditional “wait and see” approach with routine screening.

Once the concept of chemoprevention becomes a reality and an agent is proven to reduce the risk of breast cancer, the cost–benefit ratio to the health care system must be advantageous. Only half the women who develop breast cancer can be identified using the Gail model (9), and identification of specific women for intervention is based on large populations with only a small percentage of women developing the disease. At present, therefore, large numbers of women must be treated to benefit the few. As a result, the preventive treatment must be of low cost, highly effective, and without serious side effects if health care is to be improved, and these qualities are critical to widespread acceptance by National Managed Health Care systems.

The feasibility of reducing breast cancer incidence in high-risk pre- and postmenopausal women has been established by the pioneering work of Fisher et al. and the National Surgical Adjuvant Breast and Bowel Project (NSABP) P-1 Study (10,11). Tamoxifen administered at a daily dose of 20 mg for 5 years reduced breast cancer incidence by about 50%. This milestone achievement in translational research was the result of a century of laboratory and clinical studies to understand the genesis of breast cancer (12). STAR trial now establishes the practicality of chemoprevention of breast cancer, but the knockout blow has yet to be delivered. My purpose in this commentary is to explain how cancer research evolved to result in the STAR trial and to

---

**Correspondence to:** V. Craig Jordan, OBE, PhD, DSc, Fox Chase Cancer Center, 333 Cottman Ave, Philadelphia, PA 19111 (e-mail: v.craig.jordan@fccc.edu).

**See** “Notes” following “References.”

**DOI:** 10.1093/jnci/djk062

© 2007 The Author(s).

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/2.0/uk/>), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

present options available to future progress in breast cancer chemoprevention.

### **Tamoxifen, the First Selective Estrogen Receptor Modulator**

The story of the “reinvention” of tamoxifen—at one time a failed contraceptive discovered in the fertility control department at ICI Pharmaceuticals Division (now AstraZeneca)—as the first targeted therapy for the treatment of ER-positive breast cancer has recently been described (13,14). The scientific approach to cancer drug development used in the 1960s and 1970s is adequately summarized elsewhere (13), but the clinical focus at that time was directed exclusively to breast cancer therapy rather than chemoprevention. The scientific principles established in the laboratory—of targeting ER-positive tumors for treatment, while coupling the treatment with long-term adjuvant treatment regimens—produced a substantial increase in patient survivorship. It has been estimated that 500 000 women are alive today because of long-term adjuvant therapy being appropriately administered to patients with ER-positive tumors (13).

However, the transition of tamoxifen from a treatment to chemopreventive was already occurring in the 1970s and 1980s during the era of breast cancer treatment. The discovery that tamoxifen could prevent the initiation and promotion of rat mammary carcinogenesis (15–18), coupled with the clinical finding that tamoxifen treatment reduced the anticipated increase of contralateral breast cancer (19), acted as a catalyst for consideration of tamoxifen as a potential chemopreventive in women at elevated risk (20–22). The major obstacles to progress through the 1980s were in deciding whom to treat and when to deploy a chemopreventive agent, along with safety concerns. Up until the early 1980s, tamoxifen was classified as a nonsteroidal antiestrogen (23), which created a dilemma: if estrogen was essential to maintain bone density and could possibly protect women from coronary heart disease, how could women at risk for breast cancer be treated to reduce that risk if the result would be increases in osteoporotic fractures and deaths from coronary heart disease? The recognition of selective ER modulation during the 1980s ultimately propelled selective estrogen receptor modulators (SERMs) to center stage in efforts to improve women’s health and to the testing of two of them in the STAR trial.

### **Selective Estrogen Receptor Modulation**

The recognition of SERM activity in the target tissues of laboratory animals resulted in several successes in women’s health. Paradoxically, while tamoxifen was being recognized as an “antiestrogen” that blocked breast or mammary cancer growth (15,16,24,25), it also was found to maintain bone density in ovariectomized rats (26–28). The concept of target tissue-specific effects of tamoxifen was extended further with the discovery that in the same athymic mouse tamoxifen prevented estrogen-stimulated human breast tumor growth while increasing uterine weight and stimulating the growth of endometrial carcinoma (29,30). This concept translated immediately to improvements in health care (31,32) with the observation that tamoxifen increased the risk of endometrial cancer but decreased the incidence of contralateral breast cancer in postmenopausal patients (33). This observation was subsequently confirmed in other randomized clinical trials using tamoxifen as a treatment (34), with the results that patient care was improved and gynecolo-

gists were involved in the health care of breast cancer patients. Most important, the new knowledge about the SERM action of tamoxifen allowed measures to be put in place to detect increases in early-stage, low-grade endometrial cancer in the NSABP P-1 chemoprevention trial (10,11).

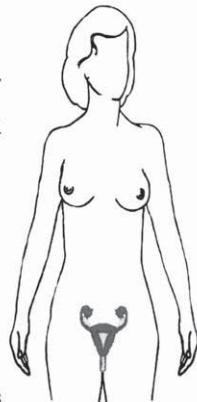
Data showing that bone density was maintained in rats treated with tamoxifen (24) were used to support the evaluation of the actions of tamoxifen on postmenopausal bone density in the Wisconsin Tamoxifen Study. A secondary endpoint in this trial was the levels of circulating lipids because it had been established that tamoxifen reduces circulating cholesterol levels in the ovariectomized rat (35). In fact, the drug already possessed a patent in the United Kingdom for use as a hypocholesterolemic agent (13). The Wisconsin Tamoxifen Study demonstrated that tamoxifen maintained bone density in postmenopausal women (36) and reduced the circulating levels of low-density lipoprotein (LDL) cholesterol but did not reduce the levels of beneficial high-density lipoprotein cholesterol (37,38). Thus, there were grounds to conclude that bone density would be maintained in postmenopausal women treated with tamoxifen and to suggest that the drug might reduce the risk of coronary heart disease (39).

These predictions were addressed in the results of the NSABP P-1 study (10,11): tamoxifen reduced the risk of breast cancer by 50%, elevated the risk of endometrial cancer in postmenopausal women fivefold, and reduced (though not to a statistically significant extent) the incidence of fractures. However, it did not reduce the incidence of coronary heart disease. In the P-1 trial, tamoxifen also reduced the incidence of breast cancers in women with hyperplasia by 80% and reduced ductal carcinoma in situ by 50%. Tamoxifen also increased the risk of deep-vein thrombosis and pulmonary emboli, and these results were confirmed subsequently in the first International Breast Intervention Study, in which Cuzick et al. (40,41) found an increase in deep-vein thrombosis with tamoxifen and an increase in the death rate caused by pulmonary emboli as a result of elective surgical procedures. The results of the NSABP P-1 trial are summarized in Fig. 1, which shows the incidence of recorded endpoints in the control and treatment arms at 48 and 74 months of follow-up. Tamoxifen was approved by the US Food and Drug Administration (FDA) for reduction of the risk of breast cancer in high-risk women in 1998.

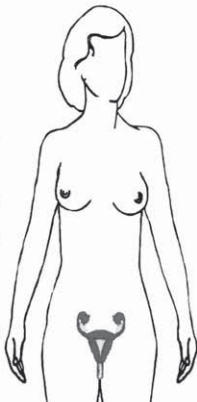
### **Raloxifene as a Multifunctional Medicine**

The development of raloxifene to its current status as a treatment for osteoporosis and a preventive treatment for breast cancer is summarized in Fig. 2. The findings that the failed breast cancer drug keoxifene (LY156758) (42,43) could preserve bone density in ovariectomized rats (24), could prevent rat mammary carcinogenesis (44), and was less effective than tamoxifen in stimulating the growth of human endometrial carcinomas implanted into athymic mice (45) demonstrated that the target tissue-selective actions of tamoxifen (now called selective ER modulation) were common to other drugs in the group previously referred to as nonsteroidal antiestrogens. These laboratory findings resulted in the publication of a strategy by which nonsteroidal antiestrogens related to tamoxifen such as keoxifene could be used to simultaneously prevent osteoporosis in postmenopausal women and reduce the incidence of breast cancer in the general population (46,47). It was as a

	Placebo		Tamoxifen			Raloxifene	
	48mo	74 mo	48mo	74 mo	45 mo	45mo	
Cataracts	22	22	25	28	12	10	Cataracts
Cataract Surgery	3	8	5	11	8	6.6	Cataract Surgery
Stroke	1.3	1.7	2.2	2.5	1	1.3	Stroke
Pulmonary Emboli	0.3	0.4	1	1	1.4	0.9	Pulmonary Emboli
Invasive BC	7	6	3.2	3.3	4.6	4.4	Invasive BC
Non-invasive BC	-	-	-	-	1.5	2.1	Non-invasive BC
BC from Atypical Hyperplasia	10	10	1.5	2.5	5.2	5.8	BC from Atypical Hyperplasia
Endometrial Cancer	0.8	0.6	3.1	3.1	2	1.3	Endometrial Cancer
All Fractures	7.3	4	5.8	3	2.7	2.5	All Fractures
DVT	0.9	0.9	1.5	1.3	2.3	1.7	DVT



**NSABP P-1 TRIAL**



**NSABP P-2 TRIAL**

**Fig. 1.** Comparison of the incidence per 1000 women per year for the recorded endpoints in the National Surgical Adjuvant Breast and Bowel Project (NSABP) P-1 placebo-controlled trial of tamoxifen evaluated at 48 months (10) or 74 months (11) and the Study of Tamoxifen and Raloxifene or NSABP P-2 trials evaluated at 45 months (8). The incidence rates for the NSABP P-1 trial are for postmenopausal (age > 50 years) patients only. In the NSABP P-1 trial, the results for noninvasive breast cancer were reported in as the pooled results from pre- and postmenopausal women and grouped as a cumulated risk. As a result, these data are omitted for NSABP P-1. BC = breast cancer; DVT = deep-vein thrombosis.

result of this strategy, supported by the data from animal models (26,44,45), that keoxifene (LY156758) was reinvented as raloxifene (LY139481 HCL). When the previous laboratory findings that raloxifene maintained bone density in the rat, lowered circulating cholesterol, and possessed low activity as an estrogen in the rodent uterus were confirmed (48), clinical studies were initiated to evaluate raloxifene as a novel agent to preserve bone density in osteoporotic women.

Clinical trials demonstrated that raloxifene treatment maintained bone density in women at risk for osteoporosis (49) and reduced LDL cholesterol (50) to the same degree as tamoxifen (37,38). The success of the SERM concept was underscored by the observation that raloxifene reduced spinal fractures in women at high risk for such fractures (51) and reduced the risk of breast cancer by 75% without measurable increases in endometrial cancer (52,53). Raloxifene was approved by the FDA for the treatment and prevention of osteoporosis in 1998.

The confirmation that SERMs are multifunctional medicines suggests two opportunities for chemoprevention of breast cancer. One is based on an indirect approach, i.e., introducing a novel modality to prevent osteoporosis and reduce the risk of breast cancer as a beneficial side effect (46,47). The other is a direct approach, namely reducing the risk of breast cancer in women at elevated risk by treating them with raloxifene, the feasibility of which was determined by the STAR trial. Both approaches can now be evaluated.

### The Indirect Approach to Breast Cancer Risk Reduction

It is estimated that half a million women are currently taking raloxifene for the treatment and prevention of osteoporosis (8). To preserve and build bone density, the medicine must be taken continuously, and in practice, the treatment regimen could last for 10 years or more. The pivotal antiosteoporosis trial—the Multiple Outcomes Raloxifene Evaluation (MORE)—was extended for an

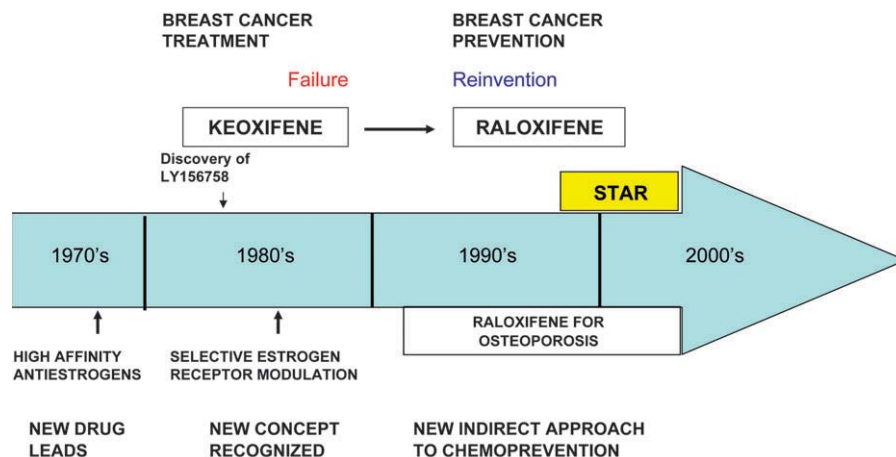
additional 4 years as a safety vanguard study; this study evolved into the Continuing Outcomes Relevant to Evista (CORE) study. The results from this long-term trial of raloxifene now provide an invaluable database to estimate reductions in age-related incidence of breast cancer. The breast cancer incidence rates among postmenopausal women at risk for osteoporosis have been estimated to be 1.4 and 4.2 cancers per 1000 women per year for women taking 60 mg raloxifene daily or placebo, respectively (54).

These data from the CORE trial (54) permit a rough calculation of the impact of raloxifene on public health. With hormone replacement therapy currently considered as a final option for the treatment and prevention of osteoporosis, the “at-risk” population is usually treated initially with a variety of formulations of bisphosphonates that have no impact on breast cancer incidence. Thus, based on the results of the CORE trial (54), if 500 000 postmenopausal women took bisphosphonates for 10 years to prevent osteoporosis, there would be an accumulation of 21 000 (500 000 women × 10 years × 4.2 breast cancers per 1000 women per year) breast cancers requiring surgery and adjuvant therapy with radiation, chemotherapy, and/or antihormone therapy. If these same women received raloxifene to prevent osteoporosis, there would be, based on current estimates, 7000 (500 000 women × 10 years × 1.4 breast cancers per 1000 women per year) breast cancers. Thus, there would be 14 000 fewer breast cancers and 14 000 fewer women who require surgery and adjuvant therapy, not to mention a 40% decrease in the number of fractures experienced by the 500 000 high-risk women (51). This advance in public health must be viewed as a clear success for the SERM concept.

### Conclusions of Study of Tamoxifen and Raloxifene Trial

Building on this advance, the recent results of the STAR trial now promise to offer additional opportunities for successful use of

**Fig. 2.** The evolution of raloxifene (LY156758 or LY139481 HCL). In the late 1970s, tamoxifen was found to be metabolically activated to a hydroxylated metabolite with a high binding affinity for the estrogen receptor. This discovery (69) created a new lead in drug discovery that resulted in the description of LY156758 in 1983 (43). Concerns about using nonsteroidal antiestrogens as long-term treatments and preventives for breast cancer caused a closer examination of the pharmacology of nonsteroidal antiestrogens. Laboratory studies, conducted during 1986-90 (70,71), described the target site estrogenic and antiestrogenic effects of the compounds now referred to as selective estrogen receptor modulators (SERMs). Raloxifene was used unsuccessfully to treat breast cancer (late 1980s) and then reinvented in the early 1990s as a SERM to treat osteoporosis that produced a lower incidence of breast cancer (52). These data were the basis for the Study of Tamoxifen and Raloxifene (STAR) trial in which raloxifene was used as a chemopreventive for breast cancer (8).



SERMs to treat women in the general population, specifically women who are at elevated risk for breast cancer but are not osteoporotic. The STAR trial is part of an ongoing exploration of the potential of medicines to reduce breast cancer incidence in populations of women at high risk, and therefore, its results cannot be considered in isolation but must be assessed based on the prior experience with the NSABP P-1 trial. However, the NSABP P-1 and the STAR trials have important differences in their populations and designs. For example, the STAR trial participants are at a higher risk for breast cancer than the women who participated in the NSABP P-1 prevention trial (8,10). Both the STAR and NSABP P-1 trials recruited women ascertained to be at elevated risk using the Gail model (55), but the STAR trial only recruited postmenopausal women. (Raloxifene has not been evaluated appropriately in premenopausal women and should not be used to treat premenopausal women at risk for breast cancer.) Thus, only relative trends can be identified in lieu of exact comparisons.

The broad conclusions of the STAR (NSABP P-2) trial are summarized and compared with the two evaluations of the NSABP P-1 trial (10,11) in Fig. 1. The incidence rates of the various endpoints in the NSABP P-1 trial are only the results for women more than 50 years of age, and, therefore, valid comparisons can be made with incidence rates in the NSABP P-2 trial. However, the data pertaining to noninvasive breast cancer in the P-1 trial were not broken down into women above or below 50 years and were represented only as cumulative rates and not as an annual rate (10,11). These data are therefore omitted from Fig. 1. The most promising results of STAR trial are, first, that raloxifene is equivalent to tamoxifen at reducing the incidence of invasive breast cancer and, second, that it is associated with a lower incidence of endometrial cancer, endometrial hyperplasia, hysterectomies, cataracts and cataract surgery, and total thromboembolic events (pulmonary emboli or deep venous thromboses) than tamoxifen. The controversial aspect of the trial appears to be the failure of raloxifene to control completely the development of noninvasive breast cancer after 2 years of treatment (8).

Examination of the breast cancer endpoints of invasive and noninvasive diseases and comparison of STAR trial data with CORE/MORE trial data shows that raloxifene actually caused a

reduction in invasive breast cancer by between 65% and 75% in osteoporotic women and not the estimated 50% decrease observed in STAR trial (8,52,54). STAR trial results (8) cannot be compared with previous studies of noninvasive breast cancer (10) because there are no reported data on the effectiveness of treatment in postmenopausal women alone in these studies and numbers are too small in CORE/MORE trial (52,54) for valid comparisons. Possible explanations for the good but suboptimal performance of raloxifene in preventing breast cancer in STAR trial are the differing pharmacologies of raloxifene and tamoxifen and the different populations of CORE/MORE and STAR trials.

The pharmacologic properties of tamoxifen and the group of benzothiophene nonsteroidal antiestrogens that include raloxifene are very different. Tamoxifen exhibits more estrogen-like properties in the rodent uterus than do benzothiophene-related compounds (43,56), and the biologic properties of the tamoxifen-ER complex are more similar to the estrogen-ER complex than the complex formed by raloxifene binding (57,58). Studies in vivo demonstrate that raloxifene-like compounds have an extremely short duration of action compared with that of tamoxifen (44,59,60). This is because the polyphenolic benzothiophene derivatives have poor bioavailability (2%) and undergo rapid phase II metabolism in the intestines and liver (61,62). In contrast, 40% of tamoxifen absorption is from the gastrointestinal tract, and the drug has a long biologic half-life so that its levels persist for up to 6 weeks after therapy stops. Tamoxifen is also metabolized to active phenolic derivatives with high affinity for the estrogen-ER (23). Thus, compliance may be critical to maintain the optimal antiestrogenic actions of raloxifene and tamoxifen, but the effectiveness of tamoxifen, the suboptimal SERM with more estrogenic properties compared to raloxifene in target tissues, will be less dependent on optimal compliance. Failure to maintain adequate raloxifene levels in noncompliant STAR trial patients would allow for promotion of breast tumor growth by endogenous estrogen.

#### Raloxifene Use for the Heart

The demonstrated effectiveness of SERM treatments in lowering circulating cholesterol levels (36,38,50) and the presumed ability of hormone replacement therapy to lower the risk of coronary

heart disease prompted the initiation of a prospective clinical trial to evaluate the ability of raloxifene to reduce the risk of coronary heart disease. The Raloxifene Use for the Heart (RUTH) trial (63) randomly assigned 10 101 postmenopausal women with coronary heart disease or multiple risk factors for coronary heart disease to either 60 mg raloxifene (5044 women) or placebo (5057 women). The two primary outcomes, a coronary event (death, myocardial infarction, or hospitalization for an acute coronary syndrome) and invasive breast cancer, were evaluated after a median follow-up of 5.6 years. There was no evidence that raloxifene had a statistically significant effect on the risk of coronary heart disease (63) despite the previous tantalizing indications that both raloxifene (64) and tamoxifen (39) might have some benefit. The result, however, is consistent with conclusion of the Oxford Overview Analysis that tamoxifen does not improve survival from causes of death other than breast cancer (4). In contrast to what was observed in the MORE trial (64), death from stroke in the RUTH trial was elevated to a statistically significant extent in women taking raloxifene, with 59 deaths from stroke in those taking raloxifene compared to 39 deaths from stroke in controls.

Despite this apparent setback, the RUTH trial has provided additional important information about cancer incidence in a placebo-controlled trial of raloxifene. Endometrial cancer was not elevated in women treated with raloxifene. There were 21 endometrial cancers in 3900 nonhysterectomized women receiving raloxifene and 17 endometrial cancers in 3882 placebo-treated controls. These data clarify the results of the STAR trial where the numbers of endometrial cancers in women treated with tamoxifen increased but not to a statistically significant extent compared with the numbers among women treated with raloxifene. It is possible that the higher hysterectomy rate in women treated with tamoxifen resulted in a lower endometrial cancer rate.

The second planned outcome of the RUTH trial was the incidence of invasive breast cancer. The rate for the placebo-treated women was 2.7 per 1000 women per year, whereas the rate for raloxifene-treated women was 1.5 per 1000 per year. This 44% decrease in invasive breast cancer is consistent with the STAR trial but, again, not as impressive as that observed in the CORE/MORE trial (52,54).

### Progress in Prevention

The success for the two SERMs, tamoxifen and raloxifene, has depended on good ideas based on effective translational research, changes in the fashions of research for the past 40 years, and the development of a patenting strategy that permits a company to test an idea during the period of exclusivity. The fashions in research changed from a focus on contraception in the 1950s and 1960s to breast cancer treatment in a period that extended from the 1970s through 1990s and finally to the current focus on chemoprevention of breast cancer and the prevention of osteoporosis. Preexisting ideas about the potential of breast cancer chemoprevention (20–22) and use of SERMs to prevent osteoporosis and breast cancer (46,47) flourished as opportunities for the broad applications of SERMs were advanced, but these advances only occurred because of delays in patenting that permitted commitment by the pharmaceutical

industry. Tamoxifen was not patented for breast cancer treatment in the United States until 1985, despite the fact that FDA approval was obtained in December 1977 (13). Similarly, raloxifene was patented as a potential cancer treatment in the early 1980s, but the patent for osteoporosis did not occur until 1992 (65). It is unlikely that any progress in women's health and chemoprevention would have occurred without patent protection. But what of future progress? The academic community cannot advance women's health without optimal medicines to test. Despite the advances noted with tamoxifen and raloxifene, these were not optimal agents designed to perform the tasks they were called upon to perform. The truth is that there was nothing else available from the pharmaceutical industry.

For the moment, raloxifene is proving to be an important advance in chemoprevention because it is a multifunctional medicine that can target women at low risk for breast cancer with osteopenia and healthy women with a high risk of breast cancer. Nevertheless, new SERMs are necessary for clinical testing in postmenopausal women. The SERM concept (46) clearly works, but a long-acting SERM is required to replace raloxifene, a drug that does not appear to perform optimally in a high-estrogen environment. The long-acting drug arzoxifene is superior to raloxifene in laboratory studies for chemoprevention (66), but its development has been stalled because raloxifene has proved to be financially beneficial to treat and prevent osteoporosis.

And what of tamoxifen, the first SERM? Twenty years ago, tamoxifen was noted to increase the risk of endometrial cancer in postmenopausal women (33) but not in premenopausal women. Additionally, there are reasonable concerns about deep venous thromboses and pulmonary emboli, although these concerns do not extend to the premenopausal women who are at elevated risk for breast cancer (10). The future use of tamoxifen for chemoprevention may well be restricted to high-risk women who will develop breast cancer during their premenopausal years. The risk-benefit ratio for tamoxifen is favorable (67) in premenopausal women. However, perhaps more importantly, the antitumor actions of 5 years of adjuvant tamoxifen persist and increase for at least 10 years after treatment stops (4). The posttreatment protective effect of tamoxifen is noted in animal models (fewer tumors developed) (17), adjuvant clinical studies [decreased mortality (4) and decreased contralateral breast cancer] (3), and continuing decreases in primary breast cancer in the NSABP P-1 trial (11). A prevention strategy using tamoxifen in high-risk premenopausal women will continue to prevent tumor development after tamoxifen treatment is stopped and when side effects and quality-of-life issues disappear. However, it must be stressed that raloxifene and aromatase inhibitors cannot be used to block estrogen synthesis to reduce breast cancer risk in this patient population. Raloxifene has not been tested in premenopausal women, and the manufacturer recommends against this indication. Aromatase inhibitors are only effective in blocking the constitutive synthesis of estrogen in postmenopausal women. Ovarian estrogen synthesis in premenopausal women is regulated by a pituitary-controlled feedback system, so the blockade by an aromatase inhibitor is reversed by enhanced gonadotropin secretion. Therefore, tamoxifen remains the only proven intervention in premenopausal women.

Tamoxifen and raloxifene both specifically reduce the incidence of ER-positive breast cancer. However, as the testing of chemopreventive agents targeted to the ER progresses and evolves, cost-effectiveness issues are being addressed. A recent study by Melnikow et al. (68) illustrates the dilemma for health care management posed by the price of treatment. The authors concluded that tamoxifen-pricing differences between different health care systems in the United States and Canada are important and that tamoxifen's use as a chemopreventive becomes cost-effective only for women at the highest risk in places where the cost of the drug is extremely low. The issue of cost-effectiveness is now even more timely as the cost of switching from tamoxifen to the more expensive aromatase inhibitors for the treatment of breast cancer has become a major issue for National Health Services in Europe. The next round of chemoprevention trials will compare SERMs with the aromatase inhibitors. The issue of osteoporosis induced by aromatase inhibitors remains a health care concern because the cost of treating large populations of women with expensive agents, monitoring them with dual energy x-ray absorptiometry, and providing them with supplementation with bisphosphonates and Vitamin D only to benefit the few may ultimately be an unreasonable public health care burden. In contrast, the proven promise of raloxifene, a safer SERM targeted specifically to women for the treatment and prevention of osteoporosis but one that also reduces the incidence of breast cancer, is a major first step in developing multifunctional medicines to improve health care.

## References

- (1) Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. *Clin Cancer Res* 2003;9:1980-9.
- (2) Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science* 1987;235:177-82.
- (3) Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;354:1451-67.
- (4) 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;365:1687-717.
- (5) Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CEJ, Davidson NE, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med* 2005;353:1673-84.
- (6) Joensuu H, Kellokumpu-Lehtinen PL, Bono P, Alanko T, Kataja V, Asola R, et al. FinHer Study Investigators. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. *N Engl J Med* 2006;354:809-20.
- (7) Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Herceptin Adjuvant Trial Study Team. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med* 2005;353:1659-72.
- (8) Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. The Study of Tamoxifen and Raloxifene (STAR): report of the National Surgical Adjuvant Breast and Bowel Project P-2 trial. *JAMA* 2006;295:2727-41.
- (9) Rockhill B, Spiegelman D, Byrne C, Hunter DJ, Colditz GA. Validation of the Gail et al. model of breast cancer risk prediction and implications for chemoprevention. *J Natl Cancer Inst* 2001;93:358-66.
- (10) Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, 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.
- (11) Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, 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.
- (12) Milestone 5. (1915) Hormones and cancer. The enemy within. Comment uploaded 1 April 2006. Available at: <http://www.nature.com/milestones/milecancer/full/milecancer05.html>. [Last accessed: February 1, 2007.]
- (13) Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2003;2:205-13.
- (14) Jordan VC. Tamoxifen (ICI 46,474) as a targeted therapy to treat and prevent breast cancer. *Br J Pharmacol* 2006;147:S269-76.
- (15) Jordan VC. Antitumour activity of the antiestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. *J Steroid Biochem* 1974;5:354.
- (16) Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. *Eur J Cancer* 1976;12:419-24.
- (17) Jordan VC, Allen KE. Evaluation of the antitumour activity of the nonsteroidal antiestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 1980;16:239-51.
- (18) Jordan VC, Allen KE, Dix CJ. Pharmacology of tamoxifen in laboratory animals. *Cancer Treat Rep* 1980;64:745-59.
- (19) Cuzick J, Baum M. Tamoxifen and contralateral breast cancer [letter]. *Lancet* 1985;2:282.
- (20) Carbone PP. Breast cancer 1984: role of tamoxifen. In: Craig Jordan V, editors. *Estrogen/antiestrogen action and breast cancer therapy*. Madison (WI): The University of Wisconsin Press; 1986. p. 483-97.
- (21) Cuzick J, Wang DY, Bulbrook RD. The prevention of breast cancer. *Lancet* 1986;1:83-6.
- (22) Powles TJ, Hardy JR, Ashley SE, Farrington GM, Cosgrove D, Davey JB, et al. A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *Br J Cancer* 1989;60:126-31.
- (23) Jordan VC. Biochemical pharmacology of antiestrogen action. *Pharmacol Rev* 1984;36:245-76.
- (24) 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.
- (25) 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.
- (26) 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.
- (27) Lippman ME, Bolan G. Oestrogen-responsive human breast cancer in long term tissue culture. *Nature* 1975;256:592-3.
- (28) Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. *Br J Cancer* 1971;25:270-5.
- (29) Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. *Fed Proc* 1987;46:1870-4.
- (30) 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.
- (31) Hardell L. Tamoxifen as risk factor for carcinoma of corpus uterus. *Lancet* 1988;2:563.
- (32) Jordan VC. Tamoxifen and endometrial cancer [letter]. *Lancet* 1988; 2:1019.
- (33) Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* 1989;1:117-20.
- (34) Fisher B, Costantino JP, Redmond CK, Fisher ER, Wickerham DL, Cronin WM. 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* 1994;86:527-37.
- (35) 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.
- (36) Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, et al. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med* 1992;326:852-6.
- (37) 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.
- (38) Love RR, Wiebe DA, Newcomb PA, Cameron L, Leventhal H, Jordan VC, et al. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Ann Intern Med* 1991;115:860–4.
- (39) McDonald CC, Stewart HJ. Fatal myocardial infarction in the Scottish adjuvant tamoxifen trial. The Scottish Breast Cancer Committee. *BMJ* 1991;303:435–7.
- (40) Cuzick J, Forbes J, Edwards R, Baum M, Cawthorn S, Coates A, et al. First results from the International Breast Cancer Intervention Study (IBIS-I): a randomised prevention trial. *Lancet* 2002;360:817–24.
- (41) Cuzick J, Powles T, Veronesi U, Forbes J, Edwards R, Ashley S, et al. Overview of the main outcomes in breast-cancer prevention trials. *Lancet* 2003;361:296–300.
- (42) Buzdar A, Marcus C, Holmes F, Hug V, Hortobagyi G. Phase II evaluation of Ly156758 in metastatic breast cancer. *Oncology* 1988;45:344–5.
- (43) Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothioephene derived antiestrogen. *Life Sci* 1983;32:1031–6.
- (44) 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.
- (45) 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.
- (46) Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 1988;6:589–95.
- (47) Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: eighth Cain memorial award lecture. *Cancer Res* 1990;50:4177–89.
- (48) Black LJ, Sato M, Rowley ER, Magee DE, Bekele A, Williams DC, et al. Raloxifene (LY139481 HCl) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. *J Clin Invest* 1994;93:63–9.
- (49) Delmas PD, Bjarnason NH, Mitlak BH, Ravoux AC, Shah AS, Huster WJ, et al. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. *N Engl J Med* 1997;337:1641–7.
- (50) Walsh BW, Kuller LH, Wild RA, Paul S, Farmer M, Lawrence JB, et al. Effects of raloxifene on serum lipids and coagulation factors in healthy postmenopausal women. *JAMA* 1998;279:1445–51.
- (51) Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, 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 [erratum in *JAMA* 1999;282:2124]. *JAMA* 1999;282:637–45.
- (52) Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, 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.
- (53) Cauley JA, Norton L, Lippman ME, Eckert S, Krueger KA, Purdie DW, et al. 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* 2001;65:125–34.
- (54) Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, et al. CORE Investigators. 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.
- (55) Gail MH, Brinton LA, Byar DP, Corle DK, Green SB, Schairer C, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst* 1989;81:1879–86.
- (56) Black LJ, Goode RL. Uterine bioassay of tamoxifen, trioxifene and a new estrogen antagonist (LY117018) in rats and mice. *Life Sci* 1980;26:1453–8.
- (57) Levenson AS, Jordan VC. The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. *Cancer Res* 1998;58:1872–5.
- (58) MacGregor-Shafer JI, Liu H, Bentrem D, Zapf J, Jordan VC. Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex by substituting glycine for aspartate at amino acid 351. *Cancer Res* 2000;60:5097–105.
- (59) Jordan VC, Gosden B. Inhibition of the uterotrophic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. *Endocrinology* 1983;113:463–8.
- (60) Jordan VC. 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* 1983;3(Suppl):S73–86.
- (61) Snyder KR, Sparano N, Malinowski JM. Raloxifene hydrochloride. *Am J Health Syst Pharm* 2000;57:1669–75;quiz 1676–8.
- (62) 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.
- (63) Barrett-Connor E, Mosca L, Collins P, Geiger MJ, Grady D, Kornitzer M, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Engl J Med* 2006;355:125–37.
- (64) Barrett-Connor E, Grady D, Sashegyi A, Anderson PW, Cox DA, Hozowski K, et al. Raloxifene and cardiovascular events in osteoporotic postmenopausal women. *JAMA* 2002;287:847–57.
- (65) Lewis JS, Jordan VC. Case histories: raloxifene. In: *Comprehensive medicinal chemistry II*. Vol 8. 2006:83–102.
- (66) Suh N, Lamph WW, Glasebrook AL, Grese TA, Palkowitz AD, Williams CR, et al. 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* 2002;8:3270–5.
- (67) Gail MH, Costantino JP, Bryant J, Croyle R, Freedman L, Helzlsouer K, et al. Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J Natl Cancer Inst* 1999;91:1829–46.
- (68) Melnikow J, Kuenneth C, Helms LJ, Barnato A, Kuppermann M, Birch S, et al. Chemoprevention: drug pricing and mortality: the case of tamoxifen. *Cancer* 2006;107:950–8.
- (69) Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antiestrogenic activity. *J Endocrinol* 1977;75:305–16.
- (70) Jordan VC. Selective estrogen receptor modulation: a personal perspective. *Cancer Res* 2001;61:5683–7.
- (71) Jordan VC, Gapstur S, Morrow M. Selective estrogen receptor modulation and reduction in risk of breast cancer, osteoporosis, and coronary heart disease. *J Natl Cancer Inst* 2001;93:1449–57.

## Notes

Dr V. C. Jordan is supported by the Department of Defense Breast Program under Center of Excellence award number BC050277, SPORE in Breast Cancer CA 89018, R01 GM067156, Fox Chase Cancer Center (FCCC) Core Grant NIH P30 CA006927, the Avon Foundation, and the Weg Fund of FCCC. Views and opinions of and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense.

Funding to pay the Open Access publication charges for this article was provided by the Weg Fund of Fox Chase Cancer Center, Philadelphia, PA.

Manuscript received August 8, 2006; revised December 21, 2006; accepted January 18, 2007.

## COMMENTARY

# Beyond raloxifene for the prevention of osteoporosis and breast cancer

VC Jordan

*Fox Chase Cancer Center, Philadelphia, USA*

Selective oestrogen receptor modulators (SERMs) can build bone in the postmenopausal woman and lower circulating cholesterol. These oestrogen-like properties contrast with the anti-oestrogenic properties observed in the breast where SERMs inhibit the oestrogen-mediated development and growth of ER positive breast cancers. The two clinically useful SERMs, tamoxifen and its chemical cousin raloxifene, are currently used successfully either for the treatment and prevention of breast cancer (tamoxifen) or the treatment and prevention of osteoporosis (raloxifene). However, raloxifene has the beneficial side-effect of breast cancer prevention. These multifunction medicines provide proof of concept that novel molecules can be selectively targeted to diseases mediated by the endocrine system.

*British Journal of Pharmacology* (2007) **150**, 3–4. doi:10.1038/sj.bjp.0706962; published online 20 November 2006

**Keywords:** tamoxifen; raloxifene; selective oestrogen receptor modulator; breast cancer; chemoprevention; antioestrogen; osteoporosis; mammary cancer; arzoxifene

**Abbreviations:** ER, oestrogen receptor; HRT, hormone replacement therapy; SERM, selective oestrogen receptor modulator

It is less than 20 years ago that raloxifene (then known as keoxifene) was found to preserve bone density in the ovariectomized rat (Jordan *et al.*, 1987), but also prevent the induction of rat mammary carcinogenesis (Gottardis and Jordan, 1987). These data catalysed the idea that non-steroidal antioestrogens (Jordan, 1984) were in fact selective oestrogen receptor modulators (SERMs) that were oestrogenic or antioestrogenic at different target sites around the body. The proposed application of this knowledge was to develop medicines to treat and prevent osteoporosis and to prevent breast cancer as a beneficial side effect (Jordan, 1988; Lerner and Jordan, 1990). The goal would be to develop a safer hormone replacement therapy (HRT). Traditional HRT, a mixture of orally active conjugated oestrogens and a synthetic progestin, is effective at preventing osteoporosis, but it is currently believed that the risks of developing an excess of breast cancer are unacceptable as a national health-care strategy (Million Women Study Collaborators, 2003).

The new SERM strategy has been successfully evaluated in clinical trials. Raloxifene dramatically reduces breast cancer incidence in postmenopausal women being treated for osteoporosis (Cummings *et al.*, 1999; Martino *et al.*, 2004). Additionally, raloxifene also reduces the incidence of breast cancer in high-risk women to the same extent as tamoxifen, that is about 50% (Vogel *et al.*, 2006). However, although the

translational research has effectively advanced therapeutics, questions now arise about optimizing the targeting of raloxifene to the breast. Raloxifene has a high binding affinity for the oestrogen receptor (ER) that is equivalent to that of oestradiol (Black *et al.*, 1983), so the molecule should perform extremely well as an antioestrogen in the breast. Unfortunately, raloxifene appears to perform suboptimally. The goal of advancing therapeutics is to deliver the medicine to the right place at the right time; or in the case of breast cancer prevention – all of the time.

In this issue, Ning *et al.* (2007) study the structure–activity relationships and binding characteristics of raloxifene derivatives at their cognate receptors ER $\alpha$  and ER $\beta$ . What is encouraging is that a derivative of raloxifene called Y134 is identified, that appears to be superior to raloxifene with regard to mammary gland selectivity.

Raloxifene is an agent that was originally destined to be a drug to treat breast cancer, but it failed in that application (Buzdar *et al.*, 1988). It appears that the pharmacokinetics and bioavailability of raloxifene are a challenge. Only about 2% of administered raloxifene is bioavailable (Snyder *et al.*, 2000), but despite this, the drug is known to have a long biological half-life of 27 h. The reason for this disparity is that raloxifene is a polyphenolic drug, and this creates a complex dimension of glucuronidation and sulphation in the gut (Kemp *et al.*, 2002; Jeong *et al.*, 2005) which controls enterohepatic recirculation and ultimately prevents the drug from reaching 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

Correspondence: Professor VC Jordan, Fox Chase Cancer Center, 333 East Cottman Avenue, Philadelphia, PA 19111, USA.

E-mail: v.craig.jordan@fccc.edu

Received 22 September 2006; accepted 3 October 2006; published online 20 November 2006

chemopreventive in rat mammary carcinogenesis (Suh *et al.*, 2002). Nevertheless, arzoxifene has not performed well as a treatment for breast cancer (Baselga *et al.*, 2003; Buzdar *et al.*, 2003), but the results of trials evaluating the effects of arzoxifene as a drug to treat osteoporosis are eagerly awaited. Perhaps, arzoxifene will be a better breast cancer preventive than a treatment.

Unfortunately, keeping phenolic drugs in the target is another complication. 4-Hydroxytamoxifen, an active metabolite of tamoxifen (Jordan *et al.*, 1977), is only sulphated by three of seven sulphotransferase isoforms, whereas raloxifene is sulphated by all seven (Falany *et al.*, 2006). Maybe local phase II metabolism plays a role in neutralizing the antioestrogen action of raloxifene in the breast. Falany *et al.* (2006) further demonstrate that SULT1E1 that sulphates raloxifene in the endometrium is only expressed in the secretory phase. In contrast, 4-hydroxytamoxifen is sulphated at all stages of the uterine cycle. If the emerging knowledge of this target site-specific phase II metabolism is applied to the breast, then perhaps an ideal agent can be designed to be retained in the breast and create optimal antioestrogenic activity. Studies such as those described by Ning *et al.* (2007) should be encouraged and expanded to identify specific SERMs, targeted to specific organs to treat and prevent numerous oestrogen-mediated diseases thereby improving women's health.

## Acknowledgements

VCJ is supported by the Department of Defense Breast Program under award number BC050277 Center of Excellence (Views and opinions of and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense), Specialized Program of Research Excellence in Breast Cancer CA 89018, R01 GM067156, FCCC Core Grant NIH P30 CA006927, the Avon Foundation and the Weg Fund of Fox Chase Cancer Center.

## References

- Baselga J, Llombart-Cussac A, Bellet M, Guillem-Porta V, Enas N, Krcy K *et al.* (2003). Randomized, double-blind multicenter trial comparing two doses of arzoxifene (LY353381) in hormone-sensitive advanced or metastatic breast cancer patients. *Annals of Oncology* **14**: 1383–1390.
- Black LJ, Jones CD, Falcone JF (1983). Antagonism of estrogen action with a new benzothioephene derived antiestrogen. *Life Sci* **32**: 1031–1036.
- Buzdar A, Marcus C, Holmes F, Hug V, Hortobagyi G (1988). Phase II evaluation of Ly156758 in metastatic breast cancer. *Oncology* **45**: 344–345.
- Buzdar A, O'Shaughnessy JA, Booser DJ, Pippin Jr JE, Jones SE, Munster PN *et al.* (2003). Phase II, randomized double-blind study of two dose levels of arzoxifene in patients with locally advanced or metastatic breast cancer. *Journal of Clinical Oncology* **21**: 1007–1014.
- Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA *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.
- 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.
- 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.
- 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.
- Jordan VC (1984). Biochemical pharmacology of antiestrogen action. *Pharmacol Rev* **36**: 245–276.
- Jordan VC (1988). Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* **6**: 589–595.
- Jordan VC, Collins MM, Rowsby L, Prestwich G (1977). A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* **75**: 305–316.
- 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.
- 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.
- Lerner LJ, Jordan VC (1990). The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res* **50**: 4177–4189.
- Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D *et al.* (2004). For the CORE Investigators 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.
- Million Women Study Collaborators (2003). Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet* **362**: 419–427.
- Ning M, Zhang S, Zhou C, Chen D, Weng J, Yang C *et al.* (2007). Biological activities of a novel selective oestrogen receptor modulator derived from raloxifene (Y134). *Br J Pharmacol* **150**: 19–28 (this issue).
- Snyder KR, Sparano N, Malinowski JM (2000). Raloxifene hydrochloride. *Am J Health Syst Pharm* **57**: 1669–1675 quiz 1676–1678.
- Suh N, Lamph WW, Glasebrook AL, Grese TA, Palkowitz AD, Williams CR *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.
- Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN *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.



ELSEVIER

available at [www.sciencedirect.com](http://www.sciencedirect.com)journal homepage: [www.elsevier.com/locate/steroids](http://www.elsevier.com/locate/steroids)

## Review

# Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer

V. Craig Jordan<sup>a,\*</sup>, Angela M.H. Brodie<sup>b</sup>

<sup>a</sup> Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111-2497, United States

<sup>b</sup> Department of Pharmacology & Experimental Therapeutics, University of Maryland School of Medicine, University of Maryland Greenebaum Cancer Center, Baltimore, MD, United States

## ARTICLE INFO

## Article history:

Received 3 October 2006

Received in revised form

25 October 2006

Accepted 26 October 2006

Published on line 13 December 2006

## Keywords:

Tamoxifen

Raloxifene

Estrogen receptor

Aromatase inhibitors

4-Hydroxyandrostenedione

Breast cancer

Antihormone resistance

## ABSTRACT

This article describes the origins and evolution of “antiestrogenic” medicines for the treatment and prevention of breast cancer. Developing drugs that target the estrogen receptor (ER) either directly (tamoxifen) or indirectly (aromatase inhibitors) has improved the prognosis of breast cancer and significantly advanced healthcare. The development of the principles for treatment and the success of the concept, in practice, has become a model for molecular medicine and presaged the current testing of numerous targeted therapies for all forms of cancer. The translational research with tamoxifen to target the ER with the appropriate duration (5 years) of adjuvant therapy has contributed to the falling national death rates from breast cancer. Additionally, exploration of the endocrine pharmacology of tamoxifen and related nonsteroidal antiestrogen (e.g. keoxifene now known as raloxifene) resulted in the laboratory recognition of selective ER modulation and the translation of the concept to use raloxifene for the prevention of osteoporosis and breast cancer.

However, the extensive evaluation of tamoxifen treatment revealed small but significant side effects such as endometrial cancer, blood clots and the development of acquired resistance. The solution was to develop drugs that targeted the aromatase enzyme specifically to prevent the conversion of androstenedione to estrone and subsequently estradiol. The successful translational research with the suicide inhibitor 4-hydroxyandrostenedione (known as formestane) pioneered the development of a range of oral aromatase inhibitors that are either suicide inhibitors (exemestane) or competitive inhibitors (letrozole and anastrozole) of the aromatase enzyme. Treatment with aromatase inhibitors is proving effective and is associated with reduction in the incidence of endometrial cancer and blood clots when compared with tamoxifen and there is also limited cross resistance so treatment can be sequential. Current clinical trials are addressing the value of aromatase inhibitors as chemopreventive agents for postmenopausal women.

© 2006 Elsevier Inc. All rights reserved.

\* Corresponding author. Tel.: +1 215 728 7410; fax: +1 215 728 7034.

E-mail addresses: [v.craig.jordan@fcc.edu](mailto:v.craig.jordan@fcc.edu) (V.C. Jordan), [abrodie@umaryland.edu](mailto:abrodie@umaryland.edu) (A.M.H. Brodie).

0039-128X/\$ – see front matter © 2006 Elsevier Inc. All rights reserved.

doi:10.1016/j.steroids.2006.10.009

---

**Contents**

1. V. Craig Jordan: ICI 46,474 to tamoxifen .....	9
1.1. Translational research with tamoxifen .....	10
2. Angela M.H. Brodie—aromatase inhibitors: developing 4-hydroxyandrostenedione .....	11
2.1. Efforts to bring 4-OHA into the clinic .....	11
2.2. The estrogen ER signal transduction pathway as a model for molecular targeting .....	12
2.3. Mechanism of estrogen synthesis .....	12
2.4. Steroidal aromatase inhibitors .....	13
3. Non-steroidal aromatase inhibitors .....	14
3.1. Selective modulation of aromatase .....	14
3.2. The estrogen receptor signal transduction pathway .....	15
3.3. Adjuvant therapy with tamoxifen .....	16
3.4. The road to adjuvant treatment with aromatase inhibitors .....	16
3.5. Selective estrogen receptor modulation and chemoprevention .....	17
4. Aromatase inhibitors as chemopreventive agents .....	19
4.1. Drug resistance to antihormones .....	19
4.2. Perspective .....	20
Acknowledgements .....	20
References .....	20

---

The enthusiasm with which the clinical community has embraced the use of antiestrogenic therapy to treat breast cancer is based upon the proven record of success that first the nonsteroidal antiestrogen tamoxifen and then the aromatase inhibitor have demonstrated in clinical trial. The reasons for the enthusiasm are obvious. Antihormonal therapy, particularly aromatase inhibition to create a “no estrogen state” in the postmenopausal breast cancer patient is effective, saves women’s lives, is contributing successfully to reducing the national mortality from breast cancer, is relatively cheap and has fewer side effects and easy administration (oral) than any other anticancer strategy. However, the successful application of a therapeutic strategy to block the known growth stimulation property of estrogen in breast cancer was not greeted with such enthusiasm 40 years ago.

Estrogen is essential for life. Without the critical role of estrogenic steroids, reproduction would not be possible. Based on emerging knowledge from laboratory studies, the value of modulating the steroid environment during the menstrual cycle was advanced to clinical testing during the 1950s as a means of oral contraception. The results of these studies were to change society forever.

The Worcester Foundation for Experimental Biology is the place where Gregory Pincus established the scientific principles necessary to propose clinical testing of the oral contraceptive and M.C. Chang subsequently established the first protocols to perform *in vitro* fertilization. Simply stated, the Worcester Foundation was, at that time, the world center for steroid endocrinology and reproductive biology. Over the years, hundreds of scientists have trained at the Foundation and subsequently spread their knowledge throughout the world [1]. However, fashions in research change and new opportunities emerge.

In 1971, President Nixon made a national commitment to seek a cure for cancer by signing the National Cancer Act. Mahlon Hoagland, the President of the Worcester Foundation,

responded to the initiative by appointing Professor Elwood V. Jensen, Director of the Ben May Cancer Research Laboratory at the University of Chicago, to be a member of the Foundation’s Scientific Advisory Board. Jensen had discovered the estrogen receptor (ER) as the putative mechanism of estrogen action in its target tissues [2]. The known link between estrogen and breast cancer suggested that “antiestrogenic strategies” might have potential as therapeutic agents [3]. Jensen applied knowledge of ER action to breast cancer treatment by devising the ER assay to identify breast cancers that would respond to endocrine ablation [4] but not all breast cancers responded. Hoagland’s plan was to encourage the exploitation of the rich resources in endocrinology at the Foundation to be used for cancer research. The scene was set for independent investigators to work in cancer endocrinology but it is fair to say no one in academic medical oncology was interested in development of new antihormone therapies. Combination cytotoxic chemotherapy was king. Industry and clinical trial groups were respectively convinced that (1) developing anticancer drugs was a very risky business and (2) the right combination of cytotoxic agents applied at the right time would cure cancer. The principle was working in childhood leukemia, why not breast cancer?

The authors first met at the Worcester Foundation during the closing months of 1972. By coincidence, we were both English and grew up in the same county of Cheshire. One of us (VCJ) had conducted a PhD (1968–1972) on the structure activity relationships of a group of failed contraceptives, the nonsteroidal antiestrogens, the other (AMHB) had worked on hormones and breast cancer at the Christie Hospital in Manchester where the first preliminary study of ICI 46,474 was subsequently completed [5]. This was before ICI 46,474 was renamed tamoxifen (Fig. 1).

We have started this review with an account of our individual experiences that led to the development of tamoxifen and the aromatase inhibitors. Our perspective is followed by a

description of the therapeutic target, the estrogen signal transduction system and we close with current clinical advances in antihormonal therapy.



**General Motors Prize Awards Ceremony—Washington, DC, 2005**

The Charles F. Kettering Prize from the General Motors Cancer Research Foundation is awarded annually for the most outstanding recent contributions to the diagnosis or treatment of cancer. V. Craig Jordan (VCJ) and Angela H. Brodie (AMHB) are recognized on separate occasions for their pioneering studies that defined the scientific principles used clinically for the targeted treatment and prevention of breast cancer. Their body of work using the selective estrogen receptor modulators, tamoxifen and raloxifene (VCJ) and the first suicide inhibitor of the aromatase enzyme 4-hydroxyandrostendione (AMHB) has been individually recognized by several of the world's leading prizes including the Brinker International Award from the Susan G. Komen Breast Cancer Foundation (VCJ 1993, AMHB 2000), the Dorothy P. Landon/American Association for Cancer Research Prize for Translational Research (VCJ 2002, AMHB 2006) and the Charles F. Kettering Prize from the General Motors Cancer Research Foundation (VCJ 2003, AMHB 2005) (see photograph). Jordan and Brodie have been members and attended the Endocrine Society since 1981 and 1962, respectively.

## 1. V. Craig Jordan: ICI 46,474 to tamoxifen

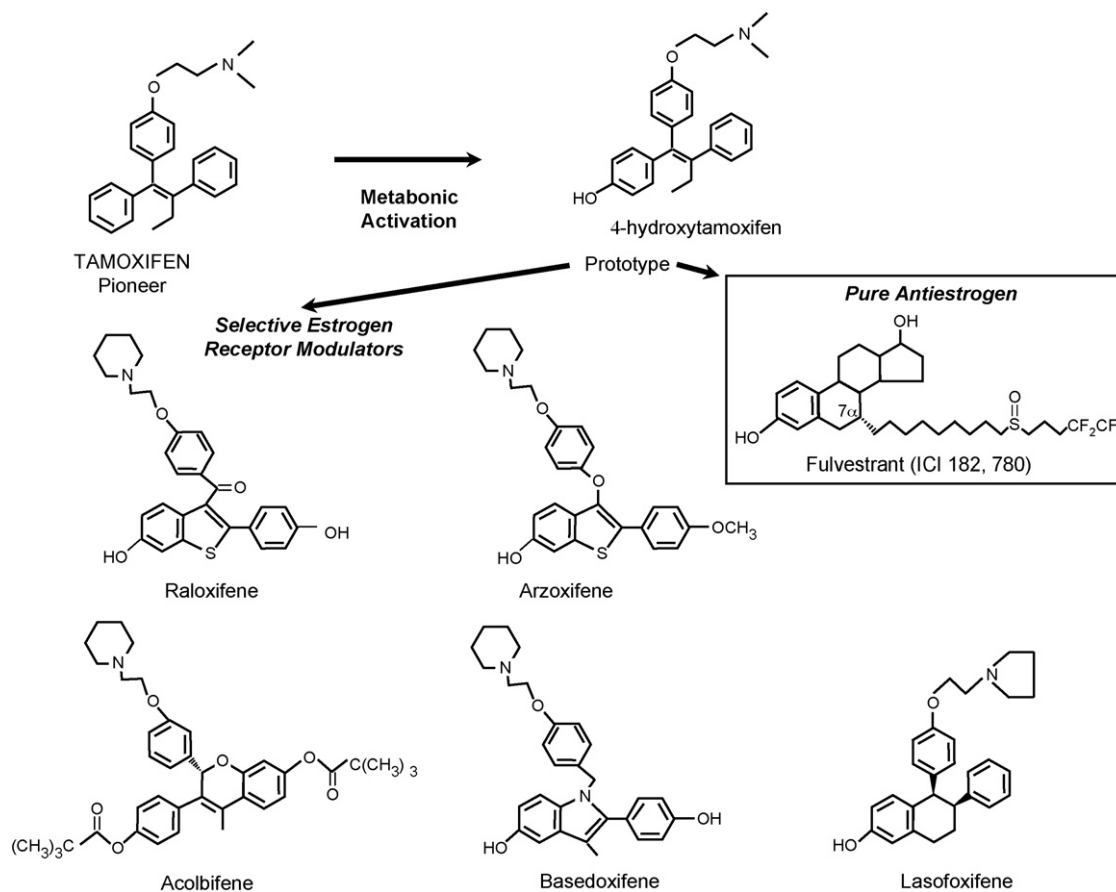
In 1967 Arthur Walpole and Mike Harper at the Imperial Chemical Industries (ICI) Pharmaceutical Division in Alderley Park, Cheshire reported the antiestrogenic and antifertility prop-

erties of a substituted triphenylethylene ICI 46,464 [6,7]. The Alderley Park team had been tasked during the 1960s to discover compounds to modulate fertility. Although Walpole also had an interest in anticancer chemotherapy, [8] as head of the fertility control program, he did not conduct any laboratory investigations of ICI 46,474 as an anticancer agent. He did, however, ensure that ICI Pharmaceuticals Division patented the compound with the statement, "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." Nevertheless, there was no patent for ICI 46,474 in the United States in 1972.

Harper had moved to the Worcester Foundation in the late 1960s and was investigating the potential of prostaglandins to be used as a once a month contraceptive. Although it was clear that prostaglandins were too toxic for systemic use, it is perhaps relevant to point out that a prostaglandin is currently used with mifepristone (RU486) as an abortifacient.

In 1972 I had completed my PhD on the "Structure Activity Relations of Substituted Triphenylethylenes and Triphenylethanes" but the University of Leeds was having difficulty securing an appropriate external examiner for my thesis. Nobody cared about the topic and it was only after considerable negotiation that Arthur Walpole (from industry!) was permitted to undertake the task. This experience started a collaboration that only ended with his untimely death in 1977.

In that same year, I took a 2 year leave of absence from the Department of Pharmacology at the University of Leeds originally to work with Mike Harper on the contraceptive properties of prostaglandins but when I arrived, he had left to work in the World Health Organization in Geneva and I was told—do anything you like, as long as some of it involves prostaglandins! My passion was the application of chemistry to medicine and I had always wanted to develop targeted anti-cancer drugs. Two events occurred in 1972–1973 that permitted me to pursue my passion. A meeting between Jensen and me at the Worcester Foundation in November 1972 would solve the problem of how to conduct a systematic laboratory examination of the antitumor actions of ICI 46,474. Jensen offered to teach me the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model [9] and techniques to measure estrogen receptors (ERs) in animal and human tumors. These techniques were essential to reinvent ICI 46,474 (tamoxifen) as an antitumor agent targeted to the ER. A phone call to Arthur Walpole in the United Kingdom secured funding to support the work and introduced me to Lois Trench, the newly appointed drug monitor for ICI 46,474 at the recently acquired Stuart Pharmaceuticals in Wilmington, Delaware. The company quickly evolved into ICI Americas and now 30 years later is known as AstraZeneca. Lois Trench provided human breast cancers for me to establish that tamoxifen blocked, the binding of estradiol to the ER [10], and I was also asked to introduce ICI 46,474 first to the Eastern Cooperative Oncology Group (ECOG), [11] and subsequently to the National Surgical and Bowel Project (NSABP) [12]. The NSABP particularly would propel ICI 46,474 from obscurity in the 1960/1970s to center stage



**Fig. 1 – The activation of the pro drug tamoxifen to 4-hydroxytamoxifen, which has a high binding affinity for ER [103]. This knowledge resulted in the development of numerous new agents for use as selective estrogen ER modulations (SERMs) for the prevention of breast cancer and osteoporosis or the pure antiestrogen fulvestrant used as a treatment for ER positive advanced breast cancer following the failure of either tamoxifen treatment or an aromatase inhibitor.**

in the treatment and prevention of breast cancer during the 1980/1990s.

### 1.1. Translational research with tamoxifen

A scientific strategy for the appropriate clinical application of tamoxifen was developed in the laboratory during the 1970s to target the drug to the tumors that were the most likely to respond [13,14]. Tamoxifen blocked the binding of estradiol to human breast and rat mammary tumor ERs and prevented the induction and growth of ER positive carcinogen-induced rat mammary carcinomas [10,15–17]. These early studies raised the question of whether tamoxifen could prevent the majority of breast cancers, i.e.: ER positive breast cancer. However, the finding that long term tamoxifen treatment in animals with early mammary cancer, i.e., a low tumor burden [18–20] could create a tumor-free state suggested longer was going to be better than shorter durations of adjuvant therapy. The laboratory observations and pilot clinical studies [21,22] were to prove remarkably effective as an approach to treat women with early node positive and node negative ER positive breast cancer. However, the original clinical strategy in the 1970s for the evaluation of tamoxifen was to use 1 year of adjuvant treatment after surgery. The reason for this was that tamoxifen

was only effective for the treatment of advanced breast cancer for about a year and there was a sincere concern that longer adjuvant treatment durations would result in premature drug resistance. This approach was to change.

An enormous advance in medicine is the introduction of meta-analysis or Overview analysis of small randomized clinical trials that individually show little or no benefits for agents under investigation but together provide a statistically secure result. The Overview analysis of breast cancer clinical trials was first conducted at Heathrow airport in 1984 [23]. The results when they were published in full in 1988 demonstrated a significant advantage for postmenopausal patients receiving tamoxifen [24]. Most importantly, a Consensus Conference held in Bethesda, MD recommended that tamoxifen should be used as an adjuvant therapy for postmenopausal ER positive, node positive patients with breast cancer [25]. The year 1985 was a good time for ICI Pharmaceuticals Division (now AstraZeneca) to be awarded a use patent for tamoxifen from the US Court of Appeals. The award of a patent for tamoxifen in 1985 started a 17 year exclusivity use patent in the US just at the time when the patent for tamoxifen had expired worldwide and just at the time that tamoxifen was poised to change healthcare. Thus, the accumulative 40-year patent for tamoxifen was to be the financial engine that facilitated

the development of a whole range of cancer therapies including aromatase inhibitors from AstraZeneca and subsequently other companies.

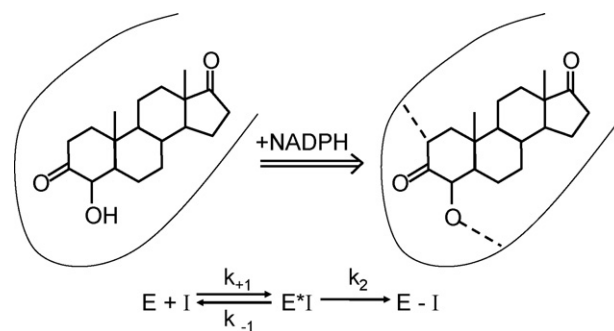
## 2. Angela M.H. Brodie—aromatase inhibitors: developing 4-hydroxyandrostenedione

I had received my PhD degree from Manchester University and was awarded an NIH Postdoctoral Training Fellowship, which brought me to the Worcester Foundation in 1962. The exciting atmosphere of cutting edge research enticed me to remain there after my fellowship. By the early 1970s, I had married a fellow scientist, Harry Brodie, and joined his lab working on the biochemistry of aromatase, the key enzyme in the biosynthesis of estrogens. Harry, an organic chemist, had begun developing inhibitors of aromatase as potential contraceptive agents and reported the first of these compounds in 1973 [26].

With my background in breast cancer at the Christie Hospital in the UK and the death of the previous lab director from the disease still on my mind, I was very interested in the possibility that aromatase inhibitors might be of value in the treatment of breast cancer. Clinical trials with ICI 46,474 (tamoxifen) had begun about this time. Although the antiestrogen was effective, it clearly did not yet have the impact on the disease that eventually brought it to the important position it has today. However, at that time, tamoxifen and other antiestrogens were known to be partial estrogen agonists as well as antagonists [6], raising concerns that they may not be optimally effective against breast cancer and may have adverse estrogenic effects. We reasoned that by using a different approach, compounds that blocked the production of estrogen without having significant estrogenic activity themselves might be identified. Results showed this to be the case. For the same reason, the possibility existed that aromatase inhibitors might also be more effective in treating breast cancer than antiestrogens. A number of laboratory studies were carried out which demonstrated the efficacy of the most potent aromatase inhibitor, 4-hydroxyandrostenedione (4-OHA) [27]. Some time later, we found that this compound acts not only by rapid competitive inhibition but also by inactivation of the enzyme. This effect is long lasting or irreversible (Fig. 2); see further below on steroidal inhibitors [28]. With some help from Craig, who had become proficient in developing mammary tumors with DMBA in rats, we showed that 4-OHA was effective in suppressing ovarian estrogen levels and causing regression of rodent mammary tumors [27]. In contrast to tamoxifen, 4-OHA was not estrogenic on other tissues such as the rat uterus. 4-OHA also inhibited peripheral (non-ovarian) estrogen synthesis in non-human primates in studies carried out in collaboration with Chris Longcope at the Worcester Foundation [29].

### 2.1. Efforts to bring 4-OHA into the clinic

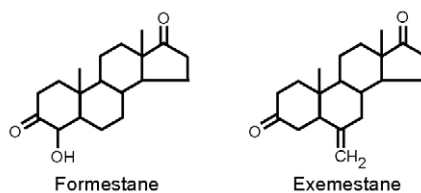
Although these studies demonstrated that 4-OHA is highly effective, clinical studies were difficult to initiate, despite encouragement from the Decision Network Group at NCI who set aside funds to carry out toxicity studies. By 1978, Harry



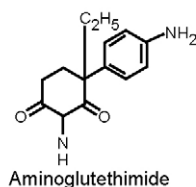
**Fig. 2 – Aromatase inactivators: several steroidal inhibitors have been demonstrated to bind irreversibly or very tightly to the active site of aromatase e.g., 4-hydroxyandrostenedione (4-OHA).**

had left research, and I had moved to the University of Maryland in 1979. I was hopeful that the Cancer Center would be interested in bringing 4-OHA into the clinic. Tamoxifen had by then been shown to be effective and there was a lack of enthusiasm to investigate other approaches, not only at the University of Maryland but also in pharmaceutical companies. One approach, however, that did help the cause of aromatase inhibitors was the use of aminoglutethimide (AG) (Fig. 3) in treating breast cancer patients. Aminoglutethimide was a drug developed for treating epilepsy. However, it was found to cause adrenal suppression by inhibiting multiple cytochrome P-450 enzymes [30]. As adrenalectomy had been shown to be effective in treating breast cancer by Charles Huggins, Richard Santen and colleagues in the late 1970s began using aminoglutethimide as a medical approach to suppressing adrenal steroids in breast cancer patients [31–33]. Because aminoglutethimide inhibited a number of steroidogenic P-450 enzymes including CYP11, patients were given cortisol replacement. Santen was able to show that the main beneficial effect of aminoglutethimide then was inhibition of estrogen synthesis. However, aminoglutethimide had a number of significant side effects. Thus, it was my good fortune that some oncologists experienced with using aminoglutethimide were receptive to testing our selective aromatase inhibitor, 4-OHA. In the fall of 1981, I was invited to a conference in Rome to give a presentation about my research. Afterwards, an oncologist from London, Charles Coombes expressed interest in testing 4-OHA in breast cancer patients. Soon after my return to Maryland, a letter arrived from the Royal Marsden Hospital in London suggesting collaboration to bring 4-OHA, the first selective aromatase inhibitor into the clinic. In my laboratory at the University of Maryland we were able to produce a kilogram of 4-OHA by combining several batches of material. The toxicology was carried out through the Cancer Research Campaign in the UK. Paul Goss joined Charles Coombes as a PhD student and cared for the first patients that were treated with 4-OHA. Mitch Dowsett was also an important part of the team and measured estrogen and drug levels in the patients. Significant responses were seen in these first series of patients many of whom had relapsed from tamoxifen treatment. With these exciting results, Charles Coombes and I traveled to Horesham to Ciba-Geigy with the proposition that they take on 4-OHA

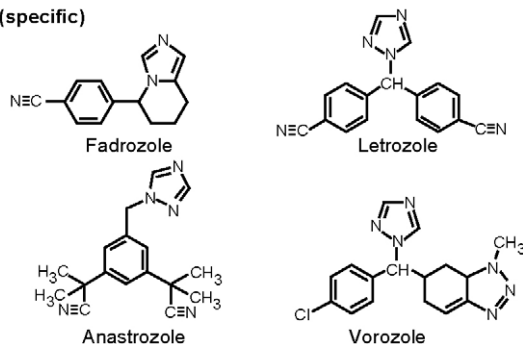
## Steroidal Irreversible Inhibitors



## Non-Steroidal Reversible Inhibitor (nonspecific)



## Non-Steroidal Reversible Inhibitors (specific)



**Fig. 3 – The structures of various aromatase inhibitors tested clinically for the treatment of breast cancer. The compounds are classified based on their mode of action and specificity for the aromatase enzyme.**

and expand the clinical trials. Ciba-Geigy produced AG and it was quickly appreciated by the late Stuart Hughes that selective aromatase inhibitors such as 4-OHA would have distinct advantages. Clinical trials proceeded and Formestane (4-OHA) was the first selective aromatase inhibitor to become available and was the first new treatment of breast cancer in 10 years at that time.

As 4-OHA was of benefit in patients who had relapsed on tamoxifen, interest gradually grew in the possible benefits of using additional “hormonal” agents that are well tolerated. Before long, a number of pharmaceutical companies began producing aromatase inhibitors. Several of these were highly effective in inhibiting estrogen synthesis and some were more potent than 4-OHA. Although several US companies had produced excellent inhibitors, these did not come to clinical trials, largely due to internal company decisions. The field eventually thinned to three companies who had developed highly potent inhibitors.

One of these, exemestane, was a steroidal compound similar to formestane and developed by Farmitalia. The company had a history of making steroid and androgenic compounds mostly for anabolic activity. Exemestane (Fig. 3) has proved to be potent and effective in patients and is now approved by the FDA for breast cancer treatment. The two other FDA approved aromatase inhibitors came from pharmaceutical companies who investigated existing drug types for example, antifungal agents that inhibited cytochrome P-450 enzymes. The challenge was to modify such agents to be selective for aromatase. The result of these endeavors initially included vorazole (Fig. 3)

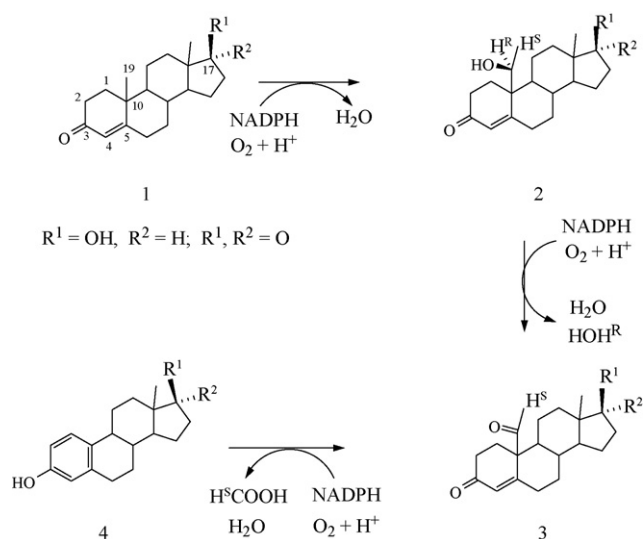
also an inhibitor in this class. It was later discontinued despite good efficacy in breast cancer patients. However, the third generation agents letrozole and anastrozole (Fig. 3) were shown to be highly selective, yet reversible inhibitors of the aromatase enzyme.

## 2.2. The estrogen ER signal transduction pathway as a model for molecular targeting

Because of the importance of estrogen as a stimulus to the development and progression of breast cancer, estrogen synthesis (via aromatase) and action (via ER) continue to be exceptional targets for the treatment and chemoprevention of breast cancer [34]. Thirty years ago the idea of targeting and blocking estrogen action to treat breast cancer with tamoxifen or the idea of blocking the estrogen synthetase (aromatase) enzyme appeared to be simple and straightforward concepts. Today, these simple approaches have become multifaceted with many layers of complexity that are being explored to enhance tissue selectivity, address intrinsic resistance and block the development of acquired antihormonal resistance.

## 2.3. Mechanism of estrogen synthesis

Aromatase mediates the conversion of the steroidal C-19 androgens to C-18 estrogens, which is the critical step in the biosynthesis of estrogens. This enzyme, therefore, has important functions in female development and reproduction. In the human, aromatase is expressed primarily by the



**Fig. 4 – Aromatase mediates conversion of androgens to estrogens. Three hydroxylation steps are postulated.**

ovary in premenopausal women [35]. However, central aromatization is necessary for the manifestations of many sex behavioral, neuroendocrine and developmental responses of several species [36,37]. In addition, aromatase is expressed in a number of other tissues throughout the body. The most important sites of non-gonadal estrogen synthesis are muscle and adipose tissue [38], where production increases with age in both sexes. Peripheral aromatization is the main source of estrogens in postmenopausal women [39] with significant production equivalent to premenopausal levels occurring in the breast [40].

Aromatization of androgens to estrogens occurs via a series of reactions (Fig. 4). An understanding of the mechanisms involved is important to the development of effective aromatase inhibitors. The aromatase complex consists of a cytochrome P450 hemoprotein and a flavoprotein, NADPH-cytochrome P450 reductase. The latter is common to most cell types and functions to donate electrons to the cytochrome P450. The P450 aromatase (P450 arom) binds the C-19 androgen substrates, androstenedione and testosterone and catalyzes their conversion to estrone and estradiol. This reaction is thought to involve three steps, each utilizing 1M equiv. of NADPH and oxygen [41]. The first step is hydroxylation at the C-19 of the androgen substrate. This appears to be a char-

acteristic cytochrome P450 hydroxylation [42,43]. Based on site-directed mutagenesis studies of the enzyme [44–46], it is suggested that hydrogen bonding of the 19-hydroxylation intermediate to an acidic side-chain residue Glu-302 is of critical importance in the aromatization process [46]. Hydrogen bonding of the 3-ketone may also occur at a polar active site (His-128 residue). This anchors the intermediate and assures stereospecific removal of the C-19 pro-R hydrogen by a heme iron-oxo species during the second hydroxylation step. Because of the high electrophilicity of the aldehyde, the usual ferric peroxide breakdown may be circumvented and the normal hydroxylation cycle altered. A number of theories have been postulated to explain the mechanisms involved in the last step. The C10–C19 bond is cleaved resulting in aromatization of the steroid A-ring and release of formic acid. Recently, Hackett et al. [46] showed that the 1 $\beta$ -hydrogen atom removal by an iron-oxo intermediate from the substrate in the presence of the 2,3-enol meets little resistance (5.3–7.8 kcal/mol), whereas in the keto tautomer, this same process encounters barriers of 17.0–27.1 kcal/mol. Although the residues involved in the enolization of C-3 toward C-2 have not yet been identified, they would be essential for the final catalytic step.

Aromatization is a unique reaction in steroid biosynthesis and may therefore be inhibited by selective compounds that do not interfere with other P450 enzymes. Since aromatization is the last step in the biosynthetic sequence of steroid production, blockade of aromatization should not affect production of other steroids. For these reasons, aromatase is a particularly suitable target for inhibition (Table 1).

#### 2.4. Steroidal aromatase inhibitors

The first selective aromatase inhibitors were reported in 1973 and were a number of C-19 steroids [26]. These compounds were substrate analogs and exhibited properties typical of competitive inhibitors. They included 1,4,6-androstatriene-3,17-dione [47] 4-hydroxyandrostenedione (4-OHA) [48] and 4-acetoxyandrostenedione [49]. Interestingly, some of these inhibitors were later found to cause inactivation of the enzyme [28] and appear to be functioning as mechanism-based inhibitors. While not intrinsically reactive, inhibitors of this type are thought to compete rapidly with the natural substrate and subsequently interact with the active site of the enzyme (Fig. 2). They bind either very tightly or irreversibly to the enzyme, thus causing its inactivation [50]. Because they bind to the active site, these inhibitors should be quite specific and should also have lasting effects *in vivo* as a result of

**Table 1 – Randomized Phase III trials of aromatase inhibitors vs. tamoxifen as first-line therapy in metastatic breast Cancer**

Efficacy results, A/I tamoxifen	ORR (%)	Clinical benefit (%)	TTP (months)
Anastrozole, N = 1021 (pooling)	29/27	57/52	8.5/7.0
Letrozole, N = 907 (1 trial)	30/20 <sup>a</sup>	49/38 <sup>a</sup>	9.4/6.0 <sup>a</sup>
Exemestane, N = 382 (randomized Phase II/III trial)	44/29 <sup>a</sup>	72/66	10.9/6.7 <sup>a</sup>

Data from: Bonnetterre et al. [86], Mouridsen et al. [87], Paridaens et al. [89] and Pritchard [90].

<sup>a</sup> Statistically significant; ORR = overall response; TTP = time to progression.

inactivating the enzyme. Thus, the continued presence of the drug to maintain inhibition is not necessary and the chance of toxic side effects, therefore, will be low.

Dr. Chen and co-workers [51] have expressed a structurally stable and functionally active human aromatase in *E. coli*. Using this purified preparation, molecular features of the interaction of androstenedione (substrate) and exemestane (steroidal inhibitor) with aromatase have been studied by UV/vis spectral analysis. In addition, proteomic studies combined with MOLDJ-TOF MS revealed a 3-D overall folding of human aromatase, similar to that of the recently published 3-D theoretical computer model [52]. Proteomic results suggest that aromatase forms a symmetric dimer in solution through the interaction of the F helix and the F-G loop. The B and C helices and the B-C loop of aromatase appear to undergo major conformational changes when the enzyme binds to substrate or steroidal inhibitors. Reaction intermediate analysis suggested that residues E134, D309, T310, 5478, and 1-1480 are involved in enzyme catalysis. From inhibitory profile analysis and time-dependent inhibitory studies, residues E302, D309, and 5478 are thought to participate in the mechanism of the suicide inhibition of aromatase with exemestane.

A number of steroidal aromatase inhibitors in addition to 4-OHA and exemestane have been shown to cause inactivation. Bruggemeier et al [53,54] studied a number of 7 $\alpha$ -substituted androstenedione derivatives, several of which cause inactivation of aromatase. The 1-methylandrosta-1,4-diene-3,17-dione (SH 489) [55] was shown to cause inactivation *in vitro*. Metcalf et al. [56] reported 10-(2-propynyl)estr-4-ene-3, 17-dione (MDL 18962) as the most potent aromatase inhibitor in their series. Two compounds with demonstrated biological activity are 6-methylen-androsta-1,4-diene-3,17-dione (Exemestane, FCE 24304) and 4-aminoandrosta-1,4,6-triene-3,17-dione (FCE 24928), [57] also cause inactivation of aromatase.

In rats, a single oral dose of 25 mg exemestane was found to cause a long-lasting reduction in plasma and urinary estrogen levels. Maximal suppression of circulating estrogens occurred 2-3 days after dosing and persisted for 4-5 days [58]. The lengthy duration of estrogen suppression is thought to be related to the irreversible nature of the drug-enzyme interaction rather than pharmacokinetic properties of the compound. Exemestane causes a marked decrease in serum and urine estrogen levels, and has no effect on other endocrine factors [59-61].

### 3. Non-steroidal aromatase inhibitors

Non-steroidal aromatase inhibitors contain a heteroatom (e.g., N, S, O) possessing a free electron pair for coordination with the heme iron ( $\text{Fe}^{3+}$ ) and a substituent for interaction with other regions of the enzyme (Fig. 5). This type of binding is reflected in Soret band changes (usually bathochromic with respect to Type I inhibitors). Compounds that carry a nitrogen heteroatom have been the most studied and their binding with cytochrome P-450 enzymes give rise to a Type II difference spectrum with Soret maximum at 421-430 nm and minimum at 390-410 nm [62,63]. Intrinsically, nonsteroidal inhibitors are likely to be less enzyme specific than steroidal substrate analogs and can inhibit other cytochrome P450-mediated

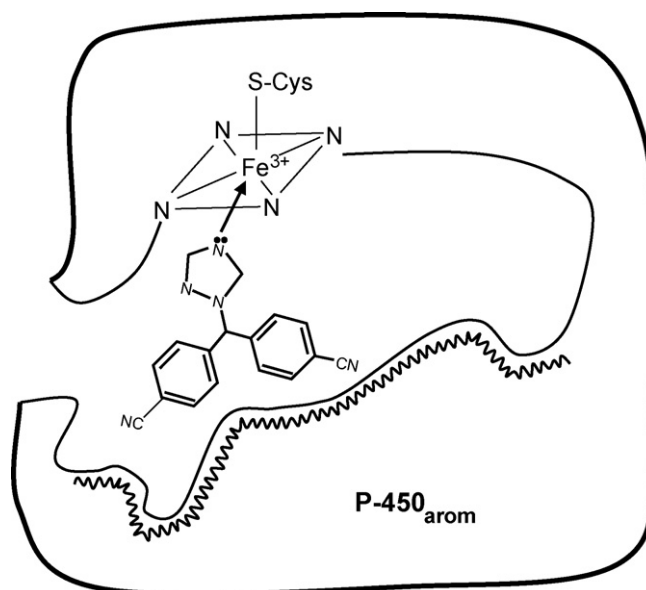


Fig. 5 – Interaction of non-steroidal inhibitors with aromatase.

hydroxylations as was the case for aminoglutethimide [30,64]. The newer non-steroidal inhibitors, anastrozole and letrozole, are triazole derivatives are potent, reversible inhibitors with high specificity. As they do not interact significantly with other P450 enzymes, they have few side effects in patients and have low toxicity.

#### 3.1. Selective modulation of aromatase

The value of aromatase inhibitors as chemopreventives for postmenopausal women would be enhanced by tissue targeting. Remarkably, the regulation of aromatase appears to be different in different tissues. Several tissue-specific promoter regions have been identified upstream from the CYP19 gene [65-67]. Promoter PI.1 is the major promoter used in placental tissues and is the farthest upstream. Promoter II is utilized in the ovary. PII contains a cAMP response element and is predominant in breast cancer tissue as a result of a switch in promoters. Thus, aromatase can be stimulated by prostaglandin  $\text{PGE}_2$  by increasing cAMP levels. Promoters PI.3, PI.4, PI.6, and PI.7 are the promoters used in other extraglandular sites. Promoter PI.3 is also present in adipose tissues such as normal breast tissue, and is increased in breast cancer tissue. Promoter PI.4 is the main promoter used in normal adipose tissue and responds to glucocorticoids and cytokines (e.g., IL-1 $\beta$ , IL-6 and TNF $\alpha$ ). Because of this tissue specific regulation, there is interest in the possibility of identifying aromatase inhibitors that are selective for breast cancer by acting via promoter regulation [68]. Safi et al., [68] reports that orphan nuclear receptor liver receptor homolog-1 (LRH-1) is a specific transcriptional activator of aromatase in human breast preadipocytes and proposes LRH-1 as a target for selective inhibition of aromatase in the breast. Recently, Bruggemeier and colleagues found that a novel series of sulfonilide analogs could suppress aromatase activity and transcription indepen-

dent of Cox-2 [69]. This separation of activities may provide a basis for developing tissue specific aromatase inhibitors.

3.2. The estrogen receptor signal transduction pathway

A model of the current thinking about estrogen action is illustrated in Fig. 6. There are two ligand activated ER signal transduction molecules (ER alpha and ER beta) located at sites through the body. The assumption that is made is that specific receptors can modify the activation or suppression of genes in a particular target site thereby creating cellular homeostasis. The developmental and growth of breast cancer appears to be mediated through a balance of ER alpha and ER beta molecules. There is evidence to suggest that in an environment with an excess of ER beta this will create a growth inhibiting state and modifying the action of tamoxifen by enhancing antiestrogenic properties of the complex. In contrast, when ER alpha dominates the equation, as in breast cancer, estrogen can enhance breast cancer cell survival and tumor growth. Simply stated, the development of drugs to block estrogen regulated tumor growth (tamoxifen, raloxifene [SERMS]) or aromatase inhibitors (4-hydroxyandrostenedione) was predicted to prevent tumor growth, but the molecular biology of the transduction system has now provided a wealth

of new information about drug resistance (see later section) and receptor modulation that is currently being applied to develop new medicines. It is clear from the investigation of the "nonsteroidal antiestrogens" tamoxifen and raloxifene that the receptor is modulated to enhance or suppress gene activation in different target tissues [70]. One proposed mechanism [71] is the balance of corepressor or coactivator proteins that can bind to the SERM ER protein complex to switch off (corepressor) or switch on (coactivator) the transcriptional potential of the complex. Alternatively in an estrogen-deprived environment, the unliganded ER would initially remain inactivated bound to a corepressor molecule.

The natural turnover of ER is accomplished by ubiquitination and destruction through the proteasome system. Fulvestrant, a pure antiestrogen is a derivative of the natural hormone estradiol with a long hydrophobic side chain substituted at the 7 $\alpha$  position on the B ring of the steroid nucleus [72]. The steroid binds to the ligand binding domain of the ER in an inverted configuration with the hydrophobic side chain disrupting the ER shape by binding with the groove that can be occupied by Helix 12 of the ER [73] As a result of the unusual shape of the ER complex, it is rapidly ubiquitinated and destroyed by the proteasome [74]. Pure antiestrogens cause a down regulation of ER so no genes can be activated. In contrast, a promiscuous SERM-like tamoxifen binds to ER

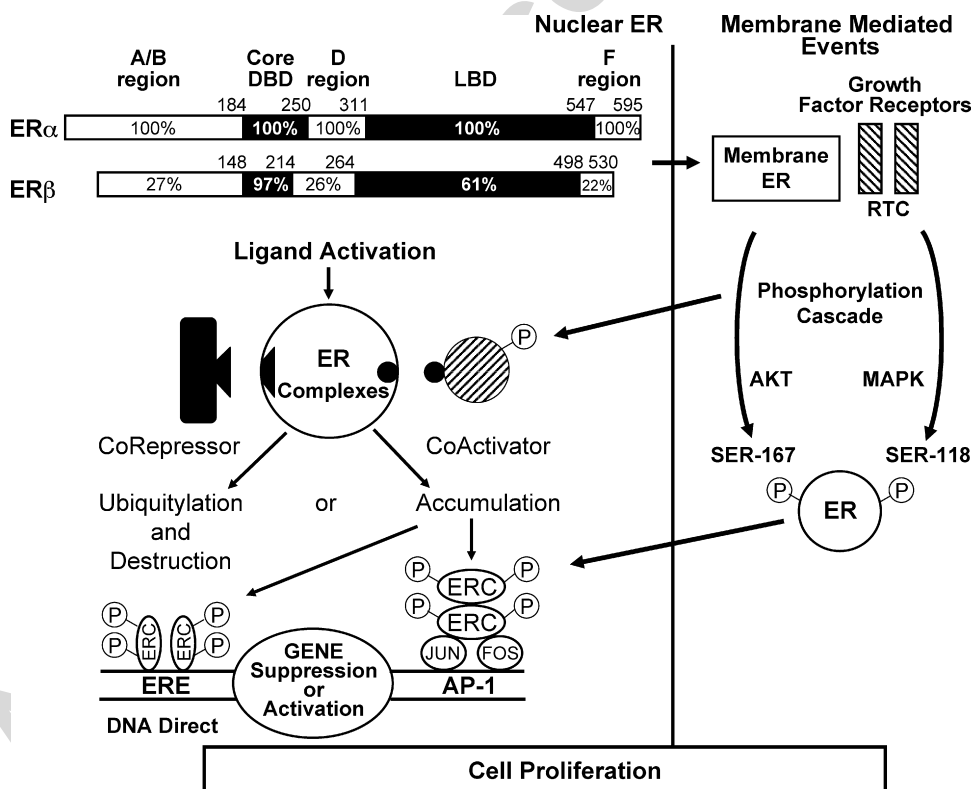


Fig. 6 – A diagrammatic representation of the estrogen signal transduction pathway. The ligand estrogen receptor (either alpha or beta) (ER) complex is modulated in its actions by coactivators (CoA) or the unoccupied receptor is neutralized by corepressors (CoR). Overall the pathway can be further modified by phosphorylation of the ER or modulator molecules via growth factor receptor signaling pathways at the cell membrane (and membrane bound ER). These phosphorylation pathways are enhanced in antihormone resistance to allow the ER to promote unregulated gene activation through genomic and tethered mechanisms. This aids survival by preventing cancer cell apoptosis. Previously published in [14] and reprinted and adapted with permission.

and the complexes can accumulate thereby activating multiple genes via genomic and tethered mechanisms with the promoter region of target genes (Fig. 6).

The simple signal transduction pathway of estrogen action is also modified in cancer to cause either intrinsic or acquired resistance to antihormonal agents. The ER can be phosphorylated in cells with high levels of growth factor (HER2, EGFR, IGF1R) signaling and redeploying from the nucleus to interact with protein kinases at the cell membrane. The ER then becomes part of the phosphorylation signal cascade that auto activates the ER and coactivators. It is essential to appreciate that a current understanding of the integration of the cross talk between ER and growth factor signal transduction pathways is important in antihormonal drug resistance and can be addressed logically in the treatment of breast cancer. This knowledge now provides a window of opportunity to apply new treatment approaches to control tumor growth. Unfortunately, the ER system eventually becomes redundant and the tumor is unresponsive to further antihormonal therapies. This survey of therapeutic target for the treatment of breast cancer can now be melded into a description of the advance in breast cancer treatment and prevention that, 30 years ago, would have seemed unlikely.

### 3.3. Adjuvant therapy with tamoxifen

Based on the successive analysis of accumulative randomized worldwide clinical trials, it is possible to summarize the main conclusions for tamoxifen therapy. At the time 20 years ago, when the Overview analysis first occurred, tamoxifen was the only universally used antihormonal agent. With no other competition, tamoxifen became the "gold standard" and established the principles of tumor targeting and identified the appropriate treatment strategy to aid survivorship [24,75-77].

- Five years of adjuvant tamoxifen enhances disease free survival. There is a 50% decrease in recurrences observed in ER positive patients 15 years after diagnosis.
- Five years of adjuvant tamoxifen enhances survival with a decrease in mortality 15 years after diagnosis.
- Adjuvant tamoxifen does not provide an increase in disease free or overall survival in ER negative breast cancer.
- Five years of adjuvant tamoxifen alone is effective in premenopausal women with ER.
- The benefits of tamoxifen in lives saved from breast cancer, far outweighs concerns about an increased incidence of endometrial cancer in postmenopausal women.
- Tamoxifen does not increase the incidence of second cancers other than endometrial cancer.
- No non-cancer related overall survival advantage is noted with tamoxifen when given as adjuvant therapy.

It is known that cytotoxic chemotherapy causes ovarian ablation by destroying the supply of follicles. However in young women the follicles are too numerous for complete destruction and menses continue after chemotherapy. Although tamoxifen is known to increase ovarian steroidogenesis in women with intact ovaries, [78,79] the antiestrogen is effective and approved for the treatment of premenopausal patients. That being the case, the question arises "should

women without ovarian failure following chemotherapy receive antihormonal therapy?" The evidence suggests that this is a reasonable course of action and tamoxifen profoundly increases the control of recurrences in young premenopausal women following chemotherapy [80].

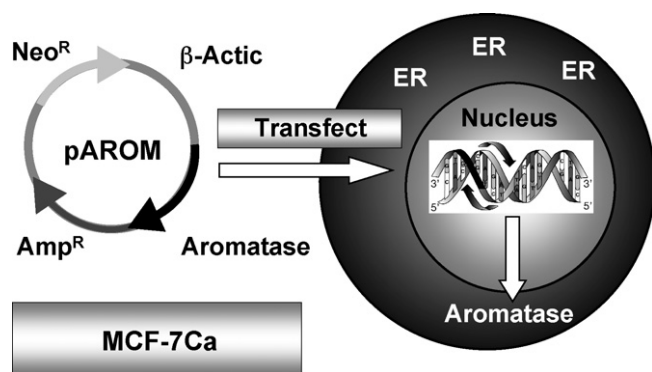
### 3.4. The road to adjuvant treatment with aromatase inhibitors

Two important observations stimulated further clinical development of aromatase inhibitors. The first was the meta-analysis discussed above showing the benefits of tamoxifen treatment [24] in ER+ breast cancer patients. The second was that it was later determined that after 5 years of adjuvant tamoxifen, no further benefit was observed [81]. This opened the door for the introduction of other treatment approaches. Interest was already turning towards aromatase inhibitors to fill the void.

The first clinical studies with formestane (4-OHA) were in patients who had relapsed from all available treatment. This included tamoxifen and second-line treatment with megestrol (medroxyprogesterone acetate) and aminoglutethimide. Although patients were often heavily pre-treated, response to 4-OHA was equal to or better than that of other agents. In addition, the compound was well tolerated and had fewer side effects than either Megestrol or aminoglutethimide. Eventually, 4-OHA was tested against tamoxifen as first-line treatment and was found to have equivalent efficacy [82]. The compound, 4-OHA was approved in most countries for treating patients with advanced breast cancer. Although 4-OHA was more effective in inhibiting peripheral aromatase and better tolerated than aminoglutethimide, subsequent aromatase inhibitors were able to block estrogen synthesis almost completely. Nevertheless, 4-OHA led the way for more potent aromatase inhibitors.

By the early 1990s, clinical trials had begun with aromatase inhibitors produced by several pharmaceutical companies. Novartis had developed oral non-steroidal aromatase inhibitors, fadrozole and letrozole, in addition to having 4-OHA. Letrozole proved especially potent. Oral doses as low as 0.1 and 0.25 mg in patients with metastatic breast cancer caused marked suppression of plasma estradiol, estrone and estrone sulfate levels and were observed within 24 h of the first administration [83]. These results suggest that this compound is a very powerful and selective aromatase inhibitor *in vivo*.

All three FDA approved compounds inhibited peripheral aromatization between 97 and 99%. Two randomized, double-blind studies demonstrated that anastrozole (1 mg daily) was slightly more effective than tamoxifen (20 mg daily) as first-line therapy in postmenopausal women with advanced breast cancer. Among those with ER+ tumors [84-86], the benefit was significant in terms of partial and complete responses including stable disease as well as time to progression. In a multicenter, randomized, double-blind study in advanced breast cancer, letrozole proved to be significantly better than tamoxifen in response rate, clinical benefit, time to progression, and time to treatment failure [87]. Exemestane was also significantly more effective than tamoxifen as first-line therapy in postmenopausal women with advanced breast cancer [88].



**Fig. 7 – A human breast cancer cell line stably transfected with aromatase is sensitive to aromatase inhibitors and antiestrogens.**

Once it was evident that aromatase inhibitors were more effective than tamoxifen, the focus of clinical trials soon moved to the use of the agents in the adjuvant setting for the treatment of early breast cancer [91,92]. The trials studied the effectiveness of aromatase inhibitors following tamoxifen, of aromatase inhibitors alone, and/or of the combination of aromatase inhibitors and tamoxifen in adjuvant therapy. These trials had their foundations in preclinical studies. We had developed a xenograft model that simulated the postmenopausal breast cancer patient. In this model, tumors are grown in ovariectomized, immunodeficient mice from MCF-7 human breast cancer cells stably transfected with the aromatase gene (MCF-7Ca) (Fig. 7). The possibility that blockade of estrogen action and estrogen synthesis may be synergistic was explored by treating mice with the aromatase inhibitor letrozole and the antiestrogen tamoxifen alone and in combination. However, the results in the model indicated that letrozole alone was better than tamoxifen or combined treatments [93]. This result was analogous to results later reported for the ATAC trial [94]. In addition, when tamoxifen treatment was no longer effective, tumor growth was significantly reduced in mice switched to letrozole treatment. Similar conclusions were reached in the MA-17 trial with letrozole [95] and the IES trial with exemestane following tamoxifen in early breast cancer [96]. Based on data from these and other multiple, large randomized trials, it was recommended by the American Society of Clinical Oncology (ASCO) technology assessment panel [97] that optimal adjuvant hormonal therapy for a postmenopausal woman with receptor-positive breast cancer include an aromatase inhibitor as initial therapy or after treatment with tamoxifen.

The aromatase inhibitors are all well tolerated. Patients experienced less gynecologic symptoms such as endometrial cancer, vaginal bleeding, and vaginal discharges. There were fewer cerebrovascular and venous thromboembolic events in patients receiving aromatase inhibitors than in those on tamoxifen. However, a low incidence of bone toxicity and musculoskeletal effects are associated with aromatase inhibitors. The latter includes small but significant increases in arthritis, arthralgia, and/or myalgia with aromatase inhibitors compared to tamoxifen. Fractures were increased with all three

aromatase inhibitors compared to tamoxifen or placebo. The ATAC trial reported a fracture incidence of 7.1% in the anastrozole arm and 4.4% in the tamoxifen arm [94,98]. The fracture rate of letrozole-treated patients in the MA-17 trial was 3.6% versus 2.9% in placebo, following 5 years of tamoxifen treatment [95]. Similar small increases in osteoporosis and/or fractures (7.41%) were associated with the steroidal aromatase inhibitor, exemestane compared to tamoxifen (5.7%) in the IES trial [96].

It is suggested that patients are evaluated for baseline bone mineral density and receive bisphosphonate therapy if indicated [99]. No significant changes in serum cholesterol, HDL cholesterol, LDL Cholesterol, triglycerides or Lp(a) occur in non-hyperlipidemic postmenopausal women treated for 3 years following 5 years of adjuvant tamoxifen [100].

Other studies are on-going to compare the three aromatase inhibitors and/or combination therapies in early stage breast cancer or in the chemoprevention setting. A randomized, Phase III, double-blind trial (BIG 1-98) of the Breast International Group is comparing several adjuvant endocrine therapies in postmenopausal women with ER+ breast cancer. Letrozole versus tamoxifen treatment was compared in the first analysis of the monotherapy arms of the BIG1-98 study. After a median follow-up of 25.8 months, adjuvant treatment with letrozole was found to reduce the risk of recurrences significantly compared with tamoxifen [101]. The MA-27 study is a Phase III adjuvant trial in postmenopausal women with primary breast cancer comparing exemestane to anastrozole, with or without celecoxib, a COX-2 inhibitor. Overall, the aromatase inhibitors are proving to be superior agents to tamoxifen in the treatment of postmenopausal women with all stages of breast cancer. However, the pharmacology of tamoxifen and other non-steroidal antiestrogens was to provide new therapeutic opportunities for improving women's health.

### 3.5. Selective estrogen receptor modulation and chemoprevention

Twenty years ago, tamoxifen was classified as a non-steroidal antiestrogen [102]. Of interest, however, was the observation that tamoxifen was metabolically activated to 4-hydroxytamoxifen, a nonsteroidal antiestrogen with a high binding affinity for the ER [103,104] (Fig. 1). This observation would provide a scientific foundation for future structure activity studies and subsequent drug development in this area. Now a whole range of new compounds with high affinity for the ER are available as therapeutic agents (Fig. 1).

In pharmacological terms tamoxifen was described as a partial agonist (estrogen-like) in target tissues such as the immature rat uterus but it was antiestrogenic because it blocked the full action of estradiol alone. In 1986, it was plausible that if estrogen was necessary to fend off osteoporosis and coronary heart disease the long-term administration of an antiestrogen to node negative women could eventually have a deleterious effect on bone density and produce a potential increase in the incidence of coronary heart disease for the majority of women. The potential side effects would be even worse for women only at high risk to develop breast cancer. Only a small minority of women would have a reduced risk of

breast cancer, but all women would be exposed to potential "antiestrogenic" toxicities. However, the classification of nonsteroidal antiestrogens was to change just after 1986. Today the concept is known as selective ER modulation (SERM).

In 1986, virtually nothing was known about the actions of nonsteroidal antiestrogens on bone density. A single report from NASA scientists showed that clomiphene, an impure isomeric mixture of a nonsteroidal estrogen and antiestrogen used for the induction of ovulation, would preserve bone density in ovariectomized rats [105]. Clearly there were efforts to prevent osteoporosis during space flight but the choice of experimental compound was flawed and frankly, a little bizarre. Since nonsteroidal antiestrogen such as tamoxifen reduce libido in men maybe that was the rationale!

However, the interpretation of the NASA results was not that simple. If clomiphene is an impure mixture of estrogenic and antiestrogenic isomers, which isomer is affecting bone? The consistent laboratory finding that tamoxifen the pure *trans* antiestrogenic isomer of a triphenylethylene maintained bone density in ovariectomized rats [106–108] seemed to translate to postmenopausal women [109], but would prospective clinical studies really show benefit? The Wisconsin Tamoxifen Study was started in 1986 to explore the potential toxicity of tamoxifen on bone density. The study demonstrated, in a double blind placebo controlled clinical trial, that tamoxifen could preserve bone in the postmenopausal woman [110]. Bone building would clearly be an advantage for chemoprevention studies, thereby enhancing the possibility that the worth of tamoxifen to prevent breast cancer could be tested safely. In the same studies, tamoxifen lowered low density lipoprotein [111,112] and, by inference, would appear not to increase the risk of coronary heart disease. These results were good. The bad was the laboratory discovery that although tamoxifen prevented the estrogen-stimulated growth of human breast cancers, the drug stimulated the growth of human endometrial cancers grown in the same athymic mouse [113]. This again was selective ER modulation. Stimulate one target site to produce growth and block the growth of another target site.

There was a very quick response from the clinical community to the warnings [113] that long-term tamoxifen treatment could be associated with an increase in the incidence of endometrial cancer [114–116] but not all reported studies [117,118] found increases in endometrial cancer associated with tamoxifen treatment. These studies were either too small or data was just not collected. There was also a question of whether the high dose of tamoxifen (40 mg. daily) used by Fornander and coworkers [116] was responsible for their findings but the report by Fisher [119] neutralized the argument because NSABP studies all use 20 mg. tamoxifen daily. Endometrial cancer again became an issue during recruitment to the pioneering tamoxifen chemoprevention study by Fisher and the NSABP when it was suggested that extremely dangerous endometrial cancer could be caused by tamoxifen treatment [120]. Nevertheless, results from the prospective chemoprevention study with tamoxifen actually showed that only postmenopausal women developed an excess of early stage mainly grade one endometrial cancers. There were no fatalities from endometrial cancer associated with tamoxifen in the study. [121,122].

The recognition that the so called "nonsteroidal antiestrogens" had estrogenic and antiestrogenic actions at different sites in the ovariectomized female rat and that these data translated to women to prevent bone loss and breast cancer created a new dimension in drug development. The fact that tamoxifen and the failed breast cancer drug keoxifene (LY156,758) [123] both prevented the development of carcinogen-induced rat mammary carcinomas [124] and maintained bone density in ovariectomized rates [106] indicated that this was a class effect. The significance of these observations for public health and chemoprevention of breast cancer was immediately recognized. At the first International Chemoprevention Meeting hosted by Dr. Ezra Greenspan, a group of scientists and clinicians were invited to New York in 1987 to share their vision of the possibilities and potential of chemoprevention [125]. The future of drug development was clear.

"The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused upon 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 upon the developing clues provided by an extensive clinical investigation of available antiestrogens. Could analogs 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 could be treated for these conditions as soon as menopause occurred. Should the agent also retain anti-breast tumor actions then it might be expected to act as a chemosuppressive. 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" [125]. This blueprint to improve healthcare was subsequently restated at the annual meeting of the American Association of Cancer Research in San Francisco, 1989 [126].

Compounds of the keoxifene class (LY117018 and LY156758) were obvious candidates for study despite the fact that the program to develop the drugs to treat breast cancer had been abandoned by Eli Lilly in 1988. The compounds were known to be less uterotrophic than tamoxifen in rodents [127] but they were short acting [128], which could explain their poor antitumor properties when compared with tamoxifen. Interestingly enough, keoxifene was already known to partially inhibit the growth of tamoxifen-stimulated human endometrial tumors under laboratory conditions [129].

Keoxifene, an estrogen that had failed to be developed as a drug to treat breast cancer [123] was reinvented in the early 1990s as raloxifene, a SERM. A use patent for the treatment and prevention of osteoporosis was filed by Eli Lilly in 1992. Raloxifene has now been available for the treatment and prevention of osteoporosis in postmenopausal women since 1999 based on the prospective clinical trials demonstrating an approximately 40% decrease in spinal fractures [130] with the advantage over hormone replacement therapy of causing a 70% decrease in the incidence of breast cancer [131,132]. The anticipated result in reducing the risk of breast cancer as a beneficial side effect of treating osteoporosis propelled raloxifene into clinical trial versus tamoxifen for the prevention of breast cancer as the primary endpoint. The results

from the study of tamoxifen and raloxifene (STAR)<sup>1</sup> in high risk postmenopausal women show that tamoxifen and raloxifene are equivalent in reducing the incidence of breast cancer but there is a decrease in the incidence of endometrial cancer, pulmonary emboli, deep vein thrombosis and endometrial hyperplasia noted with raloxifene. Raloxifene and tamoxifen, as would be expected for two SERMs, both have equivalent activity in preventing fractures [133].

Raloxifene also causes decreases in circulating low density lipoprotein cholesterol [134] and for this reason was evaluated as a preventive for coronary heart disease in the study named raloxifene use for the heart (RUTH) (see footnote 1). Although raloxifene prevents an increase in breast cancer incidence in the RUTH trial, there is no benefit in protecting against coronary heart disease and myocardial infarction [135]. Clearly, further studies with different agents will be needed to rethink the SERM strategy for a multifunctional drug that can prevent cancer (breast/uterus), osteoporosis and coronary heart disease.

Nevertheless, results with raloxifene are part fulfillment of the predicted promise [125,126] of the SERMs as medicines to prevent cancer and osteoporosis. As a result, there are now numerous new SERMs (e.g. lasofoxifene, basedoxifene, arzoxifene, etc.) being evaluated [136,137] (Fig. 1). Additionally, the concept is being applied throughout the steroid receptor super family so the impact on medicine, in the years to come with selective androgens, glucocorticoids or progestins will be considerable.

#### 4. Aromatase inhibitors as chemopreventive agents

Aromatase inhibitors have potential for chemoprevention in women with increased risk of developing breast cancer for many of the same reasons as tamoxifen. Thus, reducing the number of proliferative events by inhibiting the stimulatory effects of estrogen will reduce the number of mutations that would otherwise occur. Evidence to support the value of aromatase inhibitors in the prevention setting comes from the adjuvant clinical trials that compare and contrast tamoxifen with an aromatase inhibitor. All studies show that an aromatase inhibitor is more effective than tamoxifen at preventing the development of contralateral breast cancer [95,101,138]. Based on the recent findings from the STAR trial [133], there is active interest in comparing an aromatase inhibitor with raloxifene. The National Surgical Adjuvant Breast and Bowel Project will compare raloxifene with letrozole in their next clinical trial in postmenopausal women at high risk for breast cancer. The success of such chemoprevention trials will depend on not only reduction in tumor incidence but also the long-term tolerability of the agents. The International Breast Cancer Intervention Group is

currently comparing anastrozole versus placebo in a prevention study, and the accompanying DCIS study is comparing tamoxifen versus anastrozole in women with locally excised ductal carcinoma *in situ* (DCIS) [139]. A three-arm prevention study organized by the National Cancer Institute of Canada, will compare placebo versus exemestane versus exemestane and celecoxib [139]. Although increased risk of stroke and endometrial hyperplasia have not been associated with aromatase inhibitors, other potential side effects such as increased osteoporosis will also be important criteria in considering aromatase inhibitors for preventing breast cancer. Bisphosphonates are currently used by many postmenopausal women to prevent osteoporosis and could be used with aromatase inhibitors if bone loss is a concern.

The possibility that aromatase inhibitors can prevent breast cancer by an additional mechanism has been proposed by several investigators [140]. This hypothesis suggests that estrogens are metabolized to catechol estrogens, 2-hydroxy-estradiol, and 4-hydroxy-estradiol. Evidence exists that 4-hydroxy-estradiol but not 2-hydroxy-estradiol is potentially carcinogenic by forming depurinating estrogen-DNA adducts. Therefore, preventing the formation of estrogens and of their subsequent metabolism to catechol estrogen 3,4-quinones, may provide better protection from breast cancer than targeting the estrogen receptor. Recent studies by Russo et al., [141] demonstrated that exposure of the MCF-10F ER negative cell line to four 24 h alternate periods of 70 nM estradiol induced anchorage-independent growth, loss of ductulogenesis in collagen, invasiveness in Matrigel, and loss of 9p11-13. Only invasive cells that exhibited a 4p15.3-16 deletion were tumorigenic in nude mice. Tumors were poorly differentiated ER- $\alpha$  and PR-negative adenocarcinomas and expressed keratins, EMA, and E-cadherin. The complete transformation of the ER negative MCF-10F cells *in vitro* that resulted in tumor formation *in vivo* supports the concept that estrogen may act as an initiator of breast cancer in women.

##### 4.1. Drug resistance to antihormones

Overall, the long-term, or perhaps indefinite use of antihormonal therapy will result in the development of antihormone resistance. To investigate the mechanisms of resistance to aromatase inhibitors, one approach is to use the xenograft model with tumors of human breast cancer MCF-7Ca cells stably transfected with aromatase [142,143] (Fig. 7). Mice were treated with letrozole until tumors eventually began to grow. The expression of signaling proteins was determined in tumors during the course of letrozole treatment compared to tumors of control mice. Tumors initially upregulated the ER while responding to treatment, but subsequently receptor levels decreased in tumors unresponsive to letrozole. Nevertheless, p-ER (Ser167) was increased suggesting that ligand independent activation of ER may enhance proliferation in tumors treated with letrozole. Tyrosine kinase receptor protein HER-2 and adapter proteins (p-Shc and Grb-2) as well as the signaling proteins in the MAPK cascade (p-Raf, p-Mek1/2, and p-MAPK), but not in the PI3/Akt pathway, were all increased in tumors no longer responsive to letrozole, suggesting the possibility that ER may be phosphorylated by MAPK in the absence of ligand. To investigate whether sensitivity to

<sup>1</sup> The STAR trial compared and contrasted the efficacy of tamoxifen and raloxifene to reduce the incidence of breast cancer in postmenopausal women at high risk for breast cancer. Side effect profiles were a secondary endpoint. The RUTH trial is a placebo-controlled study to determine whether raloxifene would reduce the risk of coronary heart disease in high risk women.

letrozole could be regained, cells were isolated from the letrozole resistant tumors (LTLT) and treated with inhibitors of the MAPK pathway (P098059 and U0126). These compounds reduced MAPK activity and increased ER expression. EGFR/HER-2 inhibitors, gefitinib and AEE78S although not effective in the parental MCF-7Ca cells, also restored the sensitivity of LTLT cells to letrozole. Since there appears to be cross-talk between the ER and the tyrosine kinase receptor, the hypothesis was tested that degrading the ER with fulvestrant may prevent development of resistance. In xenografts, beginning treatment with letrozole plus fulvestrant to down regulate the ER prevented increases in HER-2, activation of MAPK, and was highly effective in inhibiting tumor growth throughout 29 weeks of treatment. These results suggest that disrupting the ER or blocking growth factor-mediated transcription may delay development of resistance to aromatase inhibitors and maintain growth inhibition of ER+ breast cancer by hormonal agents.

Numerous studies have been published on the estrogen deprivation of ER positive breast cancer cells *in vitro*. Two types of cellular response can occur based on the organization of the ER system [144]. In MCF-7 cells, ER is upregulated during conditions of estrogen deprivation whereas T47D cells undergo ER down regulation. As a result MCF-7 cells eventually grow spontaneously in the absence of estrogen [144-146], remain unresponsive to estrogen, but retain ER. In contrast T47D cells lose ER and become hormone independent [147].

There have been extensive laboratory investigations of the development of drug resistance to SERM (tamoxifen and raloxifene) treatment [148]. These studies have established some general principles that not only have applications in the treatment of breast cancer but also provide some unanticipated insights into the potential failure of second or third line treatment with the pure antiestrogen fulvestrant. Additionally, long-term antihormone action has also exposed a vulnerability of the breast cancer cells. Although estrogen is considered to be a survival signal for breast cancer cells, physiologic estrogen can cause rapid apoptosis of estrogen deprived breast cancer cells [149,150], tamoxifen resistant breast cancer cells [151] and raloxifene resistant breast cancer cells [152]. Most interestingly, combinations of physiologic estrogen and fulvestrant can cause a reversal of the apoptotic action of estrogen and robust tumor growth. [153]. Clearly, these observations can have potential implications for the treatment of advanced breast cancer once multiple antihormonal therapies have failed. There are current proposals to use physiological estrogen to reduce tumor burden in sensitive tumors that have responded and failed two consecutive antihormonal therapies. The clinical basis for estrogen responsiveness has already been demonstrated in this patient group [154] but the use of a 12 week course of low dose estrogen followed by the reintroduction of aromatase inhibition plus fulvestrant would not only incorporate the current laboratory results for the design of a clinical trial but also improve patient disease control prior to chemotherapy [155].

#### 4.2. Perspective

We have described our personal experiences over the past 35 years with the investigation and development of antihor-

mones for the treatment and prevention of breast cancer. We have been fortunate to have both been at the right places at the right times to be able to contribute to the advance in therapeutics. However, it should be clear from our narrative that we have also been unfashionable and proposed solutions to treatment at the wrong times. Nevertheless fashions in cancer research often take a decade to change. In our case, the philosophy that cytotoxic combination chemotherapy would cure breast cancer was tested by the clinical community and failed. Unfortunately, the clinical trials community can only test what is provided by the pharmaceutical industry and the idea of killing cancer cells seemed "a good idea at the time". Fortunately, when alternatives to chemotherapy were sought and changes in fashion were occurring, new agents and treatment strategies were in place to fill a potential treatment void. We independently target the same signal transduction pathway but at different points to provide an alternative approach to cytotoxic chemotherapy. Today, the concept of drug targeting has come of age as every new agent being developed by the pharmaceutical industry is designed to target the tumor specifically thereby potentially reducing toxicity for the patient. It remains for a future generation to integrate the new menu of targeted medicines into the treatment plan and to ensure that the appropriate affordable drug is made available to the appropriate woman.

#### Acknowledgements

Dr. Jordan is supported by the Department of Defense Breast Program under award number BC050277 Center of Excellence (Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense), SPORE in Breast Cancer CA 89018, R01 GM067156, FCCC Core Grant NIH P30 CA006927, the Avon Foundation and the Weg Fund of Fox Chase Cancer Center. Dr. Angela Brodie is supported by NIH R01CA-62483, NIH R01CA-27440, and DOD Centers of Excellence "Center for Molecular Targeting of Breast Cancer Metastasis. Parts of this history have been published in Jordan VC: Improvements in Tumor Targeting, Survivorship, and Chemoprevention Pioneered by Tamoxifen." *Oncology* 20(6):553-562, 2006. Reprinted with permission from CMP Healthcare Media, Copyright 2006.

#### REFERENCES

- [1] Hoagland H. The road to yesterday. Worcester MA; 1974.
- [2] Jensen EV, Jacobson HI. Basic guides to the mechanism of estrogen action. *Recent Prog Hormone Res* 1962;18:387-414.
- [3] Kennedy BJ. Hormone therapy for advanced breast cancer. *Cancer* 1965;18:1551-7.
- [4] Jensen EV, Block GE, Smith S, Kyser K, DeSombre ER. Estrogen receptors and breast cancer response to adrenalectomy. *Natl Cancer Inst Monogr* 1971;34:55-70.
- [5] Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. *Br J Cancer* 1971;25:270-5.
- [6] 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.

- [7] Harper MJ, Walpole AL. Mode of action of I.C.I. 46,474 in preventing implantation in rats. *J Endocrinol* 1967;37:83-92.
- [8] Jordan VC. The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. *Breast Cancer Res Treat* 1988;11:197-209.
- [9] Huggins C, Grand LC, Brillantes FP. Mammary cancer induced by a single feeding of polynuclear hydrocarbons and their suppression. *Nature* 1961;189:204.
- [10] Jordan VC, Koerner S, Tamoxifen. (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *Eur J Cancer* 1975;11:205-6.
- [11] Jordan VC. The antioestrogen tamoxifen (ICI 46,474) as an antitumour agent. In: Proceedings Eastern Cooperative Oncology Group Meeting. 1974.
- [12] Jordan VC. Antiestrogenic and antitumor properties of tamoxifen in laboratory animals. *Cancer Treat Rep* 1976;60:1409-19.
- [13] Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discovery* 2003;2:205-13.
- [14] Jordan VC, Tamoxifen. (ICI 46,474) as a targeted therapy to treat and prevent breast cancer. *Br J Pharmacol* 2006;147:S269-76.
- [15] Jordan VC, Dowse LJ. Tamoxifen as an anti-tumour agent: effect on oestrogen binding. *J Endocrinol* 1976;68:297-303.
- [16] Jordan VC, Jaspan T. Tamoxifen as an antitumour agent: oestrogen binding as a predictive test for tumour response. *J Endocrinol* 1976;68:453-60.
- [17] Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. *Eur J Cancer* 1976;12:419-24.
- [18] Jordan VC. Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. *Rev Endocr Relat Cancer* 1978;49-55. October Supplement.
- [19] Jordan VC, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. *Adj Ther Cancer* 1979;2:19-26.
- [20] 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.
- [21] Tormey DC, Jordan VC. Long-term tamoxifen adjuvant therapy in node-positive breast cancer: a metabolic and pilot clinical study. *Breast Cancer Res Treat* 1984;4:297-302.
- [22] Tormey DC, Rasmussen P, Jordan VC. Long-term adjuvant tamoxifen study: clinical update. *Breast Cancer Res Treat* 1987;9:157-8.
- [23] Anonymous. Review of mortality results in randomized trials in early breast cancer. *Lancet* 1984;ii:1205.
- [24] EBCTCG. 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* 1988; 319:1681-92.
- [25] Consensus Conference. Adjuvant chemotherapy for breast cancer. *J Am Med Assoc* 1985;254:3461-3.
- [26] Schwarzel WC, Kruggel WG, Brodie HJ. Studies on the mechanism of estrogen biosynthesis. 8. The development of inhibitors of the enzyme system in human placenta. *Endocrinology* 1973;92:866-80.
- [27] Brodie AMH, Schwarzel WC, Shaikh AA, Brodie HJ. The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen dependent processes in reproduction and breast cancer. *Endocrinology* 1977;100:1684-95.
- [28] Brodie AM, Garrett WM, Hendrickson JR, Tsai-Morris CH, Marcotte PA, Robinson CH. 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* 1981;38:693-702.
- [29] Brodie AM, Longcope C. Inhibition of peripheral aromatization by aromatase inhibitors, 4-hydroxy- and 4-acetoxy-androstene-3,17-dione. *Endocrinology* 1980;106:19-21.
- [30] Samojlik E, Santen RJ, Wells SA. Adrenal suppression with aminoglutethimide. II. Differential effects of aminoglutethimide on plasma androstenedione and estrogen levels. *J Clin Endocrinol Metab* 1977;45:480-7.
- [31] Lipton A, Santen RJ. Medical adrenalectomy using aminoglutethimide and dexamethasone in advanced breast cancer. *Cancer* 1974;33:503-12.
- [32] Santen RJ, Worgul TJ, Samojlik E, Interrante A, Boucher AE, Lipton A, et al. A randomized trial comparing surgical adrenalectomy with aminoglutethimide plus hydrocortisone in women with advanced breast cancer. *N Engl J Med* 1981;305:545-51.
- [33] Santen RJ, Wells SA. The use of aminoglutethimide in the treatment of patients with metastatic carcinoma of the breast. *Cancer* 1980;46:1066-74.
- [34] Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. *Clin Cancer Res* 2003;9:1980-9.
- [35] Inkster SE, Brodie AM. Expression of aromatase cytochrome P-450 in premenopausal and postmenopausal human ovaries: an immunocytochemical study. *J Clin Endocrinol Metab* 1991;73:717-26.
- [36] MacClusky NJ, Naftolin F. Sexual differentiation of the central nervous system. *Science* 1981;211:1294-303.
- [37] Balthazart, J. Steroid metabolism and the activation of social behavior. In: Balthazart J, editor. *Advances in comparative and environmental physiology*, vol. 3. Germany, Berlin: Springer; 1989. p. 105-9.
- [38] Longcope C, Pratt JH, Schneider SH, Fineberg SE. Aromatization of androgens by muscle and adipose tissue in vivo. *J Clin Endocrinol Metab* 1978;46:146-52.
- [39] Hemsell DL, Grodin JM, Brenner PF, Siiteri PK, MacDonald PC. Plasma precursors of estrogen. II. Correlation of the extent of conversion of plasma androstenedione to estrone with age. *J Clin Endocrinol Metab* 1974;38:476-9.
- [40] van Landeghem AA, Poortman J, Nabuurs M, Thijssen JH. Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue. *Cancer Res* 1985;45:2900-6.
- [41] Blankenstein MA, Szymczak J, Daroszewski J, Milewicz A, Thijssen JH. Estrogens in plasma and fatty tissue from breast cancer patients and women undergoing surgery for non-oncological reasons. *Gynecol Endocrinol* 1992;6:13-7.
- [42] Covey DF, Hood WF, Beusen DD, Carrell HL. Hydroperoxides as inactivators of aromatase: 10 beta-hydroperoxy-4-estrene-3,17-dione, crystal structure and inactivation characteristics. *Biochemistry* 1984;23:5398-406.
- [43] Hahn EF, Fishman J. Immunological probe of estrogen biosynthesis. Evidence for the 2 beta-hydroxylative pathway in aromatization of androgens. *J Biol Chem* 1984;259:1689-94.
- [44] Cole PA, Bean JM, Robinson CH. Conversion of a 3-desoxy steroid to 3-desoxyestrogen by human placental aromatase. *Proc Natl Acad Sci USA* 1990;87:2999-3003.
- [45] Kao YC, Korzekwa KR, Laughton CA, Chen S. Evaluation of the mechanism of aromatase cytochrome P450. A site-directed mutagenesis study. *Eur J Biochem* 2001;268:243-51.
- [46] Hackett JC, Brueggemeier RW, Hadad CM. The final catalytic step of cytochrome p450 aromatase: a density functional theory study. *J Am Chem Soc* 2005;127:5224-37.

- [47] Brodie A, Garrett W, Hendrickson JM, Marsh DA, Brodie HJ. The effect of 1,4,6-androstatriene-3,17-dione (ATD) on DMBA-induced mammary tumors in the rat and its mechanism of action in vivo. *Biochem Pharmacol* 1982;31:2017-23.
- [48] Brodie AMH, Schwarzel WC, Brodie HJ. Studies on the mechanism of estrogen biosynthesis in the rat ovary-1. *J Steroid Biochem* 1976;7:787-93.
- [49] Brodie AMH, Marsh DA, Brodie HJ. Aromatase inhibitors IV. Regression of estrogen-dependent mammary tumors in the rat with 4-acetoxy-4-androstene-3,17-dione. *J Steroid Biochem* 1979;10:423-9.
- [50] Sjoerdsma A. Suicide inhibitors as potential drugs. *Clin Pharm Ther* 1981;30:3-22.
- [51] Hong Y, Yu B, Sherman M, Yuan YC, Zhou D, Chen S. Molecular basis for the aromatization reaction and exemestane-mediated irreversible inhibition of human aromatase. *Mol Endocrinol* 2006; in press.
- [52] Favia AD, Cavalli A, Masetti M, Carotti A, Recanatini M. Three-dimensional model of the human aromatase enzyme and density functional parameterization of the iron-containing protoporphyrin IX for a molecular dynamics study of heme-cysteinato cytochromes. *Proteins* 2006;62:1074-87.
- [53] Brueggemeier R, Li P-K, Snider CE, Darby MV, Katlic NE. 7 $\alpha$ -Substituted androstenediones as effective in vitro and in vivo inhibitors of aromatase. *Steroids* 1987;50:163-78.
- [54] Brueggemeier R, Li P-K, Chen HH, Moh PP, Katlic NE. Biochemical pharmacology of new 7-substituted androstenediones as inhibitors of aromatase. *J Steroid Biochem Mol Biol* 1990;37:379-85.
- [55] Henderson D, Norbith G, Kerb U. 1-Methyl-1,4-androstadiene-3, 17-dione (SH 489): Characterization of an irreversible inhibitor of estrogen biosynthesis. *J Steroid Biochem Mol Biol* 1986;24:303-6.
- [56] Metcalf BW, Wright CL, Burkhart JP, Johnston JO. Substrate-induced inactivation of aromatase by allenic and acetylenic steroids. *J Am Chem Soc* 1981;103:3221-2.
- [57] Zaccheo T, Giudici D, Ornati G, Panzeri A, DiSalle E. Comparison of the effects of the irreversible aromatase inhibitor exemestane with atamestane and MDL-18962 in rats with DMBA-induced mammary tumors. *Eur J Cancer* 1991;27:1145-50.
- [58] Evans T, DiSalle E, Ornati G, et al. Phase I and endocrine study of exemestane (FCE 24304), a new aromatase inhibitor, in postmenopausal women. *Cancer Res* 1992;52:5933-9.
- [59] Bajetta EZN, Noberasco C, et al. The minimal effective exemestane dose for endocrine activity in advanced breast cancer. *Eur J Cancer* 1997;33:587-91.
- [60] Geisler JKN, Anker G, et al. In vivo inhibition of aromatization by exemestane, a novel irreversible aromatase inhibitor, in postmenopausal breast cancer patients. *Clin Cancer Res* 1998;4:29-093.
- [61] Lonning P, Bajetta E, Murray R, et al. Activity of exemestane in metastatic breast cancer after failure of nonsteroidal aromatase inhibitors: a phase II trial. *J Clin Oncol* 2000;18:2234-44.
- [62] Jefcoate CR. Measurement of substrate and inhibitor binding to microsomal cytochrome P-450 by optical-difference spectroscopy. *Meth Enzymol* 1978;52:258-79.
- [63] Dawson JH, Andersson LA, Sono M. Spectroscopic investigations of ferric cytochrome P-450-CAM ligand complexes. Identification of the ligand trans to cysteinate in the native enzyme. *J Biol Chem* 1982;257:3606-17.
- [64] Cash R, Brough AJ, Cohen MNP, Satoh PS. Aminoglutethimide (Elipten-CIBA) as an inhibitor of adrenal steroidogenesis. Mechanism of action and therapeutic trial. *J Clin Endocrinol Metab* 1967;27:1239-48.
- [65] Simpson E, Mahendroo MS, Means GD, et al. Tissue-specific promoters regulate aromatase cytochrome P450 expression. *J Steroid Biochem Mol Biol* 1993;44:321-30.
- [66] Zhao Y, Agarwal VR, Mendelson CR, et al. Transcriptional regulation of CYP19 gene (aromatase) expression in adipose stromal cells in primary culture. *J Steroid Biochem Mol Biol* 1997;61:203-10.
- [67] Simpson E, Clyne C, Rubin G, et al. Aromatase—a brief overview. *Annu Rev Physiol* 2002;64:93-127.
- [68] Safi R, Kovacic A, Gaillard S, Murata Y, Simpson ER, McDonnell DP, et al. Coactivation of liver receptor homologue-1 by peroxisome proliferator-activated receptor gamma coactivator-1alpha on aromatase promoter II and its inhibition by activated retinoid X receptor suggest a novel target for breast-specific antiestrogen therapy. *Cancer Res* 2005;65:11762-70.
- [69] Su B, Diaz-Cr ES, Landini S, Brueggemeier RW. Novel sulfonanilide analogues suppress aromatase expression and activity in breast cancer cells independent of COX-2 inhibition. *J Med Chem* 2006;49:1413-9.
- [70] Jordan VC. Selective estrogen receptor modulation: a personal perspective. *Cancer Res* 2001;61:5683-7.
- [71] Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr Rev* 2004;25:45-71.
- [72] Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. *Cancer Res* 1991;51:3867-73.
- [73] Pike AC, Brzozowski AM, Walton J, Hubbard RE, Thorsell A, Li Y, et al. Structural insights into the mode of action of a pure antiestrogen. *Structure* 2001;9:145-53.
- [74] Wijayarathne 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.
- [75] EBCTCG. 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* 1992;339:1-15.
- [76] EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;354:1451-67.
- [77] EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005;365:1687-717.
- [78] Ravdin PM, Fritz NF, Tormey DC, Jordan VC. Endocrine status of premenopausal node-positive breast cancer patients following adjuvant chemotherapy and long-term tamoxifen. *Cancer Res* 1988;48:1026-9.
- [79] Jordan VC, Fritz NF, Langan-Fahey S, Thompson M, Tormey DC. Alteration of endocrine parameters in premenopausal women with breast cancer during long-term adjuvant therapy with tamoxifen as the single agent. *J Natl Cancer Inst* 1991;83:1488-91.
- [80] Colleoni M, Gelber S, Goldhirsch A, Aebi S, Castiglione-Gertsch M, Price KN, et al. Tamoxifen after adjuvant chemotherapy for premenopausal women with lymph node-positive breast cancer: International Breast Cancer Study Group Trial 13-93. *J Clin Oncol* 2006;24:1332-41.
- [81] Fisher B, Dignam J, Bryant J, Wolmark N. Five versus more than five years of tamoxifen for lymph node-negative breast cancer: updated findings from the National Surgical Adjuvant Breast and Bowel Project B-14 randomized trial. *J Natl Cancer Inst* 2001;93:684-90.

- [82] Perez-Carrion R, Alberola V, Calabresi F, Michel R-T, Santos R, Fehling M, et al. Randomized trial of the selective aromatase inhibitor formestane (Lentaron) versus tamoxifen as first-line hormonal therapy in postmenopausal patients with advanced breast cancer. *Breast* 1993;3:198–9.
- [83] Demers LM, Lipton A, Harvey HA, Kambic KB, Grossberg H, Brady C, et al. The efficacy of CGS 20267 in suppressing estrogen biosynthesis in patients with advanced stage breast cancer. *J Steroid Biochem Mol Biol* 1993;44:687–91.
- [84] Bonnetterre J, Thurlimann B, Robertson JF, Krzakowski M, Mauriac L, Koralewski P, et al. Anastrozole versus tamoxifen as first-line therapy for advanced breast cancer in 668 postmenopausal women: results of the Tamoxifen or Arimidex Randomized Group Efficacy and Tolerability study. *J Clin Oncol* 2000;18:3748–57.
- [85] Nabholz 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;18:3758–67.
- [86] Bonnetterre J, Buzdar A, Nabholz JM, Robertson JF, Thurlimann B, von Euler M, et al. Anastrozole is superior to tamoxifen as first-line therapy in hormone receptor positive advanced breast carcinoma. *Cancer* 2001;92:2247–58.
- [87] Mouridsen H, Gershanovich M, Sun Y, Perez-Carrion R, Boni C, Monnier A, et al. Superior efficacy of letrozole versus tamoxifen as first-line therapy for postmenopausal women with advanced breast cancer: results of a phase III study of the International Letrozole Breast Cancer Group. *J Clin Oncol* 2001;19:2596–606.
- [88] Paridaens R, Dirix L, Lohrisch C, Beex L, Nooij M, Cameron D, et al. Mature results of a randomized phase II multicenter study of exemestane versus tamoxifen as first-line hormone therapy for postmenopausal women with metastatic breast cancer. *Ann Oncol* 2003;14:1391–8.
- [89] Paridaens R, Therasse P, Dirix L. First-line hormonal treatment (HT) for metastatic breast cancer (MBC) with exemestane (E) or tamoxifen (T) in postmenopausal patients (pts)—a randomized phase III trial of the EORTC Group. *J Clin Oncol* 2004;22.
- [90] Pritchard KI. Endocrine therapy of advanced disease: analysis and implications of the existing data. *Clin Cancer Res* 2003;9:460S–7S.
- [91] Brodie A, Lu Q, Long B. Aromatase and its inhibitors. *J Steroid Biochem Mol Biol* 1999;69:205–10.
- [92] Santen RJ, Harvey HA. Use of aromatase inhibitors in breast carcinoma. *Endocr Relat Cancer* 1999;6:75–92.
- [93] Long BJ, Jelovac D, Handratta V, Thiantanawat A, MacPherson N, Ragaz J, et al. Therapeutic strategies using the aromatase inhibitor letrozole and tamoxifen in a breast cancer model. *JNCI* 2004;96:456–65.
- [94] 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 randomized trial. *Lancet* 2002;359:2131–9.
- [95] Goss PE, Ingle JN, Marino S, Robert NJ, Muss HB, Piccart MJ, 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;349:1–10.
- [96] Coombes RC, Hall E, Gibson LJ, Paridaens R, Jassem J, Delozier T, et al. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. *N Engl J Med* 2004;350:1081–92.
- [97] Winer EP, Hudis C, Burstein HJ, Wolff AC, Pritchard KI, Ingle JN, et al. American Society of Clinical Oncology technology assessment on the use of aromatase inhibitors as adjuvant therapy for postmenopausal women with hormone receptor-positive breast cancer: status report 2004. *J Clin Oncol* 2005;23:619–29.
- [98] Baum M, Buzdar A, Cuzick J, Forbes J, Houghton J, Howell A, et al. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early-stage breast cancer: results of the ATAC (Arimidex, Tamoxifen Alone or in Combination) trial efficacy and safety update analyses. *Cancer* 2003;98:1802–10.
- [99] Ingle J. Aromatase inhibition and antiestrogen therapy in early breast cancer treatment and chemoprevention. *Oncology (Huntingt)* 2001;15:28–34.
- [100] Wasan KM, Goss PE, Pritchard PH, Shepherd L, Palmer MJ, Liu S, et al. The influence of letrozole on serum lipid concentrations in postmenopausal women with primary breast cancer who have completed 5 years of adjuvant tamoxifen (NCIC CTG MA.17L). *Ann Oncol* 2005;16:707–15.
- [101] Thurlimann B, Keshaviah A, Coates AS, Mouridsen H, Mauriac L, Forbes JF, et al. BIG (Breast International Group) and Group, I-C. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med* 2005;353:2747–57.
- [102] Jordan VC. Biochemical pharmacology of antiestrogen action. *Pharmacol Rev* 1984;36:245–76.
- [103] Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* 1977;75:305–16.
- [104] 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.
- [105] Beall PT, Misra KL, Young RL, Spiut HJ, Evan HJ, LeBlanc A. Clomiphene protects against osteoporosis in the mature ovariectomized rat. *Calcif Tissue Int* 1985;36:123–5.
- [106] 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.
- [107] 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.
- [108] 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.
- [109] Turken S, Siris E, Seldin D, Flaster E, Hyman G, Lindsay R. Effects of tamoxifen on spinal bone density in women with breast cancer. *J Natl Cancer Inst* 1989;81:1086–8.
- [110] Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, et al. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med* 1992;326:852–6.
- [111] 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.
- [112] Love RR, Wiebe DA, Newcomb PA, Cameron L, Leventhal H, Jordan VC, et al. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Ann Intern Med* 1991;115:860–4.
- [113] 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.

- [114] Hardell L. Tamoxifen as risk factor for carcinoma of corpus uterus. *Lancet* 1988;ii:563.
- [115] Jordan VC. Tamoxifen and endometrial cancer [letter]. *Lancet* 1988;2:1019.
- [116] Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* 1989;1:117-20.
- [117] Neven P, DeMylder X, VanBelle Y, Vanderick G, DeMylder E. Tamoxifen and the uterus and endometrium. *Lancet* 1989:375.
- [118] Stewart HJ, Knight GM. Tamoxifen and the uterus and endometrium. *Lancet* 1989;i:375-6.
- [119] Fisher B, Costantino JP, Redmond CK, Fisher ER, Wickerham DL, Cronin WM. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14 [see comments]. *J Natl Cancer Inst* 1994;86:527-37.
- [120] Magriples U, Naftolin F, Schwartz PE, Carcangui ML. High grade endometrial carcinoma in tamoxifen treated breast cancer patients. *J Clin Oncol* 1993;11:485-90.
- [121] Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, 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.
- [122] Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, 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.
- [123] Buzdar A, Marcus C, Holmes F, Hug V, Hortobagyi G. Phase II evaluation of Ly156758 in metastatic breast cancer. *Oncology* 1988;45:344-5.
- [124] 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.
- [125] Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 1988;6:589-95.
- [126] Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res* 1990;50:4177-89.
- [127] Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothioephene derived antiestrogen. *Life Sci* 1983;32:1031-6.
- [128] Jordan VC, Gosden B. Inhibition of the uterotrophic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. *Endocrinology* 1983;113:463-8.
- [129] 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.
- [130] Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, 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 [see comments] [published erratum appears in *JAMA* 1999 Dec 8;282(22):2124]. *JAMA* 1999;282:637-45.
- [131] Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, 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.
- [132] Cauley JA, Norton L, Lippman ME, Eckert S, Krueger KA, Purdie DW, et al. 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* 2001;65:125-34.
- [133] Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. The study of tamoxifen and raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA* 2006;295:2727-41.
- [134] Walsh BW, Kuller LH, Wild RA, Paul S, Farmer M, Lawrence JB, et al. Effects of raloxifene on serum lipids and coagulation factors in healthy postmenopausal women. *JAMA* 1998;279:1445-51.
- [135] Barrett-Connor E, Mosca L, Collins P, Geiger MJ, Grady D, Kornitzer M, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Engl J Med* 2006;355:125-37.
- [136] Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *J Med Chem* 2003;46:1081-111.
- [137] Ariazi EA, Ariazi JL, Cordera F, Jordan VC. Estrogen receptors as therapeutic targets in breast cancer. *Curr Top Med Chem* 2006;6:195-216.
- [138] 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;365:60-2.
- [139] Goss PE. Breast cancer prevention—clinical trials strategies involving aromatase inhibitors. *J Steroid Biochem Mol Biol* 2003;86:487-93.
- [140] Cavaliere E, Chakravarti D, Guttenplan J, Hart E, Ingle J, Jankowiak R, et al. Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. *Biochim Biophys Acta* 2006;1766:63-78.
- [141] Russo J, Fernandez SV, Russo PA, Fernbaugh R, Sheriff FS, Lareef HM, et al. 17-Beta-estradiol induces transformation and tumorigenesis in human breast epithelial cells. *FASEB J* 2006;20:1622-34.
- [142] Jelovac D, Sabnis G, Long BJ, Goloubeva OG, Brodie AMH. Activation of MAPK in xenografts and cells during prolonged treatment with aromatase inhibitor letrozole. *Cancer Res* 2005;65:5380-9.
- [143] Jelovac D, Macedo L, Goloubeva OG, Handratta V, Brodie AMH. Additive antitumor effect of aromatase inhibitor letrozole and antiestrogen fulvestrant in a postmenopausal breast cancer model. *Cancer Res* 2005;65:5439-44.
- [144] Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 1996;56:2321-30.
- [145] Welshons WV, Jordan VC. Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *Eur J Cancer* 1987;232:1935-9.
- [146] 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 Endocr* 1992;90:77-80.
- [147] Pink JJ, Bilimoria MM, Assikis J, Jordan VC. Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. *Br J Cancer* 1996;74:1227-36.
- [148] Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;5:207-13.
- [149] Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen induced apoptosis in breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 2005;94:131-41.

- [150] Lewis JS, Meeke K, Osipo C, Bell E, Kidawi N, Chandel NS, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *JNCI* 2005;97:1746-59.
- [151] 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.
- [152] 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.
- [153] Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant on estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* 2003;95:1597-607.
- [154] 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;67:111-6.
- [155] Jordan VC, Lewis JS, Osipo C, Cheng D. The apoptotic action of estrogen following exhaustive antihormonal therapy: a new clinical treatment strategy. *Breast* 2005;14:624-30.

Author's personal copy

# Problems With the Progesterone Receptor in Practice?

V. Craig Jordan, *Fox Chase Cancer Center, Philadelphia, PA*  
Richard D. Gelber, *Dana-Farber Cancer Institute, Boston, MA*

The estrogen receptor (ER) has proven to be the best target for the treatment and prevention of breast cancer.<sup>1</sup> The link between ER status and response to endocrine ablation originally was observed in women with metastatic breast cancer<sup>2</sup> long before tamoxifen was first marketed in the United States in 1978. The development of tamoxifen for long-term adjuvant therapy<sup>3</sup> and the evaluation of tamoxifen efficacy in worldwide randomized clinical trials led to a substantial increase in disease-free survival and overall survival, but only in the patients with ER-positive tumors.<sup>4,5</sup> Unfortunately, as with all targets in cancer, not all ER-positive tumors respond, despite the fact that initially, the assay was rigorously quality controlled in cooperative groups.

The solution seemed so easy because estrogen is necessary to induce the progesterone receptor (PgR), and therefore, those patients with ER+/PgR+ breast tumors should be more likely to respond to tamoxifen therapy.<sup>6,7</sup> Extrapolation of these data from the metastatic breast cancer setting to adjuvant therapy has been less rewarding. There was initial promise that PgR status correlated well with disease-free and overall survivorship in stage II breast cancer.<sup>8</sup> However, the Early Breast Cancer Trialists' Collaborative Group Overview analysis of randomized clinical trials has found strong correlation between ER status and response to adjuvant tamoxifen but no further benefit associated with positive PgR status.<sup>4,5</sup>

The development of therapeutic agents targeted specifically to block the aromatase enzyme, thereby creating a "no estrogen state," has introduced a new dimension in breast cancer therapeutics.<sup>9</sup> A multitude of recent clinical studies have compared and contrasted several new aromatase inhibitors to adjuvant tamoxifen.

In this issue of the *Journal*, Goss et al<sup>10</sup> report an analysis of ER/PgR status and breast cancer responsiveness to extended adjuvant antihormonal therapy. Following the successful completion of 5 years of adjuvant tamoxifen treatment, the MA.17 trial evaluated 5 additional years of letrozole compared with a placebo control. Patients with ER+/PgR+ breast tumors constituted 73% of the patient population, whereas patients with ER+/PgR- tumors constituted 12% of the study population. The authors found that patients whose tumors are ER+/PgR+ are more likely to benefit from an additional 5 years of letrozole than are those with ER+/PgR- tumors. Should we be surprised?

The authors contend that their result is controversial in light of the fact that a recent retrospective analysis of the ATAC

(Arimidex, Tamoxifen, Alone or in Combination) trial<sup>11</sup> showed that patients with ER+/PgR- tumors are more likely to benefit from anastrozole than tamoxifen. However, the BIG (Breast International Group) 1-98 trial<sup>12</sup> showed that the PgR status did not influence the magnitude of benefit of letrozole compared with tamoxifen.

Patients with advanced breast cancer have a higher response rate to endocrine therapy if they have ER+/PgR+ tumors, compared with ER+/PgR- tumors.<sup>13,14</sup> There is also a strong inverse relationship between S phase fraction (SPF) and steroid receptor classification. Tumors with both ER and PgR expression have low SPF, and SPF increases significantly with loss of PgR.<sup>15</sup> What is the mechanism? We have known for nearly two decades that enhanced epidermal growth factor signaling reduces PgR levels<sup>16</sup> and these ER+ tumor cells respond less completely to antiestrogen treatment than ER+/PgR+ tumor cells.<sup>17,18</sup> These concepts have recently been confirmed and extended with reference to human epidermal growth factor receptor 2-*neu*<sup>19</sup> and insulin-like growth factor receptor signaling.<sup>20</sup> Laboratory studies also suggest that when drug resistance develops during long-term tamoxifen treatment<sup>21</sup> and treatment is stopped, the undetected nascent tumors will still respond to either estrogen or tamoxifen for growth.<sup>22</sup> These data explain the effectiveness of the letrozole after tamoxifen treatment was stopped in MA.17.

The controversy arises when the sequential adjuvant study MA.17 is compared with adjuvant antihormone treatments that are initiated immediately after surgery. Unlike either the comparative ATAC or BIG 1-98 study populations, the MA.17 study investigates responsiveness in an enriched population after tamoxifen. The enrichment is evidenced by the high proportion of ER+/PgR+ tumors (73%) compared to either the ATAC (62%) or BIG 1-98 studies (63%). In other words, the ER+/PgR- tumors are more likely to recur during tamoxifen treatment. A patient can only be included in MA.17 if tamoxifen therapy is successful.

The controversy really centers on the apparent conflict in outcomes of subgroup analyses between ATAC and BIG 1-98. Based on a small study of neoadjuvant therapy<sup>23</sup> in which aromatase inhibitors performed better than tamoxifen in a growth factor-rich environment, the analysis of the ATAC data according to ER/PgR status<sup>11</sup> supported the hypothesis that PgR- status could be used to select a cohort of patients with ER+ disease who would benefit most from adjuvant aromatase inhibitors. The

observation in BIG 1-98 that the degree of benefit from letrozole compared with tamoxifen did not differ according to PgR status at this point seemed to be counterintuitive. Central pathology review of ER and PgR in tumor samples for 58% of randomized cases<sup>24</sup> (subsequently increased to 79% [M. Regan, personal communication, January 2007]) confirmed this lack of enhanced effectiveness of letrozole compared with tamoxifen for the ER+/PgR- cohort in BIG 1-98. In fact, the benefit of letrozole compared with tamoxifen was numerically larger for the ER+/PgR+ cohort compared with the ER+/PgR- cohort based on the centrally reviewed receptor values. Furthermore, 2.3% of the BIG 1-98 cases enrolled on the basis of locally determined receptor-positive breast cancer were found to have no expression of either ER or PgR on central review. This small cohort of false-positive receptor cases had a substantially worse disease-free survival compared with the centrally reviewed receptor-positive cohort, and did not benefit from letrozole compared with tamoxifen. Thus, misclassification of true receptor status can influence observed results. Obtaining quality-controlled quantification of ER and PgR values is essential to assure that patients enrolled in endocrine therapy trials have the targeted disease and also that those cared for outside of clinical trials receive proper adjuvant therapy.

Recently, Dowsett and Allred<sup>25</sup> presented time-to-recurrence results according to a centrally reviewed assessment of ER and PgR conducted on 32% of patients enrolled on the monotherapy arms in the ATAC trial. In contrast to the original ATAC report, which was based on data provided on the case report forms,<sup>11</sup> the centrally reviewed ER+/PgR- cohort did not demonstrate a differentially greater benefit of anastrozole. Notably, the centrally reviewed cases were highly selected according to geographic region, with almost 80% of United Kingdom cases included, but with less than 10% of the cases from the United States and other non-United Kingdom centers submitted for central review. It is possible, therefore, that the method of PgR determination in different parts of the world could have influenced the results.

It is also quite reasonable to conclude that the apparent differences in outcome between ATAC and BIG 1-98 reported initially are due primarily to the play of chance. The importance of PgR status to predict markedly superior response to aromatase inhibitor compared with tamoxifen may have been exaggerated in the original ATAC subgroup analyses. The current report from MA.17 shows that the ER+/PgR+ cohort benefits more from letrozole following tamoxifen than the ER+/PgR- cohort, while BIG 1-98 suggests little difference in the magnitude of the letrozole effect according to PgR status. We fully support the recommendation of Goss et al<sup>10</sup> who, in their study in this issue of the *Journal*, "caution against using these results for clinical decision-making."

#### AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author or immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

**Employment:** N/A **Leadership:** N/A **Consultant:** N/A **Stock:** N/A  
**Honoraria:** N/A **Research Funds:** Richard Gelber, Novartis **Testimony:** N/A **Other:** N/A

#### AUTHOR CONTRIBUTIONS

**Conception and design:** V. Craig Jordan, Richard D. Gelber

**Administrative support:** V. Craig Jordan

**Collection and assembly of data:** V. Craig Jordan

**Data analysis and interpretation:** V. Craig Jordan

**Manuscript writing:** V. Craig Jordan, Richard D. Gelber

**Final approval of manuscript:** V. Craig Jordan, Richard D. Gelber

#### REFERENCES

- Jensen EV, Jordan VC: The estrogen receptor: A model for molecular medicine—The Dorothy P. Landon AACR Prize for Translational Research. *Clin Cancer Res* 9:1980-1989, 2003
- McGuire WL, Carbone PP, Sears ME, et al: Estrogen receptors in human breast cancer: An overview, in: McGuire WL, Carbone PP, Volmer EP (eds): *Estrogen Receptor in Human Breast Cancer*. Raven Press, New York, NY, 1975, pp 1-7
- Jordan VC: Tamoxifen: A most unlikely pioneering medicine. *Nat Rev Drug Discov* 2:205-213, 2003
- Tamoxifen for early breast cancer: An overview of the randomised trials. *Lancet* 351:1451-1467, 1998
- 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, 2005
- Horwitz KB, McGuire WL, Pearson OH, et al: Predicting response to endocrine therapy in human breast cancer: A hypothesis. *Science* 189:726-727, 1975
- Elledge RM, Green S, Pugh R, et al: Estrogen receptor (ER) and progesterone receptor (PgR), by ligand-binding assay compared with ER, PgR and pS2, by immuno-histochemistry in predicting response to tamoxifen in metastatic breast cancer: A Southwest Oncology Group Study. *Int J Cancer* 89:111-117, 2000
- McGuire WL, Clark GM, Dressler LG, et al: Role of steroid hormone receptors as prognostic factors in primary breast cancer. *NCI Monogr* 19-23, 1986
- Jordan VC, Brodie AMH: Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* 72:7-25, 2007
- Goss PE, Ingle JN, Martino S, et al: Efficacy of letrozole extended adjuvant therapy according to estrogen receptor and progesterone receptor status on the primary tumor. *J Clin Oncol* 25:2006-2011, 2007
- Dowsett M, Cuzick J, Wale C, et al: Retrospective analysis of time to recurrence in the ATAC trial according to hormone receptor status: An hypothesis-generating study. *J Clin Oncol* 23:7512-7517, 2005
- Thurlimann B, Keshaviah A, Coates AS, et al: A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med* 353:2747-2757, 2005
- Bloom ND, Robin EH, Schreiberman B, et al: The role of progesterone receptor in the management of advanced breast cancer. *Cancer* 45:2992-2997, 1980
- Osborne CK, Yochmowitz MG, Knight WA, et al: The value of estrogen and progesterone receptors in the treatment of breast cancer. *Cancer* 46:2884-2888, 1980
- Wenger CR, Beardslee S, Owens MA, et al: DNA ploidy, S-phase, and steroid receptors in more than 127,000 breast cancer patients. *Breast Cancer Res Treat* 28:9-20, 1993
- Cormier EM, Wolf MF, Jordan VC: Decrease in estradiol-stimulated progesterone receptor production in MCF-7 cells by epidermal growth factor and possible clinical implication for paracrine-regulated breast cancer growth. *Cancer Res* 49:576-580, 1989
- Cormier EM, Jordan VC: Contrasting ability of antiestrogens to inhibit MCF-7 growth stimulated by estradiol or epidermal growth factor. *Eur J Cancer Clin Oncol* 25:57-63, 1989
- Robinson SP, Jordan VC: The paracrine stimulation of MCF-7 cells by MDA-MB-231 cells: Possible role in antiestrogen failure. *Eur J Cancer Clin Oncol* 25:493-497, 1989
- 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* 95:353-361, 2003
- Cui X, Zhang P, Deng W, et al: Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: Progesterone receptor as a potential indicator of growth factor activity in breast cancer. *Mol Endocrinol* 17:575-588, 2003
- Jordan VC: Selective estrogen receptor modulation: Concept and consequences in cancer. *Cancer Cell* 5:207-213, 2004

22. Gottardis MM, Jordan VC: Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 48:5183-8187, 1988

23. Ellis MJ, Coop A, Singh B, et al: Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: Evidence from a Phase III randomized trial. *J Clin Oncol* 19:3808-3816, 2001

24. Viale G, Regan M, Dell'Orto P, et al: Central review of ER, PgR and HER-2 in BIG 1-98 evaluating letrozole versus tamoxifen as adjuvant endocrine therapy for postmenopausal women with receptor-positive breast cancer. *Breast Cancer Res Treat* 94:S13, 2005 (suppl 1; abstr 44)

25. Dowsett M, Allred D, on behalf of the TransATAC Investigators: Relationship between quantitative ER and PgR expression and HER2 status with recurrence in the ATAC trial. *Breast Cancer Res Treat* 100:S21, 2006 (suppl 1; abstr 48)

---

■ ■ ■

### **Acknowledgment**

V. Craig Jordan is supported by the Department of Defense Breast Program under award no. BC050277 Center of Excellence (views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense), SPORE in Breast Cancer CA 89018, R01 GM067156, FCCC Core Grant NIH P30 CA006927, the Avon Foundation, and the Weg Fund of Fox Chase Cancer Center.

Richard D. Gelber is supported in part by Grant No. U24 CA-075362 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

---

## Special Review

---

# Optimizing the Antihormonal Treatment and Prevention of Breast Cancer

Roshani R. Patel, Catherine G. N. Sharma, and V. Craig Jordan

Fox Chase Cancer Center, Philadelphia

---

The incidence of breast cancer is rising throughout the world. Breast cancer is slowly becoming more prevalent in countries which previously had low rates of cancer as well as becoming a leading cause of cancer death in some countries. Fortunately, a large number of these tumors are estrogen receptor (ER) positive and respond to anti-hormonal adjuvant therapy which until recently has been 5 years of tamoxifen treatment. Unfortunately, a significant number of patients develop recurrent cancers and the recurrent tumors are resistant to tamoxifen treatment. In addition, because of tamoxifen's selective estrogenic actions, there have been reports of venous thrombosis, endometrial cancer, and strokes in patients receiving tamoxifen therapy. Thus, there are other novel therapies such as aromatase inhibitors that block estrogen production in postmenopausal women or fulvestrant that destroys the estrogen receptor. This paper will summarize the therapeutic options for anti-hormonal therapy, the role of anti-hormonal agents in advanced breast cancer, and adjuvant therapy and the current status of chemoprevention with selective ER modulators.

*Breast Cancer 14:113-122, 2007.*

Key words: Tamoxifen, Aromatase inhibitors, Fulvestrant, Estrogen receptor

---

### Introduction

There are approximately 1,000,000 new cases of breast cancer in the world each year<sup>1)</sup>. Unfortunately, as we increase our understanding of the biologic behavior of these tumors, the incidence of breast cancer continues to rise throughout the world.

There are currently over 2 million breast cancer survivors in the United States. This year, there will be an estimated 212,920 new cases of invasive breast cancer with the rate of invasive breast cancer increasing by 0.3% per year since 1987<sup>2)</sup>. According to the surveillance, epidemiology, and end results (SEER) from the 1998-2002 National Institutes of Health databases, there was an incidence of 501.8 cases of breast cancer per 100,000 women in the United States during this time period. The mortality rate was 103.8 per 100,000 women<sup>2)</sup>. On a stage by stage basis, 55%

of women had stage 0-1 breast cancer. The next largest group had stage 2 breast cancer and constituted 30% of the patients. Stage 3, 4, and unstaged breast cancer each constituted 5 % of breast cancer patients<sup>3)</sup>.

In Japan, the incidence of breast cancer is much lower than the United States. However, the incidence has been rising steadily. Since 1975, the incidence of breast cancer has more than doubled. In 1975, approximately 15/100,000 women developed breast cancer. By the year 2000, approximately 45/100,000 women developed breast cancer. The majority of the women with breast cancer were 50 years of age or older<sup>4)</sup>. Additionally, from 1970-1999, out of approximately 15 million women who were screened, 2,340 cases of breast cancer were detected. In the time period between 1999-2000 alone, there were 1,168 new cases of breast cancer out of 986,913 patients screened<sup>5)</sup>. In 2001, approximately 9,654 women died of breast cancer in Japan<sup>6)</sup>. By the year 2020, the Japanese Cancer Registry estimates that the annual incidence of breast cancer will be 127,000 in Japan if current trends continue<sup>7)</sup>.

The discrepancies in breast cancer incidence and the trends in an increased incidence in Japan

---

Reprint requests to V. Craig Jordan OBE, PhD, DSc, Vice President and Research Director for Medical Science, Alfred G. Knudson Chair of Cancer Research, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111-2497  
Email: v.craig.jordan@fccc.edu

lead one to question the underlying cause. Throughout history, scientists have noted that breast cancer occurred at a higher frequency in nuns and nulliparous women. Also, first childbirth at a later age correlates with breast cancer risk. In western countries, childbearing occurs at a later age. Traditionally, Japanese women tend to have more children and start having children at a younger age. Obesity and diets high in saturated fats such as American diets also correlate with higher rates of breast cancer. Traditional Japanese diets are 10-25% fat, whereas U.S diets are 40-45% fat<sup>8</sup>. In the past decade, more Japanese women are having children at a later age, fewer children, and have adopted more of a "western" diet which may explain the rise in breast cancer incidence<sup>9</sup>. What does this mean? Progesterone causes maturation of the glandular breast tissue during pregnancy, so early pregnancy can be viewed as chemoprevention. In nulliparous women, the breast tissue is exposed to unopposed estrogen, which causes proliferation of the gland. Higher fat stores in the body lead to higher levels of peripheral estrogen, particularly during the postmenopausal period. Thus, estrogen can be viewed as promoting estrogen responsive breast cancer development. In the United States, this is evidenced by a rise in estrogen receptor (ER) positive breast cancer and a decrease in ER negative breast cancers<sup>10</sup>. Since 1990, the SEER database in the United States has been updated to include ER positive and ER negative breast cancers. Out of 82,488 breast cancer patients from the 1992-1998 databases, 25% of the women had ER negative cancer and 75% of the women had ER positive cancer. Of those patients with ER negative cancer, 21% were greater than 50 years of age, while 37% were less than 50 years of age<sup>11</sup>.

In Japan, a few small studies have been undertaken at various hospitals looking at cases from the 1970's to determine the percentage of ER positive cancer. No recent studies have been found. One early study demonstrated that 55% of 456 patients had ER positive breast cancer. The distribution was similar between pre-menopausal and post-menopausal patients<sup>12</sup>. Another paper compared international studies of ER positive cancer in the 1970's<sup>13</sup>. The rate of ER positive breast cancer in a study from 1977 was 58% (1060 patients) in Japanese and 71% in American women. The patients were further categorized into premenopausal and postmenopausal patients. At this time,

57% of premenopausal Japanese women had ER positive cancer and 59% of premenopausal American women studied had ER positive cancer. The discrepancy in ER positive status was primarily between postmenopausal patients in both countries. Postmenopausal Japanese women had 59% ER positivity, while 71% of postmenopausal American women were ER positive<sup>14</sup>. Another study compared 260 patients from Japan with 410 patients in Western countries. The regression rate of ER positive tumors to endocrine therapy was 48% and 55%, respectively<sup>15</sup>. Regardless of the difference in ER positive tumors, the response to endocrine therapy was similar.

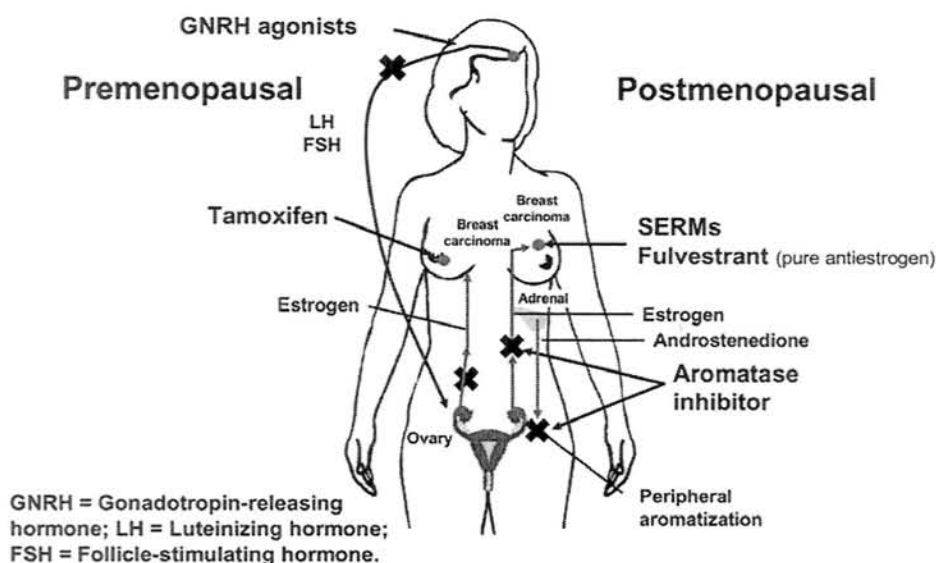
Clearly, it would be interesting to compare the current incidence of ER positive tumors between Japan and Western countries. Since the incidence of breast cancer is rising in Japan and many Japanese women have adopted "western" lifestyles, one would hypothesize that the incidence of ER positive breast cancer has risen in Japan.

The pioneering work of Elwood Jensen<sup>16</sup> identified the ER as the signal transduction pathway that controls the growth of the majority of breast cancers. The ER subsequently became the therapeutic target for the development of antiestrogenic drugs<sup>17</sup>. Thus, antihormonal therapy plays an important role in the therapeutic armamentarium for high risk patients and those patients with a diagnosis of breast cancer. Several established options to treat ER positive breast cancer are now available since the introduction of tamoxifen to Japan more than 20 years ago.

### ***The Strategic Application of Endocrine Therapy:***

Estrogen plays a key role in the development and proliferation of milk glands in the breast. Until recently, it was generally accepted that estrogen does not directly cause breast cancer. However, recent laboratory studies indicate that estrogen has an oncogenic action in breast cells<sup>18, 19</sup>. Nevertheless, because of the strong signal that estrogen has on mediators of the cell cycle, cells that have oncogenic mutations may continue to divide in the presence of estrogen<sup>20</sup>. Eventually, these cells develop into a palpable tumor. For patients who have ER positive breast cancer, there are important therapeutic options a clinician can implement based on the well developed strategy of anti-estrogenic therapy.

The intent of antihormonal therapy in women



**Fig 1.** The sites of action of various classes of endocrine agents used to treat ER positive breast cancer. SERMs such as tamoxifen and toremifene inhibit breast cancer cell proliferation and act as antagonists in the breast. Aromatase inhibitors block the aromatization of androstenedione into estradiol in peripheral tissues, hence preventing production of estrogen. GNRH agonists such as goserelin prevent release of luteinizing hormone and thus inhibit the activation of estrogen production in the ovary. Fulvestrant destroys the ER in breast cancer cells and prevents ER mediated cell replication.

is based upon blocking the ER signal transduction pathway or inhibiting the synthesis of estrogen (Fig 1). One option is to use selective estrogen receptor modulators (SERMs) that block the ER and stop breast cancer growth. Tamoxifen, the prototypical SERM, exerts its effect by binding to the ER receptor and altering the conformation of the complex, thereby inhibiting signal transduction cascades that stimulate cell replication. Unfortunately, tamoxifen expresses its SERM action by promoting cell replication in endometrial cancer cells. This is an estrogen like action that results in a small but significant increase in the detection of endometrial cancer during long term adjuvant therapy or when used in chemoprevention. Nevertheless, it is important to stress that the increase in endometrial cancer noted with tamoxifen only occurs in postmenopausal women<sup>21, 22</sup>. Tamoxifen remains the treatment of choice for premenopausal patients.

An alternative to SERMs is to block the synthesis of estrogen in the body with an aromatase inhibitor. Anastrozole, letrozole, and exemestane have all been shown to be effective in treating advanced breast cancer in postmenopausal women. These drugs inhibit the synthesis of estrogen in peripheral tissues<sup>23</sup>. It is important to stress that aromatase inhibitors should not be

used in premenopausal women because ovarian estrogen production may resume through feedback mechanisms<sup>24</sup>. Finally, another option for hormonal therapy in post-menopausal women with advanced breast cancer and recurrent cancer is to use an ER down-regulator called fulvestrant. Fulvestrant is administered as a monthly injection to provide a slow, continuous release of the drug to the patient. The active drug binds to and alters the shape of the ER in the tumor cell. The peculiar shape of the fulvestrant-ER complex then results in the rapid destruction of the ER. As a result of the destruction of the ER signal transduction pathway, fulvestrant has no agonist activity on the ER<sup>25</sup>.

An antihormonal option for premenopausal women is the long acting gonadotropin releasing hormone, goserelin. The sustained release of goserelin from the implantation of a rice grain sized depot lasts for one month and desensitizes the hypothalamic-pituitary axis to produce the equivalent of a medical oophorectomy<sup>26</sup>.

In this paper, we will discuss the agents currently available in the United States to treat ER positive breast cancer. We will first discuss the use of antihormonal agents to treat metastatic breast cancer. This will serve as a basis for our discussion of current adjuvant therapies and

recent results of chemoprevention studies.

### ***Tamoxifen, the Pioneer for Hormonal Therapy for Advanced Breast Cancer and Adjuvant Therapy***

The treatment for advanced breast cancer has changed dramatically over the past three decades. Initially, advanced breast cancer therapeutics focused on nonspecific cytotoxic agents. The reinvention of tamoxifen from a failed "morning after pill" to the first targeted therapy for breast cancer provided the clinical research community with an invaluable new therapeutic tool to pioneer the strategy of long term antihormonal therapy and chemoprevention<sup>27</sup>. Not surprisingly, enthusiasm from the medical community and pharmaceutical industry was not high. The early trials have been summarized with approximately a 30% response rate in affected patients<sup>28</sup>. This improves if ER positive patients are selected for targeted treatment. Tamoxifen responses were the same as any other endocrine approach to breast cancer; however, the advantage of tamoxifen was the low incidence of side effects compared to other endocrine therapies<sup>29</sup>.

The use of tamoxifen as the pioneering agent as an adjuvant to surgery has its origins in studies from the 1970's<sup>27</sup>. The laboratory studies demonstrated the feasibility of targeting the ER and using long term anti-hormonal therapy<sup>30, 31</sup> so that tamoxifen could be reasonably considered for use as an adjunct to surgery in node positive and then node negative patients. The reason for using tamoxifen as an adjuvant following surgery is to prevent recurrence. The clinical trials ultimately showed both increases in disease-free and overall survival with adjuvant tamoxifen treatment<sup>21</sup>. The studies demonstrated that there is a 50% decrease in recurrence in ER positive patients with 5 years of tamoxifen. Even 15 years after a diagnosis of ER positive breast cancer, treatment with 5 years of tamoxifen continues to decrease mortality<sup>32</sup>. However, there is no increase in disease free or overall survival with ER negative cancer.

The transition of tamoxifen from a short term treatment to a long term therapy for node positive and node negative breast cancer increased awareness of the pharmacology and side effects of tamoxifen<sup>33</sup>. Tamoxifen is a SERM, so the drug preserves bone density and potentially reduces the risk of fractures secondary to its agonist effects on the ER receptors in bone. Additionally,

tamoxifen decreases circulating cholesterol, but this side effect is thought to greatly improve patient prognosis. The negative side effects include an increase in the incidence of endometrial cancer and venous thrombosis. However, the benefits of tamoxifen treatment greatly outweigh the risks of endometrial cancer and venous thrombosis<sup>32</sup>.

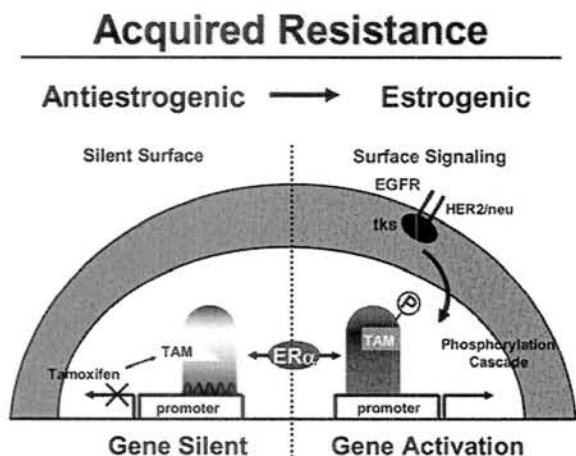
The ubiquitous use of tamoxifen in the treatment plan for breast cancer has improved patient prognosis and enhanced survivorship dramatically. Nevertheless, the knowledge of the estrogen-like side effects of tamoxifen has focused research efforts on understanding tamoxifen induced drug resistance and the development of new and safer agents to treat breast cancer.

### ***Drug resistance to Tamoxifen:***

Resistance to therapy is most often observed during the treatment of advanced breast cancer. After 1-3 years of tamoxifen treatment, breast tumors start to grow despite continuing tamoxifen therapy. However, what is unique about tamoxifen resistant tumors is that a withdrawal response occurs if tamoxifen treatment is stopped<sup>34</sup>. The tumor is dependent on tamoxifen. This phenomenon is best illustrated in the laboratory. Animal models have demonstrated that ER + breast cancer lines can eventually develop resistance to tamoxifen and the tumors then grow in response to tamoxifen<sup>35</sup>. The resistance to tamoxifen is believed to occur because of an increase in cell surface signaling through the HER2/neu/EGFR or Insulin like growth factor receptors that promote phosphorylation of the ER and its coactivators<sup>36</sup>. This in turn activates breast cancer cell growth (Fig 2). Based on the recognition that tamoxifen has limitations and that less estrogenic like drugs would be useful therapeutic agents, it is reasonable to examine the rational application of aromatase inhibitors and fulvestrant.

### ***Second Line Therapy Following the Failure of Tamoxifen***

In addition to stopping tamoxifen, there are multiple ways to address failure of tamoxifen therapy and subsequent resistance to tamoxifen (Fig 3). At present, the first two options are hypothetical. Clinicians have an intense interest in the feasibility of blocking the cell surface receptors with monoclonal antibodies or blocking the tyrosine kinases that cause tamoxifen resistance. At present there are suggestions that blocking the HER2/

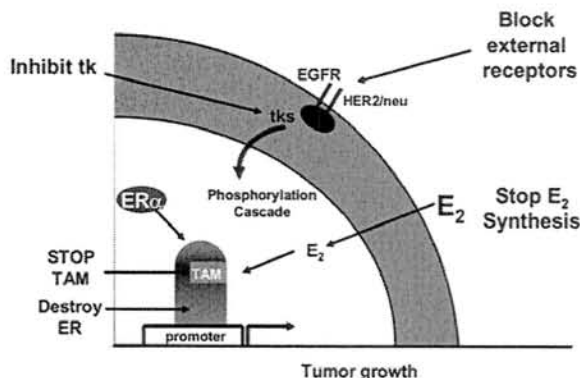


**Fig 2.** The proposed mechanism of tamoxifen resistance. With a silent surface, tamoxifen successfully prevents ER stimulated proliferation by altering the shape of the ER. With surface signaling, an increase in cell surface signaling through the HER2/neu/EGFR receptor promotes phosphorylation of the ER. This leads to proliferation of breast cancer cells in the presence of tamoxifen or estrogen.

neu pathway has promise, but only in patients with gene amplification. In contrast, the second two options for second line therapy are based on the mechanism of tamoxifen resistance. Laboratory studies show that tamoxifen resistant tumors will grow both with tamoxifen and estrogen<sup>35</sup>. Estrogen is produced by aromatization in postmenopausal women. Therefore, a plan to stop the reactivation of tumor growth after tamoxifen is reasonable. Aromatase inhibitors are the agent of choice to create a “no estrogen” state<sup>37, 38</sup>. Fulvestrant can also be used to destroy the estrogen receptor completely. In the absence of the ER, estrogen stimulated proliferation cannot occur, regardless of the cellular signaling mechanisms that activate the ER. Clinical studies of second line therapies after the development of tamoxifen resistance show that anastrozole and fulvestrant are equally effective in controlling breast tumor growth<sup>39, 40</sup>. Thus, for the purposes of clinical clarity, the treatment paradigm for patients who fail SERM therapy can be summarized as shown in figure 4.

**Adjuvant Therapy with Aromatase Inhibitors**

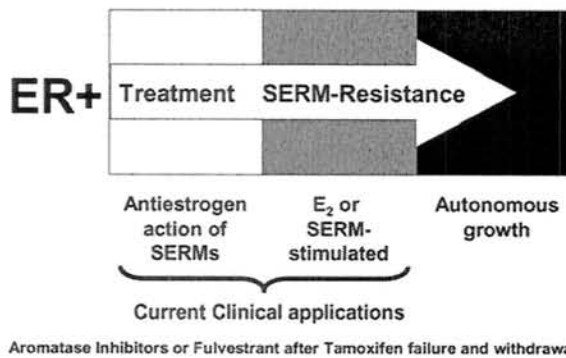
Since aromatase inhibitors do not have the estrogen-like side effects noted with tamoxifen and there is clinical evidence that they can be used once resistance to tamoxifen occurs, the logical question arises of whether they are an improvement over tamoxifen in the clinical setting. Five different studies (Fig 5) have shown



**Fig 3.** Possible strategies to prevent ER receptor activation in SERM resistant breast cancer. Herceptin, a monoclonal antibody blocks Her2/neu/EGFR to prevent phosphorylation of the ER. Alternatively, a number of small molecules that inhibit the receptor tyrosine kinase are being explored. Aromatase inhibitors prevent the formation of estrogen in a postmenopausal woman’s body. Without estrogen available to bind the receptor, the tamoxifen resistant cells cannot proliferate. Another option is to destroy the receptor completely with fulvestrant, a pure antiestrogen that binds to the ER. The strange shape of the complex programs the ER complex for rapid destruction. Thus, there will not be any ER mediated replication of cancer cells.

that the aromatase inhibitors, anastrozole, letrozole, and exemestane are better than tamoxifen at preventing contralateral breast cancer, improving disease-free survival, decreasing the risk of endometrial cancer, and have no additional risk of blood clots. In all of the studies, the patient demographics, stages of cancer, and hormone receptor status were well matched between control and experimental groups. The primary endpoint was locoregional and distant recurrences. Disease-free survival was compared between treatment and control groups as well as side effect profiles, adverse events, and deaths related or unrelated to breast cancer.

Several questions were addressed by the different studies. One question that was addressed by Boccardo *et al.*<sup>41</sup> was whether or not switching to anastrozole after two to three years of tamoxifen would help prevent relapse. The median follow up time was 36 months. One group received tamoxifen for 5 years (225 patients) and the other group received tamoxifen for 2-3 years followed by anastrozole for 3 years (223 patients). Patients who switched to anastrozole had a longer disease free survival. The difference between switching to anastrozole and continuing tamoxifen was 5.8%<sup>41</sup>. In addition, another larger international study examined the use of exemestane after 2-3 years of



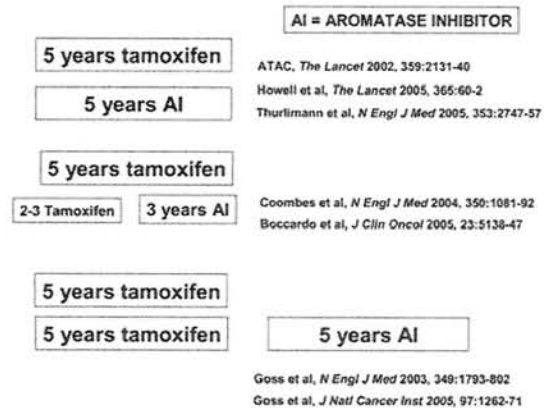
**Fig 4.** The development of SERM resistance noted in the clinical setting. During the treatment phase, tamoxifen effectively blocks ER mediated cell proliferation. During the development of tamoxifen resistance, estrogen or SERMs can stimulate breast cancer cell growth. The cancer cells need tamoxifen or estrogen to grow. Finally, resistant breast cancer cells can acquire autonomous growth (ie. The tumor is not dependent on either estrogen or the SERM for survival).

tamoxifen (2380 patients) vs. continuing tamoxifen (2362 patients), The design was similar to the study design by Boccardo *et al.* and the disease free survival was improved by 4.7 % when patients were switched to exemestane<sup>42)</sup>.

However, the question has been asked “can an aromatase inhibitor improve prognosis after the full five years of tamoxifen treatment?” Goss and his coworkers have compared letrozole (2575 patients) and placebo (2582 patients) after 5 years of tamoxifen in breast cancer survivors. The median follow up was 2.4 years and the disease free survival was 93% in the letrozole group and 87% in the placebo group, with an absolute difference of 6%<sup>43)</sup>.

Since there are benefits to switching to aromatase inhibitors, why not use an aromatase inhibitor immediately following surgery? This next question was addressed by two large multinational clinical trials. The ATAC trial compared anastrozole, tamoxifen or a combination of tamoxifen and anastrozole to see if disease free survival improved<sup>44)</sup>. There were over 9000 patients enrolled to receive tamoxifen alone (3116 patients), anastrozole alone (3125 patients), or anastrozole plus tamoxifen (3125 patients). The disease free survival after 3 years of anastrozole, tamoxifen, or a combination of the two was 89.4%, 87.4%, and 87.2%. Additionally, after 3 years, hazard ratios favored anastrozole over tamoxifen for node negative and node positive disease (0.85 for node positive and 0.7 for node negative)<sup>44)</sup>. Since there was no difference between the combination arm and

## Long Term Estrogen Deprivation Treatment



**Fig 5.** A summary of all 5 of the major trials that were conducted to compare tamoxifen and aromatase inhibitors as adjuvant therapy following surgery. The first group examined the use of aromatase inhibitors instead of 5 years of tamoxifen. The next group compared 5 years of tamoxifen with 2-3 years of tamoxifen followed by 3 years of an aromatase inhibitor. The last group compared 5 years of tamoxifen followed by 5 years of aromatase inhibitors.

the tamoxifen arm, the combination arm was closed and the anastrozole (n=2618) and tamoxifen patients (n=2598) were followed further for 2.7 years. While overall survival was the same between the two groups, the disease free survival (absolute difference of 3%), time to recurrence (absolute difference of 3.7%), and time to distant recurrence (absolute difference of 2%), were better in the group that received anastrozole vs. tamoxifen. Patients who received anastrozole (n=3092) instead of tamoxifen (n=3094) had a decrease in the risk of contralateral breast cancer (35 vs. 59 patients). In addition, the patients who took anastrozole also had a decrease in endometrial cancer (5 vs. 17 patients) and blood clots (87 vs. 140 patients)<sup>45)</sup>.

The initial portion of the BIG study addressed the use of letrozole (n=4003) in comparison with tamoxifen (n=4007)<sup>46)</sup>. An additional study will compare letrozole followed by tamoxifen, and tamoxifen followed by letrozole. At five years, patients taking letrozole had less recurrence than those on tamoxifen as demonstrated by an absolute difference of 3.4%. In the letrozole group, 16 patients developed a contralateral breast cancer, while 27 patients in the tamoxifen group developed a contralateral breast cancer. Most importantly, the BIG trial further stratified patients into node negative and node positive groups as far as disease free survival rates. When looking at dis-

ease-free survival alone, the patients with node positive cancer benefited the most from letrozole (hazard ratio = .71). The 5 year disease-free survival was 77.9% in those patients who received letrozole vs. 71.4% in those who received tamoxifen. Patients with node negative cancer had an 88.7% rate of disease-free survival (hazard ratio of 0.96) regardless of the use of letrozole or tamoxifen. Fewer women had endometrial cancer in the letrozole group (4/3089 patients) when compared to those patients taking tamoxifen (10/3157 patients). In addition, the patients taking letrozole had a significant decrease in thromboembolic events (61/3975 patients) in comparison to the patients who received tamoxifen (140/3988)<sup>46</sup>.

Overall, the trials have shown that aromatase inhibitors are favorable to tamoxifen in postmenopausal, ER positive patients, secondary to decreased recurrence and a decrease in undesirable side effects such as endometrial cancer and venous thrombosis. The concern with aromatase inhibitors is the higher rate of osteoporosis and subsequent fractures as well as higher serum cholesterol levels. Practitioners must be proactive and monitor patients for joint pain and do regular cardiovascular risk stratification. Fortunately, there are medicines such as statins to lower cholesterol and bisphosphonates to maintain bone density that can now be a part of the patient's treatment plan.

### ***Is Prevention Better Than Cure?***

The antihormonal therapy of breast cancer has probably reached its zenith, but the application of SERMs for the chemoprevention of breast cancer is providing a new dimension for the consideration of public health. Despite progress in the treatment of breast cancer, prevention remains a viable strategy. In the 1970's laboratory studies showed that tamoxifen prevented carcinogen induced rat mammary cancer, probably via an ER mediated mechanism<sup>47</sup>. Gradually, this work was translated to clinical trial and the results demonstrated that tamoxifen would be a useful agent to test as a chemo-preventative<sup>22</sup>. The NSABP-1 trial subsequently showed that in high risk women, tamoxifen significantly decreased the risk of breast cancer by 50% in pre and postmenopausal volunteers. Tamoxifen is approved in the United States for risk reduction of breast cancer in high risk premenopausal and postmenopausal women. Despite the fact that chemoprevention is a pioneering

application for tamoxifen, there are justifiable concerns about toxic side effects. The side effects (primarily venous thrombosis and endometrial cancer, though the incidence is small) have limited the use of tamoxifen by primary care practitioners. Nevertheless, it must be stressed that the side effects are limited to postmenopausal women. There are no significant increases in endometrial cancer and thrombosis in postmenopausal women. Tamoxifen remains the chemo-preventative agent of choice in this risk group<sup>48, 49</sup>. To address the issue of side effects (e.g. endometrial cancer) with tamoxifen, a novel SERM strategy was devised. If SERMs can prevent bone loss and prevent recurrent cancer at the same time<sup>50, 51</sup>, why not develop a SERM to prevent osteoporosis and prevent breast cancer at the same time? This evidenced based hypothesis<sup>52</sup> has now been evaluated with the application of raloxifene to prevent osteoporosis. Breast cancer and endometrial cancer are reduced when raloxifene is used to treat osteoporosis<sup>53, 54</sup>. It is now possible to state that thousands of women treated with raloxifene to prevent osteoporosis will have significant reductions in their breast cancer incidence<sup>49, 55, 56</sup>. Based on the successful evaluation of raloxifene as an osteoporosis drug, raloxifene was then targeted to postmenopausal women at high risk for breast cancer. The studies of tamoxifen and raloxifene (STAR) trial was designed to compare and contrast tamoxifen and raloxifene for the reduction in the incidence of breast cancer in high risk women and to improve the side effect profiles<sup>57</sup>. The results of the trial demonstrated that the SERMs are equivalent in the prevention of invasive breast cancer, but it appears that tamoxifen is slightly better in preventing noninvasive breast cancer. Nevertheless, the results do not reach statistical significance. The side effect profile of raloxifene is better than tamoxifen. Raloxifene treated women have a reduced incidence of endometrial cancer, hysterectomies, cataracts and cataract surgery. Overall, raloxifene can be stated to be an effective agent to reduce breast cancer risk in postmenopausal women.

It is important to emphasize that no other classes of hormonal therapy have been evaluated successfully for prevention other than SERMs. Aromatase inhibitors are slightly superior to SERMs for patients with breast cancer. Unfortunately, aromatase inhibitors cannot be used in premenopausal women. Therefore, the future consid-

eration for the aromatase inhibitors is the balance of side effects between SERMs and aromatase inhibitors. The side effect profile of osteoporosis, fractures, and musculoskeletal pain in high risk patients will be addressed by an international clinical trial by the International Breast Intervention Study (IBIS II). Anastrozole is being evaluated for prevention in high risk post menopausal women. The study involves 6,000 women who will be randomized to receive anastrozole or placebo. Another group of 4,000 women with locally excised DCIS will be randomized to receive anastrozole or tamoxifen. After 5 years of treatment, rates of breast cancer and side effect profiles will be examined between the groups<sup>58</sup>. The important trial (P4) to compare and contrast raloxifene with the aromatase inhibitor letrozole is being conducted by the National Surgical Adjuvant Breast and Bowel Project (NSABP). These data will not be available until 2014.

### Overall Summary

When treating patients with ER positive breast cancer, there are many new choices available for patients. Recurrent cancer which is SERM resistant and side effects such as venous thrombosis and endometrial cancer prompted investigation into aromatase inhibitors. Currently, clinical trials indicate that aromatase inhibitors should be used for initial hormonal therapy. One must keep in mind that although aromatase inhibitors are superior for prevention of recurrence, some patients still get recurrent breast cancer and the absolute difference between aromatase inhibitors and tamoxifen is a small percentage when comparing individual studies. Furthermore, the BIG trial is the only trial that stratified patients into node negative and node positive patients in terms of disease free survival rates. Letrozole was significantly more beneficial with node positive patients, and there was no difference between node negative patients. If fractures, bone pain and other side effects are intolerable, tamoxifen is still a viable option for prevention of recurrence, especially in node negative patients with no uterus and without a history of clotting. In node positive patients, aromatase inhibitors are probably the best initial treatment option. Treatment for recurrent cancer should be tailored for individual patients based on disease characteristics and tolerance of side effects such as osteopenia and musculoskeletal pain, cardiac risk, risk of venous thrombosis, and

risk of uterine cancer. For the future, prevention of breast cancer remains the most ideal situation and a significant number of patients will continue to benefit from the use of tamoxifen or raloxifene, with an additional benefit of maintaining bone density. The era of multifunctional medicines has arrived.

### Acknowledgements

Supported by 5T32CA10365-03 (RRP) and by the Department of Defense Breast Program under award number BC050277 Center of Excellence (Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense) (VCJ), SPORE in Breast Cancer CA 89018 (VCJ), R01 GM067156 (VCJ), FCCC Core Grant NIH P30 CA006927 (VCJ), the Avon Foundation and the Weg Fund of Fox Chase Cancer Center (VCJ).

### References

- 1) McPherson K, Steel CM, Dixon JM: ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *BMJ* 321(7261):624-628, 2000.
- 2) Breast Cancer Facts and Figures. *American Cancer Society*. 2005-2006.
- 3) Kols A: Breast Cancer; increasing incidence, limited options. *Outlook* 19:1-8, 2002.
- 4) Marugame T, Kamo K, Katanoda K, Ajiki W, Sobue T: Cancer incidence and incidence rates in Japan in 2000: estimates based on data from 11 population-based cancer registries. *Jpn J Clin Oncol* 36:668-675, 2006.
- 5) Japanese Cancer Society: <http://www.jcancer.jp/en/screening.html>. 2005.
- 6) Vital Statistics of Japan: Statistics and Information Department. Minister's Secretariat, Ministry of Health, Labour and Welfare. 2001.
- 7) Japanese Cancer Society: <http://www.ncc.go.jp/en/statistics/2005/index.html>. 2005.
- 8) Mettlin C: Global breast cancer mortality statistics. *CA Cancer J Clin* 49:138-144, 1999.
- 9) Tung HT, Tsukuma H, Tanaka H, Kinoshita N, Koyama Y, Ajiki W, Oshima A, Koyama H: Risk factors for breast cancer in Japan, with special attention to anthropometric measurements and reproductive history. *Japanese Journal of Clinical Oncology* 29:137-146, 1999.
- 10) Li CI, Daling JR, Malone KE: Incidence of invasive breast cancer by hormone receptor status from 1992 to 1998. *J Clin Oncol* 21:3-4, 2003.
- 11) Anderson WF, Chatterjee N, Ershler WB, Brawley OW: Estrogen receptor breast cancer phenotypes in the Surveillance, Epidemiology, and End Results database. *Breast Cancer Research and Treatment* 76:27-36, 2002.
- 12) Nomura Y, Tashiro H, Hamada Y, Shigematsu T: Relationship between estrogen receptors and risk factors of breast cancer in Japanese pre- and post-menopausal patients. *Breast Cancer Res Treat* 4:37-43, 1984.
- 13) Matsumoto K, Sakamoto G, Nomura Y: International

- comparisons concerning breast cancer and steroid receptors. *Anticancer Research* 6:621-624, 1986.
- 14) McGuire WL, Carbone PP, Sears ME, Escher GC: Estrogen receptors in human breast cancer: an overview. In: Estrogen Receptor in Human Breast Cancer. McGuire WL, Carbone PP, Volmer EP eds Raven Press, New York. pp 1-7, 1975.
  - 15) Nomura Y, Kobayashi S, Takatani O, Sugano H, Matsumoto K, McGuire WL: Estrogen receptor and endocrine responsiveness in Japanese versus American breast cancer patients. *Cancer Res* 37:106-110, 1977.
  - 16) Jensen EV, Jacobson HI: Basic guides to the mechanism of estrogen action. *Recent Progr Hormone Res* 18:387-414, 1962.
  - 17) Jensen EV, Jordan VC: The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. *Clin Cancer Res* 9:1980-1989, 2003.
  - 18) Russo J, Fernandez SV, Russo PA, Fernbaugh R, Sheriff FS, Lareef HM, Garber J, Russo IH: 17-Beta-estradiol induces transformation and tumorigenesis in human breast epithelial cells. *Faseb J* 20:1622-1634, 2006.
  - 19) Cavaliere E, Chakravarti D, Guttenplan J, Hart E, Ingle J, Jankowiak R, Muti P, Rogan E, Russo J, Santen R, Sutter T: Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. *Biochim Biophys Acta* 1766:63-78, 2006.
  - 20) NCI Website: <http://cancer.gov.cancertopics/understandingcancer>.
  - 21) EBCTCG: Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 354:1451-1467, 1998.
  - 22) Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N: 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, 1998.
  - 23) Buzdar AU, Jones SE, Vogel CL, Wolter J, Plourde P, Webster A: A phase III trial comparing anastrozole (1 and 10 milligrams), a potent and selective aromatase inhibitor, with megestrol acetate in postmenopausal women with advanced breast carcinoma. Arimidex Study Group. *Cancer* 79:730-739, 1997.
  - 24) de Ziegler D, Mattenberger C, Luyet C, Romoscanu I, Irion NF, Bianchi-Demicheli F: Clinical use of aromatase inhibitors (AI) in premenopausal women. *J Steroid Biochem Mol Biol* 95(1-5):121-127, 2005.
  - 25) Johnston S: Fulvestrant and the sequential endocrine cascade for advanced breast cancer. *British J Cancer* 90 Suppl 1:S15-18, 2004.
  - 26) Jonat W: Goserelin (Zoladex)-its role in early breast cancer in pre- and perimenopausal women. *Br J Cancer* 85 Suppl 2:1-5, 2001.
  - 27) Jordan VC: Tamoxifen: a most unlikely pioneering medicine. *Nature Reviews Drug Discovery* 2:205-213, 2003.
  - 28) Furr BJ, Jordan VC: The pharmacology and clinical uses of tamoxifen. *Pharmacol Ther* 25:127-205, 1984.
  - 29) Cole MP, Jones CT, Todd ID: A new anti-estrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. *Br J Cancer* 25:270-275, 1971.
  - 30) 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* 16:239-251, 1980.
  - 31) Jordan VC: Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. *Reviews on Endocrine-Related Cancer* (October Supplement):49-55, 1978.
  - 32) EBCTCG: 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, 2005.
  - 33) Osborne CK: Tamoxifen in the treatment of breast cancer. *N Engl J Med* 339:1609-1618, 1998.
  - 34) Howell A, Dodwell DJ, Anderson H, Redford J: Response after withdrawal of tamoxifen and progestogens in advanced breast cancer. *Ann Oncol* 3:611-617, 1992.
  - 35) Gottardis MM, Jordan VC: Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 48:5183-8187, 1988.
  - 36) Jordan VC: Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 5:207-213, 2004.
  - 37) Miller WR, Dixon JM: Antiaromatase agents: preclinical data and neoadjuvant therapy. *Clin Breast Cancer Suppl* 1:S9-14, 2000.
  - 38) Buzdar AU, Jonat W, Howell A, Jones SE, Blomqvist CP, Vogel CL, Eiermann W, Wolter JM, Steinberg M, Webster A, Lee D: Anastrozole versus megestrol acetate in the treatment of postmenopausal women with advanced breast carcinoma: results of a survival update based on a combined analysis of data from two mature phase III trials. Arimidex Study Group. *Cancer* 83:1142-1152, 1998.
  - 39) Howell A, Robertson JFR, Quaresma Albano J, Aschermannova A, Mauriac L, Kleeberg UR, Vergot I, Erikstein B, Webster A, Morris C: 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, 2002.
  - 40) Osborne CK, Pippen J, Jones SE, Parker LM, Ellis M, Come S, Gertler SZ, May JT, Burton G, Dimery I, Webster A, Morris C, Elledge R, Buzdar A: 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, 2002.
  - 41) Boccardo F, Rubagotti A, Puntoni M, Guglielmini P, Amoroso D, Fini A, Paladini G, Mesiti M, Romeo D, Rinaldini M, Scali S, Porpiglia M, Benedetto C, Restuccia N, Buzzi F, Franchi R, Massidda B, Distanti V, Amadori D, Sismondi P: Switching to anastrozole versus continued tamoxifen treatment for early breast cancer: Preliminary results of the Italian Tamoxifen Anastrozole Trial. *J Clin Oncol* 23:5138-5147, 2005.
  - 42) Coombes RC, Hall E, Gibson LJ, Paridaens R, Jassem J, Delozier T, Jones SE, Alvarez I, Bertelli G, Ortmann O, Coates AS, Bajetta E, Dodwell D, Coleman RE, Fallowfield LJ, Mickiewicz E, Andersen J, Lonning PE, Cocconi G, Stewart A, Stuart N, Snowdon CF, Carpentieri M, Massimini G, Bliss JM: 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, 2004.
- 43) Goss PE, Ingle JN, Marino S, Robert NJ, Muss HB, Piccart MJ, Castiglione M, Tu D, Shepherd LE, Pritchard KI, Livingston RB, Davidson NE, Norton L, Perez EA, Abrams JS, Therasse P, Palmer MJ, Pater JL: A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. *N Engl J Med* 349:1-10, 2003.
  - 44) ATAC Trialists' Group: 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 randomized trial. *Lancet* 359:2134-3139, 2002.
  - 45) Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, Forbes JF, Hochtin-Boes G, Houghton J, Locker GY, Tobias JS, Group AT: 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, 2005.
  - 46) Thurlimann B, Keshaviah A, Coates AS, Mouridsen H, Mauriac L, Forbes JF, Paridaens R, Castiglione-Gertsch M, Gelber RD, Rabaglio M, Smith I, Wardly A, Price KN, Goldhirsch A, BIG (Breast International Group), Group I-C: A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med* 353:2747-2757, 2005.
  - 47) Jordan VC: Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. *Eur J Cancer* 12:419-424, 1976.
  - 48) Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, Atkins JN, Margolese RG, Runowicz CD, James JM, Ford LG, Wolmark N: 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, 2005.
  - 49) Jordan VC: Optimising endocrine approaches for the chemoprevention of breast cancer. Beyond the Study of Tamoxifen and Raloxifene (STAR) Trial. *European Journal of Cancer* 42:2909-2913, 2006.
  - 50) Jordan VC, Phelps E, Lindgren JU: Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 10:31-35, 1987.
  - 51) Gottardis MM, Jordan VC: Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* 47:4020-4024, 1987.
  - 52) Jordan VC: Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 6:589-595, 1988.
  - 53) 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: 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, 1999.
  - 54) Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, Secrest RJ, Cummings SR: For the CORE Investigators 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, 2004.
  - 55) Jordan VC: Chemoprevention of breast cancer with selective oestrogen-receptor modulators. *Nature Reviews Cancer* (in press) 2007.
  - 56) Jordan VC: SERMs: meeting the promise of multifunctional medicines. (Commentary). *Journal of the National Cancer Institute* (in press) 2007.
  - 57) Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon (jr) ER, Wade I, J.L., Robidoux A, Margolese RG, James J, Lippman SM, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N: The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA* 295:2727-2741, 2006.
  - 58) <http://www.ibcsg.org/public/general-pages/trials/open/IBIS-II/trial-IBISII.html>.

# Successful Translation Research with Selective Oestrogen Receptor Modulators to Treat and Prevent Breast Cancer

## Erfolgreiche translationale Forschung über selektive Östrogenrezeptor-Modulatoren in der Behandlung und Prävention des Mammakarzinoms

### Author

V. Craig Jordan

### Affiliation

Fox Chase Cancer Center, Philadelphia

### Schlüsselwörter

- Mammakarzinom
- Prävention
- Tamoxifen
- SERM
- DICS

### Key words

- breast cancer
- prevention
- tamoxifen
- SERM
- DICS

### Zusammenfassung

Vor 30 Jahren spielte die Antiöstrogentherapie mit Tamoxifen nur eine zweitrangige Rolle in der Mammakarzinomtherapie. Die Hoffnungen, die Heilung des metastasierten Mammakarzinoms erreichen zu können, fokussierten sich auf die Entwicklung neuer Zytostatika und der Hochdosischemotherapie mit Knochenmarktransplantation. Ähnliche Strategien wurden in der adjuvanten Behandlung des Mammakarzinoms verfolgt. Vereinfacht gesagt, das Ziel war es, die Krebszellen mit unspezifischen zytotoxischen Substanzen zu töten, während der Patient durch supportive Maßnahmen am Leben gehalten wurde. Aber die medizinische Forschung verläuft nicht nur gradlinig und alternative Ansätze, die die Kontrolle des Tumorwachstums mit minimalen Nebenwirkungen erlauben, wurden entwickelt – die sogenannte targeted therapy. Die Elemente dieser neuen Strategien waren seit 20 Jahren gestützt durch die Ergebnisse der Grundlagenforschung etabliert. Der Ansatz der Langzeitantihormontherapie zur Kontrolle des Zellwachstums über „targeting“ des Tumor-Östrogenrezeptors hat die Behandlung des Mammakarzinoms revolutioniert. Der Erfolg dieser Strategie wird an der Abnahme der Mortalität des Mammakarzinoms abgelesen. Die translationale Forschung über den Tumor-Östrogenrezeptor mit einer ganzen Bandbreite neuer antiöstrogenen Substanzen (Aromatasehemmer, reine Antiöstrogene) lässt neue Therapiemodelle der „targeted therapy“ prophezeien. Wenn man sich die Pharmakologie der „Antiöstrogene“ genauer anschaut, wird man erstaunt sein. Die nicht steroidalen „Antiöstrogene“ sind selektive Östrogenmodulatoren (SERMs). Mit anderen Worten, die Substanzen wirken an der Mamma als Antiöstrogene, als Östrogene im Knochen und vermindern den Cholesterinspiegel. Dieses Wissen ermöglicht einen praktischen Ansatz in der Chemoprävention des Mammakar-

### Abstract

Thirty years ago, antiestrogen therapy with tamoxifen played only a secondary role in breast cancer care. All hopes to cure metastatic breast cancer were still pinned on either the discovery of new cytotoxic drugs or a dose dense combination of available cytotoxic drugs with bone marrow transplantation. A similar strategy with combination chemotherapy was employed as an adjuvant for primary breast cancer. Simply stated, the goal was to kill the cancer with non-specific cytotoxic drugs while keeping the patient alive with supportive care. However, medical research does not travel in straight lines and alternative approaches emerged to solve the problem of controlling tumour growth with minimal side effects. The approach was targeted therapy. All the elements of the new strategy were in place twenty years ago supported by scientific principles derived from laboratory research. The approach of using long-term antihormone therapy to control early stage breast cancer growth would revolutionise cancer care by targeting the tumour oestrogen receptor (OER). The success of the strategy would be evidenced by lives saved and contribute to a decrease in the national mortality figures for breast cancer. More importantly, translational research that targeted the tumour OER with a range of new antioestrogenic drugs (aromatase inhibitors, pure antioestrogens) would presage the current fashion of blocking survival pathways for the tumour by developing novel targeted treatments. But a surprise was in store when the pharmacology of “antioestrogens” was studied in detail. The nonsteroidal “antioestrogens” are selective oestrogen modulators (SERMs). In other words, the compounds are antioestrogens in the breast, oestrogens in the bone and lower circulating cholesterol. This knowledge would establish a practical approach to breast cancer chemopre-

received 14.3.2007  
accepted 2.4.2007

### Bibliography

DOI 10.1055/s-2007-965287  
Geburtsh Frauenheilk 2007; 67:  
443–450 © Georg Thieme  
Verlag KG Stuttgart · New York ·  
ISSN 0016-5751

### Correspondence

V. Craig Jordan, OBE, PhD, DSc  
Vice President and Research  
Director for Medical Sciences  
333 Cottman Avenue  
Philadelphia, PA 19111-2497  
v.craig.jordan@fccc.edu

zinoms bei Frauen mit hohem Risiko (Tamoxifen) und niedrigem Risiko (Raloxifene).

## Widmung

Das Manuskript wurde Herrn Professor Dr. Dr. h.c. Manfred Kaufmann, Direktor der Universitäts-Frauenklinik Frankfurt, zu seinem 60. Geburtstag gewidmet.

## Introduction

I am delighted to be invited to celebrate the 60th birthdays of Professor Manfred Kaufmann and his wife, Brigitte. Our careers are intertwined, so I wish to offer my personal observations on the specific changes that have occurred in the approach to the endocrine treatment of breast cancer over this period. These changes are considerable. As a new PhD graduate in pharmacology who had studied "antioestrogens" as an academic exercise between 1969–72, I was told I could now do anything I wanted. I chose to contribute to the reinvention of an orphan drug, ICI 46474 into tamoxifen.

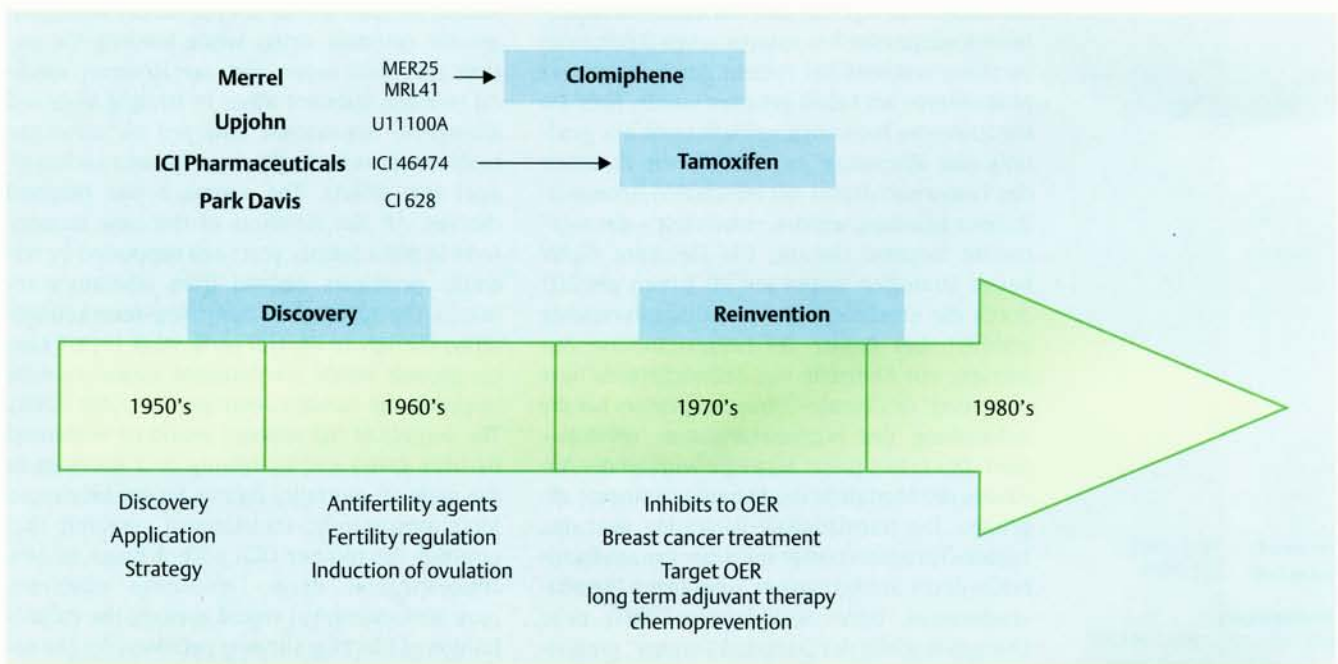
When I was a teenager, my passion was chemistry. However, I wanted to use chemistry to treat cancer because I was inspired by Paul Ehrlich whose work succeeded in treating disease selectively. For this reason, I chose to study pharmacology at Leeds University and take the unfashionable step, in the late 1960's, of exploring the possibility of using targeted drugs to treat cancer

selectively. Unfortunately, the obstacles in place to prevent progress were enormous.

Each generation creates and defends its own fashion in cancer research and this concept is well illustrated by the early resistance to change that occurred in the approach to the treatment of breast cancer. Virtually no one was interested in "another endocrine therapy" as combination cytotoxic chemotherapy was predicted to cure cancer.

In contrast, it is now clear that the approach to health care has changed in the past 30 years not once but twice as a result of advances in endocrine therapy. Tamoxifen is the first targeted treatment for breast cancer based on the successful translational work focused on the oestrogen receptor (OER) that has helped extend the lives of millions of women [1]. Perhaps equally important in this advance was the fact that tamoxifen has a beneficial therapeutic ratio that facilitated its use as a long-term adjuvant therapy. The application of the laboratory strategy of long-term antihormonal therapy targeted to the OER [2, 3] saved lives which in turn has contributed significantly to the national reductions in mortality [4, 5].

Secondly, the knowledge gained with tamoxifen propelled the drug forward for testing as the first chemopreventive for any cancer, created a new drug group, the selective oestrogen receptor modulators (SERMs) and resurrected keoxifene, a failed breast cancer drug [6] to be the first SERM for the treatment



**Fig. 1** The reinvention of nonsteroidal antioestrogens as pioneering agents for the effective treatment of breast cancer. Nonsteroidal antioestrogens were discovered by scientists in the pharmaceutical industry during the late 1950's and early 1960's. Initial optimism that they would be effective morning after pills were found to be invalid when clinical trials demonstrated that the compounds induced ovulation in subfertile women. Clomiphene and tamoxifen were both marketed for the induction of ovulation in subfertile women. During the 1960's, interest in using nonsteroidal antioestrogens in

the clinic declined. During the 1970's, the nonsteroidal antioestrogen ICI 46474 was reinvented to be a targeted therapy for the long-term treatment of breast cancer [2]. The target was the oestrogen receptor and the principles established in the laboratory in the 1970's were to provide the basis for successful long-term adjuvant therapy targeted to patients with oestrogen receptor (OER) positive breast cancers and to explore the possibility of chemoprevention in high risk populations.

and prevention of osteoporosis but with the ability to reduce the risk of breast and endometrial cancer [7].

The story of the discovery of tamoxifen as a "morning after pill" in the 1960's to then being reinvented as a targeted breast cancer therapy in the 1970's, has been recounted recently [2,3]. Nevertheless, the tale is important to retell as it illustrates how the changing fashions in research can influence progress. The development of the oral contraceptive by Pincus and colleagues at the Worcester Foundation during the 1950's changed society forever. Despite enthusiasm to create new ways to manipulate reproduction, the fashion of research in reproductive biology declined steadily throughout the 1960's with a decreased investment in the development of new contraceptive methods. Political and legal fashions were changing. But, in its place, the "War on Cancer" was declared in the United States in 1971.

The tale of tamoxifen also illustrates the length of time that must be taken to evaluate successful treatment strategies to affect changes in healthcare (♦ Fig. 1). Nevertheless, momentum to change the approach for the treatment of breast cancer, and indeed of any cancer accelerated with tamoxifen. The drug became a ubiquitous tool to test targeting in breast cancer. More importantly, tamoxifen set the stage for the current optimism that important advances in cancer research are within our grasp and the sincere belief that clever people will solve problems and develop practical ways to kill cancer cells selectively with minimal side effects for the patient.

However, the question that surfaces is "why did the process of concept to changes in healthcare take such a long time for a novel targeted therapy with few side effects?"

### The Lost Decade – Tamoxifen on Life Support

In April 1972, all the preliminary clinical data on ICI 46474 was reviewed by scientists at ICI Pharmaceuticals Division at Alderley Park but there was reluctance to pursue the development of the drug as a short term palliative treatment for breast cancer. Several factors were considered in the decision not to develop ICI 46474. The compound had no patent protection in the United States; this would only be granted in 1985! Most importantly at the time, there was estimated to be no significant market for a palliative drug that would only be effective for about a year for one out of three metastatic breast cancer patients. In the early 1970's, the total incidence of metastatic breast cancer in the United Kingdom was only a few thousand patients per year. Worldwide figures were obviously larger but the drug was to be priced 10 times more expensive than the standard endocrine treatment (diethylstilbestrol). The turnover of the drug was estimated to be £ 500 000 per annum, at most, with only £ 50 000 profit. It would always be an orphan drug and clinical development was stalled. With the wisdom of hindsight, there was also no infrastructure at Alderley Park to support a breast cancer programme. ICI Pharmaceuticals Division was not a "cancer company" and there was also no pipeline of compounds to replace ICI 46474 should subsequent studies (as they did 20 years later with rat liver carcinogenesis) reveal unacceptable toxicities. Development could not be taken seriously. In the clinical community, it was generally accepted that another endocrine therapy would add almost nothing to the medical armamentarium of breast cancer therapies. Overall, there was little initial enthusiasm for the use of a new antihormonal therapy that benefited a minority of patients for a short period.

A.L. Walpole, Head of the Fertility Control Programme at ICI Pharmaceuticals Division discovered the antifertility properties of the molecule ICI 46474 in the early 1960's. The compound was an effective "morning after pill" in rats [8] but induced ovulation in women [9]. The project did not achieve its goal. Nevertheless, the observations that ICI 46474 had equivalent antitumour activity but reduced side effects compared to standard endocrine therapies used to treat advanced breast cancer [10,11] convinced Walpole that the drug should be marketed at least as an option for treatment. The drug was available for experimentation but looking for applications. Although no studies were conducted by scientists at ICI Pharmaceuticals Division, Walpole ensured that my laboratory would be supported to find those applications. Regrettably, Walpole died suddenly in 1977 and never saw the benefits that tamoxifen was to bring [2,12].

A scientific strategy for the appropriate clinical application of tamoxifen was developed in the laboratory to target the drug to the tumours that were the most likely to respond. Tamoxifen blocked the binding of estradiol to human breast and rat mammary tumour ER's and prevented the induction and growth of ER positive carcinogen-induced rat mammary carcinomas [2,3]. These early studies raised the question of whether tamoxifen could prevent the majority of breast cancers i.e.: ER positive breast cancer. However, the finding that long-term tamoxifen treatment in animals with early mammary cancer i.e., a low tumour burden [2] could create a tumour-free state suggested longer was going to be better than shorter durations of adjuvant therapy. On a personal note, I am extremely proud of the fact that the regulatory authorities in Germany required information on my work prior to granting permission for the application of tamoxifen to treat breast cancer.

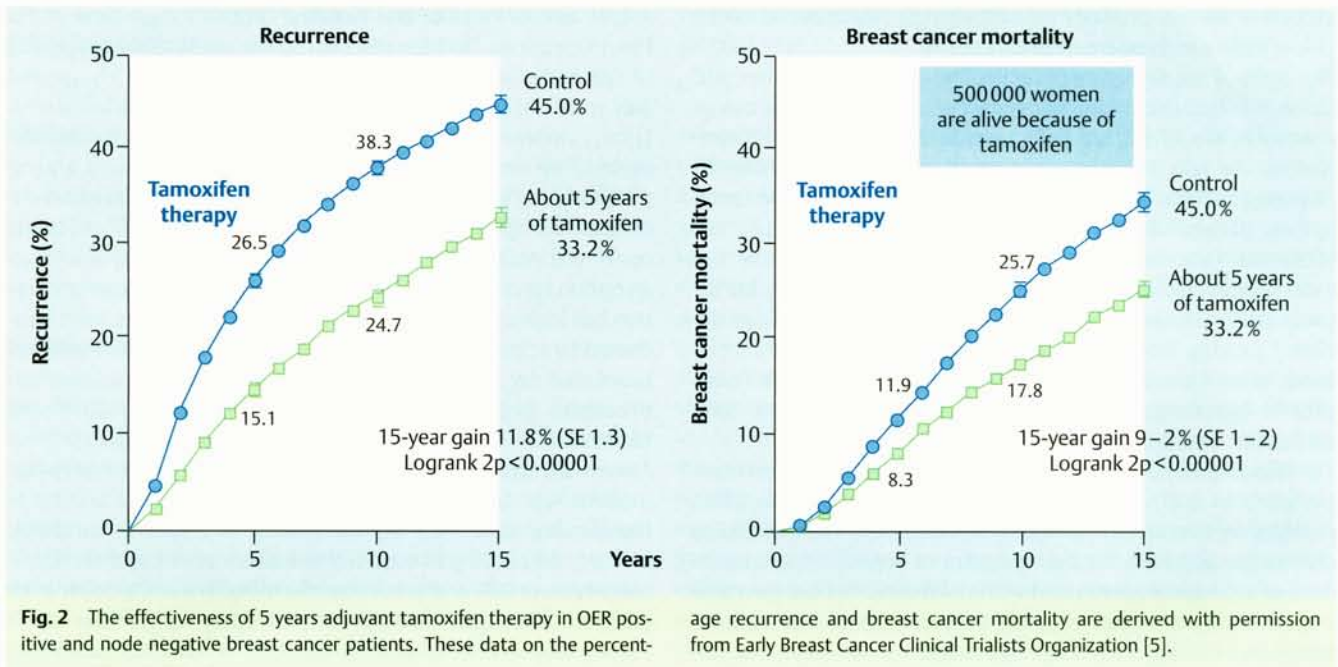
The laboratory observations were to prove remarkably effective as an approach to treat women with early node positive and node negative ER positive breast cancer. However, the original clinical strategy in the 1970's for the evaluation of tamoxifen was to use one year of adjuvant treatment after surgery. The reason for this was that tamoxifen was only effective for the treatment of advanced breast cancer for about a year and there was a sincere concern that longer adjuvant treatment durations would result in premature drug resistance. This approach was to change.

With these observations as background, all of the pieces of the puzzle were about to come together in 1986 (20 years ago) to create a significant advance that would change healthcare twice.

### An Overview of Adjuvant Clinical Trials

An enormous advance in medicine is the introduction of meta-analysis or Overview analysis of small randomised clinical trials that individually show little or no benefits for agents under investigation but together provide a valid result. The Overview analysis of breast cancer clinical trials was first conducted at Heathrow airport in 1984 [13]. The results when they were published in full in 1988 demonstrated a significant advantage for postmenopausal patients receiving tamoxifen [14].

Based on the successive analysis of accumulative randomized worldwide clinical trials, it is possible to summarise the main conclusions for tamoxifen. At the time 20 years ago, when the Overview analysis first occurred, tamoxifen was the only universally used antihormonal agent. With no other competition, tamoxifen became the "gold standard" and established the princi-



**Fig. 2** The effectiveness of 5 years adjuvant tamoxifen therapy in OER positive and node negative breast cancer patients. These data on the percent-

age recurrence and breast cancer mortality are derived with permission from Early Breast Cancer Clinical Trialists Organization [5].

ples of tumour targeting and identified the appropriate treatment strategy to aid survivorship [4, 5, 14, 15].

- ▶ Five years of adjuvant tamoxifen enhances disease free survival. There is a 50% decrease in recurrences observed in ER positive patients fifteen years after diagnosis (● Fig. 2).
- ▶ Five years of adjuvant tamoxifen enhances survival with a decrease in mortality fifteen years after diagnosis (● Fig. 2).
- ▶ Adjuvant tamoxifen does not provide an increase in disease free or overall survival in ER negative breast cancer.
- ▶ Five years of adjuvant tamoxifen alone is effective in premenopausal women with ER positive breast cancer.
- ▶ The benefits of tamoxifen in lives saved from breast cancer far outweighs concerns about an increased incidence of endometrial cancer in postmenopausal women.
- ▶ Tamoxifen does not increase the incidence of second cancers other than endometrial cancer.
- ▶ No non-cancer related overall survival advantage is noted with tamoxifen when given as adjuvant therapy.

Overall, the clinical conclusions obtained by the Overview process based on worldwide experience were all consistent with the parallel laboratory studies that guided clinical care. This might have been the end of the tale but for the continuing examination of tamoxifen and related drugs in the laboratory.

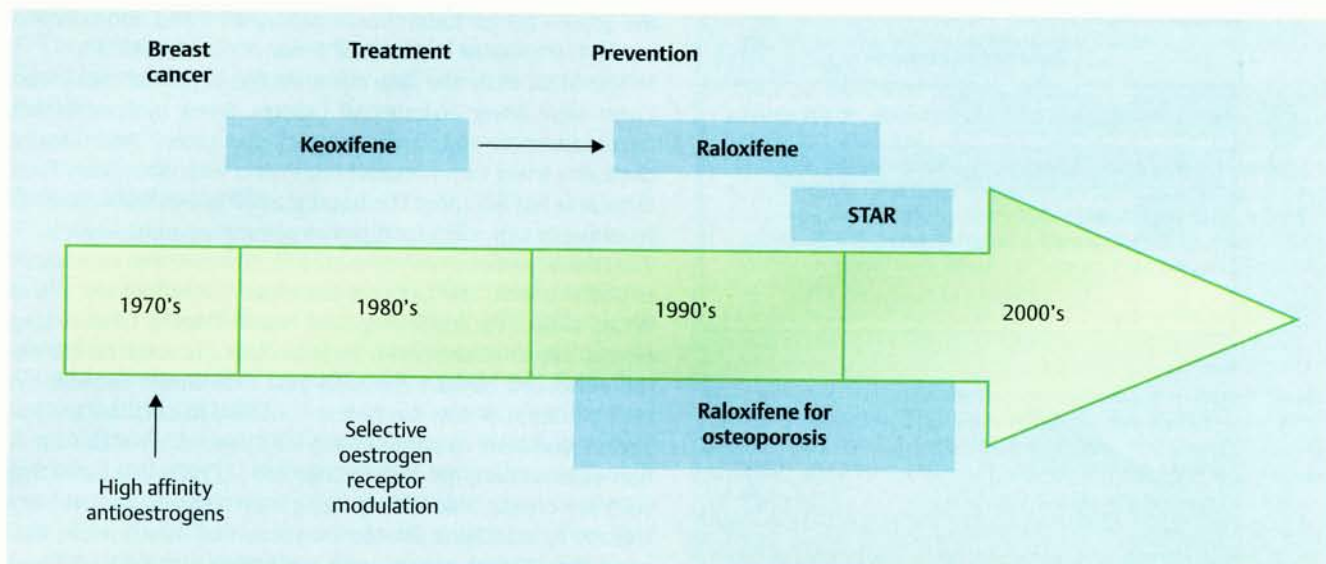
### The Changing Pharmacology of Antioestrogens

Twenty years ago, tamoxifen was classified as a non-steroidal antioestrogen [16]. In pharmacological terms tamoxifen was described as a partial agonist (oestrogen-like) in target tissues such as the immature rat uterus but it was antioestrogenic because it blocked the full action of estradiol alone. In 1986, it was plausible that if oestrogen was necessary to fend off osteoporosis and coronary heart disease the long-term administration of an antioestrogen to node negative women could eventually have a deleterious effect on bone density and produce a potential increase in the incidence of coronary heart disease for the majority of

women. The potential side effects would be even worse for women only at high risk to develop breast cancer. Only a small minority of women would have a reduced risk of breast cancer, but all women would be exposed to potential "antioestrogenic" toxicities. However, the classification of nonsteroidal antioestrogens was to change just after 1986. Today the concept is known as selective oestrogen receptor modulation.

In 1986, virtually nothing was known about the actions of nonsteroidal antioestrogens on bone density. A single report showed that clomiphene, a drug used for the induction of ovulation, would preserve bone density in ovariectomized rats [17]. However, the interpretation of these data was not that simple. Clomiphene is an impure mixture of oestrogenic and antioestrogenic isomers. Which isomer was affecting bone? The consistent laboratory finding that tamoxifen the pure *trans* antioestrogenic isomer of a triphenylethylene maintained bone density in ovariectomized rats [18–20] seemed to translate to postmenopausal women [21], but would prospective clinical studies really show benefit? The Wisconsin Tamoxifen Study was started in 1986 to explore the potential toxicity of tamoxifen on bone density. The study demonstrated in a double blind placebo controlled clinical trial that tamoxifen could preserve bone in the postmenopausal woman [22]. Bone building would clearly be an advantage for chemoprevention studies, thereby enhancing the possibility that the worth of tamoxifen to prevent breast cancer could be tested safely. In the same studies, tamoxifen lowered low density lipoprotein [23, 24] and, by inference, would appear not to increase the risk of coronary heart disease. These results were good. The bad was the laboratory discovery that although tamoxifen prevented the oestrogen-stimulated growth of human breast cancers, the drug stimulated the growth of human endometrial cancers grown in the same athymic mouse [25]. This again was selective oestrogen receptor modulation. Stimulate one target site to produce growth but at the same time block another target site.

There was a very quick response from the clinical community to the warnings [25] that long-term tamoxifen treatment could be



**Fig. 3** Development of the concepts of selective oestrogen receptor modulations and the reinvention of keoxifene, a failed breast cancer drug to raloxifene, a successful agent for the prevention of osteoporosis and breast cancer. During the 1970's, a metabolite of tamoxifen called 4 hydroxy-tamoxifen was found to have a high binding affinity to the oestrogen receptor [48]. This discovery led to the synthesis of keoxifene (LY156758) [34]. The drug was initially used as a treatment for breast cancer but was unsuccessful and Eli Lilly discontinued investigation of this application. In the laboratory, the nonsteroidal antioestrogens were found to actually be selective oestrogen receptor modulators (SERMs). The compounds maintained bone density

and oestrogenic action but prevented the development of mammary cancers and antioestrogenic action. This knowledge [32] was used to advance the evaluation of SERMs for the prevention of osteoporosis but with the prevention of breast cancer as a beneficial side effect. Keoxifene was reinvented as raloxifene and has now been successfully shown to prevent both osteoporosis in high risk women. Also, the recent study of tamoxifen and raloxifene (STAR trial) [39] has demonstrated that tamoxifen and raloxifene are equivalent in preventing the development of invasive breast cancer in high risk postmenopausal women.

associated with an increase in the incidence of endometrial cancer [26–28]. The advance in patient care was that women taking tamoxifen to treat breast cancer were also forewarned about gynecological complications.

By the 1990's, it was clear that the revelations about tamoxifen were not going to be helpful in bringing a proven agent that reduces the risk of breast cancer in pre and postmenopausal women by 50% [29,30] to a broad constituency of high risk women. However, a new chemopreventive strategy was already in place by the end of the 1980's.

### Selective OER Modulators

The recognition that the so called “nonsteroidal antioestrogens” had oestrogenic and antioestrogenic actions at different sites in the ovariectomized female rat and that these data translated to women to prevent osteoporosis and breast cancer created a new dimension in drug development. The fact that tamoxifen and the failed breast cancer drug keoxifene (LY156,758) [6] both prevented the development of carcinogen-induced rat mammary carcinomas [31] and maintained bone density in ovariectomized rates [18] indicated that this was a class effect. The significance of these observations for public health and chemoprevention of breast cancer was immediately recognized. The future of SERM drug development was clear.

“The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused upon 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 upon the developing clues pro-

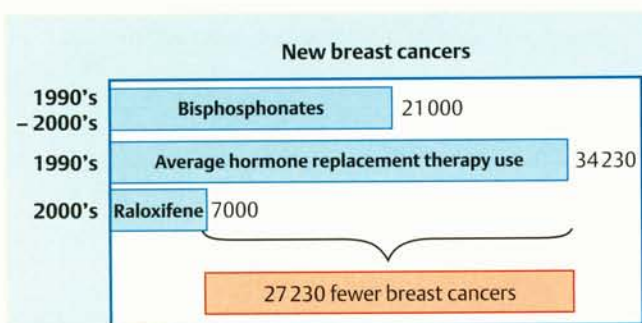
vided by an extensive clinical investigation of available antioestrogens. Could analogues be developed to treat osteoporosis or even retard the development of atherosclerosis? If this proved to be true then a majority of women in general could be treated for these conditions as soon as menopause occurred. Should the agent also retain anti-breast tumour actions then it might be expected to act as a chemosuppressive. 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” [32].

This blueprint to improve healthcare was subsequently restated at the annual meeting of the American Association of Cancer Research in San Francisco, 1989 [33].

Compounds of the keoxifene class (LY117018 and LY156758) were obvious candidates for study despite the fact that the programme to develop the drugs to treat breast cancer had been abandoned by Eli Lilly in 1988. The compounds were known to be less uterotrophic than tamoxifen in rodents [34] but they were short acting [35] which could explain their poor antitumour properties when compared with tamoxifen. Interestingly enough, keoxifene was already known to partially inhibit the growth of tamoxifen-stimulated human endometrial tumours under laboratory conditions [36].

Keoxifene, the failed breast cancer drug was reinvented in the early 1990's as raloxifene, a SERM (● Fig. 3). A use patent for the treatment and prevention of osteoporosis was filed by Eli Lilly in 1992.

Raloxifene has now been available for the treatment and prevention of osteoporosis in postmenopausal women since 1999 based on the prospective clinical trials demonstrating an approximately 40% decrease in spinal fractures [37] with the ad-



**Fig. 4** Progress in the chemoprevention of breast cancer over the past 20 years. About half a million women are currently taking raloxifene for the prevention of osteoporosis but with the beneficial side effect of preventing breast cancer [39]. During the 1990's, a woman at risk for osteoporosis could have taken either bisphosphonate or legitimately used hormone replacement therapy to maintain bone density. If half a million women were taking bisphosphonates for a decade with risk factors, based upon the osteoporosis study conducted by Martino and colleagues [41], then those patients would have accumulated 21 000 new breast cancers. The bisphosphonates have no effect on breast cancer incidence. In contrast, based on the elevated risk factors observed in the Women's Health Initiative, [49] or the Million Women's Study [50], the average elevation in breast cancer incidence for the half million women taking hormone replacement therapy for 10 years would be 34 230 new breast cancers. In contrast, if half a million women give up hormone replacement therapy and take raloxifene for 10 years, there will only be an incidence of 7000 breast cancers. In other words, 27 230 fewer breast cancers would be observed in this population over a decade. (Taken with permission from the European Journal of Cancer [42]).

vantage over hormone replacement therapy of causing a 70% decrease in the incidence of breast cancer [7,38]. The anticipated result in reducing the risk of breast cancer as a beneficial side effect of treating osteoporosis propelled raloxifene into clinical trial vs. tamoxifen for the prevention of breast cancer as the primary endpoint. The study of tamoxifen and raloxifene (STAR) in high risk postmenopausal women now shows equivalent effects of tamoxifen and raloxifene to prevent breast cancer but raloxifene is safer [39].

### A STAR is Born

The NSABP recruitment organisation randomised a total of 19 747 postmenopausal women with an increase 5 year risk of breast cancer (mean 4.03%) to receive either tamoxifen (20 mg daily) or raloxifene (60 mg daily) for 5 years to be followed by a 2 year observation period. The results were clear cut. Tamoxifen and raloxifene are equivalent as agents to reduce the risk of invasive breast cancer and no statistical significance was noted with the incidence of non invasive breast cancers (ductal carcinoma in situ plus lobular carcinoma in situ). Thus, based on the placebo controlled trial referred to as P-1 [30] where tamoxifen produced a 50% decrease in invasive breast cancer, raloxifene can be stated to do the same. In fact, the placebo controlled trial, Raloxifene use for the Heart (RUTH), demonstrated just that. The study [40] was designed to evaluate the value of raloxifene to reduce death from coronary heart disease. However, the trial showed no advantage for raloxifene. Nevertheless, raloxifene

did reduce breast cancer incidence by 50% and, most importantly, there was no elevation in endometrial cancer.

In the STAR trial, the side effect profile benefited raloxifene. There were fewer endometrial cancers, fewer hysterectomies, fewer cataracts and fewer cataract operations. Additionally, there was fewer thromboembolic events with raloxifene. Thus, raloxifene has advanced chemoprevention as a suitable, safer alternative to tamoxifen for high risk postmenopausal women. The clinical advance with raloxifene is, however, also an advance in public health. The fact that raloxifene can reduce the risk of breast cancer in postmenopausal women being treated long term to prevent fractures [41] is important and validates the initial evidenced based hypothesis that this would become the reality [32,33]. It now has become possible to calculate the impact of raloxifene on public health for a population of half a million women taking the drug for a decade [42]. It is estimated that with prescribing practices changing from hormone replacement therapy to raloxifene for the prevention of osteoporosis, that more than 27 000 women with *not* have a diagnosis of breast cancer (● Fig. 4).

### Progress and Lessons Learned

Patents produce progress and the tales of tamoxifen and raloxifene are prime examples of this principle. Both drugs failed in their primary application and were successfully reinvented which, in turn, produced wide clinical usage. Tamoxifen did not break into the headlines one day but rather sneaked up on the cancer community and was established as the "standard of care" for endocrine treatment by the early 1980's. So much so that the World Health Organization declared tamoxifen an essential drug for the treatment of breast cancer. It is cheap, remarkably non-toxic, easily administered and saves lives. Progress is measured by the hundreds of thousands of women who are alive today who would have died if they had been diagnosed with breast cancer in the 1970's. The principle of long-term antihormonal therapy targeted to the OER has dramatically improved survivorship in breast cancer.

But tamoxifen might not have happened. There was no programme or pipeline to replace tamoxifen at the beginning. With no progress with tamoxifen in the 1970's, how long would it have been before aromatase inhibitors were developed with no lead adjuvant agent to pioneer targeted antihormonal therapy and develop the market? Lives would have been lost [43] and it might have been another decade before another approach of receptor targeting was shown to save lives. Reinvention became the path to progress. The reinvention of keoxifene to become raloxifene reinforces the lesson that "observations in one field of science become major discoveries in another" [33]. The SERM field is following closely on the heels of raloxifene with many new medicines [44].

Overall, it is clear that there are no short-term solutions to therapeutic changes in healthcare. Drug targeting, clinical trials and advances in chemoprevention require decades of dedicated effort. However, the lessons learned from endocrine therapy demonstrate the principle that there are also consequences to the patient when new treatments are introduced and constant re-examination of clinical results and persistent challenge to dogma are required. Laboratory research is currently defining the evolution of long-term antihormonal therapy [45] with the remarkable discovery that minute concentrations of oestrogen can kill

breast cancers following years of antihormone treatment [46, 47] Learning to use our new knowledge of oestrogen action in clinical trials may be an unanticipated bonus of antihormone therapy.

## Acknowledgements

Dr. Jordan is supported by the Department of Defense Breast Program under award number BC050277 Center of Excellence (Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense), SPORC in Breast Cancer CA 89018, R01 GM067156, FCCC Core Grant NIH P30 CA006927, the Avon Foundation and the Weg Fund of Fox Chase Cancer Center. Parts of this history have been published in Jordan VC. Improvements in tumor targeting, survivorship, and chemoprevention pioneered by tamoxifen. *Oncology* 2006; 20: 553–562. Reprinted with permission from CMP Healthcare Media, Copyright 2006.

## References

- Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. *Clin Cancer Res* 2003; 9: 1980–1989
- Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nature Reviews Drug Discovery* 2003; 2: 205–213
- Jordan VC. Tamoxifen (ICI 46474) as a targeted therapy to treat and prevent breast cancer. *Brit J Pharmacol* 2006; 147: S269–S276
- Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998; 354: 1451–1467
- Early Breast Cancer Trialists' Collaborative Group (EBCTCG). 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
- Buzdar A, Marcus C, Holmes F, Hug V, Hortobagyi G. Phase II evaluation of LY156758 in metastatic breast cancer. *Oncology* 1988; 45: 344–345
- 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. 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: 2189–2197
- Harper MJ, Walpole AL. Mode of action of I.C.I. 46474 in preventing implantation in rats. *J Endocrinol* 1967; 37: 83–92
- Williamson JG, Ellis JP. The induction of ovulation by tamoxifen. *J Obstet Gynaec Br Commonw* 1973; 80: 844–847
- Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. *Br J Cancer* 1971; 25: 270–275
- Ward HW. Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. *Br Med J* 1973; 1: 13–14
- Jordan VC. The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. *Breast Cancer Res Treat* 1988; 11: 197–209
- Anonymous. Review of mortality results in randomized trials in early breast cancer. *Lancet* 1984; ii: 1205
- EBCTCG. 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* 1988; 319: 1681–1692
- EBCTCG. 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* 1992; 339: 1–15
- Jordan VC. Biochemical pharmacology of antiestrogen action. *Pharmacol Rev* 1984; 36: 245–276
- Beall PT, Misra KL, Young RL, Spiut HJ, Evan HJ, LeBlanc A. Clomiphene protects against osteoporosis in the mature ovariectomized rat. *Calcif Tissue Int* 1985; 36: 123–125
- Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 1987; 10: 31–35
- 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: 449–456
- Turner RT, Wakley GK, Hannon KS, Bell NH. Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. *Endocrinology* 1988; 122: 1146–1150
- Turken S, Siris E, Seldin D, Flaster E, Hyman G, Lindsay R. Effects of tamoxifen on spinal bone density in women with breast cancer. *J Natl Cancer Inst* 1989; 81: 1086–1088
- Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, Carbone PP, DeMets DL. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med* 1992; 326: 852–856
- Love RR, Newcomb PA, Wiebe DA, Surawicz TS, Jordan VC, Carbone PP, DeMets DL. Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. *J Natl Cancer Inst* 1990; 82: 1327–1332
- Love RR, Wiebe DA, Newcomb PA, Cameron L, Leventhal H, Jordan VC, Feyzi J, DeMets DL. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Ann Intern Med* 1991; 115: 860–864
- 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: 812–815
- Hardell L. Tamoxifen as risk factor for carcinoma of corpus uterus [letter]. *Lancet* 1988; 2(8610): 563
- Jordan VC. Tamoxifen and endometrial cancer [letter]. *Lancet* 1988; 2(8618): 1019
- Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, Skoog L, Somell A, Theve T, Wilking N, Hjalmar M-L. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* 1989; 1: 117–120
- Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, Atkins JN, Margolese RG, Runowicz CD, James JM, Ford LG, Wolmark N. 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: 1652–1662
- Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N. 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: 1371–1388
- Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* 1987; 47: 4020–4024
- Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 1988; 6: 589–595
- Lerner IJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res* 1990; 50: 4177–4189
- Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothiofene derived antiestrogen. *Life Sci* 1983; 32: 1031–1036
- Jordan VC, Gosden B. Inhibition of the uterotrophic activity of estrogens and antiestrogens by the short acting antiestrogen LY117018. *Endocrinology* 1983; 113: 463–468
- Gottardis MM, Jiang SY, Jeng MH, 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
- Ettlinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, Christiansen C, Delmas PD, Zanchetta JR, Stakkestad J, Gluer CC, Krueger K, Cohen FJ, Eckert S, Ensrud KE, Avioli LV, Lips P, Cummings SR. 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 [see comments] [published erratum appears in *JAMA* 1999; 282: 2124]. *JAMA* 1999; 282: 637–645
- Cauley JA, Norton L, Lippman ME, Eckert S, Krueger KA, Purdie DW, Farrerons J, Karasik A, Mellstrom D, Ng KW, Stepan JJ, Powles TJ, Morrow M, Costa A, Silfen SL, Walls EL, Schmitt H, Muchmore DB, Jordan VC, Ste-Marie LG. 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* 2001; 65: 125–134
- 39 Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon (jr) 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. The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial *JAMA* 2006; 295: 2727–2741
- 40 Barrett-Connor E, Mosca L, Collins P, Geiger MJ, Grady D, Kornitzer M, McNabb MA, Wenger NK. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Engl J Med* 2006; 355: 125–137
- 41 Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, Secrest RJ, Cummings SR. For the CORE Investigators Continuing Outcomes Relevant to Evista: Breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J Natl Cancer Inst* 2004; 96: 1751–1761
- 42 Jordan VC. Optimising endocrine approaches for the chemoprevention of breast cancer. Beyond the Study of Tamoxifen and Raloxifene (STAR) Trial. *European Journal of Cancer* 2006; 42: 2909–2913
- 43 Jordan VC. What if tamoxifen (ICI 46474) had been found to produce rat liver tumors in 1973? A personal perspective. *Ann Oncol* 1995; 6: 29–34
- 44 Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *J Med Chem* 2003; 46: 1081–1111
- 45 Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004; 5: 207–213
- 46 Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM, Jordan VC. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000; 6: 2028–2036
- 47 Lewis JS, Meeke K, Osipo C, Bell E, Kidawi N, Chandel NS, Jordan VC. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *JNCI* 2005; 97: 1746–1759
- 48 Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* 1977; 75: 305–316
- 49 Writing Group for the Women's Health Initiative Investigators: Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J. Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women. *JAMA* 2002; 288: 321–332
- 50 Million Women Study Collaborators. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet* 2003; 362: 419–427



## Original Article

# Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients

V. Craig Jordan<sup>a,\*</sup>, Joan Lewis-Wambi<sup>a</sup>, Helen Kim<sup>a</sup>, Heather Cunliffe<sup>b</sup>, Eric Ariazi<sup>a</sup>, Catherine G.N. Sharma<sup>a</sup>, Heather A. Shupp<sup>a</sup>, Ramona Swaby<sup>a</sup>

<sup>a</sup>Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA

<sup>b</sup>Translational Genomics, 445 N Fifth Street, Phoenix, AZ 85004, USA

## Abstract

The ubiquitous application of selective oestrogen receptor modulators (SERMs) and aromatase inhibitors for the treatment and prevention of breast cancer has created a significant advance in patient care. However, the consequence of prolonged treatment with antihormonal therapy is the development of drug resistance. Nevertheless, the systematic description of models of drug resistance to SERMs and aromatase inhibitors has resulted in the discovery of a vulnerability in tumour homeostasis that can be exploited to improve patient care. Drug resistance to antihormones evolves, so that eventually the cells change to create novel signal transduction pathways for enhanced oestrogen (GPR30 + OER) sensitivity, a reduction in progesterone receptor production and an increased metastatic potential. Most importantly, antihormone resistant breast cancer cells adapt with an ability to undergo apoptosis with low concentrations of oestrogen. The oestrogen destroys antihormone resistant cells and reactivates sensitivity to prolonged antihormonal therapy. We have initiated a major collaborative program of genomics and proteomics to use our laboratory models to map the mechanism of subcellular survival and apoptosis in breast cancer. The laboratory program is integrated with a clinical program that seeks to determine the minimum dose of oestrogen necessary to create objective responses in patients who have succeeded and failed two consecutive antihormonal therapies. Once our program is complete, the new knowledge will be available to translate to clinical care for the long-term maintenance of patients on antihormone therapy.

© 2007 Published by Elsevier Ltd.

**Keywords:** Aromatase inhibitors; Tamoxifen; Raloxifene; Gene array analysis

## Introduction

The translation and application of long-term antihormonal strategies, aimed at the tumour oestrogen receptor (OER), has significantly improved the prognosis of patients with breast cancer.<sup>1</sup> Long-term adjuvant tamoxifen treatment not only enhances survival and disease-free survival in patients with OER positive tumours during treatment but also reduces mortality for at least 10 years after treatment has stopped.<sup>2,3</sup> Building on the success of long-term tamoxifen therapy, a number of aromatase inhibitors have been shown to improve prognosis and reduce side effects (blood clots and endometrial cancer) if given instead of tamoxifen<sup>4-6</sup> or after tamoxifen treatment.<sup>7,8</sup> Thus, the

original scientific strategy<sup>9</sup> of long-term antihormonal adjuvant therapy targeted to patients with OER positive disease<sup>10,11</sup> has emerged as the standard of care for breast cancer patients worldwide.

The new dimension of chemoprevention has advanced significantly during the past decade.<sup>12</sup> Preliminary studies were initiated in the 1980s to explore the safety and suitability of administering tamoxifen to women only at risk for breast cancer.<sup>13-15</sup> The rationale of these studies was based on the wide clinical experience using tamoxifen to treat all stages of breast cancer, the reduction of contralateral breast cancer noted in patients receiving adjuvant tamoxifen treatment<sup>16-18</sup> and laboratory studies that repeatedly demonstrated that tamoxifen can prevent mammary cancer in animal models.<sup>19-22</sup>

The current status and results of the worldwide efforts to quantitate and evaluate the value of tamoxifen as a

\*Corresponding author. Tel.: +1 215 728 7410; fax: +1 215 728 7034.

E-mail address: v.craig.jordan@fccc.edu (V. Craig Jordan).

chemopreventive have been summarized recently<sup>23</sup> but it is the P-1 trial completed by Fisher and the National Surgical Adjuvant Breast and Bowel Project (NSABP)<sup>24,25</sup> that is considered to be the landmark.<sup>26</sup> The results can be summarized simply. Tamoxifen reduced the incidence of breast cancer by 50%<sup>24</sup> in pre and postmenopausal women at high risk.<sup>27</sup> Side effects noted were increases in early stage low grade endometrial cancer, blood clots, and cataracts<sup>24,25</sup> but only in postmenopausal women receiving long-term tamoxifen treatment. Tamoxifen is available in the United States for risk reduction in pre and postmenopausal women. However, the consensus today is that tamoxifen is better deployed as a chemopreventive for premenopausal women to reduce the risk of OER positive breast cancer.<sup>28–32</sup> There are no increases in the side effects of endometrial cancer or blood clots but tamoxifen keeps preventing breast cancer long after treatment stops<sup>31</sup> consistent with earlier treatment results.<sup>3</sup>

The concern that tamoxifen was going to be associated with the risk of endometrial cancer<sup>33</sup> and the recognition that the drugs called nonsteroidal antioestrogens<sup>34</sup> were in fact selective OER modulators (SERMs) led to a paradigm change for chemoprevention. SERMs were oestrogenic in ovariectomized rat bone<sup>35</sup> but at the same time prevented mammary cancer.<sup>21</sup> These data led to the evidence-based hypothesis that SERMs could prevent breast cancer as a beneficial side effect during the treatment and prevention of osteoporosis.<sup>36,37</sup> Based on this laboratory-based hypothesis, raloxifene was subsequently shown to reduce fractures in postmenopausal women with or at high risk for osteoporosis<sup>38</sup> but at the same time caused a 75% reduction in the incidence of breast cancer.<sup>39</sup> A follow-up trial P-2 by the NSABP<sup>40</sup> established that raloxifene was equivalent to tamoxifen at preventing invasive breast cancer in high risk postmenopausal women but with significantly fewer side effects (hysterectomies, cataracts, overall thrombotic events). However, although lower numbers of endometrial cancer were noted in raloxifene treated women compared to tamoxifen treated women, this was not significant because of a higher hysterectomy rate.<sup>40</sup> Nevertheless, a related trial called Raloxifene use for the Heart or RUTH, showed no increase in endometrial cancers during raloxifene treatment compared to placebo arm.<sup>41</sup>

Thus from this brief introduction, it can be appreciated that significant clinical advances have been made through the application of the principle of long-term antihormone therapy<sup>9,36</sup> for the treatment and prevention of breast cancer. All of the advances can now be applied in clinical practice to improve patient care. Nevertheless, despite these advances through the use of sustained administration of antihormonal drugs, there are consequences for the tumour with the eventual development of drug resistance. In the case of SERMs, the type of resistance is unique and is expressed as SERM stimulated growth.<sup>42</sup> But, it is the consistent study of the process of drug resistance to antihormones that resulted in the discovery<sup>43</sup> of a weakness

in the mechanisms of antihormonal drug resistance that has potential for the future exploitation in clinical practice.

### Classification of SERM resistance

During the past 20 years we have focused our laboratory research program on developing models of SERM resistance in vivo to replicate events that could potentially occur clinically. The models were initially developed in vivo to avoid problems with cell culture where cells that become resistant to short term SERM treatment do not develop the essential requirements for angiogenesis that are necessary to survive and grow in patients. We now have a range of models that have been evaluated for growth in vivo (athymic mice) and that have been passaged in vivo for more than 5–10 years to replicate the long-term antihormonal therapy routinely used to treat patients (Table 1).

Initial studies of resistance to tamoxifen treatment demonstrated the unique feature of SERM stimulated growth. Resistant tumours that develop in athymic mice from both OER positive breast and endometrial cells grow in response to either a SERM or estradiol.<sup>33,44</sup> This is why an aromatase inhibitor or the pure antioestrogen fulvestrant (that binds to OER and facilitates the rapid destruction of the complex)<sup>45</sup> are successful second line therapies.<sup>46,47</sup> This form of resistance is referred to as Phase I resistance.<sup>42</sup>

However, these models represent only a few years of SERM treatment which is inconsistent with clinical experience of 5 years of adjuvant tamoxifen or possibly 10 years or more of raloxifene treatment to maintain bone density. The discovery that long-term SERM treatment exposes a vulnerability in the cancer cell that could have potential therapeutic applications was first reported at the St. Gallen meeting in the early 1990s.<sup>43</sup> Simply stated, long-term SERM treatment creates an absolute dependency on the SERM for tumour growth but small physiologic doses of oestradiol cause tumour cell death. Small tumours respond more readily to the apoptotic action of oestrogen but when tumours regrow during continuous oestrogen

Table 1  
The available SERM resistant OER positive tumours used to investigate drug resistance in our laboratory.

Phase	Organ site	SERM	Cell line	Reference
I	Breast	tamoxifen	MCF-7	44, 67, 68
	Breast	tamoxifen	T47D	69
	Endometrial	tamoxifen	human tumour	33
	Endometrial	tamoxifen	ECC-1	70
II	Breast	tamoxifen	MCF-7	43, 48, 71
	Breast	raloxifene	MCF-7	72
	Endometrial	raloxifene	ECC-1	(unpublished)

Phase I resistance refers to tumours that can be stimulated to grow into oestrogen or a SERM whereas Phase II resistance refers to tumours stimulated to grow only with a SERM. Oestrogen causes Phase II tumors to undergo apoptosis and regrow.<sup>42</sup>

therapy, the tumours again respond to the SERM or no treatment<sup>48</sup> (equivalent to treatment with an aromatase inhibitor for patients). This form of resistance is referred to as Phase II resistance.<sup>42</sup> The models for SERM resistance are summarized in Table 1. Thus, it is plausible to consider a clinical strategy whereby limited duration, low dose oestrogen treatment could be used to purge and destroy Phase II resistant breast cancer cells but then patients could be treated again with antihormonal therapy to control tumour growth. However, a case could be made that the ubiquitous use of tamoxifen is declining and over the next decade the standard of care will be long-term treatment with one of several aromatase inhibitors. The question we have addressed in the laboratory is whether long-term

Table 2  
The basic characteristics of the MCF-7 cell lines developed from long-term oestrogen deprivation.

	Cell Line	
	MCF-7:2A	MCF-7:5C
OER	++	++
Oestrogen induced PgR	++	-
GPR30	++++	++++
Growth inhibitory response to SERMs	++	-
Growth inhibitory response to fulvestrant	+++	++
Invasion proteins	++	++++

Results are replicate (5) data from the affymetrix U-133 gene arrays relative to wild type MCF-7 cells. However, the biology of responses to antioestrogens are based on cell growth experiments where no effect is—and 100% response is + + + +.

oestrogen deprivation of breast cancer cells will expose the vulnerability to the apoptotic actions of oestrogen.

### Resistance of breast cancer to oestrogen deprivation

There are two laboratory approaches to developing models of drug resistance to aromatase inhibitors. The traditional model is to study the impact of oestrogen withdrawal on the growth of OER positive breast cancer cells. In contrast, there is a model in vivo employing athymic mice transplanted with MCF-7 cells stably transfected with the aromatase enzyme. Without oestrogen tumours do not grow but when animals are treated with the enzyme substrate androstenedione to make oestrogen, tumour growth occurs. Simultaneous treatment with a number of aromatase inhibitors results in initial control of oestrogen-stimulated tumour growth but then the inhibitors fail and tumour growth occurs despite continuing treatment. This approach has been most instructive about strategies for antihormonal sequencing and the rationale of avoiding a combination of a SERM and an aromatase inhibitor for breast cancer therapy.<sup>49,50</sup>

The traditional approach of oestrogen withdrawal using breast cancer cells not engineered in any way, was not possible until Berthois and coworkers<sup>51</sup> discovered that cell culture media contained significant quantities of oestrogen found to increase the growth rate of MCF-7 cells. In other words, despite the fact that investigators were adding charcoal stripped serum to remove endogenous oestrogen, the media already contained oestrogenic chemical contaminants from the phenol red pH indicator.

Initial studies of the short and long-term effects of oestrogen deprivation of MCF-7<sup>52,53</sup> and T47D<sup>54</sup> breast

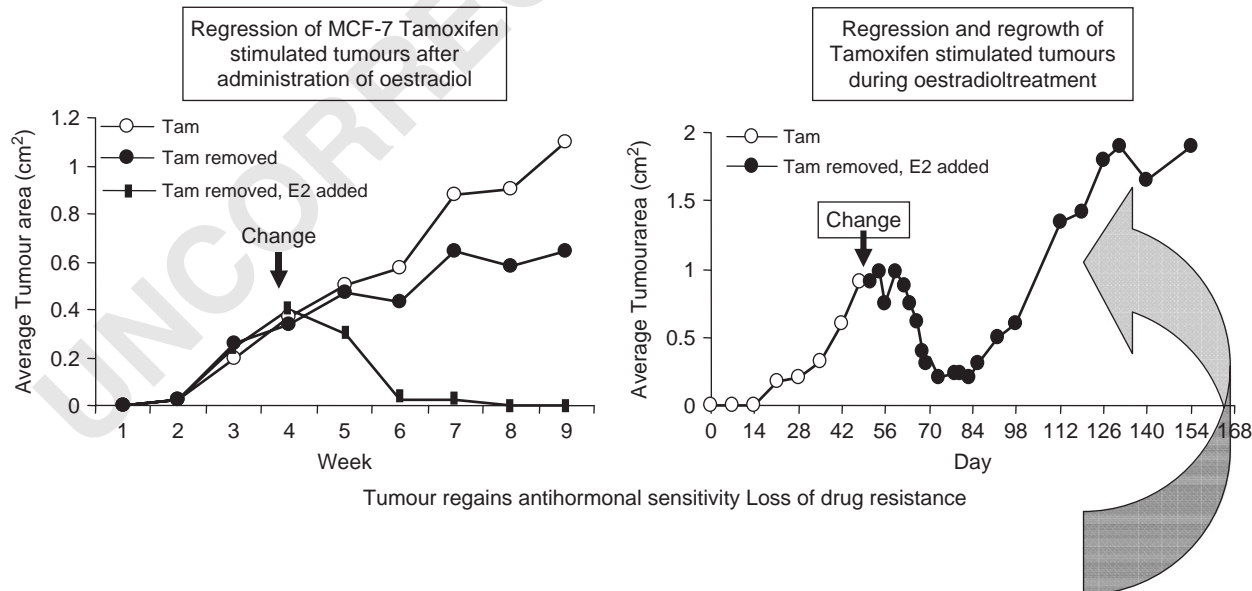


Fig. 1. Diagrammatic representation of the actions of physiologic oestradiol (E2) on the growth of small phase II MCF-7 tamoxifen resistant tumors in ovariectomized athymic mice. A larger tumour will regress with oestradiol treatment but will eventually display oestrogen-stimulated growth. If tumours are retransplanted into a new generation of ovariectomized athymic mice and treated with oestradiol, tamoxifen will block oestrogen-stimulated tumour growth.<sup>48</sup> First presented in St. Gallen, 1993.<sup>43</sup>

cancer cells noted some interesting differences based on the regulation of OER in the different cell types.<sup>55</sup> The MCF-7 cells that are obtained following long-term oestrogen deprivation remain OER positive (Table 2) whereas the T47D lose the OER.<sup>56</sup> The levels of OER increase in the oestrogen deprived MCF-7 cells (Table 2) and also there are increases in GPR30<sup>57</sup> noted in our gene array data. Thus, the oestrogen-deprived cells have an enhanced signal transduction pathway to support survival. Since breast cancers seem to rarely lose the OER efforts to study antihormonal drug resistance have focused on the MCF-7 line.

Our program to develop MCF-7 cell lines resistant to oestrogen withdrawal successfully described two clones of cells: the MCF-7:5C and the MCF-7:2A line. The MCF-7:5C line<sup>58</sup> is OER positive but progesterone receptor (PgR) negative and unresponsive to both oestrogen and SERM treatment. In contrast, the MCF-7:2A cell line<sup>59</sup> did respond to SERM therapy with a reduction in growth rate but oestrogen did not affect the growth rate, except at high concentrations. We have known for nearly 20 years that activation of growth factor receptor pathways can create intrinsic SERM resistance<sup>60,61</sup> and a down regulation of PgR induction.<sup>62</sup> These data would be consistent with the finding for the MCF-7:5C cells (Table 2). The laboratory observation that deactivation of the OER signal transduction pathway with fulvestrant is consistent with clinical observation that fulvestrant produces reasonable control of aromatase resistant breast cancer.<sup>63</sup> However, the models of oestrogen deprivation we developed in the early 1990s were to take center stage once the SERM resistant models were found to be reproducible<sup>48</sup> and worthy of further development (Table 1). The key to the value of the two MCF-7 clones (5C, 2A) was that they could be studied in vitro to understand the mechanism of oestrogen-induced apoptosis using genomics.

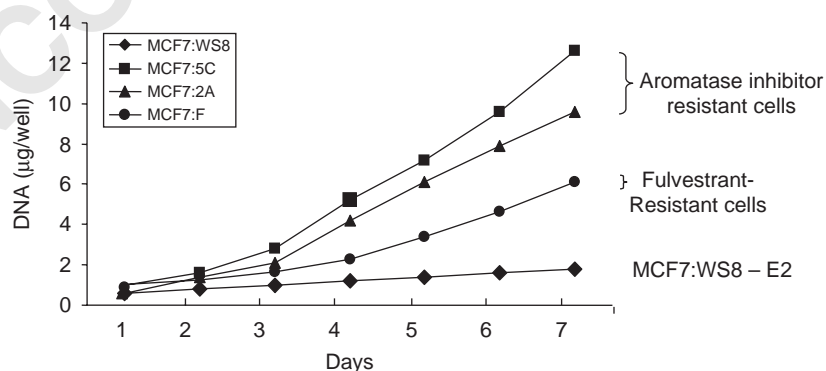


Fig. 2. The growth of wild type MCF-7 cells (WS8) and various antihormonally resistant sublines in an oestrogen-free environment. The cells MCF-7:5C and 2A grow spontaneously and could be considered to represent aromatase inhibitor resistant cells. These remain OER positive. In contrast, MCF-7F are fulvestrant resistant (MCF-7 cells grown for over a year in an oestrogen-deprived environment containing fulvestrant). These cells grow spontaneously but have no OER.

### The new biology of oestrogen action

A re-examination of MCF-7 clones 5C and 2A occurred at the time when clinical investigators were re-examining the value of high dose oestrogen therapy in those patients who had been treated exhaustively with successive anti-hormonal therapies.<sup>64</sup> The clinical studies demonstrated that high dose oestrogen therapy could cause tumour regression or stasis (30%) in patients treated exhaustively

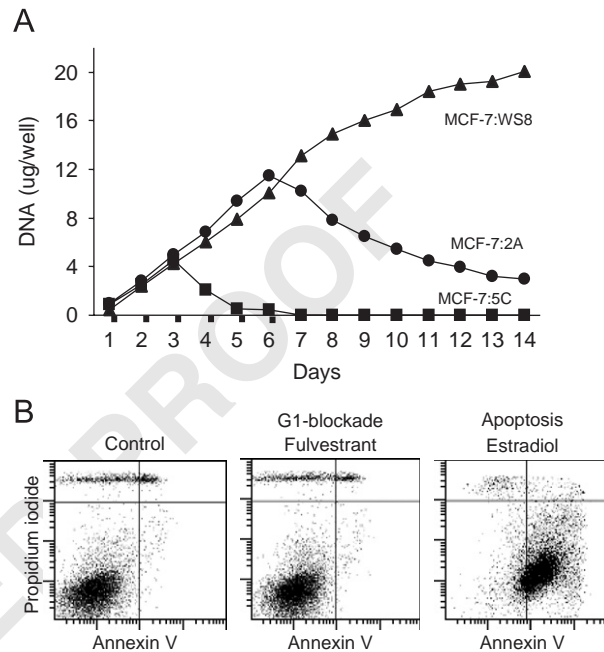


Fig. 3. The action of oestradiol (1 nM) on the growth of wild type MCF-7 cells (WS8) or long-term oestrogen-deprived MCF-7 cells (5C and 2A). In Panel A the MCF-7:5C cells undergo rapid apoptosis during the first few days of oestradiol exposure whereas the MCF-7:2A cells slowly initiate apoptosis during the days after 6 of oestradiol treatment. In panel B MCF7:5C cells respond to fulvestrant (1 µM) with a G1 blockade at 72 h whereas oestradiol (1 nM) causes massive and complete apoptosis. These results were obtained using flow cytometry.

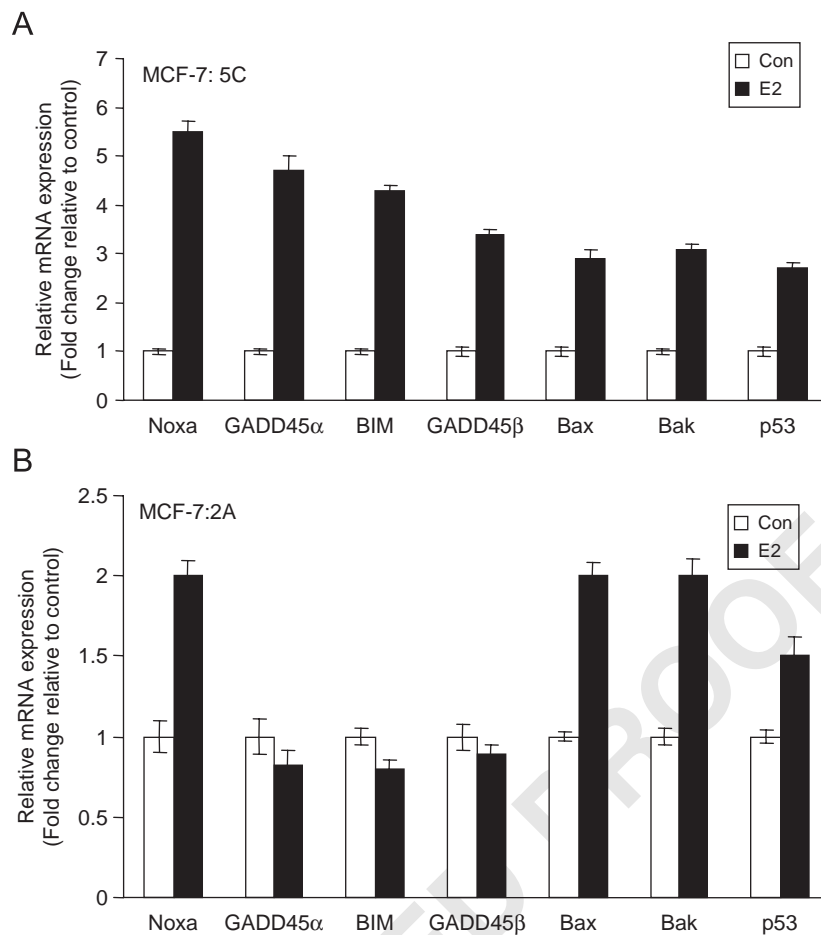


Fig. 4. Oestrogenic regulation of apoptotic genes in long term estrogen-deprived MCF-7:5C and MCF-7:2A breast cancer cells as determined by Affymetrix gene microarrays. For experiment, cells were treated with 1 nM oestradiol for 48 h and total RNA was prepared using the Qiagen Rneasy Mini kit. cRNA was generated, labeled, and hybridized to the Affymetrix Human Genome U133 plus 210 arrays containing 54,300 probe sets. Chips were then scanned and analysed using the Affymetrix Microarray Analysis Suite version 5.0. Assessment of data quality was conducted following default guidelines in the Affymetrix's GeneChip<sup>®</sup> Expression Analysis Data Analysis Fundamentals Training Manual. Global scaling for average signal intensity for all arrays was set to 500. Four biological replicates from each of the two cell lines were arrayed to determine consistent and reproducible patterns of gene expression. The above figure shows that oestradiol treatment caused 3 to 6 fold induction of the proapoptotic genes NOXA, GADD45 $\alpha$ , GADD45 $\beta$ , BIM, BAX, BAK and p53 in (A) MCF-7:5C cells but only a 2-fold induction of NOXA, BAX, and BAK in (B) MCF-7:2A cells.

with antihormones.<sup>64</sup> Additionally, high concentrations of oestrogen could induce apoptosis in long-term oestrogen deprived cells in culture.<sup>65</sup> In contrast, we pursued our original hypothesis that the apoptotic supersensitization of breast cancer cells by long-term antihormonal therapy could occur with physiologic or a very low concentration of oestrogen treatment.<sup>43,48</sup>

Two important observations, that were made during the re-evaluation of the MCF-7:5C and 2A cells, reinforced the view that oestrogen-induced apoptosis could be applied to reverse resistance to aromatase inhibitors. The first observation occurred by changing the charcoal stripped serum from the original 5% charcoal stripped calf serum<sup>58</sup> to 10% developed stripped fetal bovine serum.<sup>66</sup> This caused a dramatic increase in the growth rate of the 5C cells to be comparable to the MCF-7:2A cells (Figs. 1 and 2). Remarkably, physiologic oestradiol (1nM) now caused a massive apoptotic response in the MCF-7:5C cells (Fig. 3A,B). The MCF-7:2A cells had previously<sup>59</sup> been found to

be responsive to antioestrogens by inhibiting growth and oestrogen by inducing progesterone receptor synthesis. The 2A cells, however, only weakly responded to the growth inhibitory effects of high concentrations 1  $\mu$ M oestradiol. This original assumption is not true if the time course is extended (Fig. 3A). The 2A cells appear to have a survival mechanism that is able to protect them initially from the apoptotic actions of oestradiol. Nevertheless, this survival mechanism eventually fails. Overall, our models now create an interesting opportunity to interrogate the time courses with genomics and proteomics to find the precise oestrogen-induced mechanisms for protecting the cell from apoptosis.

#### Analysis of apoptotic pathways

A number of U-133 Affymetrix gene arrays were completed using the MCF-7, MCF-7:5C and 2A cell lines to define the early events of oestrogen action. A 48 h time

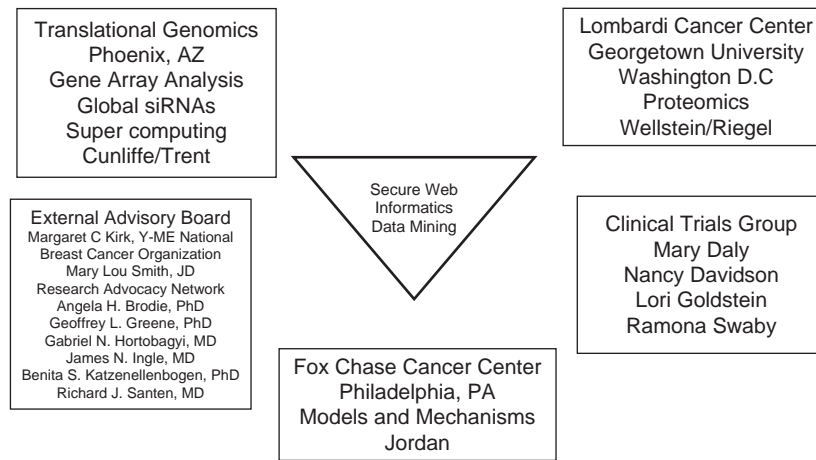


Fig. 5. The organization of our Department of Defence Center of Excellence Grant entitled “A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis.” The model systems to study the survival and apoptosis induced with oestrogen are used for time course experiments at the Fox Chase Cancer Center. The materials are distributed to Translational Genomics for siRNA analysis or gene array and the Vincent T. Lombardi Cancer Center is involved to conduct proteomics. All results are uploaded into a shared secure web for data processing and target identification by our informatics and biostatistical group. Each laboratory is able to validate emerging pathways and study individual genes of interest. Our program is integrated with a clinical trials program that provides patient samples for validation of apoptotic or survival pathways. We are grateful to our external advisory board of Patient Advocates and professional colleagues for their continuing advice and support.

point was used in our preliminary studies and five replicates were analysed to ensure statistical veracity. All gene array analyses were completed at Translational Genomics, AZ. Results illustrated in Fig. 4 show the 48 h increase in proapoptotic genes that are activated by oestrogen in the MCF-7:5C cells. This is consistent with the time course for the apoptotic death response of the MCF-7:5C cells noted in Fig. 3. In contrast, oestrogen had not yet activated the full apoptotic response in MCF-7:2A cells that become apoptotic over a much longer time course (Fig. 3).

Overall, we have confirmed our novel observations that breast cancer and endometrial cancer cells (unpublished observation) become resistant to long-term antihormonal interventions by reconfiguring the oestrogen signal transduction pathway to induce an apoptotic response rather than enhancing survival and further growth. These data plus the emerging anecdotal results of clinical case reports (James Ingle, MD and Mr. Michael Dixon personal communications) prompted us to develop a multicenter program to explore our unique model systems systematically so that we can describe the mechanisms of oestrogen-induced survival and apoptosis in breast cancer. Completion of these studies would then provide an invaluable database to translate to patient care. The goal would be to determine the lowest dose of oestrogen necessary to cause apoptosis in a significant number of women whose tumours no longer respond to antihormonal therapy. This would reverse antihormone resistance in a significant proportion of patients.

#### Translation of laboratory results to patient care

We have established a multi-center collaborative translational research grant with headquarters at the Fox Chase

Cancer Center (FCCC) (Figs. 5 and 6). The five year program is sponsored by the US Department of Defense Breast Cancer Program BC050277 entitled “A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis.”

Our goal is to create maps of the survival and apoptotic responses to oestrogen noted in our models in vivo and in vitro. Biological samples from our time course experiments using our models at the FCCC are being distributed to Translational Genomics in Arizona for Agilent gene array analysis, CGH and CpG methylation arrays. Total human genome siRNA analysis is also being completed on our cell lines. Additionally, samples for proteomics are being dispatched to Georgetown University (Vincent T. Lombardi Cancer Center, PIs Anton Wellstein and Anna T. Riegel). All processed data are then being uploaded into a secure website for data mining and target identification, so that verification and validation studies can occur at each of the collaborating sites. A clinical program is exploring the clinical applications of our laboratory observation with two successive protocols:

- (1) A single arm phase II study of pharmacologic dose oestrogen in postmenopausal women with hormone receptor-positive metastatic breast cancer after failure of sequential endocrine therapies.
- (2) Reversal of anti-estrogen resistance with sequential dose de-escalation of pharmacologic oestrogen in a single arm phase II study of postmenopausal women with hormone receptor-positive metastatic breast cancer after failure of sequential endocrine therapies.

Our clinical studies are in place (1) to confirm the clinical finding<sup>64</sup> that high dose oestrogen treatment following exhaustive antihormonal treatment of OER positive breast

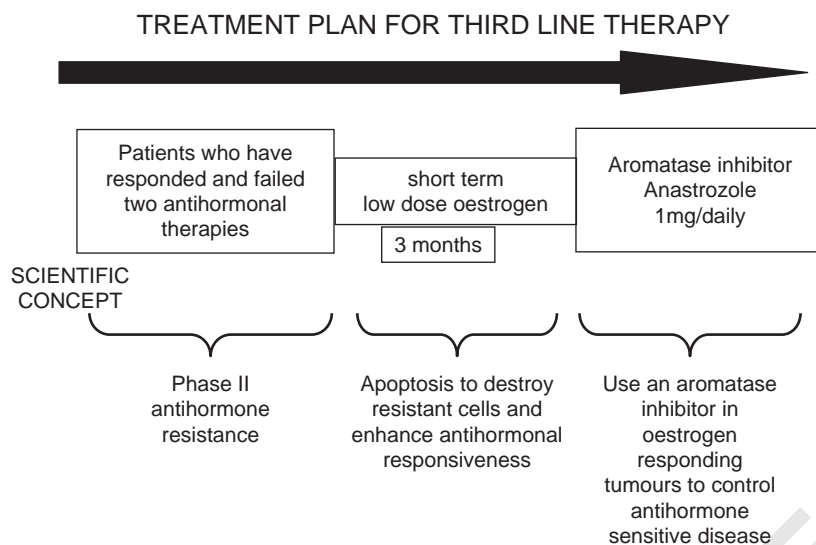


Fig. 6. An anticipated treatment plan for third line endocrine therapy. Patients must have responded and failed two successive antihormonal therapies to be eligible for a course of low dose oestradiol therapy for 3 months. The anticipated response rate is 30%<sup>64</sup> and responding patients will be treated with anastrozole until relapse. Validation of the treatment plan via the Center of Excellence grant (Fig. 5) will establish a platform to enhance response rates with apoptotic oestrogen by integrating known inhibitors of tumour survival pathways into the 3 month debulking treatment plan. The overall goal is to increase response rates and maintain patients for longer on antihormonal strategies before chemotherapy is required.

cancer will give a 30% response rate and (2) to determine the lowest dose of oestrogen that will induce an equivalent tumour regression as high dose oestrogen (30 mg. oestradiol daily). All patients will be monitored weekly using the Apoptosense<sup>®</sup> serum assay to detect apoptotic markers in responding and non-responding patients. Additionally, where possible, patients will have biopsies of accessible tumour tissue before and after 12 weeks of oestrogen therapy (or shorter if patients rapidly progress). Responding patients will be retreated with 1 mg anastrozole daily until progression.

Overall, the map of survival and apoptotic pathways we create from our laboratory models will be invaluable to guide our selection of target genes in biopsies using real time RTPCR. This will provide clues as to our future strategy of improving response rates with agents that selectively block survival pathways which can then be used in combination with our apoptotic oestrogen purge. It is our long term goal to improve oestrogen-induced response rates in patients refractory to antihormonal therapies. In so doing, select patients with metastatic breast cancer can anticipate longer disease control before chemotherapy is necessary. Most importantly, the new knowledge will provide an in silico platform to identify the apoptotic target so effectively located by the OER.

#### Conflict of Interest

None declared.

#### Acknowledgements

Supported (VCJ) by the Department of Defense Breast Program under award number BC050277 Center of Excellence (Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense), SPORE in Breast Cancer CA 89018, R01 GM067156, FCCC Core Grant NIH P30 CA006927, the Avon Foundation and the Weg Fund of Fox Chase Cancer Center. Ramona Swaby, MD is the recipient of the clinical trials grant from AstraZeneca.

#### References

- 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.
- EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;**354**:1451–67.
- EBCTCG. 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**:1687–717.
- Howell A, Cuzick J, Baum M, 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;**365**:60–2.
- Thurlimann B, Keshaviah A, Coates AS, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med* 2005;**353**:2747–57.
- Coates AS, Keshaviah A, Thurlimann B, et al. 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* 2007;**25**(5):486–92.
- Coombes RC, Hall E, Gibson LJ, et al. A randomized trial of exemestane after two to three years of tamoxifen therapy in

- 1 postmenopausal women with primary breast cancer. *N Engl J Med* 2004;**350**(11):1081–92.
- 3 8. Goss PE, Ingle JN, Martino S, et al. 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* 2005;**97**:1262–71.
- 5 9. 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.
- 7 10. Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *Eur J Cancer* 1975;**11**:205–6.
- 9 11. Jordan VC, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. *Adjuvant Therapy of Cancer* 1979;**2**:19–26.
- 11 12. Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. *Nat Rev Cancer* 2007;**7**:46–53.
- 13 13. 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. *Br J Cancer* 1989;**60**(1):126–31.
- 15 14. 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**(13):852–6.
- 17 15. Love RR, Wiebe DA, Newcomb PA, et al. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Ann Intern Med* 1991;**115**(11):860–4.
- 19 16. Cuzick J, Baum M. Tamoxifen and contralateral breast cancer letter. *Lancet* 1985;**2**(8449):282.
- 21 17. Fornander T, Rutqvist LE, Cedermark B, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* 1989;**1**(8630):117–20.
- 23 18. Fisher B, Costantino J, Redmond C, et al. 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* 1989;**320**(8):479–84.
- 25 19. Jordan VC. Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. *J Steroid Biochem* 1974;**5**:354.
- 27 20. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA- induced rat mammary carcinoma. *Eur J Cancer* 1976;**12**:419–24.
- 29 21. Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea- induced rat mammary carcinoma model. *Cancer Res* 1987;**47**(15):4020–4.
- 31 22. Jordan VC, Lababidi MK, Langan-Fahey S. Suppression of mouse mammary tumorigenesis by long-term tamoxifen therapy. *J Natl Cancer Inst* 1991;**83**(7):492–6.
- 33 23. Cuzick J, Powles T, Veronesi U, et al. Overview of the main outcomes in breast-cancer prevention trials. *Lancet* 2003;**361**:296–300.
- 35 24. 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.
- 37 25. 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.
- 39 26. <<http://www.nature.com/milestones/milecancer/full/milecancer05.html>>.
- 41 27. Gail MH, Brinton LA, Byar DP, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst* 1989;**81**(24):1879–86.
- 43 28. Gail MH, Costantino JP, Bryant J, et al. Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J Natl Cancer Inst* 1999;**91**(21):1829–46.
- 45 29. Jordan VC. Optimising endocrine approaches for the chemoprevention of breast cancer. Beyond the Study of Tamoxifen and Raloxifene (STAR) Trial. *Eur J Cancer* 2006;**42**:2909–13.
- 47 30. Veronesi U, Maisonneuve P, Decensi A. Tamoxifen: an enduring star. *J Natl Cancer Inst* 2007;**99**(4):258–60.
- 49 31. 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**(4):272–82.
- 51 32. 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**(4):283–90.
- 53 33. 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.
- 55 34. Jordan VC. Biochemical pharmacology of antiestrogen action. *Pharmacol Rev* 1984;**36**(4):245–76.
- 57 35. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 1987;**10**(1):31–5.
36. Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 1988;**6**(5):589–95.
37. Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res* 1990;**50**:4177–89.
38. 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 [see comments] [published erratum appears in JAMA 1999 Dec 8;282(22):2124]. *J Am Med Assoc* 1999;**282**(7):637–45.
39. 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. *J Am Med Assoc* 1999;**281**:2189–97.
40. Vogel VG, Costantino JP, Wickerham DL, et al. The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *J Am Med Assoc* 2006;**295**:2727–41.
41. 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**(2):125–37.
42. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;**5**:207–13.
43. Wolf DM, Jordan VC. *A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent results in cancer research*, 127. Heidelberg: Springer; 1993 23–33.
44. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 1988;**48**:5183–8187.
45. 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.
46. Osborne CK, Pippen J, Jones SE, et al. 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* 2002;**20**(16):3386–95.
47. Howell A, Robertson JFR, Quaresma Albano J, et al. 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* 2002;**20**:3396–403.
48. Yao K, Lee ES, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;**6**(5):2028–36.
49. Jelovac D, Macedo L, Golubeva OG, Handratta V, Brodie AMH. additive antitumor effect of aromatase inhibitor letrozole and

- 1 antiestrogen fulvestrant in a postmenopausal breast cancer model. *Cancer Res* 2005;**65**:5439–44.
- 3 50. Long BJ, Jelovac D, Handratta V, et al. therapeutic strategies using  
the aromatase inhibitor letrozole and tamoxifen in a breast cancer  
5 model. *JNCI* 2004;**96**:456–65.
- 7 51. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red  
in tissue culture media is a weak estrogen: implications concerning the  
9 study of estrogen-responsive cells in culture. *Proc Natl Acad Sci USA*  
1986;**83**(8):2496–500.
- 11 52. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y.  
Proliferation, hormonal responsiveness, and estrogen receptor content  
13 of MCF-7 human breast cancer cells grown in the short-term and  
long-term absence of estrogens. *Cancer Res* 1987;**47**:4355–60.
- 15 53. Welshons WV, Jordan VC. Adaptation of estrogen-dependent MCF-7  
cells to low estrogen (phenol red-free) culture. *Eur J Cancer*  
1987;**232**:1935–9.
- 17 54. Murphy CS, Meisner LF, Wu SQ, Jordan VC. Short- and long-term  
estrogen deprivation of T47D human breast cancer cells in culture.  
19 *Eur J Cancer Clin Oncol* 1989;**25**(12):1777–88.
- 21 55. Pink JJ, Jordan VC. Models of estrogen receptor regulation by  
estrogens and antiestrogens in breast cancer cell lines. *Cancer Res*  
23 1996;**56**(10):2321–30.
- 25 56. Pink JJ, Bilimoria MM, Assikis J, Jordan VC. Irreversible loss of the  
oestrogen receptor in T47D breast cancer cells following prolonged  
27 oestrogen deprivation. *Br J Cancer* 1996;**74**(8):1227–36.
- 29 57. Bologa CG, Revankar CM, Young SM, et al. Virtual and  
biomolecular screening converge on a selective agonist for GPR30.  
31 *Nat Chem Biol* 2006;**2**(4):207–12.
- 33 58. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen  
receptor positive MCF-7 clone that is resistant to antiestrogens and  
35 estradiol. *Mol Cell Endo* 1992;**90**:77–80.
59. Pink JJ, Jiang SY, Fritsch M, 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**:2583–90.
60. Cormier EM, Jordan VC. Contrasting ability of antiestrogens to  
inhibit MCF-7 growth stimulated by estradiol or epidermal growth  
factor. *Eur J Cancer Clin Oncol* 1989;**25**(1):57–63.
61. Robinson SP, Jordan VC. The paracrine stimulation of MCF-7 cells  
by MDA-MB-231 cells: possible role in antiestrogen failure. *Eur J  
Cancer Clin Oncol* 1989;**25**:493–7.
62. Cormier EM, Wolf MF, Jordan VC. Decrease in estradiol-stimulated  
progesterone receptor production in MCF-7 cells by epidermal growth  
factor and possible clinical implication for paracrine-regulated breast  
cancer growth. *Cancer Res* 1989;**49**:576–80.
63. Ingle JN, Suman VJ, Rowland KM, et al. Fulvestrant in women with  
advanced breast cancer after progression on prior aromatase inhibitor  
therapy: North Central Cancer Treatment Group Trial N0032. *J Clin  
Oncol* 2006;**24**(7):1052–6.
64. Lonning PE, Taylor PD, Anker G, et al. High-dose estrogen  
treatment in postmenopausal breast cancer patients heavily exposed  
to endocrine therapy. *Breast Cancer Res Treat* 2001;**67**:111–6.
65. Song RX, Mor G, Naftolin F, et al. Effect of long-term estrogen  
deprivation on apoptotic responses of breast cancer cells to 17beta-  
estradiol. *J Natl Cancer Inst* 2001;**93**:1714–23.
66. Lewis JS, Osipo C, Meeke K, Jordan VC. Estradiol induced apoptosis  
in a breast cancer cell line resistant to estrogen deprivation. *J Steroid  
Biochem* 2005;**94**(1–3):131–41.
67. Gottardis MM, Jiang SY, Jeng MH, Jordan VC. Inhibition of  
tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic  
mice by novel steroidal antiestrogens. *Cancer Res* 1989;**49**(15):4090–3.
68. Wolf DM, Langan-Fahey SM, Parker CJ, McCague R, Jordan VC.  
Investigation of the mechanism of tamoxifen-stimulated breast tumor  
growth with nonisomerizable analogues of tamoxifen and metabolites.  
*J Natl Cancer Inst* 1993;**85**(10):806–12.
69. MacGregor-Schafer JJ, Lee E-S, O'Regan RM, Yao K, Jordan VC.  
Rapid development of tamoxifen stimulated mutant p53 breast  
tumors (T47D) in athymic mice. *Clin Cancer Res* 2000;**6**:4373–7380.
70. Osipo C, Meeke K, Liu H, et al. Trastuzumab therapy for tamoxifen-  
stimulated endometrial cancer. *Cancer Res* 2005;**65**(18):8504–13.
71. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of  
fulvestrant on estradiol-induced regression of tamoxifen-stimulated  
breast cancer. *J Natl Cancer Inst* 2003;**95**:1597–607.
72. Liu H, Lee ES, Gajdos 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**(21):1586–97.

# Selective Estrogen-Receptor Modulators and Antihormonal Resistance in Breast Cancer

AQ:A,B

V. Craig Jordan and Bert W. O'Malley

From the Fox Chase Cancer Center, Philadelphia, PA; Baylor College of Medicine, Houston, TX.

Submitted April 12, 2007; accepted July 11, 2007; published online ahead of print at www.jco.org on September XX, 2007.

Supported by the Department of Defense Breast Program under award number BC050277 Center of Excellence, SPORE in Breast Cancer CA 89018, R01 GM067156, FCCC Core Grant, NIH P30 CA006927, the Avon Foundation, and the Weg Fund of Fox Chase Cancer Center (V.J.C.); and National Institute of Health grants from the National Institute of Child Health and Human Development and National Institute of Diabetes and Digestive and Kidney Diseases, and Nuclear Receptor Signaling Atlas (B.W.O.).

Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Address reprint requests to V. Craig Jordan, OBE, PhD, DSc, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111-2497; e-mail: v.craig.jordan@fccc.edu.

© 2007 by American Society of Clinical Oncology

0732-183X/07/2536-1/\$20.00

DOI: 10.1200/JCO.2007.11.3886

## ABSTRACT

Selective estrogen-receptor (ER) modulators (SERMs) are synthetic nonsteroidal compounds that switch on and switch off target sites throughout the body. Tamoxifen, the pioneering SERM, blocks estrogen action by binding to the ER in breast cancers. Tamoxifen has been used ubiquitously in clinical practice during the last 30 years for the treatment of breast cancer and is currently available to reduce the risk of breast cancer in high-risk women. Raloxifene maintains bone density (estrogen-like effect) in postmenopausal osteoporotic women, but at the same time reduces the incidence of breast cancer in both high- and low-risk (osteoporotic) postmenopausal women. Unlike tamoxifen, raloxifene does not increase the incidence of endometrial cancer. Clearly, the simple ER model of estrogen action can no longer be used to explain SERM action at different sites around the body. Instead, a new model has evolved on the basis of the discovery of protein partners that modulate estrogen action at distinct target sites. Coactivators are the principal players that assemble a complex of functional proteins around the ligand ER complex to initiate transcription of a target gene at its promoter site. A promiscuous SERM ER complex creates a stimulatory signal in growth factor receptor-rich breast or endometrial cancer cells. These events cause drug-resistant, SERM-stimulated growth. The sometimes surprising pharmacology of SERMs has resulted in a growing interest in the development of new selective medicines for other members of the nuclear receptor superfamily. This will allow the precise treatment of diseases that was previously considered impossible.

*J Clin Oncol* 25. © 2007 by American Society of Clinical Oncology

## INTRODUCTION

The estrogen receptor (ER) is the trigger<sup>1</sup> that initiates estrogen action in its target tissues (eg, uterus, vagina, and pituitary gland). The subsequent identification of the ER in some breast cancers created a mechanistic link to explain the hormonal dependence of some breast cancers.<sup>2</sup> Ultimately, this knowledge was used to reinvent a failed postcoital contraceptive, ICI 46474,<sup>3</sup> as tamoxifen, the first targeted antiestrogenic therapy for breast cancer.<sup>4</sup> The clinical strategy of targeting ER-positive breast tumors with long-term adjuvant therapy has saved hundreds of thousands of lives.<sup>5</sup> As a result, the evolving use of tamoxifen therapy during the last three decades has proved to be the cornerstone for the treatment and prevention of breast cancer.<sup>6</sup>

However, the recognition<sup>7</sup> that the “nonsteroidal antiestrogens” were, in fact, selective estrogens and antiestrogens at different target tissues around the body, created a new dimension in drug development and enhanced therapeutic possibilities. The selective estrogenic properties of tamoxifen and raloxifene maintained bone density<sup>8</sup> but the selective antiestrogenic properties prevented rat mam-

mary carcinogenesis.<sup>9</sup> These laboratory data were used to develop an evidence-based therapeutic strategy<sup>10,11</sup> that has now become a clinical reality with the development of raloxifene. This second-generation selective ER modulator (SERM) prevents osteoporosis but also prevents breast cancer as a beneficial side effect.<sup>12</sup> With this significant advance in therapeutics, it has become clear that the action of SERMs at different target sites can no longer be explained by an ER model that simply turns estrogen action on or off. Other physiologic factors must be involved.

In this article, we will describe our evolving understanding of SERM action at its target sites. Although the ER complex is programmed by the shape of the SERM buried inside the receptor, it is the new protein players called coactivators and corepressors<sup>13</sup> that are now known to modulate and control the dynamics of the complex as it turns on or turns off subcellular signaling networks at target sites around the body. However, we believe it is important to state at the outset that although we have, by necessity, chosen to explain the molecular mechanism of SERMs to retain therapeutic relevance in oncology, we prefer to use the term steroid

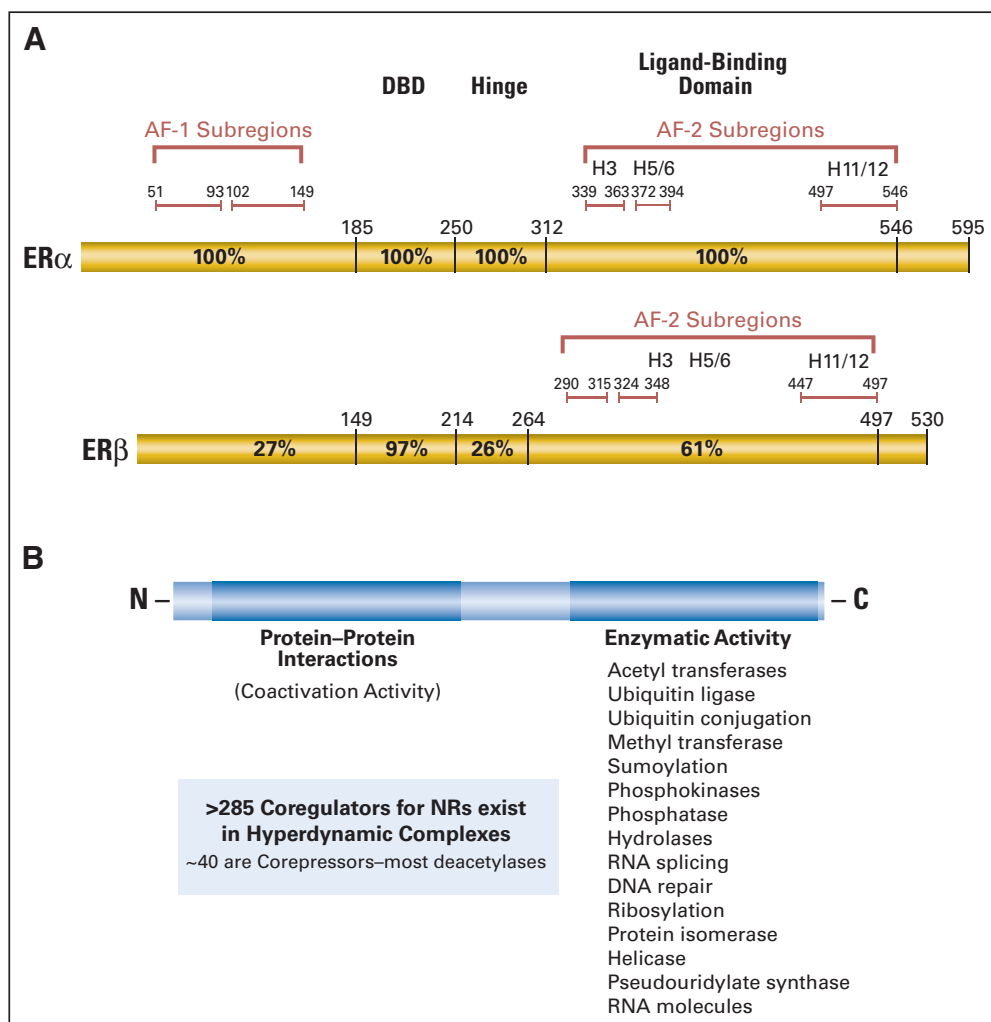
receptor modulators (SRMs) when considering mechanisms. The molecular biology of selective activity is clearly universal within the steroid receptor superfamily.<sup>13</sup> This fact has important therapeutic implications for future drug discovery.

### MECHANISMS OF SELECTIVE RECEPTOR MODULATOR ACTION

Of the 48 members of the nuclear receptor (NR) family, approximately half have been determined to be regulatable by ligands.<sup>14,15</sup> The remaining molecules are regulated by signaling pathways that impart post-translational modifications to these endocrine/metabolic transcription factors. The nuclear receptors are signal-dependent transcription factors that have two main purposes: (1) to locate target genes by binding at specific DNA sequences (termed hormone response elements [HREs]) that are located at these genes; and then, (2) to recruit transcriptional coregulators to the gene.<sup>16</sup> Ligands can induce both activation and repression of target genes. NRs recruit coactivators to activate genes, and corepressors to repress genes.<sup>17,18</sup> These two functionally different classes of molecules comprise the totality of 285-member coregulator superfamily, most of which are coactivators. The general

domain structure of coactivators is shown schematically in Figure 1, and a great deal of additional basic and clinical information is provided on the Nuclear Receptor Signaling Atlas Web site ([www.nursa.org](http://www.nursa.org)). Although the NR coregulators were identified only approximately 11 years ago,<sup>19</sup> they are generally accepted as the rate-limiting components of transcriptional control in mammals.

The molecular mechanisms by which distinct ligands can bind to the same nuclear receptor and yet exert tissue-specific actions, has been somewhat of a mystery until the last decade, when the contributions of basic receptor research have led to an enlightened viewpoint.<sup>13</sup> We now realize the complexities and the relative importance of the fundamental elements that factor into the equations for tissue-selective SRM actions. These elements are (1) receptor isoform subtypes; (2) ligand-induced conformations of the receptor; (3) precise sequence compositions of the HREs; (4) nuclear receptor coregulators (coactivators and corepressors), which are recruited by the active or inactive conformation of the receptor to the gene site; and (5) cell and signaling context. Although the coregulator recruitment is of paramount importance, under most conditions, all five of the preceding events can have a modulating influence on the actions of an SRM.



**Fig 1.** (A) The structure of the known estrogen receptors (ERs) with the identified (red) activating functions (AFs) that bind coactivators. Also identified is the DNA-binding domain (DBD) and the ligand binding domain. (B) A typical domain structure of a nuclear receptor (NR) coactivator is shown. There are two main domains: (1) a protein-protein interacting domain that binds other coactivators in the functional high molecular weight coactivator complex and (2) an enzymatic domain that either has intrinsic enzyme activity or binds a protein that has enzyme activity. Numerous enzyme activities have been demonstrated in the many coactivators discovered to date.

## RECEPTOR ISOFORMS

Multiple function-specific isoforms have been discovered for a number of receptors, including those for progesterone receptor (PR; PR $\alpha$ , PR $\beta$ ), ER (ER $\alpha$ , ER $\beta$ ), and GR (GR $\alpha$ , GR $\beta$ ).<sup>15</sup> These isoforms have different primary structures and therefore beget different gene functions. Since the tissue concentration of receptor isoforms can vary in a tissue-specific manner, the functions of the cognate receptor ligand in a given tissue can vary also. Perhaps the ER $\alpha$  and the ER $\beta$  isoforms have the most contradictory functions, with ER $\alpha$  having a growth promoting action and ER $\beta$  having a growth-inhibitory action in certain tissues.<sup>20</sup> Consequently, the tissue-selective ratio of ER $\alpha$ /ER $\beta$  can provide a tissue-selective function.

## LIGAND-INDUCED RECEPTOR CONFORMATION

For many years it was suspected that a transcription-inducing ligand acted simply by shifting the equilibrium of its cognate NR from an inactive to an active conformation. Two complimentary experimental approaches helped to clarify receptor-mediated modulations. A comprehensive pharmacologic evaluation of the structure function relationship of estrogens and antiestrogens both at an ER-regulated prolactin gene target<sup>21,22</sup> and by regulating breast cancer cell replication,<sup>23</sup> built up a hypothetical model of molecular modulation. The pharmacologic studies concluded the size and position of the "antiestrogenic" side chain of the then nonsteroidal antiestrogens controlled the folding of the ER at an antiestrogenic region of the ER.<sup>21,24,25</sup> Simply stated, the "crocodile" model proposed equilibrium mixtures of receptor jaws closed (estrogenic complex) or propped open by the ligand (partial estrogenic/antiestrogenic complex) to modulate gene function at target sites.<sup>26,27</sup> Complementary early biochemical studies utilized protease structural mapping and antibody epitope mapping techniques to demonstrate that progesterone and estrogen bound to their cognate receptors and induced a conformational alteration in the carboxy-terminal tail of the receptor, whereby the tail flipped back over the ligand pocket and the active form was stabilized.<sup>28,29</sup> It was the

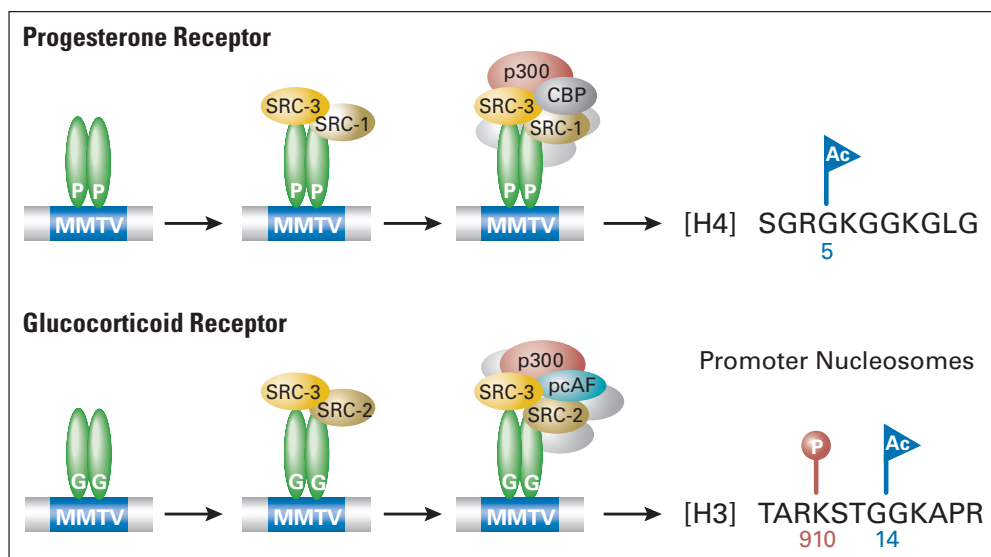
eventual x-ray crystallography of these molecules, however, that provided a more detailed picture of this model, whereby a c-terminal helix 12 was the lid that covered the ligand pocket and formed a landing platform for newly recruited coactivators (or corepressors).<sup>30-32</sup> The newly recruited coregulators then carry out all of the reactions required for the entire transcriptional process (discussed further herein). Different receptors binding to the same genetic sequence can recruit different coactivators and thereby provide quantitatively or qualitatively different gene responses (Fig 2). Similarly, different ligands occupying the same receptor at a gene site can induce different structural conformations in that receptor and lead to recruitment of different coactivators, and consequently, different gene expression patterns.

## DNA BINDING ELEMENT (HRE) OF THE TARGET GENE

The precise composition of different genomic HREs in mammals varies. HREs are usually composed of short inverted or direct repeats of approximately 7 deoxynucleotides each. When minor variations in the receptor contact sequence occur, and in combination with other surrounding transcription factors, the receptor can be forced into an altered conformation that in turn recruits different coregulators and provides distinct functions for these genes, if they are expressed in that tissue.<sup>33</sup> This basic principle has been demonstrated, but it is unclear as to how often this is a significant factor in SRM actions. What is clear is that recruitment of the receptor complex to the HRE is cyclical with binding and destruction.<sup>34</sup>

## NUCLEAR RECEPTOR COREGULATORS

Current opinions place the coregulators in the driving seat of tissue-specific actions of SRMs. The potency and selectivity for all subreactions of transcription reside in these coregulators, and thus, they are critically important for not only gene function, but also tissue-selective gene function. Currently there are approximately



**Fig 2.** Differential recruitment of coactivators to same gene by receptors determine specific promoter chromatin modifications and transcription initiations. The top panel contains a schematic of the progesterone receptor bound to the hormone response element (HRE) of the *MMTV* gene. The bottom panel demonstrates that when the glucocorticoid receptor is bound to the same HRE of the *MMTV*, it accumulates different coactivator proteins. Each of the receptors induce different patterns of histone modifications and subsequent transcription. The panels illustrate that the ligand-bound receptor itself plays the dominant role in what coactivators are recruited, and thus can modify target gene transcription accordingly. Ac, acetylation; P, phosphorylation.

285 NR coregulators, of which the vast majority are coactivators (approximately 40 are corepressor according to the Nuclear Receptor Signaling Atlas). Most occur in the majority of tissues, but at different individual concentrations in each tissue. Consequently, each tissue has a “quantitative finger print” of coactivators based on the relative concentrations of each molecule in that tissue.<sup>16</sup> This inherited complement of coregulators provides a basis for tissue-selective actions by a given NR.

Coregulators function as large, high-molecular weight complexes of approximately six to seven coregulator proteins.<sup>17</sup> Most of the coregulators are enzymes that participate in remodeling the local chromatin structure at the target promoter, initiating transcription by RNA polymerase, encouraging efficient elongation of RNA chain synthesis, regulating alternative RNA splicing, and, finally, destroying the active transcription factors at the promoter site. These series of substeps of transcription occur in rapid sequence (approximately 15 seconds apart) and are controlled by sequential occupation of the promoter by specific coregulator complexes that direct the transcriptional substep reactions.

For the most part, the coregulators are themselves regulated at the post-transcriptional level.<sup>17</sup> Their intracellular concentrations are determined by their proteasomal degradation rates. Levels are raised by inhibiting the rate of degradation, and vice versa for lowering levels. Traditional ubiquitin-mediated degradation occurs, as well as an ubiquitin-independent turnover by 11S cap proteins such as REGγ.<sup>35</sup> Degradation can be inhibited by post-translational modification of a coactivator at certain sites; alternatively, specific kinases can phosphorylate these sites to promote higher cellular levels of coactivator.

**CELL AND SIGNALING CONTEXT**

The cell context plays a role in selective gene responses to ligand because differentiation produces cells with specific available gene complements for expression. The cell also has a predetermined basal concentration of each of the coregulators and their cognate activating/inactivating enzymes, thereby establishing a threshold of available regulatory molecules. This cellular concentration of coregulators provides the potential for activity. For actual conversion to active functional molecules, however, the coregulators must be regulated by a variety of post-translational modifications, such as phosphorylation, ubiquitylation, acetylation, SUMOylation, methylation, etc. In general, coactivators are activated by phosphorylations and mono-ubiquitylations.<sup>36</sup> Protein-protein interactions in the large coactivator complexes are regulated by acetylations and methylations. Coactivators are inactivated by SUMOylation and degraded after poly-ubiquitylations. These general rules often vary for a given coactivator. Considering the crucial role that post-translational modifications play in coactivator function, it is logical to assume that the roles of signaling pathways that contain these modifying enzymes also play important roles. Since the signaling pathways have certain cell specificities and are subject to environmental stimuli for their regulation, cell context can play a role in selective activities of SRMs.

**OTHER REGULATORY INFLUENCES**

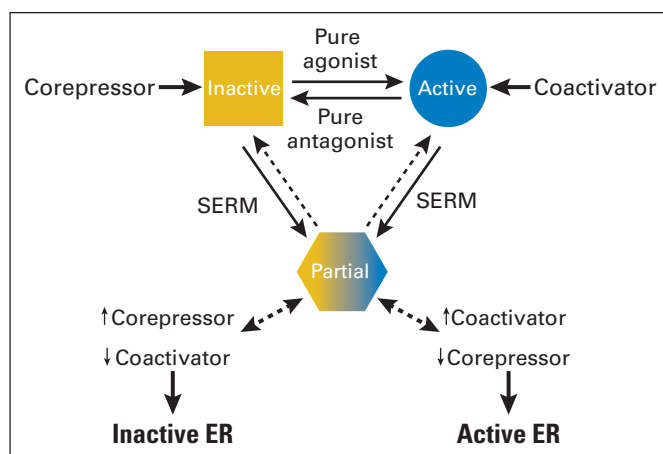
Because equilibrium reactions are the basis for biology, the promotional and contradictory influences inherent to the cell can affect

coregulator function and transcriptional potency. As discussed above, coregulator concentrations are subject to turnover by ubiquitin-dependent and ubiquitin-independent proteasomal degradation pathways, whose activities can be abrogated by certain counteracting kinases. Therefore the cell concentrations and activation of degradation pathways for coregulators can play a role in SRM actions. In addition, in vivo systemic metabolism and selective cellular uptake or metabolism of ligands can sometimes modify SRM activities.

**WHAT ARE THE MOST IMPORTANT FACTORS FOR TISSUE-SPECIFIC SRM ACTIVITY?**

The cell levels of activated coregulators are the primary determinant of tissue-specific SRM activity.<sup>13</sup> Having described herein the complete interacting equations and complexities of coregulator function, it remains that (1) the cellular complement of coregulators and (2) the cell and signaling context are the primary determinants of coregulator function. Consequently, they are the primary determinants of SRM functions.

SRMs are generally mixed antagonist/agonist ligands for receptors. When a receptor is occupied by a mixed antagonist/agonist ligand, the conformation generated in the receptor is neither purely antagonistic nor purely agonistic for activity. Rather, the conformation is intermediate for both functions (Fig 3). A pure agonist induces a receptor conformation that has a strong affinity for coactivators. A pure antagonist induces a receptor conformation that has a strong affinity for corepressors. The mixed antagonist/agonist ligand induces an intermediate conformation that, in turn, is intermediate in its affinity for both coactivators and corepressors. In other words, this receptor conformation is programmed by the local concentrations of activated coactivators and corepressors. The mechanism will obey the laws of physical chemistry. If the cellular concentration of preferred



**Fig 3.** Hypothesis for tissue specific effects of selective estrogen-receptor modulators (SERMs). A schematic is shown for the contributions of coactivators and corepressors to the tissue-specific antagonist/agonist activities of a steroid receptor modulator (SRM). In the presence of a pure antagonist, a receptor is stabilized in the inactive conformational state and binds corepressor tightly. In the presence of a pure agonist, it conforms to a fully active conformation and binds coactivator tightly. In the presence of a mixed antagonist/agonist SRM, the receptor adopts an intermediate partial conformation that is neither fully inactive or fully active. In this intermediate conformation, the SRM-bound receptor is then even more subject to interactions with the relative intracellular concentrations of preferred coactivators or corepressors for its activity.

coactivators is high (or corepressors low), then the receptor is forced into the active conformation by the excess of coactivators and receptor dependent gene expression takes place. If the cellular concentration of preferred corepressors is high (or coactivators low), then the receptor is forced into the inactive conformation by the excess of corepressors and receptor-dependent gene expression is shut down. Since activation of coregulators occurs by post-translational modifications, the status of the cell signaling pathways that produces these post-translational modifications is an overlying modulator of SRM activity.

With this background of the physiologic basis for SERM action, it is now appropriate to meld these emerging data with the current applications of SERMs in the clinic and the evolving ideas about drug resistance to SERMs.

### CURRENT THERAPY WITH SERMs

The clinical application<sup>5</sup> of the laboratory strategy of long-term anti-hormonal therapy<sup>37-39</sup> as an adjuvant to treat breast cancer has now become the standard of care. Two approaches to antihormonal therapy have occurred during the last three decades: long-term treatment to block estrogen-stimulated growth at the level of the tumor ER<sup>39</sup> and, subsequently, the use of aromatase inhibitors to block estrogen biosynthesis in postmenopausal patients.<sup>6</sup> It is clear that the aromatase inhibitors offer advantages over tamoxifen as adjuvant treatments for postmenopausal patients; there are fewer adverse effects (blood clots and endometrial cancer), and aromatase inhibitors have a small but significant improved efficacy.<sup>40,41</sup> However, substantial numbers of postmenopausal patients continue to receive tamoxifen treatment either for economic reasons or because they are hysterectomized and at low risk for blood clots (low body mass index or they are athletically active). Postmenopausal women who have completed 2 to 5 years of adjuvant tamoxifen are also eligible for a further 5 years of antihormonal therapy with an aromatase inhibitor.<sup>42-44</sup> However, the veteran SERM tamoxifen is still the antihormonal treatment of choice for premenopausal patients and the antihormonal treatment for ductal carcinoma in situ (DCIS),<sup>45</sup> and remains the appropriate treatment to reduce breast cancer risk in premenopausal women at elevated risk.<sup>46</sup> It is important to stress that premenopausal women treated with tamoxifen do not experience elevations in endometrial cancer and blood clots, so the risk:benefit ratio is strongly in favor of tamoxifen treatment.<sup>47</sup>

The development of raloxifene<sup>48</sup> has created a new therapeutic dimension. Raloxifene is used either as a treatment and preventive for osteoporosis but with a quantifiable decrease in the incidence of breast cancer,<sup>49,50</sup> or as an agent for the reduction of breast cancer incidence in high-risk postmenopausal women.<sup>51</sup> The advantage of raloxifene as a SERM is that there are no increases in endometrial cancer<sup>51,52</sup> incidence previously noted with tamoxifen in postmenopausal women.<sup>46,53</sup>

The target site-specific actions of tamoxifen and raloxifene in breast and endometrial cancer were first noted in the laboratory,<sup>54,55</sup> but the question to be asked is why. On the basis of our earlier arguments about the mechanism of actions of SERMs, studies of the cellular context and coactivator content demonstrate the tissue-specific actions of tamoxifen and raloxifene in the uterine cancer cell.<sup>56</sup>

Overall, the SERM concept<sup>10,11</sup> clearly works in clinical practice, but the use of long-term SERM treatment regimens raises the important issue of the eventual development of drug resistance.

Laboratory studies have already shown that long-term SERM treatment changes the pharmacology from an antiestrogen- to SERM-stimulated growth.<sup>57,58</sup> This acquired resistance is a topic of immediate clinical concern.

### THE DIMENSION OF DRUG RESISTANCE TO SERMs

There are currently three possible mechanisms for drug resistance to tamoxifen. Either the patient can influence the effectiveness of tamoxifen via alterations in metabolism, or the ER-positive tumor is or can become refractory to treatment. These mechanisms are illustrated in Figure 4.

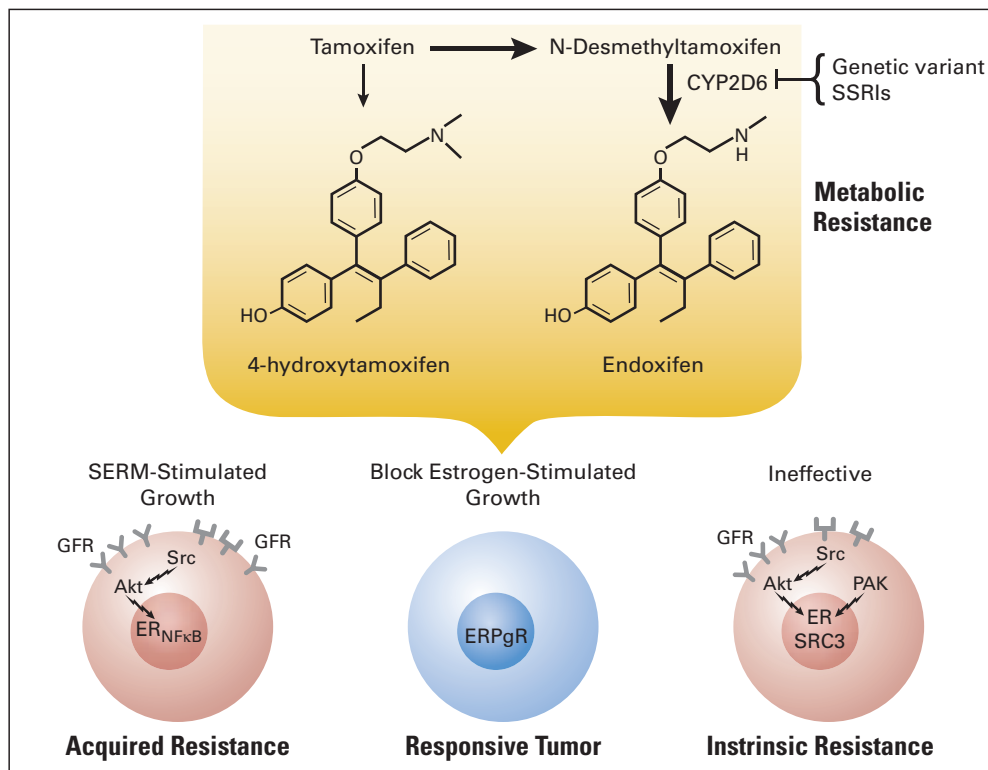
F4

#### Metabolic Resistance

The metabolic activation of tamoxifen occurs via demethylation to *N*-desmethyltamoxifen and subsequently transformation to the hydroxy metabolite endoxifen.<sup>59,60</sup> This topic has recently been reviewed<sup>61</sup> and will therefore be mentioned only briefly. Metabolic activation appears to be important for tamoxifen to acquire potent antiestrogenic and antitumor activity. Although large-scale prospective clinical trials have not been completed to prove the hypothesis definitively in large populations, there is sufficient preliminary data to warrant further study. Extensive laboratory studies demonstrate<sup>62</sup> that endoxifen is formed by the CYP2D6 enzyme system. However, there are wide variations in the CYP2D6 enzyme in the population that can influence drug metabolism. The wild-type CYP2D6 enzyme is referred to as CYP2D61\*, whereas CYP2D64\*/4\* is a null variant. It is estimated that approximately 10% of the population have CYP2D6 variants, so the case can be made that these patients should be considered for other antiestrogenic interventions (eg, aromatase inhibitors). Another dimension for consideration is the control of menopausal symptoms, especially hot flashes. If tamoxifen is a prodrug and needs to be converted to endoxifen to achieve maximal antitumor activity at the tumor ER, then these same patients may have severe hot flashes. The selective serotonin reuptake inhibitors (SSRIs) have been found to be of value to treat hot flashes. The widespread use of tamoxifen as a long-term adjuvant therapy, especially in premenopausal patients, has naturally increased SSRI use. Unfortunately, the SSRIs such as fluoxetine and paroxetine are potent inhibitors of the CYP2D6 enzyme.<sup>63</sup> Therefore, symptom treatment has the potential to undermine the efficacy of tamoxifen if the incorrect SSRI is employed. Venlafaxine has a very low affinity for the CYP2D6 enzyme system and may be the agent of choice for treatment of hot flashes.<sup>63</sup> It should, however, be pointed out that there is no substantial clinical evidence to support this conclusion. A larger body of prospective clinical data is required to confirm the admittedly compelling preliminary studies.

#### Intrinsic Resistance

A proportion of ER-positive tumors are intrinsically resistance to tamoxifen therapy. Historically, metastatic breast cancer that is ER and PR positive is approximately 80% responsive to antihormonal therapy (endocrine ablation or tamoxifen) whereas tumors that are ER positive but PR negative are only 40% responsive to antihormonal therapy.<sup>64,65</sup> We have known for about 20 years that enhanced growth factor signaling via the human epidermal growth factor receptor 1 (HER-1; EGFR) pathway improves estrogen to induce PR in breast cancer cells<sup>66</sup> and enhanced paracrine growth factor stimulation undermines that effectiveness of antiestrogen treatment at the ER.<sup>67,68</sup>



**Fig 4.** The possible types of drug resistance to the selective estrogen-receptor modulators (SERMs), particularly tamoxifen. Tamoxifen is a prodrug that needs to be metabolically activated by CYP2D6 to the active antiestrogen endoxifen. Mutation of CYP2D6 or the administration of specific selective serotonin reuptake inhibitors (SSRIs; eg, paroxetine or fluoxetine) to reduce hot flashes impairs metabolic activation and reduces the efficacy to tamoxifen. Tamoxifen and its hydroxylated metabolites are most effective at blocking estrogen-stimulated tumor growth if the cells contain both estrogen receptor (ER) and progesterone receptor (PgR). In contrast, tamoxifen is much less effective in controlling the growth of tumors, have high levels of membrane growth factor receptors (GFRs) that can activate phosphorylation cascades via Src, Akt and PAK. The ER and coactivator SRC3 could be targets for phosphorylation in these PgR-negative tumors. The tumor has intrinsic resistance to tamoxifen treatment. In contrast, tumors that initially respond to tamoxifen can acquire resistance to tamoxifen by increasing the level of GFR that phosphorylates Src and Akt. These SERM (tamoxifen)-stimulated tumors have increased nuclear levels of nuclear factor  $\kappa$  B (NF $\kappa$ B) but the tumors still rely on the ER for survival as second line treatments with either aromatase inhibitors (to block local estrogen production) or fulvestrant (to block the ER and cause preventative destruction) can result in the control of tumor growth.

These earlier observations have recently been confirmed and extended using breast cancer cells artificially transfected with insulin-like growth factor receptor<sup>69</sup> and using large tumor databases.<sup>70</sup> Tumor cell drug resistance to tamoxifen develops very quickly (8 weeks) in athymic mice with HER-2/*neu* engineered MCF-7 cells<sup>71</sup> compared with the natural process of more than 6 months.<sup>57</sup> Tamoxifen acts as an agonist in experimentally engineered breast cancer cells with high levels of the HER-2/*neu* growth factor receptor and the coactivator SRC3 (AIB1).<sup>72</sup>

In another approach, the possible connection between HER-2/*neu*, ER, PR and tamoxifen resistance has been evaluated in a tissue database linked to clinical outcomes. Intrinsic tamoxifen resistance is associated with HER-2/*neu*<sup>-</sup>, ER-positive, PR-negative tumors that have an increase in coactivator SRC3 (AIB1) levels.<sup>73</sup> Although the actual number is a small group of approximately 10% to 15% breast cancer patients, it does perhaps provide a clue to test who should avoid tamoxifen treatment.

The idea that growth factor receptor could be a predictor of SERM resistance has recently<sup>74,75</sup> been extrapolated to explain the reason for aromatase inhibitors being superior to tamoxifen as adjuvant therapy. A retrospective analysis<sup>76</sup> shows that patients with ER-positive, PR-negative tumors are more likely to respond to aromatase inhibitors than to tamoxifen. However, the conclusions, though attractive, require confirmation with prospective studies because of inconsistencies with the results from other direct trial databases comparing tamoxifen with an aromatase inhibitor and the recent reevaluation of the steroid receptor database in the original study of tamoxifen and anastrozole.<sup>77</sup>

### Acquired Resistance

Laboratory studies show that the treatment of athymic mice implanted with ER-positive, PR-positive MCF-7 tumors with contin-

uous tamoxifen will eventually develop tamoxifen-stimulated tumors that will grow in response to either tamoxifen or estradiol.<sup>57</sup> Either no treatment or treatment with the pure antiestrogen fulvestrant<sup>57,78,79</sup> results in no tumor growth. Because no treatment in the ovariectomized athymic mouse is equivalent to treatment with an aromatase inhibitor and fulvestrant destroys the ER,<sup>80</sup> one could conclude that tumor growth is prevented in the absence of a stimulatory signal transduction pathway. This hypothesis is consistent with the clinical observation that anastrozole and fulvestrant treatment are equivalent after the failure of tamoxifen therapy.<sup>81,82</sup>

Goss et al<sup>42</sup> demonstrated that patients with ER-positive tumors and treated for 5 years with tamoxifen continue to be responsive to subsequent treatment with 5 years of the aromatase inhibitor letrozole.<sup>83</sup> This result could be interpreted as the slow development of acquired resistance by the breast cancer micrometastases during 5 years of tamoxifen so that these patients respond to a non-cross-resistant therapy that prevents tumor growth by blocking the ability of the patient to synthesize estrogen. Thus, the use of letrozole after tamoxifen is incrementally building on the already established long-term antitumor effect of tamoxifen that lasts for at least 10 years after the cessation of adjuvant therapy.<sup>5</sup>

### CONSEQUENCES OF LONG-TERM ANTIHORMONE THERAPY

Laboratory models of drug resistance should replicate the duration of SERM administration to patients. Most laboratory models of antihormone resistance are either engineered with stable transfection of the HER-2/*neu* gene into MCF-7 cells<sup>72,84</sup> or reflect the early development of resistance (SERM-stimulated growth)<sup>57</sup> to treatment. This later form of resistance is consistent with tamoxifen failure during the

treatment of metastatic disease. Under these clinical circumstances, tamoxifen treatment is effective for approximately 1 year. This form of SERM resistance is referred to as phase I.<sup>85</sup> However, tamoxifen is used as an adjuvant therapy for 5 years,<sup>86</sup> and it is reasonable to suggest that raloxifene will need to be administered for 10 years or more to maintain effectiveness as an antiosteoporosis medicine. Current studies<sup>49</sup> show that up to 8 years of raloxifene reduces the majority of (65%) but not all ER-positive breast cancers. Some tumors must, therefore, become raloxifene resistant.

The repeated transplantation of MCF-7 breast tumors into successive generations of tamoxifen-treated ovariectomized athymic mice for more than 5 years replicates the exposure of tumor cells to adjuvant tamoxifen. This approach to study SERM resistance results in a continuing dependence on tamoxifen to produce growth, but cross-resistance with the SERMs toremifene and raloxifene develops<sup>79,87,88</sup> and a significant change in the response of tamoxifen or raloxifene resistant cells to physiologic estradiol.<sup>87,89,90</sup> The signaling pathways for estrogen no longer support growth, but initiate apoptosis by inducing fas receptor, rapidly reducing levels of HER-2/*neu* and reducing nuclear factor  $\kappa$  B (NF $\kappa$ B) levels.<sup>91</sup> This form of SERM resistance is referred to as phase II resistance.<sup>85</sup> As might be expected, the pure antiestrogen fulvestrant can completely prevent tumor growth in animals. Paradoxically, when combined with physiological estrogen, fulvestrant not only reverses the apoptotic actions of estrogen but also causes robust tumor growth.<sup>91</sup> The mechanism for this therapeutically relevant observation is unclear, but may involve a dramatic upregulation of HER-2 and HER-3<sup>92</sup> but may also involve the recently described ligand (estrogen, SERM, fulvestrant) activator G protein GPR30.<sup>93</sup> It is possible that this novel observation may have value to plan an appropriate strategy to use fulvestrant plus an aromatase inhibitor as a third-line endocrine therapy.<sup>94</sup> The widespread clinical use of aromatase inhibitors now brings up the question of the consequences of the long-term use of aromatase inhibitors as adjuvant therapies. There will be an eventual development of drug resistance.

Early studies of estrogen deprivation in cell culture demonstrated that cellular ER levels and spontaneous cell replication increase.<sup>95,96</sup> Subsequent studies demonstrated that the cells initially become supersensitized to the growth properties of minute quantities of estrogen,<sup>97,98</sup> but as the duration of estrogen deprivation is extended, the cells respond to estrogen with the initiation of apoptosis.<sup>99</sup> This observation<sup>99</sup> has been used to explain the earlier application of high-dose estrogen therapy to treat postmenopausal women with metastatic breast cancer.<sup>100</sup> However, estrogen-deprived cell lines only need very low concentrations of estrogen in the postmenopausal range (lnM) to initiate apoptosis.<sup>101,102</sup> Cell death occurs through an increase in proapoptotic genes<sup>103</sup> and can be enhanced by specifically reducing the synthesis of bcl-2.<sup>104</sup> These preclinical studies are being translated to clinical trials by destroying phase II antihormone-resistant breast cancer cells with limited low-dose estrogen therapy followed by maintenance with further treatment with an aromatase inhibitor treatment.<sup>103</sup>

An alternate approach to study the development of drug resistance to aromatase inhibitors in vivo utilizes ER-positive MCF-7 breast cancer cells stably transfected with the CYP19 aromatase enzyme gene.<sup>105</sup> The cells grow into tumors in athymic mice treated with the enzyme substrate androstenedione that is converted to estrone.<sup>106</sup> The model has been used effectively to examine the integration of

SERM and aromatase inhibitor therapy and has effectively replicated the clinical experience.<sup>107-110</sup> Results not only clearly demonstrate the efficacy of aromatase inhibitor when compared with tamoxifen but also demonstrate the development of resistance to aromatase inhibitors.<sup>111</sup> Aromatase resistant tumors become more dependent on growth factor receptor pathways via mitogen-activated protein kinase.<sup>112,113</sup>

Overall, the basic knowledge of SERM action and the development of laboratory models of antihormonal resistance are proving invaluable to identify molecular targets for future advances in cancer therapeutics. Important clues about the pivotal role of SRCs in SERM drug resistance and tumor cell survival are already apparent. We predict that further progress in cancer cell biology will occur through an enhanced investment to understand the modulatory mechanisms of NRs and their coactivator partners. The new knowledge will create unanticipated opportunities to control cancer in the future.

### FUTURE POTENTIAL FOR NEW SRM DEVELOPMENT

With the advent of this recent knowledge of the molecular mechanisms of action of transcriptional regulators such as NRs and coregulators, new insights to drug development are rapidly becoming available. The discovery of tamoxifen as a SERM and the successful development of additional SERMs such as raloxifene, have encouraged exploitation of the SERM concept<sup>10,11</sup> by pharmaceutical companies to discover additional new SRM ligands for other NRs. Some examples are selective progestin modulators (SPRMs)<sup>114,115</sup> that inhibit uterine cancer but are devoid of stimulatory action in the breast; selective androgen receptor modulators (SARMs)<sup>116,117</sup> that are anabolic for muscle and bone, but spare the prostate; selective glucocorticoid receptor modulators (SGRMs)<sup>118</sup> that are strongly anti-inflammatory but do not induce glucose intolerance and connective tissue destruction; and selective peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) receptor modulators (SPARMs)<sup>119-121</sup> that promote insulin sensitivity.<sup>13</sup> All of the foregoing examples are under current development or are being tested in clinical trials. In the case of each of these SRMs, the molecular mechanisms and pathways for their efficacy described herein represent the guiding principles for their tissue-specific actions and represent a substantial health care return for the investment in basic mechanistic scientific research.

### AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

### AUTHOR CONTRIBUTIONS

**Conception and design:** V. Craig Jordan, Bert W. O'Malley  
**Administrative support:** V. Craig Jordan  
**Provision of study materials or patients:** Bert W. O'Malley  
**Collection and assembly of data:** V. Craig Jordan, Bert W. O'Malley  
**Data analysis and interpretation:** V. Craig Jordan, Bert W. O'Malley  
**Manuscript writing:** V. Craig Jordan, Bert W. O'Malley  
**Final approval of manuscript:** V. Craig Jordan, Bert W. O'Malley

REFERENCES

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

2. McGuire WL, Carbone PP, Sears ME, et al: Estrogen receptors in human breast cancer: An overview, in McGuire WL, Carbone PP, Volmer EP (eds): *Estrogen Receptor in Human Breast Cancer*. New York, NY, Raven Press, 1975, pp 1-7

3. Harper MJ, Walpole AL: A new derivative of triphenylethylene: Effect on implantation and mode of action in rats. *J Reprod Fertil* 13:101-119, 1967

4. Jordan VC: Tamoxifen: A most unlikely pioneering medicine. *Nat Rev Drug Discov* 2:205-213, 2003

5. EBCTCG: 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, 2005

6. Jordan VC, Brodie AMH: Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* 72:7-25, 2007

7. Jordan VC: Selective estrogen receptor modulation: A personal perspective. *Cancer Res* 61:5683-5687, 2001

8. Jordan VC, Phelps E, Lindgren JU: Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 10:31-35, 1987

9. Gottardis MM, Jordan VC: Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* 47:4020-4024, 1987

10. Jordan VC: Chemosuppression of breast cancer with tamoxifen: Laboratory evidence and future clinical investigations. *Cancer Invest* 6:589-595, 1988

11. Lerner LJ, Jordan VC: The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res* 50:4177-4189, 1990

12. 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* 281:2189-2197, 1999

13. Smith CL, O'Malley BW: Coregulator function: A key to understanding tissue specificity of selective receptor modulators. *Endocr Rev* 25:45-71, 2004

14. Mangelsdorf DJ, Thummel C, Beato M, et al: The nuclear receptor superfamily: The second decade. *Cell* 83:835-839, 1995

15. Tsai MJ, O'Malley BW: Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 63:451-486, 1994

16. McKenna NJ, O'Malley BW: Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* 108:465-474, 2002

17. Lonard DM, O'Malley BW: The expanding cosmos of nuclear receptor coactivators. *Cell* 125:411-414, 2006

18. Glass CK, Rosenfeld CS: The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* 14:121-141, 2000

19. Oñate SA, Tsai SY, Tsai MJ, et al: Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* 270:1354-1357, 1995

20. Weihua Z, Andersson S, Cheng G, et al: Update on estrogen signaling. *FEBS Lett* 546:17-24, 2003

21. Lieberman ME, Gorski J, Jordan VC: An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. *J Biol Chem* 258:4741-4745, 1983

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

23. Murphy CS, Langan-Fahey SM, McCague R, et al: 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, 1990

24. Wolf DM, Jordan VC: 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, 1994

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

26. Jordan VC, Koch R, Mittal S, et al: Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: Modulation of prolactin synthesis in vitro. *Br J Pharmacol* 87:217-223, 1986

27. Jordan VC: Laboratory models of breast cancer to aid the elucidation of antiestrogen action. *J Lab Clin Med* 109:267-277, 1987

28. Allan GF, Leng X, Tsai SY, et al: Hormone and antihormone induce distinct conformational changes which are central to steroid receptor activation. *J Biol Chem* 267:19513-19520, 1992

29. Vegeto E, Allan GF, Schrader WT, et al: The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. *Cell* 69:703-713, 1992

30. Brzozowski AM, Pike AC, Dauter Z, et al: Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 389:753-758, 1997

31. Shiau AK, Barstad D, Loria PM, et al: The structural basis of estrogen receptor/co-activator recognition and the antagonism of this interaction by tamoxifen. *Cell* 95:927-937, 1998

32. Togashi M, Borngraeber S, Sandler B, et al: Conformational adaptation of nuclear receptor ligand binding domains to agonists: Potential for novel approaches to ligand design. *J Steroid Biochem Mol Biol* 93:127-137, 2005

33. Lefstin JA, Yamamoto KR: Allosteric effects of DNA on transcriptional regulators. *Nature* 392:885-888, 1998

34. Shang Y, Hu X, DiRenzo J, et al: Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* 103:843-852, 2000

35. Li X, Lonard DM, Jung SY, et al: The SRC-3/AIB1 coactivator is degraded in a ubiquitin- and ATP-independent manner by the REGgamma proteasome. *Cell* 124:381-392, 2006

36. O'Malley BW: Little molecules with big goals. *Science* 313:1749-1750, 2006

37. Jordan VC, Allen KE: Evaluation of the anti-tumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 16:239-251, 1980

38. Jordan VC, Allen KE, Dix CJ: Pharmacology of tamoxifen in laboratory animals. *Cancer Treat Rep* 64:745-759, 1980

39. Jensen EV, Jordan VC: The estrogen receptor: A model for molecular medicine: The Dorothy P Landon AACR Prize for Translational Research. *Clin Cancer Res* 9:1980-1989, 2003

40. Howell A, Cuzick J, Baum M, et al: 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, 2005

41. Thürlimann B, Keshaviah A, Coates AS, et al: A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med* 353:2747-2757, 2005

42. Goss PE, Ingle JN, Martino S, et al: Randomized trial of letrozole versus tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: Updated findings from NCIC CTG MA.17. *J Natl Cancer Inst* 97:1262-1271, 2005

43. Boccardo F, Rubagotti A, Puntoni M, et al: Switching to anastrozole versus continued tamoxifen treatment for early breast cancer: Preliminary results of the Italian Tamoxifen Anastrozole Trial. *J Clin Oncol* 23:5138-5147, 2005

44. Coombes RC, Hall E, Gibson LJ, et al: 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, 2004

45. Fisher B, Dignam J, Wolmark N, et al: Tamoxifen in treatment of intraductal breast cancer: National Surgical Adjuvant Breast and Bowel Project B-24 randomised controlled trial. *Lancet* 353:1993-2000, 1999

46. 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* 90:1371-1388, 1998

47. Gail MH, Costantino JP, Bryant J, et al: Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J Natl Cancer Inst* 91:1829-1846, 1999

48. Lewis JS, Jordan VC: Case histories: Raloxifene, in Taylor J, Triggler D (eds): *Comprehensive Medicinal Chemistry II, Volume 8*. Oxford, United Kingdom, Elsevier Limited, 2007, pp 103-121

49. Martino S, Cauley JA, Barrett-Connor E, et al: For the CORE Investigators 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, 2004

50. Jordan VC: Optimising endocrine approaches for the chemoprevention of breast cancer: Beyond the Study of Tamoxifen and Raloxifene (STAR) trial. *Eur J Cancer* 42:2909-2913, 2006

51. Vogel VG, Costantino JP, Wickerham DL, et al: The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA* 295:2727-2741, 2006

52. 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* 355:125-137, 2006

53. 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* 97:1652-1662, 2005

54. Gottardis MM, Robinson SP, Satyaswaroop PG, et al: Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 48:812-815, 1988

55. Gottardis MM, Ricchio ME, Satyaswaroop PG, et al: Effect of steroidal and nonsteroidal antiestrogens

SERMs and Antihormonal Resistance in Breast Cancer

on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. *Cancer Res* 50:3189-3192, 1990

56. Shang Y, Brown M: Molecular determinants for the tissue specificity of SERMs. *Science* 295: 2465-2468, 2002

57. Gottardis MM, Jordan VC: Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 48:5183-8187, 1988

58. O'Regan RM, Osipo C, Ariazi E, et al: Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. *Clin Cancer Res* 12:2255-2263, 2006

59. 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* 95:1758-1764, 2003

60. Jin Y, Desta Z, Stearns V, et al: CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. *JNCI* 97:30-39, 2005

61. Jordan VC: New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer. *Steroids* (in press)

62. Desta Z, Ward BA, Soukhova NV, et al: 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, 2004

63. 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* 80:61-74, 2006

64. Bloom ND, Robin EH, Schreiber B, et al: The role of progesterone receptor in the management of advanced breast cancer. *Cancer* 45:2992-2997, 1980

65. Osborne CK, Yochmowitz MG, Knight WA, et al: The value of estrogen and progesterone receptors in the treatment of breast cancer. *Cancer* 46: 2884-2888, 1980

66. Cormier EM, Wolf MF, Jordan VC: Decrease in estradiol-stimulated progesterone receptor production in MCF-7 cells by epidermal growth factor and possible clinical implication for paracrine-regulated breast cancer growth. *Cancer Res* 49:576-580, 1989

67. Cormier EM, Jordan VC: Contrasting ability of antiestrogens to inhibit MCF-7 growth stimulated by estradiol or epidermal growth factor. *Eur J Cancer Clin Oncol* 25:57-63, 1989

68. Robinson SP, Jordan VC: The paracrine stimulation of MCF-7 cells by MDA-MB-231 cells: Possible role in antiestrogen failure. *Eur J Cancer Clin Oncol* 25:493-497, 1989

69. Cui X, Zhang P, Deng W, et al: Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: Progesterone receptor as a potential indicator of growth factor activity in breast cancer. *Mol Endocrinol* 17:575-588, 2003

70. Arpino G, Weiss H, Lee AV, et al: Estrogen receptor-positive, progesterone receptor-negative breast cancer: Association with growth factor receptor expression and tamoxifen resistance. *J Natl Cancer Inst* 97:1254-1261, 2005

71. Benz CC, Scott GK, Sarup JC, et al: Estrogen-dependent, tamoxifen-resistant tumorigenic growth

of MCF-7 cells transfected with HER2/neu. *Breast Cancer Res Treat* 24:85-95, 1992

72. 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* 96:926-935, 2004

73. 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* 95:353-361, 2003

74. Dowsett M, Harper-Wynne C, Boeddinghaus I, et al: HER-2 amplification impedes the antiproliferative effects of hormone therapy in estrogen receptor-positive primary breast cancer. *Cancer Res* 61:8452-8458, 2001

75. Ellis MJ, Coop A, Singh B, et al: Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: Evidence from a phase III randomized trial. *J Clin Oncol* 19:3808-3816, 2001

76. Dowsett M, Cuzick J, Wale C, et al: Retrospective analysis of time to recurrence in the ATAC trial according to hormone receptor status: An hypothesis-generating study. *J Clin Oncol* 23:7512-7517, 2005

77. Jordan VC, Gelber R: Problems with the progesterone receptor in practice? *J Clin Oncol* 25:1957-1959, 2007

78. Osborne CK, Coronado-Heinsohn EB, Hilsenbeck SG, et al: Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. *J Natl Cancer Inst* 87:746-750, 1995

79. Lee ES, MacGregor-Schafer JI, Yao K, et al: Cross resistance of triphenylethylene-type antiestrogen but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 6:4893-4899, 2000

80. 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* 276:35684-35692, 2001

81. Osborne CK, Pippen J, Jones SE, et al: 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, 2002

82. Howell A, Robertson JFR, Quaresma Albano J, et al: 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, 2002

83. Goss PE, Ingle JN, Martino S, et al: Efficacy of letrozole extended adjuvant therapy according to estrogen receptor and progesterone receptor status on the primary tumor. *J Clin Oncol* 25:2006-2011, 2007

84. Massarweh S, Osborne CK, Jiang S, et al: Mechanisms of tumor regression and resistance to estrogen deprivation and fulvestrant in a model of estrogen receptor-positive, HER-2/neu-positive breast cancer. *Cancer Res* 66:8266-8273, 2006

85. Jordan VC: Selective estrogen receptor modulation: Concept and consequences in cancer. *Cancer Cell* 5:207-213, 2004

86. EBCTCG: Tamoxifen for early breast cancer: An overview of the randomised trials. *Lancet* 354: 1451-1467, 1998

87. Wolf DM, Jordan VC: A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy, in *Recent Results in Cancer Research*. Heidelberg, Germany, Springer-Verlag, 1993, pp 23-33

88. O'Regan RM, Gajdos C, Dardes RC, et al: Effects of raloxifene after tamoxifen on breast and endometrial tumor growth in athymic mice. *J Natl Cancer Inst* 94:274-283, 2002

89. Yao K, Lee ES, Bentrem DJ, et al: Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 6:2028-2036, 2000

90. Liu H, Lee ES, Gajdos C, et al: Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. *J Natl Cancer Inst* 95:1586-1597, 2003

91. Osipo C, Gajdos C, Liu H, et al: Paradoxical action of fulvestrant on estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* 95:1597-1608, 2003

92. Osipo C, Meeke K, Cheng D, et al: Role for HER2/neu and HER3 in fulvestrant-resistant breast cancer. *Int J Oncol* 30:509-520, 2007

93. Prossnitz ER, Arterburn JB, Sklar LA: GPR30: A G protein-coupled receptor for estrogen. *Mol Cell Endocrinol* 265-266:138-142, 2007

94. Jordan VC, Lewis JS, Osipo C, et al: The apoptotic action of estrogen following exhaustive anti-hormonal therapy: A new clinical treatment strategy. *Breast* 14:624-630, 2005

95. Katzenellenbogen BS, Kendra KL, Norman MJ, et al: 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 Res* 47: 4355-4360, 1987

96. Welshons WV, Jordan VC: Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *Eur J Cancer* 23:1935-1939, 1987

97. Berstein LM, Wang JP, Zheng H, et al: Long-term exposure to tamoxifen induces hypersensitivity to estradiol. *Clin Cancer Res* 10:1530-1534, 2004

98. Shim WS, Conaway M, Masamura S, et al: Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. *Endocrinology* 141: 396-405, 2000

99. Song RX, Mor G, Naftolin F, et al: Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *J Natl Cancer Inst* 93:1714-1723, 2001

100. Haddow A, Watkinson JM, Paterson E: Influence of synthetic oestrogens upon advanced malignant disease. *BMJ* 2:393-398, 1944

101. Lewis JS, Osipo C, Meeke K, et al: Estrogen induced apoptosis in breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 94:131-141, 2005

102. Lewis JS, Meeke K, Osipo C, et al: Intracellular mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *JNCI* 97:1746-1759, 2005

103. Jordan VC, Lewis-Wambi J, Kim H, et al: Exploiting the apoptotic actions of oestrogen to reverse anti-hormonal drug resistance in oestrogen receptor positive breast cancer patients. *Breast* (in press)

104. Song RX, Zhang Z, Mor G, et al: Down-regulation of Bcl-2 enhances estrogen apoptotic action in long-term estradiol-depleted ER+ breast cancer cells. *Apoptosis* 10:667-678, 2005

AQ: J

- 105.** Zhou DJ, Pompon D, Chen SA: Stable expression of human aromatase complementary DNA in mammalian cells: A useful system for aromatase inhibitor screening. *Cancer Res* 50:6949-6954, 1990
- 106.** Brodie AH, Jelovac D, Long B: The intratumoral aromatase model: Studies with aromatase inhibitors and antiestrogens. *J Steroid Biochem Mol Biol* 86:283-288, 2003
- 107.** Brodie AH, Mouridsen HT: Applicability of the intratumor aromatase preclinical model to predict clinical trial results with endocrine therapy. *Am J Clin Oncol* 26:S17-26, 2003
- 108.** Jelovac D, Macedo L, Handratta V, et al: Effects of exemestane and tamoxifen in a postmenopausal breast cancer model. *Clin Cancer Res* 10:7375-7381, 2004
- 109.** Long BJ, Jelovac D, Handratta V, et al: Therapeutic strategies using the aromatase inhibitor letrozole and tamoxifen in a breast cancer model. *J Natl Cancer Inst* 96:456-465, 2004
- 110.** Jelovac D, Macedo L, Goloubeva OG, et al: Additive antitumor effect of aromatase inhibitor letrozole and antiestrogen fulvestrant in a postmenopausal breast cancer model. *Cancer Res* 65:5439-5444, 2005
- 111.** Long BJ, Jelovac D, Thiantanawat A, et al: The effect of second-line antiestrogen therapy on breast tumor growth after first-line treatment with the aromatase inhibitor letrozole: Long-term studies using the intratumoral aromatase postmenopausal breast cancer model. *Clin Cancer Res* 8:2378-2388, 2002
- 112.** Jelovac D, Sabnis G, Long BJ, et al: Activation of mitogen-activated protein kinase in xenografts and cells during prolonged treatment with aromatase inhibitor letrozole. *Cancer Res* 65:5380-5389, 2005
- 113.** Sabnis GJ, Jelovac D, Long B, et al: The role of growth factor receptor pathways in human breast cancer cells adapted to long-term estrogen deprivation. *Cancer Res* 65:3903-3910, 2005
- 114.** Chabbert-Buffet N, Meduri G, Bouchard P, et al: Selective progesterone receptor modulators and progesterone antagonists: Mechanisms of action and clinical applications. *Hum Reprod Update* 11:293-307, 2005
- 115.** Spitz IM: Progesterone receptor antagonists and selective progesterone receptor modulators (SPRMs). *Semin Reprod Med* 23:3-7, 2005
- 116.** Gao W, Dalton JT: Expanding the therapeutic use of androgens via selective androgen receptor modulators (SARMs). *Drug Discov Today* 12:241-248, 2007
- 117.** Trump RP, Blanc JB, Stewart EL, et al: Design and synthesis of an array of selective androgen receptor modulators. *J Comb Chem* 9:107-114, 2007
- 118.** Kym PR, Kort ME, Coghlan MJ, et al: Nonsteroidal selective glucocorticoid modulators: The effect of C-10 substitution on receptor selectivity and functional potency of 5-allyl-2,5-dihydro-2,2,4-trimethyl-1H-[1]benzopyrano[3,4-f]quinolines. *J Med Chem* 46:1016-1030, 2003
- 119.** Sporn MB, Suh N, Mangelsdorf DJ: Prospects for prevention and treatment of cancer with selective PPARgamma modulators (SPARMs). *Trends Mol Med* 7:395-400, 2001
- 120.** Kopelovich L, Fay JR, Glazer RI, et al: Peroxisome proliferator-activated receptor modulators as potential chemopreventive agents. *Mol Cancer Ther* 1:357-363, 2002
- 121.** Schupp M, Clemenz M, Gineste R, et al: Molecular characterization of new selective peroxisome proliferator-activated receptor gamma modulators with angiotensin receptor blocking activity. *Diabetes* 54:3442-3452, 2005

**Tamoxifen or Raloxifene for Breast Cancer Chemoprevention:  
A tale of two choices**

**V. Craig Jordan**

**Contact Information:**

V. Craig Jordan, OBE, PhD, DSc  
Vice President and Research Director  
for Medical Science  
Alfred G. Knudson Chair of Cancer Research  
Fox Chase Cancer Center  
333 Cottman Avenue  
Philadelphia, PA 19111-2497  
Phone: (215) 728-7410  
Fax: (215) 728-7034  
Email: [v.craig.jordan@fccc.edu](mailto:v.craig.jordan@fccc.edu)

The stated goal for an investment in cancer research is the eradication of cancer. But this is just talk. A world with no cancer is a noble goal but the problem becomes where to start. In other words, how to put ideas into action and move forward from rhetoric. The task is enormous, but one solution where there has been much talk is cancer prevention. In the case of lung cancer, the solution is simple – stop smoking. But the social engineering that is required to prevent one sector of society from creating a massive health care crisis in another appears to be insoluble. It is now clear that women have been the victims here through a callous campaign to recruit smokers. Lung cancer is the disease that kills more women with cancer than any other. Based on this inconvenient truth of modern society, is there any reason to believe that the cancer research community has made any progress with practical help for people? In contrast to lung cancer, progress is quantifiable in another major killer of women – breast cancer.

In 1971, President Nixon signed the National Cancer Act and declared war on cancer, but there were no serious plans to prevent breast cancer. Nevertheless, the first experiments were being conducted to prevent breast cancer with antihormones but, regrettably, at that time no one cared (1). All efforts were focused on the application of combinations of cytotoxic chemotherapy to treat and cure cancer by killing the last cancer cell. Despite heroic attempts to kill the cancer without killing the patient, progress has been modest but significant improvements in survival did occur in premenopausal patients (2). Unfortunately, this is a hollow victory that on the face of it cannot be applied to cancer prevention; or can it?

We have known for more than a century that there is a link between the growth of breast cancer in patients and sex steroids secreted from the ovary (3) or produced

peripherally in a women's body fat. Furthermore, we have known for more than 30 years that combination cytotoxic chemotherapy will destroy ovarian function (reviewed in (4)) and stop estrogen production. Indeed, we now know that younger women who do not have a premature menopause and who do not take antiestrogen therapy have shorter survival than women who have ovarian failure(5-7). We also know that adjuvant oophorectomy produces disease free survival comparable to the use of adjuvant cytotoxic chemotherapy in premenopausal women (8, 9). Thus, based on these clinical observations, one would be drawn to the conclusion that preventing hormone action might be a valuable line of future investigation for prevention if one could only work out the mechanism. But research does not travel in straight lines; a parallel universe of knowledge had already developed to address chemoprevention with antihormones.

An ovarian link between spontaneous breast (mammary) cancer in laboratory mice was demonstrated in 1916 (10) but it was Professor Antoine Lassasagne (11) in 1936 who proposed that “a therapeutic antagonist should be sought to prevent the congestion of oestrone in the breast”. In other words, an antiestrogen could be a valuable chemopreventive agent but at the time there was no scientific foundation to support this strategy. The discovery of the estrogen receptor (ER) as the putative mechanism of estrogen action in its target tissues (12) opened the door to reinvent tamoxifen from a failed contraceptive (13) to become the first targeted therapy for breast cancer treatment (14). Tamoxifen, a nonsteroidal antiestrogen, was discovered in the 1960's as part of a worldwide effort by the pharmaceutical industry to exploit the serendipitous discovery of the drug group (15). Applications were sought based on *in vivo* studies and without reference to receptor mechanisms (16). The compounds were excellent post coital

contraceptive in rats but failed in this application because they induced ovulation in women i.e., it could guarantee pregnancy, exactly the opposite effect that was being sought. As a result, tamoxifen was briefly marketed for the induction of ovulation (17). Although numerous compounds were discovered, only tamoxifen was reinvented as a long-term receptor targeted breast cancer treatment (18) (and potential preventive (19)). A decade later the drugs described as nonsteroidal antiestrogens (20) were recognized as selective ER modulators (SERMs) that could be estrogen-like at one site i.e. bone or endometrial cancer but antiestrogenic at another i.e. breast (21-23). This discovery of SERM action (24) led to the proposition that it was plausible to prevent osteoporosis with SERMs in women but prevent breast cancer at the same time (15, 25). Raloxifene, a failed breast cancer drug (26), emerged as the first SERM used to prevent osteoporosis with the beneficial side effect of preventing both breast and endometrial cancer (27-29). This was perfect timing as hormone replacement therapy (HRT) used to prevent osteoporosis was shown to breast cancer incident (30, 31).

The practical application of using tamoxifen for breast chemoprevention was pioneered by Trevor Powles (32, 33), Bernard Fisher (34, 35) and Umberto Veronesi (36, 37) who created a fundamental change in health care. There were no surprises as the “good, the bad and the ugly” of laboratory research coupled with the vast resource of clinical experience with tamoxifen **that reduced contralateral breast cancer when used as an adjuvant (38-40)** were, in the main, predictive for the results in the chemoprevention trials. The “good” news was that tamoxifen reduced the risk of breast cancer in the large trials (34, 35, 41). Cuzick provided additional clinical trials data with the International Breast Intervention Study (IBIS-1)(42) and performed an “overview analysis” of all

tamoxifen trials (plus the osteoporosis study with raloxifene) (43). Tamoxifen is currently the only medicine that will reduce breast cancer risk safely and for prolonged periods (five and probably ten years) after therapy is stopped (35, 44, 45). This is remarkable and occurs at a time when there are no side effects. The advance with tamoxifen, now FDA approved for risk reduction in high risk women for almost a decade, does have problems but these appear to be overplayed by the media. Concerns about the “bad” side effects of endometrial cancer (generally good grade and curable) or blood clots and stroke are, in the main associated with use in postmenopausal women. There is, however, a very small concern about uterine sarcomas (46, 47). Obviously, hysterectomized women are an appropriate target population for breast chemoprevention with tamoxifen.

The “bad” for some women is the increased incidence of menopausal symptoms. As it turns out this may in fact be “good”. Tamoxifen needs to be metabolically activated to endoxifen by the *CYP2D6* gene product so patients with a variant *CYP2D6* usually have fewer hot flashes but have a higher recurrent rate (48, 49). Ironically, women who use the selective serotonin reuptake inhibitors (SSRI) paroxetine or fluoxetine to suppress hot flashes have a poor response to tamoxifen (48-50). This is because these SSRIs block tamoxifen metabolism. Venlafaxine is the SSRI of choice because it does not block endoxifen production.

The “ugly” concern with tamoxifen was liver cancer induced in rats but this did not translate to an increased incidence of hepatocellular carcinoma in women. It seemed to be obvious that this property, unique to rats, was not going to affect women, as the drug had already been marketed for 20 years at the time the hepatic toxicity was noted

(51). No elevation in hepatocellular carcinoma are observed currently (8). Clinicians, however, do have another choice, raloxifene. This compound does not produce heptaoellular carcinomas in rats.

The SERM raloxifene had been rigorously investigated as a drug to prevent osteoporosis and translational research predicted that this SERM would reduce the risk of breast cancer (21, 22, 25, 27). Based on this evaluation, the NSABP chose to initiate the landmark SERM trial, the study of tamoxifen and raloxifene or STAR (28). The results were clear and predictable: tamoxifen and raloxifene were equivalent at reducing the risk of invasive breast cancer in high risk women. There were trivial differences in ductal carcinoma in situ in favor of tamoxifen (probably due to the failure of compliance and the short duration of action of raloxifene when compared with tamoxifen (52)) but the safety profile of the two SERMs favored raloxifene. Tamoxifen-treated women had more blood clots, more endometrial cancer, hysterectomies, and cataract operations compared with raloxifene-treated women. Supportive evidence for the value of raloxifene for the chemoprevention of breast cancer in postmenopausal women without a concern about an elevation of endometrial cancer comes from the trial named raloxifene use for the heart (RUTH) (29). This trial was established to test the worth of raloxifene to prevent deaths from coronary heart disease but did not show an advantage for raloxifene over placebo. However, the trial did show a significant decrease in breast cancer and no elevation in endometrial cancer (29). Raloxifene is now a new weapon in the clinician's armamentarium to prevent breast cancer in osteoporotic women as well as postmenopausal women at high risk for breast cancer.

In closing, the question that needs to be addressed is why clinicians and women at high risk chose to avoid using approved medicines for appropriate indications? We have seen a dramatic change in the approach to breast cancer treatment and prevention in the past 30 years. Drugs can now be targeted to specific populations. In the case of prevention, tamoxifen is fully tested and is best used for high risk premenopausal women with wild type *CYP2D6* gene product and venlafaxine can be used to control hot flashes. Raloxifene cannot be used in premenopausal women. Raloxifene is the agent of choice in postmenopausal women. Raloxifene is being used by an estimated 500,000 women to prevent osteoporosis which will also prevent the development of tens of thousands of breast cancers over the next decade (53). The recent approval of raloxifene to prevent breast cancer in high risk postmenopausal women will add to a reduction in breast cancer incidence while enhancing bone strength. The SERM concept (15, 25, 54, 55) works in medical practice and agents are available now to help the right patient. Only clinician and patient prejudice, convinced by negative media messages, is preventing progress in chemoprevention.

Returning to my original arguments about lung cancer. It is hard to believe that it is acceptable to smoke cigarettes with the attendant list of known health hazards and the highest death rate for cancer among women, but it is unacceptable to employ approved medicines to reduce the risk of breast cancer. Fortunately, research is not static and new ideas will evolve and new SERMs will be developed, but, regrettably, progress will not occur in the near future. This is compounded by a lack of will by government to support clinical research in chemoprevention and to support the training of a new generation of innovative clinical investigators. In the face of these obstacles, it is essential for the

physicians to make the right choices for the appropriate patient. Interventions validated by decades of clinical and laboratory research and approved by the FDA **can** help reduce the risk of breast cancer now. After all, it's a once around life.

**Acknowledgements:**

Dr. Jordan is supported by the Department of Defense Breast Program under award number BC050277 Center of Excellence (Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense), R01 GM067156, FCCC Core Grant NIH P30 CA006927, and the Weg Fund of Fox Chase Cancer Center

## References

1. Jordan, V. C., and Brodie, A. M. H. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids*, 72: 7-25, 2007.
2. EBCTCG. 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: 1681-92, 1988.
3. Beatson, C. T. On treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. *Lancet*, 2: 162-165, 1896.
4. Jordan, V. C. Chemotherapy is antihormonal therapy--how much proof do oncologists need? *Eur J Cancer*, 34: 606-8, 1998.
5. Dellapasqua, S., Colleoni, M., Gelber, R. D., and Goldhirsch, A. Adjuvant endocrine therapy for premenopausal women with early breast cancer. *J Clin Oncol*, 23: 1736-50, 2005.
6. Colleoni, M., Gelber, S., Goldhirsch, A., Aebi, S., Castiglione-Gertsch, M., Price, K. N., Coates, A. S., and Gelber, R. D. Tamoxifen after adjuvant chemotherapy for premenopausal women with lymph node-positive breast cancer: International Breast Cancer Study Group Trial 13-93. *J Clin Oncol*, 24: 1332-41, 2006.
7. Colleoni, M., Rotmensz, N., Peruzzotti, G., Maisonneuve, P., Orlando, L., Ghisini, R., Viale, G., Pruneri, G., Veronesi, P., Luini, A., Intra, M., Cardillo, A., Torrioni, R., Rocca, A., and Goldhirsch, A. Role of endocrine responsiveness and adjuvant therapy in very young women (below 35 years) with operable breast cancer and node negative disease. *Ann Oncol*, 17: 1497-503, 2006.
8. EBCTCG. 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, 2005.
9. Schmid, P., Untch, M., Kosse, V., Bondar, G., Vassiljev, L., Tarutinov, V., Lehmann, U., Maubach, L., Meurer, J., Wallwiener, D., and Possinger, K. Leuprorelin acetate every-3-months depot versus cyclophosphamide, methotrexate, and fluorouracil as adjuvant treatment in premenopausal patients with node-positive breast cancer: the TABLE study. *J Clin Oncol*, 25: 2509-15, 2007.
10. Lathrop, A. E., and Loeb, L. Further investigations on the origins of tumors in mice III on the part played by internal secretions in the spontaneous development of tumors. *J Cancer Res*, 1: 1-19, 1916.

11. Lacassagne, A. Hormonal pathogenesis of adenocarcinoma of the breast. *Am J Cancer*, 27: 217-225, 1936.
12. Jensen, E. V., and Jacobson, H. I. Basic guides to the mechanism of estrogen action. *Recent Progr Hormone Res*, 18: 387-414, 1962.
13. Harper, M. J., and Walpole, A. L. Mode of action of I.C.I. 46,474 in preventing implantation in rats. *J Endocrinol*, 37: 83-92, 1967.
14. Jensen, E. V., and Jordan, V. C. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. *Clin Cancer Res*, 9: 1980-1989, 2003.
15. Lerner, L. J., and Jordan, V. C. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res*, 50: 4177-4189, 1990.
16. Harper, M. J., and Walpole, A. L. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. *J Reprod Fertil*, 13: 101-119, 1967.
17. Klopper, A., and Hall, M. New synthetic agent for the induction of ovulation. Preliminary trial in women. *Br Med J*, 1: 152-154, 1971.
18. Jordan, V. C. Tamoxifen: a most unlikely pioneering medicine. *Nature Reviews Drug Discovery*, 2: 205-213, 2003.
19. Jordan, V. C. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA- induced rat mammary carcinoma. *Eur J Cancer*, 12: 419-424, 1976.
20. Jordan, V. C. Biochemical pharmacology of antiestrogen action. *Pharmacol Rev*, 36: 245-76, 1984.
21. Jordan, V. C., Phelps, E., and Lindgren, J. U. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat*, 10: 31-35, 1987.
22. Gottardis, M. M., and Jordan, V. C. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea- induced rat mammary carcinoma model. *Cancer Res*, 47: 4020-4024, 1987.
23. Gottardis, M. M., Robinson, S. P., Satyaswaroop, P. G., and Jordan, V. C. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res*, 48: 812-815, 1988.
24. Jordan, V. C. Selective estrogen receptor modulation: a personal perspective. *Cancer Res*, 61: 5683-5687, 2001.

25. Jordan, V. C. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest*, 6: 589-595, 1988.
26. Buzdar, A., Marcus, C., Holmes, F., Hug, V., and Hortobagyi, G. Phase II evaluation of Ly156758 in metastatic breast cancer. *Oncology*, 45: 344-5, 1988.
27. Cummings, S. R., Eckert, S., Krueger, K. A., Grady, D., Powles, T. J., Cauley, J. A., Norton, L., Nickelsen, T., Bjarnason, N. H., Morrow, M., Lippman, M. E., Black, D., Glusman, J. E., Costa, A., and Jordan, V. C. 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, 1999.
28. Vogel, V. G., Costantino, J. P., Wickerham, D. L., Cronin, W. M., Cecchini, R. S., Atkins, J. N., Bevers, T. B., Fehrenbacher, L., Pajon (jr), E. R., Wade, J. L., Robidoux, A., Margolese, R. G., James, J., Lippman, S. M., Runowicz, C. D., Ganz, P. A., Reis, S. E., McCaskill-Stevens, W., Ford, L. G., Jordan, V. C., and Wolmark, N. The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial *JAMA*, 295: 2727-2741, 2006.
29. Barrett-Connor, E., Mosca, L., Collins, P., Geiger, M. J., Grady, D., Kornitzer, M., McNabb, M. A., and Wenger, N. K. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Engl J Med*, 355: 125-137, 2006.
30. Million Women Study Collaborators. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet*, 362: 419-427, 2003.
31. Ravdin, P. M., Cronin, K. A., Howlader, N., Berg, C. D., Chlebowski, R. T., Feuer, E. J., Edwards, B. K., and Berry, D. A. The decrease in breast-cancer incidence in 2003 in the United States. *N Engl J Med*, 356: 1670-4, 2007.
32. Powles, T. J., Hardy, J. R., Ashley, S. E., Farrington, G. M., Cosgrove, D., Davey, J. B., Dowsett, M., McKinna, J. A., Nash, A. G., and Sinnott, H. D. A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *Br J Cancer*, 60: 126-131, 1989.
33. Powles, T., Eeles, R., Ashley, S., Easton, D., Chang, J., Dowsett, M., Tidy, A., Viggers, J., and Davey, J. Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomised chemoprevention trial [see comments]. *Lancet*, 352: 98-101, 1998.
34. Fisher, B., Costantino, J. P., Wickerham, D. L., Redmond, C. K., Kavanah, M., Cronin, W. M., Vogel, V., Robidoux, A., Dimitrov, N., Atkins, J., Daly, M.,

- Wieand, S., Tan-Chiu, E., Ford, L., and Wolmark, N. 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, 1998.
35. Fisher, B., Costantino, J. P., Wickerham, D. L., Cecchini, R. S., Cronin, W. M., Robidoux, A., Bevers, T. B., Kavanah, M. T., Atkins, J. N., Margolese, R. G., Runowicz, C. D., James, J. M., Ford, L. G., and Wolmark, N. 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, 2005.
  36. Veronesi, U., Maisonneuve, P., Costa, A., Sacchini, V., Maltoni, C., Robertson, C., Rotmensz, N., and Boyle, P. Prevention of breast cancer with tamoxifen: preliminary findings from the Italian randomised trial among hysterectomised women. Italian Tamoxifen Prevention Study. *Lancet*, 352: 93-7, 1998.
  37. Veronesi, U., Maisonneuve, P., Rotmensz, N., Bonanni, B., Boyle, P., Viale, G., Costa, A., Sacchini, V., Travaglini, R., D'Aiuto, G., Oliviero, P., Lovison, F., Gucciardo, G., del Turco, M. R., Muraca, M. G., Pizzichetta, M. A., Conforti, S., and Decensi, A. Tamoxifen for the prevention of breast cancer: late results of the Italian Randomized Tamoxifen Prevention Trial among women with hysterectomy. *J Natl Cancer Inst*, 99: 727-37, 2007.
  38. Cuzick, J., and Baum, M. Tamoxifen and contralateral breast cancer [letter]. *Lancet*, 2: 282, 1985.
  39. Fornander, T., Rutqvist, L. E., Cedermark, B., Glas, U., Mattsson, A., Silfversward, C., Skoog, L., Somell, A., Theve, T., Wilking, N., and Hjalmar, M.-L. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet*, 1: 117-120, 1989.
  40. Fisher, B., Costantino, J., Redmond, C., Poisson, R., Bowman, D., Couture, J., Dimitrov, N. V., Wolmark, N., Wickerham, D. L., Fisher, E. R., and et al. 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: 479-84., 1989.
  41. Gail, M. H., Costantino, J. P., Bryant, J., Croyle, R., Freedman, L., Helzlsouer, K., and Vogel, V. Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J Natl Cancer Inst*, 91: 1829-1846, 1999.
  42. Cuzick, J., Forbes, J., Edwards, R., Baum, M., Cawthorn, S., Coates, A., Hamed, A., Howell, A., and Powles, T. First results from the International Breast Cancer Intervention Study (IBIS-I): a randomised prevention trial. *Lancet*, 360: 817-824, 2002.

43. Cuzick, J., Powles, T., Veronesi, U., Forbes, J., Edwards, R., Ashley, S., and Boyle, P. Overview of the main outcomes in breast-cancer prevention trials. *Lancet*, 361: 296-300, 2003.
44. Powles, T. J., Ashley, S., Tidy, A., Smith, I. E., and Dowsett, M. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. *J Natl Cancer Inst*, 99: 283-90, 2007.
45. Cuzick, J., Forbes, J. F., Sestak, I., Cawthorn, S., Hamed, H., Holli, K., and Howell, A. Long-term results of tamoxifen prophylaxis for breast cancer--96-month follow-up of the randomized IBIS-I trial. *J Natl Cancer Inst*, 99: 272-82, 2007.
46. Wickerham, D. L., Fisher B, Wolmark N, Bryant J, Costantino J, Bernstein L, Runowicz CD. Association of tamoxifen and uterine sarcoma. *J Clin Oncol*, 20: 2758-60, 2002.
47. Bergman, L., Beelen, M. L., Gallee, M. P., Hollema, H., Benraadt, J., and van Leeuwen, F. E. Risk and prognosis of endometrial cancer after tamoxifen for breast cancer. Comprehensive Cancer Centres' ALERT Group. Assessment of Liver and Endometrial cancer Risk following Tamoxifen. *Lancet*, 356: 881-7, 2000.
48. Goetz, M. P., Rae, J. M., Suman, V. J., Safgren, S. L., Ames, M. M., Visscher, D. W., Reynolds, C., Couch, F. J., Lingle, W. L., Flockhart, D. A., Desta, Z., Perez, E. A., and Ingle, J. N. Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. *J Clin Oncol*, 23: 9312-9318, 2005.
49. Goetz, M. P., Knox, S. K., Suman, V. J., Rae, J. M., Safgren, S. L., Ames, M. M., Visscher, D. W., Reynolds, C., Couch, F. J., Lingle, W. L., Weinshilboum, R. M., Fritcher, E. G., Nibbe, A. M., Desta, Z., Nguyen, A., Flockhart, D. A., Perez, E. A., and Ingle, J. N. The impact of cytochrome P450 2D6 metabolism in women receiving adjuvant tamoxifen. *Breast Cancer Res Treat*, 101: 113-21, 2007.
50. Stearns, V., Johnson, M. D., Rae, J. M., Morocho, A., Novielli, A., Bhargava, P., Hayes, D. F., Desta, Z., and Flockhart, D. A. Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. *J Natl Cancer Inst*, 95: 1758-1764, 2003.
51. Jordan, V. C. What if tamoxifen (ICI 46,474) had been found to produce rat liver tumors in 1973? A personal perspective. *Ann Oncol*, 6: 29-34, 1995.
52. Snyder, K. R., Sparano, N., and Malinowski, J. M. Raloxifene hydrochloride. *Am J Health Syst Pharm*, 57: 1669-1675; quiz 1676-1678., 2000.

53. Jordan, V. C. Optimising endocrine approaches for the chemoprevention of breast cancer. Beyond the Study of Tamoxifen and Raloxifene (STAR) Trial. *European Journal of Cancer*, 42: 2909-2913, 2006.
54. Jordan, V. C. Designer estrogens. *Sci Am*, 279: 60-7, 1998.
55. Jordan, V. C. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. *Nature Reviews Cancer*, 7: 46-53, 2007.

# SERMs for the treatment and prevention of breast cancer

Ramona F. Swaby · Catherine G. N. Sharma ·  
V. Craig Jordan

© Springer Science + Business Media, LLC 2007

**Abstract** Tamoxifen and raloxifene are both selective estrogen receptor modulators (SERMs). The medicines can block estrogen mediated breast cancer growth and development but will also maintain bone density in postmenopausal women and lower circulating cholesterol. Tamoxifen has remained the antihormonal therapy of choice for the treatment of ER positive breast cancer for the last 30 years. However, although adjuvant tamoxifen produces profound increases in disease-free and overall survival in patients with ER positive breast cancer, concerns about drug resistance, blood clots and endometrial cancer have resulted in a change to the use of aromatase inhibitors for the treatment of postmenopausal women. Nevertheless, tamoxifen remains the antihormonal treatment of choice for premenopausal women with ER positive breast cancer and for risk reduction in premenopausal women who are at high risk for developing breast cancer. The risk of endometrial cancer and thromboembolic disorders during tamoxifen therapy is not elevated in premenopausal women. It is important to note that aromatase inhibitors or raloxifene should not be used in premenopausal women. Raloxifene is used to prevent osteoporosis in postmenopausal women and, unlike tamoxifen, does not increase the risk of endometrial cancer. However, raloxifene does reduce breast cancer risk by 50–70% in both low risk and high risk postmenopausal women. Comparisons of raloxifene with tamoxifen show equal efficacy as a chemopreventive for breast cancer but there is a reduction in thromboembolic disorders, fewer endometrial

cancers, hysterectomies, cataracts and cataract surgeries in women taking raloxifene. Overall, SERMs continue to fulfill their promise as appropriate medicines that target specific populations for the treatment and prevention of breast cancer.

**Keywords** Tamoxifen · Raloxifene · Estrogen receptor · Selective estrogen receptor modulator · Osteoporosis · Endometrial cancer

## 1 Introduction

Schinzinger [1, 2] first proposed, whereas Beatson [3, 4] first reported, performing oophorectomy for the treatment of metastatic breast cancer in 1896. It has now become accepted that ovarian hormones, particularly estrogen, are central to the development of breast cancer. Laboratory evidence identified estrogen as the trophic hormone in estrogen target tissues (e.g. the uterus and some breast cancers) [5] so naturally “anti-estrogen” therapy became a central theme for the treatment and now prevention of breast cancer [6]. One medicine, tamoxifen [7], originally classified as a nonsteroidal antiestrogen [8] but now reclassified as a selective estrogen receptor modulator (SERM) [9] has proved to be a pioneering intervention that not only produced dramatic survival advantages when used as an adjuvant therapy [10] but also became the first chemopreventive for any cancer [11, 12]. However, the recognition of SERM action [13, 14] actually opened the door to new opportunities in therapeutics and advanced the idea of multifunctional medicines to address a number of prevention issues pertinent to postmenopausal women’s health. Osteoporosis is a major health care problem but emerging information about the inappropriateness of long-

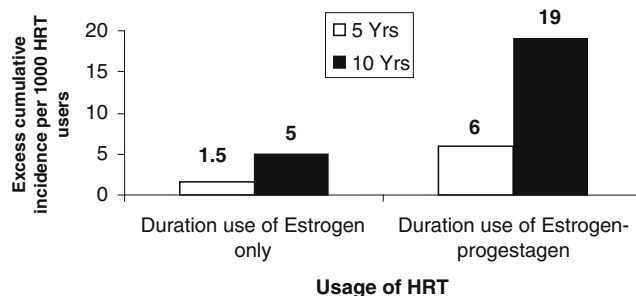
---

R. F. Swaby · C. G. N. Sharma · V. C. Jordan (✉)  
Fox Chase Cancer Center,  
333 Cottman Avenue,  
Philadelphia, PA 19111-2497, USA  
e-mail: v.craig.jordan@fccc.edu

term hormone replacement therapy (HRT) to prevent osteoporosis has acted as a catalyst for the development of new, safer SERMs.

In the United States alone, approximately 90 million prescriptions for HRT were dispensed annually from 1999 through 2002 [15]. Indeed, records suggest that hormonal replacement therapy was the most commonly prescribed medicine in the world during the late 1990s and early 2000s [16]. Despite epidemiologic data suggesting the overwhelming benefits of HRT, data regarding hormonal therapy use and breast cancer incidence were unconfirmed. Therefore, as part of the Women's Health Initiative (WHI), a large randomized controlled primary prevention trial to determine the risk benefit ratio of HRT in postmenopausal women was undertaken. In July of 2002, the principal results from the WHI study examining the effects of HRT were reported [17]. In this trial in which approximately 16,000 women were treated either with estrogen/progesterone combination HRT or placebo, an approximately 26% increase in the incidence of breast cancer was detected among the women treated with HRT. This data was subsequently confirmed and extended in the Million Women Study [18]. The Million Women Study, while not a randomized prospective clinical trial, followed cohorts of post-menopausal women during the same time frame as the WHI and collected information about their use of HRT. These cohorts were followed for cancer incidence and any death due to breast cancer. The overall conclusion was that users of HRT were more likely than never users of HRT to develop breast cancer and die from it [18]. The profound excess of new breast cancers that accumulated populations of women taking 5 or 10 years of HRT is illustrated in Fig. 1. As soon as these data were reported, the use of HRT dropped dramatically both within the United States and in Europe [15, 19].

Recently, a 7% decrease in the age-adjusted incidence of breast cancer has been observed from 2002–2003 [20]. This



**Fig. 1** Diagrammatic representation of the estimated cumulative incidence of breast cancer in 1,000 postmenopausal women taking hormone replacement therapy (HRT) in excess of the incidence observed in women not taking HRT. The Million Women's Study [18] compared and contrasted women using estrogen only preparations for 5 or 10 years with those women taking a combination of estrogen and a progestin for the same period

decline, not attributable to changes in mammography screening, represents a decline of approximately 14,000 breast cancer cases in the United States in 2003 when compared with 2002. The effect was found to be important for women age 50 or greater and specifically, statistically significant for women aged 50–74. Most importantly, this effect was essentially confined to hormonally responsive breast cancers. While these data do not speak to the initiation and development of breast cancers, the time course suggests that estrogen may play a role in propagating sub-clinical ER breast cancers that in a less estrogenic environment may have remained sub-clinical and/or eliminated through the body's usual tumor surveillance system. Clearly it would be advantageous to have targeted specific agents to treat and ultimately prevent breast cancer.

The story of SERM recognition and development [21, 22] has its origins in the study of tamoxifen (ICI 46,474) a drug originally discovered at the laboratories of ICI Pharmaceuticals Division, UK, in their fertility control program [23] as a potential post coital contraceptive. The drug failed in its primary application but slowly succeeded in a secondary application as a treatment for breast cancer [7, 24].

## 2 Tamoxifen, the first SERM

Tamoxifen is a pioneering medicine [7] because it became one of the first targeted treatments for cancer where the treatment strategy used today translated from the laboratory to clinical practice. The pharmacology of tamoxifen was studied extensively in animal models of mammary carcinogenesis to explore appropriate strategies to enhance disease control in patients. Tamoxifen was found to inhibit binding of estrogen to the ER mammary carcinomas both *in vitro* and *in vivo* [25–27]. *In vitro*, tamoxifen was demonstrated to have low affinity for the estrogen receptor [28], however, tamoxifen acts as a prodrug and is rapidly converted in the liver to a metabolite with high affinity to block the ER [29]. Tamoxifen, as well as its active metabolites, achieve stable, steady-state levels within the serum that remain constant during treatment ranging from months to years (over 7 years) [30].

An examination of tamoxifen treatment during the early stages of tumorigenesis in the rat mammary carcinoma model demonstrated that longer rather than shorter durations of tamoxifen would be necessary to use as a strategy for the adjuvant treatment of breast cancer [31–33]. However, there was initial concern that long-term adjuvant tamoxifen would cause premature drug resistance. Nevertheless, clinical trial strategies eventually explored the optimal duration for tamoxifen therapy. It is now possible to assess the value of the idea of targeting tamoxifen to treat

women with ER positive tumors with long-term therapy. The Oxford Overview Analysis has established treatment trends based on the results from worldwide randomized clinical trials.

When the Overview analyses were first initiated, tamoxifen was the only universally used antihormonal agent. With no other competition, tamoxifen became the “gold standard” and established the principles of tumor targeting and identified the appropriate treatment strategy to aid survivorship [10, 34–36].

- Five years of adjuvant tamoxifen enhances disease free survival. There is a 50% decrease in recurrences observed in ER positive patients 15 years after diagnosis.
- Five years of adjuvant tamoxifen enhances survival with a decrease in mortality 15 years after diagnosis.
- Adjuvant tamoxifen does not provide an increase in disease free or overall survival in ER negative breast cancer.
- Five years of adjuvant tamoxifen alone is effective in premenopausal women with ER positive breast cancer; tamoxifen is ineffective in ER negative breast cancer.
- The benefits of tamoxifen in lives saved from breast cancer, far outweighs concerns about an increased incidence of endometrial cancer in postmenopausal women.

- Tamoxifen does not increase the incidence of second cancers other than endometrial cancer.
- No non-cancer related overall survival advantage is noted with tamoxifen when given as adjuvant therapy.

The Overview analysis process is now being applied to the numerous new aromatase inhibitors [6] that are being compared to tamoxifen directly, after a few years of tamoxifen or after a full five years of tamoxifen (Fig. 2). As a group, the aromatase inhibitors are superior to tamoxifen with improved overall survival and a reduced incidence of estrogen-like side effects.

Once antihormonal therapy had started to achieve optimal success in the treatment of node positive and node negative disease during the last decade, the trend for clinical research during the 1990s was to build on the successes of SERMs as treatments for disease so that breast incidence could be reduced in specific populations of women.

### 3 Tamoxifen and primary prevention

Early laboratory observations [37, 38] plus the finding that tamoxifen decreases contralateral breast cancer by 50% when the drug is used as an adjuvant therapy [39], made tamoxifen the agent of choice for evaluation as a chemopreventive agent. A series of clinical trials aimed at primary

**Fig. 2** A comparison of the strategies used to compare and contrast the therapeutic efficacy and side effects of various aromatase inhibitors with adjuvant tamoxifen in populations of postmenopausal women diagnosed with ER positive breast cancer

## Long Term Estrogen Deprivation Treatment

**AI = AROMATASE INHIBITOR**

**5 years tamoxifen**

ATAC, *The Lancet* 2002, 359:2131-40

**5 years AI**

Howell et al, *The Lancet* 2005, 365:60-2

Thurlimann et al, *N Engl J Med* 2005, 353:2747-57

**5 years tamoxifen**

Coombes et al, *N Engl J Med* 2004, 350:1081-92

**2-3 Tamoxifen 3 years AI**

Boccardo et al, *J Clin Oncol* 2005, 23:5138-47

**5 years tamoxifen**

**5 years tamoxifen**

**5 years AI**

Goss et al, *N Engl J Med* 2003, 349:1793-802

Goss et al, *J Natl Cancer Inst* 2005,97:1262-71

breast cancer prevention established tamoxifen as the first drug to be approved for risk reduction of any cancer. The trials have been compared and contrasted [40] so only the conclusions will be considered after presenting the two main studies.

The National Surgical Adjuvant Breast and Bowel Project (NSABP) initiated the Breast Cancer Prevention Trial (P-1) in 1993 [11]. Approximately 13,000 pre and postmenopausal women were recruited because they were at high risk for developing breast cancer either due to age close to the peak incidence age of breast cancer, a high Gail score [41], or that had a history of lobular carcinoma *in situ*. The volunteers were randomized to receive placebo or 5 years of tamoxifen at the previously established daily dose of 20 mg/day. Tamoxifen produced a 49% (two-sided  $p < 0.0001$ ) decrease in the development of invasive breast cancers and a 50% (two-sided  $p < 0.002$ ) decrease in the development of non-invasive breast cancers. This effect was restricted to ER positive tumors (a 69% reduction), with no effect on the development of ER negative tumors [11]. The NSABP P-1 clinical trial was important in that it once again confirmed the requirement of the ER in a tumor for tamoxifen to be effective. The NSABP P-1 Trial, of all the prevention clinical trials, was the only one that did not incorporate the use of HRT in either of the trial arms. Allowing for the use of HRT in other prevention clinical trials may explain the blunted efficacy results when compared to the NSABP P-1 trial.

The International Breast Cancer Intervention Study (IBIS-I) was an international phase III chemoprevention trial comparing tamoxifen vs. placebo [42]. This trial enrolled approximately 7,000 pre- and post-menopausal women recruited on several continents. Their age was between 35–70 years prospectively determined to be at increased risk for breast cancer development [42]. Risk factors for breast cancer included at least a two-fold relative risk for patients ages 45–70 years, a four-fold relative risk for ages 40–44 and an approximately ten-fold relative risk for ages 35–39. Therefore, almost all participants (97%) had a family history of breast cancer. Approximately one-third of all patients used HRT while being treated on this clinical trial. At a median follow-up of 50 months, a 32% reduction in the development of breast cancers was documented (69 vs. 101,  $p = 0.01$ ). The risk reduction was demonstrated among the occurrence of both invasive (25% reduction, 64 vs. 85) and non-invasive breast cancers (69% reduction, 5 vs. 16), although these subset analyses did not achieve statistical significance. There was no reduction in the occurrence of ER negative breast cancers.

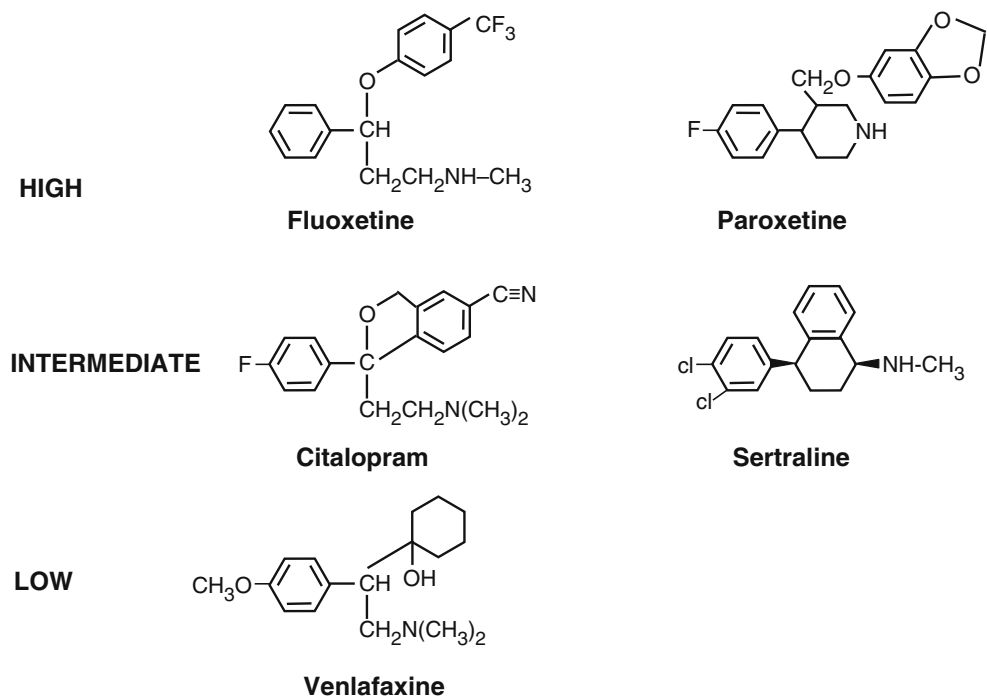
Taken together, the above data supports the conclusion that tamoxifen lowers the risk of developing ER-positive breast cancer in patients without a personal history of breast cancer, but that are at higher risk for the development of

breast cancer due to genetic and/or other established risk factors. The prevention of breast cancer comes at the expense of well documented side effects, including an approximately 2–5 fold increase in uterine cancer [12, 42], and an approximately 2–3 fold increase in thromboembolic disease but only in postmenopausal women. In addition to increased menopausal symptoms, vaginal discharge and ocular abnormalities occur with tamoxifen. These definitive clinical trial data suggest that chemoprevention with tamoxifen should focus on high risk premenopausal women [43]. It is anticipated, based on the Overview Analysis [36] 5 years of treatment will be followed by continuing protection for the following 10 years. Naturally, once tamoxifen treatment is stopped, menopausal symptoms will stop but the problem is whether women will wish to tolerate 5 years of tamoxifen. Solutions to the problem of compliance have focused on the selective serotonin reuptake inhibitors (SSRIs) (Fig. 3) but recent studies of the metabolism of tamoxifen have revealed important lessons that can potentially refine current chemoprevention strategies.

#### 4 Refining treatment and prevention with tamoxifen

Alterations in the cytochrome P450 system impact upon tamoxifen metabolism and its efficacy. Tamoxifen metabolites have been recognized to have antiestrogenic activity [44, 45]. More recently, the cytochrome P450 2D6 (CYP2D6) metabolic pathway was shown to be important in the production of the tamoxifen metabolite, 4-hydroxy-N-desmethyl-tamoxifen (endoxifen (Fig. 4)). Endoxifen has similar potency to 4-hydroxy tamoxifen [46], but an approximately ten-fold higher circulating concentration than 4-hydroxy-tamoxifen [45]. Therefore, if cytochrome CYP2D6 is metabolically inactivated due to genetic variants of this particular phenotype or through inhibition of the 2D6 enzyme from use of concomitant medications that inhibit CYP2D6, tamoxifen cannot be metabolized to its active metabolites, resulting in diminished efficacy. Jin et al. [47] examined plasma endoxifen concentrations in healthy women 4 months of beginning adjuvant tamoxifen therapy. Endoxifen concentrations in the blood were found to be statistically significantly lower in patients with a CYP2D6 homozygous or heterozygous variant genotype when compared to homozygous wild-type genotype. Similarly low concentrations of endoxifen were also identified within this same cohort of patients among subjects using concomitant potent inhibitors of CYP2D6 such as paroxetine (Fig. 3). Such diminished endoxifen levels have recently been demonstrated to correlate with worse clinical outcome [48]. SSRIs are commonly prescribed to women taking tamoxifen for the treatment of associated hot flashes but the SSRIs range from potent to mild inhibitors of the

**Fig. 3** The classification of selective serotonin reuptake inhibitors (SSRIs) used for the relief of hot flashes in women being treated with tamoxifen. The SSRIs have high, intermediate or low affinity for the CYP2D6 gene that metabolizes tamoxifen or N-desmethyl-tamoxifen to 4-hydroxytamoxifen or endoxifen respectively (See Fig. 4)

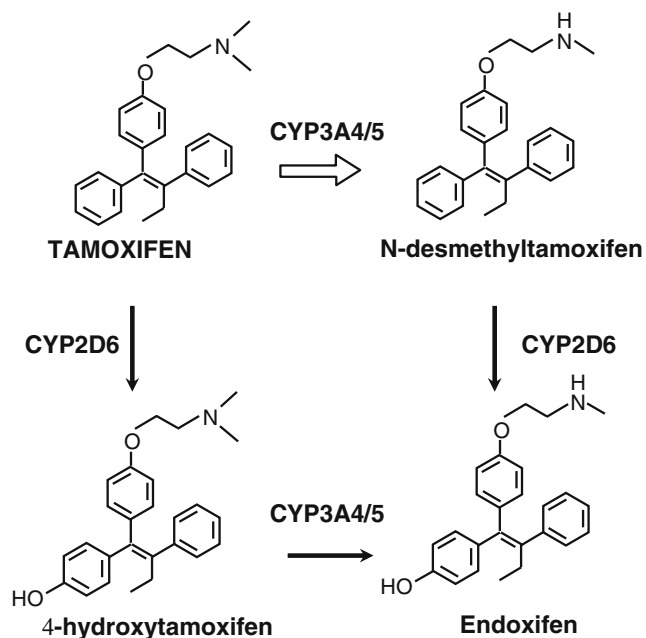


CYP2D6 cytochrome enzymes (Fig. 3). To determine whether this knowledge has clinical relevance, a retrospective analysis was performed on a North Central Cancer Treatment Group (NCCTG) randomized phase III clinical trial [48]. In this trial, postmenopausal women with ER-positive breast cancer were originally randomized to adjuvant treatment with either tamoxifen for 5 years or tamoxifen for 5 years followed by an additional year of fluoxymestron (NCCTG 89-30-52). Paraffin embedded tumor samples from the tamoxifen only arm were genotyped for CYP2D6 wildtype and polymorphisms. Additionally, utilizing chart review, use of SSRIs was also evaluated with respect to relapse-free survival (RFS), disease-free survival (DFS) and overall survival (OS). In a multivariate analysis, patients homozygous for CYP2D6 variant (CYP2D6\*4/\*4) trended towards worse RFS (HR, 1.85;  $P=0.176$ ) and DFS (HR, 1.86;  $P=0.089$ ), without affecting OS (HR 1.12;  $P=0.780$ ) compared to patients heterozygous for the CYP2D6 variant (CYP2D6 \*4/4) or had wild-type CYP2D6 (CYP2D6 4/4). Additionally, the symptoms of moderate and severe hot flashes segregated with patients who were found to have the CYP2D6 \*4/\*4 homozygous gene polymorphism [48]. When these data (NCCTG 89-30-52) were re-analyzed to include evaluation of concomitant CYP2D6 inhibitor use, multivariate analysis revealed that patients with significantly decreased tamoxifen metabolism due to either homozygous CYP2D6 \*4/\*4 variant genotype or due to concomitant use of an extensive CYP2D6 inhibitor, had a statistically significantly worse RFS (HR, adj=1.71,  $p=0.017$ ) with a statistically significant risk of breast cancer relapse (HR 3.12,  $p=0.007$ ) [49].

This suggests that in order to individualize therapy for premenopausal women with ER-positive early stage breast cancers, tamoxifen might be best for patients homozygous wildtype for CYP2D6 genotype and for those not requiring SSRI's for the treatment of hot flashes. Alternatively, Venlafaxine, which has low interaction with CYP2D6, could be used to control hot flashes. Alternative therapies such as the newer aromatase inhibitors might be considered, for example, for postmenopausal patients with diminished endoxifen metabolism either due to CYP2D6 genotyping or need for utilizing SSRIs for hot flush symptom management [50].

## 5 Recognition of selective estrogen receptor modulations

The recognition of SERM action and the realization that nonsteroidal antiestrogens were, in fact, target site specific estrogens and antiestrogens arose from the pharmacological evaluation of tamoxifen during the transition from breast cancer treatment to chemoprevention in the mid 1980s. It was reasoned that if estrogen was beneficial for maintaining bone density in postmenopausal women then perhaps the long-term administration of tamoxifen to women without cancer might prevent breast cancer but accelerate the development of osteoporosis. However, the finding that tamoxifen and the related compound raloxifene (then known as keoxifene) would prevent bone loss in ovariectomized rats [51–53] at doses that would prevent rat mammary carcinogenesis [32, 54] changed that perspective. More importantly, the simultaneous findings that tamoxifen



**Fig. 4** The principal metabolites of tamoxifen observed in the serum of women receiving adjuvant tamoxifen therapy. Demethylation occurs through CYP3A4 and aromatic hydroxylation through CYP2D6. The SSRIs (Fig. 3) block the metabolic activation of tamoxifen by binding to CYP2D6

could prevent estrogen-stimulated breast cancer growth but, at the same time, enhance the growth of the uterus or endometrial cancer [55, 56] rapidly translated to clinical practice with the finding that postmenopausal patients being treated with tamoxifen had an increased risk of developing endometrial cancer [57, 58]. This translational research resulted in gynecologists becoming involved in cancer care and safety procedures were established to avoid the progression of endometrial carcinoma stimulated to grow by tamoxifen. It was also reasoned that SERMs had opposing action in the uterus and breast and this translated to patients, why not translate the possibility of using SERMs to prevent breast cancer by treating osteoporosis?

## 6 The concept

A plan to prevent breast cancer as a public health initiative was initially described at the First International Chemoprevention meeting in New York in 1987. It is reasonable to simply state the proposal, published from the 1987 meeting and subsequently refined and presented at the annual meeting of the American Association for Cancer Research in San Francisco in 1989.

“The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused upon 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 upon the developing clues provided by an extensive clinical investigation of available antiestrogens. Could analogs 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 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 [13].” The concept was refined by 1990 [14] “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.” This concept is exactly what has been translated to clinical practice [59, 60]: use a SERM (raloxifene) to treat osteoporosis and reduce the incidence of breast cancer as a beneficial side effect.

## 7 The SERM concept into practice

The Multiple Outcomes of Raloxifene (MORE) clinical trial was a multicenter, randomized, placebo controlled clinical trial utilizing raloxifene or placebo for the prevention of osteoporosis as its primary endpoint [59, 61, 62]. One of the multiple outcomes evaluated in this clinical trial was the secondary endpoint of breast cancer incidence. Therefore, post-menopausal women who met the criteria for diagnosis of osteoporosis were randomized in a 2:1 ratio to treatment with either of two doses of raloxifene—60 or 120 mg, or placebo. This population was an older population as the mean age of participants was approximately 66 years of age with over 80% aged 60 or older. Approximately 12% of trial subjects reported a first-degree relative with breast cancer. Additionally, approximately 29% of women reported previous HRT use at baseline and approximately 12% of women used HRT while being treated. In this population, raloxifene use was associated

with a 72% reduction in the incidence of invasive breast cancer (RR=0.28, 95% CI 0.17, 0.46) without significant impact on the incidence on *in situ* disease (nine vs. five cases for raloxifene and placebo, respectively, RR=0.90, 95% CI=0.30, 2.69). Of note, raloxifene had no effect upon the incidence of invasive estrogen receptor negative tumors (RR 1.13, 95% CI 0.35, 3.66).

In the Continuing Outcomes Relevant to Evista (CORE) trials, the chemopreventive effect of raloxifene were substantiated. This trial was essentially an extension of the MORE trial above for an additional 4 years of evaluation of the effect of extended raloxifene therapy [60]. Patients initially assigned to either 60 or 120 mg of treatment with raloxifene after the 4 years of the MORE trial were offered to continue raloxifene therapy with 60 mg of raloxifene (with the exception of patients still enrolled in the CORE trial assigned to 120 mg of raloxifene, i.e. less than 4 years of treatment). Similarly, patients initially assigned to the placebo arm of the MORE trial were continued on placebo. During the additional 4 years of evaluation, the continued use of raloxifene was associated with an approximately 59% reduction in the incidence of invasive breast cancer when compared to placebo (HR=0.41, 95% CI 0.24–0.71) and a 66% reduction in the incidence of ER positive breast cancers (HR=0.34, 95% CI=0.18–0.66). Again, no protective effect was demonstrated in the development of ER negative breast cancers or *in situ* breast cancer. Over the 8 year period of evaluation from both the MORE data as well as the CORE data, raloxifene was demonstrated to reduce newly diagnosed invasive breast cancers by approximately 66% in total, when compared to placebo (HR=0.34, 95% CI=0.22–0.50). This translated into an approximately 76% reduction in the relative occurrence of ER positive breast cancers (HR=0.24, 95% CI 0.15) with no resulting effect on ER negative breast cancers and *in situ* breast cancers, essentially providing confirmation of the earlier MORE trial results.

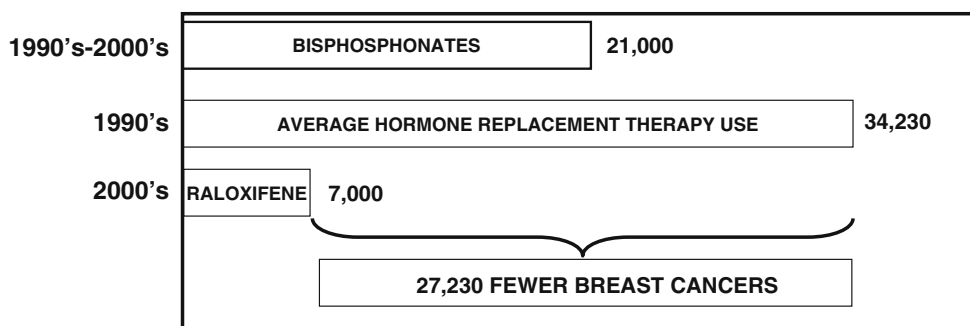
Based on analysis from the MORE trial evaluating cardiovascular risk, the Raloxifene Use for The Heart (Ruth) trial was undertaken with prevention of cardiac events and incidence of new breast cancer diagnosis as the primary objectives [63]. Approximately, 10,000 post-menopausal women with diagnosed coronary heart disease (CHD) or who were determined to be at risk for the development of CHD due to known risk factors such as diabetes mellitus, tobacco smoking and hypertension were randomized to treatment with either raloxifene 60 mg or placebo. Although raloxifene demonstrated no significant benefit for preventing primary coronary events in this patient population, (HR=0.95, 95% CI=0.84–1.07), a reduction in the development of invasive breast cancer was demonstrated. Once again, raloxifene use of approxi-

mately 5 years was associated with a 44% reduction (HR=0.56, 95% CI=0.38–0.83) in the incidence of invasive breast cancer with treatment effect limited to ER positive breast cancers only. It is worthy to note that in this trial, analysis of breast cancer risk was performed and the preventative effect of raloxifene was also limited to patients at higher risk for developing breast cancer with a Gail score of 1.66 or higher. Most importantly, there was no increase in the risk of endometrial cancer confirming preclinical reports that raloxifene was substantially less effective than tamoxifen at stimulating endometrial cancer growth [64]. The final evaluation of raloxifene that will be presented is the chemoprevention of breast cancer growth determined in high risk postmenopausal women. The comparator medicine was tamoxifen.

## 8 Raloxifene and primary prevention

Patients were recruited into the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) trial from July 1, 1999 through November 4, 2004 [65]. This clinical trial randomizing patients to treatment with either tamoxifen or raloxifene for the primary prevention of breast cancer enrolled postmenopausal patients between the ages 35 and older, deemed to be at higher risk for the development of a first invasive breast cancer (the study primary endpoint) with either a 5 year predicted breast cancer risk of 1.66% based on the Gail model, or a previous history of lobular carcinoma *in situ* (LCIS) treated by local excision alone. It is worth noting that 19% of participants reported a family history of breast cancer in two or more first-degree relatives, and more than 71% reported a history of invasive breast cancer in one or more first-degree relative. Therefore, the mean predicted 5-year risk of developing breast cancer among the study population was 4.03% (SD, 2.17%). The primary endpoint of this randomized, double-blinded trial was the development of a first invasive breast cancer. Secondary endpoints also prospectively analyzed include, *in situ* breast cancer, endometrial cancer, all other cancers, cardiovascular disease, stroke, pulmonary embolism, DVT, transient ischemic attack, osteoporotic fracture, cataracts, death, and quality of life. The data was reported at a median follow-up time of 3.9 years. Both raloxifene and tamoxifen were equally effective at preventing the development of a first invasive breast cancer (RR 1.02; 95% CI, 0.82–1.28,  $p=0.96$ ). However, although not statistically significant, tamoxifen was better at preventing the occurrence of *in situ* breast cancers (57 vs. 80 for tamoxifen and raloxifene, respectively,  $p=0.052$ ). This result is somewhat curious since the same mechanisms that would prevent an invasive breast cancer from developing could be expected to prevent

## New Breast Cancers



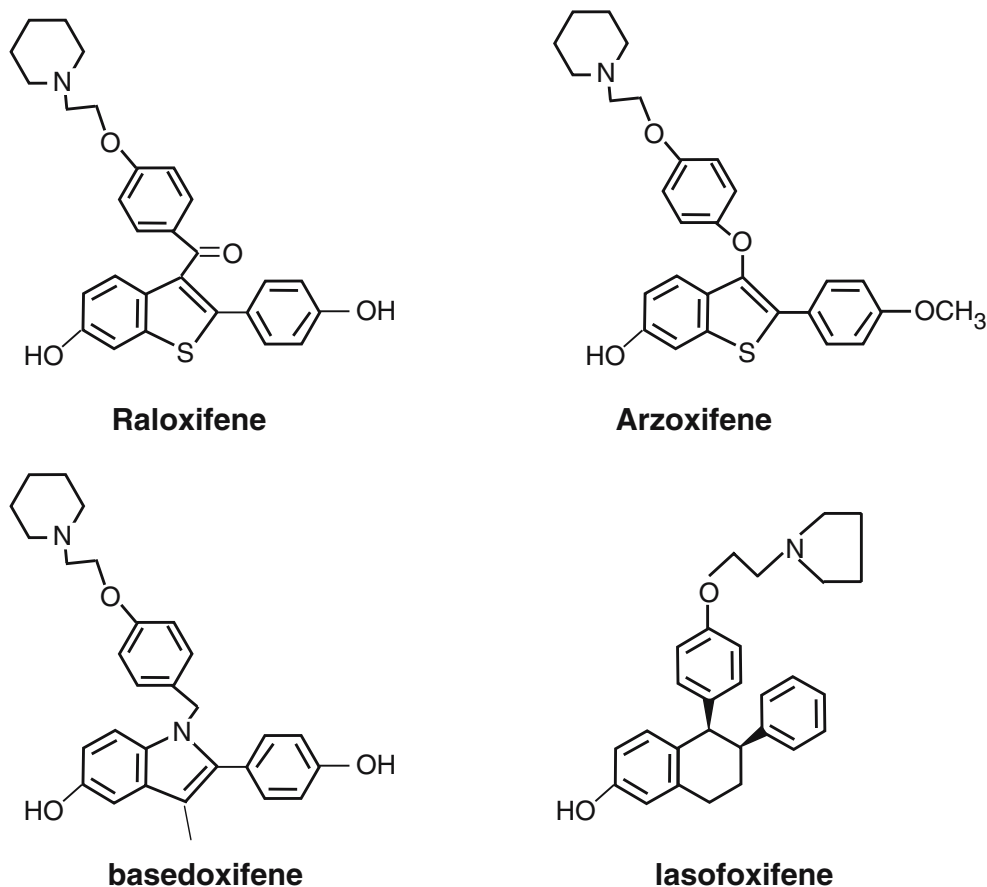
**Fig. 5** An estimation of breast cancer incidence in a population of 500,000 postmenopausal women with the same risk for osteoporotic fractures as participants in the CORE trial [60] treated for a 10 year period with a bisphosphonate, hormone replacement therapy (HRT) based on the average breast cancer risk between the Women's Health Initiative [17] and the Million Women's Study [18] or currently with

raloxifene. The overall change in prescribing practices from the former practice of using HRT to prevent osteoporosis as the standard treatment to the current practice of prescribing raloxifene would be anticipated to produce a net decrease of 27,230 breast cancers. (Reprinted with permission from the European Journal of Cancer [43])

*in situ* breast cancers. However, this finding has been reported previously in both the MORE and CORE studies where raloxifene did not appear to reduce the risk of non-invasive breast cancers, although both studies had small numbers of total events. This trial confirmed that raloxifene was less stimulatory for the uterus with less uterine

hyperplasia (RR, 0.16; 95% CI, 0.09–0.29) and although there were more reported cases of uterine cancer with tamoxifen (36 vs. 23 cases), this did not reach statistical significance (RR, 0.62; 95% CI, 0.35–1.08). Higher rates of thromboembolic disease were reported for tamoxifen with 30% less events occurring in the raloxifene treated subjects

**Fig. 6** A comparison of the structure of raloxifene with newer SERMs under development for the prevention of osteoporosis but with the potential to reduce the incidence of breast cancer as a beneficial side effect. Arzoxifene has a longer biological half life than raloxifene. Basedoxifene [74] and lasofoxifene [75] are two SERMs completing evaluation for the treatment of osteoporosis with the expectation that breast cancer incidence will be reduced



(RR, 0.70; 95% CI, 0.54–0.91). Additionally, higher rates of both cataract development ( $p=0.002$ ) and patients undergoing cataract surgery ( $p=0.03$ ) were higher in the tamoxifen arms. No difference in the rates of cardiovascular disease endpoints were reported. Interestingly, numerically there were higher numbers of unrelated cancers reported in the raloxifene arm. However, the overall numbers were small and the confidence intervals were wide suggesting that chance cannot be excluded as a possible cause. This clinical trial has now provided clinicians and post-menopausal patients with two viable options for primary prevention of breast cancer.

## 9 Direct and indirect approaches to chemoprevention

SERMs have proved to be valuable chemopreventive therapies to reduce the risk of breast cancer in both premenopausal (tamoxifen) and postmenopausal (tamoxifen and raloxifene) high risk women [66]. The approach to prevent the development of disease can be described as the direct approach for breast cancer chemoprevention. However, the changing fashion in restricting the application of HRT because of the definitive evidence that HRT increases the global incidence of breast cancer [18], and a decrease in HRT users will undoubtedly result in a fall in the incidence of breast cancer. If the availability of raloxifene to substitute for HRT for the prevention of osteoporosis is added into the equation, causing a reduction in breast cancer risk, then the SERMs will have gone some way in advancing the goal of reducing breast cancer incidence and mortality. The hypothetical benefits of the progress made in the past two decades in the chemoprevention of breast cancer are shown in Fig. 5. However, raloxifene is not an optimal drug for the prevention of breast cancer and osteoporosis. There are problems with both drug absorption and rapid Phase II metabolism [67]. In response, newer SERMs are now positioned (Fig. 6) to complete testing for the prevention of osteoporosis [68] and it is anticipated that they will also be a reduction in breast cancer incidence.

In closing, it is perhaps pertinent to state the current changes in the options for women's health that have occurred with the introduction of SERMs. Two decades ago, the concept [13] that SERMs could be useful multi-functional medicines has now become a clinically validated reality. During the past decade, there have been important changes in the evolution of ideas about women's health. HRT does not provide an easy solution to prevent coronary heart disease, osteoporosis and Alzheimer's disease. The WHI [17, 69–72] and the Million Women's Study [18] have defined the price to be paid with no decreases in coronary heart disease in the elderly, increases in breast cancer and

modest but significant increases in Alzheimer's disease. There are suitable alternatives to the prevention of osteoporosis using bisphosphonates [73] but this intervention does not affect breast cancer or coronary heart disease. Statins have proven to be effective in retarding the development of arteriosclerosis and coronary heart disease. There is, however, no firm prospective evidence that these medicines reduce the incidence of breast cancer. In contrast, SERMs such as raloxifene can reduce the risk of osteoporosis and breast cancer. Admittedly raloxifene did not fulfill the promise to reduce the risk of coronary heart disease in the Raloxifene use for the Heart (RUTH) trial [63] but it is fair to say that the menu of medicines now available to prevent diseases that develop after menopause have steadily improved the prospects retarding disease development over the past 20 years.

**Acknowledgements** This manuscript is supported by the Department of Defense Breast Program under award number BC050277 Center of Excellence(VCJ) (Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense), SPORE in Breast Cancer CA 89018(VCJ), R01 GM067156(VCJ), FCCC Core Grant NIH P30 CA006927, the Avon Foundation(VCJ) and the Weg Fund of Fox Chase Cancer Center(VCJ). Dr. Swaby is also supported by the Fox Chase Intramural Translational Research Award.

## References

- Schinzinger A. Ueber carcinoma mammae [abstract] 18th Congress of the German Society for Surgery. Beilage zum Centralblatt für Chirurgie 1889;16:55–6.
- Schinzinger A. Ueber carcinoma mammae [abstract]. 18th Kongress, Berlin, April 24–27, 1889. Berlin (Germany): Hirschwald. Verhandlungen der Deutschen Gesellschaft für Chirurgie 1889;28.
- Beatson GT. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. *Lancet* 1896;2:104–7.
- Beatson CT. On treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. *Lancet* 1896;2:162–5.
- Jensen EV, Jacobson HI. Basic guides to the mechanism of estrogen action. *Recent Progr Hormone Res* 1962;18:387–414.
- 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.
- Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nature Reviews Drug Discovery* 2003;2:205–13.
- Jordan VC. Biochemical pharmacology of antiestrogen action. *Pharmacol Rev* 1984;36:245–76.
- Jordan VC. Selective estrogen receptor modulation: a personal perspective. *Cancer Res* 2001;61:5683–7.
- EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;354:1451–67.
- Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, 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.

12. Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, 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.
13. Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 1988;6:589–95.
14. Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res* 1990;50:4177–89.
15. Hersh AL, Stefanick ML, Stafford RS. National use of postmenopausal hormone therapy. *JAMA* 2004;291:47–53.
16. Riggs BL, Hartmann LC. Selective Estrogen-Receptor Modulators—Mechanisms of Action and Application to Clinical Practice. *N Engl J Med* 2003;348:618–29.
17. Writing Group for the Women's Health Initiative Investigators, Rossouw JEAG, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women. *JAMA* 2002;288:321–32.
18. Million Women Study Collaborators. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet* 2003;362:419–27.
19. Faber A, Bouvy ML, Loskamp L, van de Berg PB, Egberts ACG, de Jong-van den Berg LTW. Dramatic change in prescribing of hormonal replacement therapy in the Netherlands after publication of the Million Women Study: a follow-up study. *Br J Clin Pharmacol* 2005;60:641–7.
20. Ravdin PM, Cronin KA, Howlander N, Chlebowski RT, Berry DA. A sharp decrease in breast cancer incidence in the United States in 2003. *Breast Cancer Res and Treatment Breast Cancer Research & Treatment* 100 Supplement 1,S6:59, 2003.
21. Jordan VC. The science of selective estrogen receptor modulators: concept to clinical practice. *Clin Cancer Res* 2006;12:5010–3.
22. Jordan VC. SERMs: meeting the promise of multifunctional medicines. (Commentary). *J Natl Cancer Inst* 2007;99:350–6.
23. 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.
24. Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. an early clinical appraisal of ICI 46474. *Br J Cancer* 1971;25:270–5.
25. Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *Eur J Cancer* 1975;11:205–6.
26. Nicholson RI, Golder MP. The effect of synthetic anti-oestrogens on the growth and biochemistry of rat mammary tumours. *Eur J Cancer* 1975;11:571–9.
27. Jordan VC, Jaspan T. Tamoxifen as an antitumour agent: oestrogen binding as a predictive test for tumour response. *J Endocrinol* 1976;68:453–60.
28. Skidmore J, Walpole AL, Woodburn J. Effect of some triphenylethylenes on oestradiol binding *in vitro* to macromolecules from uterus and anterior pituitary. *J Endocrinol* 1972;52:289–98.
29. Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* 1977;75:305–16.
30. Langan-Fahey SM, Tormey DC, Jordan VC. Tamoxifen metabolites in patients on long-term adjuvant therapy for breast cancer. *Eur J Cancer* 1990;26:883–8.
31. Jordan VC, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. *Adjuv Ther Cancer* 1979;2:19–26.
32. 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.
33. Jordan VC, Allen KE, Dix CJ. Pharmacology of tamoxifen in laboratory animals. *Cancer Treat Rep* 1980;64:745–59.
34. EBCTCG. 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* 1988;319:1681–92.
35. EBCTCG. 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* 1992;339:1–15.
36. EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005;365:1687–717.
37. Jordan VC. Antitumour activity of the antioestrogen ICI 46,474 (tamoxifen) in the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model. *J Steroid Biochem* 1974;5:354.
38. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. *Eur J Cancer* 1976;12:419–24.
39. Cuzick J, Baum M. Tamoxifen and contralateral breast cancer [letter]. *Lancet* 1985;2:282.
40. Cuzick J, Powles T, Veronesi U, Forbes J, Edwards R, Ashley S, et al. Overview of the main outcomes in breast-cancer prevention trials. *Lancet* 2003;361:296–300.
41. Gail MH, Brinton LA, Byar DP, Corle DK, Green SB, Schairer C, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst* 1989;81:1879–86.
42. IBIS Investigators. First results from the International Breast Study: a randomized prevention trial. *Lancet* 2002;360:817–24.
43. Jordan VC. Optimising endocrine approaches for the chemoprevention of breast cancer. Beyond the Study of Tamoxifen and Raloxifene (STAR) Trial. *Eur J Cancer* 2006;42:2909–13.
44. Furr BJ, Jordan VC. The pharmacology and clinical uses of tamoxifen. *Pharmacol Ther* 1984;25:127–205.
45. Stearns V, Johnson MD, Rae JM, Morocho A, Novielli A, Bhargava P, 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.
46. Johnson MD, Zuo H, Lee KH, Trebley JP, Rae JM, Weatherman RV, et al. Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. *Breast Cancer Res Treat* 2004;85:151–9.
47. Jin Y, Desta Z, Stearns V, Ward B, Ho H, Lee KH, et al. CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. *JNCI* 2005;97:30–9.
48. Goetz MP, Rae JM, Suman VJ, Safgren SL, Ames MM, Visscher DW, et al. Pharmacogenetics of tamoxifen biotransformation is associated with clinical outcomes of efficacy and hot flashes. *J Clin Oncol* 2005;23:9312–8.
49. Goetz MP, Knox SK, Suman VJ, Rae JM, Safgren SL, Ames MM, et al. The impact of cytochrome P450 2D6 metabolism in women receiving adjuvant tamoxifen. *Breast Cancer Res Treat* 2007;101:113–21.
50. Garber K. Tamoxifen pharmacogenetics moves closer to reality. *J Natl Cancer Inst* 2005;97:412–3.
51. 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.
52. 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.
53. 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.

54. 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.
55. Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. *Fed Proc* 1987;46:1870–4.
56. 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.
57. Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* 1989;1: 117–20.
58. Fisher B, Costantino JP, Redmond CK, Fisher ER, Wickerham DL, Cronin WM. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14 [see comments]. *J Natl Cancer Inst* 1994;86:527–37.
59. Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, 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.
60. Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, et al. For the CORE investigators 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.
61. Cauley JA, Norton L, Lippman ME, Eckert S, Krueger KA, Purdie DW, et al. 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* 2001;65:125–34.
62. Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, 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 [see comments] [published erratum appears in *JAMA* 1999 Dec 8;282(22):2124]. *JAMA* 1999;282:637–45.
63. Barrett-Connor E, Mosca L, Collins P, Geiger MJ, Grady D, Kornitzer M, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Engl J Med* 2006;355:125–37.
64. 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.
65. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA* 2006;295:2727–41.
66. Jordan VC. Chemoprevention of breast cancer with selective estrogen-receptor modulators. *Nat Rev, Cancer* 2007;7:46–53.
67. Jordan VC. Beyond raloxifene for the prevention of osteoporosis and breast cancer. *Br J Pharmacol.* 2007;150:3–4.
68. Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *J Med Chem* 2003;46:1081–111.
69. Espeland MA, Rapp SR, Shumaker SA, Brunner R, Manson JE, Sherwin BB, et al. Conjugated equine estrogens and global cognitive function in postmenopausal women: Women's Health Initiative Memory Study. *JAMA* 2004;291:2959–68.
70. Shumaker SA, Legault C, Kuller L, Rapp SR, Thal L, Lane DS, et al. Conjugated equine estrogens and incidence of probable dementia and mild cognitive impairment in postmenopausal women: Women's Health Initiative Memory Study. *JAMA* 2004;291:2947–58.
71. Shumaker SA, Legault C, Rapp SR, Thal L, Wallace RB, Ockene JK, et al. 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. *JAMA* 2003;289:2651–62.
72. Wassertheil-Smoller S, Hendrix SL, Limacher M, Heiss G, Kooperberg C, Baird A, et al. Effect of estrogen plus progestin on stroke in postmenopausal women: the Women's Health Initiative: a randomized trial. *JAMA* 2003;289:2673–84.
73. Licata AA. Discovery, clinical development, and therapeutic uses of bisphosphonates. *Ann Pharmacother* 2005;39:668–77.
74. Miller CP, Collini MD, Tran BD, Harris HA, Kharode YP, Marzolf JT, et al. Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. *J Med Chem* 2001;44:1654–7.
75. Rosati RL, Da Silva Jardine P, Cameron KO, Thompson DD, Ke HZ, Toler SM, 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.

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

Eric A. Ariazi<sup>1,2</sup>, Andrei Leitão<sup>3</sup>, Tudor I. Oprea<sup>3</sup>, Bin Chen<sup>1</sup>, Teresa Louis<sup>1</sup>, Anne Marie Bertucci<sup>1</sup>, Catherine G.N. Sharma<sup>2</sup>, Shaun D. Gill<sup>2</sup>, Helen R. Kim<sup>2</sup>, Heather A. Shupp<sup>2</sup>, Jennifer R. Pyle<sup>2</sup>, Alexis Madrack<sup>2</sup>, Anne L. Donato<sup>2</sup>, Dong Cheng<sup>1</sup>, James R. Paige<sup>1</sup> and V. Craig Jordan<sup>1,2</sup>

<sup>1</sup>Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, Illinois; <sup>2</sup>Fox Chase Cancer Center, Philadelphia, Pennsylvania; and <sup>3</sup>Division of Biocomputing, University of New Mexico Health Sciences Center, Albuquerque, New Mexico

**Grant support:** Department of Defense Breast Program under award number BC050277 Center of Excellence (V.C. Jordan) (Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense), SPORE in Breast Cancer CA89018 (V.C. Jordan), the Avon Foundation (V.C. Jordan), the Weg Fund (V.C. Jordan), and NIH P30 CA006927 (Fox Chase Cancer Center), an Eli Lilly Fellowship (Robert H. Lurie Comprehensive Cancer Center), the Lynn Sage Breast Cancer Research Foundation (Robert H. Lurie Comprehensive Cancer Center), the NIH Molecular Libraries Initiative award U54 MH074425-01, and by NCI CA118100 (UNM Cancer Center).

**Key Words:** Breast cancer, exemestane, bone mineral density, androgen receptor, estrogen receptor, and molecular modeling

**Requests for reprints:** V. Craig Jordan, OBE, PhD, DSc, Vice President and Research Director for Medical Sciences, Alfred G. Knudson Chair of Cancer Research, Fox Chase Cancer Center,

333 Cottman Avenue, Philadelphia, PA 19111-2497. Phone: 215-728-7410; Fax: 215-728-7034.

E-mail: [v.craig.jordan@fcc.edu](mailto:v.craig.jordan@fcc.edu)

## **Abstract**

Aromatase inhibitors (AIs) are being evaluated as long-term adjuvant therapies and chemopreventives in breast cancer. However, there are concerns about bone mineral density loss in an estrogen-free environment. Unlike non-steroidal AIs, the steroidal AI exemestane may exert beneficial effects on bone through its primary metabolite 17-hydroexemestane. We investigated 17-hydroexemestane and observed it bound estrogen receptor  $\alpha$  (ER $\alpha$ ) very weakly and androgen receptor strongly. Next, we evaluated 17-hydroexemestane in MCF-7 and T47D breast cancer cells and attributed dependency of its effects on ER or AR using the antiestrogen fulvestrant or the antiandrogen bicalutamide. 17-Hydroexemestane induced proliferation, stimulated cell cycle progression (Supplementary Material) and regulated transcription at high sub-micromolar and micromolar concentrations through ER in both cell lines, but through AR at low nanomolar concentrations selectively in T47D cells. Responses of each cell type to high and low concentrations of the non-aromatizable synthetic androgen R1881 paralleled those of 17-hydroexemestane. 17-Hydroexemestane down-regulated ER $\alpha$  protein levels at high concentrations in a cell-type specific manner similarly as 17 $\beta$ -estradiol, and increased AR protein accumulation at low concentrations in both cell types similarly as R1881. Computer docking indicated that the 17 $\beta$ -OH group of 17-hydroexemestane relative to the 17-keto group of exemestane contributed significantly more intermolecular interaction energy towards binding AR than ER $\alpha$ . Molecular modeling also indicated that 17-hydroexemestane interacted with ER $\alpha$  and AR through selective recognition motifs employed by 17 $\beta$ -estradiol and R1881, respectively. We conclude that 17-hydroexemestane exerts biological effects as an androgen. These results may have important implications for long-term maintenance of patients with AIs.

## **Introduction**

The third generation aromatase inhibitors (AIs) anastrozole (Arimidex) (1, 2), letrozole (Femara) (3, 4), and exemestane (EXE; Aromasin) (5, 6), by virtue of blocking extragonadal conversion of androgens to estrogens and giving rise to an estrogen-depleted environment, exhibit improved efficacy over tamoxifen in the adjuvant therapy of estrogen receptor (ER)-positive breast cancer in postmenopausal women (7). Clinical trials evaluating these AIs demonstrated a reduced incidence of contralateral primary breast cancer in the AI groups compared to tamoxifen (1-6), hence AIs are currently being evaluated as chemopreventives in ongoing studies (8). AIs also exhibit reduced overall toxicity compared to tamoxifen (1-6, 9), but the toxicity profiles are different: tamoxifen is associated with increased incidences of thromboembolic events and endometrial cancer, while AIs are associated with decreased bone mineral density (BMD) coupled with an increased risk of bone fractures (10-12) and severe musculoskeletal pain which limits patient compliance (13, 14). Since the available third-generation AIs all exhibit similar efficacies, the selection of a specific AI for long-term adjuvant therapy of breast cancer and as a chemopreventive in healthy women at high risk for breast cancer will likely be determined by safety and tolerability profiles.

AIs fall into two classes, steroidal as represented by EXE, which acts as a suicide inhibitor of aromatase, and non-steroidal including anastrozole and letrozole, which reversibly block aromatase activity (7). Possibly due to its steroid structure, EXE may exhibit a unique pharmacology distinct from the non-steroidal AIs. In two pre-clinical studies by Goss *et al.* (15, 16), EXE was administered to female ovariectomized rats, an animal model of osteoporosis, and found to reduce bone resorption markers and increase BMD and bone strength, while lowering serum cholesterol and low-density lipoprotein levels compared to ovariectomized controls. One of these pre-clinical studies also evaluated the non-steroidal AI letrozole, but in contrast, found no benefit of letrozole on bone or lipid profiles (16). In a clinical study investigating the effects of 2 years of EXE on bone compared to placebo without prior tamoxifen therapy in patients with surgically resected breast cancer at low risk for recurrence, EXE did not enhance BMD loss in

lumbar spine, and only modestly enhanced BMD loss in the femoral neck compared to the placebo group (17). Interestingly, in this study EXE promoted bone metabolism by increasing levels of both bone resorption and formation markers (17). However, a clear-cut advantage of EXE versus the non-steroidal AIs on bone safety has not been demonstrated in humans, possibly because all other clinical studies compared the AI to tamoxifen (9, 12, 18), or the AI to placebo with prior tamoxifen therapy (10, 11). Drawing conclusions from these studies is difficult since tamoxifen preserves BMD, thereby protecting against fractures, and withdrawal of tamoxifen may have lasting effects on BMD (19).

Maintenance of BMD in women is a known estrogenic effect (20). However, androgen receptors (ARs) are also expressed in multiple bone cell types (21, 22), and studies show that androgens maintain BMD in ovariectomized rats (23, 24) and in women (21, 25-27). In ovariectomized rats, physiological concentrations of androstenedione, a weak androgen and a substrate of aromatase, reduced loss of bone, and the antiandrogen bicalutamide (BIC) abrogated this effect (23) but anastrozole did not (23). Therefore, the protective effect of androstenedione on maintenance of BMD was androgen mediated, and not due to aromatization of androstenedione to estrogen. Further, the non-aromatizable androgen 5 $\alpha$ -dihydrotestosterone has been shown to stimulate bone growth in osteopenic ovariectomized rats (24). In pre- and postmenopausal women, endogenous androgen levels correlate with BMD (25, 26). Further, a study comparing estrogen to a synthetic androgen in postmenopausal osteoporotic women showed that both steroids were equally effective in reducing bone resorption (27). Also, a two-year double blind trial showed that estrogen plus a non-aromatizable androgen significantly improved BMD over estrogen alone in surgically menopausal women (28). Therefore, exogenous androgens promote BMD maintenance in women when used alone (27) and in conjunction with estrogen (28).

While EXE does not bind ER, it is structurally related to androstenedione, and has weak affinity for AR (29, 30). At high doses, EXE exerts possible androgenic activity *in vivo* by inducing an increase in ventral prostate weight in immature castrated rats (29). Recently, Miki *et*

*al.* (22) demonstrated in human osteoblast hFOB and osteosarcoma Saos-2 cells, that EXE promoted proliferation which was partially blocked by the antiandrogen hydroxyflutamide, and increased alkaline phosphatase activity. However, metabolites of EXE may be mediating these effects. EXE is administered orally at 25 mg per day and rapidly absorbed, showing peak plasma levels within 2 to 4 hours and a direct relationship between dosage and peak plasma levels after single (10 to 200 mg) or repeated doses (0.5 to 50 mg) (30, 31). Single dose studies suggested that EXE has a short elimination half-life, but multiple-dose studies show its terminal half-life to be about 24 hours. EXE undergoes complex metabolism, and the primary metabolite in plasma has been identified as 17-hydroxexemestane (17-H-EXE), which accumulates to a concentration of about 10% of its parent compound (30). Taking the possible action of metabolites into consideration, Goss *et al.* (16) administered 17-H-EXE to ovariectomized rats and found that it produced the same bone sparing effects and favorable changes in circulating lipid levels as EXE. Also, Miki *et al.* (22) stated that 17-H-EXE promoted proliferation of the osteoblast and osteosarcoma cells similar to EXE, but the data were not shown and the authors did not further explore 17-H-EXE activities. Additionally, Miki *et al.* (22) showed that the osteoblasts efficiently metabolized androstenedione to testosterone, which involves reduction of the androstenedione's 17-keto group to a hydroxyl group. Similar metabolism would convert EXE to 17-H-EXE and thus, EXE's activities in the osteoblasts may have been mediated by a metabolite of EXE. Hence, a thorough investigation of EXE and 17-H-EXE activities through ER and AR is warranted to provide evidence regarding whether EXE could display a more favorable safety and toxicity profile than non-steroidal AIs for long-term adjuvant use and as a chemopreventative of breast cancer in postmenopausal women. Therefore, we evaluated the pharmacological actions of EXE and its primary metabolite 17-H-EXE on ER- and AR-regulated activities in a range of cellular and molecular assays. First, we determined the relative binding affinity (RBA) of 17-H-EXE to ER $\alpha$  and AR. Next, using MCF-7 and T47D breast cancer cells, we examined the ability of 17-H-EXE to stimulate cell proliferation and cell cycle progression (Supplementary Material) via ER and AR, to regulate ER- and AR-dependent transcription, and to modulate ER $\alpha$  and AR

protein levels. Lastly, we investigated intermolecular interactions between 17-H-EXE and ER $\alpha$  and AR using molecular modeling.

## **Materials and Methods**

### **Compounds and Cell Lines.**

EXE and 17-H-EXE were provided by Pfizer (Nerviano, Italy). Fulvestrant (FUL; ICI 182,780, Faslodex) and bicalutamide (BIC; Casodex) were provided by Dr. Alan E. Wakeling and Dr. Barrington J.A. Furr, respectively (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom). All other compounds were obtained from Sigma-Aldrich (St. Louis, MO) and cell culture reagents from Invitrogen (Carlsbad, CA). All test agents were dissolved in ethanol, and added to the medium at 1:1000 (v/v). MCF-7/WS8 and T47D:A18 human mammary carcinoma cells, clonally selected from their parental counterparts for sensitivity to growth stimulation by E<sub>2</sub> (32), were used in all experiments indicating MCF-7 and T47D cells. Cells were maintained in steroid-replete RPMI-1640 medium, but three days prior to all experiments, were cultured in steroid-free media as previously described (32, 33).

### **Competitive Hormone-binding Assays**

Competitive hormone-binding assays were conducted using fluorescence polarization-based ER $\alpha$  and AR Competitor Assay kits (Invitrogen, Carlsbad, CA) as previously described (34).

### **Cellular Proliferation Assays**

Cellular proliferation following 7 days in culture was determined by DNA mass per well in 12-well plates using the fluorescent DNA dye Hoechst 33258 as previously described (32).

### **Reporter Gene Assays**

Reporter gene assays were conducted by transfecting cells with either an ERE(5x)-regulated (pERE(5x)TA-ffLuc; (33)) or ARE(5x)-regulated (pAR-Luc; Panomics, Fremont, CA) firefly luciferase expression plasmid, and co-transfected with a basal TATA promoter-regulated (pTA-srLuc) *Renilla* luciferase expression plasmid as previously described (33).

### **Quantitative Real-time Polymerase Chain Reaction (qPCR)**

qPCR was used to determine AR and ribosomal large phosphoprotein subunit P0 (RLP0; 36B4) mRNA levels as previously described (35)

### **Immunoblot Analyses**

Immunoblots, prepared as previously described (33), were probed with primary antibodies against AR (AR 441; Lab Vision, Fremont, CA), ER $\alpha$  (AER 611; Lab Vision), and  $\beta$ -actin (AC-15; Sigma-Aldrich).

### **Molecular Modeling and Virtual Docking Calculations**

The 3D-conformations for E<sub>2</sub>, 17-H-EXE, EXE, R1881 and DEX were generated with Omega version 2.1 software (OpenEye Scientific Software, Santa Fe, NM). These compounds were docked using the following X-ray crystallographic structures: 1GWR (ER $\alpha$  co-complexed with E<sub>2</sub>, 2.4 Å resolution) (36) and 1XQ3 (AR co-complexed with R1881, 2.25 Å resolution) (37). ER $\alpha$  and AR ligand binding pockets were built using a ligand-centered box and the receptor-bound conformation of the respective ligand: E<sub>2</sub> (for 1GWR) and R1881 (for 1XQ3). The volume of the cavity differs for the two receptors: 648 Å<sup>3</sup> for 1GWR, and 532 Å<sup>3</sup> for 1XQ3. All receptor and ligand bonds were kept rigid. The receptor structures were filled with water since ER $\alpha$  (38) and AR crystal structures (39) indicate that specific stable hydrogen bond (H-bond) networks form among particular water molecules, ligands, and amino acid side chains. Docking was performed with FRED version 2.2 software (OpenEye), using a short refinement step for the ligands within the receptor, and using the MMFF94 force field. The best thirty conformations for each compound were compared and ranked by FRED's Chemscore function. For each ligand-docked receptor evaluated, the docked conformation with the lowest total intermolecular interaction energy (kJ/mol) was selected. To address whether water could be displaced by a compound during the process of binding, docking calculations were also performed using receptors modeled with water removed as presented in Supplementary Table 1, and the differences between the methods in Supplementary Table 2.

### **Curve Fitting and Statistical Analyses**

All statistical tests, curve fitting and determination of half maximal inhibitory concentrations (IC<sub>50</sub>s) and half maximal effective concentrations (EC<sub>50</sub>s) were performed using

GraphPad Prism 4.03 (GraphPad Software). Significant differences were determined using 1-way ANOVA with Bonferroni multiple comparison post test.

## Results

### Experimentally determined binding of 17-H-EXE and EXE to ER $\alpha$ and AR

Structures of the compounds relevant to these studies, the steroidal AI parent compound EXE, its primary metabolite 17-H-EXE, E<sub>2</sub>, and the synthetic non-aromatizable androgen R1881, are shown in Fig. 1A. Importantly, the only difference between parental EXE and its metabolite 17-H-EXE is a hydroxyl group in the metabolite in place of a ketone in the parent compound at the 17 $\beta$  position, whereas both compounds share a 3-keto group. For steroidal estrogens, elimination or modification of the 17 $\beta$ -OH group reduces binding to ER $\alpha$ , but that of the 3-OH group is much more dramatic (40). For steroidal androgens, the trend is reversed; elimination or modification of the 17 $\beta$ -OH group is more significant for AR binding than that of the 3-keto group (41). The 3-keto group found in both EXE and 17-H-EXE also favors binding to AR (41).

We tested the binding of EXE and 17-H-EXE to ER $\alpha$  and AR using fluorescence polarization-based competitive hormone binding assays (Fig. 1B-C; Table 1). For purposes of comparison, compound affinities were arbitrarily categorized with respect to their RBAs as strong (100 to  $\geq 1$ ), moderate ( $< 1$  to  $\geq 0.1$ ), weak ( $< 0.1$  to  $\geq 0.01$ ), very weak ( $< 0.01$  to detectable binding defined as 50% competition), and inactive (compound did not compete for at least 50% binding). E<sub>2</sub> competitively bound ER $\alpha$  with an IC<sub>50</sub> of  $1.33 \times 10^{-9}$  M (RBA = 100; Fig. 1B), and R1881 competitively bound AR with an IC<sub>50</sub> of  $1.34 \times 10^{-8}$  M (RBA = 100; Fig. 1C). Considering ER $\alpha$  (Fig. 1B), both R1881 and 17-H-EXE competed for binding to ER $\alpha$  with IC<sub>50</sub>s of  $1.02 \times 10^{-6}$  M (RBA = 0.130) and  $2.12 \times 10^{-5}$  M (RBA = 0.006), respectively, which categorized R1881 as a moderate and 17-H-EXE as a very weak ER $\alpha$  ligand. Neither EXE nor DEX significantly competed for binding to ER $\alpha$ . Regarding AR (Fig. 1C), 17-H-EXE and EXE competed for binding to AR with IC<sub>50</sub>s of  $3.96 \times 10^{-8}$  M (RBA = 33.8) and  $2.03 \times 10^{-6}$  M (RBA = 0.658), respectively, which classified 17-H-EXE as a strong and EXE as a weak AR ligand. However, DEX would also be categorized as a weak AR ligand. Hence, the observed very weak ER $\alpha$  binding and strong AR binding of 17-H-EXE was consistent with what previously reported

structure activity relationships (40, 41) would have predicted due to reduction of the 17-keto group in EXE to a 17 $\beta$ -OH in the metabolite.

### **Proliferation responses to 17-H-EXE and EXE**

We examined the effects of EXE and 17-H-EXE on 7 days of proliferation in ER $\alpha$ - and AR-positive MCF-7 and T47D mammary carcinoma cells (Fig. 2). As expected, both cell lines were growth stimulated by E<sub>2</sub>, with growth EC<sub>50</sub>s of  $1.7 \times 10^{-12}$  M E<sub>2</sub> for MCF-7 cells (Fig 2A), and  $7.1 \times 10^{-12}$  M E<sub>2</sub> for T47D cells (Fig 2B). These growth responses to E<sub>2</sub> were completely blocked by FUL (all *P* values < 0.001), validating the E<sub>2</sub> responsiveness via ER in these cell lines.

Both cell lines were also growth stimulated by R1881 (Fig. 2A-B) and 17-H-EXE (Fig. 2C-D), whereas EXE did not exert any significant effect on proliferation (Fig. 2C-D). Considering MCF-7 cells, R1881 exhibited a growth EC<sub>50</sub> of  $2.4 \times 10^{-8}$  M (Fig. 2A), or approximately 4 orders of magnitude higher than that of E<sub>2</sub>. Similarly, 17-H-EXE exhibited a growth EC<sub>50</sub> of  $2.7 \times 10^{-6}$  M in MCF-7 cells (Fig. 2C), or approximately 6 orders of magnitude higher than that of E<sub>2</sub>. These growth responses to R1881 and 17-H-EXE in MCF-7 cells were completely blocked by co-treatment with FUL (Fig. 2A-B; both *P* values < 0.001). Therefore, while R1881, a non-aromatizable synthetic androgen, stimulated growth of MCF-7 cells, it did so by acting through ER. Hence, at high concentrations R1881 exerted estrogenic activity. Similarly, at high concentrations 17-H-EXE also exerted estrogenic activity and stimulated growth of MCF-7 cells by acting through ER.

Interestingly, in T47D cells, the growth response to R1881 and 17-H-EXE followed an apparent bimodal pattern, which was different than in MCF-7 cells. In T47D cells, proliferative effects of high concentrations of R1881 ( $5 \times 10^{-6}$  M, Fig. 2B) and 17-H-EXE ( $5 \times 10^{-6}$  M, Fig. 2D) were only partially blocked by FUL (both *P* values < 0.001), down to the level of growth observed at nanomolar concentrations of these compounds. However, proliferative effects of lower concentrations of R1881 ( $10^{-9}$  M) and 17-H-EXE ( $10^{-8}$  M) were completely blocked by the anti-androgen BIC (both *P* values < 0.001). Based on these observed levels of inhibition by BIC

and FUL, maximal concentrations at which R1881 and 17-H-EXE stimulated growth through AR-dependent activities were  $10^{-7}$  M and  $10^{-6}$  M respectively, and above these concentrations, R1881 and 17-H-EXE stimulated growth through ER-dependent activities. Using this information to define concentration ranges in which these compounds exert AR-mediated or ER-mediated effects in T47D cells, the growth  $EC_{50}$ s via AR of R1881 and 17-H-EXE were  $1.0 \times 10^{-10}$  M (Fig. 2B) and  $4.3 \times 10^{-10}$  M (Fig. 2D), respectively. Similarly, the growth  $EC_{50}$ s via ER of R1881 and 17-H-EXE in T47D cells were  $3.1 \times 10^{-7}$  M (Fig. 2B) and  $1.5 \times 10^{-6}$  M (Fig. 2D), respectively. Hence, in T47D cells, both R1881 and 17-H-EXE stimulated growth via AR at lower concentrations and via ER at higher concentrations. These results were consistent with the observed binding affinities of these compounds to ER $\alpha$  (Fig. 1B) and AR (Fig. 1C).

#### **Cell cycle progression responses to 17-H-EXE**

As shown in Supplementary Fig. 1, 17-H-EXE at  $10^{-8}$  M acted through AR to stimulate S-phase entry in T47D cells by 1.9-fold ( $P < 0.001$ ), but at  $5 \times 10^{-6}$  M acted through ER to stimulate S-phase entry in MCF-7 cells by 2.2-fold ( $P < 0.001$ ). Hence, 17-H-EXE effects on cell cycle progression were consistent with its effects on proliferation (Fig. 2).

#### **17-H-EXE regulation of ER $\alpha$ and AR transcriptional activities**

Next, we investigated the ability of 17-H-EXE to regulate ER and AR transcriptional activity by transfecting cells with an ERE(5x)-regulated or ARE(5x)-regulated dual-luciferase plasmid set, treating cells with test compounds, and measuring dual-luciferase activity 44 h after treatment (Fig. 3A-C). E<sub>2</sub> at  $10^{-10}$  M induced ERE(5x)-regulated transcription by 19.4-fold in MCF-7 cells (Fig. 3A;  $P < 0.001$ ), and 11.3-fold in T47D cells (Fig. 3B;  $P < 0.001$ ) compared to CON-treated cells; this E<sub>2</sub>-induced transcriptional activity was blocked by FUL (both  $P$  values  $< 0.001$ ), validating dependence on ER for ERE(5x)-regulated transcription. At high sub-micromolar and micromolar concentrations, R1881 stimulated ERE(5x)-regulated transcription in both cell lines, with maximal inductions of 22.7-fold at  $5 \times 10^{-6}$  M in MCF-7 cells (Fig. 3A;  $P < 0.001$ ), and 7.9-fold at  $5 \times 10^{-6}$  M in T47D cells (Fig. 3B;  $P < 0.001$ ) compared to CON-treated cells. R1881's ability at  $5 \times 10^{-6}$  M to induce ERE(5x)-regulated transcription was blocked by

FUL (Fig. 3A-B; both  $P$  values  $< 0.001$ ), indicating that at high concentrations, R1881 acted as an estrogen. In a similar manner as R1881, 17-H-EXE stimulated ERE(5x)-regulated transcription in a concentration dependent manner at sub-micromolar and micromolar concentrations (Fig. 3A-B). At  $5 \times 10^{-6}$  M, 17-H-EXE maximally induced ERE(5x)-regulated transcription by 7.7-fold in MCF-7 cells (Fig. 3A;  $P < 0.001$ ), and 3.3-fold in T47D cells (Fig. 3B;  $P < 0.001$ ) compared to CON-treated cells; this transcriptional activation was blocked by FUL (both  $P$  values  $< 0.001$ ). Therefore, at high concentrations, 17-H-EXE acted as an estrogen and induced ER transcriptional activity.

In like manner, AR-dependent transcriptional activity was investigated. T47D cells showed a concentration-dependent induction of ARE(5x)-regulated transcription in response to R1881, with  $10^{-9}$  M R1881 inducing transcription by 8.5-fold, and  $10^{-6}$  M R1881 maximally inducing transcription by 12.7-fold relative to CON-treated cells (Fig. 3C; both  $P$  values  $< 0.001$ ). BIC blocked  $10^{-9}$  M R1881 induction of ARE(5x)-regulated transcription (Fig. 3C;  $P < 0.001$ ), confirming dependence on AR. MCF-7 cells failed to respond to  $10^{-6}$  M R1881 with induction of ARE(5x)-regulated transcription (data not shown), though these cells express AR protein. This supports our prior results that T47D cells were growth stimulated by R1881 through an AR-dependent mechanism (Fig. 2B), but that MCF-7 cells were not (Fig. 2A). As expected,  $10^{-6}$  M  $E_2$  failed to induce ARE(5x)-regulated transcription (Fig. 3C). Next, 17-H-EXE was evaluated in T47D cells, and in a concentration-dependent manner, induced ARE(5x)-regulated transcription with maximal induction of 4.7-fold occurring at  $5 \times 10^{-6}$  M relative to CON treatment (Fig. 3C;  $P < 0.001$ ). However, since high concentrations of 17-H-EXE were needed to induce this synthetic ARE(5x)-regulated promoter, we tested whether lower concentrations of 17-H-EXE could modulate endogenous AR mRNA expression, which is known to be negatively feedback-regulated by its gene product (42). Using real-time PCR, AR mRNA levels were determined in T47D cells following 24 h of treatment with test compounds (Fig. 3D). R1881 at  $10^{-9}$  M significantly down-regulated AR mRNA expression by 48% ( $P < 0.001$ ), whereas  $10^{-9}$  M  $E_2$  did not (Fig. 3D). BIC prevented R1881-mediated decrease in AR mRNA expression (Fig. 3D),

validating that AR mRNA levels were negatively feed-back regulated. Similarly, a low  $10^{-8}$  M concentration of 17-H-EXE led to a 41% decrease in AR mRNA levels ( $P < 0.01$ ), with increased 17-H-EXE concentrations further decreasing AR mRNA expression (Fig. 3D). BIC blocked 17-H-EXE-mediated down-regulation of AR mRNA expression ( $P < 0.01$ ), whereas FUL did not (Fig. 3D). Therefore, 17-H-EXE acted as an androgen via AR to feedback regulate expression of endogenous AR mRNA in T47D cells.

### **17-H-EXE modulation of AR and ER $\alpha$ protein levels**

Androgens and estrogens modulate protein expression levels of their cognate receptors. R1881 stabilizes AR protein allowing its accumulation (43), whereas  $E_2$  promotes ER $\alpha$  degradation in a cell type dependent manner (32). Therefore, we investigated the effects of 17-H-EXE on AR and ER $\alpha$  protein levels by treating cells with test compounds for 24 h, and analyzing receptor levels by immunoblotting.  $E_2$  decreased ER $\alpha$  protein levels in MCF-7 (Fig. 4A), but not T47D cells (Fig. 4B), as we have previously demonstrated (32). As expected, FUL promoted ER $\alpha$  protein degradation in both cell lines.  $E_2$  did not significantly affect AR protein accumulation in MCF-7 cells (Fig. 4A), but did down-regulate AR protein levels in T47D cells (Fig. 4B). Also, FUL and  $E_2$  plus FUL treatments did not significantly affect AR protein levels in MCF-7 cells (Fig. 4A), but did modestly up-regulate AR protein levels in T47D cells (Fig. 4B). As expected, R1881 caused an increase in accumulation of AR protein in both cell lines (Fig. 4A-B), likely by stabilizing the protein (43). Next, we characterized the effects of low  $10^{-8}$  M and high  $5 \times 10^{-6}$  M concentrations of 17-H-EXE on ER $\alpha$  and AR expression. The high  $5 \times 10^{-6}$  M concentration of 17-H-EXE led to decreased ER $\alpha$  protein levels in MCF-7 (Fig. 4A), but not in T47D cells (Fig. 4B); this pattern indicates that  $5 \times 10^{-6}$  M 17-H-EXE acted as an estrogen to regulate ER $\alpha$  protein in a cell type-dependent manner. Similar to R1881, treatment with low  $10^{-8}$  M or high  $5 \times 10^{-6}$  M concentrations of 17-H-EXE led to increased AR protein accumulation in both cell lines (Fig. 4A-B), indicating that 17-H-EXE acted as an androgen likely by stabilizing AR protein. Therefore, 17-H-EXE modulated ER $\alpha$  and AR protein accumulation as would an estrogen and an androgen, respectively.

## Molecular docking of 17-H-EXE and EXE into ER $\alpha$ and AR

To investigate the mechanism by which 17-H-EXE binds ER $\alpha$  as a very weak ligand, and AR as a strong ligand, molecular models were constructed *in silico*. The trends in the computed intermolecular interaction energies match the experimentally determined RBAs (Table 1). Superimposition of the docked and crystallographic structures of E<sub>2</sub> complexed with ER $\alpha$  (Fig. 5A) and of R1881 complexed with AR (Fig. 5B) showed the docking models recapitulate the molecular recognition patterns of the crystal structures.

Considering ER $\alpha$ , the intermolecular interaction energies of R1881 and 17-H-EXE were less favorable than E<sub>2</sub> by 1.94 and 2.76 kJ/mol, respectively, due to decreased H-bond interactions and increased steric clash (Table 1). EXE was much less favorable than E<sub>2</sub> by 4.57 kJ/mol (Table 1). Hence, the 17 $\beta$ -OH group of 17-H-EXE compared to the 17-keto group of EXE contributed -1.81 kJ/mol toward increased affinity for ER $\alpha$ . Interestingly, the docking calculations suggested that 17-H-EXE's higher affinity over EXE for ER $\alpha$  was not due to increased H-bonding mediated by the 17 $\beta$ -OH group, but rather increased lipophilic interactions (Table 1) due to a slight repositioning of the compound as a consequence of 17 $\beta$ -OH-group. In the E<sub>2</sub> docked to ER $\alpha$  model, H-bonds between E<sub>2</sub> and Glu353, Arg394, and His524 side-chains were observed (Fig. 5A). In the docked 17-H-EXE to ER $\alpha$  model (Fig. 5C), the same Arg394 and His524 interactions were maintained, except there was a loss of the Glu353 interaction. The R1881 docked to ER $\alpha$  model is shown in Supplementary Fig. 2A.

Considering AR, the intermolecular interaction energy of 17-H-EXE was only 0.8 kJ/mol less favorable than R1881, while EXE was significantly less favorable than R1881 by 6.27 kJ/mol (Table 1). Docking of 17-H-EXE to AR, compared to the parent drug EXE, indicated that 17-H-EXE exhibited improved lipophilic interactions by -2.11 kJ/mol, more favorable H-bonding interactions by -2.65 kJ/mol, and decreased steric clash by -1.08 kJ/mol. Hence, the 17 $\beta$ -OH group in 17-H-EXE compared to the 17-keto group in EXE contributed -5.47 kJ/mol towards higher affinity for binding AR (Table 1). In the R1881 docked to AR model, H-bonds between R1881 and Asn705, Gln711 and Arg752 were observed (Fig. 5B). The OH side-chain of

Thr877 was in close proximity to both docked R1881 (Fig. 5B) and 17-H-EXE (Fig. 5D), but the angle was not favorable for H-bonding. Docking of 17-H-EXE to AR (Fig. 5D) indicated a short 2.78 Å H-bond between the ligand's 17β-OH group and Asn705, but not between the ligand's 3-keto group and Gln711 and Arg752. Hence, the short 2.78 Å H-bond observed in the 17-H-EXE docked to AR model was important in mediating high affinity binding. The EXE docked to AR model is shown in Supplementary Fig. 2B.

## Discussion

We observed that 17-H-EXE, the primary metabolite of EXE, bound to ER $\alpha$  as a very weak ligand, and acted through ER at high sub-micromolar and micromolar concentrations to stimulate growth, induce ERE-regulated reporter gene expression, and down-modulate ER $\alpha$  protein levels in breast cancer cells. However, we also observed that 17-H-EXE bound to AR as a strong ligand, and found in T47D cells that 17-H-EXE stimulated growth, down-modulated AR mRNA expression, and stabilized AR protein levels, with all of these effects occurring at low nanomolar concentrations and blocked by BIC. Moreover, computer docking indicated that the 17 $\beta$ -OH group of 17-H-EXE versus the 17-keto group of EXE contributed significantly more towards increasing affinity to AR than to ER $\alpha$ . Molecular modeling also indicated that 17 $\beta$ -OH group of 17-H-EXE interacted with AR through an important H-bond of Asn705, a conserved recognition motif employed by R1881. Therefore, we propose that the primary mechanism of action of EXE *in vivo* is mediated by 17-H-EXE regulating AR activities.

The FDA label for EXE (Aromasin; Pfizer) reports that in postmenopausal women with advanced breast cancer, the mean AUC (area under the curve) values of EXE following repeated doses was 75.4 ng·h/ml (254 nmol·h/l), which was almost twice that in healthy postmenopausal women (41.4 ng·h/ml; 140 nmol·h/l) (31). Since circulating levels of 17-H-EXE can reach about one-tenth the level of the parent compound (30), we hypothesize that circulating levels of 17-H-EXE are sufficient to bind AR and regulate AR-dependent activities. Further, a subpopulation of patients may exist who metabolize EXE at higher rates, leading to correspondingly higher circulating 17-H-EXE levels. For instance, one patient administered 800 mg of EXE achieved 17-H-EXE plasma levels approximately one-half the level of the parent compound (30). Based on our results, we would predict that higher circulating levels of 17-H-EXE would associate with decreased rates of BMD loss and risk of bone fractures in postmenopausal women. We suggest that circulating levels of 17-H-EXE and EXE should be determined in clinical trials and correlated to disease outcome and toxicity profiles such as BMD loss.

While the clinical studies reported thus far were not designed to directly compare one AI versus another, comparisons in the rate of BMD loss from baseline to year 1, and from year 1 to 2 can be made. In the bone safety subprotocol of the IES (Intergroup Exemestane Study) trial, the rate of BMD loss was greatest within 6 months of switching from tamoxifen to EXE at -2.7% in the lumbar spine and -1.4% in the hip, but thereafter, BMD loss progressively slowed in months 6-12 and again in months 12-24 to only -1.0% and -0.8% in the lumbar spine and hip, respectively (10), which is in the same range as would be expected for postmenopausal women in general. However, in the bone safety substudy of the MA.17 trial, patients administered letrozole experienced a relatively constant rate of BMD loss for 2 years: at 12 months the rate of BMD loss from baseline was -3.3% and -1.43% in lumbar spine and hip, respectively, and from year 1 to year 2, -2.05% and -2.17% in lumbar spine and hip, respectively (11). In the bone substudy of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial, the rate of BMD loss from baseline to year 1 was -2.2% in lumbar spine and -1.5% in hip, and from year 1 to year 2, -1.8% in lumbar spine and -1.9% in hip (18). Collectively, these results suggest that after the initial 12 months of AI therapy, EXE may be associated with slower rates of BMD loss compared to non-steroidal AIs. Furthermore, although not directly comparable, the fracture rate per 1000 woman-years in the ATAC trial was 22.6 for anastrozole and 15.6 for tamoxifen (1), while in the IES trial, the incidence rate per 1000 woman-years for multiple fractures was 19.2 for EXE and 15.1 for tamoxifen (10). These results show that while both anastrozole and EXE were associated with higher fracture rates than tamoxifen, they also suggest that EXE may be associated with a lower fracture rate than anastrozole. Clinical trials now underway to directly compare the different AIs will hopefully provide clear results.

Androgens regulate growth of normal and neoplastic mammary cells in a cell type-specific manner, either by inhibiting or stimulating growth (44). However, the mechanisms by which androgens via AR regulate breast cancer growth remain elusive. Female AR knockout mice exhibit decreased ductal branching and terminal end buds in prepubertal animals and retarded lobuloalveolar development in adult animals (45). Likewise, targeted disruption of AR

in MCF-7 cells also leads to severe inhibition of proliferation (45). Epidemiological analyses indicate a positive correlation between androgen levels and the incidence of breast cancer; meta-analysis from 9 prospective studies showed that a doubling in testosterone concentrations in postmenopausal women translated into an increased relative risk of 1.42 unadjusted and 1.32 adjusted for E<sub>2</sub> (46). AR status in breast cancer associates with both positive and negative indicators and clinical outcome. AR expression has been found in 84% (47) to 91% (48) of clinical breast cancers, and associated with ER status, but has also been found in 49% of ER-negative tumors (49). Patients with tumors that co-express AR with ER and progesterone receptor have shown longer disease-free survival (DFS) than patients whose tumors were negative for all three receptors (48), but AR protein levels have also served as an independent predictor of axillary metastases in multivariate analysis (47). Further, AR expression has correlated with decreased histopathological grade, greater age, and postmenopausal status, but also lymph node-positive status (50). In AR-positive/ER-negative tumors, AR expression again associated with positive and negative indicators/outcome such as increased age, postmenopausal status, and longer DFS but also tumor grade, tumor size, and HER-2/neu overexpression (49).

Patients who fail AI therapy, whether the AI was steroidal or non-steroidal, likely harbor tumor cells which have been selected for growth in an estrogen-depleted environment, and hence are not dependent on ER activity for survival. Not all androgens are metabolized by aromatase to estrogens; for instance, dihydrotestosterone cannot be converted to an estrogen by aromatase (44). Thus, a possible mechanism for failure of AI therapy in the clinic is androgen-stimulated breast cancer growth, a largely unrecognized alternative mechanism. We observed cellular proliferation of T47D cells in response to R1881 and 17-H-EXE, and these effects were blocked by BIC. Therefore, T47D cells contain a functional AR signaling pathway that promoted growth in the absence of estrogen. Since functional AR signaling could be etiologically involved in a subpopulation of clinical breast cancers, those patients who have AR-positive tumors and achieve high circulating levels of 17-H-EXE, yet whose disease progresses while on EXE therapy, may respond to AR-based therapy such as the antiandrogen BIC.

## **Acknowledgements**

We thank Dr. Alan E. Wakeling and Dr. Barrington J.A. Furr, both of AstraZeneca Pharmaceuticals (Macclesfield, United Kingdom) for providing FUL and BIC, respectively. We also thank members of the Jordan laboratory for helpful discussions, and Dr. Jennifer L. Ariazi (GlaxoSmithKline, Collegeville, PA) for critical review of the manuscript.

## References

1. Howell A, Cuzick J, Baum M, 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;365:60-2.
2. Jonat W, Gnant M, Boccardo F, et al. Effectiveness of switching from adjuvant tamoxifen to anastrozole in postmenopausal women with hormone-sensitive early-stage breast cancer: a meta-analysis. *Lancet Oncol* 2006;7:991-6.
3. Coates AS, Keshaviah A, Thurlimann B, et al. 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* 2007;25:486-92.
4. Goss PE, Ingle JN, Martino S, et al. 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* 2005;97:1262-71.
5. Coombes RC, Hall E, Gibson LJ, et al. A Randomized Trial of Exemestane after Two to Three Years of Tamoxifen Therapy in Postmenopausal Women with Primary Breast Cancer. *N Engl J Med* 2004;350:1081-92.
6. Coombes RC, Kilburn LS, Snowdon CF, et al. Survival and safety of exemestane versus tamoxifen after 2-3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial. *Lancet* 2007;369:559-70.
7. 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.
8. Howell A, Clarke RB, Evans G, et al. Estrogen deprivation for breast cancer prevention. *Recent Results Cancer Res* 2007;174:151-67.
9. Buzdar A, Howell A, Cuzick J, et al. Comprehensive side-effect profile of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: long-term safety analysis of the ATAC trial. *Lancet Oncol* 2006;7:633-43.

10. Coleman RE, Banks LM, Girgis SI, et al. Skeletal effects of exemestane on bone-mineral density, bone biomarkers, and fracture incidence in postmenopausal women with early breast cancer participating in the Intergroup Exemestane Study (IES): a randomised controlled study. *Lancet Oncol* 2007;8:119-27.
11. Perez EA, Josse RG, Pritchard KI, et al. Effect of letrozole versus placebo on bone mineral density in women with primary breast cancer completing 5 or more years of adjuvant tamoxifen: a companion study to NCIC CTG MA.17. *J Clin Oncol* 2006;24:3629-35.
12. Eastell R, Hannon RA, Cuzick J, Dowsett M, Clack G, Adams JE. Effect of an aromatase inhibitor on bmd and bone turnover markers: 2-year results of the Anastrozole, Tamoxifen, Alone or in Combination (ATAC) trial (18233230). *J Bone Miner Res* 2006;21:1215-23.
13. Morales L, Pans S, Paridaens R, et al. Debilitating musculoskeletal pain and stiffness with letrozole and exemestane: associated tenosynovial changes on magnetic resonance imaging. *Breast Cancer Res Treat* 2006.
14. Mackey J, Gelmon K. Adjuvant aromatase inhibitors in breast cancer therapy: significance of musculoskeletal complications. *Curr Opin Oncol* 2007;19 Suppl 1:S9-S18.
15. Goss PE, Qi S, Josse RG, et al. The steroidal aromatase inhibitor exemestane prevents bone loss in ovariectomized rats. *Bone* 2004;34:384-92.
16. Goss PE, Qi S, Cheung AM, Hu H, Mendes M, Pritzker KP. Effects of the steroidal aromatase inhibitor exemestane and the nonsteroidal aromatase inhibitor letrozole on bone and lipid metabolism in ovariectomized rats. *Clin Cancer Res* 2004;10:5717-23.
17. Lonning PE, Geisler J, Krag LE, et al. Effects of exemestane administered for 2 years versus placebo on bone mineral density, bone biomarkers, and plasma lipids in patients with surgically resected early breast cancer. *J Clin Oncol* 2005;23:5126-37.
18. Coleman RE, Group AT. Effect of anastrozole on bone mineral density: 5-year results from the 'Arimidex', Tamoxifen, Alone or in Combination (ATAC) trial. *J Clin Oncol (Meeting Abstracts)* 2006;24:511.

19. 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.
20. Cummings SR, Browner WS, Bauer D, et al. Endogenous Hormones and the Risk of Hip and Vertebral Fractures among Older Women. *N Engl J Med* 1998;339:733-8.
21. Hofbauer LC, Khosla S. Androgen effects on bone metabolism: recent progress and controversies. *Eur J Endocrinol* 1999;140:271-86.
22. Miki Y, Suzuki T, Hatori M, et al. Effects of aromatase inhibitors on human osteoblast and osteoblast-like cells: a possible androgenic bone protective effects induced by exemestane. *Bone* 2007;40:876-87.
23. Lea CK, Flanagan AM. Physiological plasma levels of androgens reduce bone loss in the ovariectomized rat. *Am J Physiol* 1998;274:E328-35.
24. Tobias JH, Gallagher A, Chambers TJ. 5 alpha-Dihydrotestosterone partially restores cancellous bone volume in osteopenic ovariectomized rats. *Am J Physiol* 1994;267:E853-9.
25. Buchanan JR, Hospodar P, Myers C, Leuenberger P, Demers LM. Effect of excess endogenous androgens on bone density in young women. *J Clin Endocrinol Metab* 1988;67:937-43.
26. Dixon JE, Rodin A, Murby B, Chapman MG, Fogelman I. Bone mass in hirsute women with androgen excess. *Clin Endocrinol (Oxf)* 1989;30:271-7.
27. Riggs BL, Jowsey J, Goldsmith RS, Kelly PJ, Hoffman DL, Arnaud CD. Short- and long-term effects of estrogen and synthetic anabolic hormone in postmenopausal osteoporosis. *J Clin Invest* 1972;51:1659-63.
28. Barrett-Connor E, Young R, Notelovitz M, et al. A two-year, double-blind comparison of estrogen-androgen and conjugated estrogens in surgically menopausal women. Effects on bone mineral density, symptoms and lipid profiles. *J Reprod Med* 1999;44:1012-20.
29. di Salle E, Giudici D, Ornati G, et al. 4-Aminoandrostenedione derivatives: a novel class of irreversible aromatase inhibitors. Comparison with FCE 24304 and 4-hydroxyandrostenedione. *J Steroid Biochem Mol Biol* 1990;37:369-74.

30. Evans TR, Di Salle E, Ornati G, et al. Phase I and endocrine study of exemestane (FCE 24304), a new aromatase inhibitor, in postmenopausal women. *Cancer Res* 1992;52:5933-9.
31. Pfizer Inc. Product Information: Aromasin, exemestane tablets. NY, NY 10017; 2007.
32. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 1996;56:2321-30.
33. Ariazi EA, Kraus RJ, Farrell ML, Jordan VC, Mertz JE. Estrogen-related receptor alpha1 transcriptional activities are regulated in part via the ErbB2/HER2 signaling pathway. *Mol Cancer Res* 2007;5:71-85.
34. Abdelrahim M, Ariazi E, Kim K, et al. 3-Methylcholanthrene and other aryl hydrocarbon receptor agonists directly activate estrogen receptor alpha. *Cancer Res* 2006;66:2459-67.
35. Bauer JA, Thompson TA, Church DR, Ariazi EA, Wilding G. Growth inhibition and differentiation in human prostate carcinoma cells induced by the vitamin D analog 1alpha,24-dihydroxyvitamin D2. *Prostate* 2003;55:159-67.
36. Warnmark A, Treuter E, Gustafsson JA, Hubbard RE, Brzozowski AM, Pike AC. Interaction of transcriptional intermediary factor 2 nuclear receptor box peptides with the coactivator binding site of estrogen receptor alpha. *J Biol Chem* 2002;277:21862-8.
37. He B, Gampe RT, Jr., Kole AJ, et al. Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance. *Mol Cell* 2004;16:425-38.
38. Brzozowski AM, Pike AC, Dauter Z, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 1997;389:753-8.
39. Matias PM, Donner P, Coelho R, et al. Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. *J Biol Chem* 2000;275:26164-71.
40. Fang H, Tong W, Shi LM, et al. Structure-activity relationships for a large diverse set of natural, synthetic, and environmental estrogens. *Chem Res Toxicol* 2001;14:280-94.

41. Fang H, Tong W, Branham WS, et al. Study of 202 natural, synthetic, and environmental chemicals for binding to the androgen receptor. *Chem Res Toxicol* 2003;16:1338-58.
42. Wolf DA, Herzinger T, Hermeking H, Blaschke D, Horz W. Transcriptional and posttranscriptional regulation of human androgen receptor expression by androgen. *Mol Endocrinol* 1993;7:924-36.
43. Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM. Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. *Mol Endocrinol* 1995;9:208-18.
44. Somboonporn W, Davis SR. Testosterone Effects on the Breast: Implications for Testosterone Therapy for Women. *Endocr Rev* 2004;25:374-88.
45. Yeh S, Hu Y-C, Wang P-H, et al. Abnormal Mammary Gland Development and Growth Retardation in Female Mice and MCF7 Breast Cancer Cells Lacking Androgen Receptor. *J Exp Med* 2003;198:1899-908.
46. Group EHaBCC. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. *J Natl Cancer Inst* 2002;94:606-16.
47. Soreide JA, Lea OA, Varhaug JE, Skarstein A, Kvinnsland S. Androgen receptors in operable breast cancer: relation to other steroid hormone receptors, correlations to prognostic factors and predictive value for effect of adjuvant tamoxifen treatment. *Eur J Surg Oncol* 1992;18:112-8.
48. Kuenen-Boumeester V, Van der Kwast TH, Claassen CC, et al. The clinical significance of androgen receptors in breast cancer and their relation to histological and cell biological parameters. *Eur J Cancer* 1996;32A:1560-5.
49. Agoff SN, Swanson PE, Linden H, Hawes SE, Lawton TJ. Androgen receptor expression in estrogen receptor-negative breast cancer. Immunohistochemical, clinical, and prognostic associations. *Am J Clin Pathol* 2003;120:725-31.

50. Bieche I, Parfait B, Tozlu S, Lidereau R, Vidaud M. Quantitation of androgen receptor gene expression in sporadic breast tumors by real-time RT-PCR: evidence that MYC is an AR-regulated gene. *Carcinogenesis* 2001;22:1521-6.

## Tables

**Table 1.** Compound affinity for ER $\alpha$  and AR determined experimentally using a competitive hormone binding assay (Fig. 1B-C), and by computer docking in which receptors were modeled as filled with water.

Compound	Receptor	Competitive Hormone Binding			Intermolecular Interaction Energy (kJ/mol)				
		IC <sub>50</sub> (M)	95% CI (M)	RBA (%)	Total Score	Lipophilic	H-Bond	Steric Clash	RTB Penalty
E <sub>2</sub>	ER $\alpha$	1.33×10 <sup>-9</sup>	1.18 – 1.49×10 <sup>-9</sup>	100	-31.90	-25.96	-6.00	0.06	0
R1881	ER $\alpha$	1.02 × 10 <sup>-6</sup>	0.90 – 1.15×10 <sup>-6</sup>	0.130	-29.96	-26.01	-4.32	0.37	0
17-H-EXE	ER $\alpha$	2.12 × 10 <sup>-5</sup>	1.73 – 2.61×10 <sup>-5</sup>	0.006	-29.14	-27.73	-3.34	1.93	0
EXE	ER $\alpha$	NA*			-27.33	-25.98	-3.34	1.99	0
DEX	ER $\alpha$	NA*			-23.71	-29.70	-4.18	9.07	1.10
R1881	AR	1.34 × 10 <sup>-8</sup>	1.00 – 1.79×10 <sup>-8</sup>	100	-32.75	-28.47	-4.56	0.28	0
17-H-EXE	AR	3.96 × 10 <sup>-8</sup>	2.74 – 5.71×10 <sup>-8</sup>	33.8	-31.95	-30.54	-4.76	3.35	0
EXE	AR	2.03 × 10 <sup>-6</sup>	1.39 – 2.97×10 <sup>-6</sup>	0.658	-26.48	-28.80	-2.11	4.43	0
DEX	AR	1.03 × 10 <sup>-5</sup>	0.75 – 1.43×10 <sup>-5</sup>	0.130	-24.53	-32.21	-2.49	9.07	1.10

\*NA, not applicable; test compound did not compete for at least 50% binding of ER $\alpha$ .

RBA, relative binding affinity; H-bond, hydrogen bond; RTB Penalty, rotatable bond penalty.

## Figure Legends

**Figure 1.** Compounds examined in this study and their RBAs for ER $\alpha$  and AR. **(A)** Structures of EXE, its primary metabolite 17-H-EXE, E<sub>2</sub>, and R1881. **(B)** ER $\alpha$  and **(C)** AR fluorescence polarization-based competitive hormone binding assays. Baculovirus-produced human ER $\alpha$  and rat AR ligand-binding domain tagged with a His-GST epitope (His-GST-AR<sub>LBD</sub>) were used at final concentrations of 15 nM and 25 nM, respectively. The fluorescently-labeled ER $\alpha$  and AR ligands, Fluormone ES2 and Fluormone AL Green, respectively, were both used at a final concentration of a 1 nM. The competing test compounds were E<sub>2</sub>, R1881, 17-H-EXE, EXE and DEX, as indicated. Each data point represents the mean of triplicate determinations and error bars are 95% confidence intervals (CIs). Curve fitting was performed using GraphPad Prism software (version 4.03). IC<sub>50</sub>s corresponding to a half-maximal shift in polarization values of the test compounds were determined using the maximum and minimum polarization values of the E<sub>2</sub>-competitive binding curve for ER $\alpha$  or of the R1881-competition binding curve for AR, as appropriate.

**Figure 2.** 17-H-EXE and R1881 stimulate cellular proliferation. DNA-based cellular proliferation assays of **(A)** MCF-7 cells treated with E<sub>2</sub> and R1881, **(B)** T47D cells treated with E<sub>2</sub> and R1881, **(C)** MCF-7 cells treated with EXE and 17-H-EXE, and **(D)** T47D cells treated with EXE and 17-H-EXE. Cells were cultured in steroid-free medium for 3 days before the assays. MCF-7 cells were seeded at 15,000 cells per well, and T47D cells at 20,000 cells per well in 12-well plates. Cells were treated on days 0 (the day after seeding), 3 and 6, and then collected on day 7. Cellular DNA quantities were determined using the fluorescent DNA-binding dye Hoechst 33258 and compared against a standard curve. Data shown represent the mean of 4 replicates and standard deviations. DNA values were fitted to a sigmoidal dose response curve and growth EC<sub>50</sub>s calculated using GraphPad Prism 4.03 software. At high concentrations, 17-H-

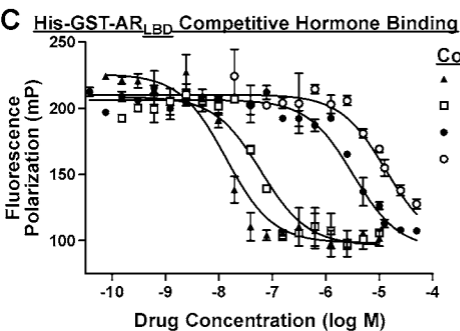
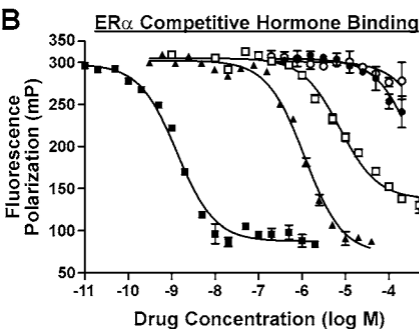
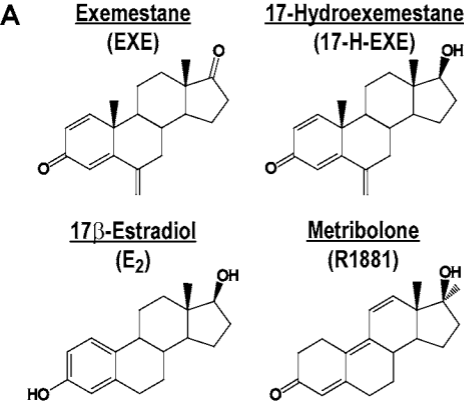
EXE and R1881 increased growth via ER in both cell lines, but at low concentrations, stimulated growth via AR selectively in T47D cells.

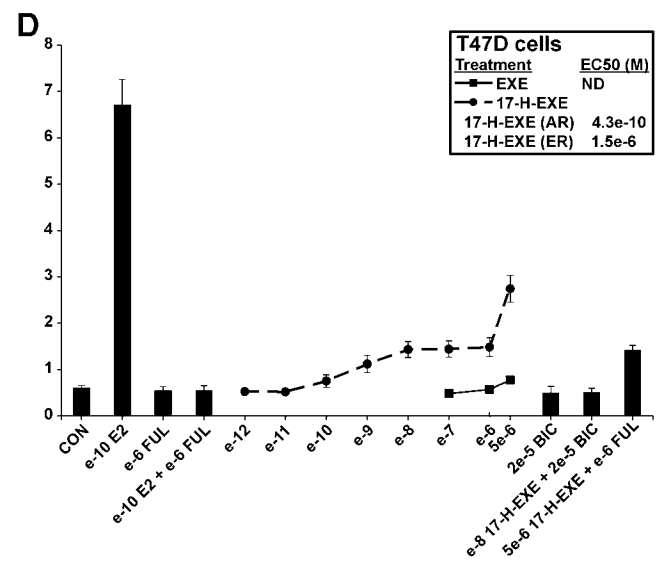
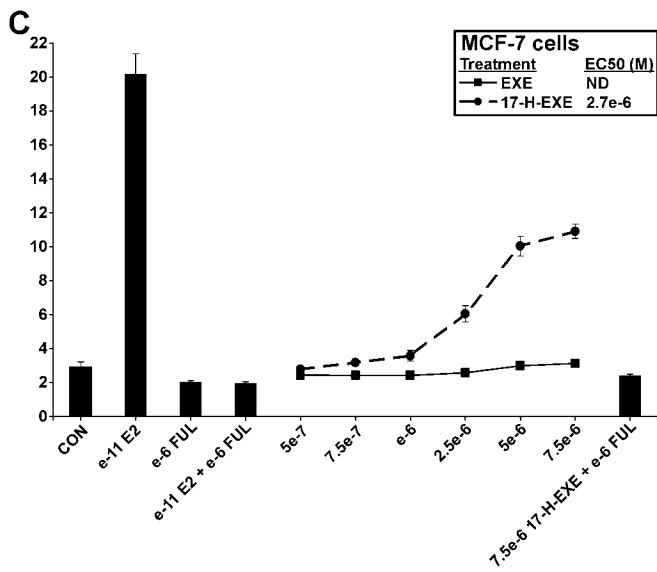
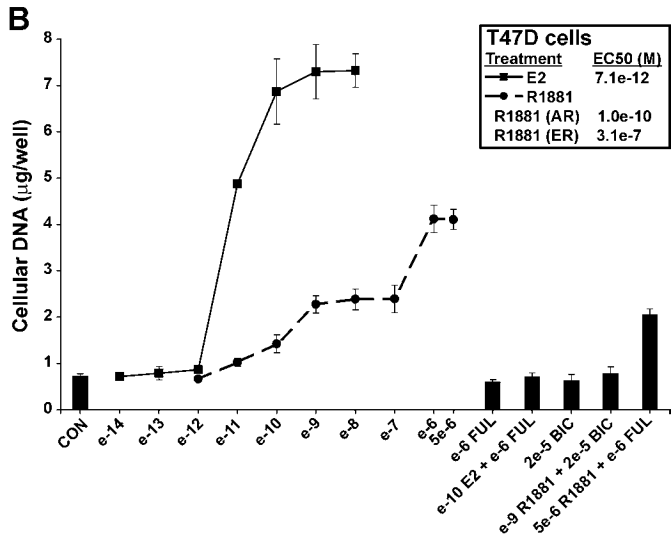
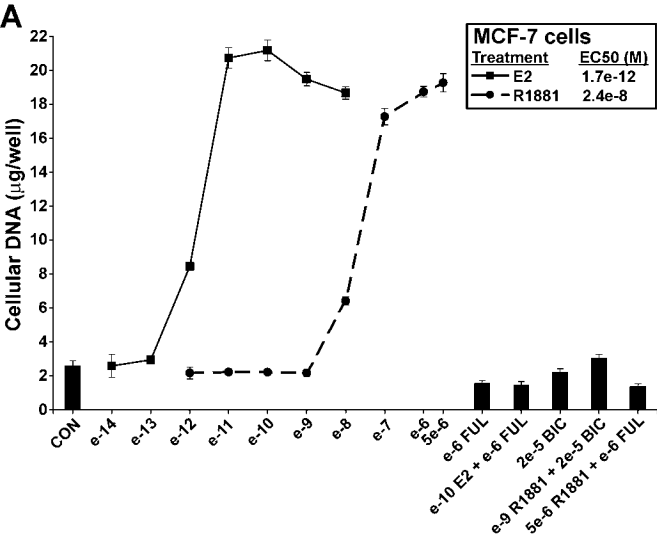
**Figure 3.** 17-H-EXE and R1881 regulate ER transcriptional activity at high concentrations and AR transcriptional activity at low concentrations. ERE(5x)-regulated dual-luciferase activity in (A) MCF-7 cells and (B) T47D cells. (C) ARE(5x)-regulated reporter gene activity in T47D cells. A-C, Under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

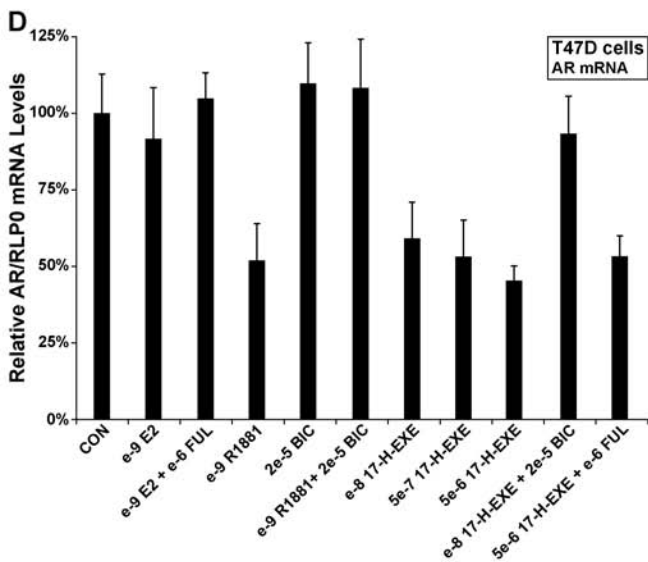
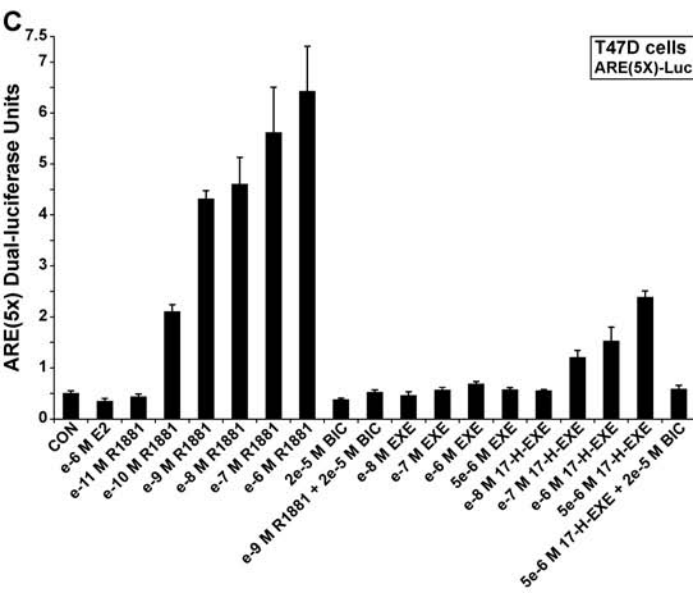
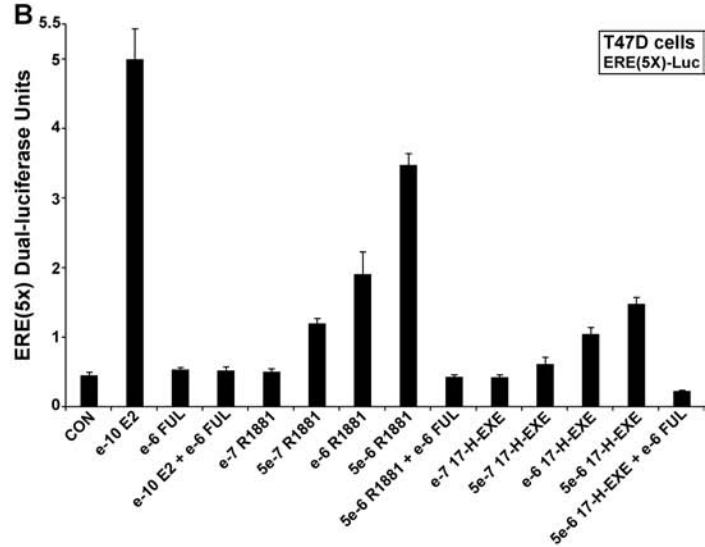
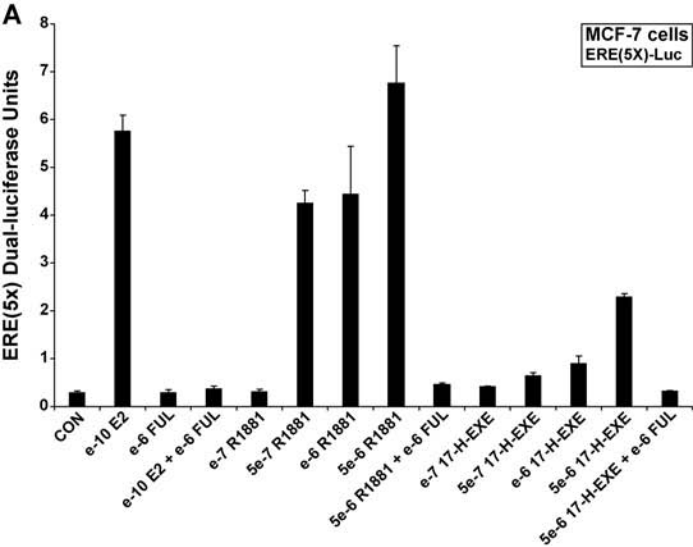
**Figure 4.** 17-H-EXE modulates AR and ER $\alpha$  protein levels. Immunoblot analysis of AR and ER $\alpha$  in (A) MCF-7 cells and (B) T47D cells. Cells were treated as indicated for 24 h and 20  $\mu$ g of cellular protein were resolved by 4-12% SDS-PAGE, and then transferred to a nylon membrane. Membranes were probed for AR, ER $\alpha$  and  $\beta$ -actin, and immunoreactive bands were visualized by chemiluminescence and autoradiography. Cropped blots are shown. 17-H-EXE up-

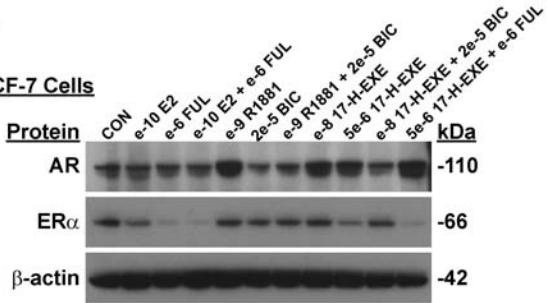
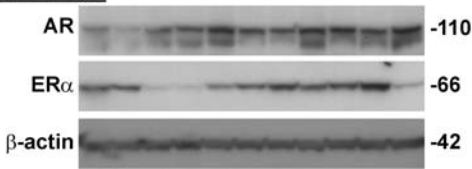
regulated AR protein levels at  $10^{-8}$  M in both cell lines, and down-regulated ER $\alpha$  in MCF-7 cells at  $5 \times 10^{-6}$  M.

**Figure 5.** Intermolecular interactions of ligands complexed with ER $\alpha$  and AR by computer docking. **(A)** Superposition of E<sub>2</sub> from the X-ray crystal structure (gray) and modeled E<sub>2</sub> (yellow) docked to ER $\alpha$ . **(B)** Superposition of R1881 from the crystal structure (gray) and modeled R1881 (yellow) docked to AR. **(C)** Modeled 17-H-EXE docked to ER $\alpha$ . **(D)** Modeled 17-H-EXE docked to AR. Hydrogen, oxygen, and nitrogen atoms are shown in cyan, red, and blue, respectively. The carbon backbone of the protein is shown in green. Hydrogens from the X-ray crystal conformations of E<sub>2</sub> (A) and R1881 (C) were omitted. H-bonds were shown to the modeled compound conformations only. Intermolecular H-bonds up to 3.5 Å are shown as black dashed lines and their length in angstroms indicated.

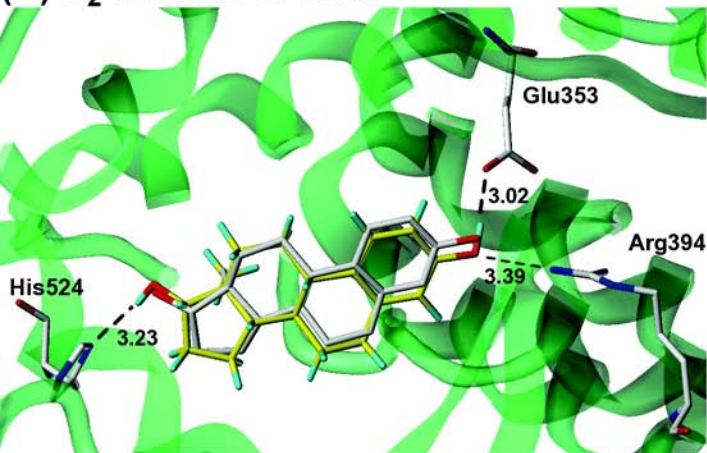




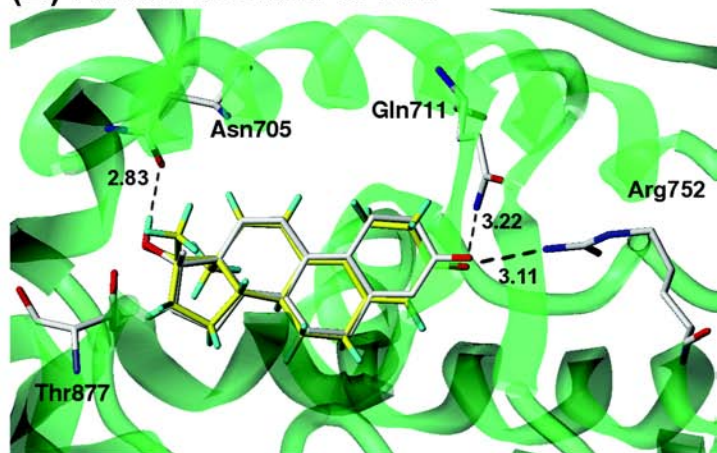


**A****MCF-7 Cells****B T47D Cells**

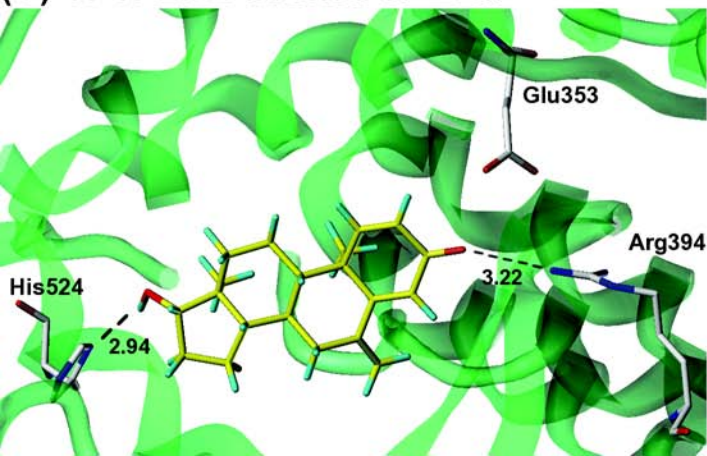
**(A) E<sub>2</sub> docked to ER $\alpha$**



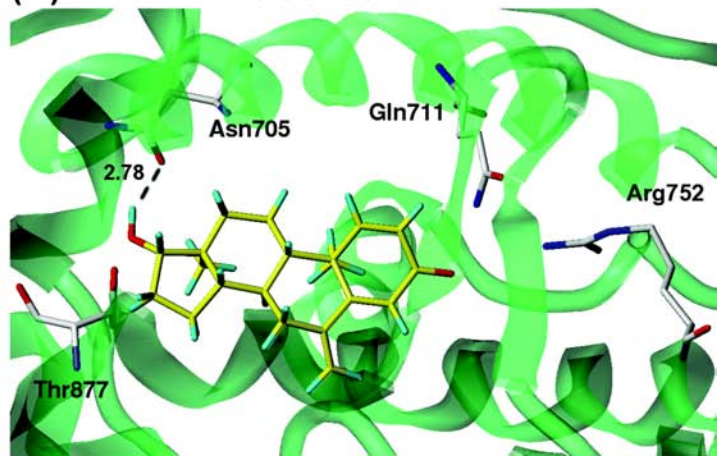
**(B) R1881 docked to AR**



**(C) 17-H-EXE docked to ER $\alpha$**



**(D) 17-H-EXE docked to AR**



## Comparative Global Gene Expression Profiling to Identify Unifying and Selective Pathways Involved in Tamoxifen, Raloxifene, and Aromatase Inhibitor-resistant Breast Cancer Xenograft Tumors

Eric A. Ariazi<sup>1</sup>, Heather E. Cunliffe<sup>2</sup>, Amanda L. Willis<sup>2</sup>, Catherine M. Mancini<sup>2</sup>,  
Yoganand Balagurunathan<sup>2</sup>, Shaun D. Gill<sup>1</sup>, Jennifer R. Pyle<sup>1</sup>, Heather A. Shupp<sup>1</sup>, V.  
Craig Jordan<sup>1</sup>

<sup>1</sup>Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, <sup>2</sup>Translational Genomics Research Institute, 445 N Fifth Street, Phoenix, AZ 85004

We compared global gene expression profiles across multiple *in vivo* breast cancer models of resistance to the selective estrogen receptor modulators (SERMs) tamoxifen (TAM) and raloxifene (RAL), and of aromatase inhibitor (AI) resistance to identify unifying and selective pathways involved in their etiology. The *in vivo* models of antihormone resistance were developed by either 1) serially transplanting MCF-7 tumors into SERM-treated immunocompromised mice for several years to mimic SERM treatment in the clinic and resulting in the MCF-7/RAL1 and MCF-7/TAM2 models, or 2) culturing MCF-7 cells in the presence of a SERM or under estrogen-deprived conditions (as a surrogate for AIs) for several years *in vitro*, and then injecting the resistant cells into immunocompromised mice to generate xenograft MCF-7/RAL2 and AI-resistant MCF-7/5C tumors. These tumor models have allowed us to define Phase I and Phase II antihormonal resistance. Phase I SERM-resistant (*i.e.* MCF-7/RAL1) tumors were growth stimulated in response to either the SERM or E<sub>2</sub>. Phase II SERM (MCF-7/RAL2 and MCF-7/TAM2) and AI-resistant (MCF-7/5C) tumors, had paradoxically underwent rapid regression due to apoptosis when treated with E<sub>2</sub>. Array-based gene expression profiling was conducted using Agilent 22K expression arrays. RNA from each antihormone-resistant model treated with the SERM or deprived of estrogen as appropriate was hybridized against wild-type estrogen-stimulated MCF-7 tumor RNA. Genes with a high coefficient of variation computed across all samples were examined by hierarchical clustering and multi-dimensional scaling analyses. Each of the models showed a distinct pattern of gene expression, yet the Phase 2 models clustered together. There were 650 genes in common to all the antihormone-resistant tumor models, and 453 genes selective to the Phase 2 resistant tumors, that were 2-fold differentially expressed compared to wild-type MCF-7 tumors. We are currently investigating molecular pathways indicated by the gene expression changes to understand mechanisms associated with Phase 1 and Phase 2 antihormone resistance. Phase II antihormonal resistance has not yet been widely recognized, but could be exploited by developing a novel treatment based on short-term, low-dose estrogen for patients who fail exhaustive endocrine therapy.

Grant support: Department of Defense Breast Program under award number BC050277 Center of Excellence (V.C. Jordan) (Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense), SPORE in Breast Cancer CA89018 (V.C. Jordan), the Avon Foundation (V.C. Jordan), the Weg Fund (V.C. Jordan), and NIH P30 CA006927 (Fox Chase Cancer Center).

**Long-term estrogen deprivation of breast cancer cells causes significant genomic evolution and development of enhanced malignant behavior.**

**Catherine M. Mancini<sup>1</sup>, Joan S. Lewis-Wambi<sup>2</sup>, Eric A. Ariazi<sup>2</sup>, Helen R. Kim<sup>2</sup>,  
Amanda L. Willis<sup>1</sup>, V. Craig Jordan<sup>2</sup> and Heather Cunliffe<sup>1</sup>**

Suppression of estrogen synthesis using aromatase inhibitors is highly effective in the treatment of postmenopausal women with estrogen-receptor alpha (ER $\alpha$ )-positive breast cancer. Third generation aromatase inhibitors are superior to adjuvant tamoxifen resulting in improved disease-free survival and a lower incidence of side effects. Unfortunately, one of the consequences of long-term estrogen deprivation or exhaustive endocrine therapy is the development of drug resistance. Previous studies have shown the acquisition of tamoxifen resistance in wild-type MCF-7 breast cancer cells is accompanied by a dramatic and significant increase in malignant cell behavior. At present, however, it is not known whether acquired resistance to long-term estrogen deprivation increases the malignant phenotype of breast cancer cells.

MCF-7:5C and MCF-7:2A are two ER $\alpha$ -positive human breast cancer cell lines derived from long-term estrogen deprivation of wild type hormone-dependent MCF-7 cells. MCF-7 cells grown in estrogen-depleted growth medium grew slowly (6-day doubling time) whereas MCF-7:5C and MCF-7:2A cells grew robustly in the absence of estrogen (doubling times of 2.7 and 3.4 days, respectively). Importantly, MCF-7:5C and MCF-7:2A cells were found to be significantly more invasive and migratory than MCF-7.

To investigate the global molecular evolution of MCF-7:5C and MCF-7:2A, whole genome expression and array-based comparative genomic hybridization (aCGH) analysis were performed on all three cell lines. Analysis of genes differentially expressed between MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells suggested altered signaling pathway regulation including increased AKT signaling and deregulated cell cycle control. The expectation of long-term estrogen deprivation resulting in significant genomic evolution was confirmed by aCGH. MCF-7:5C and MCF-7:2A cells displayed both common and unique chromosomal breaks, as well as shared and unique regions of DNA gain and loss, suggesting estrogen deprivation can cause repeated genomic alterations associated with development of endocrine resistance. Interestingly, amplification of ESR1, BRCA1 and CDK4 genes were found to be overexpressed by both endocrine resistant lines.

Currently, a comprehensive integrated genomic and gene expression analysis is being performed for MCF-7, MCF-7:5C and MCF-7:2A cells to identify consistent potential biomarkers of aromatase inhibitor resistance in addition to possible biological drivers of enhanced malignant behavior in endocrine resistant breast cancer cells.

## **Overexpression of CEACAM6 promotes invasion and migration of aromatase inhibitor-resistant breast cancer cells**

Joan S. Lewis-Wambi, Helen R. Kim, Heather Cunliffe, and V. Craig Jordan, Fox Chase Cancer Center, Philadelphia, PA and Translational Genomics Research Institute (TGen), Phoenix, AZ, USA.

Estrogen deprivation using aromatase inhibitors (AIs) has proven highly effective in the treatment of postmenopausal women with estrogen-receptor (ER)-positive breast cancer. Unfortunately, one of the consequences of long term estrogen deprivation (i.e. long-term use of AI) is the development of drug resistance (estrogen-independent growth). We have previously reported the development of a long-term estrogen deprived (LTED) breast cancer cell line (MCF-7:5C) which grows in the absence of estrogen but is sensitive to estrogen-induced apoptosis (Lewis et al, JNCI 2005). In the present study, we used a Chemicon cell invasion assay kit to quantify cellular invasiveness of LTED cell. We found that MCF-7:5C cells along with another LTED cell line (MCF-7:2A) were highly invasive and more metastatic than wild-type MCF-7 breast cancer cells with the order of invasiveness being MCF-7:5C > MCF-7:2A > MCF-7. Microarray analysis of MCF-7:5C, MCF-7:2A, and MCF-7 breast cancer cells showed significant differences in the gene expression profiles between the cell lines with ~900 genes altered based on a change of twofold or greater. Invasion and metastatic genes such as carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), chemokine (C-X-C motif) receptor 4 (CXCR4), CD44, MMP3, and cadherins were dramatically upregulated in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells. In particular, CEACAM6 transcript was upregulated by 112-fold in MCF-7:5C cells and 21-fold in MCF-7:2A cells and its protein by 40-fold and 10-fold, respectively, relative to wild-type MCF-7 cells. Small interfering RNA (siRNA)-mediated downregulation of CEACAM6 expression in MCF-7:5C and MCF-7:2A cells dramatically reduced migration and invasion of these cells. Interestingly, we found that 17 $\beta$ -estradiol also blocked the invasiveness and migration of MCF-7:5C and MCF-7:2A cells and it downregulated CEACAM6 mRNA and protein expression in these cells. These findings identify CEACAM6 as a unique mediator of invasion and migration of aromatase inhibitor resistant breast cancer cells and suggest that it might be an important biomarker for metastasis and a possible target for treatment of patients with metastatic disease. This work is supported by the Department of Defense Breast Program under award number BC050277 Center of Excellence (Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense).

*The above abstract (abstract #5710) was selected for an oral presentation in a Minisymposium session at the 2007 AACR Annual Meeting in Los Angeles, CA and was published in the 2007 Proceedings of the American Association for Cancer Research.*



**Grant Number:** 1K01CA120051-01A2

**Principal Investigator(s):**  
Joan LEWIS-WAMBI, PHD

**Project Title:** The new biology of estrogen action in aromatase resistance breast cancer cells

CHIEF OPERATING OFFICER  
FOX CHASE CANCER CENTER  
333 COTTMAN AVENUE  
PHILADELPHIA, PA 19111

**Award e-mailed to:** awardnotices@fcc.edu

**Budget Period:** 08/27/2007 – 07/31/2008

**Project Period:** 08/27/2007 – 07/31/2012

Dear Business Official:

The National Institutes of Health hereby awards a grant in the amount of \$120,208 (see “Award Calculation” in Section I and “Terms and Conditions” in Section III) to FOX CHASE CANCER CENTER in support of the above referenced project. This award is pursuant to the authority of 42 USC 241 42 CFR 52 and is subject to the requirements of this statute and regulation and of other referenced, incorporated or attached terms and conditions.

Acceptance of this award including the “Terms and Conditions” is acknowledged by the grantee when funds are drawn down or otherwise obtained from the grant payment system.

Each publication, press release or other document that cites results from NIH grant-supported research must include an acknowledgment of NIH grant support and disclaimer such as “The project described was supported by Grant Number K01CA120051 from the National Cancer Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.”

Award recipients are strongly encouraged to submit to PubMed Central (PMC), upon acceptance for publication, an electronic version of peer-reviewed, original research publications, resulting from research supported in whole or in part, with direct costs from National Institutes of Health. The author’s final manuscript is defined as the final version accepted for journal publication, and includes all modifications from the publishing peer review process. For additional information, please visit <http://publicaccess.nih.gov/>.

If you have any questions about this award, please contact the individual(s) referenced in Section IV.

Sincerely yours,

Carol Perry  
Grants Management Officer  
NATIONAL CANCER INSTITUTE

Additional information follows

---

**SECTION I – AWARD DATA – 1K01CA120051-01A2****Award Calculation (U.S. Dollars)**

Salaries and Wages	\$58,916
Fringe Benefits	\$22,388
Supplies	\$13,291
Travel Costs	\$4,680
Other Costs	\$12,029

Federal Direct Costs	\$111,304
Federal F&A Costs	\$8,904
Approved Budget	\$120,208
Federal Share	\$120,208
<b>TOTAL FEDERAL AWARD AMOUNT</b>	<b>\$120,208</b>

<b>AMOUNT OF THIS ACTION (FEDERAL SHARE)</b>	<b>\$120,208</b>
----------------------------------------------	------------------

SUMMARY TOTALS FOR ALL YEARS		
YR	THIS AWARD	CUMULATIVE TOTALS
1	\$120,208	\$120,208
2	\$122,842	\$122,842
3	\$125,555	\$125,555
4	\$128,350	\$128,350
5	\$131,229	\$131,229

Recommended future year total cost support, subject to the availability of funds and satisfactory progress of the project

**Fiscal Information:**

CFDA Number:	93.398
EIN:	1232003072A1
Document Number:	KCA120051A
Fiscal Year:	2007

IC	CAN	2007	2008	2009	2010	2011
CA	8473570	\$120,208	\$122,842	\$125,555	\$128,350	\$131,229

Recommended future year total cost support, subject to the availability of funds and satisfactory progress of the project

**NIH Administrative Data:**

PCC: 5YMB / OC: 415L / Processed: PERRYCA 08/20/2007

---

**SECTION II – PAYMENT/HOTLINE INFORMATION – 1K01CA120051-01A2**

For payment and HHS Office of Inspector General Hotline information, see the NIH Home Page at <http://grants.nih.gov/grants/policy/awardconditions.htm>

---

**SECTION III – TERMS AND CONDITIONS – 1K01CA120051-01A2**

This award is based on the application submitted to, and as approved by, NIH on the above-titled project and is subject to the terms and conditions incorporated either directly or by reference in the following:

- The grant program legislation and program regulation cited in this Notice of Award.
- The restrictions on the expenditure of federal funds in appropriations acts to the extent those restrictions are pertinent to the award.
- 45 CFR Part 74 or 45 CFR Part 92 as applicable.
- The NIH Grants Policy Statement, including addenda in effect as of the beginning date of the budget period.
- This award notice, INCLUDING THE TERMS AND CONDITIONS CITED BELOW.

(See NIH Home Page at '<http://grants.nih.gov/grants/policy/awardconditions.htm>' for certain references cited above.)

An unobligated balance may be carried over into the next budget period without Grants Management Officer prior approval.

This grant is subject to Streamlined Noncompeting Award Procedures (SNAP).

**Treatment of Program Income:**  
Additional Costs

---

**SECTION IV – CA Special Terms and Conditions – 1K01CA120051-01A2**

**RESTRICTION** National Institutes of Health research or training grant funds (both direct costs and associated facilities and administrative costs) released as a result of this Career Development Award may not be retained by the awardee institution without written prior approval of the NIH awarding unit.

**REQUIREMENT** This award is subject to the conditions set forth in PAR-06-220, "NCI Mentored Career Development Award to Promote Diversity," NIH Guide to Grants and Contracts, March 8, 2006, which are hereby incorporated by reference as special terms and conditions of this award.

Copies of this PAR may be accessed at the following internet address:  
<http://www.nih.gov/grants/guide/index.html>

Copies may also be obtained from the Grants Management Contact indicated in the terms of award.

**INFORMATION** Mentored career award recipients are now eligible to receive concurrent support in the last two years of the career award. Concurrent support must be in accordance with NOT-OD-007, "Mentored Career Development Awards: Change in NIH Policy Concerning Concurrent Support From Career Development Award and a Research Grant"  
<http://grants2.nih.gov/grants/guide/notice-files/NOT-OD-04-007.html>.

**INFORMATION** Although the budget period start date for this award is August 27, 2007, this award includes funds for 12 months of support. Future year budget periods will cycle on August 1. Allowable preaward costs may be charged to this award, in accordance with the conditions outlined in the NIH Grants Policy Statement, (December 2003), and with institutional requirements for prior approval. The NIH GPS can be found on the internet at  
[http://grants1.nih.gov/grants/policy/nihgps\\_2003/index.htm](http://grants1.nih.gov/grants/policy/nihgps_2003/index.htm)

**INFORMATION** This award, including the budget and the budget period, has been discussed between Michael Zarkin of the National Cancer Institute and Diane Alexander on August 2, 2007.

**INFORMATION** In a continuing effort to provide exceptional customer service, the NCI Office of Grants Administration has set up a Feedback address on its web site (<http://www.nci.nih.gov/admin/gab/index.htm>). General concerns and issues related to NCI grants policies, procedures, and practices can be sent to the Customer Liaison using this feature. Specific questions or concerns related to this grant should be addressed to the Grants Management Specialist listed in the Terms of Award.

## **STAFF CONTACTS**

The Grants Management Specialist is responsible for the negotiation, award and administration of this project and for interpretation of Grants Administration policies and provisions. The Program Official is responsible for the scientific, programmatic and technical aspects of this project. These individuals work together in overall project administration. Prior approval requests (signed by an Authorized Organizational Representative) should be submitted in writing to the Grants Management Specialist. Requests may be made via e-mail.

**Grants Management Specialist:** Michael Zarkin  
**Email:** [zarkinm@mail.nih.gov](mailto:zarkinm@mail.nih.gov) **Phone:** 301-496-2261 **Fax:** 301-496-8601

**Program Official:** Belinda Locke  
**Email:** [lockeb@mail.nih.gov](mailto:lockeb@mail.nih.gov) **Phone:** 301-496-7344 **Fax:** 301-402-4551

## **SPREADSHEET SUMMARY**

**GRANT NUMBER:** 1K01CA120051-01A2

**INSTITUTION: FOX CHASE CANCER CENTER**

<b>Budget</b>	<b>Year 1</b>	<b>Year 2</b>	<b>Year 3</b>	<b>Year 4</b>	<b>Year 5</b>
Salaries and Wages	\$58,916	\$60,683	\$62,504	\$64,379	\$66,310
Fringe Benefits	\$22,388	\$23,060	\$23,751	\$24,464	\$25,198
Supplies	\$13,291	\$13,820	\$13,335	\$12,835	\$12,320
Travel Costs	\$4,680	\$3,790	\$3,904	\$4,021	\$4,142
Other Costs	\$12,029	\$12,390	\$12,761	\$13,144	\$13,538
TOTAL FEDERAL DC	\$111,304	\$113,743	\$116,255	\$118,843	\$121,508
TOTAL FEDERAL F&A	\$8,904	\$9,099	\$9,300	\$9,507	\$9,721
TOTAL COST	\$120,208	\$122,842	\$125,555	\$128,350	\$131,229

<b>Facilities and Administrative Costs</b>	<b>Year 1</b>	<b>Year 2</b>	<b>Year 3</b>	<b>Year 4</b>	<b>Year 5</b>
F&A Cost Rate 1	8%	8%	8%	8%	8%
F&A Cost Base 1	\$111,304	\$113,743	\$116,255	\$118,843	\$121,508
F&A Costs 1	\$8,904	\$9,099	\$9,300	\$9,507	\$9,721

**Overexpression of CEACAM6 promotes migration and invasion of estrogen deprived breast cancer cells**

**Joan S. Lewis-Wambi<sup>1</sup>, Heather E. Cunliffe<sup>2</sup>, Helen R. Kim<sup>1</sup>, Amanda L. Willis<sup>2</sup>, V. Craig Jordan<sup>1</sup>**

<sup>1</sup>Department of Medical Sciences, Fox Chase Cancer Center, Philadelphia, PA and <sup>2</sup>Translational Genomics Research Institute, Phoenix, AZ.

**Request for reprints:** V. Craig Jordan, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111 (e-mail: [V.Craig.Jordan@fccc.edu](mailto:V.Craig.Jordan@fccc.edu)).

**Running Title:** CEACAM6 overexpression and breast cancer invasiveness

**Key words:** CEACAM6, breast cancer, invasion, estrogen deprivation, drug-resistance

**Footnotes:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

## Abstract

Suppression of estrogen synthesis using aromatase inhibitors is highly effective in the treatment of postmenopausal women with estrogen-receptor alpha (ER $\alpha$ )-positive breast cancer, however, acquisition of resistance is inevitable and a major clinical concern. In this study, we investigated the effects of long-term estrogen deprivation on invasion and migration of two ER $\alpha$ -positive human breast cancer cell lines (MCF-7:5C and MCF-7:2A) that have acquired resistance to estrogen withdrawal. We found that MCF-7:5C and MCF-7:2A cells were significantly more invasive and migratory than wild-type MCF-7 breast cancer cells. Microarray analysis of MCF-7:5C and MCF-7:2A cells revealed that several invasive and metastatic genes were significantly upregulated in these cells compared to MCF-7 cells. In particular, carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) mRNA was upregulated by 27-fold in MCF-7:5C cells and 14-fold in MCF-7:2A cells and its protein by 40-fold and 10-fold, respectively, relative to MCF-7 cells. Suppression of CEACAM6 expression using small interfering RNA (siRNA) inhibited migration and invasion of MCF-7:5C and MCF-7:2A cells. We observed that 17 $\beta$ -estradiol abolished the invasiveness and migration of MCF-7:5C and MCF-7:2A cells while downregulating CEACAM6 expression. Elucidation of the mechanism of CEACAM6 mediated invasiveness in MCF-7:5C and MCF-7:2A cells suggests a possible involvement of the c-Src and Akt signaling pathways. These findings identify CEACAM6 as a unique mediator of invasion and migration in aromatase inhibitor resistant breast cancer cells and suggest that this protein could be an important biomarker of metastasis as well as a possible target for treatment of patients with metastatic disease.

## Introduction

Tamoxifen has traditionally been the primary adjuvant endocrine therapy for postmenopausal women with ER $\alpha$ -positive breast cancer; however, aromatase inhibitors (AIs) have challenged this standard in postmenopausal women. AIs (letrozole, anastrozole and exemestane) are anti-estrogen agents that suppress estrogen production in peripheral tissues and breast tumors by inhibiting or inactivating aromatase, the enzyme which catalyses the conversion of androgens to estrogens in postmenopausal women (1). Several randomized trials (2-5) have shown that third generation AIs are superior to adjuvant tamoxifen in terms of improved disease-free survival and less side effects. Unfortunately, one of the consequences of prolonged estrogen deprivation or exhaustive endocrine therapy is the development of drug resistance (6, 7). Previous studies have shown that acquisition of tamoxifen resistance in wild-type MCF-7 breast cancer cells is associated with a significant increase in invasiveness and migration of cancer cells (8-10). However, it is not known whether acquired resistance to prolonged estrogen deprivation affects invasiveness in breast cancer cells.

Invasion and metastasis are the hallmarks of cancer malignancy and they are the primary cause of patient mortality during breast cancer progression (11). Invasion refers to the ability of cancer cells to penetrate through the membranes that separate them from healthy tissues and blood vessels and metastasis refers to the spreading of cancer cells to other parts of the body (12). In order for a transformed cell to metastasize, it must first lose adhesion, penetrate and invade the surrounding extracellular matrix (ECM), enter the vascular system, and adhere to distant organs (12). These processes require extensive alterations in gene expression profiles,

including the down-regulation of genes involved in cell anchorage and the up-regulation of genes involved in cell motility and matrix degradation (11, 13, 14).

CEACAM6 is a glycosylphosphatidylinositol-anchored cell surface protein that functions as a homotypic intercellular adhesion molecule (15). It is overexpressed in a number of human malignancies including pancreatic cancer, gastrointestinal cancer, and breast cancers (16, 17) and increased levels of CEACAM6 are inversely correlated to the differentiation state of cancer cells. Previous studies have shown that overexpression of CEACAM6 in pancreatic adenocarcinoma cells is associated with greater *in vivo* metastatic ability and increased invasiveness and migration (18-20). More recently, Poola and coworkers (21) reported that the expression of CEACAM6 protein in atypical ductal hyperplastic (ADH) tissues was associated with the development of invasive breast cancer (IBC) and it was suggested that CEACAM6 can potentially be applied as a diagnostic marker either singly or in combination with other markers to predict IBC development in women with ADH lesions. Currently, the role of CEACAM6 overexpression in breast cancer migration and invasion is not known.

In the present study, we investigated the role of CEACAM6 in cellular invasion and migration of long-term estrogen deprived human breast cancer cells. We found that CEACAM6 was significantly overexpressed in estrogen-deprived MCF-7:5C and MCF-7:2A breast cancer cells compared to wild-type MCF-7 cells and that these cells were markedly more invasive and migratory than MCF-7 cells. Suppression of CEACAM6 expression by small interfering RNA (siRNA) completely reversed the invasive phenotype of MCF-7:5C and MCF-7:2A cells. E-cadherin and  $\beta$ -catenin were also significantly reduced in these cells. The mechanism of action of CEACAM6 appears to involve the c-Src and Akt signalling pathways.

## Materials and Methods

**Antibodies and reagents.** Mouse anti-CEACAM6 (clone MUS) and anti-CEACAM5 antibodies were purchased from Signet Laboratories (Dedham, MA ); rabbit anti-ER $\alpha$  (G20), mouse anti-N-cadherin (H-63), mouse anti-alpha-catenin (G11), mouse anti-beta-catenin (9F2), rabbit anti-chemokine C-X-C receptor 4 (CXCR4) (H118), rabbit anti-tissue inhibitor of metalloproteinases 3 (TIMP3), mouse anti-matrix metalloproteinase 9 (MMP9) (IB4), mouse anti-E-cadherin (67A4), and mouse anti-CD44 (DF1485) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-fibronectin , rabbit anti-integrin alpha5beta1, rabbit anti-integrin alphavbeta3, and mouse anti-vitronectin antibodies were purchased from Chemicon International (Temecula, CA); rabbit anti-c-Src and rabbit anti-phospho-Src (Tyr<sup>529</sup>) antibodies were purchased from Biosource International (Carmarillo, CA); rabbit anti-AKT, rabbit anti-phospho-AKT (Ser<sup>473</sup>), and rabbit anti-PTEN antibodies were purchased from Cell Signaling Technology (Beverly, MA); mouse anti-beta-actin (clone AC-15) antibody and 17 beta-estradiol were purchased from Sigma Chemical Co. (St Louis, MO); PP2 was purchased from EMD Biosciences, Inc. (La Jolla, CA); LY294002 was purchased from Promega (Madison, WI); fulvestrant was obtained as a generous gift from AstraZeneca (Macclesfield, United Kingdom); Affymetrix Human Genome U133 Plus 2.0 Arrays were purchased from Affymetrix (Santa Clara, CA); fetal bovine serum (FBS), cell culture medium, and other reagents were purchased from Invitrogen (Carlsbad, CA).

**Cell lines and culture conditions.** The MCF-7:WS8 cells (22) used in this study were cloned from wild-type MCF-7 human breast cancer cells originally obtained from Dr. Dean Edwards (University of Texas, San Antonio, TX), and were maintained in full serum medium (RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, 100  $\mu$ g/mL

streptomycin, 1X non-essential amino acids, and bovine insulin at 6 ng/mL (Sigma-Aldrich, St. Louis, MO). MCF-7:5C cells (23, 24) were clonally isolated from wild-type MCF-7 cells and were maintained in phenol red-free RPMI media containing 10% dextran-coated charcoal stripped FBS (SFS), 2 mM glutamine, 6 ng/ml bovine insulin, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1X non-essential amino acids. MCF-7:2A cells (25) were also cloned from MCF-7 cells and were maintained in estrogen-free RPMI 1640 medium supplemented with 10% SFS and other supplements as in the full serum medium.

**RNA preparation and microarray hybridization.** Wild-type MCF-7:WS8 cells were cultured in estrogen-free RPMI plus 10% SFS for 4 days to deplete any residual estrogen. On day 4, MCF-7:WS8, MCF-7:5C, and MCF-7:2A cells were seeded in T75 flasks containing estrogen-free RPMI plus 10% SFS. Total RNA was prepared using the Qiagen RNeasy Mini kit. A DNase I digestion step was included to eliminate DNA contamination. cRNA was generated, labeled, and hybridized to the Affymetrix Human Genome U133 Plus 2.0 Arrays by the Northwestern University Genomics Core (Chicago, IL). Arrays were washed, stained, and scanned according to the directions detailed in the Affymetrix GeneChip® Expression Analysis Technical Manual.

**Microarray Data Analysis.** Assessment of data quality was conducted following default guidelines in the Affymetrix GeneChip® Expression Analysis Data Analysis Fundamentals Training Manual. Data was extracted and normalized using *Affymetrix Microarray Suite* (MAS5.0) following recommended protocols for background and chip-correction. Global scaling for average signal intensity for all arrays was set to 500. Four biological replicates from each of the three cell lines were arrayed to determine consistent and reproducible patterns of gene expression. All but one array showed a high degree of reproducibility within a set of replicate

hybridizations, leaving at least three array replicates per cell line for further analysis. Data was filtered as follows. Genes across all arrays with an expression intensity  $<70$  were removed. Genes with a present call (consistently within replicates) for at least one cell line were included. To eliminate genes with variable expression within a group of replicates (intra-set deviation), normalized gene intensity ratios (signal intensities divided by the median gene intensity all hybridizations) were derived, then the standard deviation of the log-transformed normalized intensity ratios were calculated for each group of replicates. Genes with a standard deviation  $>0.15$  were excluded. Lastly, to filter for genes with variable expression between cell lines (inter-set deviation), genes were retained that showed a standard deviation of  $>0.3$ . A total of 904 genes met the filtering criteria described and were examined by hierarchical clustering using resources available at TGen<sup>1</sup>. Uncentered Pearson's correlation with average linkage was used on log<sub>2</sub>-transformed data, with induced genes indicated in red and repressed genes in green.

**Cell proliferation assay.** Cell proliferation assay was performed as previously described (24). The DNA content of the cells was determined using a Fluorescent DNA Quantitation kit (Bio-Rad Laboratories, Hercules, CA). For each analysis, three replicate wells were used, and at least three independent experiments were performed.

**Western Blot Analysis.** Cells were washed twice with PBS and whole cell lysates were harvested, as previously described (24). Fifty micrograms of protein per sample in SDS buffer were resolved on 4-12% polyacrylamide gels using a mini-PROTEAN II Electrophoresis Cell (Bio-Rad). Separated proteins were transferred onto nitrocellulose membranes (Millipore) using the submarine electrophoretic transfer unit in the same apparatus. Membranes were then

---

<sup>1</sup> Internet address: <http://biodiscovery.tgen.org/microarray/>

incubated overnight at 4°C with the respective primary antibodies. Secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) were used with an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL) to visualize the resolved proteins.

**Real-time reverse transcription-PCR for human ER $\alpha$  and CEACAM6 mRNAs in AI-resistant breast cancer cells.** Total RNA was extracted from MCF-7:WS8, MCF-7:5C and MCF-7:2A cells using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA was converted to first-strand cDNA using SuperScript III with a combination of random hexamers and oligo(dT) as primers (Invitrogen). Quantitative real-time PCR assays were done as previously described (24) with the Taqman Universal or SYBR Green PCR Master Mixes and an ABI 7700 sequence detection system (PE Applied Biosystems, Foster City, CA). The *ER $\alpha$*  forward and reverse primers were 5'-AAGAGGGTGCCAGGCTTTGT-3', and 5'-CAGGATCTCTAGCCAGGCACAT-3', respectively. The *ER $\alpha$*  probe was 5'-[FAM]-ATTTGACCCTCCATGATCAGGTCCACC-[TAMRA]-3'. The forward and reverse primers for CEACAM6 were synthesized by Sigma Genosys (Sigma-Aldrich). The sequences for *CEACAM6* forward and reverse primers were 5'-GACGTTTGTGTGGATTGCTGGAACGC-3' and 5'-TGCCACGCAGCCTCTAACC-3'. The reporter dye at the 5'-end of each probe was FAM and the quencher dye at the 3'-end was TAMRA. Eukaryotic 18S ribosomal RNA (Applied Biosystems) was used as an internal control, and each total cDNA sample was normalized to the content of 18S rRNA.

**Cell migration and invasion assays.** Cell migration was measured in a Boyden chamber using Transwell filters (6.5-mm diameter, 8- $\mu$ m pore size polycarbonate membrane) obtained from

Corning (Cambridge, MA). Cells ( $1 \times 10^5$ ) in 0.5 mL serum-free medium were placed in the upper chamber, and the lower chamber was loaded with 0.8 mL medium containing 10% dextran-coated charcoal treated FBS. Cells that migrated to the lower surface of filters were stained with Wright Giemsa solution, and five fields of each well were counted after 24 or 48 hours of incubation at 37°C with 5% CO<sub>2</sub>. Three wells were examined for each condition and cell type, and the experiments were repeated in triplicate. Cell invasion assay was performed using the Chemicon cell invasion kit (Chemicon International, Temecula, CA) in accordance with the manufacturer's protocol. Cells ( $1 \times 10^5$ /ml) were seeded onto 12-well cell culture chamber using inserts with 8 µm pore size polycarbonate membrane over a thin layer of extracellular matrix. The ECM layer occludes the membrane pores, blocking non-invasive cells from migrating through. Following incubation of the plates for 48 h at 37°C, cells that had invaded through the ECM layer and migrated to the lower surface of the membrane were stained and counted under the microscope in at least five different fields and photographed.

**CEACAM6 siRNA-mediated gene knockdown.** CEACAM6-specific siRNA (Silencer™ Predesigned siRNA; sense: GCCCUGGUGUAUUUUCAUtt, antisense: AUCGAAAAUACAC CAGGGCtg) (AM16704) and negative control siRNA (Silencer™ Negative Control siRNA) were purchased from Ambion (Austin, TX). Transfection complexes were prepared in Opti-MEM serum-free medium (Invitrogen) by mixing 0.3 µL of siPORT NeoFX transfection reagent (Ambion) and 10 nM CEACAM6 siRNA or negative control siRNA (Ambion). Cells ( $9 \times 10^4$  cells per well) were reverse-transfected in 12-well plates simultaneously with addition of transfection complexes. The medium was replaced with phenol red-free RPMI supplemented with 10% SFS 24 h after transfection. The cultures were harvested for CEACAM6 protein analysis 48 and 72 hours after transfection.

**Statistical Analyses.** Statistical analyses were performed using Microsoft Excel (Seattle, WA). Differences between groups were evaluated using Student's *t* test. Data were considered significant if  $p < 0.05$ .

## RESULTS

### **Growth rate, cell morphology, and ER $\alpha$ status of long term estrogen deprived breast**

**cancer cells.** We first examined the effect of long term estrogen deprivation on cell growth and ER $\alpha$  expression using two estrogen deprived breast cancer cell clones, MCF-7:5C and MCF-7:2A. As shown in **Fig. 1A**, MCF-7:5C and MCF-7:2A cells grew normally in the absence of estrogen, whereas, wild-type hormone-dependent MCF-7 cells, grew minimally without estrogen. The doubling times for MCF-7:5C, MCF-7:2A and MCF-7 cells were 2.7, 3.4, and 6 days, respectively. We also examined cell morphology changes associated with resistance to long term estrogen deprivation. **Fig. 1B** shows that wild-type MCF-7 cells grew as a uniform monolayer of tightly associated cells with limited cell spreading but distinct cellular boundaries whereas estrogen deprived MCF-7:5C and MCF-7:2A cells grew in a less uniform monolayer with cellular boundaries that were obscured. Analysis of ER $\alpha$  status demonstrated that mRNA and protein expression were significantly increased in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells. We also observed that treatment with estradiol or the antiestrogen fulvestrant significantly down-regulated ER $\alpha$  expression in all three cell lines (**Fig. 1C and D**). Of note, MCF-7:2A cells also expressed a high molecular weight ER $\alpha$  protein (80kDa) which was previously cloned and characterized by our laboratory (26). It contains an in-frame duplication of exons 6 and 7 in the steroid-binding domain of the receptor and it has abrogated ligand binding but no DNA binding. Taken together, these results show that long-term estrogen deprivation significantly increases the growth rate and ER $\alpha$  expression of MCF-7:5C and MCF-7:2A cells.

### **Global gene expression profiles associated with acquired resistance to long term estrogen**

**deprivation.** To investigate whether aberrant gene expression patterns contributed to resistance to estrogen deprivation, we performed microarray analyses on wild-type MCF-7 cells and

estrogen-deprived MCF-7:5C and MCF-7:2A cells. Subconfluent cells grown in phenol red-free RPMI 1640 media supplemented with 10% SFS were analyzed with the Affymetrix Human Genome U133 Plus 2.0 Array. Two-dimensional hierarchical clustering was performed to analyze differences in gene expression patterns between wild-type MCF-7 cells and clonal derivative MCF-7:5C and MCF-7:2A cells. Data filtering identified 904 genes that were significantly altered between estrogen deprived MCF-7:5C and MCF-7:2A cells and wild-type MCF-7 cells (**Fig. 2A**; Supplemental Fig. S1). The sample dendrogram showed that MCF-7:2A cells and wild-type MCF-7 cells clustered more closely, whereas MCF-7:5C cells clustered on a more distant branch, suggesting that MCF-7:2A cells are more similar to the parental MCF-7 cells than MCF-7:5C cells (**Fig. 2A**). The extent of overlap in identity of the genes upregulated or downregulated greater than 2-fold between any of the three cell lines is presented in **Fig. 2B** in the form of a Venn diagram. Of the 14,056 genes that met minimal quality metrics, a total of 2476 genes were upregulated (**Fig. 2B**, upper diagram), a total of 3156 genes were downregulated (**Fig. 2B**, lower diagram) and 8424 genes remained unchanged (not shown). Gene Ontology showed that many of the genes significantly altered between the three cell lines are involved in cell cycle control, proliferation, cell death/apoptosis, growth factor signaling, nucleotide and amino acid metabolism, cell adhesion, and motility and invasion (**Fig. 2C**; Supplemental Table 1). A graphical representation of some of the invasive genes overexpressed in estrogen deprived cells relative to parental MCF-7 cells is shown in **Fig. 2D**. A complete list of differentially expressed genes between each cell line is provided in Supplemental Table 2.

**Estrogen deprivation increases CEACAM6 expression and enhances migration and invasion of breast cancer cells.** The microarray studies showed that several genes associated with invasion were overexpressed in estrogen-deprived MCF-7:5C and MCF-7:2A cells

compared to wild-type MCF-7 cells. In particular, CEACAM6 gene transcript was overexpressed 27-fold in MCF-7:5C cells and 14-fold in MCF-7:2A cells (**Fig. 2D**). Quantitative real-time PCR was performed on MCF-7, MCF-7:5C and MCF-7:2A cells. **Fig. 3A** shows that CEACAM6 mRNA was upregulated 2-fold in MCF-7 cells, 17-fold in MCF-7:5C cells, and 10-fold in MCF-7:2A cells relative to the internal control 18S rRNA, thus validating the microarray results. Western blot analysis was performed to assess CEACAM6 protein expression. **Fig. 3B**, CEACAM6 protein was significantly upregulated in MCF-7:5C and MCF-7:2A cells compared to wild-type MCF-7 cells which expressed minimal levels of the protein. CEACAM5 (CEA), MMP-9, CXCR4, and CD44 were also markedly elevated in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells.

Since the estrogen-deprived cells appeared to overexpress several genes that are linked to metastatic invasion, we assessed the migratory and invasive potential of these cells. Cell migration was measured using a modified Boyden chamber assay with 10% SFS as a chemoattractant. As shown in **Fig. 3C**, both MCF-7:5C and MCF-7:2A cells had the highest numbers of migrating cells compared to MCF-7 cells; a phenotype that correlated with CEACAM6 expression. Similar results were obtained when the different cell lines were tested for their ability to invade through membranes coated with Matrigel. **Fig. 3D** shows that MCF-7:5C and MCF-7:2A cells had the highest number of invading cells while MCF-7 cells were noninvasive. The overall ability of the cell lines to migrate and invade was as follows: MCF-7:5C > MCF-7:2A > MCF-7.

To verify that the invasiveness of MCF-7:5C and MCF-7:2A cells was not a result of clonal selection, invasion assays were performed with MCF-7:ED cells which were derived by maintaining a population of MCF-7 cells under estrogen-deprived conditions for more than a

year. Results from these studies showed that MCF-7:ED cells were significantly more invasive than MCF-7 cells and that these cells also overexpress CEACAM6 (data not shown).

**siRNA mediated CEACAM6 knockdown inhibits invasion and migration of MCF-7:5C cells.** To test the hypothesis that CEACAM6 is required for cell migration and invasion, we used siRNA to suppress CEACAM6 expression in MCF-7:5C cells. These cells were used because they expressed the highest level of CEACAM6 and were the most invasive. MCF-7:5C cells were transfected with CEACAM6-specific or control siRNA and Western blot analysis was performed 48 hours following siRNA exposure. **Fig. 4A** shows that CEACAM6 protein was significantly suppressed in MCF-7:5C cells transfected with the CEACAM6-specific siRNA but not the control siRNA. Suppression of CEACAM6 expression by CEACAM6-specific siRNA was also confirmed at the transcript level using quantitative real-time PCR at 48 hours following transfection (**Fig. 4B**). To clarify the role of CEACAM6 siRNA in cell invasion, MCF-7:5C cells were pretreated with CEACAM6 siRNA or control siRNA for 48 hours and invasiveness was measured over the subsequent 48 hours. **Fig. 4C** shows that CEACAM6 siRNA almost completely reversed the invasiveness of MCF-7:5C cells whereas control siRNA did not affect cell invasion. The invasiveness of MCF-7:5C cells was inhibited by nearly 80% when CEACAM6 expression was suppressed. A similar trend was observed for cell migration (data not shown). Suppression of CEACAM6, however, did not alter the growth of MCF-7:5C cells. These results demonstrate that CEACAM6 expression is an important determinant of cell migration and invasion in estrogen-deprived breast cancer cells and therefore, modulation of CEACAM6 expression may be a potential therapeutic target for metastatic breast cancer.

**Estradiol down-regulates CEACAM6 expression and blocks invasion and migration of MCF-7:5C cells.** Previous studies have shown that estradiol increases the ability of MCF-7 cells to form tumors (27, 28) and it enhances the ability of these cells to invade through an artificial reconstituted basement membrane *in vitro* (29). Since estrogen-deprived MCF-7:5C cells overexpress CEACAM6 and are invasive, we examined whether CEACAM6 expression was hormonally regulated in these cells. MCF-7:5C cells were treated with 1 nM estradiol for 48 hours and CEACAM6 mRNA and protein levels were analyzed by quantitative realtime PCR and Western blot, respectively. As shown in **Fig. 5A**, estradiol completely down-regulated CEACAM6 mRNA level in MCF-7:5C cells. This finding is consistent with the microarray studies which showed a significant reduction in CEACAM6 transcript in MCF-7:5C cells following 48 hours of estradiol treatment (data not shown). The ability of estradiol to down-regulate CEACAM6 protein levels was found to be maximal at 48 hours (**Fig. 5B**). To determine whether the down-regulation of CEACAM6 protein by estradiol was an ER $\alpha$ -mediated event, MCF-7:5C cells were pretreated with the antiestrogen fulvestrant which is known to degrade ER $\alpha$  (30, 31). **Fig. 5B** shows that pretreatment with 1  $\mu$ M fulvestrant reversed the inhibitory effect of estradiol on CEACAM6 protein in MCF-7:5C and MCF-7:2A cells. Fulvestrant also completely counteracted the anti-invasive effects of estradiol in MCF-7:5C cells (**Fig. 5C**). Interestingly, estradiol enhanced the invasiveness of wild-type MCF-7 cells without significantly changing CEACAM6 protein levels in these cells (data not shown).

**c-Src, E-cadherin, and Akt are important for CEACAM6-mediated invasion of MCF-7:5C and MCF-7:2A cells.** The Src family of nonreceptor tyrosine kinases, most notably c-Src, is frequently overexpressed or aberrantly activated in epithelial cancers (32). Previous studies have reported that CEACAM6 cross-linking initiates c-Src-dependent cross-talk between CEACAM6

and  $\alpha_v\beta_3$  integrin, leading to increased ECM-adhesion and invasion (33). We therefore examined the role of c-Src in invasion of MCF-7:5C and MCF-7:2A cells by measuring c-Src expression. **Fig. 6A** shows that total and phosphorylated c-Src<sup>tyr529</sup> were significantly upregulated in MCF-7:5C and MCF-7:2A cells relative to MCF-7 cells and that treatment with the c-Src kinase inhibitor PP2 significantly reduced the invasiveness of MCF-7:5C ( $p<.001$ ) and MCF-7:2A cells ( $p<.01$ ) (**Fig. 6B**). We also found that E-cadherin and  $\beta$ -catenin were significantly reduced in MCF-7:5C and MCF-7:2A cells whereas N-cadherin was significantly increased in comparison to MCF-7 cells (**Fig. 6C**). Phosphorylated Akt<sup>Ser473</sup> was also significantly upregulated in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells (**Fig. 6C**). Furthermore, we found that inhibition of Akt phosphorylation using the PI3K inhibitor LY294002 significantly reduced cell growth and invasion of MCF-7:5C and MCF-7:2A cells (**Fig. 6D**), thereby suggesting a potential role for the Akt/PI3K signaling pathway in invasion of these cells.

## Discussion

Estrogen suppression using aromatase inhibitors is currently the standard of care for patients with ER-positive breast cancer; however, effective treatment is limited by the inevitable acquisition of resistance. In the present study, we showed that long-term estrogen deprivation significantly increased the migration and invasiveness of two ER $\alpha$ -positive human breast cancer cell lines that have acquired resistance to estrogen deprivation. MCF-7:5C and MCF-7:2A cells were 15- and 6-times, respectively, more invasive than wild type MCF-7 cells and these cells showed significant upregulation of several invasion genes. In particular, CEACAM6 was significantly overexpressed in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells. Furthermore, blockade of CEACAM6 expression using siRNA completely inhibited the invasiveness of MCF-7:5C and MCF-7:2A cells and caused a reduction in phosphorylated c-Src and Akt expression (Supplemental Figure S2). Other invasion genes such as CXCR4, CEACAM5/CEA, CD44, MMP9 and N-cadherin were also overexpressed in MCF-7:5C and MCF-7:2A cells whereas E-cadherin and  $\beta$ -catenin were significantly reduced in both clones relative to wild-type MCF-7 cells. Interestingly, we found that 17 $\beta$ -estradiol, which enhanced the invasive ability of wild-type MCF-7 cells, completely reduced CEACAM6 expression and blocked migration and invasion of estrogen-deprived MCF-7:5C cells. To our knowledge, this study is the first to demonstrate a critical role for CEACAM6 in migration and invasiveness of aromatase inhibitor resistant breast cancer cells and it suggests that c-Src and Akt, through cross-talk with CEACAM6, may be important mediators of this phenotype.

Previous studies have reported that overexpression of CEACAM6 in pancreatic adenocarcinoma cells is associated with enhanced cellular invasiveness and increased metastatic

potential *in vivo* and that this effect is completely attenuated by suppression of CEACAM6 expression (18). We found that CEACAM6 was overexpressed in MCF-7:5C and MCF-7:2A cells (**Fig. 3A** and **B**) and that suppression of CEACAM6 expression completely reversed the invasive phenotype of these cells *in vitro* (**Fig. 4C**). Interestingly, both MCF-7:5C (24) and MCF-7:2A cells are capable of forming tumors *in vivo* and these tumors overexpress CEACAM6 (data not shown). The metastatic potential of these cells *in vivo*, however, has not been determined. Currently, the mechanism by which CEACAM6 facilitates invasion is not fully understood. However, there is evidence that CEACAM6, along with other GPI-anchored proteins, are capable of modulating the activity of intracellular tyrosine kinases such as c-Src (34, 35). In particular, studies by Duxbury and coworkers (33, 36) showed that c-Src activity was increased in CEACAM6-overexpressing BxPC3 human pancreatic cancer cells and decreased following suppression of CEACAM6 expression and that inhibition of c-Src activity significantly suppressed CEACAM6-mediated cellular invasiveness. We also found that c-Src and phosphorylated c-Src were significantly elevated in MCF-7:5C and MCF-7:2A cells and that suppression of CEACAM6 expression reduced phosphorylated c-Src in these cells (Supplemental Figure S2). Similarly, we found that pharmacological blockade of c-Src using the Src tyrosine kinase inhibitor pyrazolopyrimidine (PP2) inhibited the invasiveness of MCF-7:5C and MCF-7:2A cells (**Fig. 6D**) and significantly reduced cell growth (data not shown). Src family kinases are capable of modulating matrix metalloproteinases which are proteolytic enzymes that degrade ECM proteins and basement membrane. c-Src has been shown to enhance MMP-9 activity (37) which is known to degrade type IV collagen, the main component of basement membranes. We found that MMP-9 expression was significantly elevated in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells, however, the significance of this

observation is not known since we also observed elevated levels of tissue inhibitor of metalloproteinases (TIMP)-3 in these cells. TIMP-3 is an extracellular matrix-bound protein that has inhibitory actions on MMPs and its expression affects tumor growth, invasion and angiogenesis (38, 39). It is worth noting that several other invasion-related genes (i.e. CEACAM5, CXCR4, and CD44) (**Fig. 2D**) were also significantly increased in MCF-7:5C and MCF-7:2A cells relative to wild-type MCF-7 cells, however, their exact contribution to migration and invasion is not known. The fact that blockade of CEACAM6 expression almost completely reversed the invasiveness of MCF-7:5C and MCF-7:2A cells suggests a dominant role in this phenotype, however, a possible contribution by the other invasive genes cannot be ruled out and warrants further investigation.

In addition to c-Src, we also found elevated levels of phosphorylated Akt<sup>ser473</sup> in CEACAM6-overexpressing MCF-7:5C and MCF-7:2A cells. Akt is a serine/threonine protein kinase that was first identified as the cellular homologue of viral Akt and found to mediate cell survival, proliferation, and differentiation (40, 41). Regulation of Akt has been extensively investigated, and the phosphatidylinositol 3-kinase (PI-3K)–3-phosphoinositide-dependent protein kinase 1 (PDK-1) pathway has been clearly shown to be essential for Akt activation (40). Akt activation plays a major role in tumor cell migration and invasion and metastasis (42). In our study, suppression of CEACAM6 expression significantly reduced phosphorylated Akt expression in MCF-7:5C and MCF-7:2A cells (Supplemental Figure S2) and blockade of the PI3K/Akt signaling pathway with the pharmacologic inhibitor LY294002 inhibited cell growth and reduced invasion (**Fig. 6D**). We also found that PTEN signaling was significantly altered in MCF-7:5C and MCF-7:2A cells (**Fig. 2C**). PTEN is a powerful negative regulator of the PI3K/Akt pathway and it plays an important role in regulating cell cycle and apoptosis (43, 44). Loss of PTEN

staining by immunohistochemistry has been associated with lymph node metastasis with higher rates of disease recurrence and disease-related death (45). These findings suggest that the Akt/PI3K signaling pathway might be an important target for CEACAM6-mediated migration and invasion of MCF-7:5C and MCF-7:2A cells.

In many types of epithelial cancers, the ability to undergo metastasis is associated with a loss of epithelial features and acquisition of mesenchymal properties, a process known as epithelial-to-mesenchymal transition (EMT). During EMT, cancer cells lose expression of proteins that promote cell-cell contact such as E-cadherin and  $\gamma$ -catenin and acquire mesenchymal markers such as vimentin, fibronectin, and N-cadherin (46), which promote cell migration and invasion. We found that E-cadherin and  $\beta$ -catenin were significantly decreased whereas N-cadherin was markedly increased in invasive MCF-7:5C and MCF-7:2A cells compared to noninvasive MCF-7 cells (**Fig 6C**). This shift in expression from E- to N-cadherin has previously been shown to reflect a dedifferentiation from an epithelial to a mesenchymal phenotype which is associated with an increased invasive state. Interestingly, our cell morphology studies (**Fig. 1B**) did not show significant EMT-like changes in MCF-7:5C or MCF-7:2A cells despite their invasiveness. A possible explanation might be the high expression of ER $\alpha$  found in these cells (**Fig. 1A and C**). Previous studies have reported that enhanced ER $\alpha$  activity inhibits EMT in cancer cells whereas reduced ER $\alpha$  activity is associated with an induction of invasive growth (29, 47). There is also clinical evidence which shows that ER $\alpha$ -positive tumors have lower metastatic potential than ER $\alpha$ -negative tumors (48, 49). The fact that both MCF-7:5C and MCF-7:2A cells express high levels of functional ER $\alpha$  suggests a lack of correlation between ER $\alpha$  expression and the ability to invade in our *in vitro* model system.

In summary, we have identified CEACAM6 as a critical gene in the regulation of migration and invasiveness of breast cancer cells that have acquired resistance to estrogen deprivation. CEACAM6 is an adhesion molecule that is frequently overexpressed in solid tumors where it is associated with advanced disease stage, tumor invasion and metastatic potential. The fact that CEACAM6 overexpression is linked to enhanced migration and invasion of long-term estrogen deprived breast cancer cells has possible clinical implications for breast cancer patients who have been or are currently being treated with aromatase inhibitors for an extended period. Future studies will explore whether CEACAM6 interacts with other invasion genes and whether it affects tumor growth *in vivo* as well as metastasis in mice.

## **Acknowledgments**

**Grant support:** The Department of Defense Breast Program under award number BC050277 Center of Excellence; Fox Chase Cancer Center Core Grant NIH P30 CA006927; Weg Fund of Fox Chase Cancer Center; the American Cancer Society Grant IRG-9202714; and the Hollenbach Family Fund.

The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

We thank Dr Chris Wambi (Department of Radiation Oncology, University of Pennsylvania, Philadelphia, PA) for his valuable comments and critical review of this manuscript.

## References

1. Brodie AM, Coombes RC, Dowsett M. Aromatase inhibitors: basic and clinical studies. *J Steroid Biochem* 1987;27:899-903.
2. Baum M, Budzar AU, Cuzick J, 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;359:2131-9.
3. Thurlimann B, Keshaviah A, Coates AS, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med* 2005;353:2747-57.
4. Conte P, Frassoldati A. Aromatase inhibitors in the adjuvant treatment of postmenopausal women with early breast cancer: Putting safety issues into perspective. *Breast J* 2007;13:28-35.
5. Coombes RC, Kilburn LS, Snowdon CF, et al. Survival and safety of exemestane versus tamoxifen after 2-3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial. *Lancet* 2007;369:559-70.
6. Osipo C, Liu H, Meeke K, Jordan VC. The consequences of exhaustive antiestrogen therapy in breast cancer: estrogen-induced tumor cell death. *Exp Biol Med (Maywood)* 2004;229:722-31.
7. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer cell* 2004;5:207-13.
8. Hiscox S, Morgan L, Barrow D, Dutkowskil C, Wakeling A, Nicholson RI. Tamoxifen resistance in breast cancer cells is accompanied by an enhanced motile and invasive phenotype: inhibition by gefitinib ('Iressa', ZD1839). *Clin Exp Metastasis* 2004;21:201-12.
9. Hiscox S, Jiang WG, Obermeier K, et al. Tamoxifen resistance in MCF7 cells promotes EMT-like behaviour and involves modulation of beta-catenin phosphorylation. *Int J Cancer* 2006;118:290-301.
10. Hiscox S, Morgan L, Green TP, Barrow D, Gee J, Nicholson RI. Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. *Breast Cancer Res Treat* 2006;97:263-74.
11. Chau NM, Ashcroft M. Akt2: a role in breast cancer metastasis. *Breast Cancer Res* 2004;6:55-7.
12. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* 2003;3:453-8.

13. Vleminckx K, Vakaet L, Jr., Mareel M, Fiers W, van Roy F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* 1991;66:107-19.
14. van den Brule FA, Engel J, Stetler-Stevenson WG, Liu FT, Sobel ME, Castronovo V. Genes involved in tumor invasion and metastasis are differentially modulated by estradiol and progesterin in human breast-cancer cells. *Int J Cancer* 1992;52:653-7.
15. Kuroki M, Matsuo Y, Kinugasa T, Matsuoka Y. Three different NCA species, CGM6/CD67, NCA-95, and NCA-90, are comprised in the major 90 to 100-kDa band of granulocyte NCA detectable upon SDS-polyacrylamide gel electrophoresis. *Biochem Biophys Res Commun* 1992;182:501-6.
16. Scholzel S, Zimmermann W, Schwarzkopf G, Grunert F, Rogaczewski B, Thompson J. Carcinoembryonic antigen family members CEACAM6 and CEACAM7 are differentially expressed in normal tissues and oppositely deregulated in hyperplastic colorectal polyps and early adenomas. *Am J Pathol* 2000;156:595-605.
17. Blumenthal RD, Leon E, Hansen HJ, Goldenberg DM. Expression patterns of CEACAM5 and CEACAM6 in primary and metastatic cancers. *BMC Cancer* 2007;7:2.
18. Duxbury MS, Ito H, Benoit E, Ashley SW, Whang EE. CEACAM6 is a determinant of pancreatic adenocarcinoma cellular invasiveness. *Br J Cancer* 2004;91:1384-90.
19. Duxbury MS, Matros E, Clancy T, et al. CEACAM6 is a novel biomarker in pancreatic adenocarcinoma and PanIN lesions. *Ann Surg* 2005;241:491-6.
20. Blumenthal RD, Hansen HJ, Goldenberg DM. Inhibition of adhesion, invasion, and metastasis by antibodies targeting CEACAM6 (NCA-90) and CEACAM5 (Carcinoembryonic Antigen). *Cancer Res* 2005;65:8809-17.
21. Poola I, Shokrani B, Bhatnagar R, DeWitty RL, Yue Q, Bonney G. Expression of carcinoembryonic antigen cell adhesion molecule 6 oncoprotein in atypical ductal hyperplastic tissues is associated with the development of invasive breast cancer. *Clin Cancer Res* 2006;12:4773-83.
22. 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.
23. 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;94:131-41.
24. Lewis JS, 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;97:1746-59.

25. Pink JJ, Jiang SY, Fritsch M, Jordan VC. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* 1995;55:2583-90.
26. Pink JJ, Wu SQ, Wolf DM, Bilimoria MM, Jordan VC. A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. *Nucleic Acids Res* 1996;24:962-9.
27. Gottardis MM, Robinson SP, Jordan VC. Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumoristatic action of tamoxifen. *J Steroid Biochem* 1988;30:311-4.
28. Osborne CK, Hobbs K, Clark GM. Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. *Cancer Res* 1985;45:584-90.
29. Thompson EW, Reich R, Shima TB, et al. Differential regulation of growth and invasiveness of MCF-7 breast cancer cells by antiestrogens. *Cancer Res* 1988;48:6764-8.
30. Gibson MK, Nemmers LA, Beckman WC, Jr., Davis VL, Curtis SW, Korach KS. The mechanism of ICI 164,384 antiestrogenicity involves rapid loss of estrogen receptor in uterine tissue. *Endocrinology* 1991;129:2000-10.
31. Dauvois S, Danielian PS, White R, Parker MG. Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover. *Proc Natl Acad Sci U S A* 1992;89:4037-41.
32. Irby RB, Yeatman TJ. Role of Src expression and activation in human cancer. *Oncogene* 2000;19:5636-42.
33. Duxbury MS, Ito H, Ashley SW, Whang EE. c-Src-dependent cross-talk between CEACAM6 and  $\alpha$ v $\beta$ 3 integrin enhances pancreatic adenocarcinoma cell adhesion to extracellular matrix components. *Biochem Biophys Res Commun* 2004;317:133-41.
34. Stefanova I, Horejsi V, Ansotegui IJ, Knapp W, Stockinger H. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* 1991;254:1016-9.
35. Skubitz KM, Campbell KD, Ahmed K, Skubitz AP. CD66 family members are associated with tyrosine kinase activity in human neutrophils. *J Immunol* 1995;155:5382-90.
36. Duxbury MS, Ito H, Ashley SW, Whang EE. CEACAM6 cross-linking induces caveolin-1-dependent, Src-mediated focal adhesion kinase phosphorylation in BxPC3 pancreatic adenocarcinoma cells. *J Biol Chem* 2004;279:23176-82.
37. Yang CM, Lin MI, Hsieh HL, Sun CC, Ma YH, Hsiao LD. Bradykinin-induced p42/p44 MAPK phosphorylation and cell proliferation via Src, EGF receptors, and PI3-K/Akt in vascular smooth muscle cells. *J Cell Physiol* 2005;203:538-46.

38. Spurbeck WW, Ng CY, Strom TS, Vanin EF, Davidoff AM. Enforced expression of tissue inhibitor of matrix metalloproteinase-3 affects functional capillary morphogenesis and inhibits tumor growth in a murine tumor model. *Blood* 2002;100:3361-8.
39. Stetler-Stevenson WG, Seo DW. TIMP-2: an endogenous inhibitor of angiogenesis. *Trends Mol Med* 2005;11:97-103.
40. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev* 1999;13:2905-27.
41. Bellacosa A, Kumar CC, Di Cristofano A, Testa JR. Activation of AKT kinases in cancer: implications for therapeutic targeting. *Advances in cancer research* 2005;94:29-86.
42. Sekharam M, Zhao H, Sun M, et al. Insulin-like growth factor 1 receptor enhances invasion and induces resistance to apoptosis of colon cancer cells through the Akt/Bcl-x(L) pathway. *Cancer Res* 2003;63:7708-16.
43. Simpson L, Parsons R. PTEN: life as a tumor suppressor. *Exp Cell Res* 2001;264:29-41.
44. Parsons R, Simpson L. PTEN and cancer. *Methods Mol Biol* 2003;222:147-66.
45. Mikhail M, Velazquez E, Shapiro R, et al. PTEN expression in melanoma: relationship with patient survival, Bcl-2 expression, and proliferation. *Clin Cancer Res* 2005;11:5153-7.
46. Kang Y, Massague J. Epithelial-mesenchymal transitions: twist in development and metastasis. *Cell* 2004;118:277-9.
47. Garcia M, Derocq D, Freiss G, Rochefort H. Activation of estrogen receptor transfected into a receptor-negative breast cancer cell line decreases the metastatic and invasive potential of the cells. *Proc Natl Acad Sci U S A* 1992;89:11538-42.
48. Clark GM, McGuire WL. Prognostic factors in primary breast cancer. *Breast Cancer Res Treat* 1983;3 Suppl:S69-72.
49. Esteva FJ, Sahin AA, Cristofanilli M, Arun B, Hortobagyi GN. Molecular prognostic factors for breast cancer metastasis and survival. *Semin Radiat Oncol* 2002;12:319-28.

## Figure legends

**Figure 1.** Characterization of long-term estrogen deprived breast cancer cells. *A*, comparison of cell growth rates among wild-type MCF-7 cells and estrogen-deprived MCF-7:5C and MCF-7:2A variant clones. To determine growth rates in basal medium, cells were seeded in 24-well dishes (30,000 per well) in estrogen-free RPMI media containing 10% SFS for the indicated time and total DNA (ng/well) was quantitated. *Points*, mean ( $n=6$ ); *bars*, SE. *B*, phase-contrast photomicrographs of MCF-7, MCF-7:5C, and MCF-7:2A cells. All cells were in log growth phase. Magnification, x10. *C*, ER $\alpha$  mRNA levels were determined by quantitative real-time PCR and normalized to 18S rRNA level. For estrogenic regulation of ER $\alpha$ , cells were treated with E<sub>2</sub> for 48 hours. *Columns*, mean ( $n = 3$ ); *bars*, SE. *p values* for comparisons are indicated. *D*, ER $\alpha$  protein levels were determined by immunoblotting with a specific ER $\alpha$  antibody. Cells were treated with ethanol vehicle (control), 1nM estradiol, or 1  $\mu$ M fulvestrant for 48 hours and 50  $\mu$ g of protein lysates were analyzed.  $\beta$ -actin was used as a loading control.

**Figure 2.** Overview of global gene expression patterns in wild-type MCF-7:WS8 cells and estrogen deprived MCF-7:5C and MCF-7:2A variant clones. *A*, unsupervised hierarchical clustering dendrogram of 904 genes most differentially expressed across the three cell lines, and consistently regulated in replicate expression arrays for each cell line. Each row represents a single gene. *Red*, genes with high expression levels; *green*, genes with low expression levels. The similarities in the expression pattern among the three cell lines are presented as a "condition tree" on the top of the matrix. *B*, venn diagram representation of genes regulated greater than 2 fold between any of the three cell lines. Of the 14,056 genes that met minimal quality metrics (as

described in the methods section for *Panel A*), a total of 2476 genes were upregulated (upper diagram), a total of 3156 genes were downregulated (lower diagram) and 8424 genes remained unchanged (not shown). Each cell line pairwise comparison is shown as a separate color (identified by the key below the lower venn diagram. Letters *a* through *g* define regions of unique and overlapping patterns of gene regulation and discussed in the main text. *C*, gene ontology list (color coded to the venn diagrams) of the unique cell processes operating in the clones relative to wild-type MCF-7:WS8 (yellow and blue), the most significantly operating processes in both clones relative to MCF-7:WS8 (green), and between the two clones (purple). Only those ontologies with a  $p < 0.01$  are listed. GeneGo software was used for ontology analysis. *D*, fold change in expression of invasion genes CEACAM6, CEACAM5/CEA, MMP-9, CXCR4, and CD44 in estrogen deprived MCF-7:5C and MCF-7:2A cells compared to parental MCF-7 cells, as identified by microarray gene analysis.

**Figure 3.** CEACAM6 promotes cell migration and invasion of long-term estrogen deprived breast cancer cells. *A*, CEACAM6 mRNA expression in MCF-7, MCF-7:5C (5C), and MCF-7:2A (2A) cells was determined by quantitative real-time PCR and normalized to 18S rRNA level. Error bars indicate SE.  $p$  values for comparisons are indicated. *B*, Western blot analysis of CEACAM6 and other invasion proteins in wild-type MCF-7 cells and estrogen-deprived MCF-7:5C and MCF-7:2A cells. *C*, the migration and invasiveness of estrogen-deprived breast cancer cells was compared with parental MCF-7 cells. For quantification of cells migrating across Transwell filters, cells were seeded onto the migration chamber ( $1 \times 10^4$  per chamber). After 24 h, chambers were stained and counted for migrated cells. *Columns*, average number of cells migrating per 10 microscope fields.  $p$  values for comparisons are indicated. *D*, Cells that invaded

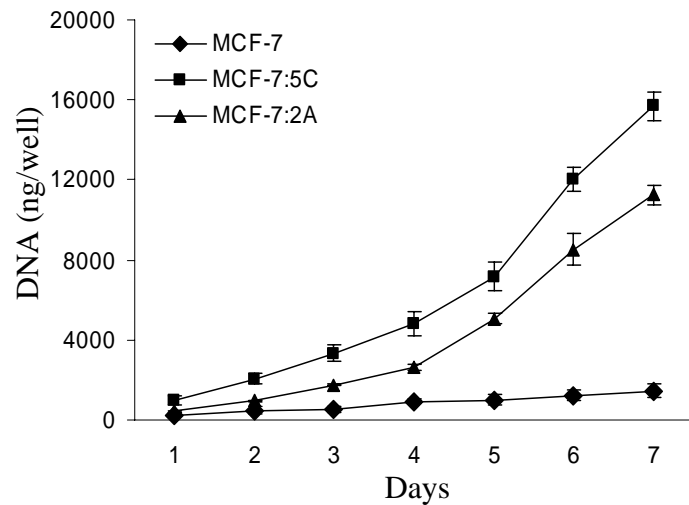
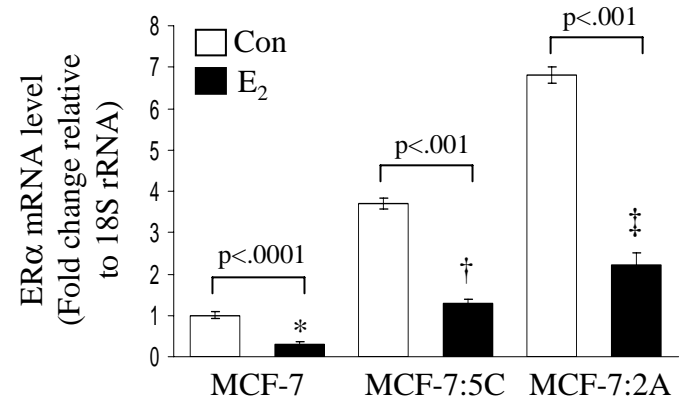
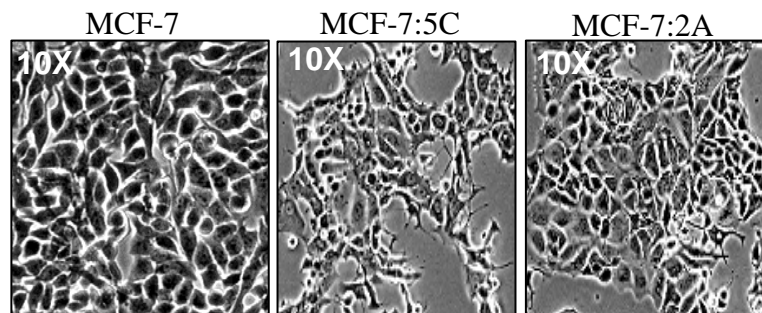
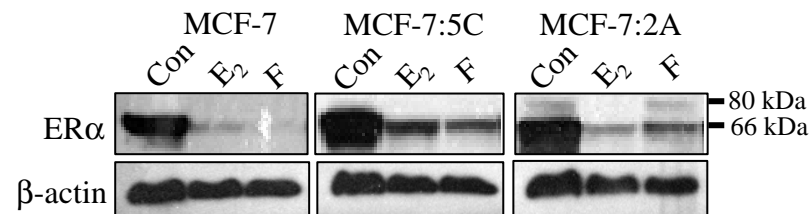
through the Matrigel-coated transwells were fixed, stained, visualized at x10 magnification by light microscopy and photographed with a digital camera after 48 hours; each panel represents an example of three replicates. *Columns*, average number of cells invading per 10 microscope fields. \* $p < .0001$ , † $p < .000001$ .

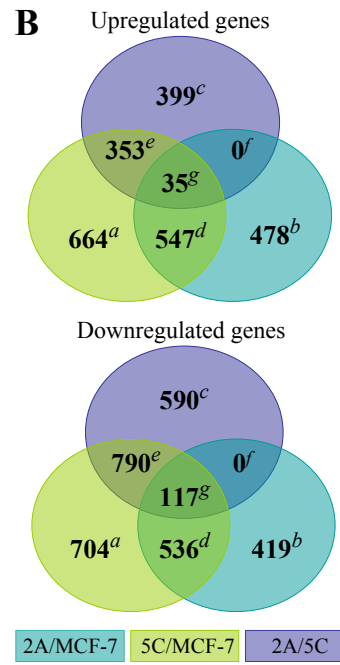
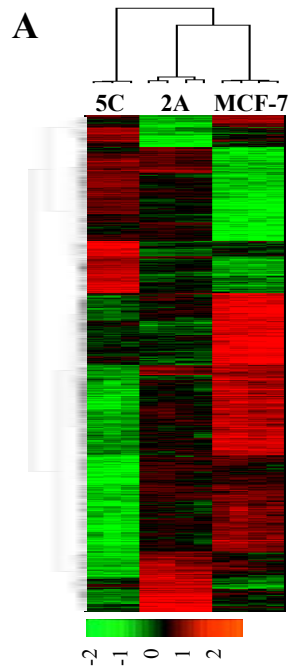
**Figure 4.** CEACAM6 is critical for invasion of estrogen deprived MCF-7:5C breast cancer cells. *A*, siRNA-mediated gene knockdown of CEACAM6 in MCF-7:5C invasive breast cancer cells. Immunoblot analysis of MCF-7:5C cells transfected with siRNA against CEACAM6, siCEACAM6, and scrambled sequence control siRNA, siControl for 48 hours. Cell lysates were probed for CEACAM6 with the corresponding antibody and  $\beta$ -actin, as loading control. *B*, relative amounts of CEACAM6 mRNA expression normalized to 18S rRNA level was quantified in transfected cells by quantitative real-time PCR. Error bars indicate SE. \* $p < .0001$ . *C*, matrigel invasion assay of siControl and siCEACAM6-transfected MCF-7:5C cells. Transfected cells were seeded onto the invasion chamber ( $1 \times 10^4$  per chamber). After 48 h, cells that invaded through the Matrigel-coated transwells were fixed, stained, and photographed; each panel represents an example of three replicates. *Columns*, average number of cells invading per 10 microscope fields. Error bars indicate SE. \* $p < .0001$ .

**Figure 5.**  $17\beta$ -estradiol suppresses CEACAM6 expression and blocks invasion of estrogen-deprived breast cancer cells. *A*, estrogenic regulation of CEACAM6 mRNA expression in MCF-7:5C and MCF-7:2A cells. Cells were treated with 1nM estradiol ( $E_2$ ) for 48 hours. CEACAM6 mRNA level was determined by quantitative real-time PCR and normalized to 18S rRNA and CEACAM6 mRNA in MCF-7 cells. Error bars indicate SE. \* $p < .0001$ . *B*, Western blot analysis of CEACAM6 protein in MCF-7, MCF-7:5C, and MCF-7:2A cells following treatment with  $E_2$

alone or E<sub>2</sub> plus fulvestrant (pure antiestrogen) for 48 h. *Line graph*, time-dependent effect of E<sub>2</sub> on CEACAM6 protein level in MCF-7:5C cells. *C*, the invasiveness of MCF-7:5C cells is blocked by E<sub>2</sub> but not the pure antiestrogen fulvestrant. Cells were seeded onto the invasion chamber (1 x 10<sup>4</sup> per chamber) and after 24 hours, they were treated with E<sub>2</sub> alone or E<sub>2</sub> plus fulvestrant for an additional 48 hours. Cells that invaded through the Matrigel-coated transwells were fixed, stained, and photographed; each panel represents an example of three replicates. *Columns*, average number of cells invading per 10 microscope fields. \**p*<.0001.

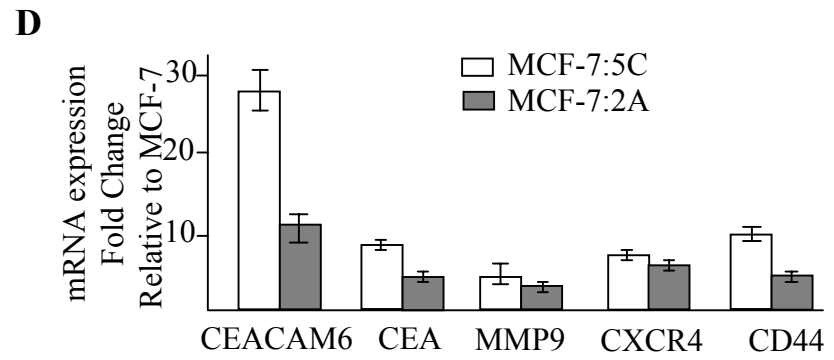
**Figure 6.** *A*, Effect of the Src inhibitor PP2 on Src phosphorylation and invasion of breast cancer cells. *A*, Wild-type MCF-7 cells and estrogen deprived MCF-7:5C and MCF-7:2A were treated with 10 μM PP2 for 24 h and Western blot analysis was performed on lysates for detection of total c-Src and c-Src-Tyr<sup>529</sup>. Anti-β-actin antibody was used as loading control. *B*, MCF-7:5C and MCF-7:2A cells were incubated for 48 h in the absence (control) or presence of 10 μM PP2 and matrigel invasion chambers were used to measure the effect of c-Src inhibition of cellular invasion. Cells that invaded through the Matrigel were stained, visualized using light microscopy, and counted. Each experiment was done in triplicate. The mean values of invasive cells were graphed versus 10 μM PP2. Error bars indicate SE. *p* values are indicated. *C*, total cell lysates were prepared from MCF-7, MCF-7:5C, and MCF-7:2A cells and equivalent amounts of protein were resolved by SDS-PAGE, blotted, and probed with the indicated antibodies *D*, functional assays. The indicated cells were treated with 10 μM PI3K inhibitor LY294002 for 7 days (*top panel*) or 48 hours (*bottom panel*) and cell growth was determined by DNA quantitation assay and cell invasion was determined by Matrigel invasion assay, as previously described. The experiments were repeated thrice in triplicates. Error bars indicate SE. \**p*<.001 (*top panel*) ; \**p*<.01 (*bottom panel*).

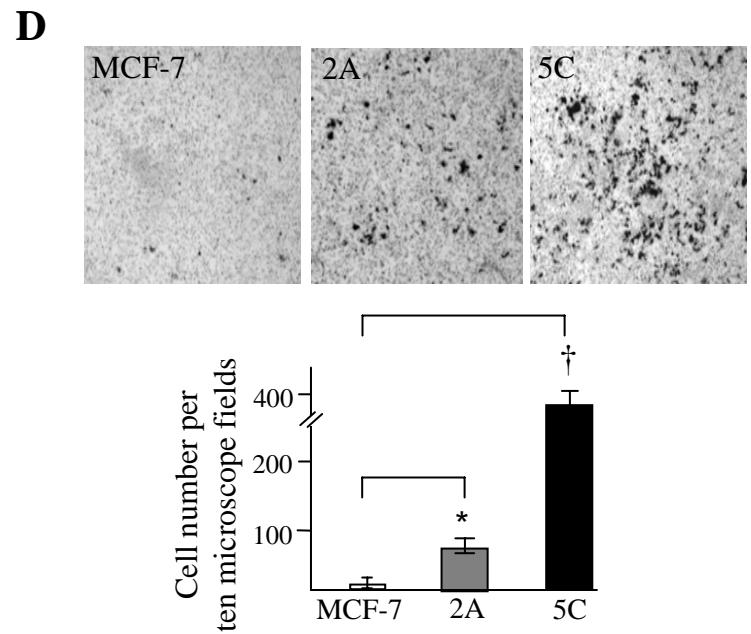
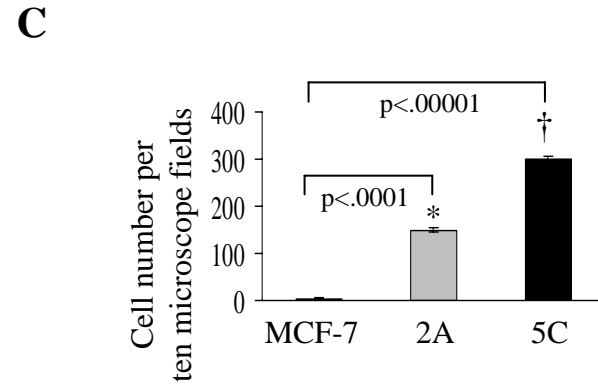
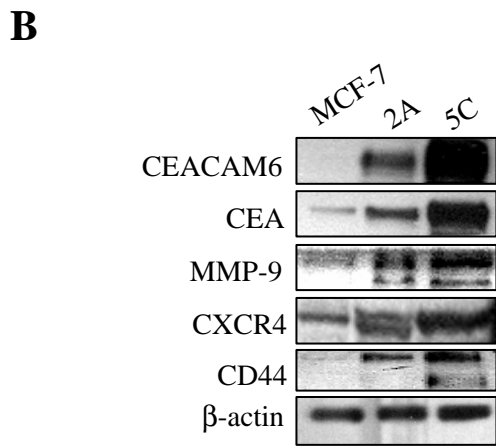
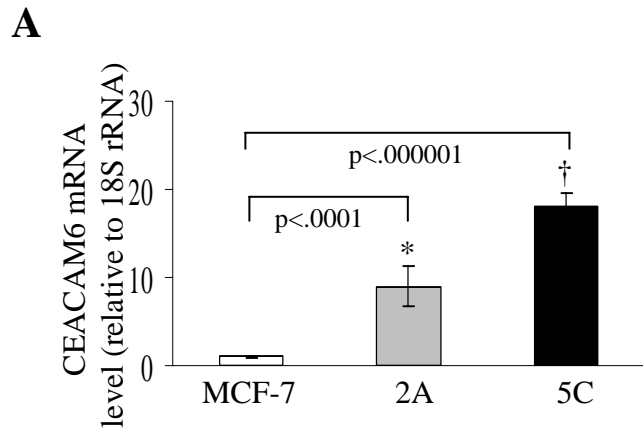
**A****C****B****D**

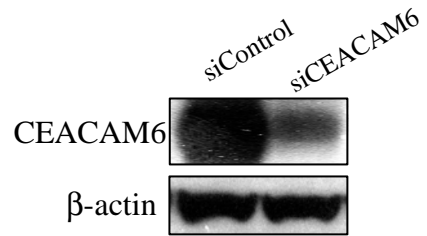
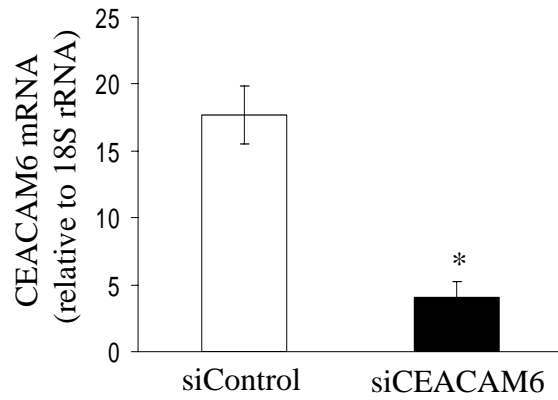
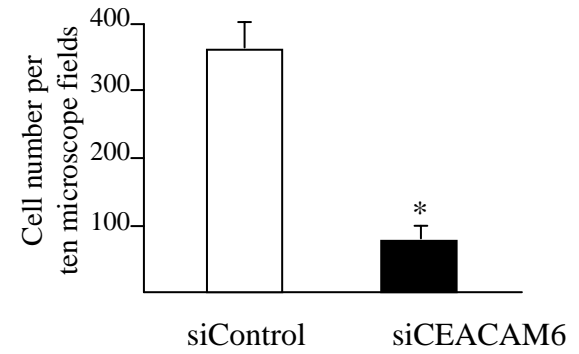
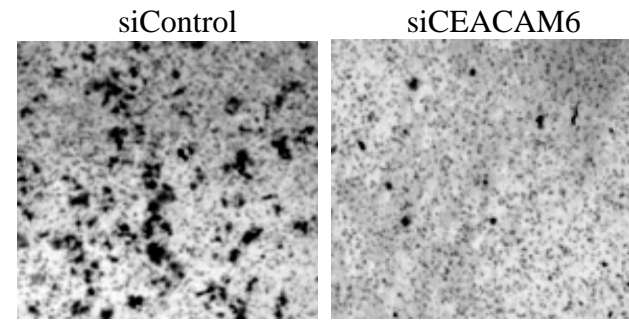


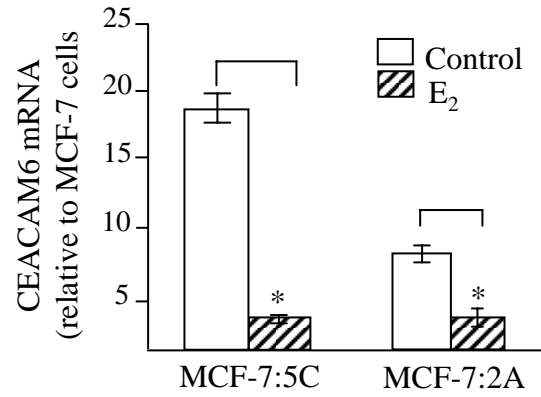
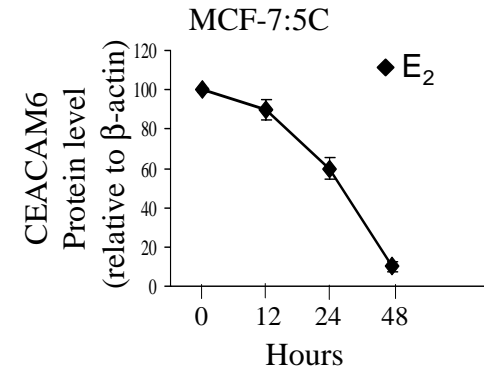
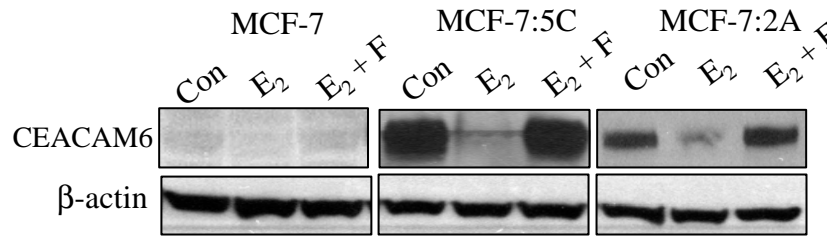
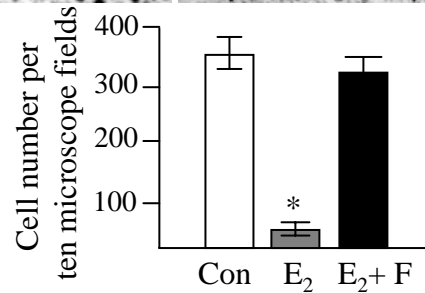
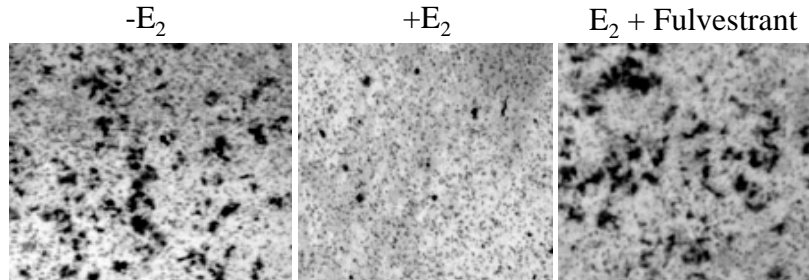
**C Gene Ontology**

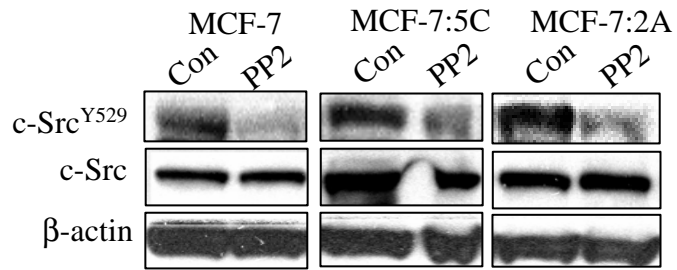
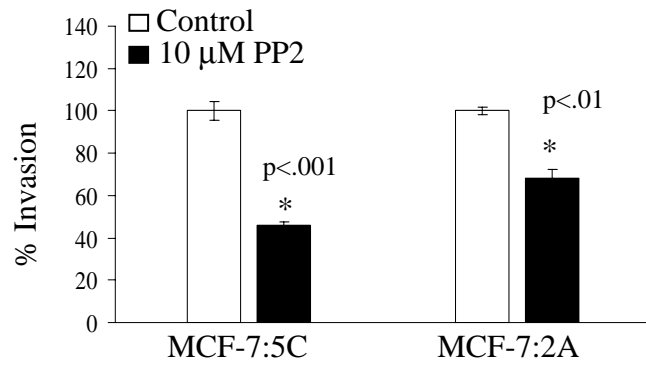
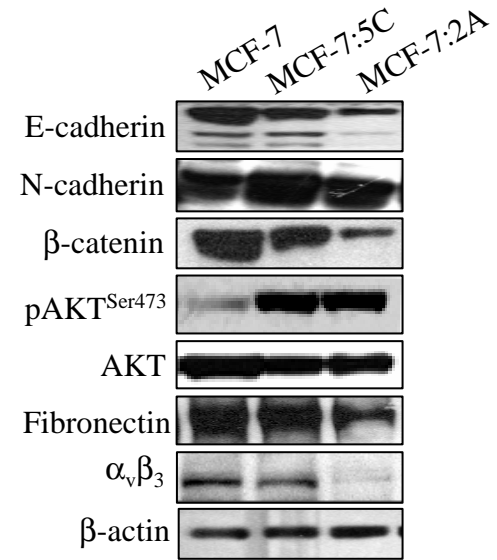
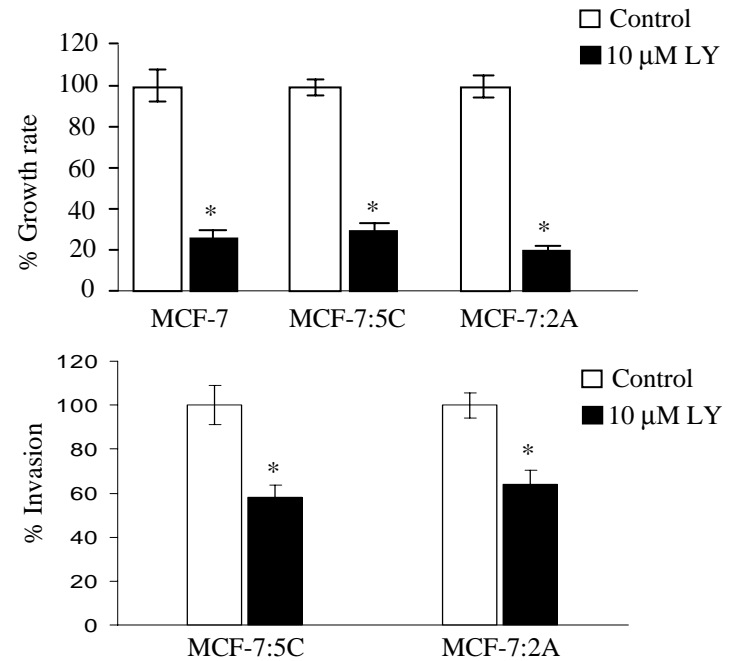
Cellular Process	p-value
Cell Cycle Control	5.28E-11
Growth and Differentiation	1.55E-04
Amino Acid/Vitamin Metabolism	1.75E-04
Apoptosis	1.63E-03
Growth Factor Signaling	5.18E-03
Cell Cycle Control	3.52E-24
Apoptosis	7.32E-05
Amino Acid/Nucleotide/Vitamin Metabolism	2.56E-04
Growth Factor Signaling	7.52E-04
Growth and Differentiation	1.25E-03
Cell Cycle Control	1.43E-16
Vitamin Metabolism	4.33E-03
Growth and Differentiation	5.23E-03
PTEN Signaling	5.14E-04
Proteolysis	7.69E-04
Growth and Differentiation	1.04E-03
Steroid Metabolism	5.33E-03





**A****B****C**

**A****B****C**

**A****B****C****D**

Clinical Breast Cancer

**Low Dose Estrogen Therapy to Reverse Acquired  
Antihormonal Resistance in the Treatment of Breast Cancer**

**Ramona F. Swaby and V. Craig Jordan**

**Affiliation:**

Fox Chase Cancer Center, Philadelphia, PA

**Correspondence:**

V. Craig Jordan, OBE, PhD, DSc  
Vice President and Research Director  
For Medical Science  
Alfred G. Knudson Chair of Cancer Research  
Fox Chase Cancer Center  
333 Cottman Avenue  
Philadelphia, PA 19111-2497  
Phone: (215) 728-7410  
Fax: (215) 728-7034  
Email: [v.craig.jordan@fccc.edu](mailto:v.craig.jordan@fccc.edu)

## **Abstract**

Estrogen is a potent stimulus for growth in its target organs; the uterus, vagina and some estrogen receptor positive breast cancers. However, estrogen is also able to control menopausal symptoms and maintain bone density in postmenopausal women. Until recently, there was also believed to be a link between estrogen and the prevention of cardiovascular disease. For these reasons, hormone replacement therapy (HRT) with an orally active estrogen and progestin has been used routinely for more than 50 years to maintain physiologic homeostasis after menopause. Not surprisingly, HRT increases the risk of developing breast cancer. The link between estrogen and breast cancer growth served as the incentive to develop long term tamoxifen therapy and subsequently the aromatase inhibitors, as successful “antiestrogenic” treatments. Unfortunately, the consequence of exhaustive therapy is drug resistance. Laboratory studies have defined the evolution of tumor drug resistance to tamoxifen, raloxifene (used for breast and osteoporosis chemoprevention), and the aromatase inhibitors. Remarkably, the long term exposure of breast cancers to antihormonal therapy also exposes a vulnerability that is being exploited in the clinic. Years of antihormonal therapy alters the cellular response mechanism to estrogen. Normally, estrogen is classified as a survival signal in breast cancer but in sensitive antihormone resistant cells, estrogen induces apoptosis. Once resistant cells are killed, antihormonal therapy is once again effective. This new targeted approach to the treatment of metastatic breast cancer could open the door to novel approaches to treatment with drug combinations.

## **Introduction**

Estrogen is essential for life. Without estrogen there would be no human race; reproduction would be impossible. However, with the evolution of the human race and the development of functional societies has come the promise of an extended life through the control and in some cases, conquest of disease.

The end of the 19<sup>th</sup> century was a period of important medical advances with the introduction of vaccines and the start of the chemotherapeutic era for infectious diseases. Life expectancy for women was short – 44.46 years.<sup>1</sup> After a century of implementing public health advances with vaccination and antibacterial therapies, life expectancy for women is now 80.8 years.<sup>2</sup> This is true for all developed countries, but with success in public health comes new challenges for a population that is larger than ever before.

Cancer is essentially a disease of advancing years. Specifically, breast cancer is rare in women under 30 years of age (4 per 100,000 women), but increases dramatically during the next 40 years of life. The incidence of breast cancer in a population of 70-75 year old women is 400 per 100,000 women per year. Although there is some emerging evidence that estrogen can cause transformation of breast or mammary cells,<sup>3</sup> there is evidence from prospective studies that the practice of prescribing hormone replacement therapy (HRT) to prevent osteoporosis and hypothetically to prevent aging has significantly increased breast cancer incidence.<sup>4-6</sup> A brief examination of why HRT became so fashionable and the current clinical concerns will serve as a physiological background to address the rationale for the development of endocrine therapies (high dose) sex hormones or antihormones for breast cancer treatment over the past 50 years.

## **Hormone replacement therapy**

The initial goal for estrogen replacement was to ameliorate the menopausal symptoms that occurred once ovarian estrogen synthesis ceased. Subsequently, the focus was to maintain bone density or prevent increases in coronary heart disease in women during later life. Two approaches occurred to enhance and maintain the physiologic actions of estrogen past the menopause.

Synthetic estrogens based either on the structure of triphenylethylene or the very potent, but shorter acting diethylstilbestrol<sup>7,8</sup> (Figure 1), were described in the literature proved to be a cheap source of new medicines. High dose synthetic estrogen administration was found to be effective in the treatment of breast and prostate cancer,<sup>9</sup> but even low doses of synthetic estrogens never really became accepted as HRT in postmenopausal women. Indeed, diethylstilbestrol subsequently achieved notoriety as an estrogen supplement to prevent recurrent abortion. Children of treated mothers had a high incidence of clear cell carcinoma of the vagina<sup>10, 11</sup>. In contrast, the synthetic estrogens based on triphenylethylenes were subsequently to undergo a metamorphosis and be transformed into antiestrogens used for the treatment of breast cancer<sup>12</sup> (Figure 2).

The estrogen (Figure 3) derived from pregnant mares (Premarin®) was initially used as an estrogen replacement therapy for postmenopausal women. However it found that there was a 6 fold elevation in endometrial cancer.<sup>13, 14</sup> The stimulatory action of estrogen in the uterus was neutralized by combining the orally active estrogen with the synthetic progestin medroxyprogesterone acetate (MPA) as Prem Pro®. This preparation was used by patients for up to a decade to prevent osteoporosis, menopausal symptoms,

and was also taken by many women in the belief it would prevent aging and coronary heart disease (CHD).

The actual link between HRT and breast cancer was addressed prospectively in two studies initiated during the 1990's. The Women's Health Initiative (WHI) recruited 16,608 women between the ages of 50-79 years who received either conjugated equine estrogen 0.625mg/day plus MPA 2.5 mg/d or placebo. The primary outcome was CHD with invasive breast cancer as the primary adverse outcome. The Million Women Study recruited 1,084 (10 women aged 50-64 years) to determine the effects of specific types of HRT on incidence and fatal breast cancer.

The WHI, with a mean follow up of 5.2 years, was stopped prematurely because invasive breast cancer incidence exceeded the stopping boundary.<sup>4</sup> Overall, it was found that breast cancers were diagnosed in the HRT treated women at a later stage compared to placebo, possibly because there was an increase in mammographic density.<sup>6</sup> Overall, the study investigators did not find that HRT should be used to reduce the risk of CHD.<sup>4</sup> However, a recent sub-analysis of younger women in the group indicates minor benefit.<sup>15</sup>

The Million Women's Study<sup>5</sup> concluded that HRT is associated with an increased risk of incidence of fatal breast cancer, particularly if the HRT was an estrogen/progestin combination. The authors estimated that, over the decade 1993-2003, HRT had increased the incidence of invasive breast cancer in the United Kingdom by an excess of 20,000 new breast cancers.

It is interesting to note that with the publication of both the WHI Study and the Million Women's Study in the first 5 years of the 21<sup>st</sup> Century, there has been a

significant decline in the prescribing of HRT.<sup>16-20</sup> As a result, this has been associated with a drop in the incidence of breast cancer.<sup>21</sup>

Thus, estrogen has a justified reputation as a potent stimulant of breast cancer development and growth. This reputation led to the development of antiestrogenic targeted strategies to treat and prevent breast cancer.

### **Antiestrogenic treatment strategies**

In the latter part of the 19<sup>th</sup> Century, farmers in Scotland ovariectomized their farm animals to extend milk production. The observation had also been made that the histology of the lactating breast was similar to breast cancer. This knowledge was subsequently used by George Beaston<sup>22</sup> to justify the oophorectomy of a young woman who had inoperable advanced metastatic disease breast cancer. The woman responded dramatically but further evaluation of the concept demonstrated that only one in three women would have effective disease control for about 1-3 years.<sup>23</sup> Nevertheless, the concept of endocrine ablation as a standard treatment for metastatic breast cancer was subsequently extended to postmenopausal women with the use of adrenalectomy and hypophysectomy.<sup>24</sup> The response rate remained at 30% but it was not until the pioneering work of Elwood Jensen<sup>25</sup> and the identification of the estrogen receptor (ER) that progress was made in understanding estrogen regulated growth mechanisms. The development of the ER assay that was primarily used to exclude those women who would *not* respond to endocrine ablation, was an important step forward in breast cancer treatment.<sup>26, 27</sup> Looked at in another way, the presence of the ER in a breast tumor increased the probability that endocrine ablation would be successful. Since this was the era before tamoxifen, it also suggested a use for a drug ICI 46,474, discovered in the

antifertility program at Imperial Chemical Industries (ICI) Pharmaceuticals Division (now AstraZeneca). The compound failed in its primary application as an antifertility agent<sup>28</sup> because, like clomiphene,<sup>29</sup> it induced ovulation in subfertile women.<sup>30</sup> The compound was found to have modest activity as a treatment for unselected breast cancer<sup>31</sup> but ICI 46,474 was subsequently reinvented<sup>32</sup> during the 1970's as a targeted therapy for breast cancer. A scientific foundation was established in the laboratory for the treatment and prevention of breast cancer<sup>33-35</sup> by blocking estrogen action at the level of the ER.<sup>36</sup>

Coincidentally, another approach to controlling the growth of estrogen stimulated breast cancer was also emerging in the 1970's with the specific targeting of the aromatase enzyme CYP19 that converts androstenedione or testosterone into estrone or estradiol respectively in postmenopausal patients.<sup>37</sup> The first clinically useful specific aromatase inhibitor was 4-hydroxyandrostenedione that binds irreversibly to the active site of the enzyme.<sup>38</sup> There are now numerous aromatase inhibitors that bind either irreversibly or competitively at the active site of the aromatase enzyme.

### **Transition to Tamoxifen.**

Prior to 1981, the standard of care for the palliative treatment of post-menopausal women with metastatic breast cancer included high dose estrogen treatment.<sup>24</sup> Although the mechanism of action was unknown, treatment with diethylstilbestrol (DES) was accepted as being among the most effective of the medical hormonal manipulations employed with expected response rates (RR) of approximately 36%. Other common hormonal approaches included "androgenization" with androgens (21% RR), high dose progestins, used either as a single agent or in combination with estrogen, and the use of

glucocorticosteroids as a means of chemical castration to interrupt the hormonal feedback-stimulation axis. These additional hormonal therapies resulted in expected RR ranging from 15 to 50%, with the lower figures being more realistic.

ICI 46,474, also known as tamoxifen, is a non-steroidal antiestrogen demonstrated in animal laboratory models to oppose the action of estrogens.<sup>39</sup> An early clinical appraisal of this agent was initially undertaken in 46 post-menopausal patients with metastatic breast cancer whose treatment had progressed after prior treatment with hormonal therapies.<sup>31</sup> Of the 46 patients treated with tamoxifen for at least 3 months, 10 patients (21%) demonstrated partial or complete response. Additionally, 17 patients (37%) experienced stable disease with some experiencing response of visceral metastases as well. Tamoxifen was well tolerated with few serious side effects. Hot flushes and nausea and vomiting were the most significant side effects resulting in treatment discontinuation in a few (4%).

Based on this and other encouraging data,<sup>40</sup> a randomized clinical evaluation of tamoxifen and diethylstilbestrol was undertaken.<sup>41</sup> One hundred and fifty-one post-menopausal women with metastatic breast cancer and measurable disease who may have been previously treated with chemotherapy, but had not been treated with previous hormonal therapies for metastatic disease, were randomized to treatment with either tamoxifen (10 mg 3 x daily) or diethylstilbestrol (5 mg 3 x daily). Treatment with diethylstilbestrol (RR = 41%) resulted in higher response rates (RR) than tamoxifen (RR = 33%), but the difference was not statistically significant. Clinical benefit rates (Clinical benefit = complete response + partial response + stable disease) of 84% and 78% were also similar for both tamoxifen and diethylstilbestrol, respectively. Toxicity profiles

avored tamoxifen with significantly lower rates of nausea and vomiting, edema, and vaginal bleeding. Several smaller randomized trials also confirmed these findings.<sup>42-44</sup> No significant differences between estrogen preparations and tamoxifen with respect to reported response rates (ranging from 25% to 53%), rates of clinical benefit, and/or duration of response were found. Because tamoxifen was associated with fewer side effects without loss of efficacy, it replaced DES as the first-line medical intervention of choice for post-menopausal women with metastatic breast cancer. Updated long-term follow-up analysis of greater than 14 years have confirmed the initial reported response rates.<sup>45</sup> However, of interest, with longer follow-up, 5-year survival is significantly superior (adjusted  $p = 0.039$ ) for the patients treated with DES (35%) compared to those treated with tamoxifen (16%).

Tamoxifen dosing was modeled in the laboratory to show that early chronic dosing of rats was more important at preventing mammary cancer development than larger interval doses.<sup>28, 46, 47</sup> These translational animal studies *in vivo* established the current standard use of long-term adjuvant antiestrogen therapy chronically administered to prevent breast cancer recurrence. Five years of adjuvant tamoxifen is known to reduce both the local recurrence as well as distant metastatic disease by approximately 50% in patients whose breast cancer expresses the estrogen receptor (ER).<sup>48</sup> Adjuvant tamoxifen also reduces the risk of breast cancer mortality by approximately one-third.<sup>48</sup>

### **Long term antihormonal therapy**

The scientific strategy<sup>36</sup> of targeting those breast tumors with the ER with long-term antihormonal therapy<sup>47</sup> has now reached its zenith. Long-term antihormonal adjuvant therapy is routine for patients with an ER positive tumor and several clinical

facts are now clear. Five years of adjuvant tamoxifen therapy is now considered sufficient to provide long-term survival benefits for patients<sup>49</sup> and the antitumor effects of tamoxifen extend for at least 10 years following a five year course of adjuvant therapy.<sup>48</sup> Side effects in postmenopausal women using tamoxifen are principally increases in endometrial cancer risk and blood clots. Although the risk benefit ratio is acceptable when tamoxifen is used as a therapy, this is not acceptable for postmenopausal women wishing to reduce the risk of breast cancer.<sup>50-52</sup> Aromatase inhibitors used for breast cancer treatment improve both survival and reduce concerns about blood clots and endometrial cancer,<sup>53-56</sup> but there is a potential concern about osteoporosis that can be adequately addressed with bisphosphonate treatment for women with either osteopenia or osteoporosis. No results are as yet available for the use of aromatase inhibitors as chemopreventive agents but the SERM raloxifene is available for the prevention of osteoporosis with, as predicted,<sup>57-60</sup> the prevention of breast cancer as a beneficial side effect.<sup>61, 62</sup> The use of raloxifene for this indication by one-half million osteoporotic women reduce breast cancer incidence by approximately 27,000 over ten years.<sup>63</sup> Recently, the application of raloxifene has been extended to primary chemoprevention in high risk postmenopausal women.<sup>64</sup>

Each of the applications of SERMs or aromatase inhibitors described above uses a 5 year treatment period. A small study demonstrated that longer term tamoxifen extending to 10 years did not improve recurrence rates but did increase accumulated side effects.<sup>65</sup> In contrast, the application of a non cross resistant aromatase inhibitor following 5 years of tamoxifen improves not only disease-free survival, but reduces the incidence of side effects and contralateral breast cancer.<sup>66, 67</sup> Thus, the proposal<sup>47</sup> of

using a SERM followed by estrogen deprivation has now become a clinical reality and long-term antihormonal therapy for the treatment and prevention of breast cancer is the standard of care. However, the ubiquitous application of antihormones in medicines now has consequences for breast cancer cells potentially exposed to estrogen deprivation for a decade. The treatment of antihormonal drug resistance is a challenge that needs to be addressed to develop cheap and effective future interventions.

### **Drug Resistance to Tamoxifen – Evolution from Benefit to Liability.**

With the advent of newer third generation selective aromatase inhibitors, it is common practice for post-menopausal patients to be treated with tamoxifen followed by extended adjuvant antiestrogen therapy with an aromatase inhibitor, resulting in at least 5-10 years total of chronic, continuous antiestrogen blockade<sup>66</sup>. However, antiestrogen therapy is not able to prevent all recurrences, suggesting that despite the presence of the ER, a majority of tumors become resistant. In fact, continuous extended therapy tamoxifen has consequences for initially estrogen responsive breast cancer cells. Here again, pre-clinical *in vivo* modeling has provided a scientific insight. The estrogen responsive ER positive breast cancer cell line MCF-7<sup>68</sup> has been successfully grown into tumors by inoculation into athymic mice. Subsequent treatment with long-term tamoxifen has been used to mimic the effects of adjuvant therapy. Years of treatment are replicated by serially transplanting any growing tumors into tamoxifen treated athymic, ovariectomized mice. Initially, tumors established in the presence of estrogen are growth suppressed by tamoxifen, maintaining cytostatic activity without progressive increase in size<sup>69</sup> for several months. However, eventually tamoxifen stimulated tumors start to grow but the tumors also grow in response to physiological estradiol levels.<sup>69</sup> These

characteristics are described as Phase I selective estrogen receptor modulator (SERM) resistance where either a SERM (e.g. – tamoxifen or raloxifene<sup>70</sup>) or estrogen can stimulate tumor growth in cells previously exposed to treatment with long term tamoxifen or SERM therapy (Figure 4). In the clinic, Phase I tumor resistance is usually treated with either an aromatase inhibitor or fulvestrant to destroy the ER.<sup>71, 72</sup>

### **A new biology of estrogen action**

If long-term tamoxifen treated tumors continue to be passaged for 4-5 years to mimic adjuvant tamoxifen therapy, they acquire molecular changes associated with an unanticipated vulnerability. Selective ER modulator stimulated growth is thought to be mediated by anti-apoptotic pathways.<sup>73, 74</sup> Unexpectedly, estrogen, rather than promoting growth of these long-term estrogen-deprived cells, now produces a tumoricidal effect.<sup>75, 76</sup> To confirm this laboratory finding, fresh mice were “bi-transplanted” with both newly established MCF-7 tumor as well as long term tamoxifen resistant MCF-7 tumor within the same animal on different sides of the axillary region of the mammary fat pads. When treated with tamoxifen, the wild MCF-7 tumor did not grow in response to tamoxifen treatment, while the tamoxifen resistant MCF-7 tumor grew. In contrast, estrogen stimulated the wild type MCF-7 tumor to grow but the long term tamoxifen resistance tumor did not grow. This suggested that the difference in response was not due to a difference in the host having an enhanced or altered response to estrogens and/or tamoxifen, but rather a property inherent to the ER positive breast cancer cells acquired in the setting of chronic estrogen deprivation over long periods of time.<sup>76</sup> These characteristics are described as Phase II of SERM resistance where ER positive tumors are stimulated to grow by tamoxifen, but killed by estrogen.

There is also another consequence of Phase II SERM resistance; Laboratory studies. Fulvestrant, the pure antiestrogen, is able to prevent Phase II tumor growth after tamoxifen withdrawal and the results are comparable to no treatment.<sup>74</sup> Again, these laboratory results are consistent with the clinical use of fulvestrant or an aromatase inhibitor following the development of tamoxifen resistance.<sup>71,72</sup> However, the laboratory finding that physiological estrogen plus fulvestrant causes robust tumor growth<sup>74, 77</sup> raises the question of a negative drug interaction between fulvestrant and physiologic estradiol. The inhibitor actions of each agent are cancelled out by the combination. Fulvestrant is not very active as a third line agent which raises the possibility that the estrogen already present in the postmenopausal woman may interfere in an unanticipated fashion with the inhibitory action of the pure antiestrogen. Clinical studies are ongoing, examining the efficacy of a fulvestrant/aromatase inhibitor combination.

Overall, the recognition of the new biology of estrogen action observed following the development of long-term tamoxifen treatment raises the question of the global relevance of the observation to estrogen withdrawal following treatment with aromatase inhibitors and the potential exploitation of the new knowledge of mechanisms can be identified.

### **Long-term Estrogen Withdrawal Apoptotic Mechanisms.**

The increasing clinical use of aromatase inhibitors to reduce estrogen synthesis as a strategy to treat breast cancer has resulted in increased efforts to examine drug resistance to estrogen withdrawal rather than SERM action. Early studies growing MCF-7 breast cancer cells in estrogen free media resulted in an increase in intracellular ER

levels and spontaneous cell growth.<sup>78, 79</sup> Several estrogen independent clones were isolated for study<sup>80, 81</sup> and ideas were proposed that MCF-7 cells are hypersensitized to grow in extremely low levels of estrogen: i.e. below the level that can be detected or further reduced.<sup>82</sup> However, Song and coworkers<sup>83</sup> observed that increasing concentrations of estradiol could increase apoptosis in estrogen deprived cells by increasing the concentration of FASL that activates death receptor pathways. Thus, the original observations that Phase II tamoxifen resistant tumors could be treated with physiologic estrogen<sup>75, 76</sup> were extended to aromatase inhibitor resistant cells. However, in contrast to Song's study<sup>83</sup>, Phase II tamoxifen resistant tumors respond to increasing estrogen treatment by increasing the FAS receptor, decreasing HER2/neu, and NFκB that is associated with tumor regression.<sup>74</sup> Furthermore, MCF-7 cells kept for many years under estrogen directed conditions using medium containing stripped fetal bovine serum produce rapid apoptosis via an intrinsic medium diverted at the mitochondrion.<sup>84, 85</sup> However, both Lewis<sup>85</sup> and Song<sup>86</sup> find that apoptosis is modulated through bcl-2 or bcl-2XL. A representative schema based on the studies of Lewis and coworkers<sup>85</sup> as shown in Figure 5.

It is also perhaps important to state that the new knowledge is emerging through re-examination of existing cell lines. In early publications studying the effects of estrogen withdrawal, no estrogen-induced apoptosis was noted<sup>80, 81</sup> but by altering culture conditions or extending the period of estrogen exposure, apoptosis occurs.<sup>84, 87</sup> Overall, the phenomenon observed with long-term estrogen withdrawal is similar to the Phase II resistance of the model described for SERMs.

## **Clinical Clues.**

In the clinic, patients with ER positive breast cancer are treated with exhaustive antiestrogen therapies. However, over time and with sequential antiestrogen therapy, antiestrogen resistance can be expected to occur in as many as 50%.<sup>88</sup> With each successive antiestrogen treatment of such recurrent tumors, tumor response becomes less durable. Also, the combination of tamoxifen plus DES was no better than tamoxifen alone.<sup>88</sup> Lonning and coworkers<sup>89</sup> addressed the hypothesis that patients with ER positive breast cancers who had been treated exhaustively with antihormonal therapy could potentially respond to high dose estrogen therapy. Thirty-two patients with advanced breast cancer previously exposed to between 2 and 10 (median 4) endocrine treatments were treated with DES (5 mg three times daily). Therapy was well tolerated but 4 patients terminated treatment within 2 weeks of starting and another two stopped treatment before progress. One of these patients had stable disease for 15 weeks and one a partial response for 39 weeks. Of the remainder, four patients obtained a complete response and six patients, a partial response. Two patients had stable disease for six months and one  $\geq$  one year. Overall, these extremely encouraging preliminary studies with high dose estrogen therapy are complimented by anecdotal reports of the effectiveness of low dose estrogen treatment for those women with endocrine refractory breast cancer following exhaustive antihormonal therapy (Dr. James Ingle, Mr. Michael Dixon, personal communications). As a result, several clinical studies are currently underway (Drs. Matthew Ellis and Richard Santen, personal communications).

## **Estrogen-induced apoptosis: clinical and laboratory correlations**

Based on the pre-clinical laboratory modeling, we have translated the new biology of estrogen action into a Department of Defense Center of Excellence grant with laboratory and clinical collaborators illustrated in Figure 6. Our goal is to define the pathways for estrogen induced survival and apoptosis in endocrine responsive breast and endometrial cancer and use the emerging database to guide the interpretation and development of a series of clinical trials. The ultimate goal of our clinical trial design is illustrated in Figure 7 and currently consists of two separate but interconnected therapeutic estrogen trials.

In Trial I, “A Single Arm Phase II Study of Pharmacologic Dose Estrogen in Postmenopausal Women with Hormone Receptor-Positive Metastatic Breast Cancer After Failure of Sequential Endocrine Therapies” eighty eight patients who have clearly responded and failed at least two antiestrogenic therapies will be treated for 12 weeks with 30mg estradiol (Estrace®). Patients who respond or have stable disease will be treated subsequently with 1 mg anastrozole until disease progression. Serum and, where possible, recurrent tissue biopsies will be used to determine serum apoptotic markers (Apoptosense®) and target genes in tumor material as markers of apoptosis or tumor progression. These data will be compared and contrasted with the results obtain from preclinical studies using our cell and animal models.

In Trial 2 “Reversal of Anti-Estrogen Resistance with Sequential Dose De-escalation of Pharmacologic Estrogen in a Single Arm Phase II Study of Postmenopausal Women with Hormone Receptor-Positive Metastatic Breast Cancer After Failure of Sequential Endocrine Therapies”, patients who have responded and subsequently failed two

antiestrogenic therapies will be treated as groups with successively lower doses of daily estradiol to determine the lowest dose necessary to produce an equivalent response to 30 mg estradiol in Trial 1.

On completion of the integrated research program, several questions will need be addressed to improve treatment of metastatic breast cancer:

- 1) Can a select group of patients be identified from either tumor markers or early serum apoptotic products who will respond to limited low dose estradiol treatment and who will subsequently remain under disease control with anastrozole treatment?
- 2) Can cell survival pathways be identified for tumors that do not respond to estradiol treatment?
- 3) Can survival pathways be subverted to improve response rates to estradiol-induced apoptosis?

## **Conclusions**

The development and extensive clinical application of long-term antihormonal therapy<sup>37</sup> has had consequences for the patient with the development of antihormonal drug resistance in some breast cancers.<sup>90</sup> However, with the development of drug resistance to exhaustive antihormonal therapy, a vulnerability of the cancer has been exposed. The recognition of the new biology of estrogen action that causes apoptosis in sensitive breast tumors now opens an unanticipated door of opportunity to exploit the findings to aid patients. Although the actual clinical responses may not be profound in unselected patient populations or in populations whose tumors do not have the correct (Stage II) form of breast cancer, our ability to decipher apoptotic mechanisms from

laboratory models and eventually target patients appropriately, may eventually have profound positive effects for some patients. The translational knowledge gained over the next few years may again provide unanticipated opportunities to exploit the discovery of “apoptotic triggers” for other forms of cancer.

It is perhaps pertinent to restate that for 70 years there has been an “ebb and flow” relationship in the role of estrogen in breast tumor homeostasis. We have illustrated in this review many of the changing fashions that have occurred in how estrogen is perceived as a benefit or a villain in women’s health. The effects of modulating the ER system in the breast, at one time or another, have been dismissed because they are small or believed to be of no major consequence. Nevertheless, the small observations become accumulative. By way of example, it is important to recall that initial use of tamoxifen, a failed contraceptive, to treat unselected populations showed only modest responses for some patients with metastatic breast cancer.<sup>32</sup> Years later, after deciphering the target populations and translating the appropriate treatment strategies from the laboratory to the clinic, the drug became the gold standard for endocrine therapy<sup>32</sup> and was credited with improving the survival of hundreds of thousands of women.<sup>48</sup> The challenge for the future is to exploit the profound apoptotic action of estradiol as a lead to develop innovative new therapies for cancer.

### **Acknowledgements:**

Drs. Jordan and Swaby are supported by the Department of Defense Breast Program under award number BC050277 Center of Excellence (Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense) and FCCC Core Grant NIH P30 CA006927. Dr. Jordan is also supported by the Weg Fund of Fox Chase Cancer Center.



## References

1. U.S. Department of Commerce, Bureau of the Census. Historical Statistics of the United States (Massachusetts only; white and nonwhite combined, the later being about 1% of the total.
2. Department of Health and Human Services, National Center for Health Statistics; National Vital Statistics Reports. 2006;54.
3. Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. *N Engl J Med* 2006;354(3):270-82.
4. 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(3):321-33.
5. Million Women Study Collaborators. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet* 2003;362:419-27.
6. Chlebowski RT, 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;289:3243-53.
7. Dodds EC, Goldberg L, Lawson W, Robinson R. Oestrogenic activity of certain synthetic compounds. *Nature* 1938;141:247-8.
8. Robson JM, Schonberg A, Fahim HA. Duration of action of natural and synthetic estrogens. *Nature* 1938;142:292-3.
9. Haddow A, Watkinson JM, Paterson E. Influence of synthetic oestrogens upon advanced malignant disease. *Br Med J* 1944;2:393-8.
10. Greenwald P, Barlow JJ, Nasca PC, Burnett WS. Vaginal cancer after maternal treatment with synthetic estrogens. *N Engl J Med* 1971;285(7):390-2.
11. Herbst AL, Ulfelder H, Poskanzer DC. Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. *N Engl J Med* 1971;284:878-81.
12. Lerner LJ, Jordan VC. The development of antiestrogens for the treatment of breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res* 1990;50:4177-89.
13. Smith DC, Prentice R, Thompson DJ, Herrmann WL. Association of exogenous estrogen and endometrial carcinoma. *N Engl J Med* 1975;293(23):1164-7.
14. Ziel HK, Finkle WD. Increased risk of endometrial carcinoma among users of conjugated estrogens. *N Engl J Med* 1975;293(23):1167-70.
15. Rossouw JE, Prentice RL, Manson JE, et al. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. *JAMA* 2007;297(13):1465-77.
16. Faber A, Bouvy ML, Loskamp L, van de Berg PB, Egberts TC, de Jong-van den Berg LT. Dramatic change in prescribing of hormone replacement therapy in The Netherlands after publication of the Million Women Study: a follow-up study. *Br J Clin Pharmacol* 2005;60(6):641-7.
17. de Jong-van den Berg LT, Faber A, van den Berg PB. HRT use in 2001 and 2004 in The Netherlands--a world of difference. *Maturitas* 2006;54(2):193-7.
18. Usher C, Teeling M, Bennett K, Feely J. Effect of clinical trial publicity on HRT prescribing in Ireland. *Eur J Clin Pharmacol* 2006;62(4):307-10.

19. Hing E, Brett KM. Changes in U.S. prescribing patterns of menopausal hormone therapy, 2001-2003. *Obstet Gynecol* 2006;108(1):33-40.
20. Power ML, Schulkin J, Rossouw JE. Evolving practice patterns and attitudes toward hormone therapy of obstetrician-gynecologists. *Menopause* 2007;14(1):20-8.
21. Ravdin PM, Cronin KA, Howlader N, et al. The decrease in breast-cancer incidence in 2003 in the United States. *N Engl J Med* 2007;356(16):1670-4.
22. Beatson CT. On treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. *Lancet* 1896;2:162-5.
23. Boyd S. On oophorectomy in cancer of the breast. *B M J* 1900;ii:1161-7.
24. Kennedy BJ. Hormone therapy for advanced breast cancer. *Cancer* 1965;18:1551-7.
25. Jensen EV, Jacobson HI. Basic guides to the mechanism of estrogen action. *Recent Progr Hormone Res* 1962;18:387-414.
26. Jensen EV, Block GE, Smith S, Kyser K, DeSombre ER. Estrogen receptors and breast cancer response to adrenalectomy. *Natl Cancer Inst Monogr* 1971;34:55-70.
27. McGuire WL, Carbone PP, Sears ME, Escher GC. Estrogen receptors in human breast cancer: an overview. In: *Estrogen Receptor in Human Breast Cancer*. McGuire, W.L., Carbone, P.P., Volmer, E.P. (eds) Raven Press, New York. 1975:pp 1-7.
28. 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.
29. Greenblatt RB, Roy S, Mahesh VB, Barfeld WE, Jungck EL. Induction of ovulation with clomiphene citrate. *Am J Obstet Gynecol* 1962;84:900-12.
30. Klopper A, Hall M. New synthetic agent for the induction of ovulation. Preliminary trial in women. *Br Med J* 1971;1:152-4.
31. Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. *Br J Cancer* 1971;25:270-5.
32. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nature Reviews Drug Discovery* 2003;2:205-13.
33. Jordan VC, Dowse LJ. Tamoxifen as an anti-tumour agent: effect on oestrogen binding. *J Endocrinol* 1976;68(02):297-303.
34. Jordan VC, Jaspan T. Tamoxifen as an antitumour agent: oestrogen binding as a predictive test for tumour response. *J Endocrinol* 1976;68:453-60.
35. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. *Eur J Cancer* 1976;12:419-24.
36. Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *Eur J Cancer* 1975;11:205-6.
37. 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.
38. Coombes CR, Goss P, Dowsett M, Gazet JC, Brodie A. 4-hydroxyandrostenedione in treatment of postmenopausal patients with advanced breast cancer. *The Lancet* 1984;i:1237-9.
39. Harper MJ, Walpole AL. Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. *Nature* 1966;212(57):87.
40. Ward HW. Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. *Br Med J* 1973;1(844):13-4.

41. Ingle JN, Ahmann DL, Green SJ, et al. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *N Engl J Med* 1981;304:16-21.
42. Stewart HJ, Forrest AP, Gunn JM, et al. The tamoxifen trial - a double-blind comparison with stilboestrol in postmenopausal women with advanced breast cancer. *Eur J Cancer* 1980;Suppl 1:83-8.
43. Beex L, Pieters G, Smals A, Koenders A, Benraad T, Kloppenborg P. Tamoxifen versus ethinyl estradiol in the treatment of postmenopausal women with advanced breast cancer. *Cancer Treat Rep* 1981;65(3-4):179-85.
44. Matelski H, Greene R, Huberman M, Lokich J, Zipoli T. Randomized trial of estrogen vs. tamoxifen therapy for advanced breast cancer. *Am J Clin Oncol* 1985;8(2):128-33.
45. Peethambaram PP, Ingle JN, Suman VJ, Hartmann LC, Loprinzi CL. Randomized trial of diethylstilbestrol vs. tamoxifen in postmenopausal women with metastatic breast cancer. An updated analysis. *Breast Cancer Res Treat* 1999;54(2):117-22.
46. Jordan VC, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. *Adjuvant Therapy of Cancer* 1979;2:19-26.
47. 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.
48. EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005;365:1687-717.
49. EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;354:1451-67.
50. 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.
51. 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.
52. Gail MH, Costantino JP, Bryant J, et al. Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J Natl Cancer Inst* 1999;91(21):1829-46.
53. Howell A, Cuzick J, Baum M, 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;365:60-2.
54. Thurlimann B, Keshaviah A, Coates AS, et al. A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med* 2005;353:2747-57.
55. Coombes RC, Hall E, Gibson LJ, et al. A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. *N Engl J Med* 2004;350(11):1081-92.
56. Coates AS, Keshaviah A, Thurlimann B, et al. 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* 2007;25(5):486-92.

57. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 1987;10(1):31-5.

58. Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea- induced rat mammary carcinoma model. *Cancer Res* 1987;47(15):4020-4.

59. Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 1988;6(5):589-95.

60. 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 [see comments] [published erratum appears in *JAMA* 1999 Dec 8;282(22):2124]. *JAMA* 1999;282(7):637-45.

61. 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.

62. Martino S, Cauley JA, Barrett-Connor E, et al. For the CORE Investigators 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.

63. Jordan VC. Optimising endocrine approaches for the chemoprevention of breast cancer. Beyond the Study of Tamoxifen and Raloxifene (STAR) Trial. *European Journal of Cancer* 2006;42:2909-13.

64. Vogel VG, Costantino JP, Wickerham DL, et al. The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial *JAMA* 2006;295:2727-41.

65. Fisher B, Dignam J, Bryant J, Wolmark N. Five versus more than five years of tamoxifen for lymph node-negative breast cancer: updated findings from the National Surgical Adjuvant Breast and Bowel Project B-14 randomized trial. *J Natl Cancer Inst* 2001;93:684-90.

66. Goss PE, Ingle JN, Marino 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;349:1-10.

67. Goss PE, Ingle JN, Martino S, et al. 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* 2005;97:1262-71.

68. Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Res* 1997;57(15):3071-8.

69. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 1988;48:5183-7.

70. O'Regan RM, Osipo C, Ariazi E, et al. Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. *Clin Cancer Res* 2006;12:2255-63.

71. Osborne CK, Pippen J, Jones SE, et al. 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* 2002;20(16):3386-95.
72. Howell A, Robertson JFR, Quaresma Albano J, et al. 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* 2002;20:3396-403.
73. Gomez BP, Riggins RB, Shajahan AN, et al. Human X-Box binding protein-1 confers both estrogen independence and antiestrogen resistance in breast cancer cell lines. *Faseb J* 2007.
74. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant on estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* 2003;95:1597-607.
75. Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. In: *Recent Results in Cancer Research*. Heidelberg: Springer-Verlag; 1993:23-33.
76. Yao K, Lee ES, Bentrem DJ, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;6(5):2028-36.
77. Osipo C, Meeke K, Cheng D, et al. Role for HER2/neu and HER3 in fulvestrant-resistant breast cancer. *Int J Oncol* 2007;30(2):509-20.
78. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. 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 Res* 1987;47:4355-60.
79. Welshons WV, Jordan VC. Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *Eur J Cancer* 1987;232:1935-9.
80. 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 and Cell Endo* 1992;90:77-80.
81. Pink JJ, Jiang SY, Fritsch M, Jordan VC. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* 1995;55:2583-90.
82. Masamura S, Santner SJ, Heitjan DF, Santen RJ. Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. *J Clin Endocrinol Metab* 1995;80(10):2918-25.
83. Song RX, Mor G, Naftolin F, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *J Natl Cancer Inst* 2001;93:1714-23.
84. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen induced apoptosis in breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 2005;94:131-41.
85. Lewis JS, Meeke K, Osipo C, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *JNCI* 2005;97:1746-59.
86. Song RX, Santen RJ. Apoptotic action of estrogen. *Apoptosis* 2003;8(1):55-60.

87. Jordan VC, Lewis-Wambi J, Kim H, et al. Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients. *Breast* 2007;(in press).
88. Mouridsen HT, Salimtschik M, Dombernowsky P, et al. Therapeutic effect of tamoxifen versus combined tamoxifen and diethylstilboestrol in advanced breast cancer in postmenopausal women. *Eur J Cancer* 1980;Suppl 1:107-10.
89. Lonning PE, Taylor PD, Anker G, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 2001;67:111-6.
90. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;5:207-13.
91. Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* 1977;75(2):305-16.
92. 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(1):83-91.
93. Buzdar A, Marcus C, Holmes F, Hug V, Hortobagyi G. Phase II evaluation of Ly156758 in metastatic breast cancer. *Oncology* 1988;45(5):344-5.
94. 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(2):125-37.
95. Howell A, Dodwell DJ, Anderson H, Redford J. Response after withdrawal of tamoxifen and progestogens in advanced breast cancer. *Ann Oncol* 1992;3(8):611-7.

## Figure Legends

**Figure 1.** The evolution in structure function relationships of estrogens based on diethylstilbestrol. This potent estrogen has a high affinity for the estrogen receptor (ER) and historically was used, at high doses (15 mg. daily) to treat both breast and prostate cancer. Description of the metabolic activation of tamoxifen to 4-hydroxytamoxifen<sup>91, 92</sup> was the first clue that tamoxifen was a prodrug and needed to be converted to metabolites with a high binding affinity for ER. Raloxifene (formerly the failed breast cancer drug keoxifene<sup>93</sup>) used knowledge from prior structure function studies to design an antiestrogen with low uterotrophic action but a high affinity for ER. The compound is a selective ER modulator (SERM) used for the long term treatment and prevention of osteoporosis and the prevention of breast cancer.<sup>61, 64</sup> Raloxifene, unlike tamoxifen, has not been found to increase uterine hyperplasia or increase the incidence of endometrial cancer.<sup>64, 94</sup>

**Figure 2.** The evolution in structure function relationships of antiestrogens based on triphenylchlorethylene.<sup>8</sup> The long acting estrogen triphenylchlorethylene was used as a treatment for breast cancer<sup>9</sup> and served as the basis for the discovery of clomiphene (a mixture of estrogenic and antiestrogenic *cis* and *trans* isomers (**ref**) used for the treatment of infertility.<sup>29</sup> Clomiphene was not used to treat breast cancer because of concerns about toxicity but served as the lead compound for the subsequent synthesis of tamoxifen, the pure *trans* isomer of a triphenylethylenes,<sup>28</sup> eventually developed for the targeted treatment and prevention of breast cancer.<sup>32</sup>

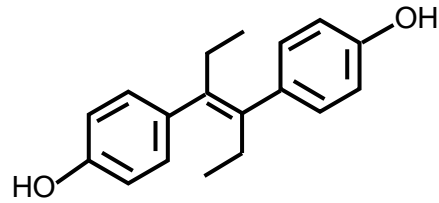
**Figure 3.** The natural estrogens 17 $\beta$  estradiol and estrone are metabolically interconverted in women. The orally active estrogen preparation Premarin® is obtained by extracting pregnant mare's urine. The principal estrogen is estrone sulphate which can be activated to estrone with sulphatase. Estrone in turn can then be converted by 17 hydroxy steroid dehydrogenase to the potent estrogen 17 $\beta$  estradiol. The other minor substitutes of Premarin® are equilin and equilinenin. Both are weak estrogens.

**Figure 4.** The evolution of antihormonal resistance in breast cancer. A. The current clinical view of drug resistance to tamoxifen or any selective estrogen receptor modulator (SERM). Long term tamoxifen treatment eventually selects for tamoxifen stimulated tumor growth. These tumors, are recognized by responding to tamoxifen withdrawal<sup>95</sup> but also grow in response to physiologic correlation of estrogen. These observations are supported by laboratory studies<sup>69</sup>. This form of tamoxifen resistance forms the basis for the response of patients to either aromatase inhibitors or fulvestrant following tamoxifen failure<sup>71, 72</sup> and the basis of the success of extended antihormonal therapy with five years of tamoxifen followed by five years of an aromatase inhibitor<sup>66</sup>. B. The emerging laboratory view of drug resistance to SERM or aromatase inhibitors. Drug resistance evolves to a point where the tumor is exclusively dependent on the SERM (Tamoxifen and raloxifene) or there is autonomous growth via the ER with long term estrogen withdrawal. The biology of estrogen changes dramatically as the tumor cell evolves from Phase I to Phase II. Estrogen now becomes an inhibitory or apoptotic signal. These emerging new laboratory data have important implications for future clinical practice.

**Figure 5.** A summary of sequence of subcellular events that occur in experimental models during estradiol-induced apoptosis in breast cancer. In some models, estradiol increases Fas ligand(L)<sup>83</sup> but in others, Fas receptor increases and there is a reduction in the survival signals from Her2/neu and NFκB<sup>74</sup>. In contrast, Lewis and coworkers<sup>85</sup> have described the actions of estradiol mediated through a mitochondrial mechanism.

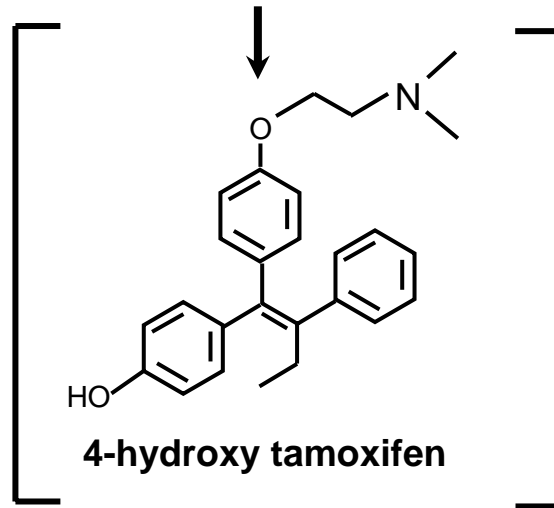
**Figure 6.** The organization of our Department of Defense Center of Excellence Grant entitled “A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis.” The model systems to study the survival and apoptosis induced with estrogen are being used for time course experiments at the Fox Chase Cancer Center. The materials are distributed to Translational Genomics for genomic analysis using CGH, siRNA analysis or agilent gene array analysis, and the Vincent T. Lombardi Cancer Center is involved to conduct proteomics. All results are uploaded into a shared secure web for data processing and target identification by our informatics and biostatistical group. Each laboratory is able to validate emerging pathways and study individual genes of interest. Our program is integrated with a clinical trials program that provides patient samples for validation of apoptotic or survival pathways. We are grateful to our external advisory board of Patient Advocates and professional colleagues for their continuing advice and support.

**Figure 7.** An anticipated treatment plan for third line endocrine therapy. Patients must have responded and failed two successive antihormonal therapies to be eligible for a course of low dose estradiol therapy for 3 months. The anticipated response rate is 30%<sup>89</sup> and responding patients will be treated with anastrozole until relapse. Validation of the treatment plan via the Center of Excellence grant (Figure 5) will establish a platform to enhance response rates with apoptotic estrogen by integrating known inhibitors of tumor survival pathways into the 3 month low dose estrogen debulking treatment plan. The overall goal is to increase response rates and maintain patients for longer on antihormonal strategies before chemotherapy is required.



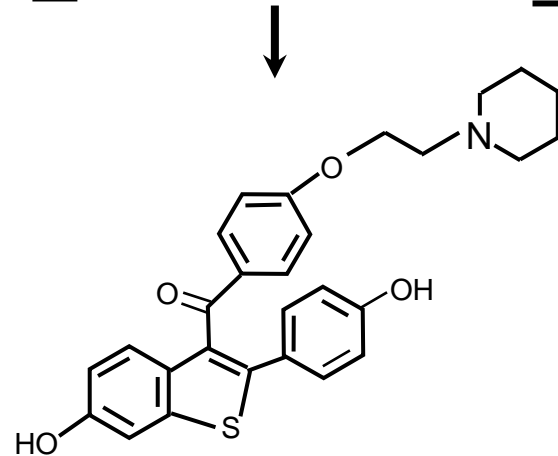
**diethylstilbestrol (DES)**

potent synthetic estrogen  
(breast and prostate cancer treatment)



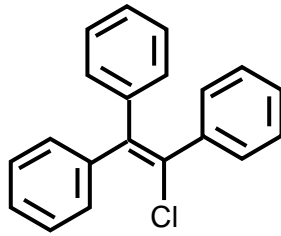
**4-hydroxy tamoxifen**

an active metabolite  
of tamoxifen



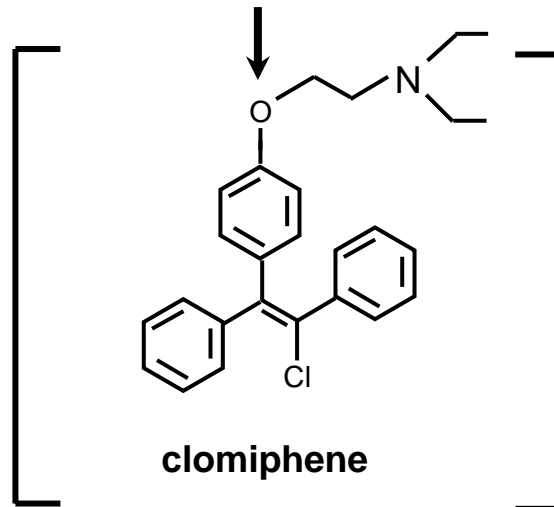
**raloxifene**

Selective estrogen receptor  
modulator for the  
treatment and prevention of  
osteoporosis and the prevention  
of breast cancer.

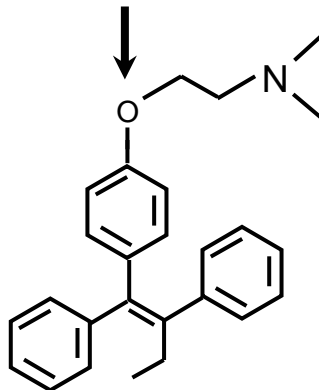


long acting estrogen  
(breast cancer treatment)

**Triphenyl chloroethylene**

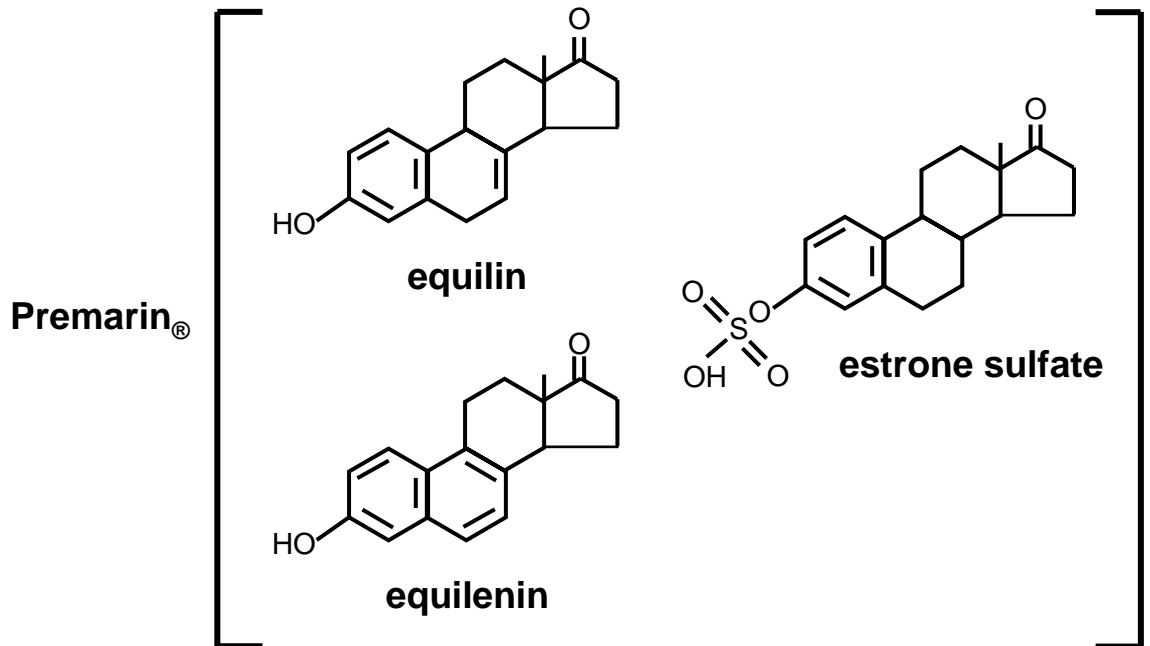
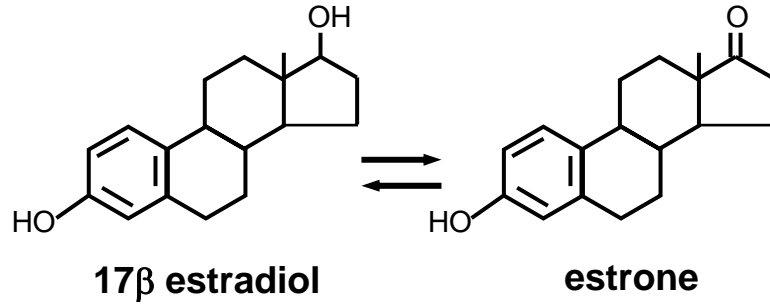


mixed cis/trans isomers  
estrogen-antiestrogen  
(induction of ovulation)

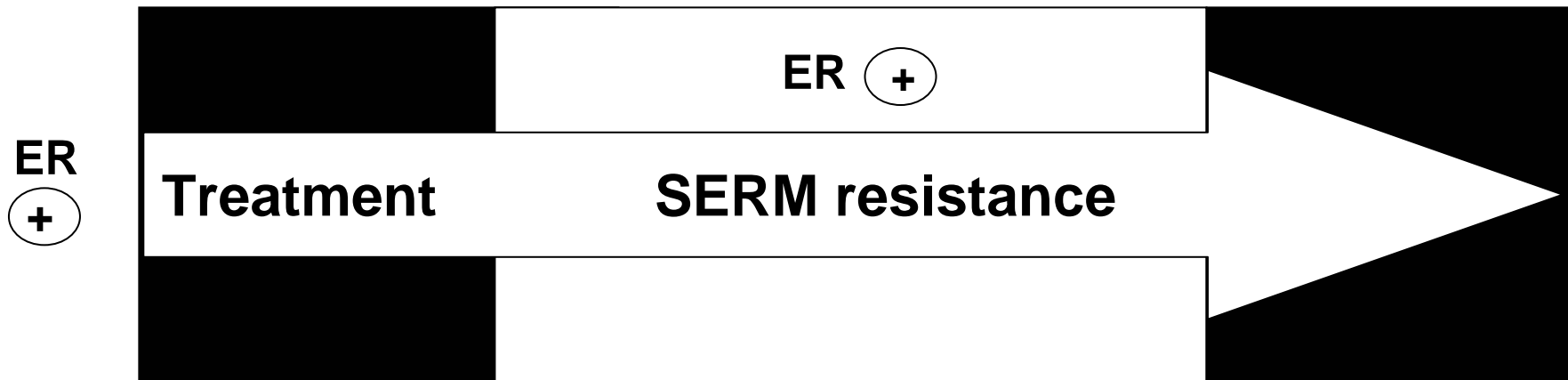


pure trans isomer  
antiestrogen  
(treatment and prevention of  
breast cancer)

**tamoxifen**



A

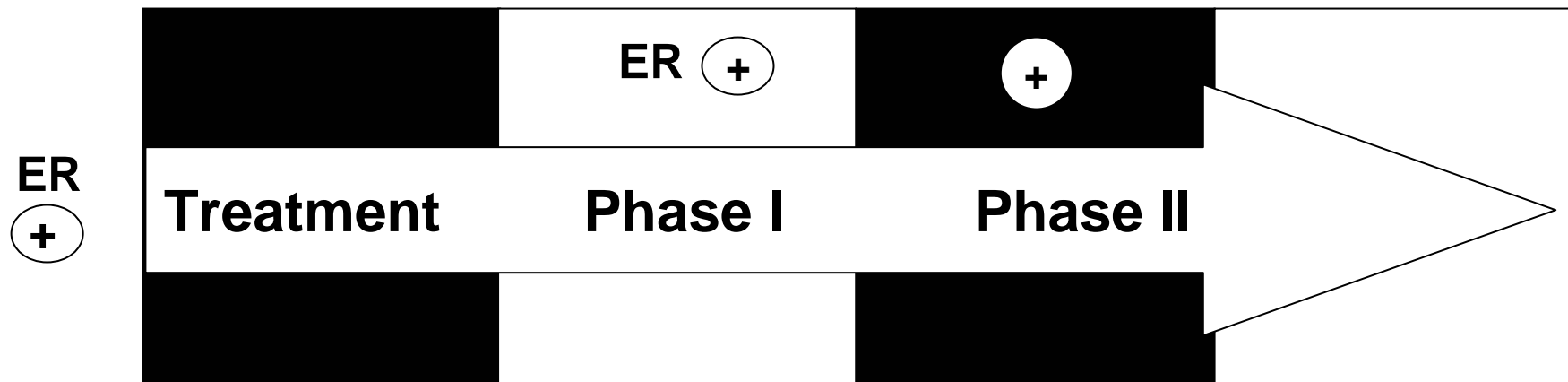


Antiestrogen action of  
SERMs

Estrogen or SERM – stimulated growth

Autonomous  
growth

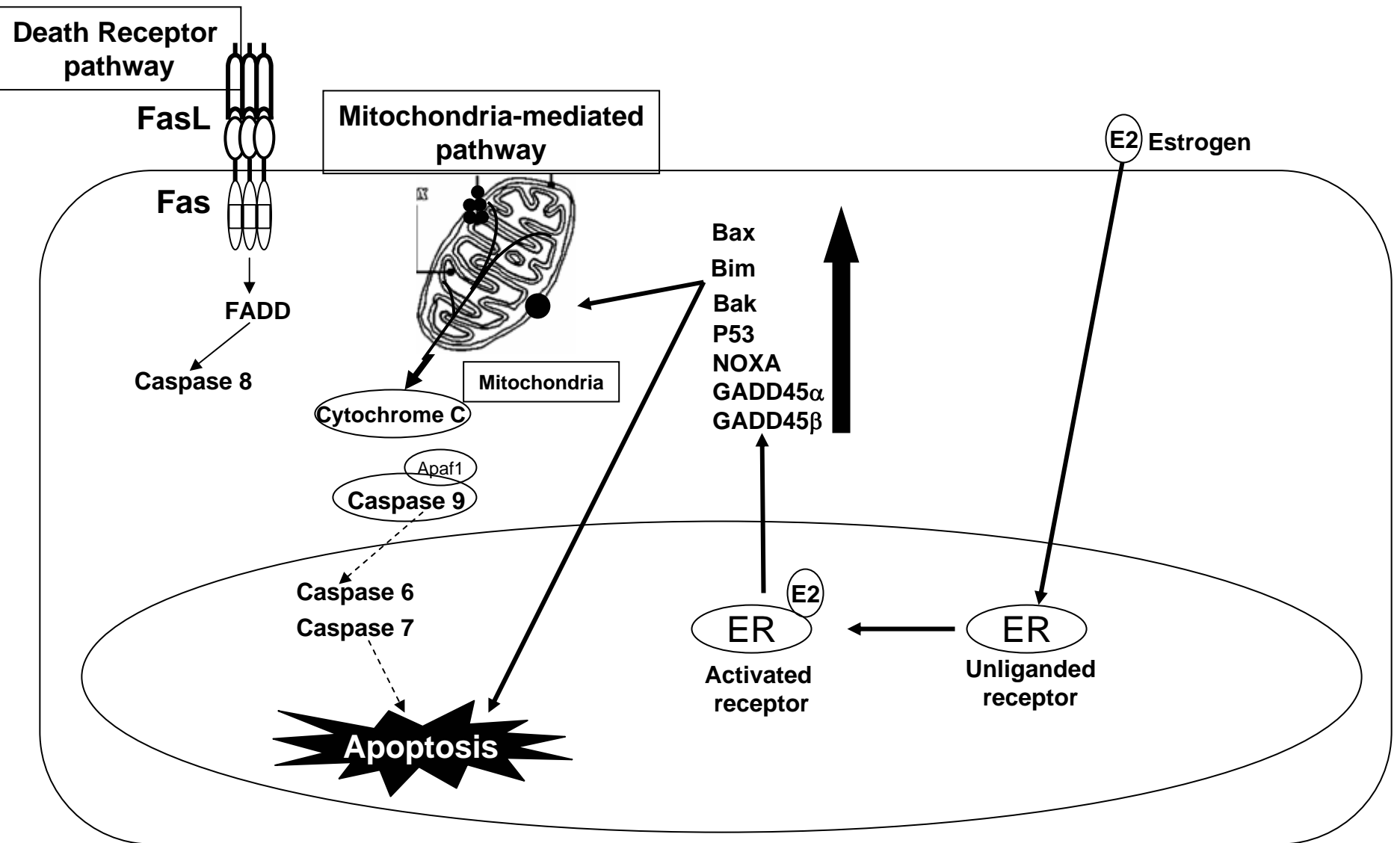
B



E<sub>2</sub> or  
SERM-  
stimulated

SERM-  
stimulated  
E<sub>2</sub> Inhibited

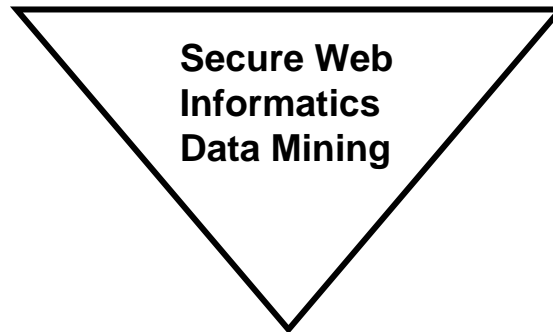
Autonomous  
growth  
E<sub>2</sub> Inhibited



**DEATH**

**Translational Genomics**  
**Phoenix, AZ**  
**Gene Array Analysis**  
**Global siRNAs**  
**Super computing**  
**Cunliffe/Trent**

**Lombardi Cancer Center**  
**Georgetown University**  
**Washington D.C**  
**Proteomics**  
**Wellstein/Riegel**



**External Advisory Board**  
**Margaret C Kirk, Y-ME National**  
**Breast Cancer Organization**  
**Mary Lou Smith, JD**  
**Research Advocacy Network**  
**Angela H. Brodie, PhD**  
**Geoffrey L. Greene, PhD**  
**Gabriel N. Hortobagyi, MD**  
**James N. Ingle, MD**  
**Benita S. Katzenellenbogen, PhD**  
**Richard J. Santen, MD**

**Clinical Trials Group**  
**Mary Daly**  
**Nancy Davidson**  
**Lori Goldstein**  
**Ramona Swaby**

**Fox Chase Cancer Center**  
**Philadelphia, PA**  
**Models and Mechanisms**  
**Jordan**

# TREATMENT PLAN FOR THIRD LINE THERAPY



**Patients who have responded and failed two antihormonal therapies**

**short term low dose estrogen**

**3 months**

**Aromatase inhibitor  
Anastrozole  
1mg/daily**

**SCIENTIFIC CONCEPT**



**Phase II antihormone resistance**



**Apoptosis to destroy resistant cells and enhance antihormonal responsiveness**



**Use an aromatase inhibitor in estrogen responding tumors to control antihormone sensitive disease**